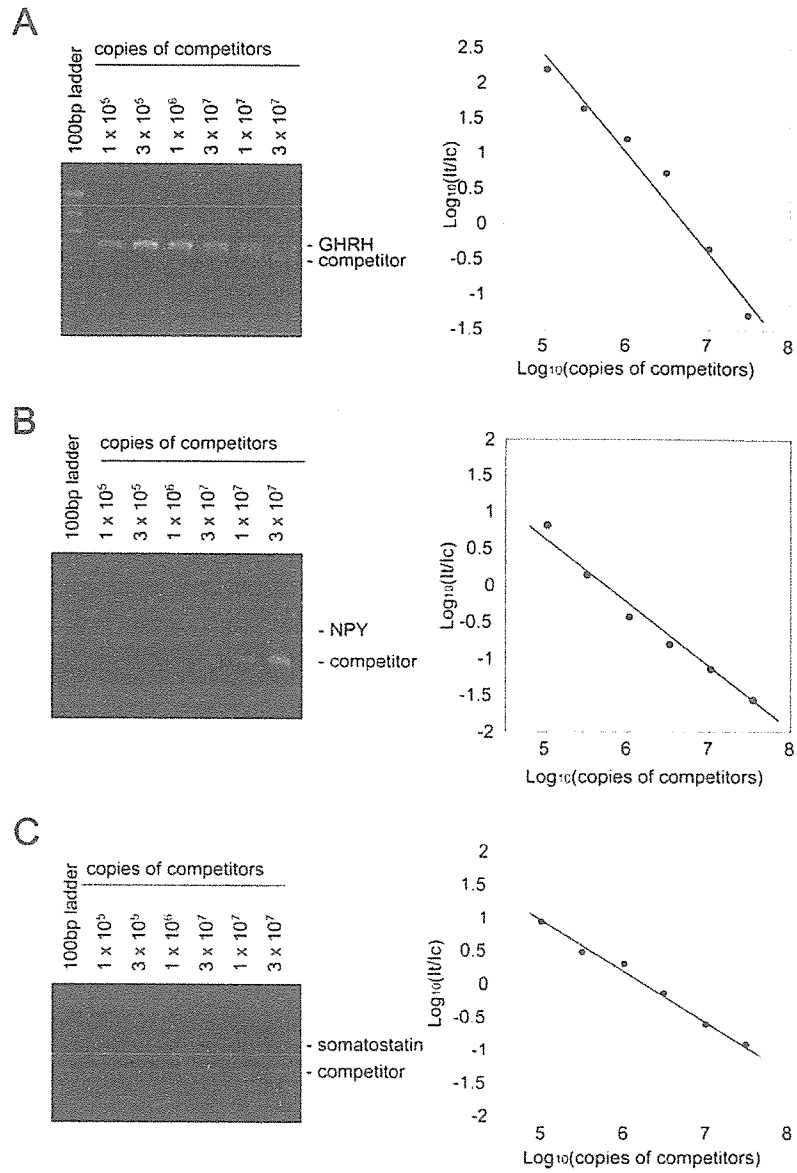

FIG. 6. Competitive RT-PCR assay for GHRH (A), NPY (B), and somatostatin (C) of cultured hypothalamic neurons. Extracted total RNAs were coamplified with serially diluted competitors. The competitor copy numbers were expressed as copies per microliter = $OD_{260} \times 40$ (ng/μl) $\times 10^{-9} \times 6 \times 10^{23}$/(length $\times$ 345). The ratio of the intensities of the target gene to the competitor was plotted against the concentration of the competitor on a log scale.

were distributed in the portion medial to that of the GHRH neurons in the Arc (Fig. 1, E and F). The mean number of NPY neurons did not differ between Tg and WT rats (Tg rats, 468 ± 49 *vs.* WT rats, 478 ± 49) (Fig. 1G).

Coexpression of GHS-R with GHRH or NPY in the Arc

GHS-R-positive neurons (*green*), GHRH neurons (*red*), and GHS-R-positive GHRH neurons (*yellow*) in the Arc of WT rats (A–C) and Tg rats (D–F) are shown in Fig. 2. The numbers of GHRH neurons and GHS-R-positive GHRH neurons in Tg rats were significantly reduced than those in WT rats, respectively (GHRH neurons, 41 ± 5, GHS-R-positive GHRH neurons, 20 ± 3 in Tg rats; GHRH neurons, 82 ± 5, GHS-R-positive GHRH neurons, 54 ± 7 in WT rats, $P < 0.05$). GHS-R-positive neurons (*green*), NPY neurons (*red*), and GHS-R-positive NPY neurons (*yellow*) in the Arc of WT rats (A–C) and Tg rats (D–F) are shown in Fig. 3. There was no difference in the number of NPY neurons and GHS-R-positive NPY neurons between Tg rats (NPY neurons, 322 ± 27, GHS-R-positive NPY neurons, 315 ± 28) and WT rats (NPY neurons, 311 ± 18, GHS-R-positive NPY neurons, 309 ± 18). The number of GHRH neurons in Fig. 2 or NPY neurons in Fig. 3 was lower than that in Fig. 1. These differences are explained by the methodological difference between glucose oxidase diaminobenzidine nickel intensify method used in Fig. 1 and double-labeled immunofluorescence used in Figs. 2 and 3, the former is more sensitive than the latter.

Fos expression in response to KP-102 in the Arc

Fos-positive neurons in the Arc of WT and Tg rats in response to vehicle are shown in Fig. 4, A and C, respectively. In response to ICV administration of KP-102, Fos-positive

neurons distributed widely in the Arc of WT and Tg rats are shown in Fig. 4, B and D, respectively. The mean number of Fos-positive neurons after ICV administration of KP-102 was significantly reduced to 46% of that seen in WT rats (Fig. 4E).

The distributions of Fos-positive GHRH neurons of the Arc in response to saline or KP-102 in the WT and Tg rats are shown in Fig. 5. The distribution of Fos-positive GHRH neurons in response to saline in WT rats is shown in Fig. 5A and that of Fos-positive GHRH neurons in response to KP-102 in WT rats is in Fig. 5B. The distribution of Fos-positive GHRH neurons in response to saline in Tg rats is shown in Fig. 5C, whereas that of Fos-positive GHRH neurons in response to KP-102 in Tg rats is in Fig. 5D. The mean number of Fos-positive GHRH neurons in response to KP-102 was significantly less in Tg rats than in WT rats (Fig. 5E).

GHRH mRNA expression in primary cultured hypothalamic neurons

The results of a typical RT-PCR analysis of GHRH, NPY, and somatostatin mRNA levels in cultured hypothalamic neurons are shown in Fig. 6. Treatment of cultured neurons with KP-102 at concentrations ranging from 2–200 nM for 2 h did not significantly affect the level of GHRH mRNA expression (Fig. 7A). KP-102 at 20 nM did not significantly affect the GHRH mRNA expression level during 1, 2, 8, and 24 h, although with 2-h incubation, there was a trend to increased GHRH mRNA expression (Fig. 7B). Treatment of cultured neurons with KP-102 for 2 h did not significantly increase the level of NPY or somatostatin mRNA expression at a concentration of 0.2, 2.0, or 20 nM (Fig. 7, C and D). However,

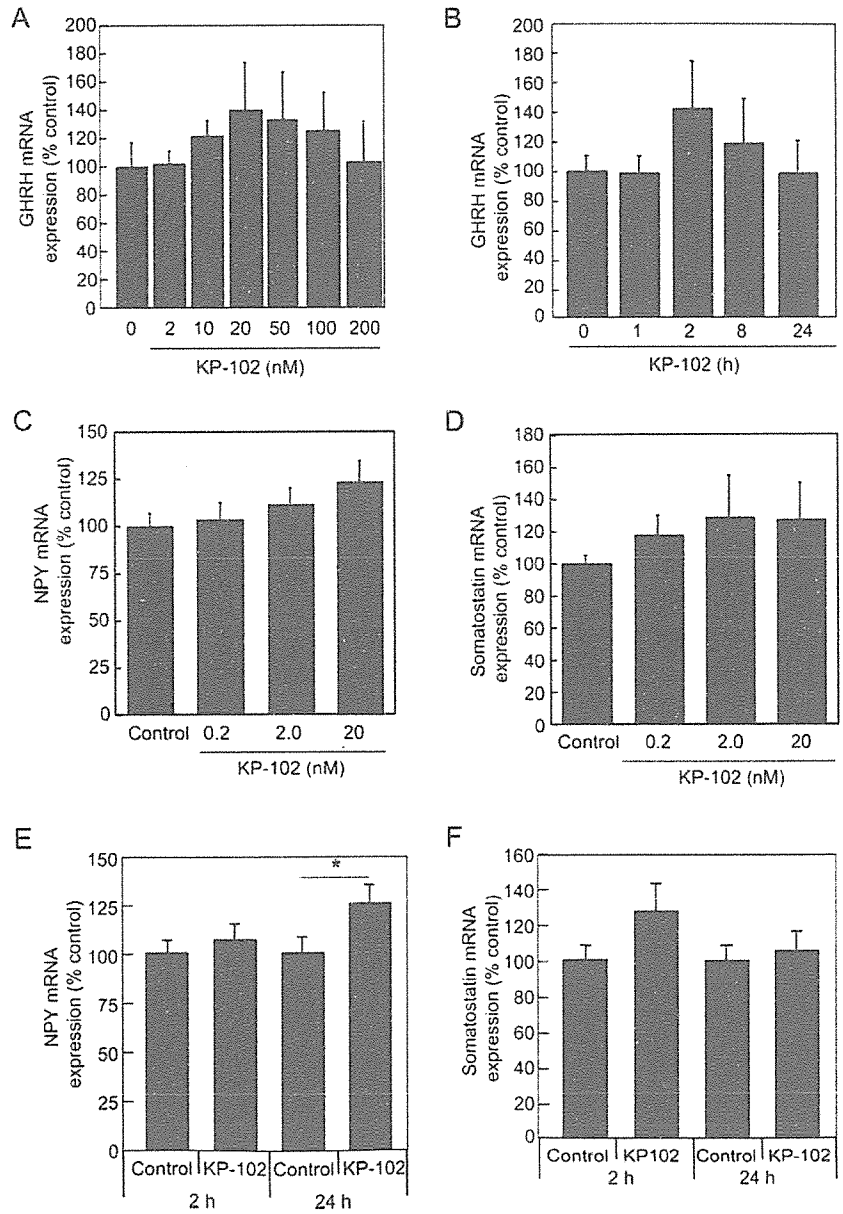

FIG. 7. Effects of KP-102 on GHRH, NPY, and somatostatin mRNA expression in cultured rat hypothalamic neurons. A, Dose-response effect of KP-102 on GHRH mRNA expression. B, Effect of duration of treatment with KP-102 on GHRH mRNA expression. C, Effect of KP-102 at concentrations of 0.2, 2.0, and 20 nM on NPY mRNA expression for 2 h. D, Effect of KP-102 on somatostatin mRNA expression at concentrations of 0.2, 2.0, and 20 nM for 2 h. E, Effect of KP-102 at a concentration of 20 nM on NPY mRNA expression for 2 or 24 h. F, Effect of KP-102 at a concentration of 20 nM on somatostatin mRNA expression for 2 or 24 h. The numbers of wells in each experimental group (A–F) were 12, 12, 9, 9, 8, and 8, respectively. *, $P < 0.05$.

treatment of cultured neurons with KP-102 at a concentration of 20 nM for 24 h but not 2 h significantly increased the NPY mRNA expression level (Fig. 7E). The level of somatostatin mRNA expression was not affected by KP-102 given at a concentration of 20 nM for either 2 or 24 h (Fig. 7F).

NPY at a concentration of 1 nM but not 0.1 nM significantly decreased the GHRH mRNA expression level (Fig. 8A). The inhibitory effect of 1 nM NPY on GHRH mRNA expression was completely blocked by anti-NPY IgG (3.6 μg/ml) (Fig. 8B). Anti-NPY IgG itself did not affect the level of GHRH mRNA expression. KP-102 significantly increased the level of GHRH mRNA expression approximately 2-fold in the presence of anti-NPY IgG during 2-h incubation, but it did not induce significant change in the expression level when given with normal rabbit serum IgG or without IgG (Fig. 8C).

Somatostatin significantly decreased the GHRH mRNA expression level at concentrations of 10 and 100 nM during the 2-h incubation period (Fig. 8D). The 10 nM somatostatin-induced suppression of GHRH mRNA expression level was completely reversed in the presence of antisomatostatin IgG (3.6 μg/ml) but not in the presence of normal rabbit serum IgG (Fig. 8E). The antisomatostatin IgG did not affect the level of GHRH mRNA expression in the presence of KP-102 (Fig. 8F). Treatment of neurons with somatostatin at a concentration of 10 nM for 2 h induced no significant change in NPY mRNA expression (Fig. 9A). The somatostatin-induced suppression of GHRH mRNA expression was partially reversed by anti-NPY IgG (Fig. 9B). GH at concentrations ranging from 10–500 ng/ml for 2 h significantly increased somatostatin mRNA expression level and suppressed GHRH mRNA expression level without influence on NPY mRNA expression (Fig. 10).

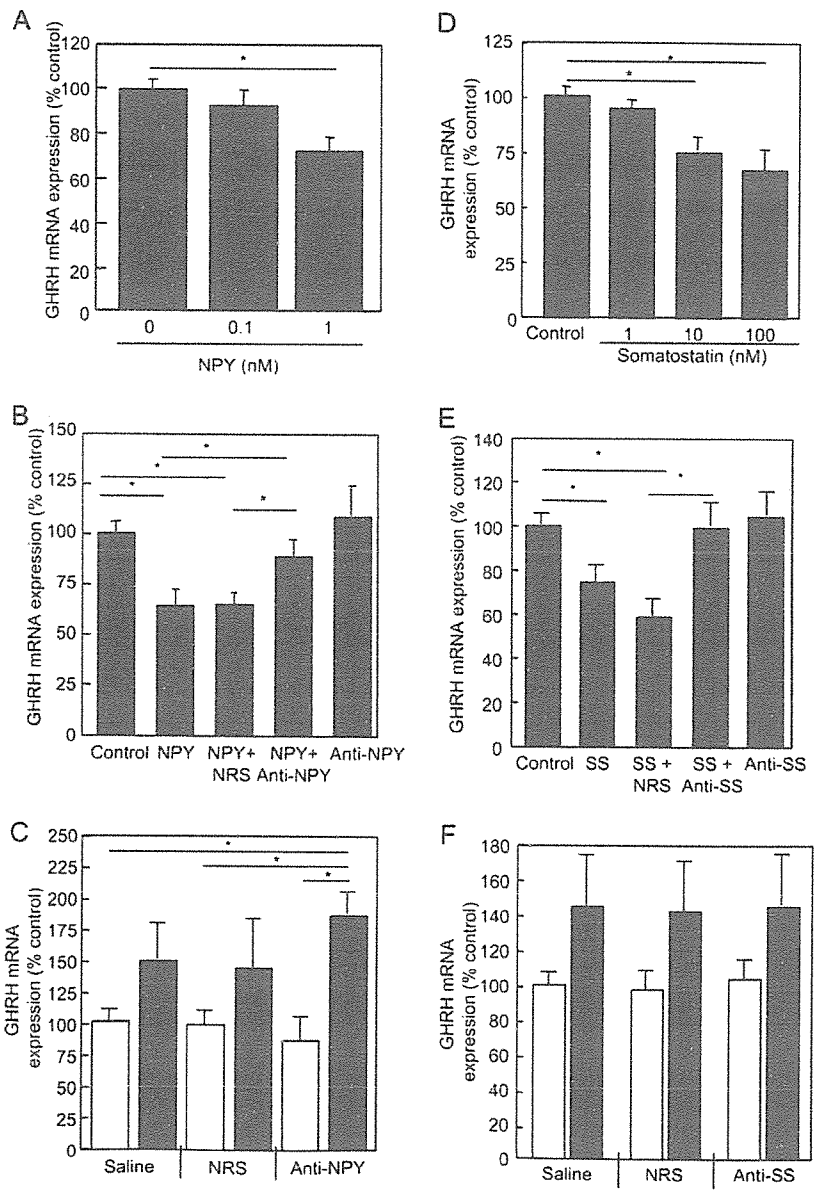

FIG. 8. Effects of NPY and somatostatin on GHRH mRNA expression and the KP-102-induced changes in GHRH mRNA expression level. A, Inhibitory effect of NPY on GHRH mRNA expression. B, Effect of anti-NPY IgG on NPY-induced inhibition of GHRH mRNA expression. C, Effect of anti-NPY IgG on KP-102-induced changes in GHRH mRNA expression. Cells were treated with vehicle (*white bar*) or 20 nM KP-102 (*black bar*) for 2 h in the presence of NRS or anti-NPY IgG. D, Inhibitory effect of somatostatin on GHRH mRNA expression. E, Effect of antisomatostatin IgG on somatostatin-induced inhibition of GHRH mRNA expression. F, Effect of antisomatostatin IgG on KP-102-induced changes of GHRH mRNA expression. Cells were treated with vehicle (*white bar*) or 20 nM KP-102 (*black bar*) for 2 h in the presence of NRS or antisomatostatin IgG. The number of wells in each experimental group (A–F) were 8, 9, 12, 8, 8, and 8, respectively. NRS, Normal rabbit serum IgG (3.6 μg/ml); Anti-NPY, anti-NPY IgG (3.6 μg/ml); Anti-SS, antisomatostatin IgG (3.6 μg/ml). *, $P < 0.05$.

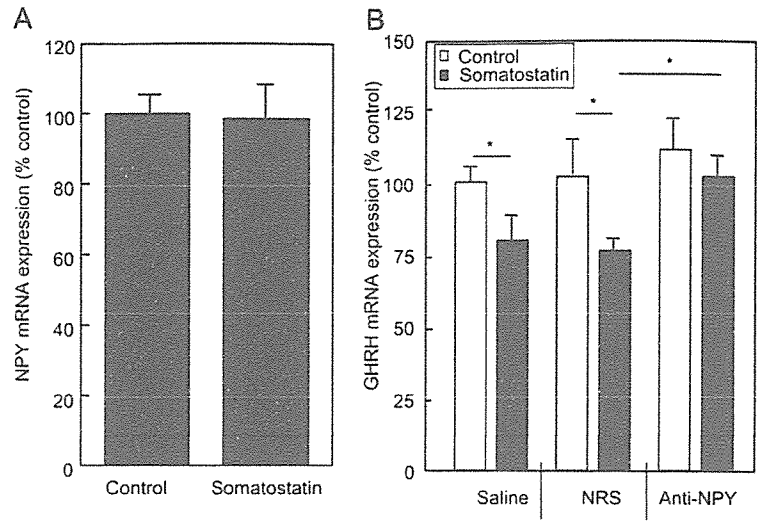

FIG. 9. Effect of somatostatin on NPY mRNA expression and effect of anti-NPY IgG on somatostatin-induced inhibition of GHRH mRNA. A, Effect of 10 nM somatostatin for 2 h on NPY mRNA expression. B, Effect of anti-NPY IgG on somatostatin-induced inhibition of GHRH mRNA expression. NRS, Normal rabbit serum IgG (3.6 μg/ml); Anti-NPY, anti-NPY IgG (3.6 μg/ml). The numbers of wells in each experimental group of A and B were nine. *, $P < 0.05$.

Concentrations of GHRH in cultured neurons

Although the basal secretion of GHRH from cultured hypothalamic neurons was undetectable level by RIA, the cellular concentrations of GHRH after 4-h treatment with KP-102 at concentrations of 2.0 and 20 nM were significantly increased (control, 567.5 ± 11.7 pg/well, 2.0 nM KP-102, 602.0 ± 13.6 pg/well, $P < 0.001$, *vs.* control, 20 nM KP-102, 651.0 ± 10.9 pg/well, $P < 0.001$ *vs.* control n = 6). The cellular concentrations of GHRH after 4-h treatment with somatostatin at a concentration of 1.0 and 10 nM did not show any significant changes.

Discussion

We have previously created Tg rats expressing an antisense GHS-R mRNA under the control of the promoter for TH and have reported that the concentrations of GHS-R protein in the Arc determined by Western blot analysis are lower in Tg rats than in WT rats (7). In the present study, we found that the numbers of GHS-R-positive neurons, GHRH neurons, and GHS-R-positive GHRH neurons were significantly lower in Tg rats compared with WT rats, whereas the number of NPY neurons or GHS-R-positive NPY neurons did not differ between the two groups. The expression level of NPY mRNA in the Arc of the Tg rats is thought not to be affected by the induction of GHS-R antisense due to the absence of TH in most of the NPY neurons located in the Arc (18, 19). The present study shows that, in response to the ICV injection of KP-102, one of the GHSs, Fos-positive neurons, and Fos-positive GHRH neurons in the Arc were reduced in Tg rats, reflecting the reduced expression of GHS-R in neurons including GHRH neurons in the Arc of Tg rats. These results suggest that the ghrelin/GHS-R system plays a role in up-regulating GHRH mRNA expression. This hypothesis is supported by a previous study, which found that Tg mice that constitutively overexpress GHS-R in the GHRH neurons show an increase in hypothalamic GHRH expression (12). However, unexpectedly, the ICV administration of ghrelin has been reported to induce no changes in the levels of GHRH mRNA expression levels, although it increases the levels of NPY and agouti-related peptide mRNA expression in the Arc (20, 21). Therefore, it seems that some mechanism masks the stimulatory effect of exogenous ghrelin on GHRH mRNA expression level in *in vivo* experiments.

Because GH, NPY, and somatostatin are involved in the feedback mechanism of the regulation of GH secretion, these hormones are the likely candidates that mask the stimulatory effect of ghrelin on GHRH mRNA expression. ICV administration of ghrelin increases GH secretion in nonanesthetized and anesthetized rats (22, 23). It is reported that iv administration of GH induces the expression of c-*fos* mRNA in NPY neurons of the Arc (24), where the GH receptor is expressed (25, 26). However, the present study showed that GH did not affect NPY mRNA expression. The difference in the effects of GH on NPY neurons may be explained by the difference of GH signal transport between *in vivo* study and *in vitro* study. Although these experiments do not show how GH modifies the release of NPY, the results of our study do not suggest a direct action of GH on NPY neurons.

When it is administered ICV, NPY inhibits GH secretion in rats (27, 28) at least in part through somatostatin release because NPY stimulates somatostatin release from the hypothalamus *in vitro* (29, 30). NPY neurons in the Arc project to the periventricular nucleus (PeV) (31), and synaptic connections between NPY axons and somatostatin neurons have been demonstrated in the PeV (32). These findings suggest that GH secreted in response to ghrelin may act on somatostatin neurons because GH receptor mRNA is present in somatostatin neurons in the PeV (33), and the iv administration of GH induces c-*fos* mRNA expression in somatostatin neurons in the PeV and Arc (24). The results of the present study actually showed that GH directly stimulated somatostatin mRNA expression and inhibited GHRH mRNA expression, suggesting the inhibitory role of GH in the GHRH expression.

Furthermore, NPY and somatostatin neurons activated by ghrelin directly may inhibit GHRH expression because somatostatin and NPY inhibit GHRH release from the hypothalamus *in vitro* (14, 30, 34). Histological connections between these neurons support this possibility; GHRH neurons are present in the ventrolateral part of the Arc where NPY

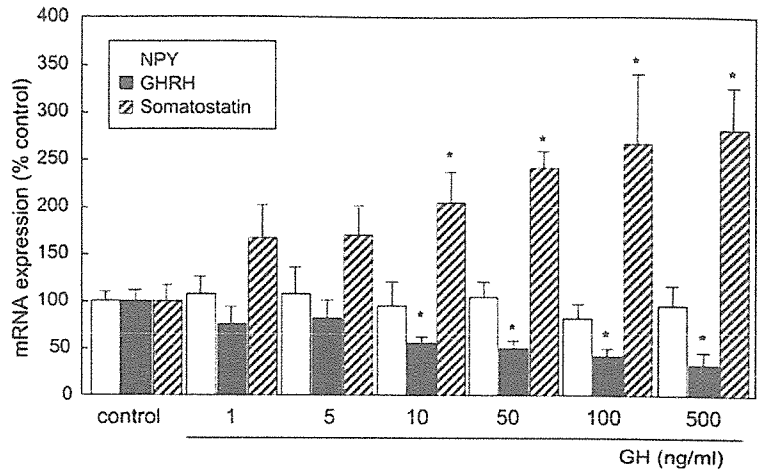

FIG. 10. Effects of GH on GHRH, NPY, and somatostatin mRNA expression in cultured rat hypothalamic neurons. The number of wells in each treatment group was six. *, $P < 0.05$.

fibers are concentrated (8, 35). GHRH neurons are innervated by somatostatin fibers, and somatostatin receptors are present on GHRH neurons (36–39). However, deleting the influence of GH, the present study showed that KP-102 significantly stimulated NPY mRNA expression and did not affect somatostatin mRNA expression, although both NPY and somatostatin inhibited GHRH mRNA expression. Therefore, NPY but not somatostatin activated by ghrelin seems to inhibit the activity of GHRH neurons. Furthermore, the inhibitory effect of somatostatin on GHRH mRNA expression was partially blocked by anti-NPY IgG, although somatostatin did not significantly affect NPY mRNA expression in the present study. Therefore, NPY released in response to somatostatin may also be involved in the inhibitory mechanism of GHRH mRNA expression by somatostatin in addition to direct action of somatostatin on GHRH neurons.

In contrast to our results, several reports showed that ICV administration of somatostatin stimulates GH release (40–42) and that ICV administration of antisense oligonucleotides against somatostatin 1 receptor suppresses GH tone in rats (43). Furthermore, somatostatin stimulates *in vitro* GHRH release in rat hypothalamic perfusion system (44). These reports suggest that somatostatin has a dual effect on GH secretion, although we found only inhibitory action of somatostatin on GHRH mRNA expression. The mechanism by which somatostatin stimulates GH secretion still remains unclear. Further studies are needed to clarify the complex mechanism.

We have found that KP-102 significantly increased the GHRH mRNA expression level only in the presence of anti-NPY rabbit IgG. Although the concentrations of NPY in the culture media were not measured in the present study, ghrelin has already been shown to stimulate *in vitro* NPY release from the rat hypothalamus (45). Therefore, these results suggest that KP-102 not only up-regulates GHRH mRNA expression but also stimulates NPY release in the Arc and that the NPY that is released by KP-102 attenuates the stimulatory effect of KP-102 on GHRH mRNA expression. The results of the present study are in agreement with a report that fasting-induced inhibition of GHRH mRNA expression in WT mice is abolished in *npy* null mice (46).

The present study showed that somatostatin inhibited the GHRH mRNA expression level. The results of the present study also showed that the effect of KP-102 on the GHRH mRNA expression level was not influenced by antisomatostatin IgG and that KP-102 did not significantly affect somatostatin mRNA expression, suggesting that somatostatin does not play a significant role downstream of the action of KP-102 on the expression of GHRH mRNA expression. These results are in agreement with other studies showing that ghrelin and GHSs have no effect on *in vitro* release of somatostatin from rat hypothalamus (30, 45).

In summary, the present study has shown that the number of GHS-R-positive GHRH neurons is reduced in Tg rats whose GHS-R expression is attenuated. It has also been shown that KP-102, one of the GHSs, stimulates the NPY mRNA expression level of cultured rat hypothalamic neurons and that NPY reduces the GHRH mRNA expression level. The present study also demonstrated that KP-102 stimulates the level of GHRH mRNA expression when NPY action is deleted. Furthermore, GH and somatostatin inhibit GHRH mRNA expression. These results indicate that GHS-R is involved in the up-regulation of GHRH and NPY expression in the Arc and that NPY as well as GH and somatostatin down-regulate GHRH mRNA expression. It is also suggested that the reduction of GHRH neurons in the Arc of Tg rats is induced by the decrease in GHS-R expression.

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All authors have nothing to declare.

References

1. **Smith RG, Van der Ploeg LHT, Howard AD, Feighner SD, Cheng K, Hickey GJ, Wyvratt Jr MJ, Fisher MH, Nargund RP, Patchett AA** 1997 Peptidomimetic regulation of growth hormone secretion. Endocr Rev 18:621–645
2. **Howard AD, Feighner SD, Cully DF, Arena JP, Liberator PA, Rosenblum CI, Hamelin M, Hreniuk DL, Palyha OC, Anderson J, Paress PS, Diaz C, Chou**

M, Liu KK, McKee KK, Pong S-S, Chaung L-Y, Elbrecht A, Dashkevicz M, Heavens R, Rigby M, Sirinathsinghji DJS, Dean DC, Melillo DG, Patchett AA, Nargund R, Griffin PR, DeMartino JA, Gupta SK, Schaeffer JM, Smith RG, Van der Ploeg LHT 1996 A receptor in pituitary and hypothalamus that functions in growth hormone release. Science 273:974–977

3. **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
4. **Guan X-M, Yu H, Palyha OC, McKee KK, Feighner SD, Sirinathsinghji DJS, Smith RG, Van der Plog LHT, Howard AD** 1997 Distribution of mRNA encoding the growth hormone secretagogue receptor in brain and peripheral tissues. Mol Brain Res 48:23–29
5. **Dickson SL, Luckman SM** 1997 Induction of c-fos messenger ribonucleic acid in neuropeptide Y and growth hormone (GH)-releasing factor neurons in the rat arcuate nucleus following systemic injection of the GH secretagogue, GH-releasing peptide-6. Endocrinology 138:771–777
6. **Kamegai J, Hasegawa O, Minami S, Sugihara H, Wakabayashi I** 1996 The growth hormone-releasing peptide KP-102 induces c-fos expression in the arcuate nucleus. Mol Brain Res 39:153–159
7. **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
8. **Meister B, Hokfelt T, Vale WW, Sawchenko PE, Swanson L, Goldstein M** 1986 Coexistence of tyrosine hydroxylase and growth hormone-releasing factor in a subpopulation of tubero-infundibular neurons of the rat. Neuroendocrinology 42:237–247
9. **Tannenbaum GS, Lapointe M, Beaudet A, Howard AD** 1998 Expression of growth hormone secretagogue-receptors by growth hormone-releasing hormone neurons in the mediobasal hypothalamus. Endocrinology 139:4420–4423
10. **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
11. **Tannenbaum GS, Epelbaum J, Bowers CY** 2003 Interrelationship between the novel peptide ghrelin and somatostatin/growth hormone-releasing hormone in regulation of pulsatile growth hormone secretion. Endocrinology 144:967–974
12. **Lall S, Balthasar N, Carmignac D, Magoulas C, Sesay A, Houston P, Mathers K and Robinson I** 2004 Physiological studies of transgenic mice overexpressing growth hormone (GH) secretagogue receptor 1A in GH-releasing hormone neurons. Endocrinology 145:1602–1611
13. **Shibasaki T, Yamauchi N, Takeuchi K, Ishii S, Sugihara H, Wakabayashi I** 1998 The growth hormone secretagogue KP-102-induced stimulation of food intake is modified by fasting, restraint stress, and somatostatin in rats. Neurosci Lett 255:9–12
14. **Yamauchi N, Shibasaki T, Ling N, Demura H** 1991 In vitro release of growth hormone-releasing factor (GRF) from the hypothalamus: somatostatin inhibits GRF release. Regul Pept 33:71–78
15. **Shuto Y, Shibasaki T, Wada K, Parhar I, Kamegai J, Sugihara H, Oikawa S, Wakabayashi I** 2001 Generation of polyclonal antiserum against the growth hormone secretagogue receptor (GHS-R): evidence that the GHS-R exists in the hypothalamus, pituitary and stomach of rats. Life Sci 68:991–996
16. **Shibasaki T, Oda T, Imaki T, Ling N, Demura H** 1993 Injection of anti-neuropeptide Y γ-globulin into the hypothalamic paraventricular nucleus decreases food intake in rats. Brain Res 601:313–316
17. **Shu S, Ju G, Fan L** 1988 The glucose oxidase-DAB-nickel method in peroxidase histochemistry of the nervous system. Neurosci Lett 85:169–171
18. **Everitt BJ, Hokfelt T, Terenius L, Tatemoto K, Mutt V, Goldstein M** 1984 Differential co-existence of neuropeptide Y (NPY)-like immunoreactivity with catecholamines in the central nervous system of the rat. Neuroscience 11:443–462
19. **Everitt BJ, Meister B, Hokfelt T, Melander T, Terenius L, Rokaeus A, Theodorsson-Norheim E, Dockray G, Edwardson J, Cuello C, Elde R, Goldstein M, Hemmings H, Ouimet C, Walaas I, Greengard P, Vale W, Weber E, Wu J-Y, Chang K-J** 1986 The hypothalamic arcuate nucleus-median eminence complex: immunohistochemistry of transmitters, peptides and DARPP-32 with special reference to coexistence in dopamine neurons. Brain Res Rev 11:97–155
20. **Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I** 2000 Central effect of ghrelin, an endogenous growth hormone secretagogue, on hypothalamic peptide gene expression. Endocrinology 141:4797–4800
21. **Seoane, LM, Lopez M, Tovar S, Casanueva FF, Senaris R, Dieguez C** 2003 Agouti-related peptide, neuropeptide Y, and somatostatin-producing neurons are targets for ghrelin actions in the rat hypothalamus. Endocrinology 144:544–551
22. **Tamura H, Kamegai J, Shimizu T, Ishii S, Sugihara H, Oikawa S** 2002 Ghrelin stimulates GH but not food intake in arcuate nucleus ablated rats. Endocrinology 143:3268–3275
23. **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
24. **Kamegai J, Minami S, Sugihara H, Higuchi H, Wakabayashi I** 1994 Growth hormone induces expression of the c-fos gene on hypothalamic neuropeptide Y and somatostatin neurons in hypophysectomized rats. Endocrinology 135: 2765–2771
25. **Chan YY, Steiner RA, Clifton DK** 1996 Regulation of hypothalamic neuropeptide-Y neurons by growth hormone in the rat. Endocrinology 137:1319–1325
26. **Kamegai J, Minami S, Sugihara H, Hasegawa O, Higuchi H, Wakabayashi I** 1996 Growth hormone receptor gene is expressed in neuropeptide Y neurons in hypothalamic arcuate nucleus of rats. Endocrinology 137:2109–2112
27. **MacDonald JK, Lumpkin MD, Samson WK, McCann SM** 1985 Neuropeptide Y affects secretion of luteinizing hormone and growth hormone in ovariectomized rats. Proc Natl Acad Sci USA 82:561–564
28. **Harfstrand A, Eneroth P, Agnati L, Fuxe K** 1987 Further studies on the effects of central administration of neuropeptide Y on neuroendocrine function in the male rat: relationship to hypothalamic catecholamines. Regul Pept 17:167–179
29. **Rettori V, Milenkovic L, Aguila MC, McCann SM** 1990 Physiologically significant effect of neuropeptide Y to stress growth hormone release by stimulating somatostatin discharge. Endocrinology 126:2296–2301
30. **Korbonits M, Little JA, Forsling ML, Tringali G, Costa A, Navarra P, Trainer PJ, Grossman AB** 1999 The effect of growth hormone secretagogues and neuropeptide Y on hypothalamic hormone release from acute rat hypothalamic explants. J Neuroendocrinol 11:521–528
31. **Bai FL, Yamano M, Shiotani Y, Emson PC, Smith AD, Powell JF, Tohyama M** 1985 An arcuato-paraventricular and -dorsomedial hypothalamic neuropeptide Y-containing system which lacks noradrenaline in the rat. Brain Res 331:172–175
32. **Hisano S, Tsuruo Y, Kagotani Y, Daikoku S, Chihara K** 1990 Immunohistochemical evidence for synaptic connections between neuropeptide Y-containing axons and periventricular somatostatin neurons in the anterior hypothalamus in rats. Brain Res 520:170–177
33. **Burton KA, Kabigting EB, Clifton DK, Steiner RA** 1992 Growth hormone receptor messenger ribonucleic acid distribution in the adult male rat brain and its colocalization in hypothalamic somatostatin neurons. Endocrinology 131:958–963
34. **Aguila MC** 1998 Somatostatin decreases somatostatin messenger ribonucleic acid levels in the rat periventricular nucleus. Peptides 19:1573–1579
35. **De Quidt ME, Emson PC** 1986 Distribution of neuropeptide Y-like immunoreactivity in the rat central nervous system: II. Immunohistochemical analysis. Neuroscience 18:545–618
36. **Liposits Zs, Merchenthaler I, Paul WK, Flerko B** 1988 Synaptic communication between somatostatinergic axons and growth hormone-releasing factor (GRF) synthesizing neurons in the arcuate nucleus of the rat. Histochemistry 89:247–252
37. **Bertherat J, Dournaud P, Berod A, Normand E, Bloch B, Rostene W, Kordon C, Epelbaum J** 1992 Growth hormone-releasing hormone-synthesizing neurons are a subpopulation of somatostatin receptor-labelled cells in the rat arcuate nucleus: a combined in situ hybridization and receptor light-microscopic radioautographic study. Neuroendocrinology 56:25–31
38. **McCarthy GF, Beaudet A, Tannenbaum GS** 1992 Colocalization of somatostatin receptors and growth hormone-releasing factor immunoreactivity in neurons of the rat arcuate nucleus. Neuroendocrinology 56:18–24
39. **Lanneau C, Peienau S, Petit F, Epelbaum J, Gardette R** 2000 Somatostatin modulation of excitatory synaptic transmission between periventricular and arcuate hypothalamic nuclei in vitro. J Neurophysiol 84:1464–1474
40. **Murakami Y, Kato Y, Kabayama Y, Inoue T, Koshiyama H, Imura H** 1987 Involvement of hypothalamic growth hormone (GH)- releasing factor in GH secretion induced by intracerebroventricular injection of somatostatin in rats. Endocrinology 120:311–316
41. **Abe H, Kato Y, Iwasaki Y, Chihara K, Imura H** 1978 Central effect of somatostatin on the secretion of growth hormone in the anesthetized rat. Proc Soc Exp Biol Med 159:346–349
42. **Lumpkin MD, Negro-Vilar A, McCann SM** 1980 Paradoxical elevation of growth hormone by intraventricular somatostatin: possible ultrashort-loop feedback. Science 211:1072–1074
43. **Lanneau C, Bluet-Pajot MT, Zizzari P, Csaba Z, Dournaud P, Helboe L, Hoyer D, Pellegrini E, Tannenbaum GS, Epelbaum J, Gardette R** 2000 Involvement of the Sst1 somatostatin receptor subtype in the intrahypothalamic neuronal network regulating growth hormone secretion: an in vitro and in vivo antisense study. Endocrinology 141:967–979
44. **Pecori Giraldi F, Frohman LA** 1995 Discordant effects of endogenous and exogenous somatostatin on growth hormone-releasing hormone secretion from perifused mouse hypothalami. Neuroendocrinology 61:566–572
45. **Wren AM, Small CJ, Fribbens CV, Neary NM, Ward HL, Seal LJ, Ghatei MA, Bloom SR** 2002 The hypothalamic mechanisms of the hypophysiotropic action of ghrelin. Neuroendocrinology 76:316–324
46. **Park S, Peng WD, Frohman LA, Kineman RD** 2005 Expression analysis of hypothalamic and pituitary components of the growth hormone axis in fasted and streptozotocin-treated neuropeptide Y (NPY)-intact ($NPY^{+/+}$) and NPY-knockout ($NPY^{-/-}$) mice. Neuroendocrinology 81:360–371

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EXPERIMENTAL STUDY

Influencing the between-feeding and endocrine responses of plasma ghrelin in healthy dogs

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Abstract

Objectives: Ghrelin has recently been isolated from rat and human stomach as an endogenous ligand for the growth hormone (GH) secretagog receptor. Using beagle dogs, we investigated the distribution of ghrelin in the stomach and its possible role.

Methods: We examined: (i) GH release in response to ghrelin injection (0.5 or 5 μg/kg, i.v.), (ii) gastric localization of ghrelin-immunostained cells, (iii) changes in daily food consumption after ghrelin injection (3, 10, and 20 μg/kg, i.v.), (iv) plasma ghrelin levels under regular, but restricted feeding conditions, and (v) variations in plasma ghrelin levels in relatively lean, normal and obese dogs.

Results: Administration of ghrelin to dogs promptly increased circulating GH concentrations, although this effect was transitory and was maintained for only 20 min. Ghrelin was localized in the stomach fundus and body, but none was detected in either the pylorus or cardia. Administration of ghrelin at a dose of 20 μg/kg increased the daily food intake of beagle dogs. Plasma ghrelin levels peaked just before meal times, and then returned to basal levels. Obese dogs had higher plasma ghrelin levels than did normal and lean dogs.

Conclusions: These results indicate that ghrelin is a potent GH secretagog in dogs. The distribution of ghrelin-immunoreactive cells in the canine stomach resembles that of both the murine and human stomach. Ghrelin participates in the control of feeding behavior and energy homeostasis in dogs and may, therefore, be involved in the development of obesity.

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Introduction

Obesity and anorexia have become serious problems in companion animals such as dogs and cats, as well as in humans. Both obesity and anorexia are characterized by abnormal feeding – in the former case, hyperphagia, and in the latter, hypophagia. Recent molecular biological approaches have led to advances in research on the mechanisms of feeding regulation, such as the discovery of new peptides that regulate feeding behavior. In the near future these peptides may be useful as clinical pharmacological substrates in the treatment of obesity and anorexia. Ghrelin, which is one of these candidate substances, was recently isolated from the rat and human stomach as an endogenous ligand for the growth hormone (GH) secretagog receptor (1). Ghrelin consists of 28 amino acids, including an O-*n*-octanoylated Ser3 residue that is essential for GH release. Central and peripheral injections of ghrelin stimulate GH release in many species such as rats, humans, Shiba goats, dogs and fish (1–6).

Immunohistochemical studies, including those involving *in situ* hybridization, have revealed that in the gastrointestinal tract, ghrelin is produced mainly from the neck to the base of the oxyntic glands, in particular in the X/A-like cells (1, 7) whose physiological role was previously unknown. This distribution of ghrelin in the stomach has been confirmed in humans, cows, pigs and horses (4, 7). The common action and distribution of ghrelin in many species suggests that the structure of ghrelin is conserved among species.

Remarkable work carried out in recent years has shown that ghrelin has various physiological functions such as stimulation of food intake, gastric acid secretion, and gastric motor activity (8–11). It is likely that the appetite-stimulating effect of peripheral ghrelin is due to its action on the afferent vagal nerve (10). On the other hand, its central effect is thought to occur via the secretion of neuropeptide Y and agouti-related peptide from the arcuate nucleus in the hypothalamus (11). These results suggest that ghrelin plays an important role in the regulation of food intake and energy expenditure in rats (9, 11, 12) and humans (13). Ghrelin levels in rats exhibit a diurnal pattern, with bimodal peaks occurring before dark and light periods (14). These two peaks are consistent

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with maximum and minimum volumes of gastric content respectively. It has been suggested that this preprandial rise and postprandial fall in circulating ghrelin levels also occurs in humans (15). Moreover, it has been shown that a transient surge in plasma ghrelin occurs in the prefeeding period in scheduled meal-fed sheep (16). These results indicate strongly that ghrelin secretion may be a trigger for endogenous hunger signals. Since the continuous administration of ghrelin to rodents results in fat deposition and obesity, ghrelin may also be involved in the development of both lean and obese conditions (12, 17).

We have, therefore, examined the relationship between ghrelin and food intake in the beagle dog. Ghrelin secretion should be affected by feeding behavior, but little is known about ghrelin-induced appetite stimulation in dogs. First, we examined whether ghrelin stimulates GH release, and whether ghrelin-immunostained cells are localized in the dog stomach. Secondly, we investigated whether intravenous (i.v.) administration of ghrelin increases food intake in dogs, and determined peripheral ghrelin levels before and after feeding. Finally, we measured and then compared the plasma ghrelin levels in lean, normal and obese dogs.

Materials and methods

Animals and experimental design

All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care. Healthy adult male and female beagle dogs were used for this study. The animals were housed individually in a roofed enclosure in cages (1.0 m × 0.8 m × 0.6 m) at ambient temperature and under natural photoperiod conditions.

In the first experiment, we used 12 healthy male beagle dogs (Kitayama Labs, Yamaguchi, Japan) weighing approximately 6.0–8.3 kg (age range 7–9 months; median 7.8 months), in which we evaluated the effect of ghrelin administration on plasma GH levels. The dogs were divided randomly into three experimental groups: a saline-treated (0.5 ml/kg) control group, and two groups that were administered rat synthetic ghrelin (Peptide Ins. Inc. Osaka, Japan) by i.v. injection, one group at a dose of 0.5 μg/kg and the other at 5 μg/kg. Samples of blood (2 ml) were drawn from conscious animals into tubes containing disodium ethylenediaminetetraacetic acid (EDTA; 1 mg/ml blood) via puncture of the jugular vein using sterile needles and syringes, before injection and 10, 20, 40, 80, and 160 min after injection. Plasma was separated by centrifugation and was kept at −80 °C until determination of GH. Plasma GH was measured by radioimmunoassay (RIA) without extraction.

In the second experiment, the immunohistochemical localization of ghrelin in the dog stomach was examined. The Animal Hospital of Miyazaki University supplied the stomach, esophagus, and duodenum from three adult beagle dogs (age range 8–9.3 months, mean 8.6 months) after euthanasia induced by an overdose injection of pentobarbital.

In the third experiment, we studied the regulation of food intake by ghrelin in six male beagle dogs (6.9–10.2 kg; age range 6–9 months; median 7.4 months). The animals were fed a sufficient quantity of commercial canine laboratory diet (DS-A; Oriental Yeast, Chiba, Japan), and water was available *ad libitum*. Food consumption was measured at 0900 h every 24 h for 10 consecutive days – the pre-experiment period. Each animal then received an i.v. injection of saline and then ghrelin in the order of dosage of 3, 10, and 20 μg/kg ghrelin at 3-day intervals. Food consumption was measured in the 24 h after each injection in each dog. The 3-day interdose interval and the increasing order of dose were imposed to avoid any potential carry-over effects of individual doses.

In the fourth experiment, 12 male beagle dogs (8.6–11.2 kg; age range 7–10 months; median 8.2 months) were randomly subdivided into 2 mealtime groups that were fed a restricted diet at 1000 or 1700 h, regularly for 10 days. On the 10th day, we collected blood samples from each dog at 0730, 0930, 1100, 1430, 1630 and 1930 h, into chilled tubes containing disodium EDTA (1 mg/ml blood) and aprotinin (500 U/ml blood). Plasma was separated by centrifugation, and we added 10% plasma volume of 0.1 mol/l HCl. Samples were stored at −80 °C until determination of ghrelin levels.

In the fifth experiment, 28 adult female beagle dogs (age range 8–13 months; median 9.6 months) were chosen from 290 dogs by 8 keepers, and divided into 4 groups under the randomized block design: relatively lean 'lean', normal 'normal', relatively light obese 'obese L', and relatively heavy obese 'obese H', and each group had mean±S.E.M. body weights of 7.2±0.2 kg ($P < 0.05$ vs normal), 10.1±0.2 kg, 12.4±0.4 kg ($P < 0.05$ vs normal) and 15.9±1.2 kg ($P < 0.05$ vs obese L) respectively. In addition, to compare the gender difference of plasma ghrelin levels, we measured the plasma ghrelin levels in each of four male and female beagle dogs at about 7 weeks of age. We collected blood samples from all of these dogs after they were fasted overnight. Sampling was carried out as described earlier and the plasma was stored at −80 °C until determination of ghrelin concentrations.

RIA of GH and ghrelin

We developed an RIA system for canine GH measurement by using a canine GH RIA kit supplied by NIDDK (National Hormone and Peptide Program, Harbor-UCLA, CA, USA). Iodination was performed by the chloramine-T method. The second antibody was goat anti-monkey IgG serum (HAC-MKA2– 02GTP88), which was supplied by the Biosignal Research Center,

Institute for Molecular and Cellular Regulation, Gunma University, Japan. After completion of the kit protocol, 25 μl plasma were diluted with 175 μl assay buffer for use in the assay. All samples were analyzed in duplicate within one RIA, and the minimum detectable mass was 0.25 ng/ml. The assay procedure was performed according to the method described by Hayashida *et al.* (4). The intra- and interassay coefficients of variation were 6.4% and 3.9% respectively.

Plasma ghrelin levels were measured with a ghrelin (human) RIA kit (Linco Research, St Louis, MO, USA). Without exception, all assay procedures were carried out in accordance with the protocol supplied by the manufacturers. Since this RIA was for use with rat and human plasma, we verified that dog plasma contained suitable matrices. The RIA technique detected rat and human ghrelin with equal accuracy in dog. The intra- and interassay coefficients of variation were 5.1% and 2.4% respectively. The sensitivity of the assay was 10 pg/ml.

Immunohistochemical staining

Tissue blocks of the canine stomach, esophagus and duodenum, taken from a total of three dogs, were rinsed with ice-cold saline and fixed in 4% paraformaldehyde plus 0.2% picric acid in 0.1 mol/l phosphate buffer for 2 days, then incubated for 24 h at 4 °C in 0.1 mol/l phosphate buffer containing 20% sucrose. Samples were then frozen and stored at −80 °C until immunohistochemical staining. Immunostaining for ghrelin in cells in the cardia, fundus, body, and pylorus of the stomach, and in the esophagus and duodenum of each dog was performed as follows. Ten-micrometer-thick sections were prepared with the aid of a cryostat at −20 °C and were then thaw-mounted onto gelatin-coated glass slides and air-dried for 10 min. After pretreatment with 0.3% hydrogen peroxidase for 1 h to inactivate endogenous peroxidases and then incubation with normal goat serum for 1 h to block nonspecific binding, all sections were incubated overnight at 4 °C with anti-ghrelin antiserum. The polyclonal antibody used in this study was produced in rabbits against the N-terminal fragment of rat ghrelin. Details of the preparation and characterization of the antibody have been described by Date *et al.* (7). The rat anti-ghrelin antibody specifically recognizes ghrelin with *n*-octanoylated Ser3, and does not recognize des-acyl ghrelin. The final dilution of the anti-ghrelin antiserum used in the immunohistochemistry was 1:10 000. After being washed with phosphate-buffered saline, the sections were stained by the avidin-biotin-peroxidase complex method (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, CA, USA), with a diaminobenzidine substrate kit (Vector Laboratories) at room temperature.

Data are expressed as the mean±S.E.M. Statistical analyses were carried out by Student's *t*-test, or one-way analysis of variance and Tukey's *post hoc* test, as required.

Results

GH release in response to ghrelin injection

Plasma GH levels increased in a monophasic manner after a single ghrelin injection (Fig. 1). An i.v. administration of 5 μg/kg ghrelin led to a peak GH value of 1545 ng/ml 10 min later, a value that is eight times higher than the preinjection level. The GH release response to ghrelin was significantly increased both 10 and 20 min after injection ($P < 0.05$). GH levels had returned to basal values by 40 min after injection. Administration of a dose of 0.5 μg/kg had no significant effect on plasma GH concentrations.

Distribution of ghrelin-immunostained cells

We detected ghrelin-immunoreactive cells in the oxyntic glands of the fundus and body of the stomach (Fig. 2). In accordance with the physiological role and the conformation changes of acid secretion that occur in the dog stomach, the scatter of ghrelin-positive cells was higher in the fundus than in the body of the stomach. Ghrelin-positive cells were restricted to the gastric mucosa and were scattered from the glandular base to the glandular neck. A comparison with hematoxylin and eosin stained sections of the same tissue revealed that these cells resemble endocrine cells, with unstained nuclei and dense granules in the cytoplasm. No immunostained cells were detected in the esophagus, cardia, pylorus, or duodenum (data not shown). All of these findings were confirmed in tissues taken from all three animals.

Change of daily food consumption after ghrelin injection

Figure 3 shows the effects of three different doses of ghrelin (3, 10, and 20 μg/kg) on food intake measured

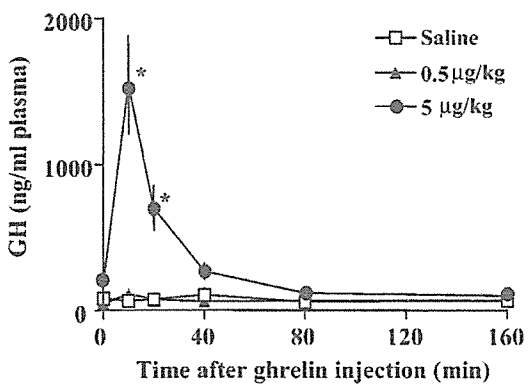

Figure 1 Time course of changes in plasma GH levels after i.v. administration of ghrelin in beagle dogs. Symbols and vertical lines represent the mean value±S.E.M. of four healthy dogs. *$P < 0.05$, significantly different from the saline-treated and 5 μg/kg ghrelin-treated groups at each time point.

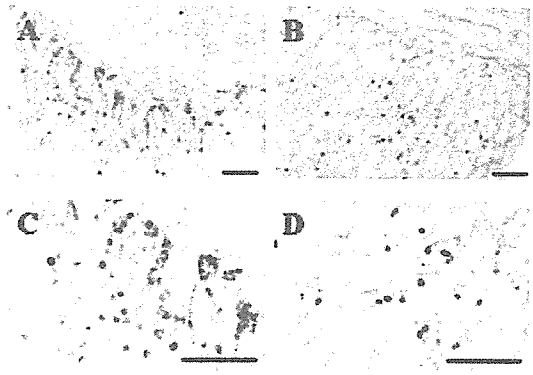

Figure 2 Ghrelin-immunoreactive cells in the stomach of beagle dogs. Immunoreactive cells were detected in the oxyntic glands from the fundus (A and C) and body (B and D) region. Ghrelin-immunoreactive cells were restricted to the gastric mucosa. Many ghrelin cells were scattered from the glandular base to the glandular neck. Each lower panel (C and D) shows a higher magnification of the panel above (A and B respectively). The data shown are representative of all three animals studied. Bars = 100 μm.

24 h after the injections. The average daily food intake was increased significantly by injection of 20 μg/kg ghrelin compared with saline treatment (Fig. 3). Food consumption gradually increased from 75 g/day up to 125 g/day after injections of 3, 10 and 20 μg/kg ghrelin, but only the highest dose resulted in a statistically significant change.

Ghrelin response to feeding

Restricting feeding times to 1000 or 1700 h resulted in the same peak patterns of plasma ghrelin levels. The peaks were observed just before feeding, at 0930 h and at 1630 h (Fig. 4). After feeding, plasma ghrelin levels immediately fell and then remained almost constantly low throughout the day.

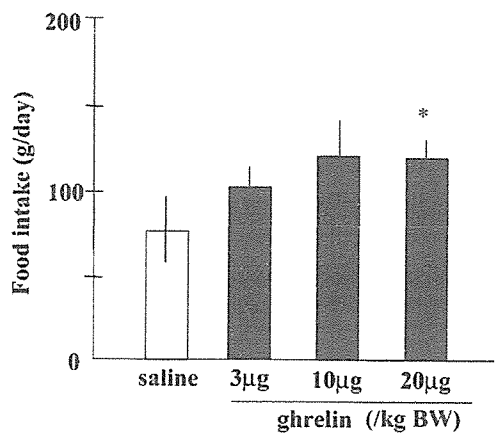

Figure 3 Effect of a single i.v. injection of ghrelin on daily food intake in dogs. Daily food intake increased after treatment with ghrelin. Bars and vertical lines represent the mean values±S.E.M. ($n = 6$). *$P < 0.05$, significantly different from the saline-treated group. BW, body weight.

Variations in plasma ghrelin levels in lean, normal and obese dogs

Relatively higher and lower plasma concentrations of ghrelin were observed in relatively light obese and lean dogs respectively (Fig. 5). Although no significant difference was observed between lean and normal or between relatively light obese and normal, relatively heavy obese dogs showed a significant increase in plasma ghrelin levels in comparison with normal and lean dogs. There was no significant difference in plasma ghrelin levels between normal female and male dogs (female 476±59.8 vs male 497±98.3, mean±S.E.M. $n = 4$).

Discussion

Injection of ghrelin at a dose of 5 μg/kg body weight caused GH secretion to rise to eight times higher than basal levels by 10 min after injection. The time course of changes in GH secretion was close to that recorded in previous reports (5). The cDNA encoding the dog ghrelin precursor has been sequenced (18), and it has been shown that there is only one amino acid sequence difference between rat and canine ghrelin. Our study demonstrates that rat synthetic ghrelin is able adequately to stimulate GH secretion in the dog, and that endogenous ghrelin may play an important role in GH secretion in this animal. The GH response to stimulation with ghrelin

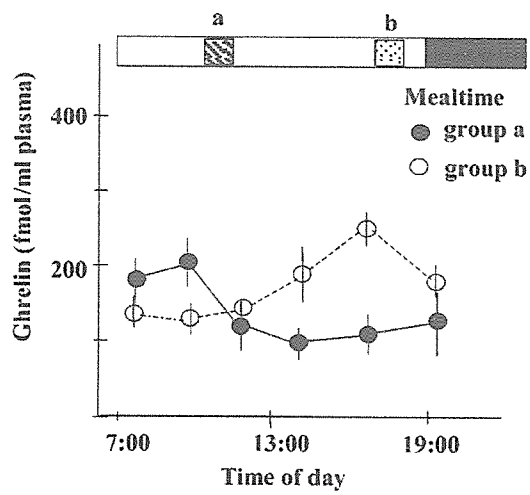

Figure 4 Influence of daily feeding time on plasma ghrelin levels in dogs. The white and black bar represents the light and dark periods. Areas a and b represent the meal time in group a and group b respectively. Plasma ghrelin peaked just before the restricted daily feeding time at 1000 h (group a; closed circles) and 1700 h (group b; open circles) and then decreased immediately after the end of feeding. Symbols and vertical lines represent the mean value±S.E.M. ($n = 6$).

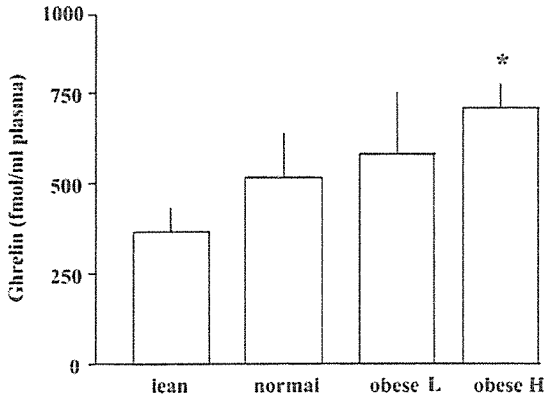

Figure 5 Plasma ghrelin concentrations in lean, normal and obese dogs after overnight food deprivation. Bars and vertical lines represent the mean values±S.E.M. ($n = 7$). Young, female, adult, lean (mean body weight 7.2±0.2 kg), normal (mean body weight 10.1±0.2 kg), light obese (obese L; mean body weight 12.4±0.4 kg) and heavy obese (obese H; mean body weight 15.9±1.2 kg) beagle dogs were studied. *$P < 0.05$, significantly different from the normal.

recorded in this study was considerably lower than that reported previously in healthy dogs in response to a dose of 2 μg/kg (5). Indeed, it has been shown that ghrelin strongly stimulates GH release in humans, whereas in dogs, either ghrelin is not be a very potent stimulator of GH release or else the results reflect methodological differences in the assays used to determine GH levels.

The immunohistochemical studies carried out in the present investigation revealed that ghrelin-immunostained cells were detected in the oxyntic glands of the canine stomach. They are particularly abundant in the fundus and body of the stomach, and are entirely lacking in the pylorus, cardia, esophagus and duodenum. This pattern of distribution correlates well with that of the expression of the ghrelin precursor, as determined by Northern blotting analysis of dog tissues (18). The dog gastric mucosa has been investigated at the electron microscope level by Rindi *et al.* (19), who found that murine and canine ghrelin-immunoreactive cells closely resemble those of the human stomach in their general ultrastructure, including the structural patterns of their compact granules. It seems, therefore, that there is some structural homogeneity of these cells among species, and we can therefore probably expect them to have a functional homogeneity similar to that of endocrine cells among various animals, including the dog.

Daily treatment with ghrelin or a single injection increases food intake and body weight gain in both rats (9, 11, 12) and humans (13). However, little is known about ghrelin-induced appetite stimulation in dogs. We have found that administration of ghrelin induces an increase in food intake, suggesting that endogenously released ghrelin is involved in the control of daily food intake. We observed that plasma ghrelin levels increase just before a meal time, and then rapidly return to the basal level after the end of feeding. In free-feeding rats, ghrelin secretion follows a diurnal pattern, with bimodal peaks occurring before the dark and light periods (14). Both peaks are consistent with the periods just before feeding, in accordance with the circadian rhythms of rats. Moreover, it has been shown that a transient surge of plasma ghrelin occurs in the prefeeding period in scheduled meal-fed sheep (16). When sheep are fed two or four times a day, the ghrelin levels rise just before each feeding (20). Plasma ghrelin levels showed a nocturnal rise that exceeded the meal-associated increase in lean human (21). These results indicate that the ghrelin secretory response to feeding in dogs is similar to that of sheep, rodents and human. A transient surge of ghrelin secretion has also been observed just before pseudofeeding in sheep (16). The regulation of this secretion seems to be complicated by the influences of the gastric contents, gastric acid secretion, and the central nervous system via the vagus nerve (22), since ghrelin signals may be involved centrally and/or peripherally via the gut–brain axis. It would be worthwhile examining further the stimulation of food intake by ghrelin and how ghrelin levels are regulated under conditions of negative and positive energy balance, such as during feeding.

From our results and those of these other studies it seems that ghrelin is involved in the regulation of eating behavior and energy metabolism in both the acute and chronic feeding states (13, 15, 23, 24). Circulating plasma ghrelin levels in healthy dogs decreased significantly after eating. Since this suggests that eating behavior influences the secretion of ghrelin, we examined ghrelin secretion in relatively lean, normal, and obese dogs to determine the ghrelin status in obesity. The obese dogs had higher plasma concentrations of ghrelin than did the lean dogs. These results contrast with those published previously for obese humans, in whom significantly lower ghrelin levels have been detected (25). Several studies have demonstrated that plasma ghrelin levels are inversely correlated with the body mass index (26–29), suggesting that ghrelin levels are downregulated in obesity. On the other hand, our results in dogs are in agreement with the ghrelin secretion patterns recorded in lean and obese Zucker rats, which show, respectively, low and high ghrelin levels in plasma (30). The Zucker fa/fa rat is a widely used model of obesity that is characterized by massive obesity, overeating, and alterations of growth hormone metabolism. Whether or not obesity can be linked to plasma ghrelin levels needs to be clarified by further characterization of the pathophysiology of obesity in dogs. Recently, abnormal circulating ghrelin levels have been reported in patients with anorexia nervosa (31) and Prader–Willi syndrome (26). Studies such as these will help us to understand whether ghrelin plays a role in the pathogenesis of simple or secondary obesity in humans, and the use of dogs with obesity-associated disease could prove an interesting approach.

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References

1 Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H & Kangawa K. Ghrelin is a growth hormone-releasing acylated peptide from stomach. *Nature* 1999 **402** 656–660.
2 Date Y, Murakami N, Kojima M, Kuroiwa T, Matsukura S, Kangawa K & Nakazato M. Central effects of a novel acylated peptide, ghrelin, on growth hormone release in rats. *Biochemical and Biophysical Research Communications* 2000 **275** 477–480.
3 Seoane LM, Tovar S, Baldelli R, Arvat E, Ghigo E, Casanueva FF & Dieguez C. Ghrelin elicits a marked stimulatory effect on GH secretion in freely moving rats. *European Journal of Endocrinology* 2000 **143** R7–R9.
4 Hayashida T, Murakami K, Mogi K, Nishihara M, Nakazato M, Mondal MS, Horii Y, Kojima M, Kangawa K & Murakami N. Ghrelin in domestic animals: distribution in stomach and its possible role. *Domestic Animal Endocrinology* 2001 **21** 17–24.
5 Bhatti SFM, De Vliegher SP, Van Ham L & Kooistra HS. Effects of growth hormone-releasing peptides in healthy dogs and in dogs with pituitary-dependent hyperadrenocorticism. *Molecular and Cellular Endocrinology* 2002 **197** 97–103.
6 Kaiya H, Kojima M, Hosoda H, Moriyama S, Takahashi A, Kawauchi H & Kangawa K. Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout. *Endocrinology* 2003 **144** 5215–5226.
7 Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K & Nakazato M. 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* 2000 **141** 4255–4261.
8 Masuda Y, Tanaka T, Inomata N, Ohnuma N, Tanaka S, Itoh Z, Hosoda H, Kojima M & Kangawa K. Ghrelin stimulates gastric acid secretion and motility in rats. *Biochemical and Biophysical Research Communications* 2000 **276** 905–908.
9 Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CL, Taheri S, Kennedy AR, Roberts GH, Morgan DGA, Ghatei MA & Bloom SR. The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. *Endocrinology* 2000 **141** 4325–4328.
10 Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K & Matsukura S. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. *Biochemical and Biophysical Research Communications* 2001 **280** 904–907.
11 Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K & Matsukura S. A role for ghrelin in the central regulation of feeding. *Nature* 2001 **409** 194–198.
12 Tschop M, Smiley DL & Heiman ML. Ghrelin induces adiposity in rodents. *Nature* 2000 **407** 908–913.
13 Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA & Bloom SR. Ghrelin enhances appetite and increases food intake in humans. *Journal of Clinical Endocrinology and Metabolism* 2001 **86** 5992–5995.
14 Murakami N, Hayashida T, Kuroiwa T, Nakahara K, Ida T, Mondal MS, Nakazato M, Kojima M & Kangawa K. Role for central ghrelin in food intake and secretion profile of stomach ghrelin in rats. *Journal of Endocrinology* 2002 **174** 283–288.
15 Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE & Weigle DS. A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. *Diabetes* 2001 **50** 1714–1719.
16 Sugino T, Hasegawa Y, Kikkawa Y, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K & Terashima Y. A transient ghrelin surge occurs just before feeding in a scheduled meal-fed sheep. *Biochemical and Biophysical Research Communications* 2002 **295** 255–260.
17 Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA & Bloom SR. Ghrelin causes hyperphagia and obesity in rats. *Diabetes* 2001 **50** 2540–2547.
18 Tomasetto C, Wendling C, Rio MC & Poitras P. Identification of cDNA encoding motilin related peptide/ghrelin precursor from dog fundus. *Peptides* 2001 **22** 2055–2059.
19 Rindi G, Necchi V, Savio A, Torsello A, Zoli M, Locatelli V, Raimondo F, Cocchi D & Solcia E. Characterisation of gastric ghrelin cells in man and other mammals: studies in adult and fetal tissues. *Histochemistry and Cell Biology* 2002 **117** 511–519.
20 Sugino T, Yamaura J, Yamagishi M, Ogura A, Hayashi R, Kurose Y, Kojima M, Kangawa K, Hasegawa Y & Terashima Y. A transient surge of ghrelin secretion before feeding is modified by different feeding regimens in sheep. *Biochemical and Biophysical Research Communications* 2002 **298** 785–788.
21 Yildiz BO, Suchard MA, Wong ML, McCann SM & Licinio J. Alterations in the dynamics of circulating ghrelin, adiponectin and leptin in human obesity. *PNAS* 2004 **101** 10434–10439.
22 Sugino T, Yamaura J, Yamagishi M, Kurose Y, Kojima M, Kangawa K, Hasegawa Y & Terashima Y. Involvement of cholinergic neurons in the regulation of the ghrelin secretory response to feeding in sheep. *Biochemical and Biophysical Research Communications* 2003 **304** 308–312.
23 Tschop M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R & Folwaczny C. Post-prandial decrease of circulating human ghrelin levels. *Journal of Endocrinological Investigation* 2001 **24** RC19–RC21.
24 Tang-Christensen M, Vrang N, Ortmann S, Bidlingmaier M, Horvath TL & Tschop M. Central administration of ghrelin and agouti-related protein (83–132) increase food intake and decreases spontaneous locomotor activity in rats. *Endocrinology* 2004 **145** 4645–4652.
25 Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E & Heiman ML. Circulating ghrelin levels are decreased in human obesity. *Diabetes* 2001 **50** 707–709.
26 Cummings DE, Clement K, Purnell JQ, Vaisse C, Foster KE, Frayo RS, Schwartz MW, Basdevant A & Weigle DS. Elevated plasma ghrelin levels in Prader–Willi syndrome. *Nature Medicine* 2002 **8** 643–644.
27 English PJ, Ghatei MA, Malik IA, Bloom SR & Wilding JPH. Food fails to suppress ghrelin levels in obese humans. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 2984–2987.
28 Hansen TK, Dall R, Hosoda H, Kojima M, Kangawa K, Christiansen JS & Jorgensen JOL. Weight loss increases circulating levels of ghrelin in human obesity. *Clinical Endocrinology* 2002 **56** 203–206.
29 Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe SI, Hosoda H, Kangawa K & Matsukura S. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. *Journal of Clinical Endocrinology and Metabolism* 2002 **87** 240–244.
30 Beck B, Richy S & Stricker-Krongrad A. Ghrelin and body weight regulation in the obese Zucker rat in relation to feeding state and dark/light cycle. *Experimental Biology and Medicine* 2003 **228** 1124–1131.
31 Tanaka M, Naruo T, Yasuhara D, Tatebe Y, Nagai N, Shiiya T, Nakazato M, Matsukura S & Nozoe S-I. Fasting plasma ghrelin levels in subtypes of anorexia nervosa. *Psychoneuroendocrinology* 2003 **28** 829–835.

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Different effects of peripheral and central ghrelin on regulation of food intake in the Japanese quail

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Abstract

When rat or human ghrelin is administered to chickens or rats it stimulates the release of growth hormone, however, its effects on food intake differ between the two species. To investigate this discrepancy, we measured plasma ghrelin concentrations before and after food intake, and determined the effects of central (intracerebroventricular, icv) and peripheral (intraperitoneal, ip) injections of various ghrelin doses on food intake and body temperature in the Japanese quail. In control quails, plasma ghrelin levels were significantly increased in the fasting state; subsequent feeding produced a reduction, suggesting that ghrelin may act as an orexigenic signal in Japanese quails as well as mammals. Food intake was stimulated by ip, but not icv, injections of small doses of ghrelin, whereas both ip and icv injections of larger doses inhibited feeding. A large dose of ghrelin also increased body temperature. These results suggest that an increase in peripheral ghrelin may act as a hunger signal to induce food intake through stimulation of the afferent vagal nerve in the Japanese quail, as in rats, whereas central ghrelin may inhibit feeding in different ways in the two species.

Keywords: Food intake; Body temperature; Ghrelin; Japanese quail

1. Introduction

Ghrelin, a novel 28-amino acid peptide, was originally isolated from the stomachs of rats and humans as an endogenous ligand for the growth hormone (GH) secretagogue receptor (GHS-R) (Kojima et al., 1999). It has a unique structure, containing a Ser^3 residue that is modified by *n*-octanoic acid. This octanoyl modification is essential for receptor binding and the subsequent expression of biological activity (Kojima et al., 1999). Ghrelin has also been isolated from chickens. This form is composed of 26 amino acids, has an octanoylated Ser^3 and shows 54% total sequence identity and 100% N-terminal-region identity [Gly1-Pro7] with rat and human ghrelin (Kaiya et al., 2002). In addition, two isoforms of the chicken GHS-R (cGHS-R1a and cGHS-R1aV) have been generated by alternative splicing of a primary transcript. cGHS-R1a shows strong amino acid sequence identity (68%) with the corresponding parts of the mammalian GHS-R1a cDNA product, while cGHS-R1aV lacks the transmembrane-6 domain due to a 48-bp deletion (Tanaka et al., 2003).

Ghrelin stimulates the release of GH from the pituitary gland both in vivo and in vitro ; the *n*-octanoyl modification is essential for this activity (Kojima et al., 1999). In addition, central and peripheral injection of ghrelin stimulates food intake in rats (Nakazato et al., 2001; Tschop et al., 2000). The central effect is mediated by neuropeptide Y and agouti-related peptide secretion from the arcuate nucleus in the hypothalamus (Nakazato et al., 2001). On the other hand, the appetite-stimulating

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activity of peripheral ghrelin is produced via stimulation of the afferent vagal nerve (Date et al., 2002).

Although accumulating evidence suggests that ghrelin plays many important roles in various physiological functions in rats and humans, for example, fetal development (Hayashida et al., 2002), gastric acid secretion (Date et al., 2001) and improvements in cardiovascular function (Nagaya et al., 2001), little is known about ghrelin in non-mammalian vertebrates. In the chicken, administration of either human or chicken ghrelin stimulates GH release (Ahmed and Harvey, 2002; Kaiya et al., 2002) but strongly inhibits feeding (Furuse et al., 2001; Saito et al., 2002a). In particular, central (intracerebroventricular, icv) injection of chicken ghrelin suppresses food intake in neonatal chicks (Saito et al., 2002a), the opposite effect to that seen in rats.

To investigate these discrepant effects of ghrelin on feeding, we measured plasma ghrelin concentrations before and after food intake, and determined the effects of both icv and peripheral (intraperitoneal, ip) injection of ghrelin on food intake, in the Japanese quail. If ghrelin is indeed orexigenic in this species, one would expect plasma ghrelin levels to be increased by fasting and decreased by subsequent feeding, as is the case in mammals. Although the regulation of appetite by ghrelin has previously been examined in 1-day-old chicks (Furuse et al., 2001; Saito et al., 2002a), we decided to use adult Japanese quails. This was mainly because the growth curve of young birds is steep, so body weight gains and food intakes vary widely from day to day, whereas adult birds have ceased growing, and therefore body weight gains and food intakes are not subject to such variability. Furthermore, it is technically easier to implant indwelling icv cannulae into adult birds.

In addition to its effects on feeding, it has been reported that central injection of ghrelin into rats transiently reduced the core body temperature (Lawrence et al., 2002) and the temperature of brown adipose tissue (Yasuda et al., 2003). Therefore, it is highly probable that ghrelin regulates adiposity by influencing both energy intake and energy expenditure. Although neonatal chicks have been reported to become hyperactive within 30 min of an icv injection of ghrelin (Saito et al., 2002b), and to exhibit sleep-like behavior thereafter (Tachibana et al., 2001), to the best of our knowledge there are no published reports concerning thermoregulation by ghrelin in adult avian species. Therefore, we also measured the effects of peripheral and central ghrelin on body temperature in Japanese quails.

2. Materials and methods

2.1. Animals

Adult male Japanese quail (*Coturnix coturnix japonica*) were housed in individual net cages (W: 14 × L: 26 × H: 17 cm) in a room with a 12-h light (300 lx) /12-h dark (dim light, 25 lx) period (lights on at 07:00 h) and a temperature of 28 ± 1 °C, and were given free access to food and water. Before the feeding experiment, the birds were weighed and assigned to an experimental group (six birds in each group) based on their body weight. The average body weight (110–120 g) in each group was kept as uniform as possible.

To examine the orexigenic or anorexic effect of ghrelin, rat ghrelin (Peptide Institute, Osaka, Japan) or 0.9% saline (vehicle control) was administered ip or icv at either 07:00 h (i.e., during the birds' active phase) or 19:00 h (i.e., during the birds' resting phase). The doses injected ip were 0.5 (1.66 μg), 1 (3.31 μg), and 3 (9.94 μg) nmol/200 μl saline, while the doses injected icv were 0.05 (0.16 μg), 0.5 (1.66 μg) and 1 (3.31 μg) nmol/10 μl saline.

These experiments were performed in duplicate. All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

2.2. Surgical procedures

For implantation of the icv cannula, each bird was anesthetized with 5% sodium pentobarbital (1.4 μl/g body weight) and placed in a stereotaxic frame. A stainless steel guide cannula (outer diameter 550 μm, length 14 mm) was stereotaxically implanted into the third cerebral ventricle using a modification of a previously reported method (Bayle et al., 1974). The coordinates were 5 mm anterior to the interaural axis and 6.5 mm below the dura at the midline. One stainless steel anchoring screw was fixed to the skull, and the guide cannula was secured in place with acrylic dental cement. The birds were returned to their individual cages and allowed to recover for at least 4 days. They were acclimatized to handling every day before the start of the experiments.

The icv injections were administered through the implanted guide cannulae without anesthesia or restraining of the birds.

At the end of the experiments, proper placement of the cannulae was verified by administering Evans Blue dye (10 μl), followed by sacrifice and brain sectioning (20 μm intervals). Data for birds lacking dye in the third ventricle were excluded from the analysis.

2.3. Measurement of food intake

Food consumption was determined 2, 4, and 12 h after administration of ghrelin or saline by measuring the disappearance of food from the pre-weighed feeder. Any spillage was also collected and weighed.

2.4. Measurement of body temperature

The quails' body temperature was measured 0 (before injection), 5, 10, 20, 40, 60, and 120 min after administering

ghrelin or saline at the doses stated above ($n = 6$ in each group) at 10:00 h, using a previously reported method (Bayle et al., 1974). Briefly, the temperature was measured electronically with a small sensor (measurable range: 25–50 °C; measurement error: 0.05 °C) connected to a line (outer diameter 0.7 mm; length 45 cm) which is connected to the monitor body. The sensor tip was inserted into the cloaca, and part of the line was fixed to the bird's body, with the digital signal transferred to the monitor body.

2.5. Measurement of plasma ghrelin

Plasma acyl-ghrelin concentrations were determined by radioimmunoassay using a specific antibody that recognizes the N-terminal region, including the octanoylated Ser^3 residue (Hosoda et al., 2000). Sixteen Japanese quails were fasted for 24 h (from 10:00 h): eight were sacrificed by decapitation after 24 h of fasting; the remaining eight were supplied with food after the fast and then sacrificed 3 h later. Untreated, free-feeding quails were sacrificed at 10:00 and 13:00 h as controls.

Following decapitation, blood from the neck was drawn into chilled polypropylene tubes containing a proteinase inhibitor, aprotinin (500 kIU/ml; Sigma–Aldrich, St. Louis, USA), and 2Na-EDTA (2 mg/ml), then centrifuged immediately before adding 0.1 N HCl (10% of the plasma volume). The samples were stored at −80 °C until required.

For determination of ghrelin levels, the plasma was diluted in 0.9% saline and applied to a Sep-Pak C-18 cartridge (Waters, Milford, MA, USA) that had been pre-equilibrated with 0.9% saline. The cartridge was washed with saline and 10% CH_3CN solution containing 0.1% trifluoroacetic acid (TFA). The adsorbed peptides were eluted with 60% CH_3CN solution containing 0.1% TFA, lyophilized, and radioimmunoassayed. The intra- and interassay coefficients of variation were 6.7 and 3.2%, respectively.

2.6. Statistical analysis

All results are expressed as means ± SEM. Data were analyzed by analysis of variance and the post hoc Fisher's test.

3. Results

3.1. Food intake

In the ip administration group, 3 nmol ghrelin significantly ($P < 0.05$) decreased food intake during both the light (Fig. 1A) and dark periods (Fig. 1B) compared with the saline-control group. Conversely, 0.5 and 1 nmol ghrelin stimulated feeding (Figs. 1A and B). This orexigenic action of small doses of ghrelin was more evident during the dark period (Fig. 1B) than the light period (Fig. 1A). During the first 2 h after the ip injection, the 1-nmol dose of ghrelin stimulated food intake more potently during the dark period (Fig. 1B) whereas the 3-nmol dose inhibited feeding more markedly during the light period (Fig. 1A).

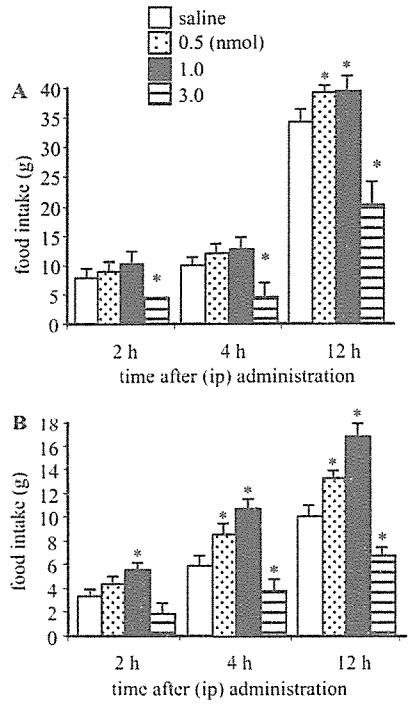

Fig. 1. Effect of intraperitoneal (ip) administration of ghrelin on food intake in the Japanese quail. Saline (vehicle control), 0.5, 1 or 3 nmol ghrelin were injected ip at 07:00 h (A) or 19:00 h (B). Each bar and vertical line represents the mean ± SEM ($n = 12$). *Significantly different from the saline-treated group: $P < 0.05$.

No significant increase in food intake was observed after icv injections of ghrelin (Figs. 2A and B). Indeed, 0.5 and 1 nmol ghrelin inhibited food intake during the both the light and dark periods (Figs. 2A and B). No significant difference was observed between the saline group and the 0.05 nmol ghrelin-treated group (Figs. 2A and B).

The effects of smaller doses of ghrelin (0.005 nmol icv and 0.01 nmol ip) were examined, but these doses effected no significant change in food intake ($n = 6$; data not shown).

3.2. Body temperature

Both ip and icv injections of ghrelin increased body temperature in a dose-dependent manner (Fig. 3). At 3 nmol ip, ghrelin significantly ($P < 0.05$) increased body temperature 5, 10, and 20 min after treatment. However, no such change was observed with 0.5 and 1.0 nmol ip (Fig. 3A). Similarly, 1.0 and 0.5 nmol ghrelin icv pro-

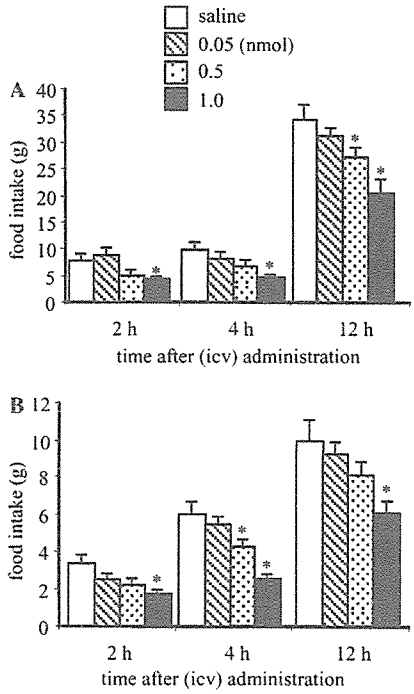

Fig. 2. Effect of intracerebroventricular (icv) administration of ghrelin on food intake in the Japanese quail. Saline (vehicle control), 0.05, 0.5 or 1 nmol ghrelin were injected icv at 07:00 h (A) or 19:00 h (B). Each bar and vertical line represents the mean ± SEM ($n = 12$). *Significantly different from the saline-treated group: $P < 0.05$.

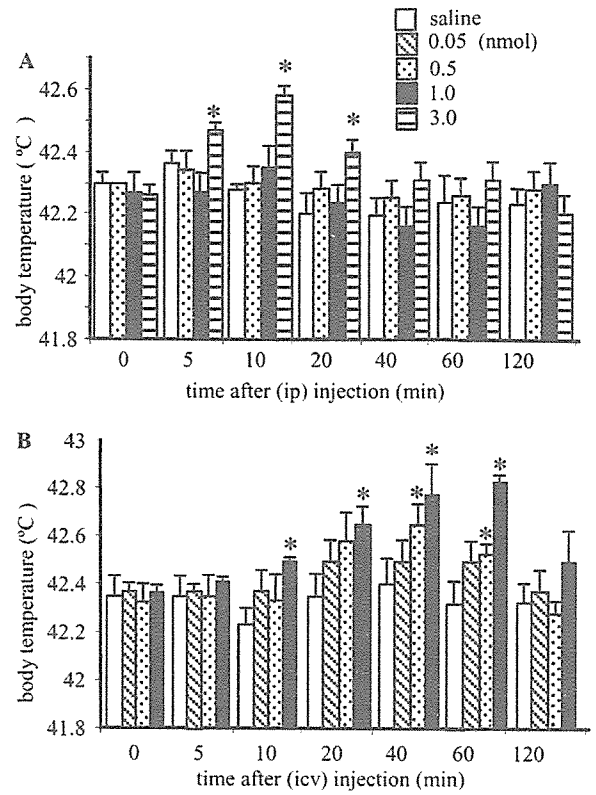

Fig. 3. Effect of ip or icv administration of ghrelin on body temperature in the Japanese quail. Saline (vehicle control), 0.5, 1 or 3 nmol of ghrelin were injected ip (A), and saline (vehicle control), 0.05, 0.5 or 1 nmol ghrelin were injected icv (B) at 10:00 h. Each bar and vertical line represents the mean ± SEM ($n = 6$). *Significantly different from the saline-treated group: $P < 0.05$.

duced a significant ($P < 0.05$) increase in body temperature 10–60 and 40–60 min after injection, respectively (Fig. 3B). Although 0.05 nmol ghrelin also caused an increase in body temperature, the change was not significantly different from that seen with saline alone (i.e., control birds).

3.3. Plasma ghrelin

Plasma ghrelin levels increased about 5-fold in quails that had been fasted for 24 h compared with the free-feeding birds. The levels were decreased 3 h after refeeding (Fig. 4). Ghrelin levels differed significantly between the two groups; levels in both groups were also significantly different from those in the free-feeding quail.

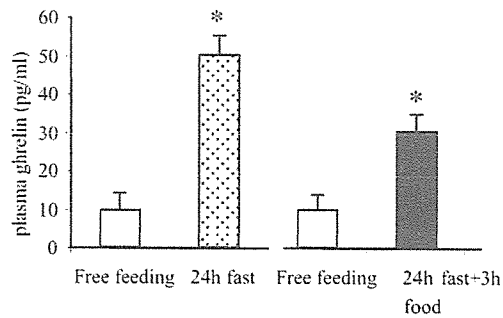

Fig. 4. Plasma ghrelin levels in free-feeding and 24-h fasted groups of Japanese quail. Sixteen Japanese quails were fasted for 24 h (from 10:00 h), after which eight were sacrificed (dotted bars). The remaining eight birds were supplied with food after the fast, then sacrificed 3 h later (black bars). Free-feeding animals were sacrificed at either 10:00 h (left-hand white bar) or 13:00 h (right-hand white bar) as controls. Each bar and vertical line represents the mean ± SEM ($n = 8$). *Significantly different from the saline-treated group: $P < 0.05$.

4. Discussion

The finding that icv injection of rat ghrelin inhibits food intake in Japanese quails is in agreement with previous observations made in chickens (Furuse et al., 2001; Saito et al., 2002a). Since the amino acid sequence of rat ghrelin is quite different from that of the chicken hormone, except for the seven N-terminal residues (Kaiya et al., 2002), rat ghrelin could possibly act as an antagonist of the chicken GHS-R, thus inhibiting the effect of endogenous chicken ghrelin. However, this inhibition might not be due to an antagonistic effect of rat ghrelin

at the chick ghrelin receptor, since icv injections of chick ghrelin also inhibit food intake. Moreover, human or rat ghrelin can also cause an increase in GH secretion in the chicken. Therefore, avian ghrelin may act to inhibit food intake. However, if this is true, one would expect the pattern of plasma ghrelin secretion before and after feeding to be the reverse of that seen in rats. In the present study, contrary to our expectations, plasma ghrelin levels were increased 5-fold compared with those in free-feeding birds following a 24-h of fast. In addition, allowing the birds to feed for 3 h after the 24-h fast produced a decrease in their plasma ghrelin levels. These results suggest that increased peripheral ghrelin levels act as a hunger signal rather than a satiety signal. The present study also demonstrated that ip administration of small doses (0.5 or 1.0 nmol) of ghrelin stimulates food intake; conversely, large doses (3.0 nmol) inhibit it (Fig. 1). However, icv ghrelin consistently decreased feeding. In agreement with this finding, previous studies have shown that neither rat nor chicken ghrelin has any transient stimulatory effect on food intake in neonatal chicks when administered centrally (Furuse et al., 2001; Saito et al., 2002a).

We do not know the reason for the discrepancy between the ip and icv effects of ghrelin on feeding regulation, or that between the effects of small and large ip doses of the peptide. However, are at least two possible explanations. First, the mechanisms underlying the central and peripheral effects of ghrelin on feeding may differ, perhaps being anorexic and orexigenic, respectively. It has been shown that ghrelin can pass through the blood–brain barrier in rats (Banks et al., 2002), so it may be that when the largest dose of ghrelin was injected ip in the present study, some of it reached central (arcuate nucleus) GHS-Rs through the blood–brain barrier, thereby inhibiting food intake. An analogous situation has been observed with neuropeptide YY (PYY) in rats; peripheral injections of PYY inhibited food intake, whereas icv injections increased it, suggesting that PYY acts both orexigenically and anorexically depending upon whether it is located centrally or peripherally (unpublished data). The existence of peripheral sensors for ghrelin is suggested by the finding that GHS-R mRNA is distributed in various peripheral organs, such as the stomach and intestine, in the adult chicken (Tanaka et al., 2003). Peripheral ghrelin acts via gastric vagal afferent nerves (Date et al., 2002). The ghrelin signals from peripheral GHS-Rs may be converted to neurotransmitter-mediated signals in the nucleus of the solitary tract. This neurotransmitter could have the opposite effect to direct release of ghrelin in the hypothalamus. Second, ghrelin may act to down-regulate the GHS-R. GHS-Rs may become saturated following the administration (either ip or icv) of a large dose of ghrelin (Figs. 1 and 2). This might in turn cause a down-regulation of feeding stimuli, thus inhibiting food intake. The stimulatory effect of ghrelin on food intake was more marked during the dark period (resting phase) (Fig. 1B) than the light period (active phase) (Fig. 1A). The inverse was found following ip injections of 1 nmol ghrelin, which inhibited food intake over the 12-h light period but stimulated it during the 12-h dark period. This difference may be attributable to differences in the number of unsaturated receptors present when hungry (i.e., during the dark period) and sated (i.e., during the light period). It appears that relatively low levels of this peptide are sufficient to stimulate food intake in the Japanese quail, but further studies are required to confirm this.

Ahmed and Harvey (2002) found that ghrelin immunoreactivity was present in the chicken hypothalamus, although not, as in rats, in the arcuate (infundibular) nucleus. Discrete parvocellular cells and neuronal fibers with ghrelin immunoreactivity were present in the anterior medial hypothalamus. This restriction of ghrelin immunoreactivity to the hypothalamus of chicks suggests that it evolved phylogenetically as a neuropeptide rather than as a gastrointestinal hormone. Since the parvocellular cells in the hypothalamus are thought to connect with portal blood vessels in the median eminence, the presence of ghrelin in the anterior medial hypothalamus suggests that it may be a hypophysiotropic releasing factor that stimulates pituitary GH release after secretion into the hypothalamo-hypophyseal circulation (Ramesh et al., 2000).

Ghrelin injected either peripherally (Fig. 3A) or centrally (Fig. 3B) caused a transient, dose-dependent increase in body temperature, suggesting that it might affect energy expenditure in birds. After ip or icv administration of large doses of ghrelin, changes in feeding may produce a transient change in body temperature. The mechanism underlying this transient thermal change is unclear, but may involve changes in metabolism because the regulatory center for body temperature is located close to the feeding center in the hypothalamus. Daily 2-h food restriction in rats has been found to induce anticipatory increases in body temperature (Boulos and Terman, 1980), suggesting that the regulatory mechanisms for temperature and feeding may be linked. Further studies are required to confirm this possibility.

Earlier studies that reported the inhibitory effect of ghrelin on food intake in domestic chickens used the lateral ventricle as the route for central injections (Furuse et al., 2001; Saito et al., 2002a) whereas in our study we used the third ventricle. We also observed an inhibitory effect of ghrelin on food intake. Therefore, although the route of injection was different from that used in the previous studies in chickens, the results for both chickens and Japanese quails were the same, and both were the opposite to those reported for mammals, suggesting that the site of injection does not influence the differences between birds and mammals.

In conclusion, the results of the present study suggest that an increase in peripheral ghrelin acts as a hunger signal to stimulate food intake in the Japanese quail.

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References

Ahmed, S., Harvey, S., 2002. Ghrelin: A hypothalamic GH-releasing factor in domestic fowl (*Gallus domesticus*). J. Endocrinol. 172, 117–125.

Banks, W.A., Tschop, M., Robinson, S.M., Heiman, M.L., 2002. Extent and direction of ghrelin transport across the blood–brain barrier is determined by its unique primary structure. J. Pharmacol. Exp. Ther. 302, 222–277.

Bayle, J.D., Ramade, F., Oliver, J., 1974. Stereotaxic topography of the brain of the quail (*Coturnix coturnix japonica*). J. Physiol. (Paris) 68, 219–241.

Boulos, Z., Terman, M., 1980. Food availability and daily biological rhythms. Neurosci. Biobehav. Rev. 4, 119–131.

Date, Y., Murakami, N., Toshinai, K., Matsukura, S., Niijima, A., Matsuo, H., Kangawa, K., Nakazato, M., 2002. The role of the gastric afferent vagal nerve in ghrelin-induced feeding and growth hormone secretion in rats. Gastroenterology 123, 1120–1128.

Date, Y., Nakazato, M., Murakami, N., Kojima, M., Kangawa, K., Matsukura, S., 2001. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem. Biophys. Res. Commun. 280, 904–907.

Furuse, M., Tachibana, T., Ohgushi, A., Ando, R., Yoshimatsu, T., Denbow, D.M., 2001. Intracerebroventricular injection of ghrelin and growth hormone releasing factor inhibits food intake in neonatal chicks. Neurosci. Lett. 301, 123–126.

Hayashida, T., Nakahara, K., Mondal, M.S., Date, Y., Nakazato, M., Kojima, M., Kangawa, K., Murakami, N., 2002. Ghrelin in neonatal rats: Distribution in stomach and its possible role. J. Endocrinol. 173, 239–245.

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.

Kaiya, H., Van Der Geyten, S., Kojima, M., Hosoda, H., Kitajima, Y., Matsumoto, M., Geelissen, S., Darras, V.M., Kangawa, K., 2002. Chicken ghrelin: Purification, cDNA cloning, and biological activity. Endocrinology 143, 3454–3463.

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.

Lawrence, C.B., Snape, A.C., Baudoin, F.M., Luckman, S.M., 2002. Acute central ghrelin and GH secretagogues induce feeding and activate brain appetite centers. Endocrinology 143, 155–162.

Nagaya, N., Uematsu, M., Kojima, M., Ikeda, Y., Yoshihara, F., Shimizu, W., Hosoda, H., Hirota, Y., Ishida, H., Mori, H., Kangawa, K., 2001. Chronic administration of ghrelin improves left ventricular dysfunction and attenuates development of cardiac cachexia in rats with heart failure. Circulation 104, 1430–1435.

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.

Ramesh, R., Kuenzel, W.J., Buntin, J.D., Proudman, J.A., 2000. Identification of growth-hormone- and prolactin-containing neurons within the avian brain. Cell Tissue Res. 299, 371–383.

Saito, E.-S., Kaiya, H., Takagi, T., Yamasaki, I., Denbow, D.M., Kangawa, K., Furuse, M., 2002a. Chicken ghrelin and growth hormone-releasing peptide-2 inhibit food intake of neonatal chicks. Eur. J. Pharmacol. 453, 75–79.

Saito, E.-S., Takagi, T., Nakanishi, T., Sashihara, K., Furuse, M., 2002b. Ghrelin activates behavior of neonatal chicks in a short period of post-intracerebroventricular injection. J. Appl. Anim. Res. 22, 33–41.

Tachibana, T., Ohgushi, A., Furuse, M., 2001. Intracerebroventricular injection of ghrelin induces sleep-like behavior in neonatal chicks. J. Poult. Sci. 38, 358–363.

Tanaka, M., Miyazaki, T., Yamamoto, I., Nakai, N., Ohta, Y., Tsushima, N., Wakita, M., Shimada, K., 2003. Molecular characterization of chicken growth hormone secretagogue receptor gene. Gen. Comp. Endocrinol. 134, 198–202.

Tschop, M., Smiley, D.L., Heiman, M.L., 2000. Ghrelin induces adiposity in rodents. Nature 407, 908–913.

Yasuda, T., Masaki, T., Kakuma, T., Yoshimatsu, H., 2003. Centrally administered ghrelin suppresses sympathetic nerve activity in brown adipose tissue of rats. Neurosci. Lett. 349, 75–78.

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Maternal Ghrelin Plays an Important Role in Rat Fetal Development during Pregnancy

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Ghrelin, an acylated peptide serving as an endogenous ligand for GH secretagogue receptor (GHS-R), was originally isolated from rat and human stomach. In this study, we report the critical role of maternal ghrelin in fetal development. High levels of ghrelin receptor (GHS-R) mRNA were detected in various peripheral fetal tissues beginning at embryonic d 14 and lasting until birth. Fetal GHS-R expression was also confirmed in fetal tissues by immunohistochemistry. Autoradiography revealed that both des-acyl ghrelin and acyl ghrelin bind to fetal tissues. Chronic treatment of mothers with ghrelin resulted in a significant increase in birth weight in comparison to newborns from saline-treated mothers. Even when maternal food intake after ghrelin treatment was restricted through paired feeding, significant stimulation of fetal development still occurred. Conversely, active immunization of mothers against ghrelin decreased fetal body weight during pregnancy. A single ghrelin injection into the mother increased circulating ghrelin levels in the fetus within 5 min of injection, suggesting that maternal ghrelin transits easily to the fetal circulation. High levels of des-acyl ghrelin were detected in fetal blood and amniotic fluid. Both acylated and des-acyl ghrelin increased [^{3}H]thymidine and 5-bromo-2′-deoxyuridine incorporation of cultured fetal skin cells in a dose-dependent manner, and calcium-imaging analysis revealed that acyl and des-acyl ghrelin increased the Ca^{2+} influx in discrete cultured fetal skin cells, respectively. These results indicate that maternal ghrelin regulates fetal development during the late stages of pregnancy. (*Endocrinology* 147: 1333–1342, 2006)

GHRELIN, RECENTLY purified from rat and human stomachs as an endogenous ligand for the GH secretagogue receptor (GHS-R), is a 28-amino acid peptide with an *n*-octanoylation modification at Ser^3 (1). This octanoylation is important for the stimulation of GH secretion from the pituitary gland (1). Although cells immunostained for ghrelin are distributed widely in the stomach, hypothalamus, pituitary gland, liver, kidney, pancreas, and placenta, the main source of circulating ghrelin is considered to be the gastrointestinal tract (2–6). Both acylated and des-acyl ghrelin are observed in the peripheral circulation, the levels of des-acyl ghrelin being higher (7, 8). Several studies on the physiological function of ghrelin have demonstrated that, in addition to stimulating GH secretion, ghrelin also stimulates food intake and body weight gain independent of GH secretion (8–13). It is likely that the appetite-stimulating effect of peripheral ghrelin is due to action via the afferent vagal nerve (14). In contrast, the central effect is thought to be via neuropeptide Y and agouti-related peptide secretion from the arcuate nucleus in the hypothalamus (11, 15). Administration of ghrelin continuously to rodents resulted in fat deposition and obesity (9). These effects of ghrelin on appetite and fat deposition are counteracted by leptin (11). These results imply that ghrelin may play an important role in the regulation of food intake and energy expenditure.

The ghrelin receptor GHS-R, highly conserved from teleost fish to humans, is expressed widely in both central and peripheral organs, including the brain, pituitary gland, and pancreas (16–19). The broad distribution of GHS-R suggests that ghrelin may play important roles in addition to those mentioned above. It has been also demonstrated that ghrelin might be involved in stomach motility (10), gastric acid secretion (20), insulin and gastrin release (21), the cardiovascular system, and stress reactions (12). In addition, we demonstrated previously that neonatal rats treated daily with ghrelin for 2 or 3 wk from birth showed faster eye and vaginal opening than those of saline-treated group (22), suggesting that ghrelin may be involved in neonatal development. Therefore, it has been assumed that ghrelin from the maternal stomach or placenta during pregnancy may play a role in fetal development. In the present study, we examined the possible involvement of maternal ghrelin in rat fetal development.

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Abbreviations: BrdU, 5-Bromo-2′-deoxyuridine; E, embryonic day; GHS-R, GH secretagogue receptor; mcKLH, mariculture keyhole limpet hemocyanin.

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Materials and Methods

Animals

Wistar rats were housed under controlled temperature (23 ± 1 C) and regulated 12-h light 12-h dark conditions (lights on at 0700 h). Female rats were mated on the day of proestrus at approximately 3 months old.

The next estrus day was considered to be d 0 of pregnancy. As reported previously, delivery usually occurs in our rat colony during the morning on d 21 of pregnancy (23). The average number (±SEM) of pups per mother at delivery was 13.10 ± 1.78 (n = 122). All procedures were performed in accordance with the Japanese Physiological Society's guidelines for animal care.

RT-PCR for GHS-R 1a mRNA

Total RNA was extracted from fetal tissues on d 14, 15, and 19 of pregnancy using Trizol reagent (Invitrogen, Carlsbad, CA) as described previously (24). First-strand cDNA was synthesized from 2 μg of total RNA by random primer RT. The resulting cDNA was subjected to PCR amplification using sense and antisense primers specific for GHS-R1a (24). PCR products were electrophoresed on a 2% agarose gel. GAPDH was used as a control housekeeping gene.

Autoradiography for [^{125}I]acyl ghrelin

Fetuses [embryonic d 17 (E17)] were embedded in Tissue-Tec OCT compound (Sakura Finetechnical Co., Ltd., Tokyo, Japan) and frozen. Sections cut using a cryostat were mounted on gelatin-coated glass slides. Autoradiography was performed as described previously (14) with the following minor modifications. After preincubation for 30 min in incubation buffer at room temperature, sections were incubated for 12 h at 4 C in buffer containing 20 ng/ml rat [^{125}I-Tyr^{29}]acylated rat ghrelin. Nonspecific binding was determined in the presence of excess unlabeled acyl or des-acyl rat ghrelin (10 μg/ml). Sections were then exposed to an IP plate (Fuji Film, Tokyo, Japan) for 12 h and analyzed on BAS-5000 (Fuji Film).

Preparation of anti-GHS-R serum

The [Cys^0]-rat GHS-R [342–364] peptide was synthesized using the Fmoc solid-phase method on a peptide synthesizer (433A; Applied Biosystems, Foster City, CA), then purified by reverse phase-HPLC. The synthesized peptide (10 mg) was conjugated to maleimide-activated mariculture keyhole limpet hemocyanin (6 mg) (mcKLH; Pierce, Rockford, IL) in conjugation buffer (Pierce). The conjugate was emulsified with an equal volume of Freund's complete adjuvant and was used to immunize New Zealand white rabbits by intracutaneous and sc injection. Animals were boosted every 2 wk and bled 7 d after each injection. The specificity of the antisera was confirmed by the immunoreactivity of GHS-R-expressing cells (CHO-GHSR62 cells) and lack thereof in control cells.

Immunohistochemistry for GHS-R

Immunohistochemical analyses for GHS-R were performed on frozen fetuses (E17 and 19) using a modification of a method that has been described previously (25). The fetuses were placed in fixative for 5 d at 4 C and then transferred to 0.1 M phosphate buffer containing 20% sucrose. They were cut into serial, 12-μm-thick sections at −20 C with a cryostat. The sections were incubated for 2 d with a rabbit-anti-GHS-R antibody at 4 C. Slides were then incubated with Alexa-546-labeled goat-antirabbit IgG antibody (Molecular Probes, Inc., Eugene, OR; dilution 1:400). Samples were observed with the aid of an Olympus AX-70 fluorescence microscope (Olympus, Tokyo, Japan). To examine the specificity of GHS-R antibody in tissue sections, the reaction was also performed using GHS-R antibody that had been preabsorbed with excess synthetic GHS-R (10 μg).

Measurement of acyl and des-acyl ghrelin, IGF-I, and corticosterone

Levels of acyl or des-acyl ghrelin were measured by specific ELISA kits for acyl or des-acyl ghrelin (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan). The ELISA can detect each acyl or des-acyl ghrelin using two specific antibodies recognizing only acyl ghrelin (octanoylation modification at [Ser^3]-ghrelin [1–11]) or only des-acyl ghrelin (nonoctanoylation modification at [Ser^3]-ghrelin [1–11]). Blood collected from pregnant rats and their fetuses was immediately put into chilled polypropylene tubes containing a protease inhibitor, aprotinin (Sigma-Aldrich, St. Louis, MO), and 2Na-EDTA and then centrifuged. We then added a 10% plasma volume of 0.1 N HCl. Maternal blood was taken at 0830 h (satiety phase) at 2-d intervals from d 11–21. Fetal blood and amniotic fluid were collected on d 17, 19, and 21.

To examine the transit of maternal acyl ghrelin to the fetal circulation, acyl ghrelin (0.2 and 20 nmol) or saline was injected into pregnant rats iv under light ether anesthesia on d 19 of pregnancy (n = 12 per group). Blood was then collected from both the mother and fetus at 5, 10, and 30 min after injection.

To determine the effect of maternal treatment with acyl ghrelin on plasma IGF-I and corticosterone levels in the fetal circulation, fetal plasma IGF-I and corticosterone levels were measured by enzyme immunoassay kit (Funakoshi, Tokyo, Japan) and [^{125}I]corticosterone RIA kit (ICN Biomedicals, Costa Mesa, CA), respectively. The limit of assay sensitivity was 5 ng/ml for IGF-I and 20 ng/ml for corticosterone. The intra and interassay coefficients of variation were 5 and 16%, respectively, for IGF-I, and 6 and 12%, respectively, for corticosterone.

Ghrelin administration and neonatal body weights

We sc injected either saline, acyl ghrelin (1.5 or 3.0 nmol), or des-acyl ghrelin (3.0 nmol) three times a day (at 0830, 1330, and 1830 h) from d 14 to delivery, or continuously infused vehicle, acyl ghrelin (0.125 or 0.5 nmol/h) or des-acyl ghrelin (0.5 nmol/h) through an osmotic minipump implanted sc from d 15 until delivery (n = 10 per group) (11, 26). We also injected 3 nmol acyl ghrelin three times a day from d 14 to delivery into pair-fed pregnant rats and the effect was compared with saline-treated pregnant rats. Neonatal body weights were measured on the day of delivery. If the pups numbered more than 15 or less than 11 they were excluded from the analyses.

Passive immunization for acyl ghrelin

Rat acyl ghrelin (3 mg) was conjugated to a carrier protein, mcKLH (3 mg), in conjugation buffer (Pierce) (7). Each conjugate was emulsified with an equal volume of Freund's adjuvant. Immunization, initiated by intradermal injection in 44-d-old female rats, was repeated six times at 2-wk intervals. As a control antigen, carrier protein alone without ghrelin was administered. Rats were mated on d 114 after the fifth immunization. The antibody titers were verified in diluted plasma every 10 d after immunization using [^{125}I]ghrelin binding capacity.

Quantitative RT-PCR of GH mRNA in fetal pituitary

The pituitary gland and blood were collected from E19 and E20 fetuses, isolated from the mothers' implanted osmotic minipump (acyl ghrelin 0.5 nmol/h and saline). GH mRNA expression was measured by real-time quantitative PCR as described previously (25). Experiments contrasted the relative levels of both GH and GAPDH transcripts in every sample. The total RNA from each tissue was extracted using an RNeasy Micro kit (Qiagen, Valencia, CA) and synthesized into first-strand cDNA using an iScript cDNA Synthesis kit (Bio-Rad Laboratories, Hercules, CA). An aliquot of the first-strand cDNA (40–100 ng tissue equivalent) was quantified on an iCycler (Bio-Rad Laboratory) using iQ SYBR Green Supermix (Bio-Rad Laboratory) with primers to amplify GAPDH (25) and GH specifically (26).

Incorporation of [^{3}H]thymidine or 5-bromo-2′-deoxyuridine (BrdU) into cultured cells

We assessed the effect of acyl and des-acyl ghrelin administration on the proliferation of fetal skin cells by measuring the incorporation of [^{3}H]thymidine (2 μCi/ml) or BrdU (10 μM). Dispersed fetal skin cells were prepared from E17 fetuses by sequential collagenase treatment, papain digestion, and mechanical desegregation. Dispersed cells were then suspended in MCDB153HAA medium (F-Peptide Co., Ltd., Yamagata, Japan) containing 2% fetal calf serum, penicillin (100 U/ml), streptomycin (100 μg/ml), and 5 ng/ml epidermal growth factor. Cells were seeded in polyethylenimine-coated 48- and 96-well dishes at densities of 5×10^5 per well and 3×10^4 per well for the [^{3}H]thymidine and BrdU experiments, respectively. BrdU was detected using a Cell Proliferation ELISA Kit (Roche Diagnostic GmbH, Mannheim, Germany), as reported by Kusunoki *et al.* (27).