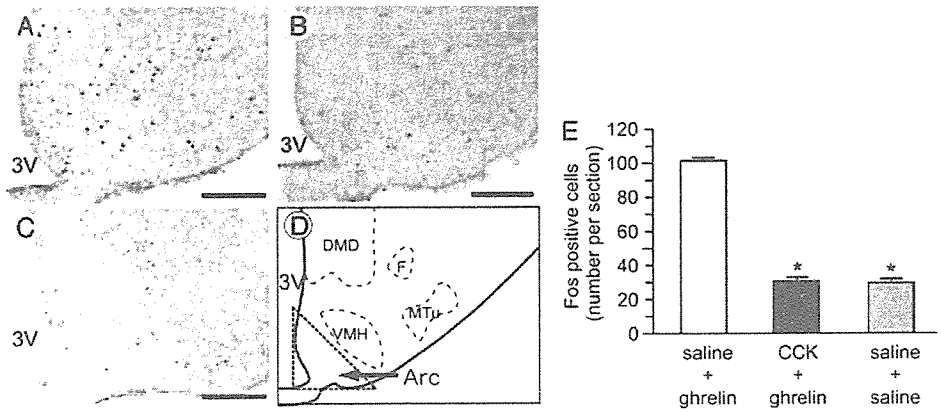

FIG. 4. Fos expression in the arcuate nucleus induced by ghrelin administration. A, Fos expression in response to iv ghrelin administration to rats after saline treatment. B, Fos expression in response to iv ghrelin administration after CCK treatment. C, Fos expression in response to iv saline to rats. D, A schematic drawing of an area in which Fos-positive neurons were counted. Fos-expressing neurons in a 0.7-mm right triangle (0.245 mm^2) were evaluated. Arc, arcuate nucleus; DMD, dorsomedial nucleus, dorsal; F, fornix; MTu, medial tuberal nucleus; VMH, ventromedial hypothalamic nucleus; 3V, third ventricle. *Scale bar*, 50 μm. C, The number of cells per section (bilateral). Data are expressed as mean ± SEM (n = 3). *, $P < 0.0001$ *vs.* rats administered ghrelin after saline treatment.

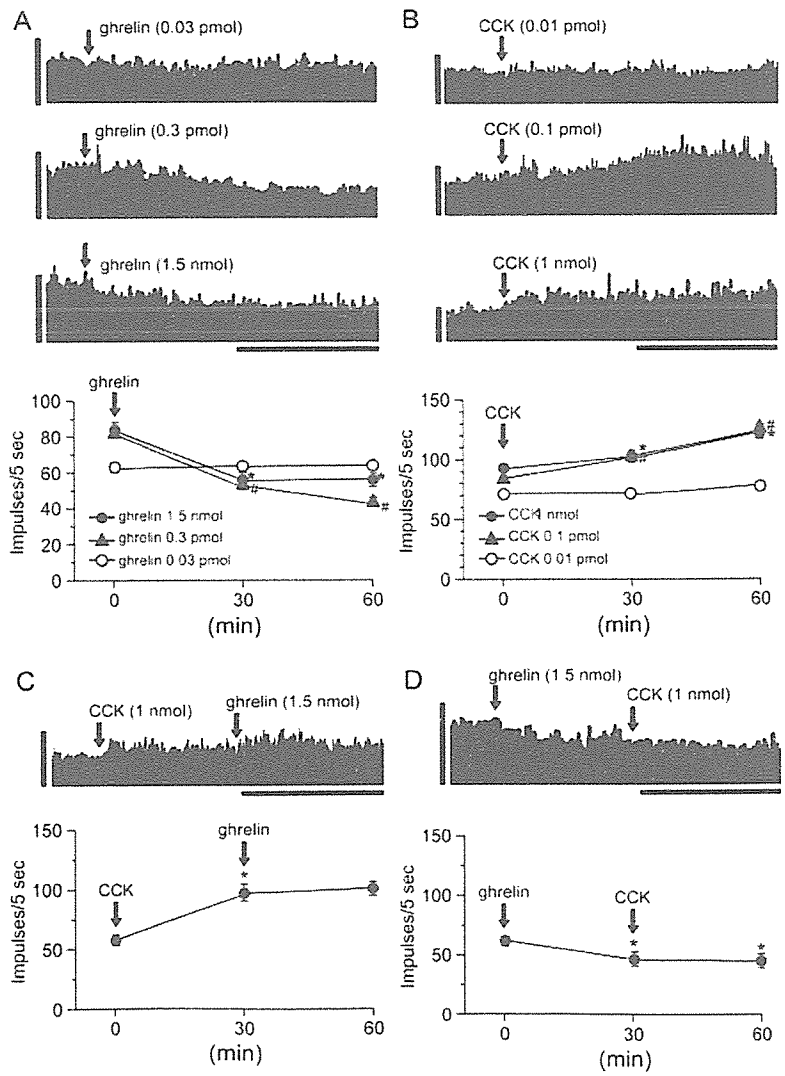

FIG. 5. The electrophysiological effect of ghrelin and CCK on gastric vagal afferent activity. A, Alterations of gastric vagal afferent discharge after a single iv administration of ghrelin (0.03 pmol–1.5 nmol). *, $P < 0.05$ *vs.* value at 0 min of 1.5 nmol ghrelin. #, $P < 0.05$ *vs.* value at 0 min of 0.3 pmol ghrelin. B, Alterations of gastric vagal afferent discharge after a single iv administration of CCK (0.01 pmol–1 nmol). *, $P < 0.05$ *vs.* value at 0 min of 1 nmol CCK. #, $P < 0.05$ *vs.* value at 0 min of 0.1 pmol CCK. C, Ghrelin (1.5 nmol) administration after CCK treatment (1 nmol) does not attenuate gastric vagal afferent activity. *, $P < 0.05$ *vs.* value at 0 min. D, CCK (1 nmol) administration after ghrelin (1.5 nmol) treatment does not activate gastric vagal afferent activity. *, $P < 0.05$ *vs.* value at 0 min. Representative data of gastric vagal afferent discharge rates are shown in the *upper panels*. *Vertical bar*, 100 impulses/5 sec; *horizontal bar*, 30 min.

These findings indicate that central ghrelin, peripheral ghrelin, or both may increase food intake and GH secretion via NPY and GHRH directly. However, we recently demonstrated that blockade of the gastric vagal afferent abolished ghrelin-induced feeding, GH secretion, and activation of NPY and GHRH neurons (38). These data suggest a possibility that ghrelin's signals for starvation and GH secretion are conveyed to the brain via the gastric vagal afferent system. Therefore, ghrelin and CCK, both produced within the gastrointestinal tract, exert opposite effects on feeding behavior through the vagal afferent, thereby regulating food intake on a short-term basis as a meal initiator and terminator, respectively.

In this study, we examined the interaction of ghrelin with CCK in the regulation of feeding behavior using CCK-AR-deficient OLETF rats. Ghrelin increased food intake in both OLETF and their lean littermates, LETO rats. In contrast, CCK decreased food intake in LETO rats fasted for 8-h period, but did not affect food intake in OLETF rats. These findings indicate that CCK-AR is required for CCK, but not ghrelin, regulation of feeding and that exogenous CCK reduces food intake of rats whose endogenous ghrelin levels are increased. Preadministration of CCK to LETO, but not to OLETF, rats blocked the food intake induced by peripheral administration of ghrelin. Conversely, the preadministration of ghrelin to LETO rats blocked the feeding reduction induced by peripheral CCK administration. These findings suggest that the effect of CCK or ghrelin administered after ghrelin or CCK, respectively, on feeding, might not be displayed, while some information to determine feeding behavior induced by exogenously preadministered ghrelin or CCK is transmitting via the vagal afferent system to the brain. When ghrelin or CCK was administered to rats, each plasma level transiently increases over their physiological ranges, which may also have cause complete blockade of the effect of serially administered ghrelin or CCK on feeding.

We also investigated the colocalization of GHS-R with CCK-AR in the rat nodose ganglion. Receptors of the vagal afferent are generated by nodose ganglion neurons and are transported to the nerve terminal through axonal transport (68, 69). Although we failed to demonstrate the colocalization of GHS-R and CCK-AR in the nerve terminal, immunohistochemical double staining of the nodose ganglion demonstrated that the majority of the GHS-R-immunoreactive neurons express CCK-AR. These findings suggest that the vagus nerve plays a major role in determining the peripheral parameters of energy balance.

Signals mediated by ghrelin secretion by the stomach are thought to be transmitted to the hypothalamus of the brain via the NTS, as iv administration of ghrelin induces Fos expression in the arcuate nucleus of the hypothalamus (38). Ghrelin suppresses gastric vagal afferent discharges when administered iv (38), whereas CCK enhances these discharges (43–48). Preadministration of CCK reduced the number of Fos-immunoreactive neurons induced by ghrelin. Very recently, Kobelt *et al.* (70) showed that peripherally administered CCK simultaneously with ghrelin inhibited ghrelin-induced feeding and ghrelin-induced Fos expression in the hypothalamic arcuate nucleus. These results are consistent with our data presented here. In addition, treatment with ghrelin after CCK administration did not affect the vagal afferent discharges induced by CCK. The effect of some peptides on vagal afferent discharge is known to be rapid (71, 72). However, in our experimental system, the changes in firing rate of the vagal afferent fibers induced by several substances continued over 60 min (38, 42, 73–79). These findings suggest that alteration of the firing rate counted by the interval and/or number of firing fibers may be caused by several messengers after peptides bound to their receptors. For example, a single somatostatin administration to rats actually increased the vagal afferent discharge for over 60 min. The afferent discharge stimulated by somatostatin was canceled by an injection of a monoclonal antibody for somatostatin before, but is ineffective after, the somatostatin injection (79). These results suggest the involvement of a unique postreceptor mechanism in the chemoreception as responsible for this long-acting effect of somatostatin on the afferent discharge. Such a postreceptor mechanism may apply to the time course of the ghrelin-induced decrease or CCK-induced increase of the vagal afferent discharge, although the precise mechanism remains to be elucidated. Recently, Królczyk *et al.* (80) performed electrophysiological recordings in both fasted and fed rats and demonstrated that the firing rate of the vagal afferent discharge in fasted rats was lower than that in fed rats. In that study, the increase in the firing rate after food administration to the fasted rats lasted for 15 min. Considering that ghrelin concentration increases in the fasting state and CCK concentration increases after feeding, exogenous administrations of ghrelin and CCK may induce in part starvation and satiety conditions on the basis of circulating hormones, respectively. The actual linkage of these peripheral signals with the vagal afferent pathway is likely to be more complicated given the remarkable number of neurotransmitters, neuropeptides, and neuromodulators. Feeding is a complicated interaction of many factors such as orexigenic or anorectic signals, emotion, learning, memory, *etc.* We believe that the alternation of the firing rate of the vagal afferent induced by ghrelin, CCK, or the combination of ghrelin with CCK is only a part of feeding regulation. Although it is difficult to clearly explain the reasons why the afferent activity lasts for such a long period after the single administration of ghrelin or CCK, we suggest that the long-acting effect on the afferent discharge may provide sufficient time for the brain to receive feeding-related conditions originating throughout the body. In addition, there may be a limitation on connecting the electrophysiological findings of rats under anesthetization with the feeding behavior of free-moving rats.

In summary, this study demonstrates that ghrelin administration after CCK treatment does not induce feeding; CCK administration after ghrelin treatment does not reduce it. We assume some mechanism whereby the intracellular signaling pathway induced by preadministered ghrelin or CCK interferes with signal transmission of serially administered CCK or ghrelin. In addition, the efficiency of ghrelin and CCK signal transport may depend on the balance in the plasma concentrations of these factors. In normal subjects, plasma ghrelin levels rise before the onset of meals and decline 30 min after feeding. In obese subjects, however, these declinations in plasma ghrelin levels are absent (81). The lack of

suppression of ghrelin secretion after a meal may be a critical factor in the pathophysiology of obesity and eating disorders. CCK is released postprandially, eliciting satiety signals (82–85). Plasma CCK concentrations in lean subjects fed a solid meal peak around 60 min after eating (86, 87). CCK also interacts synergistically in rats with other hormones released postprandially, including insulin, leptin, and glucagon (88–90). Abnormalities in the release of or sensitivity to ghrelin and/or CCK may be involved in alterations of food intake. Further investigation of the mechanisms controlling ghrelin and CCK release will help our understanding of the multifactorial regulation of feeding behavior, potentially leading to new treatments for obesity and eating disorders.

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References

1. **Bray GA** 1991 Weight homeostasis. Annu Rev Med 42:205–216
2. **Levin BE, Routh VH** 1996 Role of the brain in energy balance and obesity. Am J Physiol 271:R491–R500
3. **Keesey RE, Hirvonen MD** 1997 Body weight set-points: determination and adjustment. Nutr 127:1875S–1883S
4. **Havel PJ** 2001 Peripheral signals conveying metabolic information to the brain: short-term and long-term regulation of food intake and energy homeostasis. Exp Biol Med 226:963–977
5. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a novel growth hormone releasing acylated peptide from stomach. Nature 402:656–660
6. **Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M** 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141: 4255–4261
7. **Dornonville de la Cour C, Björkqvist M, Sandvik AK, Bakke I, Zhao C-M, Chen D, Håkanson R** 2001 A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. Regul Pept 99:141–150
8. **Smith GP, Gibbs J** 1994 Satiating effect of cholecystokinin. Ann NY Acad Sci 713:236–241
9. **Moran TH** 2000 Cholecystokinin and satiety: current perspectives. Nutrition 16:858–865
10. **Batterham RL, Cowley MA, Small CJ, Herzog H, Cohen MA, Dakin CL, Wren AM, Brynes AE, Low MJ, Ghatei MA, Cone RD, Bloom SR** 2002 Gut hormone PYY (3–36) physiologically inhibits food intake. Nature 418:650–654
11. **Flint A, Raben A, Astrup A, Holst JJ** 1998 Glucagon-like peptide 1 promotes satiety and suppresses energy intake in humans. J Clin Invest 101:515–520
12. **Naslund E, Gutniak M, Skogar S, Rossner S, Hellstrom PM** 1998 Glucagon-like peptide 1 increases the period of postprandial satiety and slows gastric emptying in obese men. Am J Clin Nutr 68:525–530
13. **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
14. **Tschöp M, Smiley DL, Heiman M** 2000 Ghrelin induces adiposity in rodents. Nature 407:908–913
15. **Wren AM, Small CJ, Ward HL, Murphy KG, Dakin CLD, Taheri S, Kennedy AR, Roberts GH, Morgan DGA, Ghatei MA, Bloom SR** 2000 The novel hypothalamic peptide ghrelin stimulates food intake and growth hormone secretion. Endocrinology 141:4325–4328
16. **Wren AM, Seal LJ, Cohen MA, Brynes AE, Frost GS, Murphy KG, Dhillo WS, Ghatei MA, Bloom SR** 2001 Ghrelin enhances appetite and increases food intake in humans. J Clin Endocrinol Metab 86:5992–5995
17. **Wren AM, Small CJ, Abbott CR, Dhillo WS, Seal LJ, Cohen MA, Batterham RL, Taheri S, Stanley SA, Ghatei MA, Bloom SR** 2001 Ghrelin causes hyperphagia and obesity in rats. Diabetes 50:2540–2547
18. **Cummings DE, Purnell JQ, Frayo RS, Schmidova K, Wisse BE, Weigle DS** 2001 A preprandial rise in plasma ghrelin levels suggests a role in meal initiation in humans. Diabetes 50:1714–1719
19. **Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, Nozoe S, Hosoda H, Kangawa K, Matsukura S** 2002 Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab 87:240–244
20. **Tschöp M, Wawarta R, Riepl RL, Friedrich S, Bidlingmaier M, Landgraf R, Folwaczny C** 2001 Post-prandial decrease of circulating human ghrelin levels. J Endocrinol Invest 24:RC19–RC21
21. **Weigle DS, Cummings DE, Newby PD, Breen PA, Frayo RS, Matthys CC, Callahan HS, Purnell JQ** 2003 Roles of leptin and ghrelin in the loss of body weight caused by a low fat, high carbohydrate diet. J Clin Endocrinol Metab 88:1577–1586
22. **Cummings DE, Frayo RS, Marmonier C, Aubert R, Chapelot D** 2004 Plasma ghrelin levels and hunger scores in humans initiating meals voluntarily without time- and food-related cues. Am J Physiol Endocrinol Metab 287:E297–E304
23. **Cummings DE, Schwartz MW** 2003 Genetics and pathophysiology of human obesity. Annu Rev Med 54:453–471
24. **Cummings DE, Shannon MH** 2003 Roles for ghrelin in the regulation of appetite and body weight. Arch Surg 138:389–396
25. **Choi K, Roh SG, Hong YH, Shrestha YB, Hishikawa D, Chen C, Kojima M, Kangawa K, Sasaki S** 2003 The role of ghrelin and growth hormone secretagogues receptor on rat adipogenesis. Endocrinology 144:754–759
26. **Thompson NM, Gill DA, Davies R, Loveridge N, Houston PA, Robinson IC, Wells T** 2004 Ghrelin and des-octanoyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of the type 1a growth hormone secretagogue receptor. Endocrinology 145:234–242
27. **Marzullo P, Verti B, Savia G, Walker GE, Guzzaloni G, Tagliaferri M, Di Blasio A, Liuzzi A** 2004 The relationship between active ghrelin levels and human obesity involves alterations in resting energy expenditure. J Clin Endocrinol Metab 89:936–939
28. **Bray GA** 1995 Nutrient intake is modulated by peripheral peptide administration. Obes Res Suppl 4:569S–572S
29. **Hirschberg AL** 1998 Hormonal regulation of appetite and food intake. Ann Med 30:7–20
30. **Gibbs J, Young RC, Smith GP** 1973 Cholecystokinin decreases food intake in rats. J Comp Physiol Psychol 84:488–495
31. **Pi-Sunyer X, Kissileff HR, Thornton J, Smith GP** 1982 C-terminal octapeptide of cholecystokinin decreases food intake in obese men. Physiol Behav 29:627–630
32. **Geary N, Kissileff HR, Pi-Sunyer FX, Hinton V** 1992 Individual, but not simultaneous, glucagon and cholecystokinin infusions inhibit feeding in men. Am J Physiol 262:R975–R980
33. **Polak JM, Bloom SR, Rayford PL, Pearse AG, Buchan AM, Thompson JC** 1975 Identification of cholecystokinin-secreting cells. Lancet 2:1016–1018
34. **Buchan AM, Polak JM, Solcia E, Capella C, Hudson D, Pearse AG** 1978 Electron immunohistochemical evidence for the human intestinal I cell as the source of CCK. Gut 19:403–407
35. **Liddle RA, Goldfine ID, Rosen MS, Taplitz RA, Williams JA** 1985 Cholecystokinin bioactivity in human plasma. Molecular forms, responses to feeding, and relationship to gallbladder contraction. J Clin Invest 75:1144–1152
36. **Liddle RA** 2000 Regulation of cholecystokinin secretion in humans. J Gastroenterol 35:181–187
37. **Crawley JN, Beinfeld MC** 1983 Rapid development of tolerance to the behavioural actions of cholecystokinin. Nature 302:703–706
38. **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
39. **Gibbs J, Smith GP** 1988 Peptides of digestive system and brain. Model of the cholecystokinin. Ann Endocrinol 49:113–120
40. **Moriarty P, Dimaline R, Thompson DG, Dockray GJ** 1997 Characterization of cholecystokinin A and cholecystokinin B receptors expressed by vagal afferent neurons. Neuroscience 79:905–913
41. **Ritter RC, Ritter S, Ewart WR, Wingate DL** 1989 Capsaicin attenuates hindbrain neuron responses to circulating cholecystokinin. Am J Physiol 257: R1162–R1168
42. **Asakawa A, Inui A, Kaga T, Yuzuriha H, Nagata T, Ueno N, Makino S, Fujimiya M, Niijima A, Fujino MA, Kasuga M** 2001 Ghrelin is an appetite-stimulatory signal from stomach with structural resemblance to motilin. Gastroenterology 120:337–345
43. **Schwartz GJ, Moran TH, White WO, Ladenheim EE** 1997 Relationships between gastric motility and gastric vagal afferent responses to CCK and GRP in rats differ. Am J Physiol 272:R1726–R1733
44. **Schwartz GJ, McHugh PR, Moran TH** 1993 Gastric loads and cholecystokinin

synergistically stimulate rat gastric vagal afferents. Am J Physiol 265:R872–R876
45. **Schwartz GJ, Moran TH** 1994 CCK elicits and modulates vagal afferent activity arising from gastric and duodenal sites. Ann NY Acad Sci 713:121–128
46. **Moran TH, Norgren R, Crosby RJ, McHugh PR** 1990 Central and peripheral vagal transport of cholecystokinin binding sites occurs in afferent fibers. Brain Res 526:95–102
47. **Corp ES, McQuade J, Moran TH, Smith GP** 1993 Characterization of type A and type B CCK receptor binding sites in rat vagus nerve. Brain Res 623:161–166
48. **Mercer JG, Lawrence CB** 1992 Selectivity of cholecystokinin (CCK) receptor antagonists, MK-329 and L-365,260, for axonally-transported CCK binding sites on the rat vagus nerve. Neurosci Lett 137:229–231
49. **Murakami N, Takamure M, Takahashi K, Utunomiya K, Kuroda H, Etoh T** 1991 Long-term cultured neurons from rat suprachiasmatic nucleus retain the capacity for circadian oscillation of vasopressin release. Brain Res 545:347–350
50. **Nakahara K, Murakami N, Nasu T, Kuroda H, Murakami T** 1997 Involvement of protein kinase A in the subjective nocturnal rise of melatonin release by chick pineal cells in constant darkness. J Pineal Res 23:221–229
51. **Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M** 1999 Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci USA 96:748–753
52. **Niijima A, Yamamoto T** 1994 The effects of lithium chloride on the activity of the afferent nerve fibers from the abdominal visceral organs in the rat. Brain Res Bull 35:141–145
53. **Bray GA** 2000 Afferent signals regulating food intake. Proc Nutr Soc 59:373–378
54. **Ritter RC, Brenner L, Yox DP** 1992 Participation of vagal sensory neurons in putative satiety signals from the upper gastrointestinal tract. In: Ritter S, Ritter RC, Barnes CD, eds. Neuroanatomy and physiology of abdominal vagal afferents. Boca Raton: CRC Press; 222–244
55. **Takaya K, Ariyasu H, Kanamoto N, Iwakura H, Yoshimoto A, Harada M, Mori K, Komatsu Y, Usui T, Shimatsu A, Ogawa Y, Hosoda K, Akamizu T, Kojima M, Kangawa K, Nakao K** 2000 Ghrelin strongly stimulates growth hormone release in humans. J Clin Endocrinol Metab 85:4908–4911
56. **Tschöp M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML** 2001 Circulating ghrelin levels are decreased in human obesity. Diabetes 50:707–709
57. **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
58. **Toshinai K, Mondal MS, Nakazato M, Date Y, Murakami N, Kojima M, Kangawa K, Matsukura S** 2001 Upregulation of ghrelin expression in the stomach upon fasting, insulin-induced hypoglycemia, and leptin administration. Biochem Biophys Res Commun 281:1220–1225
59. **Inui A** 2001 Ghrelin: an orexigenic and somatotrophic signal from the stomach. Nat Rev Neurosci 2:551–560
60. **Smith GP, Jerome C, Cushin BJ, Eterno R, Simansky KJ** 1981 Abdominal vagotomy blocks the satiety effect of cholecystokinin in the rat. Science 213:1036–1037
61. **Geracioti Jr TD, Liddle RA** 1988 Impaired cholecystokinin secretion in bulimia nervosa. N Engl J Med 319:683–688
62. **Jansen JB, Lamers CB** 1983 Radioimmunoassay of cholecystokinin in human tissue and plasma. Clin Chim Acta 131:305–316
63. **Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL** 2003 The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649–661
64. **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
65. **Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K** 2001 Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50:227–232
66. **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
67. **Mondal MS, Date Y, Yamaguchi H, Toshinai K, Tsuruta T, Kangawa K, Nakazato M** 2005 Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. Regul Pept 126:55–59
68. **Moran TH, Smith GP, Hostetler AM, McHugh PR** 1987 Transport of cholecystokinin (CCK) binding sites in subdiaphragmatic vagal branches. Brain Res 415:149–152
69. **Zarbin MA, Wamsley JK, Innis RB, Kuhar MJ** 1981 Cholecystokinin receptors: presence and axonal flow in the rat vagus nerve. Life Sci 29:697–705
70. **Kobelt P, Tebbe JJ, Tjandra I, Stengel A, Bae HG, Andresen V, van der Voort IR, Veh RW, Werner CR, Klapp BF, Wiedenmann B, Wang L, Taché Y, Mönnikes H** 2005 CCK inhibits the orexigenic effect of peripheral ghrelin. Am J Physiol Regul Integr Comp Physiol 288:R751–758
71. **Hillsley K, Grundy D** 1998 Serotonin and cholecystokinin activate different populations of rat mesenteric vagal afferents. Neurosci Lett 255:63–66
72. **Eastwood C, Maubach K, Kirkup AJ, Grundy D** 1998 The role of endogenous cholecystokinin in the sensory transduction luminal nutrient signals in the rat jejunum. Neurosci Lett 254:145–148
73. **Nishizawa M, Nakabayashi H, Uchida K, Nakagawa A, Niijima A** 1996 The hepatic vagal nerve is receptive to incretin hormone glucagon-like peptide-1, but not to glucose-dependent insulinotropic polypeptide, in the portal vein. J Auton Nerv Syst 61:149–154
74. **Niijima A** 1996 The afferent discharges from sensors for interleukin 1 beta in the hepatoportal system in the anesthetized rat. J Auton Nerv Syst 61:287–291
75. **Nakabayashi H, Niijima A, Kurata Y, Jiang ZY, Usukura N, Takeda R** 1987 Pancreatic vagal nerve is receptive to somatostatin in rats. Am J Physiol 253(1 Pt 2):R200–R203
76. **Lutz TA, Niijima A, Scharrer E** 1996 Intraportal infusion of 2,5-anhydro-D-mannitol increases afferent activity in the common hepatic vagus branch. J Auton Nerv Syst 61:204–208
77. **Niijima A, Hatanaka S, Furuhama K** 1996 Effects of DQ-2511 on neutral activity in afferent and efferent loops of gastric vago-vagal reflex pathways in the rat. J Auton Pharmacol 16:49–53
78. **Koda S, Date Y, Murakami N, Shimbara T, Hanada T, Toshinai K, Niijima A, Furuya M, Inomata N, Osuye K, Nakazato M** 2005 The role of the vagal nerve in peripheral PYY3–36-induced feeding reduction in rats. Endocrinology 146:2369–2375
79. **Nakabayashi H, Niijima A, Nishizawa M, Nakabayashi IO, Takeda R** 1994 A unique receptor-mediated mechanism in vagal chemoreception of somatostatin in the hepatoportal area. J Auton Nerv Syst 50:45–50
80. **Królczyk G, Zurowski D, Sobocki J, Laskiewicz J, Thor PJ** 2004 Encoding meal in integrated vagal afferent discharge. Physiol Pharmacol 55:99–106
81. **English PJ, Ghatei MA, Malik IA, Bloom SR, Wilding JP** 2002 Food fails to suppress ghrelin levels in obese humans. J Clin Endocrinol Metab 87:2984–2987
82. **Lee MC, Schiffman SS, Pappas TN** 1994 Role of neuropeptides in the regulation of feeding behavior: a review of cholecystokinin, bombesin, neuropeptide Y, and galanin. Neurosci Biobehav Rev 18:313–323
83. **Crawley JN, Corwin RL** 1994 Biological actions of cholecystokinin. Peptides 15:731–755
84. **Weller A, Smith GP, Gibbs J** 1990 Endogenous cholecystokinin reduces feeding in young rats. Science 247:1589–1591
85. **Lewis LD, Williams JA** 1990 Regulation of cholecystokinin secretion by food, hormones, and neural pathways in the rat. Am J Physiol 258:G512–G518
86. **Beardshall K, Deprez P, Playford RJ, Alexander M, Calam J** 1992 Effect of chymotrypsin on human cholecystokinin release: use of clostripain in the validation of a new radioimmunoassay. Regul Pept 40:1–12
87. **Rehfeld JF** 1998 Accurate measurement of cholecystokinin in plasma. Clin Chem 44:991–1001
88. **Barrachina MD, Martinez V, Wang L, Wei JY, Tache Y** 1997 Synergistic interaction between leptin and cholecystokinin to reduce short-term food intake in lean mice. Proc Natl Acad Sci USA 94:10455–10460
89. **Riedy CA, Chavez M, Figlewicz DP, Woods SC** 1995 Central insulin enhances sensitivity to cholecystokinin. Physiol Behav 58:755–760
90. **Le Sauter J, Geary N** 1987 Pancreatic glucagon and cholecystokinin synergistically inhibit sham feeding in rats. Am J Physiol 253:R719–R772

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Des-Acyl Ghrelin Induces Food Intake by a Mechanism Independent of the Growth Hormone Secretagogue Receptor

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Ghrelin, an acylated peptide produced predominantly in the stomach, stimulates feeding and GH secretion via interactions with the GH secretagogue type 1a receptor (GHS-R1a), the functionally active form of the GHS-R. Ghrelin molecules exist in the stomach and hypothalamus as two major endogenous forms, a form acylated at serine 3 (ghrelin) and a des-acylated form (des-acyl ghrelin). Acylation is indispensable for the binding of ghrelin to the GHS-R1a. Ghrelin enhances feeding via the neuronal pathways of neuropeptide Y and orexin, which act as orexigenic peptides in the hypothalamus. We here studied the effect of des-acyl ghrelin on feeding behavior. Intracerebroventricular (icv) administration of rat des-acyl ghrelin to rats or mice fed *ad libitum* stimulated feeding during the light phase; neither ip nor icv administration of des-acyl ghrelin to fasting mice suppressed feeding. The icv administration of des-acyl ghrelin induced the expression of Fos, a marker of neuronal activation, in orexin-expressing neurons of the lateral hypothalamic area, but not neuropeptide Y-expressing neurons of the arcuate nucleus. Peripheral administration of des-acyl ghrelin to rats or mice did not affect feeding. Although icv administration of ghrelin did not induce food intake in GHS-R-deficient mice, it did in orexin-deficient mice. In contrast, icv administration of des-acyl ghrelin stimulated feeding in GHS-R-deficient mice, but not orexin-deficient mice. Des-acyl ghrelin increased the intracellular calcium concentrations in isolated orexin neurons. Central des-acyl ghrelin may activate orexin-expressing neurons, perhaps functioning in feeding regulation through interactions with a target protein distinct from the GHS-R. (*Endocrinology* 147: 2306–2314, 2006)

GHRELIN IS A 28-amino-acid peptide isolated from human and rat stomach as an endogenous ligand for the GH secretagogue receptor (GHS-R) (1). The GHS-R, a G protein-coupled seven-transmembrane domain receptor, was initially identified as a receptor for small synthetic molecules termed GH secretagogues (GHSs), such as L-692,429, GHRP-6, and MK-0677, all of which act on the pituitary to stimulate GH secretion (2, 3). Two GHS-R subtypes are generated by alternative splicing of a single gene: the full-length type 1a receptor (GHS-R1a) and a carboxyl-terminally truncated form, the GHS-R type 1b (GHS-R1b), that encodes a protein containing transmembrane domain one to five (2, 3). The GHS-R1a is the functionally active, signal transducing form of the GHS-R, whereas the GHS-R1b is devoid of high-affinity ligand binding and signal transduction activity. Ghrelin molecules, predominantly produced by endocrine cells of the gastric oxyntic glands (4, 5), exist in two major molecular forms, ghrelin and des-*n*-octanoyl ghrelin (des-acyl ghrelin) (6). These two ghrelin molecules are also produced in the rat hypothalamus, as demonstrated by the combination of reverse-phase HPLC (RP-HPLC) with two separate RIAs recognizing ghrelin and des-acyl ghrelin (7, 8). All ghrelin species identified in fish, amphibians, birds, and many mammals possess a unique structural modification of the hydroxyl group of their third residue, which is either serine or threonine, by *n*-octanoic acid (9). This acylation is essential for the binding of ghrelin to the GHS-R1a (1, 10, 11); thus, the acylated form has been designated as ghrelin in our original description (1). Administration of ghrelin stimulates food intake in humans and rats (12–16) but does not change feeding behavior in GHS-R-deficient mice (17), suggesting that ghrelin enhances food intake via GHS-R-mediated signaling.

Several recent *in vitro* studies have demonstrated that radiolabeled ghrelin and des-acyl ghrelin bound to the membranes of PC-3 prostate tumor cells, H9c2 cadiomyocytes and isolated adipocytes, none of which expressed the GHS-R (18–20). This binding could be displaced by ghrelin, des-acyl ghrelin, and synthetic GHSs. Ghrelin and des-acyl ghrelin exhibit similar GHS-R-independent biological activities, including a cytoprotective effect on cultured cardiomyocytes

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Abbreviations: AgRP, Agouti gene-related protein; CRF, corticotropin-releasing factor; 2-DG, 2-deoxy-D-glucose; GHS, GH secretagogue; GHS-R, GH secretagogue receptor; HKRB, Krebs-Ringer bicarbonate buffer; icv, intracerebroventricular(ly); LHA, lateral hypothalamic area; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; PVN, paraventricular nucleus; RP-HPLC, reverse-phase HPLC.

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(18), the inhibition of cell proliferation of breast carcinoma cell lines (19), the reduction of glycerol release from rat epididymal adipocytes (20), an ionotropic effect on guinea pig papillary muscle (21), and the promotion of bone marrow adipogenesis (22). Although the signaling molecules downstream of des-acyl ghrelin remain undefined, des-acyl ghrelin appears to share a subset of biological activities with ghrelin in peripheral tissues through an unidentified receptor or a target protein unique from the GHS-R.

The coordination of the regulation of food intake and energy expenditure occurs in the hypothalamus. Glucoprivic states induced by fasting or treatment with 2-deoxy-D-glucose (2-DG), a selective inhibitor of carbohydrate metabolism, increase feeding through the activation of orexigenic peptides, neuropeptide Y (NPY) and agouti gene-related protein (AgRP) in the arcuate nucleus (23). Secretion of des-acyl ghrelin from the rat hypothalamus increased in glucoprivic states induced by fasting or treatment with 2-DG (7). The axonal terminals of neurons that produce ghrelin and des-acyl ghrelin make direct synaptic contacts with NPY- and orexin-expressing neurons, which participate in hypothalamic feeding regulation (16, 24). Both ghrelin and des-acyl ghrelin may have a direct central action on the control of feeding.

We here investigated the effect of central or peripheral administration of des-acyl ghrelin on food intake in rats and mice and Fos expression, a marker of neuronal activation (25), in neurons that produce the orexigenic hypothalamic peptides, NPY/AgRP, orexin, or melanin-concentrating hormone (MCH). We studied the functional signaling downstream of des-acyl ghrelin using orexin-deficient mice and rats pretreated with antiorexin-A and -B IgGs. We confirmed that des-acyl ghrelin increased the intracellular calcium concentrations in orexin neurons dispersed from the lateral hypothalamic area (LHA) by the calcium-imaging analysis. We demonstrated that des-acyl ghrelin increased feeding by activation of orexin neurons in the LHA. We examined whether des-acyl ghrelin-induced food intake was mediated by the GHS-R pathway using GHS-R-deficient mice. Des-acyl ghrelin appears to regulate feeding via a receptor or target protein independent of the GHS-R.

Materials and Methods

Animals

We used male Wistar rats (Charles River Japan, Inc., Shiga, Japan), weighing 300–350 g, male C57BL/6 (Charles River Japan, Inc.), weighing 24–28 g, and male ddy mice (Kiwa Laboratory Animals Co., Ltd., Wakayama, Japan), weighing 35–39 g. Orexin-deficient mice (12-wk-old, male) and GHS-R-deficient mice (12-wk-old, male) were generated by targeted mutation of embryonic stem cells as reported (17, 26). All animals were individually housed in plastic cages at a constant room temperature in a 12-h light (0800–2000 h)/12-h dark cycle and given standard laboratory chow and water *ad libitum*. All procedures were approved by University of Miyazaki Animal Care and Use Committee and were in accordance with the Japanese Physiological Society's guidelines for animal care. Anesthesia was given as an ip injection of sodium pentobarbital (Abbot Labs., Chicago, IL). Intracerebroventricular (icv) cannulae were implanted into the lateral cerebral ventricles of rats and mice. Proper placement of the cannulae was verified at the end of the experiment by dye administration. Intravenous cannulae were implanted into the rat right jugular vein. Only animals that exhibited progressive weight gain after surgery were used.

Peptide synthesis

Rat ghrelin and des-acyl ghrelin were purchased from Peptide Institute, Inc. (Osaka, Japan). Adequate purification of synthesized peptides was ascertained by RP-HPLC, ion-exchange-HPLC, capillary zone electrophoresis, amino acid sequencing, and mass spectrometry (MALDI-MS). Ghrelin and des-acyl ghrelin were separately eluted, each as single peaks by RP-HPLC performed using a TSK ODS SIL 120A column (4.6 × 150 mm) (Tosho Co., Tokyo, Japan) with a linear gradient of 10–60% acetonitrile (CH_3CN) containing 0.1% trifluoroacetic acid (Fig. 1).

Fos expression

Ghrelin (200 pmol/10 μl saline), des-acyl ghrelin (200 pmol/10 μl saline), or saline was injected icv into Wistar rats or GHS-R-deficient mice 90 min before transcardial perfusion with fixative containing 4% paraformaldehyde. The brain was sectioned into 20- or 40-μm-thick samples. Fos-specific immunohistochemistry was performed as described (27). Hypothalamic sections from rats and GHS-R-deficient mice were incubated for 2 d with goat anti-Fos antiserum (Santa Cruz Biotechnology, Santa Cruz, CA; dilution 1:1500), then stained using the avidin-biotin complex method (Vectastain Elite ABC kit; Vector Laboratories, Inc., Burlingame, CA). These sections were also stained with either rabbit antiorexin-A (dilution 1:3000) (16, 27) or rabbit anti-MCH (Phoenix Pharmaceuticals, Inc., Belmont, CA; dilution 1:200) antisera. We observed orexin- and MCH-expressing neurons by light microscopy. For immunofluorescence microscopy, we incubated hypothalamic sections of GHS-R-deficient mice with goat anti-Fos antiserum (dilution 1:1500) for 2 d at 4 C, then performed an additional 2 h incubation with

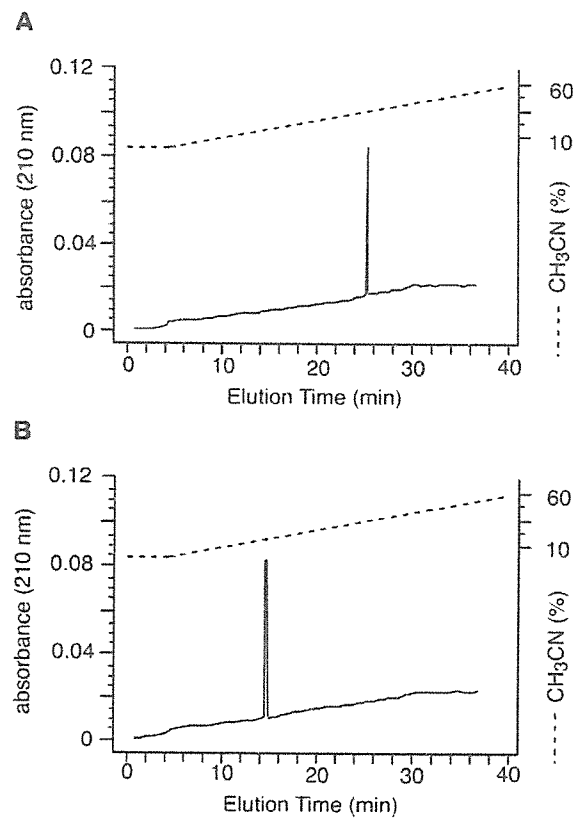


FIG. 1. RP-HPLC analysis of synthetic rat ghrelin (A) and des-acyl ghrelin (B) used for experimentation. Each peptide (0.3 nmol) was loaded onto a TSK ODS SIL 120A column using a linear gradient of 10–60% CH_3CN containing 0.1% trifluoroacetic acid at a rate of 1.0 ml/min for 40 min. Each peptide is eluted as a single peak whose elution position was identical with that of the corresponding synthetic peptide.

Alexa 488-conjugated donkey antigoat IgG antibody (Molecular Probes, Inc., Eugene, OR; dilution 1:400). After washing with PBS (pH 7.4), samples were incubated with a rabbit antiorexin-A antiserum for 2 d at 4 C and Alexa 546-labeled goat antirabbit IgG antibody (Molecular Probes; dilution 1:400) for a final 2 h. Slides were observed on a fluorescence microscope (BH2-RFC; Olympus, Tokyo, Japan).

Food intake

Experiments were performed 1 wk after the implantation of icv or iv cannulae. First, ghrelin or des-acyl ghrelin (each at 200 pmol/10 μl saline) was administered icv at 1000 h to rats fed *ad libitum* (n = 10 per group). The 1-, 2-, and 4-h food intake amounts were then measured. Second, des-acyl ghrelin (1 nmol/10 μl saline) was administered icv to rats (n = 6 per group) 10 min before the beginning of the dark phase, after which the 30-min food intake was measured. Third, ghrelin (1.5 nmol) or des-acyl ghrelin (1.5 or 5 nmol/100 μl saline) was administered iv to rats at 1000 h through an iv cannula. Fourth, des-acyl ghrelin (1 or 5 nmol/2 μl saline) was administered ip at 1000 h to C57BL/6 mice fed *ad libitum* (n = 8 per group). Fifth, 3 h after an icv administration of antiorexin-A and -B (each at 0.25 μg/2.5 μl saline), anti-NPY (0.5 μg/5 μl saline), or normal rabbit serum (0.5 μg/5 μl saline) IgGs, ghrelin or des-acyl ghrelin (each at 200 pmol/5 μl saline) was administered at 1200 h to rats (n = 10–12 per group). Sixth, ghrelin or des-acyl ghrelin (each at 200 pmol/2 μl saline) was administered icv at 1000 h to orexin-deficient mice or their wild-type littermates (n = 6–8 per group). Seventh, ghrelin (200 pmol/2 μl saline), des-acyl ghrelin (200 pmol/2 μl saline) or NPY (1 nmol/2 μl saline; Peptide Institute, Inc.) was administered icv at 1000 h to GHS-R-deficient mice or their wild-type littermates (n = 6–8 per group). With the exception of the first and second experiments, 2-h food intake was measured in all tests. Eighth, ghrelin or des-acyl ghrelin (each at 1 nmol/2 μl saline) was administered icv at 1000 h to ddy mice fed *ad libitum*. Ninth, des-acyl ghrelin (1 nmol/2 μl saline) was administered icv at 1000 h to ddy mice that had fasted for the previous 16 h (n = 8 per group). Tenth, des-acyl ghrelin (1 nmol/50 μl saline) was administered ip at 1000 h to ddy mice that had fasted for the previous 16 h (n = 8 per group). After the injections of ddy mice in the eighth, ninth, and tenth experiments outlined above, we measured 20-min, 1-h, and 2-h food intake. These feeding tests were performed using a cross-over design experiments in which animals were randomized to receive either test substance with a washout period of 3 d between each administration.

Measurement of cytosolic Ca^{2+} concentration ($[Ca^{2+}]i$)

The LHA of rat brain was punched out according to the *Atlas of the Rat Brain* (28). The tissue was washed twice with HEPES and Krebs-Ringer bicarbonate buffer (HKRB) [129 mM NaCl, 5.0 mM $NaHCO_3$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, and 10 mM HEPES (pH 7.4)] containing 10 mM glucose. The LHA was incubated in HKRB supplemented with 1 mg/ml papain (Sigma-Aldrich, St. Louis, MO), 5 mg/ml deoxyribonuclease, and 0.025% BSA for 20 min at 36 C in a shaking water bath, then LHA cells were dispersed by mechanical desegregation for 4 min. The cell suspension was diluted with HKRB and centrifuged at 100 × *g* for 5 min. The pellet was resuspended in HKRB and distributed onto the glass well (Nunc 96 Microwell Optical Bottom Plate; Nalge Nunc International, Rochester, NY). Measurement of $[Ca^{2+}]i$ was carried out 2–4 h after the preparation of cells. The cells were loaded with Fluo-3 for 20 min in HEPES buffer solution [10 mM HEPES, 140 mM NaCl, 5.0 mM KCl, 1.2 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10 mM glucose, and 2 μm Fluo-3/acetoxymethylester (Dojindo Labs, Kumamoto, Japan) (pH 7.2)]. They were washed twice with HEPES buffer, then filled with 100 μl HEPES buffer. One min after, 50 μl of 3 μM rat des-acyl ghrelin were added into the well. $[Ca^{2+}]i$ was determined by measuring fluorescence signal from the Ca^{2+} indicator Fluo-3/acetoxymethylester, with 480 nm excitation and 530 nm emission using a cooled charge-coupled device camera, and the ratio image was produced in Functional Imaging Cell-Sorting System (IMACS; Hamamatsu Photonics, Hamamatsu, Japan). The level of $[Ca^{2+}]i$ in a single neuron was recorded for 6 min after the administration of des-acyl ghrelin. After $[Ca^{2+}]i$ measurement, the neurons were fixed with 4% paraformaldehyde overnight. They were incubated with rabbit antiorexin-A antiserum (dilution 1:1500) for 2 d at 4 C, then Alexa 350-conjugated goat antirabbit IgG antibody (dilution 1:400) for 2 h (16, 27). The picture of calcium imaging was collated with the immunohistochemical picture. Fluorescence signals from Fluo-3 were converted automatically to pseudo colors in IMACS. The levels of $[Ca^{2+}]i$ were assigned pseudo colors ranging from blue of the lowest value through yellow to red of the highest value. Fluorescence signals from Alexa-350 were shown in white.

Measurement of locomotor activity

Locomotor activity of rats was measured using a rat locomotor activity recording system (Muromachi Co. Ltd., Tokyo, Japan) comprising infrared sensors, an interface and a computer. The infrared sensors were placed above the cages and measured all locomotor activity. A cage with the infrared sensor was placed in an isolated chamber with a controlled light/dark cycle. Rats were given icv des-acyl ghrelin (1 nmol/10 μl saline), human orexin-A (Peptide Institute, Inc.; 1 nmol/10 μl saline) or saline administration icv at 0900 h (n = 5 per group), then these rats were immediately returned to their individual cages. Locomotor activity counts were made 30 min and analyzed by Compact ACTAM Software (Muromachi Co. Ltd.).

GH response

Ghrelin or des-acyl ghrelin (each at 1.5 nmol/100 μl) was administered iv to rats (n = 6 per group) at 1000 h. Blood samples (80 μl) were obtained from the tail veins at 0, 15, 30, and 60 min after administration. After removal, six anterior pituitary glands of rats were immersed in Hanks' balanced salt solution, then incubated at 37 C for 30 min. Each one pituitary grand was placed in a polystyrene well (16 mm in diameter; Iwaki Glassware Co., Tokyo, Japan) filled with oxygenated medium (DMEM containing 2.5% fetal calf serum and 2.5% bovine serum). After rinsing twice in 500 μl medium for 1 min each, 750 μl medium was added into each well for 5 min. The medium was then collected into plastic microtubes to evaluate basal GH secretion. The pituitary glands were then stimulated for 5 min with medium containing either ghrelin or des-acyl ghrelin (1 μM). The medium was collected into plastic microtubes to quantify GH concentration with a Biotrak Rat GH RIA kit

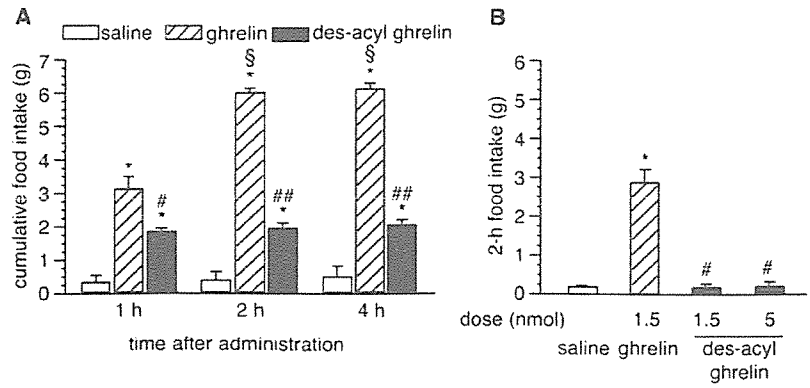


FIG. 2. Effect of ghrelin or des-acyl ghrelin on food intake in rats. A, Intracerebroventricular administration of des-acyl ghrelin or ghrelin (each at 200 pmol) at 1000 h. *, $P < 0.001$ *vs.* saline; #, $P < 0.05$; ##, $P < 0.001$ *vs.* ghrelin; §, $P < 0.001$ *vs.* 1-h food intake. B, Intravenous administration of ghrelin (1.5 nmol) or des-acyl ghrelin (1.5 or 5 nmol) at 1000 h. *, $P < 0.001$ *vs.* saline; #, $P < 0.001$ *vs.* ghrelin.

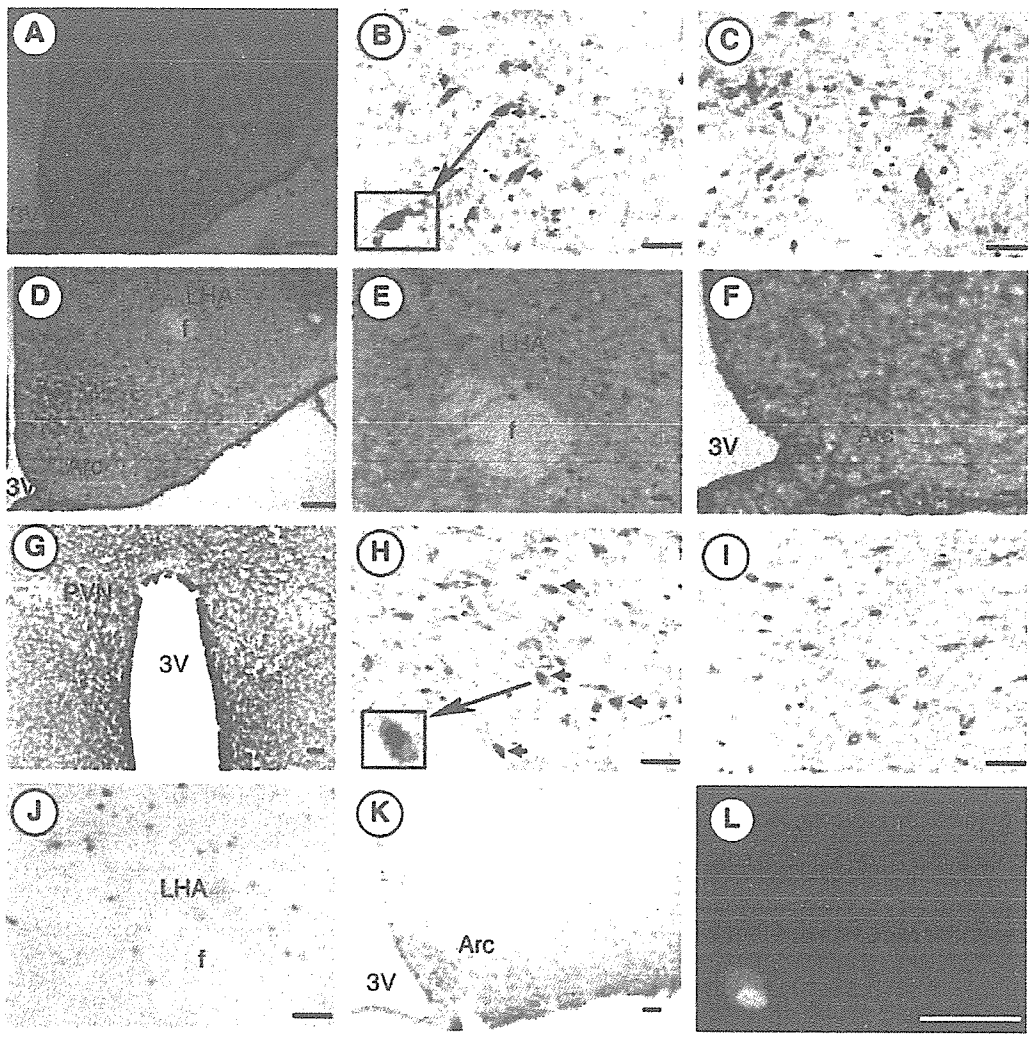


FIG. 3. Fos expression in the hypothalamus after icv administration of ghrelin or des-acyl ghrelin (each at 200 pmol). A, Fos expression (*dark blue-black*) in the LHA and arcuate nucleus of rats given ghrelin. B, Costaining (*arrows*) of Fos (*dark blue-black*) and orexin (*brown*) in rats given ghrelin. The *inset* is a higher magnification of Fos expression in an orexin-expressing neuron. C, No Fos (*dark blue-black*) expression in MCH-expressing neurons (*brown*) after ghrelin administration. D, Fos expression (*dark blue-black*) in the hypothalamus of rats given des-acyl ghrelin. E, Fos expression (*dark blue-black*) in the LHA of rats given des-acyl ghrelin. No Fos (*dark blue-black*) expression in the arcuate nucleus (F) or the PVN (G) after des-acyl ghrelin administration. H, Costaining (*arrows*) of Fos (*dark blue-black*) and orexin (*brown*) in rats given des-acyl ghrelin. The *inset* is a higher magnification of Fos expression in an orexin-expressing neuron. I, No expression of Fos (*dark blue-black*) is observed in MCH-expressing neurons (*brown*) after des-acyl ghrelin administration. J, Fos expression (*dark blue-black*) in the LHA of GHS-R-deficient mice given des-acyl ghrelin. K, No Fos expression (*brown*) in the arcuate nucleus of GHS-R-deficient mice given des-acyl ghrelin. L, Costaining of Fos (*green*) and orexin (*red*) in the LHA neurons of GHS-R-deficient mice given des-acyl ghrelin. 3V, Third ventricle; Arc, arcuate nucleus; f, fornix. *Scale bars*, A, D, 200 μm; B, C, E, F, H, G, H, I, J, K, L, 50 μm.

(Amersham, Buckinghamshire, UK). The experiment was concluded by treatment with 60 mM KCl to induce depolarization.

Statistic analysis

Data (mean ± SEM) were analyzed by ANOVA and the *post hoc* Scheffé's F test. Differences were considered to be significant when the P values were less than 0.05.

Results

Des-acyl ghrelin-induced food intake

The icv administration of either ghrelin or des-acyl ghrelin to rats stimulated food intake (Fig. 2A). Although ghrelin increased food intake for at least 2 h after administration, the effect of des-acyl ghrelin lasted for only 1 h. Des-acyl ghrelin also significantly increased the early dark-phase food intake in rats in comparison to saline administration (des-acyl ghrelin, 2.04 ± 0.37 g/30 min; saline, 0.96 ± 0.23 g/30 min, $P <$ 0.05). Next, we studied the effect of peripherally administered des-acyl ghrelin on feeding. A single iv administration of ghrelin significantly increased feeding, whereas des-acyl ghrelin did not (Fig. 2B). An ip administration of des-acyl ghrelin to C57BL/6 mice fed *ad libitum* did not increase food intake (des-acyl ghrelin 1 nmol, 0.05 ± 0.03 g/2 h; 5 nmol, 0.04 ± 0.02 g/2 h; saline, 0.05 ± 0.02 g/2 h).

Fos expression

Intracerebroventricular administration of ghrelin induced Fos expression in the LHA and arcuate nucleus (Fig. 3A). In contrast, icv administration of des-acyl ghrelin induced Fos in the LHA, but not the arcuate nucleus or the paraventricular nucleus (PVN) (Fig. 3, D–G). By double immunohistochemistry, ghrelin induced Fos in 32 ± 7% of orexin-immunoreactive neurons (Fig. 3B). Des-acyl ghrelin induced Fos in 22 ± 5% of orexin-immunoreactive neurons (Fig. 3H). In the LHA, neither ghrelin nor des-acyl ghrelin induced Fos in MCH-immunoreactive neurons (Fig. 3, C and I). Intracerebroventricular administration of des-acyl ghrelin to GHS-R-deficient mice induced Fos in the LHA, but not the arcuate nucleus (Fig. 3, J and K). In GHS-R-deficient mice, des-acyl ghrelin induced Fos expression in 28 ± 2% of orexin-immunoreactive neurons, whereas ghrelin did not induce the expression of Fos in any hypothalamic neurons (Fig. 3L).

Functional relationship between des-acyl ghrelin and orexin in feeding

We examined the effects of orexin and NPY blockades on des-acyl ghrelin-induced food intake. Both ghrelin and des-acyl ghrelin increased food intake in rats given an icv administration of control IgG (Fig. 4). Pretreatment with anti-orexin-A and -B IgGs, however, reduced ghrelin-induced food intake by 29% from the amounts seen in rats given control IgG and ghrelin, whereas pretreatment with anti-orexin-A and -B IgGs completely abolished des-acyl ghrelin-induced food intake. Whereas pretreatment with anti-NPY IgG reduced ghrelin-induced feeding in rats in comparison to rats given control IgG and ghrelin, anti-NPY IgG did not affect des-acyl ghrelin-induced feeding in comparison to rats given control IgG and des-acyl ghrelin (Fig. 4).

Orexin-deficient mice were used to verify the functional relationship between des-acyl ghrelin and orexin in feeding regulation. Although ghrelin induced food intake in orexin-deficient mice, the potency of this induction in these mice was significantly reduced from that seen in wild-type littermates (Fig. 5A). Des-acyl ghrelin stimulated feeding in wild-type mice, but not in orexin-deficient mice (Fig. 5A). To investigate whether des-acyl ghrelin regulates feeding through the GHS-R, we gave an icv administration of des-acyl ghrelin to GHS-R-deficient mice. Des-acyl ghrelin, but

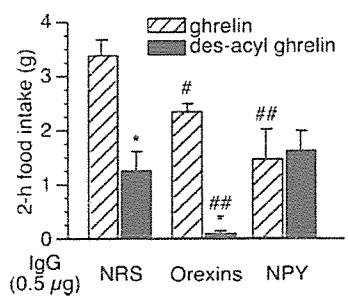


FIG. 4. The effect of antiorexin-A and -B (each at 0.25 μg) and anti-NPY (0.5 μg) IgGs on ghrelin- or des-acyl ghrelin-induced feeding. Ghrelin or des-acyl ghrelin (each at 200 pmol) was given to rats 3 h after icv administration of IgG. The 2-h food intake was then measured. NRS, Normal rabbit serum. *, $P < 0.01$ *vs.* ghrelin; #, $P < 0.05$; ##, $P < 0.01$ *vs.* NRS IgG.

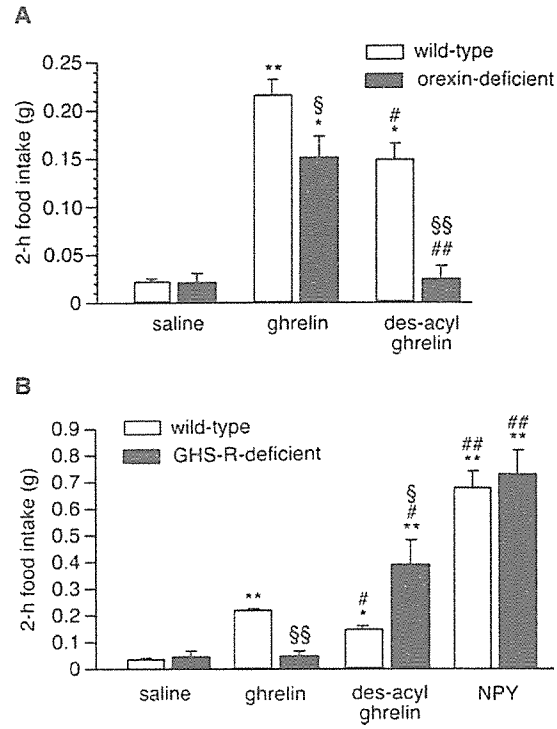


FIG. 5. A, Effect of icv administration of ghrelin or des-acyl ghrelin (each at 200 pmol) on 2-h food intake in orexin-deficient mice. *, $P < 0.05$, **; $P < 0.01$ *vs.* saline; #, $P < 0.05$; ##, $P < 0.01$ *vs.* ghrelin, §, $P < 0.05$, §§, $P < 0.01$ *vs.* wild-type mice. B, Effect of icv administration of ghrelin (200 pmol), des-acyl ghrelin (200 pmol), or NPY (1 nmol) on 2-h food intake in GHS-R-deficient mice. *, $P < 0.05$; **, $P < 0.001$ *vs.* saline; #, $P < 0.001$ *vs.* ghrelin; §, $P < 0.05$; §§, $P < 0.01$ *vs.* wild-type mice.

not ghrelin, stimulated feeding in GHS-R-deficient mice (Fig. 5B). Des-acyl ghrelin-induced feeding in GHS-R-deficient mice was more potent than that induced in wild-type littermates. NPY was used as a positive control to evaluate the orexigenic effects on GHS-R-deficient mice. NPY-induced food intake was similar in both GHS-R-deficient mice and their wild-type littermates (Fig. 5B).

Intracerebroventricular administration of des-acyl ghrelin increased food intake in ddy mice fed *ad libitum* (Fig. 6A). Neither icv nor ip administration of des-acyl ghrelin suppressed food intake in ddy mice that had fasted for 16 h (Fig. 6, B and C).

Cytosolic $[Ca^{2+}]i$ response in orexin neurons

We studied the cytosolic $[Ca^{2+}]i$ response of orexin-expressing neurons to des-acyl ghrelin. Some cells dispersed from the rat LHA showed increased cytosolic $[Ca^{2+}]i$ in response to des-acyl ghrelin administration (Fig. 7, A and B). These cells showed orexin immunoreactivity by immunohistochemistry (Fig. 7C).

Locomotor activity

We examined the effect of des-acyl ghrelin on locomotor activity. Intracerebroventricular administration of des-acyl

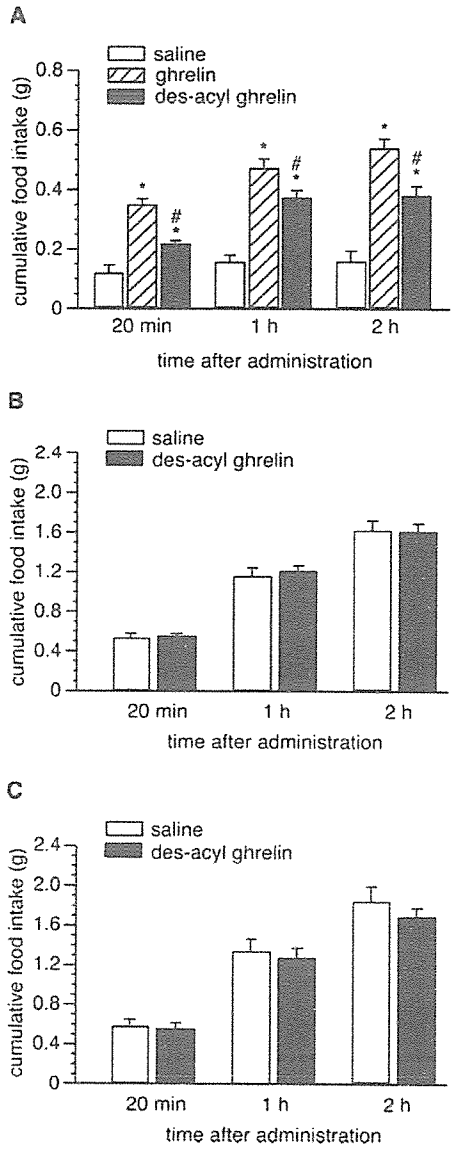


FIG. 6. Effect of des-acyl ghrelin on food intake in ddy mice. A, Intracerebroventricular administration of ghrelin or des-acyl ghrelin (each at 1 nmol) at 1000 h to mice *ad libitum*. *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ *vs.* saline; #, $P < 0.01$ *vs.* ghrelin. Neither icv (B) nor ip (C) administration of des-acyl ghrelin (1 nmol) suppressed feeding in mice that had fasted for 16 h.

ghrelin to rats significantly increased locomotor activity compared with saline administration (Fig. 8). Orexin-A also significantly increased locomotor activity in these rats.

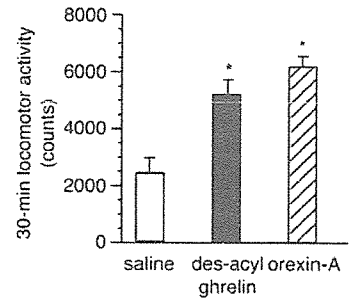


FIG. 8. Effects of icv administration of des-acyl ghrelin or orexin-A (each at 1 nmol) on locomotor activity in rats. *, $P < 0.05$ *vs.* saline.

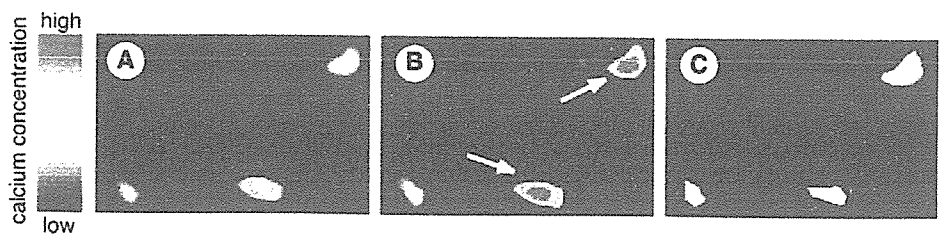
GH response

We studied the release of GH in response to peripheral des-acyl ghrelin administration. Intravenous administration of ghrelin elicited a marked increase in plasma GH levels, with the peak occurring 15 min after administration (Fig. 9A). Intravenous administration of des-acyl ghrelin did not stimulate GH release. We examined the effect of des-acyl ghrelin on GH release from isolated samples of the rat anterior pituitary. GH concentrations in the culture medium of the anterior pituitary cultures increased in response to ghrelin administration, but not to des-acyl ghrelin administration (Fig. 9B).

Discussion

At present, the amino acid sequences of ghrelin peptides in 21 species of fish, amphibians, birds, and mammals have been determined (9). All of the ghrelin molecules identified possess a serine or threonine as the third amino acid residue. A hydroxyl group of this amino acid forms an ester with a monocarboxylic acid of medium-chain fatty acid (6). Ghrelin peptide is present in the stomach of humans, rats, and mice as two major molecular forms: ghrelin and des-acyl ghrelin (6). In the plasma, ghrelin accounts for only 2–20% of total ghrelin immunoreactivity (6, 7, 28–30). This is likely due to the shorter half-life of ghrelin than that of des-acyl ghrelin because plasma ghrelin rapidly disappears from the circulation because of binding to the GHS-R in the systemic tissues (31). Deacylation of ghrelin to des-acyl ghrelin, which rapidly occurs in the plasma, is also responsible for the reduced half-life of ghrelin. Two enzymes involved in the deacylation of ghrelin have been identified: high-density lipoprotein-associated paraoxonase functions in the plasma, whereas lysophospholipase I, a thioesterase active against palmitoyl-Gsα and plamitoyl-coenzyme A, functions in the stomach (32–34). In contrast, the enzyme that catalyzes the acyl modification of ghrelin has not been identified.

FIG. 7. Effect of des-acyl ghrelin on $[Ca^{2+}]i$ response in isolated orexin-expressing neurons. A, A picture shows the basal level of $[Ca^{2+}]i$ in neurons (*blue*) before administration of des-acyl ghrelin. B, Des-acyl ghrelin increased cytosolic $[Ca^{2+}]i$ in two neurons (*arrows*). C, Immunostaining of orexin-expressing neurons (*white*) after the measurement of $[Ca^{2+}]i$ response.



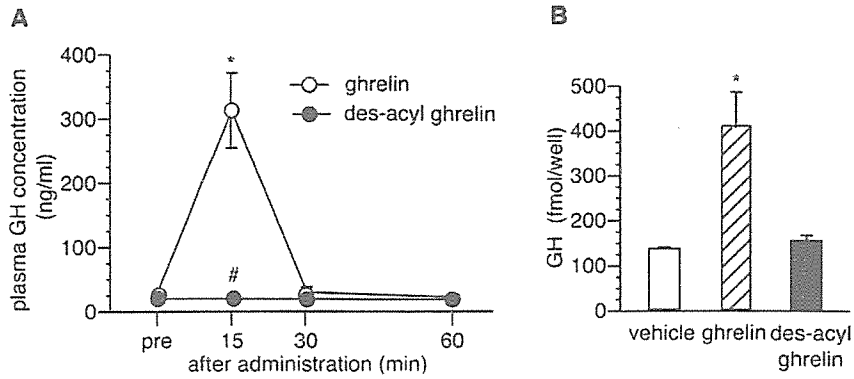


FIG. 9. A, Effect of iv administration of ghrelin or des-acyl ghrelin (each at 1.5 nmol) on the plasma GH concentration in rats. *, $P < 0.001$ *vs.* preadministration; #, $P < 0.001$ *vs.* ghrelin at the same time point. B, Effect of ghrelin or des-acyl ghrelin administration (each at 200 pmol) on GH release from rat pituitary gland (n = 6 per group). *, $P < 0.001$ *vs.* control vehicle.

Acylation of ghrelin is essential for ghrelin's GH-releasing activity (1, 9, 10); several recent *in vitro* studies have shown that des-acyl ghrelin exhibits biological activities on the cell proliferation and metabolism of cardiomyocytes, adipocytes, myocytes, and myelocytes (18–22). Although many of these cells did not express the GHS-R, des-acyl ghrelin bound to their cell membranes (18–20). We here examined the orexigenic activity of des-acyl ghrelin. We confirmed the purity of ghrelin and des-acyl ghrelin by several biochemical methods before using these substances in feeding experiments. Both ghrelin and des-acyl ghrelin were completely pure by RP-HPLC, ion-exchange-HPLC, capillary zone electrophoresis, and mass spectrometry. Des-acyl ghrelin did not stimulate GH release when either peripherally administered to rats or applied directly to the rat pituitary *in vitro*. Intracerebroventricular administration of des-acyl ghrelin significantly induced feeding during both the light and dark phases in rats. Intracerebroventricular administration of des-acyl ghrelin also increased food intake in GHS-R-deficient mice and their wild-type littermates. Two recent studies reported the anorexic activity of des-acyl ghrelin in rats and mice (35, 36). In these studies, ip administration of des-acyl ghrelin suppressed feeding in rats had fasted for 16 h (35). Both ip and icv administrations of des-acyl ghrelin suppressed feeding in ddy mice that had been fasting for 16 h; icv administration of des-acyl ghrelin did not significantly change the light phase food intake in ddy mice fed *ad libitum* (36). These studies described that icv and ip administrations of des-acyl ghrelin expressed Fos in the PVN neurons, presumably corticotropin-releasing factor (CRF) neurons (35, 36). We also examined the effect of des-acyl ghrelin on feeding in ddy mice because the anorexic effect noted above contrasted the orexigenic effect observed in C57BL/6 mice. An icv administration of des-acyl ghrelin significantly increased the light phase food intake of ddy mice fed *ad libitum*. The icv administration of des-acyl ghrelin did not suppress food intake in ddy mice that had fasted for 16 h. In addition, an icv administration of des-acyl ghrelin did not express Fos in any neurons of the PVN where CRF-producing neurons are present. Because the effects of peptides in feeding experiments are hampered by unsatisfactory habituation (37, 38), all of the rats and mice used in these experiments were satisfactorily acclimated to handling before ip and icv injections. We do not know why our findings conflicted with previous results; des-acyl ghrelin, however, reproducibly stimulated feeding in rats, C57BL/6 mice and ddy mice. Des-acyl ghrelin, which was synthesized in the rat hypothalamus, was released in response to fasting (7). The ratio of des-acyl ghrelin to ghrelin in the rat hypothalamus was 2:1 under *ad libitum* conditions, and this ratio did not change in upon fasting. Des-acyl ghrelin, as well as ghrelin, may serve as orexigenic peptides in the hypothalamus.

Ghrelin-producing neurons localize to the hypothalamic arcuate nucleus and adjacent to the third ventricle between the dorsal, ventral, paraventricular, and arcuate hypothalamic nuclei of rats and mice (1, 24). Ghrelin fibers synapse on NPY/AgRP neurons in the arcuate nucleus and orexin neurons in the LHA (16, 24, 39). We investigated the signaling events downstream of des-acyl ghrelin that stimulates feeding. Fos expression, induced by icv administration of des-acyl ghrelin, was restricted to orexin-expressing neurons in the LHA. Des-acyl ghrelin-induced food intake was completely abolished in rats by pretreatment with antiorexin IgG, but not anti-NPY IgG or control serum IgG. Des-acyl ghrelin did not stimulate feeding in orexin-deficient mice. These results indicate that des-acyl ghrelin-induced feeding is mediated by the activation of the orexin pathway. Orexin-A and -B are hypothalamic peptides functioning in the regulation of feeding, energy homeostasis, and arousal (40). Approximately 3000 orexin-expressing neurons are present in the LHA of rats and mice. Orexin-positive nerve fibers have wide projections onto a variety of brain regions, such as the arousal centers in the forebrain and brain stem and the feeding center within the hypothalamus (40). Orexin-expressing neurons are heterogeneous in their anatomical projections and physiological functions, playing multifaceted roles in the brain. Ghrelin fibers project to orexin-positive neurons (16) and ghrelin stimulated elecrophysiological activity of isolated orexin neurons in the whole-cell patch-clamp study (41); we demonstrate here that ghrelin and des-acyl ghrelin acted on orexin-expressing neurons and that des-acyl ghrelin increased intracellular calcium concentration in isolated orexin neurons. There are three possible subtypes of orexin neurons: those that express the GHS-R as a receptor for ghrelin, those expressing an as-yet unknown target protein of des-acyl ghrelin, neurons possessing both proteins. Orexin also functions to maintain wakefulness (40). We examined the effect of des-acyl ghrelin on locomotor activity. As expected, icv administration of des-acyl ghrelin increased locomotor activity, suggesting that des-acyl ghrelin may increase wake-

fulness and locomotor activity for food seeking by stimulating orexin neurons.

We next investigated the functional relationship between des-acyl ghrelin and the GHS-R using GHS-R-deficient mice. The icv administration of des-acyl ghrelin to GHS-R-deficient mice induced food intake and Fos expression in orexin-expressing neurons. The icv administration of ghrelin to GHS-R-deficient mice did not stimulate food intake. Des-acyl ghrelin did not bind to GHS-R-expressing Chinese hamster ovary cells and did not inhibit the binding of ghrelin to rat pituitary culture cells expressing the GHS-R (1, 10, 42), implying that des-acyl ghrelin does not compete with ghrelin for the binding to the GHS-R. Thus, des-acyl ghrelin is thought to stimulate feeding via a mechanism independent of the GHS-R.

A number of gastrointestinal peptides transmit satiety or starvation signals to the nucleus of the solitary tract via the vagal afferents and/or to the hypothalamus via the bloodstream (43). Although iv administration of ghrelin stimulated both vagal afferents and feeding, iv administration of des-acyl ghrelin affected neither (44). Peripheral administration of des-acyl ghrelin to rats and mice did not affect feeding. Receptors on vagal afferents are generated by nodose ganglion neurons, transported to the nerve terminals through axonal transport (45). These results indicate that a receptor or a target protein binding to des-acyl ghrelin is not expressed in nodose ganglion neurons. The plasma concentration of des-acyl ghrelin increased upon fasting (7). The peripheral des-acyl ghrelin does not act to suppress feeding.

In summary, centrally administered des-acyl ghrelin increased feeding through activation of the orexin pathway. In addition to its peripheral actions, which include cell proliferation, inhibition of apoptosis, and fat metabolism (18–22), des-acyl ghrelin may function in hypothalamic feeding regulation. Central administration of des-acyl ghrelin to GHS-R-deficient mice stimulated feeding, suggesting that des-acyl ghrelin acts on a target protein that is specific for des-acyl ghrelin and independent of the GHS-R. Ghrelin and des-acyl ghrelin act in the regulations of peripheral cell functions through a common putative target protein (18–22). Ghrelin and des-acyl ghrelin function as orexigenic peptides in the hypothalamus. Des-acyl ghrelin may have basal effects of ghrelin-related peptides. Further studies examining the physiological and neuroanatomical interactions between des-acyl ghrelin and its target will establish roles of ghrelin peptides in the regulation of feeding and energy homeostasis.

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References

1. **Kojima M, Hosoda H, Date Y, Nakazato M, Matsuo H, Kangawa K** 1999 Ghrelin is a novel growth hormone releasing acylated peptide from stomach. Nature 402:656–660
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 SS, Chaung LY, 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 rrelease. Science 273:974–977
3. **MaKee KK, Palyha OC, Feighner SD, Hreniuk DL, Tan CP, Phillips MS, Smith RG, Van der Ploeg LHT, Howard AD** 1997 Molecular analysis of rat pituitary and hypothalamus growth hormone secretagogue receptors. Mol Endocrinol 11:415–423
4. **Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, Matsukura S, Kangawa K, Nakazato M** 2000 Ghrelin, a novel growth hormone-releasing acylated peptide, is synthesized in a distinct endocrine cell type in the gastrointestinal tracts of rats and humans. Endocrinology 141: 4255–4261
5. **Dornonville de la Cour C, Björkqvist M, Sandvik AK, Bakke I, Zhao CM, Chen D, Håkanson R** 2001 A-like cells in the rat stomach contain ghrelin and do not operate under gastrin control. Regul Pept 99:141–150
6. **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 281:1220–1225
7. **Sato T, Fukue Y, Teranishi H, Yoshida Y, Kojima M** 2005 Molecular forms of hypothalamic ghrelin and its regulation by fasting and 2-deoxy-D-glucose administration. Endocrinology 146:2510–2516
8. **Mondal MS, Date Y, Yamaguchi H, Toshinai K, Tsuruta T, Kangawa K, Nakazato M** 2005 Identification of ghrelin and its receptor in neurons of the rat arcuate nucleus. Regul Pept 126:55–59
9. **Kojima M, Kangawa K** 2005 Ghrelin: Structure and function. Physiol Rev 85:495–522
10. **Muccioli G, Tschöp M, Papotti M, Deghenghi R, Heiman M, Ghigo E** 2002 Neuroendocrine and peripheral activities of ghrelin: implication in metabolism and obesity. Eur J Pharmacol 440:235–254
11. **Matsumoto M, Hosoda H, Kitajima Y, Morozumi N. Minamitake Y, Tanaka S, Matsuo H, Kojima M, Hayashi Y, Kangawa K** 2001 Structure-activity relationship of ghrelin: pharmacological study of ghrelin peptides. Biochem Biophys Res Commun 287:142–146
12. **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
13. **Shintani M, Ogawa Y, Ebihara K, Aizawa-Abe M, Miyanaga F, Takaya K, Hayashi, T, Inoue G, Hosoda K, Kojima M, Kangawa K, Nakao K** 2001 Ghrelin, an endogenous growth hormone secretagogue, is a novel orexigenic peptide that antagonizes leptin action through the activation of hypothalamic neuropeptide Y/Y1 receptor pathway. Diabetes 50:227–232
14. **Kamegai J, Tamura H, Shimizu T, Ishii S, Sugihara H, Wakabayashi I** 2001 Chronic central infusion of ghrelin increases hypothalamic neuropeptide Y and Agouti-related protein mRNA levels and body weight in rats. Diabetes 50: 2438–2443
15. **Bagnasco M, Tulipano G, Melis MR, Argiolas A, Cocchi D, Muller EE** 2003 Endogenous ghrelin is an orexigenic peptide acting in the arcuate nucleus in response to fasting. Regul Pept 111:161–167
16. **Toshinai K, Date Y, Murakami N, Shimada M, Mondal MS, Shimbara T, Guan LL, Wand QP, Funahashi H, Sakurai T, Shioda S, Matsukura S, Kanagawa K, Nakazato M** 2003 Ghrelin-induced food intake is mediated via the orexin pathway. Endocrinology 144:1506–1512
17. **Sun Y, Wang P, Zheng H, Smith RG** 2003 Ghrelin stimulation of growth hormone release and appetite is mediated through the growth hormone secretagogue receptor. Proc Natl Acad Sci USA 101:4679–4684
18. **Baldanzi G, Filigheddu N, Cutrupi S, Catapano F, Bonissoni S, Fubini A, Malan D, Baj G, Granata R, Broglio F, Papotti M, Surico N, Bussolino F, Isgaard J, Deghenghi R, Sinigaglia F, Prat M, Muccioli G, Ghigo E, Graziani A** 2004 Ghrelin and de-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and PI 3-kinase/AKT. J Cell Biol 159:1029–1037
19. **Cassoni P, Ghe C, Marrocco T, Tarabra E, Allia E, Catapano F, Deghenghi R, Ghigo E, Papotti M, Muccioli G** 2004 Expression of ghrelin and biological activity of specific receptors for ghrelin and des-acyl ghrelin in human prostate neoplasms and related cell line. Eur J Endocrinol 150:173–184
20. **Muccioli G, Pons N, Ghe C, Catapano F, Granata R, Ghigo E** 2004 Ghrelin and des-acyl ghrelin both inhibit isoproterenol-induced lipolysis in rat adipocytes via a non-type 1a growth hormone secretagogue receptor. Eur J Pharmacol 498:27–35

21. **Bedendi I, Allatti G, Marcantoni A, Malan D, Catapano F, Ghe C, Deghenghi R, Ghigo E, and Muccioli G** 2003 Cardiac effects of ghrelin and its endogenous derivatives des-octanoyl ghrelin and des-Gln14-ghrelin. Eur J Pharmacol 476: 87–95
22. **Thompson NM, Gill DAS, Davies R, Loveridge N, Houston PA, Robinson ICAF, Wells T** 2004 Ghrelin and des-octanoyl ghrelin promote adipogenesis directly *in vivo* by a mechanism independent of the type 1a growth hormone secretagogue receptor. Endocrinology 145:234–242
23. **Fraley GS, Ritter S** 2003 Immunolesion of norepinephrine and epinephrine afferents to medial hypothalamus alters basal and 2-deoxy-D-glucose-induced neuropeptide Y and agouti gene-related protein messenger ribonucleic acid expression in the arcuate nucleus. Endocrinology 144:75–83
24. **Cowley MA, Smith RG, Diano S, Tschöp M, Pronchuk N, Grove KL, Strasburger CJ, Bidlingmaier M, Esterman M, Heiman ML, Garcia-Segura LM, Nillni EA, Mendez P, Low MJ, Sotonyi P, Friedman JM, Liu H, Pinto S, Colmers WF, Cone RD, Horvath TL** 2003 The distribution and mechanism of action of ghrelin in the CNS demonstrates a novel hypothalamic circuit regulating energy homeostasis. Neuron 37:649–661
25. **Sagar SM, Sharp FR, Curran T** 1988 Expression of c-fos protein in brain: metabolic mapping at the cellular level. Science 240:1328–1331
26. **Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, Richardson JA, Williams SC, Xiong Y, Kisanuki Y, Fitch TE, Nakazato M, Hammer RE, Saper CB, Yanagisawa M** 1999 Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 98:437–451
27. **Date Y, Ueta Y, Yamashita H, Yamaguchi H, Matsukura S, Kangawa K, Sakurai T, Yanagisawa M, Nakazato M** 1999 Orexins, orexigenic hypothalamic peptides, interact with autonomic, neuroendocrine and neuroregulatory systems. Proc Natl Acad Sci USA 96:748–753
28. **Paxinos G, Watson C** 1998 The rat brain in stereotaxic coordinates. 4th ed. San Diego: Academic Press
29. **Nishi Y, Hiejima H, Hirohara M, Sato T, Kangawa K, Kojima M** 2005 Developmental changes in the pattern of ghrelin's acyl modification and the levels of acyl-modified ghrelin in murine stomach. Endocrinology 146:2709–2715
30. **Akamizu T, Shinomiya T, Irako T, Fukunaga M, Nakai Y, Nakai Y, Kangawa K** 2005 Separate measurement of plasma levels of acylated and desacyl ghrelin in healthy subjects using a new direct ELISA assay. J Clin Endocrinol Metab 90:6–9
31. **Akamizu T, Takaya K, Irako T, Hosoda H, Teramukai S, Matsuyama A, Tada H, Miura K, Shimizu A, Fukushima M, Yokode M, Tanaka K, Kangawa K** 2004 Pharmacokinetics, safety, and endocrine and appetite effects of ghrelin administration in young healthy subjects. Eur J Endocrinol 150:447–455
32. **Beaumont NJ, Skinner VO, Tan TM, Ramesh BS, Byrne DJ, MacColl GS, Keen JN, Bouloux PM, Mikhailidis DP, Bruck-dorfer KR, Vanderpump MP, Srai KS** 2003 Ghrelin can bind to a species of high density lipoprotein associated with paraxonase. J Biol Chem 278:8877–8880
33. **Shanado Y, Kometani M, Uchiyama H, Koizumi S, Teno N** 2004 Lysophospholipase I identified as a ghrelin deacylation enzyme in rat stomach. Biochem Biophys Res Commun 325:1487–1494
34. **Duncan JA, Gilman AG** 1998 A cytoplasmic acyl-protein thioesterase that removes plamitate from G protein α subunits and p21(RAS). J Biol Cehm 273:15830–15837
35. **Chen CY, Imui A, Asakawa A, Fujino K, Kato I, Chen CC, Ueno N, Fujimiya M** 2005 Des-acyl ghrelin acts by CRF type 2 receptors to disrupt fasted stomach motility in conscious rats. Gastroenterology 129:8–25
36. **Asakawa A, Inui A, Fujimiya M, Sakamaki R, Shinfuku N, Ueta Y, Meguid MM, Kasuga M** 2005 Stomach regulates energy balance via acylated ghrelin and desacyl ghrelin. Gut 54:18–24
37. **Tschöp M, Castaneda TR, Joost HG, Thone-Reineke C, Ortmann S, Klaus S, Hagan MM, Chandler PC, Oswald KD, Benoit SC, Seeley RJ, Kinzig KP, Moran TH, Beck-sickinger AG, Koglin N, Rodgers RJ, Blundell JE, Ishii Y, Beattie AH, Holch P, Allison DB, Raun K, Madsen K, Wulff BS, Stidsen CE, Birringer M, Kreuzer OJ, Schindler M, Arndt K, Rudolf K, Mark M, Deng XY, Whitcomb DC, Halem H, Taylor J, Dong J, Datta R, Culler M, Craney S, Flora D, Smiley D, Heiman ML** 2004 Physiology: does gut hormone PYY3–36 decrease food intake in rodents? Nature 430:1–4
38. **Halatchev IG, Ellacott KL, Fan W, Cone RD** 2004 Peptide YY3–36 inhibits food intake in mice through a melanocortin-4 receptor-independent mechanism. Endocrinology 145:2585–2590
39. **Jobst EE, Enriori PJ, Cowley MA** 2004 The electrophysiology of feeding circuits. Trends Endocrinol Metab 15:488–499
40. **Sakurai T** 2005 Reverse pharmacology of orexin: from an orphan GPCR to integrative physiology. Regul Pept 126:3–10
41. **Yamanaka A, Beuckmann CT, Willie JT, Hara J, Tsujino N, Mieda M, Tominaga M, Yagami K, Sugiyama F, Goto K, Yanagisawa M, Sakurai T** 2003 Hypothalamic orexin neurons regulate arousal according to energy balance in mice. Neuron 38:701–713
42. **Muccioli G, Papotti M, Locatelli V, Ghigo E, Deghenghi R** 2000 Binding of 125I-labeled ghrelin to membranes from human hypothalamus and pituitary gland. J Endocrinol Invest 24:RC7–RC9
43. **Konturek SJ, Konturek JW, Pawlik T, Brzozowski T** 2004 Brain-gut axis and its role in the control of food intake. J Physiol Pharmacol 55:137–154
44. **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. Gastroenterology 123:1120–1128
45. **Date Y, Toshinai K, Koda S, Miyazato M, Shimbara T, Tsuruta T, Niijima A, Kangawa K, Nakazato M** 2005 Peripheral interaction of ghrelin and cholecystokinin on feeding regulation. Endocrinology 146:3518–3525

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Changes in plasma ghrelin levels, gastric ghrelin production, and body weight after *Helicobacter pylori* cure

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Background. Ghrelin is a body weight-regulating peptide produced and secreted primarily by the gastric mucosa. *Helicobacter pylori* infection impairs gastric ghrelin production, leading to a lower plasma ghrelin concentration. However, the effect of *H. pylori* eradication on plasma ghrelin levels and its relation to body weight change after *H. pylori* cure are still uncertain. We examined the association of plasma ghrelin levels with gastric ghrelin production and body weight change before and after *H. pylori* eradication. ***Methods.*** Plasma ghrelin concentrations, gastric ghrelin expression, and body weight were determined in a total of 134 consecutive individuals before and 12 weeks after successful *H. pylori* eradication. Gastric ghrelin expression was evaluated by determining mRNA expression levels and the number of ghrelin-producing cells in gastric mucosa biopsy specimens by real-time reverse transcriptase-polymerase chain reaction and immunohistochemistry, respectively. ***Results.*** Plasma ghrelin concentration increased in 50 patients and decreased in 84 patients after *H. pylori* eradication. After *H. pylori* cure, however, gastric preproghrelin mRNA expression was increased nearly fourfold ($P < 0.0001$), and the number of ghrelin-positive cells was increased or unchanged. In contrast, plasma ghrelin changes after *H. pylori* cure were inversely correlated with both body weight change ($P < 0.0001$) and initial plasma ghrelin levels ($P < 0.0001$). ***Conclusions.*** Changes in plasma ghrelin concentrations before and after *H. pylori* cure were inversely correlated with body weight change and initial plasma ghrelin levels but not with gastric ghrelin production in Japanese patients.

Key words: plasma ghrelin, gastric ghrelin, body weight, *Helicobacter pylori* cure

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Introduction

Ghrelin is a strong growth hormone-releasing peptide that controls food intake, facilitates fat storage, and regulates short- and long-term body weight.[1–5] The majority of circulating ghrelin is produced in the gastric mucosa.[6] Plasma ghrelin level is regulated by multiple factors, including food intake, body weight, and gastric ghrelin production.[7] In the short term, the plasma ghrelin concentration is increased after fasting and decreased after meals.[8,9] In the long term, the plasma ghrelin concentration is lower in obese and higher in lean subjects than in normal weight subjects.[8,10] Moreover, diet-induced or exercise-induced body weight loss increases the plasma ghrelin concentration,[5,11,12] and weight gain decreases elevated plasma ghrelin concentrations in anorexia nervosa.[13] In addition, attenuation of gastric ghrelin production through gastrectomy leads to a decrease in the plasma ghrelin concentration.[5]

Helicobacter pylori is a major cause of gastritis, peptic ulcer disease, and gastric carcinoma.[14–16] Eradication of *H. pylori* improves gastritis[16,17] and decreases the recurrence rate of peptic ulcer disease.[14,18] Much attention has recently been directed to the relationship between obesity and *H. pylori* infection. Previous studies showed that *H. pylori* infection is inversely related to obesity. For example, Wu et al.[19] reported that the seropositivity of *H. pylori* infection was significantly lower in morbidly obese patients.[19] Furuta et al.[20] have shown body weight gain after *H. pylori* cure. As ghrelin is mainly synthesized and secreted by the gastric mucosa, the inverse effect of *H. pylori* infection on body weight has been attributed to the difference in plasma ghrelin concentrations in patients with and without *H. pylori* infection.[21] This hypothesis states that the increase of gastric ghrelin production after *H. pylori* cure may elevate the plasma ghrelin concentration, resulting in a body weight gain. However, the hypothesis is still controversial.[22–24] We thus attempted to examine the effect of *H. pylori* eradi-

Table 1. Clinical characteristics before eradication therapy in patients with decreased or increased plasma ghrelin levels after treatment

Variable	Total (n = 134)	Change in plasma ghrelin levels after treatment		
		Decrease (n = 84)	Increase (n = 50)	P value*
Age	48.8 ± 0.6	48.3 ± 0.7	49.7 ± 0.8	0.25
Body mass index	22.5 ± 0.3	22.2 ± 0.3	23.0 ± 0.4	0.18
Initial plasma ghrelin (fmol/ml)	127 ± 7	149 ± 8	89 ± 8	<0.0001
Pepsinogen I (ng/ml)	73 ± 3	76 ± 4	68 ± 4	0.23
Pepsinogen I/II ratio	2.69 ± 0.10	2.69 ± 0.12	2.69 ± 0.16	0.86
Gastric preproghrelin mRNA	95 ± 1.5	12.8 ± 2.8	7.4 ± 1.9	0.17
Total cholesterol (mg/dl)	196 ± 3	197 ± 4	193 ± 4	0.46
HDL-cholesterol (mg/dl)	59.0 ± 1.5	60.0 ± 1.6	58.3 ± 1.9	0.51
Triglycerides (mg/dl)	99 ± 5	98 ± 6	101 ± 6	0.79
Fasting blood sugar (mg/dl)	95 ± 1	95 ± 1	95 ± 2	0.97

Data are means ± standard error
HDL, high-density lipoprotein
*Difference in clinical data before treatment between subjects with decreased and those with increased plasma ghrelin after treatment

cation on plasma ghrelin concentrations in a large number of patients to elucidate their relationship with body weight changes after *H. pylori* cure. To this end, we compared plasma ghrelin levels before and after *H. pylori* eradication. Furthermore, we examined the correlation between changes in plasma ghrelin with those in gastric ghrelin production and body weight after *H. pylori* cure. We report here that plasma ghrelin concentrations by *H. pylori* eradication were inversely correlated with body weight change and initial ghrelin levels but not with gastric ghrelin production in Japanese patients.

Methods

Participants

Subjects were selected from 144 consecutive *H. pylori*-infected men with normal body mass index (BMI)(calculated as weight in kilograms divided by the square of height in meters) identified in the gastric cancer surveillance program from June 2001 to March 2003 at Tochigi, Japan. Subjects underwent endoscopic biopsy at enrollment and 12 weeks after *H. pylori* treatment. Five adjacent biopsy specimens from the greater curvatures at the midcorpus of the stomach as well as five from the antrum were obtained endoscopically from all subjects. One biopsy specimen from the corpus of the stomach and one from the antrum were cultured individually to evaluate for the presence of *H. pylori* infection. Three biopsy specimens from the corpus and three from the antrum were immediately snap frozen and stored in liquid nitrogen for later use. The remaining corpus and antral specimens were fixed and stained with hematoxylin and eosin, Giemsa, and anti-ghrelin antibody. Histological assessments were performed by a single observer (H. Osawa.). *Helicobacter pylori* infection was evaluated by bacterial culture and histological examination.

All subjects received eradication therapy, and the eradication was successful in 134 subjects (mean age ± SE; 49.2 ± 0.5 years). Indications for *H. pylori* eradication included chronic gastritis accompanied by either adenoma, a family history of gastric cancer, hyperplastic polyps, severe atrophic gastritis (62 patients), gastric ulcer (47 patients), duodenal ulcer (23 patients), or enlarged fold gastritis (2 patients). Patient characteristics are shown in Table 1. All subjects were clinically stable at the time of evaluation and had no history of eradication therapy before the study. No subjects had evidence of a cachectic state such as advanced cancer, thyroid disease, liver disease, or infection. Subjects with diabetes mellitus or renal dysfunction (serum creatinine ≥1.5 mg/dl) were excluded. Written informed consent was obtained from the participants in accordance with the Declaration of Helsinki and its later revision. The Ethics Committee of Jichi Medical University, Japan, approved this study.

Eradication therapy and data collection

A triple regimen, composed of lansoprazole 30 mg twice daily, clarithromycin 200 mg twice daily, and amoxicillin 750 mg twice daily, was given for 7 days after the endoscopic examination. Body weight was measured at 4 p.m., and blood was collected at 8 a.m., after an overnight fast, before and 12 weeks after the treatment. There were no educational schedules provided for reducing body weight during the course of study.

Hormone assay and immunohistochemistry

Plasma ghrelin was measured using a radioimmunoassay for total ghrelin developed in our laboratory. Inter-

and intra-assay variation was less than 8% and 6%, respectively. The limit of detection of this assay is 12 fmol/tube of human ghrelin. We have described previously the properties of the antiserum for ghrelin used in this study.[6–8] Plasma ghrelin levels were 194 ± 15 fmol/ml (mean ± standard error) in healthy *H. pylori*-negative subjects.[7]

Immunohistochemical analysis was performed using anti-ghrelin antiserum as described previously.[6–8] Briefly, paraffin-embedded sections of the biopsy samples taken from the greater curvature at the midcorpus of the stomach were deparaffinized in xylene, immersed in citrate buffer, heated at 120°C for 20 min in an autoclave, and left at room temperature for 60 min. After incubation with a blocking reagent (Dako Japan, Kyoto, Japan) for 10 min, individual sections were incubated with ghrelin antiserum (1:500) in a moist chamber at 4°C overnight. The slides were then washed five times with phosphate-buffered saline and incubated with dextran polymer system/peroxidase (EnVision+; Dako Japan) at 37°C for 60 min. Slides were viewed at 100× magnification and digitized with a digital HD microscope (VH 7000; Keyence, Tokyo, Japan). Immunoreactive cells in the gastric mucosa were counted and calculated as the number of positive cells per branch of the oxyntic gland.

Real-time quantitative reverse transcriptase-polymerase chain reaction

Total RNA was isolated from the biopsy specimen with ISOGEN (Nippon Gene, Tokyo, Japan). Two micrograms of total RNA from each sample was reverse-transcribed by using random nanomers and reverse transcriptase (Toyobo, Osaka, Japan) according to the manufacturer's protocol.

The level of gastric preproghrelin mRNA was measured by real-time quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) on an ABI 7700 sequence detector system (PE Applied Biosystems, Foster City, CA, USA) as reported previously.[7] Briefly, the reaction contained preproghrelin sense (5′-GGCA GGCTCCAGCTTCCT-3′), and antisense (5′-TGGC TTCTTCGACTCCTTTCTC-3′) primers and preproghrelin probe labeled with a 6-carboxyfluorescein (5′-AGCCCTGAACACCAGAGA-3′). The thermal cycling conditions comprised 50°C for 2 min and 95°C for 10 min, followed by 15 s of denaturing at 95°C and 1 min of annealing/extension at 60°C for 40 cycles. The levels of preproghrelin mRNA were calculated as the ratio of preproghrelin mRNA/GAPDH mRNA and are shown as the mean ratio (×1000) of three corpus samples. As gastric ghrelin is produced predominantly in the corpus mucosa, preproghrelin mRNA levels in the gastric corpus mucosa were compared in subjects before and 12 weeks after *H. pylori* cure.

Statistical analysis

Statistical analyses were performed using Stat View, version 5.0 (SAS Institute, Cary, NC, USA). The level of preproghrelin mRNA was expressed as the median (first quartile to third quartile). The number of immunoreactive cells and clinical data are presented as means ± standard error. The Wilcoxon rank sum test was used to compare gastric preproghrelin mRNA levels before and after *H. pylori* cure. A two-tailed paired *t* test was used to compare the plasma ghrelin levels before and after *H. pylori* cure. A two-tailed unpaired *t* test was used to compare clinical data before eradication therapy between two groups classified according to the direction of change in the plasma ghrelin level after *H. pylori* cure. A P value of less than 0.05 was accepted as statistically significant.

Results

Changes in plasma ghrelin after H. pylori *cure*

To examine the effect of *H. pylori* eradication on plasma ghrelin concentration, we first compared plasma ghrelin concentrations before and 12 weeks after treatment. Interestingly, mean plasma ghrelin concentrations decreased from 120 ± 6.3 fmol/ml before *H. pylori* eradication to 103 ± 5.3 fmol/ml after *H. pylori* eradication ($P < 0.0001$). However, the direction of change in levels after treatment differed among enrolled patients: levels increased in 50 patients and decreased in 84 patients (Fig. 1A). To elucidate the potential mechanisms leading to these disparate changes in plasma ghrelin levels after *H. pylori* eradication, we analyzed the relationship between the initial plasma ghrelin levels and their changes at 12 weeks after *H. pylori* cure (Fig. 1B). Interestingly, elevated initial plasma ghrelin concentrations decreased after the cure, but lower initial plasma ghrelin concentrations did not change significantly. Moreover, the change in the plasma ghrelin concentration after 12 weeks was inversely correlated with the initial plasma ghrelin level ($r = -0.52$, $P < 0.0001$).

Gastric ghrelin increases after H. pylori *cure*

We next examined the effect of *H. pylori* eradication on ghrelin production by the gastric mucosa. Since circulating ghrelin is produced and secreted mainly by the gastric mucosa, we analyzed the relation between the changes in the plasma ghrelin concentration and gastric

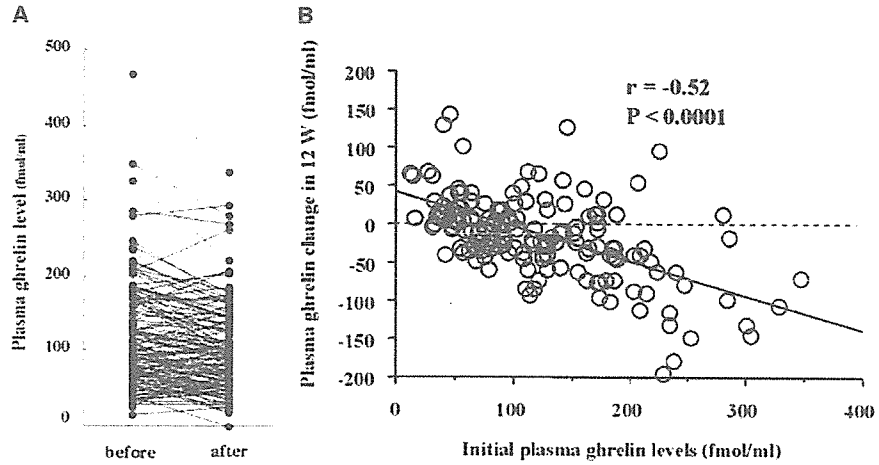

Fig. 1. A Comparison of plasma ghrelin concentrations before and 12 weeks after treatment. Plasma ghrelin levels increased in 50 patients and decreased in 84 patients. The averages before and after *Helicobacter pylori* eradication were 120 ± 6.3 and 103 ± 5.3 fmol/ml, respectively ($P < 0.0001$). **B** The relationship between the initial plasma ghrelin level and the change in plasma ghrelin at 12 weeks after *H. pylori* cure, calculated by subtracting the levels before the treatment from the levels at 12 weeks after treatment. The change at 12 weeks correlated inversely with the initial plasma ghrelin level ($r = -0.52$, $P < 0.0001$)

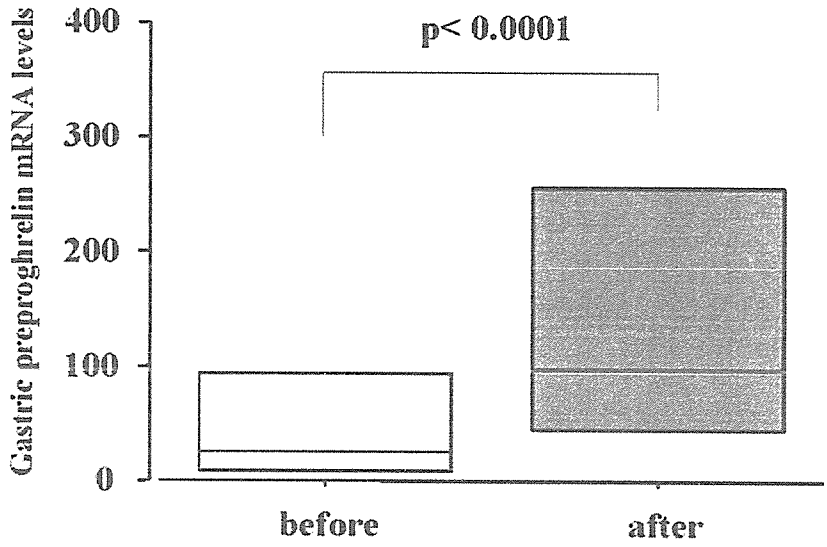

Fig. 2. Comparison of gastric preproghrelin mRNA expression levels before and 12 weeks after *H. pylori* cure. Gastric preproghrelin mRNA levels significantly increased after the eradication therapy [median (first quartile to third quartile); from 27 (8–94) to 98 (46–256); $P < 0.0001$ by Wilcoxon rank sum test]

ghrelin production after *H. pylori* eradication. We compared gastric preproghrelin mRNA expression levels in the corpus mucosa before and 12 weeks after treatment. As shown in Fig. 2, median preproghrelin mRNA expression was increased nearly fourfold ($P < 0.0001$) after *H. pylori* eradication. In addition, the number of ghrelin-positive cells was increased in 77 patients and was unchanged in 57 patients. No correlation was observed between the changes in plasma ghrelin and those in gastric preproghrelin mRNA or the number of ghrelin-positive cells after *H. pylori* cure. These data indicate that gastric ghrelin production is enhanced after *H. pylori* eradication even in patients with decreased plasma ghrelin concentrations.

Body weight changes correlate inversely with changes in the plasma ghrelin concentration

Body weight gain is a well-known effect of *H. pylori* eradication, and the plasma ghrelin concentration is influenced by body weight change.[5,12] Therefore, we examined the relationship between the changes in plasma ghrelin concentrations and body weight after *H. pylori* eradication. The change in plasma ghrelin was clearly inversely correlated with body weight change after *H. pylori* cure ($r = -0.50$, $P < 0.0001$) (Fig. 3). Plasma ghrelin decreased in 23 of 28 patients (82%) with more than 2 kg of weight gain, and in all 7 patients with more than 3 kg of weight gain. These data suggest that the plasma ghrelin concentration after *H. pylori* cure is more strongly influenced by body weight change than by the increase in gastric preproghrelin mRNA or the number of ghrelin-producing cells.

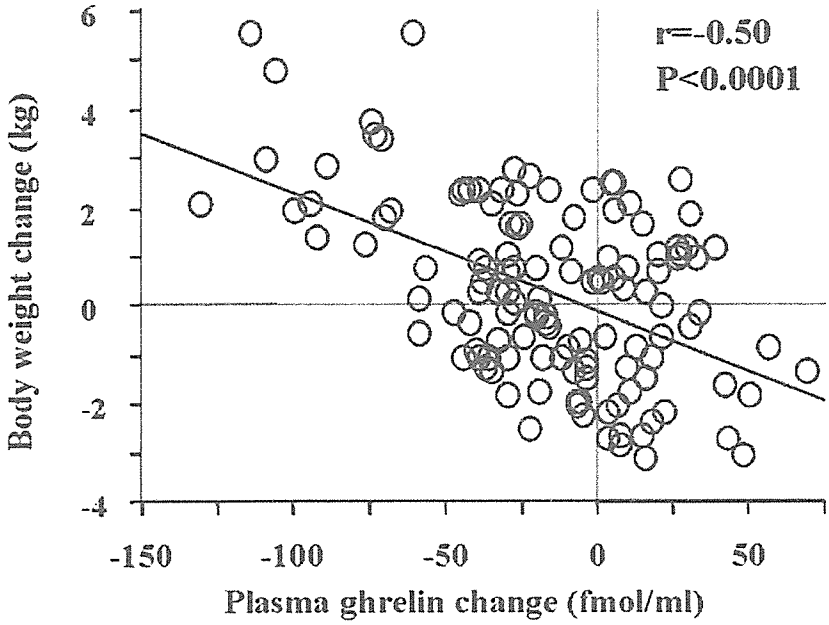

Fig. 3. The relationship between plasma ghrelin and body weight changes at 12 weeks after *H. pylori* cure. The change in plasma ghrelin levels correlated inversely with body weight changes after *H. pylori* cure ($r = -0.50$, $P < 0.0001$). Plasma ghrelin levels decreased in 82% of patients with more than 2 kg of weight gain, and in all patients with more than 3 kg of weight gain

Body weight changes correlate positively with initial plasma ghrelin concentrations

To clarify the differences in clinical characteristics among subjects in relation to changes in the plasma ghrelin level after *H. pylori* eradication, we classified the patients into two groups: patients with increased plasma ghrelin and those with decreased plasma ghrelin after *H. pylori* cure. The clinical characteristics before treatment in subjects of both groups are shown in Table 1. Initial plasma ghrelin levels were significantly higher in those whose plasma ghrelin decreased after treatment, although other clinical data showed no significant differences between the two groups. In addition, these subjects had a significantly greater increase in body weight than those with increased plasma ghrelin after treatment (0.7 ± 0.2 vs -0.3 ± 0.2 kg, $P = 0.003$), despite the short period after treatment. The initial plasma ghrelin levels and body weight changes after treatment were positively correlated ($r = 0.42$, $P < 0.0001$) (Fig. 4). In particular, 12 of 14 patients (86%) with initial ghrelin levels of more than 200 fmol/ml increased in body weight, suggesting that high levels of initial plasma ghrelin may be a predictive factor of body weight gain after *H. pylori* eradication.

Discussion

In the current study, we showed that despite an increase in gastric preproghrelin mRNA and ghrelin-producing cells after *H. pylori* eradication, the mean plasma ghrelin concentration decreased after treatment, but with wide variation. Moreover, we demonstrated that changes in plasma ghrelin concentrations after *H. pylori* eradication were inversely correlated with weight changes as well as with initial plasma ghrelin levels.

The majority of circulating ghrelin is synthesized in the gastric mucosa.[6] Gastric ghrelin production is decreased by *H. pylori* infection[7] and increased by eradication therapy.[25] As ghrelin is a body weight-regulating peptide, much attention has been paid to nutritional status and the dynamics of gastric and plasma ghrelin in response to *H. pylori* infection.[19,21] Nwokolo et al.[26] reported that plasma ghrelin levels increased after *H. pylori* cure in ten patients, a result that is inconsistent with our data. Since their report, it has been believed that plasma ghrelin concentrations increase after *H. pylori* cure owing to an increase in gastric ghrelin production, leading to body weight gain.[21,25] For example, Tatsuguchi et al.[25] reported that the number of gastric ghrelin-positive cells increased after *H. pylori* eradication, consistent with our present data. Although they did not measure plasma ghrelin concentrations or body weight after *H. pylori* eradication, they speculated, in accordance with the report of Nwokolo et al.,[26] that the increase in gastric ghrelin-positive cells would lead to increased plasma ghrelin levels, resulting in obesity. Another study, however, found that plasma ghrelin levels were unaffected.[27] In fact, the plasma ghrelin concentration is not regulated simply by the amount of gastric ghrelin production. Even in healthy humans, the plasma ghrelin concentration is tightly correlated with body weight.[8-10] Therefore, Peeters proposed in his re-

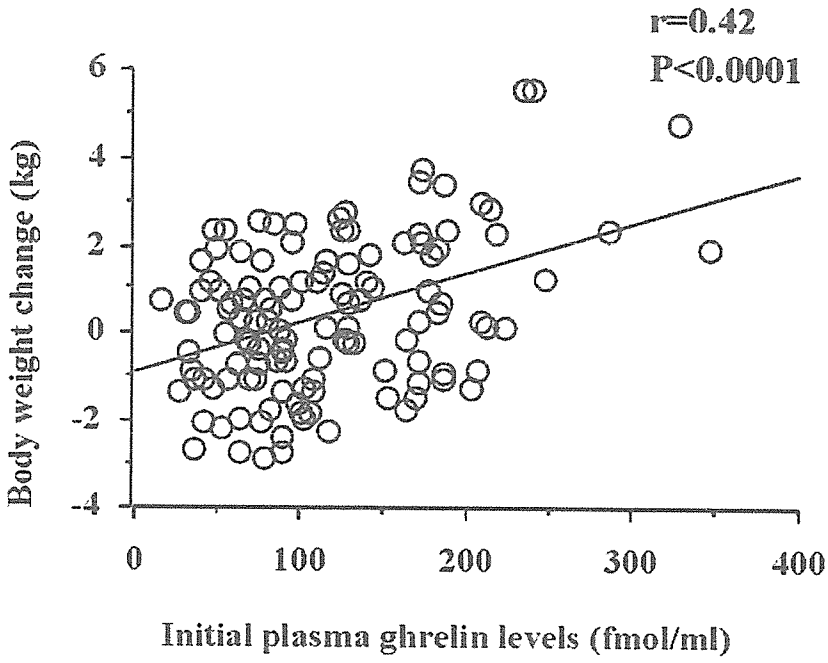

Fig. 4. The relationship between the initial plasma ghrelin level and body weight change. Initial plasma ghrelin levels correlated positively with body weight changes at 12 weeks after *H. pylori* cure ($r = 0.42$, $P < 0.0001$). Body weight increased in 86% of patients with initial plasma ghrelin levels of more than 200 fmol/ml

cent review[24] that the questions as to whether there is a rise in ghrelin following *H. pylori* eradication and whether such a rise can be an important determinant of body weight increase be reexamined. He also suggested that only a subpopulation of infected patients might show a rise in ghrelin following eradication. Thus, to clarify how plasma ghrelin concentrations changed after *H. pylori* cure and to elucidate how those changes affected body weight changes, we conducted the present study, in which we examined changes of plasma ghrelin concentration, gastric ghrelin production, and body weight before and after *H. pylori* eradication.

We clearly showed a significant inverse correlation between changes in plasma ghrelin and body weight, and found that these changes were not related to changes in gastric ghrelin production. Plasma ghrelin decreased in many patients with weight gain, in particular in all those who gained more than 3 kg of weight after treatment. Although the apparent discrepancy between the observations of Nwokolo's group and our group is difficult to explain, one might speculate that the discrepancy is related to differences in the study designs. The number of enrolled patients in our study was more than ten times the number in their study. In addition, racial differences in the enrolled subjects may account for the discrepancy. In this respect, Asians including Japanese are more prone to central adiposity than are Caucasians.[28–32] As body fat storage is closely associated with plasma ghrelin levels,[4] the racial difference in body fat distribution may account for the discrepancy.

It is important to note that, in our study, initial plasma ghrelin levels were negatively correlated with the change in plasma ghrelin levels and positively with the weight change after *H. pylori* eradication. Weight gain is a major effect of *H. pylori* eradication.[20,33,34] Those patients with high initial levels of plasma ghrelin gained in weight after *H. pylori* eradication. Thus, initial ghrelin levels can be a predictive factor of weight gain induced by *H. pylori* eradication. Previous studies have addressed the question as to whether ghrelin is involved in weight gain after *H. pylori* cure.[25,26] Although the correlation between initial plasma ghrelin levels and weight change suggests that ghrelin participates in the weight gain after *H. pylori* eradication, our present data do not definitely resolve this question. However, we suggest that the weight gain after *H. pylori* eradication does not result simply from an increase in plasma ghrelin by the recovery of gastric ghrelin production.

It is intriguing that plasma ghrelin concentrations decreased in many patients with weight gain after *H. pylori* cure in spite of the enhancement of gastric ghrelin production. We measured total plasma ghrelin, including octanoyl ghrelin and des-octanoyl ghrelin, using a radioimmunoassay.[6,7] Recently, the physiological roles of the two isoforms of ghrelin have been discussed. The discrepancy between gastric ghrelin transcription and plasma ghrelin concentration may be explained by ghrelin isoforms. Thompson et al.[35] reported that ghrelin and des-octanoyl ghrelin are present in plasma and affect growth hormone secretagogue receptors differently. Furthermore, des-octanoyl ghrelin, a major

circulating form of ghrelin, has adipogenic activity but does not stimulate growth hormone production, whereas octanoyl ghrelin does stimulate growth hormone production. Thus, it is reasonable to suggest that increased expression of preproghrelin mRNA in the stomach does not directly reflect the total plasma ghrelin level. However, the precise mechanism and regulation of gastric ghrelin secretion are yet to be elucidated. Further study on the ghrelin secretory machinery of gastric mucosal cells is warranted.

In conclusion, we have shown that changes in plasma ghrelin concentrations after *H. pylori* cure are inversely correlated with weight changes and initial ghrelin levels but not with gastric ghrelin production in Japanese patients. These observations provide novel insights for understanding ghrelin and its functions as it relates to various diseases.

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;402:656–60.
2. Nakazato M, Murakami N, Date Y, Kojima M, Matsuo H, Kangawa K, et al. A role for ghrelin in the central regulation of feeding. Nature 2001;409:194–8.
3. Date Y, Nakazato M, Murakami N, Kojima M, Kangawa K, Matsukura S. Ghrelin acts in the central nervous system to stimulate gastric acid secretion. Biochem Biophys Res Commun 2001; 280:904–7.
4. Tschop M, Smiley DL, Heiman ML. Ghrelin induces adiposity in rodents. Nature 2000;407:908–13.
5. Cummings DE, Weigle DS, Frayo RS, Breen PA, Ma MK, Dellinger EP, et al. Plasma ghrelin levels after diet-induced weight loss or gastric bypass surgery. N Engl J Med 2002;346: 1623–30.
6. Date Y, Kojima M, Hosoda H, Sawaguchi A, Mondal MS, Suganuma T, et al. 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;141:4255–61.
7. Osawa H, Nakazato M, Date Y, Kita H, Ohnishi H, Ueno H, et al. Impaired production of gastric ghrelin in chronic gastritis associated with *Helicobacter pylori*. J Clin Endocrinol Metab 2005;90: 10–6.
8. Shiiya T, Nakazato M, Mizuta M, Date Y, Mondal MS, Tanaka M, et al. Plasma ghrelin levels in lean and obese humans and the effect of glucose on ghrelin secretion. J Clin Endocrinol Metab 2002;87:240–4.
9. 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; 50:1714–9.
10. Tschop M, Weyer C, Tataranni PA, Devanarayan V, Ravussin E, Heiman ML. Circulating ghrelin levels are decreased in human obesity. Diabetes 2001;50:707–9.
11. Foster-Schubert KE, McTiernan A, Frayo RS, Schwartz RS, Rajan KB, Yasui Y, et al. Human plasma ghrelin levels increase during a one-year exercise program. J Clin Endocrinol Metab 2005;90:820–5.
12. Leidy HJ, Gardner JK, Frye BR, Snook ML, Schuchert MK, Richard EL, et al. Circulating ghrelin is sensitive to changes in body weight during a diet and exercise program in normal-weight young women. J Clin Endocrinol Metab 2004;89:2659–64.
13. Otto B, Cuntz U, Fruehauf E, Wawarta R, Folwaczny C, Riepl RL, et al. Weight gain decreases elevated plasma ghrelin concentrations of patients with anorexia nervosa. Eur J Endocrinol 2001; 145:669–73.
14. Marshall BJ, Goodwin CS, Warren JR, Murray R, Blincow ED, Blackbourn SJ, et al. Prospective double-blind trial of duodenal ulcer relapse after eradication of Campylobacter pylori. Lancet 1988;2:1437–42.
15. Uemura N, Okamoto S, Yamamoto S, Matsumura N, Yamaguchi S, Yamakido M, et al. *Helicobacter pylori* infection and the development of gastric cancer. N Engl J Med 2001;345:784–9.
16. Goodwin CS, Mendall MM, Northfield TC. *Helicobacter pylori* infection. Lancet 1997;349:265–9.
17. Blaser MJ. Hypotheses on the pathogenesis and natural history of *Helicobacter pylori*-induced inflammation. Gastroenterology 1992;102:720–7.
18. Graham DY, Lew GM, Klein PD, Evans DG, Evans DJ Jr, Saeed ZA, et al. Effect of treatment of *Helicobacter pylori* infection on the long-term recurrence of gastric or duodenal ulcer. A randomized, controlled study. Ann Intern Med 1992;116:705–8.
19. Wu MS, Lee WJ, Wang HH, Huang SP, Lin JT. A case–control study of association of *Helicobacter pylori* infection with morbid obesity in Taiwan. Arch Intern Med 2005;165:1552–5.
20. Furuta T, Shirai N, Xiao F, Takashima M, Hanai H. Effect of *Helicobacter pylori* infection and its eradication on nutrition. Aliment Pharmacol Ther 2002;16:799–806.
21. Blaser MJ, Atherton JC. *Helicobacter pylori* persistence: biology and disease. J Clin Invest 2004;113:321–33.
22. Kawano S, Kawahara A, Nakai R, Fu HY, Tsuji S, Tsujii M. *Helicobacter pylori* infection does not affect serum leptin concentration and body mass index (BMI) in asymptomatic subjects. J Gastroenterol 2001;36:579–80.
23. Kyriazanos ID, Sfiniadakis I, Gizaris V, Hountis P, Hatziveis K, Dafnopoulou A, et al. The incidence of *Helicobacter pylori* infection is not increased among obese young individuals in Greece. J Clin Gastroenterol 2002;34:541–6.
24. Peeters TL. Ghrelin: a new player in the control of gastrointestinal functions. Gut 2005;54:1638–49.
25. Tatsuguchi A, Miyake K, Gudis K, Futagami S, Tsukui T, Wada K, et al. Effect of *Helicobacter pylori* infection on ghrelin expression in human gastric mucosa. Am J Gastroenterol 2004;99:2121–7.
26. Nwokolo CU, Freshwater DA, O'Hare P, Randeva HS. Plasma ghrelin following cure of *Helicobacter pylori*. Gut 2003;52:637–40.
27. Isomoto H, Nakazato M, Ueno H, Date Y, Nishi Y, Mukae H, et al. Low plasma ghrelin levels in patients with Helicobacter pylori-associated gastritis. Am J Med 2004;117:429–32.
28. Ramachandran A, Snehalatha C, Viswanathan V, Viswanathan M, Haffner SM. Risk of noninsulin dependent diabetes mellitus conferred by obesity and central adiposity in different ethnic groups: a comparative analysis between Asian Indians, Mexican Americans and Whites. Diabetes Res Clin Pract 1997;36:121–5.
29. Yamamoto A, Horibe H, Mabuchi H, Kita T, Matsuzawa Y, Saito Y, et al. Analysis of serum lipid levels in Japanese men and women according to body mass index. Increase in risk of atherosclerosis in postmenopausal women. Research Group on Serum Lipid Survey 1990 in Japan. Atherosclerosis 1999;143:55–73.
30. Masaki KH, Curb JD, Chiu D, Petrovitch H, Rodriguez BL. Association of body mass index with blood pressure in elderly Japanese American men. The Honolulu Heart Program. Hypertension 1997;29:673–7.
31. Davies MJ, Ammari F, Sherriff C, Burden ML, Gujral J, Burden AC. Screening for Type 2 diabetes mellitus in the UK Indo-Asian population. Diabet Med 1999;16:131–7.
32. McNeely MJ, Boyko EJ, Shofer JB, Newell-Morris L, Leonetti DL, Fujimoto WY. Standard definitions of overweight and central