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

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

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

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

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

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Materials and Methods

Animals

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

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

RT-PCR for GHS-R 1a mRNA

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

Autoradiography for [^{125}I]acyl ghrelin

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

Preparation of anti-GHS-R serum

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

Immunohistochemistry for GHS-R

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

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

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

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

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

Ghrelin administration and neonatal body weights

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

Passive immunization for acyl ghrelin

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

Quantitative RT-PCR of GH mRNA in fetal pituitary

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

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

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

Statistics

Values are given as means ± SEM. Comparisons between two groups were made by ANOVA with the *post hoc* Fisher test. Differences at $P < 0.05$ were accepted as statistically significant.

Results

Expression of GHS-Rs in fetal tissue

GHS-R1a mRNA expression was detected in various fetal tissues with a high density in the spinal cord from E14 until birth (Fig. 1A). GHS-R mRNA expression in the fetal pituitary was also detected at E19. To confirm the expression of GHS-R in fetal tissues at the protein level, we performed immunohistochemistry on E17 fetuses using an antibody specific for GHS-R. Positive cells were distributed extensively in fetal tissues; the skin, bone, intestine, tongue, and muscle being stained particularly strongly (Fig. 1, C–G). Immunoreactivity was not detected in sections that were incubated with GHS-R antiserum that had been preabsorbed with excess synthetic GHS-R (Fig. 1, D-2 and F-2). Although RT-PCR analysis demonstrated the expression of GHS-R mRNA in sections of the brain, pituitary, stomach, and lung, only relatively weak staining was observed in these organs.

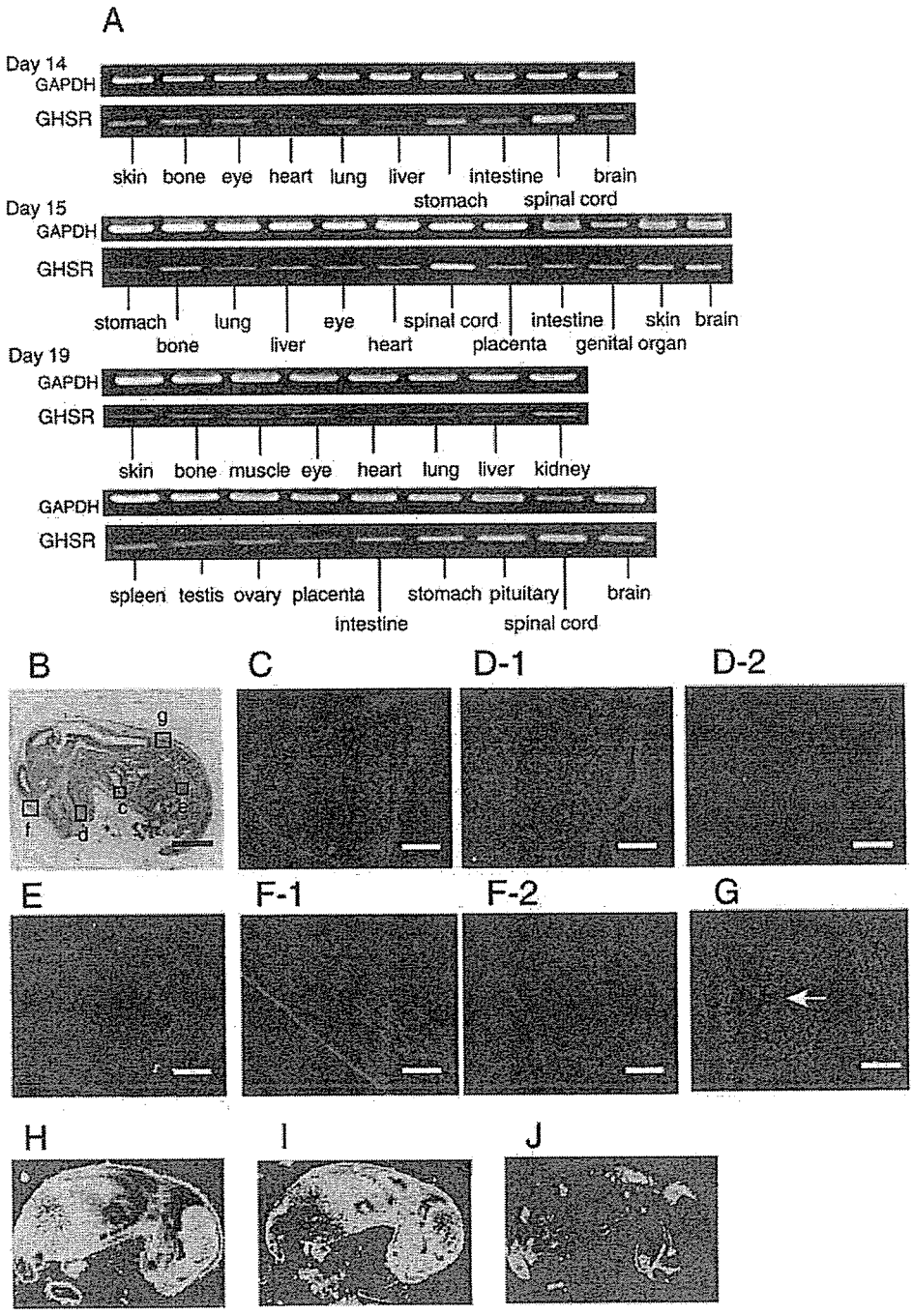

FIG. 1. A, GHS-R 1a mRNA expression in various fetal tissues. Fetal (E14, 15, 19) tissue cDNA fragments were amplified by PCR in the presence of oligonucleotide primers specific for GHS-R 1a. B, Sagittal section of E17 fetuses immunostained for GHS-R by avidin-biotin-peroxidase complexes (Vectastain Elite ABC Kit; Vector Laboratories, Burlingame, CA) using a diaminobenzidine substrate kit (Vector Laboratories). C–G, Immunofluorescence staining for GHS-R in fetal ribs (C), tongue (D), intestine (E), skin (F), and the muscle between the two transverse processes of a thoracic vertebra (G). D-2 and F-2 represent the immunostaining using absorbed antiserum with excess of synthetic GHS-R. *Bar* scales are: 3.7 mm (B); 75 μm (C); 65 μm (D); 75 μm (E); 100 μm (F); 75 μm (G). H–J, [^{125}I]acyl ghrelin autoradiograph of a sagittally sectioned fetus (E17) (H). Replacement was examined in the presence of excess unlabeled acyl (I) or des-acyl (J) ghrelin. The *red color* indicates the highest binding state with [^{125}I]acyl ghrelin (high binding order: *red > yellow > green > blue colors*).

[^{125}I]Acyl ghrelin autoradiography revealed dense binding to bone, skin, heart, and tongue (Fig. 1H); similar to the immunohistochemistry, the brain and digestive tract bound the isotope only weakly. In addition, excess unlabeled acyl ghrelin (Fig. 1I) and des-acyl ghrelin (Fig. 1J) could displace with [^{125}I]acyl ghrelin binding. More potent replacement was observed in excess unlabeled des-acyl ghrelin treatment (Fig. 1J).

Circulating ghrelin levels during late pregnancy

We measured the circulating levels of acyl and des-acyl ghrelin in pregnant rats and their fetuses, respectively. The levels of acyl ghrelin in maternal plasma exhibited a gradual but not significant decline in late pregnancy (Fig. 2A). In contrast, des-acyl ghrelin increased significantly during late pregnancy (Fig. 2A). Both ghrelin forms, acyl and des-acyl ghrelin, were also present in the fetal circulation; these levels decreased gradually as the time for delivery approached (Fig. 2B). We noticed a significant difference in des-acyl ghrelin levels when compared between the maternal and fetal plasma: the fetal levels of des-acyl ghrelin were 5- to 10-fold higher than the maternal levels (Fig. 2, A and B). On d 17 and 19 of pregnancy, we detected a large quantity of des-acyl ghrelin in the amniotic fluid (Fig. 2C). Acyl ghrelin levels increased rapidly in fetal blood within 5 min of administration of either 0.2 or 20 nmol acyl ghrelin (iv) into the mother (Fig. 2D). In the case of the 20-nmol dose, although maternal trunk ghrelin levels declined 30 min after injection, fetal trunk ghrelin levels were still increased at the sampling time.

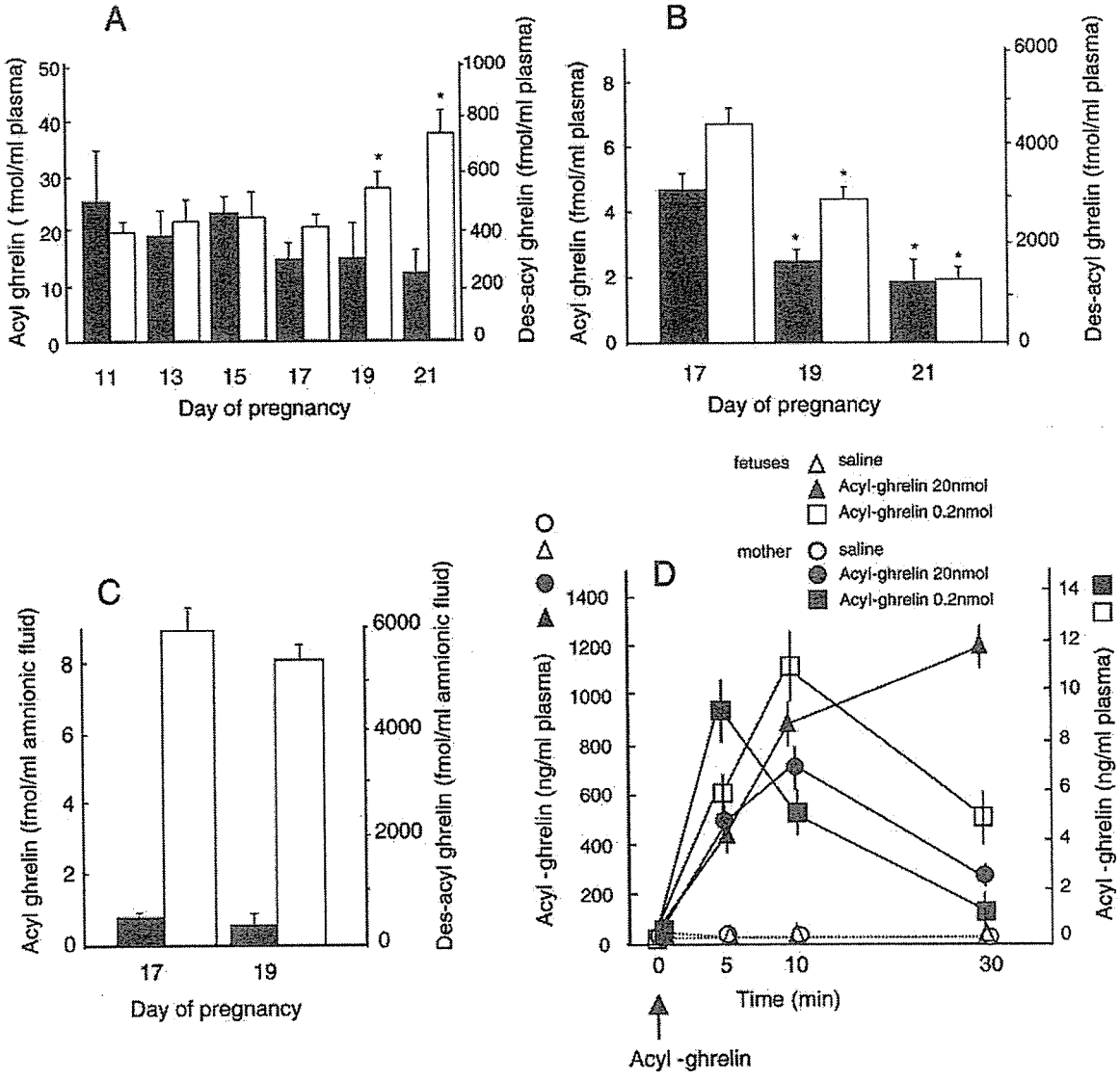

FIG. 2. A, Plasma acyl and des-acyl ghrelin levels in pregnant rats during late pregnancy. B, Fetal plasma levels of acyl and des-acyl ghrelin. C, Acyl and des-acyl ghrelin levels in amniotic fluid. All *black and white bars* represent the levels of acyl and des-acyl ghrelin, respectively. Each *bar* and *vertical line* represent the mean ± SEM (n = 8). *Asterisks* indicate significant differences (A, $P < 0.05$ *vs.* E11; B, $P < 0.05$ *vs.* E17). D, Maternal and fetal levels of circulating acyl ghrelin after iv injection of saline or 0.2 and 20 nmol acyl ghrelin into mothers at d 19 of pregnancy. The *right and left vertical scales* correspond to 0.2 and 20 nmol acyl ghrelin treatments, respectively.

Effect of chronic ghrelin treatment on fetal body weight at birth

We examined the effect of prolonged maternal treatment with ghrelin, beginning at d 14 or 15 of pregnancy and lasting until delivery, on neonatal body weight at birth. Chronic treatment with acyl ghrelin, either by injection three times per day (Fig. 3A) or constant infusion through an osmotic mini-pump (Fig. 3B), significantly increased the average neonatal body weight at birth in comparison to that of neonates delivered by a saline-treated group. We observed more than a 10% body weight gain, and the increase was dose-dependent. No significant changes were observed after treatment with des-acyl ghrelin.

We investigated the effect of acyl ghrelin injection on food intake of pregnant females. Daily treatment with acyl ghrelin significantly increased daily maternal food intake (Fig. 3C). However, a paired feeding study demonstrated that even when pregnant females treated with acyl ghrelin consumed the same amount of food as saline-treated pregnant females, neonatal body weight was significantly greater in the ghrelin-treated group (Fig. 3D).

To examine the effect of endogenous maternal ghrelin on fetal development, we compared the birth weight of pups born to mothers passive-immunized against a complex of acyl ghrelin and mcKLH (carrier protein) with that of pups born to mothers passive-immunized against mcKLH. After six immunizations at 2-wk intervals beginning at 44 d after birth, rats were mated when the relative ghrelin binding titer was maximally increased (Fig. 3E). Although body weight gain was temporarily lower, it was not significantly so. The body weights of ghrelin-immunized females recovered gradually to normal levels at 104 d of age (Fig. 3E). The body weights of neonates born to mothers passive-immunized against acyl ghrelin were lower than those of neonates born to saline-treated mothers (Fig. 3F).

Effect of ghrelin on GH mRNA levels in fetal pituitary tissue, and IGF-I and corticosterone levels in fetal plasma

If GH, prolactin, or corticosterone secretions from fetal pituitary or adrenal tissues were stimulated by maternal ghrelin, the released hormone might stimulate fetal development. We examined the effect on fetal pituitary GH mRNA levels and fetal plasma IGF-I or corticosterone levels by administering acyl ghrelin to pregnant females. However, pituitary GH mRNA at E19 and E20 was not affected by this treatment (Fig. 4A). In addition, fetal plasma IGF-I and corticosterone concentrations at E19 and E20 were not affected by maternal ghrelin treatment (Fig. 4, B and C). We found no significant change in fetal prolactin levels (data not shown).

Effect of ghrelin on proliferation of cultured fetal skin cells

To examine a possibility of direct effect of circulating ghrelin on fetal development, we examined the fetal cell proliferation by ghrelin using [^{3}H]thymidine and BrdU incorporation. We used primary cultured fetal skin cells at E17, because abundant cells at this stage were easy to collect. Both [^{3}H]thymidine (Fig. 5A) and BrdU (Fig. 5, B–E) incorporation increased significantly after treatment with acyl ghrelin in a dose-dependent or time-dependent manner. Des-acyl ghrelin was more potent than acyl ghrelin at stimulating the proliferation of fetal skin cells (Fig. 5E). The GHS-R antagonist [D-Lys^3]-GHRP-6 inhibited acyl ghrelin- and des-acyl ghrelin-stimulated cell proliferation (Fig. 5E).

Calcium-imaging analysis revealed two types of fetal skin cells (Fig. 5F): one type responding to des-acyl ghrelin, but not to acyl ghrelin, and the other responding to acyl ghrelin, but not to des-acyl ghrelin. No. 21 and 23 cells were shown as examples, respectively.

Discussion

The present study clearly demonstrated that maternal ghrelin would play an important role in fetal development during pregnancy; first, exogenous chronic treatment of the mother with ghrelin increased fetal body weight at birth; second, mothers immunized against ghrelin delivered fetuses with a lower body weight; and third, proliferation of cultured fetal skin cells was stimulated by ghrelin. Both GHS-R1a mRNA expression and GHS-R protein were detected in various fetal tissues. Autoradiography using [^{125}I]acyl ghrelin also demonstrated dense binding to the bone, skin, heart, and tongue. This distribution of functional GHS-R throughout peripheral fetal tissues suggests that ghrelin acts on such fetal peripheral tissues. Surprisingly, excess unlabeled des-acyl ghrelin could displace completely with [^{125}I]acyl ghrelin binding, suggesting that the acyl modification is dispensable for ghrelin function in binding site of fetal tissues. Because des-acyl ghrelin does not bind to GHS-R (1), we presume that fetal tissues may express a GHS-R subtype for des-acyl ghrelin. In support of this supposition (28, 29), it has been shown that the increases in plasma glucose and decreases in insulin, but not increases in GH secretion, induced by acyl ghrelin administration can be counteracted by coadministration of des-acyl ghrelin (28). In addition, ghrelin and des-acyl ghrelin inhibit cell death in cardiomyocytes and endothelial cells through ERK1/2 and phosphatidylinositol 3-kinase/AKTJ (30).

Plasma total ghrelin levels have been measured in pregnant women, rats, and human fetuses (31–34). In pregnant rats, plasma total ghrelin, determined with an antibody recognizing the C-terminal region, was shown to decrease at around the middle to late stage of pregnancy (31). Total ghrelin increases at around mid-gestation in human pregnancy (32, 33). Human fetuses exhibit levels of total ghrelin in umbilical venous blood that are not correlated with either gestational age or maternal ghrelin levels (34). In addition, ghrelin mRNA expression has been observed in the placenta and ovary of pregnant rats, and in the fetal pancreas (3, 35, 36). It has also been reported that ghrelin might play an important role in the regulation of blood pressure and the development of preimplantation embryos (37, 38). In the present study, both acyl and des-acyl ghrelin were present in the maternal and fetal circulations during the last half of pregnancy, and there was a significant difference in des-acyl ghrelin levels between the maternal and fetal plasma. The fetal levels of plasma des-acyl ghrelin were 5- to 10-fold higher than the maternal levels. In addition, we detected a large quantity of des-acyl ghrelin in the amniotic fluid. As

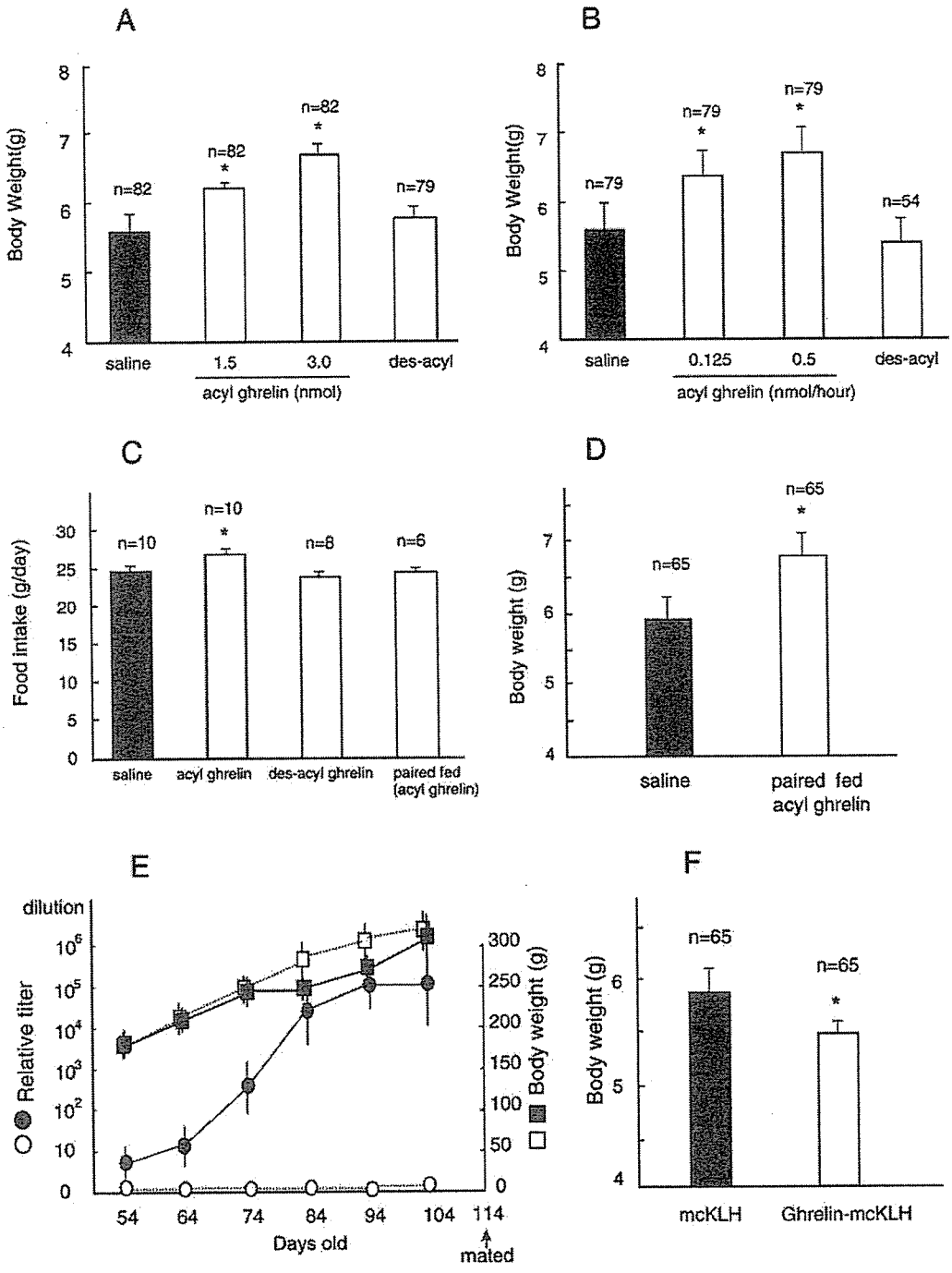

FIG. 3. A and B, Effect on neonatal body weight at birth of daily sc injections of ghrelin (A) or continuous infusion of ghrelin with an osmotic minipump (B) into pregnant rats. Treatment with ghrelin was performed from d 14 until delivery. C, Daily food intake in pregnant rats (A) and in pair-fed pregnant rats treated with acyl ghrelin on the same schedule as A. D, The effect of daily sc injection of 3 nmol acyl ghrelin into pair-fed pregnant rats on neonatal body weight at birth. Acyl ghrelin was injected three times daily from d 14 until delivery. Paired feeding for the saline-treated group also begun on d 15 of pregnancy. E, Comparison of body weight and relative antibody titer between rats immunized against ghrelin-mcKLH complex (●, ■) or carrier protein alone (○, □). The relative titer (●, ○) was expressed as a dilution rate at 50% binding capacity. Each *bar* (A–E) and *symbol* (F) and *vertical line* represent the mean ± SEM. The *upper numbers* represent the total number of newborn rats compared in each group. *Asterisks* indicate significant differences ($P < 0.05$ *vs.* control). F, Comparison of the body weight of newborn rats delivered from rats passive-immunized with the ghrelin-mcKLH complex or with carrier protein alone (mcKLH).

demonstrated previously, ghrelin-positive cells were not evident in the fetal stomach until E19 by immunohistochemistry using an antibody recognizing the N-terminal of acyl ghrelin, suggesting that fetal plasma ghrelin originates from the maternal placenta and/or the maternal blood (3, 22). Indeed, acyl ghrelin levels in fetal plasma increased rapidly

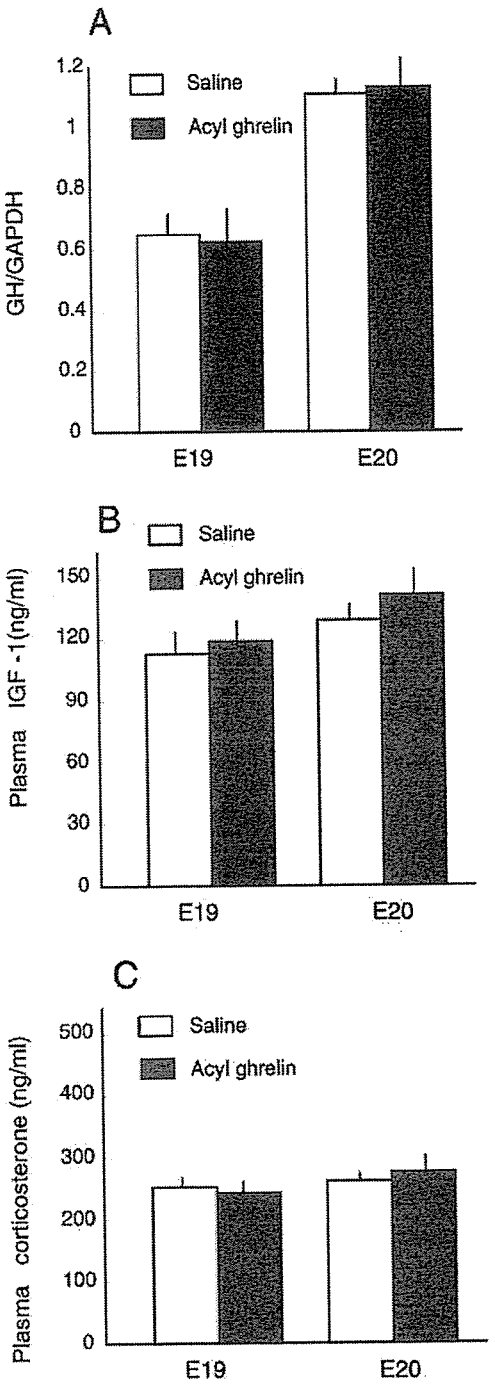

FIG. 4. A, The effect of continuous infusion of acyl ghrelin into pregnant rats on fetal pituitary GH mRNA levels (A) and fetal plasma IGF-I (B) and corticosterone levels (C). Acyl ghrelin was infused at a rate of 0.5 nmol per hour with an osmotic minipump beginning on d 14 and lasting until delivery. The fetal pituitary and plasma were collected at E19 and E20. Each *bar and vertical line* represent the mean ± SEM (n = 6).

within 5 min after administration of acyl ghrelin to the mother, indicating that maternal ghrelin easily transits to the fetal circulation. Although maternal trunk ghrelin levels declined 30 min after injection, fetal trunk ghrelin increased at the time, probably resulting from a longer half-life of ghrelin in fetuses than in adults, and high levels of des-acyl ghrelin might accumulate in the fetal circulation. The existence of GHS-R and an additional GHS-R subtype in fetal tissues, combined with both acyl ghrelin and large quantities of des-acyl ghrelin in the fetal circulation and amniotic fluid, supports the hypothesis that maternal ghrelin plays a critical role in fetal development.

Fetal growth is mainly influenced by the nutrition provided by the mother through the arteria umbilicalis (39, 40). Decreases in the amount of food given to pregnant mothers during the gestational period tend to decrease the size of their neonatal pups in comparison with pups born to mothers fed *ad libitum*. Daily treatment with acyl ghrelin significantly increased daily maternal food intake. The stimulation of fetal growth by maternal ghrelin injection would result from increased nutrition provided by the mother. However, a paired feeding study demonstrated that even when pregnant females treated with acyl ghrelin consumed the same amount of food as saline-treated pregnant females, neonatal body weight was significantly greater in the ghrelin-treated group. This result indicates that maternal ghrelin affected fetal development through a mechanism independent of increased nutrition.

In rats, a rapid increase in fetal body weight occurs during the last quarter of pregnancy. The somatotroph, a GH-secreting cell, appears in the fetal pituitary near E18 (41). Pituitary GH mRNA at E19 and E20 was not altered by ghrelin treatment, indicating that maternal ghrelin-induced fetal development is not due to increased release of fetal GH. The stimulation of maternal GH secretion by daily treatment of ghrelin, leading to the transition of maternal GH to fetal circulation, may stimulate fetal development. Garcia-Aragon and colleagues (42) provided evidence for the wide distribution of GH receptor in the mid-late gestation of rat fetus. The receptor expression markedly increased between E12 and E18; the receptor was present in all major organ systems at E18. Genetically manipulated model mice, Laron dwarfs, with inactivating GH receptor mutations, were shorter in length than normal at birth. Congenitally GH-deficient newborn babies are also much shorter (43, 44). In contrast, the fetuses of GH-deficient dwarf rats were proportionately smaller in size (45). However, we previously reported that continuous infusion of ghrelin to rats stimulated GH secretion for several days, but that the effect decreased after prolonged administration (26). Levels of GH mRNA within the pituitary were also decreased by these treatments (26), probably due to transcriptional down-regulation. In addition, fetal plasma IGF-I levels were not affected by maternal treatment with ghrelin. We found no significant change in fetal circulating levels of corticosterone and prolactin during maternal ghrelin administration. Therefore, the stimulation of fetal development by maternal ghrelin administration is probably not due to the maternal GH and fetal circulating IGF-I and corticosterone levels.

Both [^{3}H]thymidine and BrdU incorporation increased significantly after treatment with acyl ghrelin in a dose-dependent and time-dependent manner. Interestingly, des-acyl ghrelin stimulated proliferation more potently than acyl ghrelin. The GHS-R antagonist [D-Lys^3]-GHRP-6 inhibited

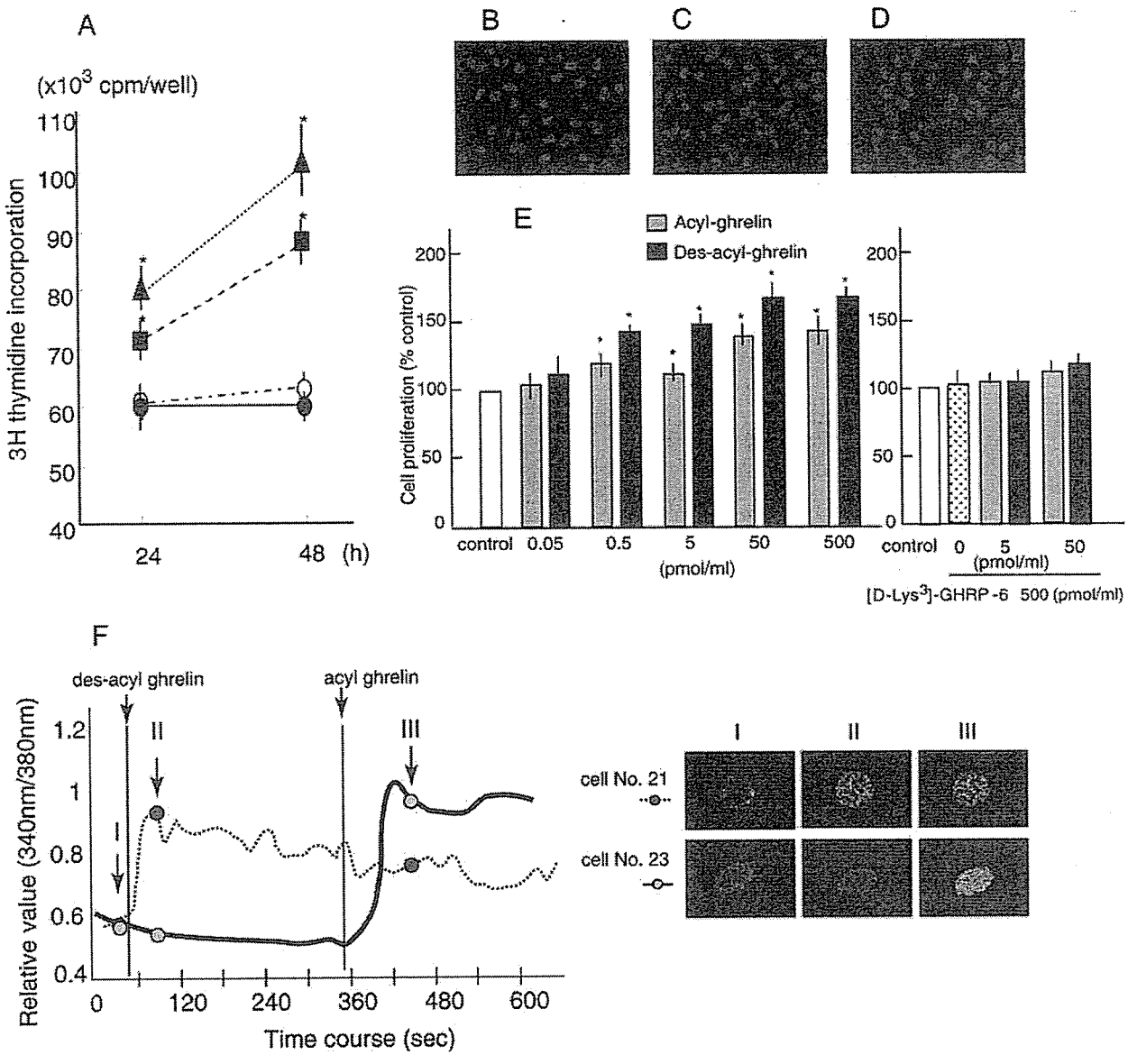

FIG. 5. A, The effect of acyl ghrelin on [^{3}H]thymidine incorporation by cultured fetal (E17) skin cells. One day after initiation of culture, acyl ghrelin (●, 0 pmol; ○, 0.5 pmol; ■, 5 pmol; ▲, 50 pmol/ml) and [^{3}H]thymidine (2 μCi/ml) were added for a 24- or 48-h incubation period. Each *symbol* and *vertical line* represents the mean ± SEM (n = 16 wells). *Asterisks* indicate significant differences ($P < 0.05$ *vs.* ●). B–D, Example of BrdU incorporation (shown in E) into the nucleus of cultured fetal (E17) skin cells incubated for 24 h with 50 pmol/ml acyl ghrelin (C), 50 pmol/ml des-acyl ghrelin (D), or without ghrelin (B). E (*left*), The effect of various doses of acyl and des-acyl ghrelin on BrdU incorporation by cultured fetal skin cells. Fetal (E17) skin cells were cultured for 24 h with or without ghrelin. E (*right*), Effect of [D-Lys3]-GHRP-6, a GHS-R antagonist, on the ghrelin-stimulated proliferation of fetal skin cells. Various doses of acyl or des-acyl ghrelin were added to the culture medium together with 500 pmol [D-Lys3]-GHRP-6. F, Calcium imaging analysis of fetal skin cells. The skin cells were prepared from E17 fetuses. Cell no. 21 responded to des-acyl ghrelin, but not to acyl ghrelin. In contrast, cell no. 23 responded to acyl ghrelin, but not to des-acyl ghrelin. Photographs on the *right* represent the calcium response in cells at points I, II, and III. The *red and yellow color spots* indicate high intracellular Ca^{2+} concentration. The *blue color* shows the basal condition. Des-acyl ghrelin and acyl ghrelin were added to the culture medium at the point indicated by the *arrow*. The *graph on the left* represents the transition of the relative value of Ca^{2+} concentration analyzed by an imaging scanner.

acyl ghrelin- and des-acyl ghrelin-stimulated cell proliferation. These results clearly indicate that both acyl ghrelin and des-acyl ghrelin stimulate proliferation of fetal skin cells. Acyl ghrelin induces neurogenesis in the dorsal motor nucleus (46) and stimulates bone formation (47). During pregnancy, maternal ghrelin is likely transferred to the fetal circulation, and then would prompt fetal growth through stimulation of cell proliferation. Calcium-imaging analysis revealed that two types of cells exist in cultured fetal skin cells: one responds only to des-acyl ghrelin, and the other one responds only to acyl ghrelin. These results strongly suggest that fetal skin cells have different type of receptors: one is a classical receptor for acyl ghrelin, GHS-R 1a, and the other is a novel receptor for des-acyl ghrelin that mediates intracellular calcium mobilization.

In this study, we detected high levels of des-acyl ghrelin

in the fetal circulation and amniotic fluid. These findings suggest that amniotic fluid serves, in part, as an incubation medium to provide des-acyl ghrelin to the fetus. In this way, des-acyl ghrelin may act on fetal development by direct stimulation of proliferation. If this is true, however, the lack of an effect of des-acyl ghrelin treatment on neonatal body weight at birth (Fig. 3) remains to be explained. We speculate that, late in pregnancy, high endogenous quantities of des-acyl ghrelin in the fetal circulation and amniotic fluid saturate the GHS-R 1a subtype des-acyl ghrelin receptors, effectively preventing the exogenous des-acyl ghrelin from exerting an effect. It has been reported that ghrelin knockout mice do not exhibit any changes in development (probably including fetal development) (13). We do not know the reason for the discrepancy of neonatal body weights between mothers passive-immunized against acyl ghrelin and ghrelin knockout mice. Further studies are required to elucidate this discrepancy.

In conclusion, the present study has demonstrated that maternal ghrelin is easily transferred to the fetal circulation, and then prompts fetal growth through stimulation of cell proliferation during the late half of pregnancy. Recent reports that ghrelin directly stimulates bone formation (47) also supports this hypothesis. These findings may have implications for the clinical application of ghrelin for pregnant subjects.

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Ghrelin Improves Renal Function in Mice with Ischemic Acute Renal Failure

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Growth hormone and IGF-1 have been suggested to have tissue-protective effects. Ghrelin is a stomach-derived growth hormone secretagogue. The effects of ghrelin on ischemia/reperfusion-induced renal failure in mice were examined. Ischemic acute renal failure was induced by bilateral renal artery clamping for 45 min and reperfusion for 24 h. Ghrelin (100 μg/kg mouse) or vehicle was injected subcutaneously six times before surgery and three times after surgery every 8 h. Twenty-four hours after reperfusion, the right kidney was isolated and perfused. Acetylcholine (ACh)- and adrenomedullin-induced endothelium-dependent vasorelaxation of renal vessels significantly improved in ghrelin-pretreated mice (%Δ renal perfusion pressure by 10^{-7} M ACh −63.5 ± 3.7 *versus* −41.2 ± 5.5%; $P < 0.05$). This change was associated with significant increases of nitric oxide release in the kidneys of ghrelin-treated mice (10^{-7} M ACh 35.5 ± 5.8 *versus* 16.9 ± 3.5 fmol/g kidney per min; $P < 0.05$). Serum concentration of urea nitrogen (53 ± 7 *versus* 87 ± 15 mg/dl; $P < 0.05$) and renal injury score were significantly lower in the ghrelin group (2.5 ± 0.8 *versus* 5.3 ± 1.5; $P < 0.01$). Tubular apoptotic index was significantly lower in the ghrelin group (5 ± 5 *versus* 28 ± 4; $P < 0.05$). Furthermore, the survival rate after the 60-min ischemic period was higher in the ghrelin group (80 *versus* 20%; $P < 0.05$). Ghrelin treatment significantly increased the serum level of IGF-1. However, such renal protective effects of ghrelin on ischemia/reperfusion injury were not observed in insulin receptor substrate-2 knockout mice. These results suggest that ghrelin may protect the kidneys from ischemia/reperfusion injury and that this effect is related to an improvement of endothelial function through an IGF-1–mediated pathway.

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Ghrelin has a growth hormone (GH)-releasing effect. It was first isolated from the stomach and is known as an endogenous ligand for GH secretagogue receptor (GHSR) (1). Ghrelin is a peptide of 28 amino acids with an *n*-octanoyl modification at serine-3, and this modification is closely related to its physiologic activity. Studies in healthy volunteers have shown that ghrelin increases the cardiac index and stroke volume and decreases the mean arterial pressure. These effects were associated with upregulation of GH and IGF-1 (2). Ghrelin also has beneficial effects on left ventricular systolic function and energy metabolism in severe heart failure and improves cardiac cachexia (3–5). GH and IGF-1 improve severe heart failure caused by dilated cardiomyopathy and ischemic heart disease (6–8). These findings suggest that the cardiac effect of ghrelin is exerted through an increase of GH release. There have also been reports on the renal protective effects of GH and IGF-1 against various types of renal damage (9), although several other reports did not confirm this effect (10). As for the renal protective mechanism, induction of nitric oxide (NO) and cGMP in the kidney by GH and IGF-1 were suggested to improve renal circulation (11,12).

It is still controversial whether ghrelin has a GH-independent effect on cardiovascular function. GHSR widely distributes and exists in the heart and vessels (13). Moreover, intra-arterial infusion of ghrelin in healthy individuals dose-dependently increased blood flow without changes in serum IGF-1 concentration (14), indicating the possibility of a direct cardiovascular action of ghrelin.

Despite the reports on the cardiac effects of ghrelin, there are no data on the protective effect of ghrelin in organs other than the heart. In this study, we investigated whether ghrelin improved ischemic acute renal failure (iARF) and whether ghrelin influenced vascular endothelial function in mice. Furthermore, to explore the role of IGF-1 in the effects of ghrelin, we studied the effects of ghrelin in insulin receptor substrate-2 (IRS-2) knockout (KO) mice.

Materials and Methods

Animals

All studies were performed in concordance with the university guidelines for animal experiments. Adult male BALB/C mice that weighed 30 to 35 g were obtained from Charles River Laboratories

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(Yokohama, Japan). The IRS-$2^{-/-}$ mice were maintained on the original C57BL6/CBA hybrid background and were prepared by IRS-$2^{+/-}$ mouse intercrosses (15).

iARF

iARF was induced in 8- to 12-wk-old BALB/C and IRS-$2^{-/-}$ mice as described elsewhere (16). In brief, after anesthesia with pentobarbital (40 mg/kg, intraperitoneally), a middle abdominal incision was made and bilateral renal arteries were clamped for 45 min. After declamping, we confirmed the restoration of renal blood flow and closed the incision. Twenty-four hours after the start of reperfusion, 1.0 ml of blood was drawn to measure the serum level of blood urea nitrogen (BUN), creatinine, and IGF-1. Thereafter, the right kidney was used for isolated perfusion and the left kidney was used for histologic examination and analysis of renal tubular cells apoptosis.

Administration of Ghrelin

Rat ghrelin was obtained from the Peptide Institute (Osaka, Japan). Ghrelin (100 μg/kg mouse) was dissolved in 0.9% saline that contained BSA and was subcutaneously injected six times before ischemia every 8 h and three times after ischemia. An equal volume of the vehicle was injected into the control mice. To confirm the rationality of our protocol, we also examined ghrelin's effect after a single injection given just before ischemia and 8 h after reperfusion.

Isolated Perfused Kidney

Male BALB/C and IRS-$2^{-/-}$ mice that were treated with vehicle or ghrelin were anesthetized with pentobarbital (40 mg/kg, intraperitoneally), then the right kidney was isolated and perfused as described previously (17). In brief, after an abdominal incision, a 24-G needle was inserted into the right renal artery and then renal perfusion was started with Krebs-Henseleit buffer. The buffer was saturated with 95% O_2/5% CO_2 and contained 10^{-6} mol/L angiotensin II and 10^{-5} mol/L indomethacin to maintain the renal perfusion pressure (RPP) at approximately 100 mmHg. After a 60-min equilibrium period, graded doses of acetylcholine (ACh; 10^{-8} to 10^{-7} M) and adrenomedullin (AM; 10^{-10} to 10^{-7} M) were added to the buffer at 10-min intervals, and RPP was monitored through a pressure transducer (Datex-Ohmeda K.K., Tokyo, Japan). The renal vein was also cannulated to drain the perfusate into the NO assay system.

Measurement of NO Released from Kidney

We measured NO concentration in the perfusate from the renal vein using a chemiluminescence assay as described previously (17–19). The venous effluent was introduced into a rotatory mixer with a chemiluminescence assay probe of 10 mmol/L H_2O_2, 18 mmol/L recrystallized luminol, 2 mmol/L potassium carbonate, and 150 mmol/L desferrioxamine. The mixture of the perfusate and probe then entered a chemiluminescence detector. The chemiluminescent signal was measured continuously and was recorded using a pen recorder. The NO signal was calibrated using an NO solution. NO release was normalized by kidney weight and expressed as femtomoles per minute per gram of renal tissue.

Measurement of cGMP Level in the Mouse Kidney

After the NO measurement, we perfused the kidney for 15 min with 10^{-8} M AM through the renal artery. Then the kidneys were homogenized in 4% TCA (pH 4.0) on ice. After centrifugation, the supernatant was extracted four times with water-saturated ether and then evaporated. The pellets were redissolved in a buffer solution. The cGMP content was assayed using an ELISA kit according to the manufacturer's recommendation (Amersham Biosciences Corp., Piscataway, NJ) (20).

Histologic Studies

Tissue samples were fixed in 4% paraformaldehyde and embedded in paraffin. We obtained 5-mm sections and stained them with the periodic acid-Schiff reagent. We conducted a semiquantitative histologic analysis. Twenty tubules or glomeruli in each kidney were randomly selected at a ×400 magnification, and the degree of renal damage was scored using the scoring system for renal injury reported by Solez *et al.* (21). We calculated the mean renal injury score in each mouse and then averaged the scores for each group. The sections were examined by a pathologist in a blinded manner. We examined the tissues for the presence of expansion of Bowman's space, interstitial edema, epithelial detachment, and tubular cells casts. Renal morphologic changes were graded on a scale of 0 to 3+: 0, normal; 1+, slight; 2+, moderate; and 3+, severe.

Detection of Apoptotic Cells

To examine the antiapoptotic effect of ghrelin, we performed terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) staining of renal tubular cells. Nuclei were also counterstained with propidium iodine and mounted with ProLong Antifade Kit (Molecular Probes, Eugene, OR). The sections were observed using a confocal microscope (FLUOVIEW FV300, Olympus, Tokyo). The apoptotic index was calculated as the number of TUNEL-positive nuclei per high-power field (×400).

Survival Rate of Mice with iARF

To examine the effect of ghrelin on the survival of mice with ARF, we prolonged the duration of renal arterial clamping from 45 to 60 min. After removal of the clamp, we closely observed the mice during a 36-h reperfusion period.

Statistical Analyses

All data are expressed as the mean value ± SEM. Statistical comparisons were made by ANOVA followed by the Student-Neumann-Keuls test. To compare renal injury scores, we used the nonparametric Kruskal-Wallis test. The survival rate of mice after 60 min of ischemia and 36 h of reperfusion was estimated with the Kaplan-Meier method. $P < 0.05$ was considered statistically significant.

Results

Effects of Ghrelin on Renal Vascular Endothelial Function

Body weight, kidney weight, and RPP of the four groups of mice are summarized in Table 1. Bilateral kidneys from BALB/C mice were macroscopically normal. The kidney weight was significantly greater in iARF mice than in sham-operated mice. Baseline RPP in the iARF group was higher than in the sham-operated group. Vehicle-treated mice with iARF showed significantly higher RPP than ghrelin-treated mice with iARF (Table 1).

The effect of ACh and AM on RPP and NO release in the four groups are shown in Figure 1. They lowered RPP of kidneys in all groups in a dose-dependent manner. The endothelium-dependent vasodilatory effect of them was significantly greater in the sham-operated mice than in the iARF mice. In sham-operated mice, ghrelin did not modify the renal vascular response. However, in iARF mice, treatment with ghrelin significantly increased ACh- and AM-induced vasodilation. The ACh- and AM-induced NO release from the kidney was greater in the ghrelin group of iARF mice than in the vehicle group (Figure 1).

Table 1. Baseline characteristics of mice that had iARF and were treated with vehicle or ghrelin[a]

	n	BW (g)	KW (g)	KW/BW (%)	Baseline RPP (mmHg)
BALB/C					
sham + vehicle	8	27.6 ± 2.6	0.137 ± 0.042	0.496 ± 0.125	79.6 ± 5.5
sham + ghrelin	8	28.6 ± 2.1	0.132 ± 0.058	0.462 ± 0.117	87.6 ± 4.2
iARF + vehicle	8	25.9 ± 3.5	0.173 ± 0.079[b]	0.668 ± 0.098[b]	110.3 ± 10.9[c]
iARF + ghrelin	8	27.8 ± 2.2	0.159 ± 0.059[d]	0.572 ± 0.143[e]	95.3 ± 7.5[e,f]
IRS-2 KO					
iARF + vehicle	4	29.5 ± 2.1	0.158 ± 0.009	0.54 ± 0.04	110.6 ± 5.4
iARF + ghrelin	4	28.8 ± 0.8	0.165 ± 0.006	0.57 ± 0.03	111.3 ± 7.5

[a]Values are means ± SEM. BW, body weight; KW, kidney weight; RPP, renal perfusion pressure; KO, knockout.
[b]$P < 0.05$ *versus* sham + vehicle.
[c]$P < 0.01$ *versus* sham + vehicle.
[d]$P < 0.05$ *versus* sham + ghrelin.
[e]$P < 0.05$ *versus* iARF + vehicle.
[f]$P < 0.01$ *versus* sham + ghrelin.

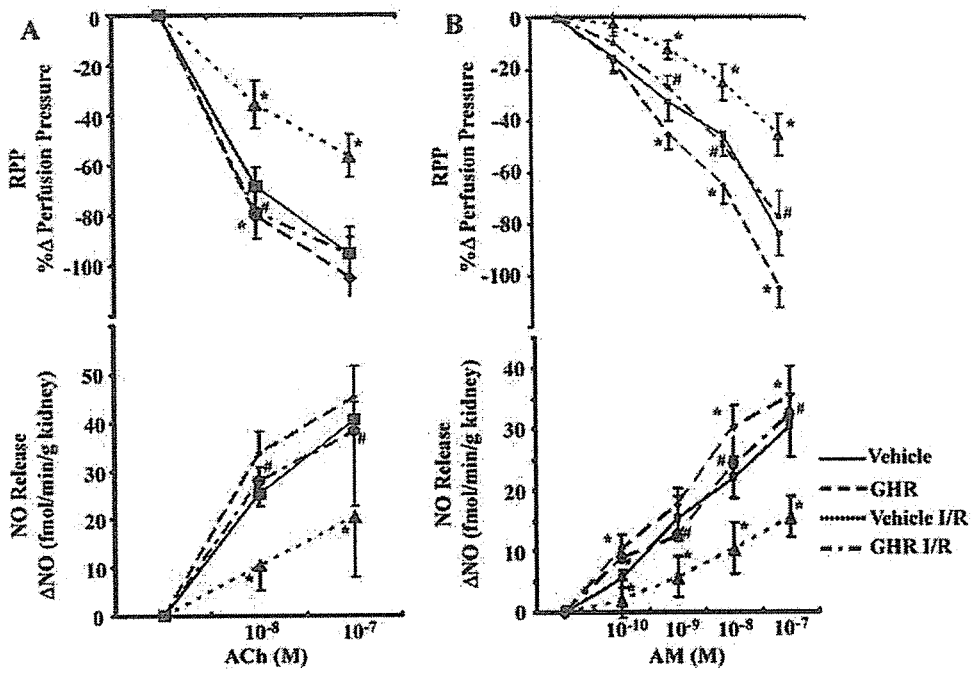

Figure 1. Effects of acetylcholine (ACh; A) and adrenomedullin (AM; B) on renal perfusion pressure (RPP) and nitric oxide (NO)-releasing activity in the vehicle, ghrelin, vehicle-ischemia/reperfusion (I/R) and ghrelin-I/R groups. NO concentration in the venous effluent was measured by luminol chemiluminescence assay. *$P < 0.05$ *versus* vehicle; #$P < 0.05$ *versus* vehicle-I/R. Bars indicate means ± SEM; $n = 8$.

To examine the involvement of the NO-cGMP pathway, we measured cGMP in the kidneys of mice in the two groups. The renal content of cGMP was significantly greater in the ghrelin group (Figure 2).

Effects of Ghrelin on Ischemia/Reperfusion Injury of the Kidney

None of the mice died of iARF when the renal arteries were clamped for 45 min. Figure 3 shows the renal histology stained with periodic acid-Schiff reagent. In the vehicle group, remarkable damage, particularly in the tubuli, was observed. Renal damage included detachment of epithelial cells of the tubuli, interstitial edema, and many tubular cell casts. Bowman's space was also remarkably expanded. The kidneys of the mice that were administered ghrelin were also damaged, but the extent of the injuries was less than that of injuries observed in the control mice. The renal injury scores of the four groups are shown in Figure 4. The ischemia/reperfusion (I/R) procedures resulted in significantly greater increases in the injury scores, and administration of ghrelin reduced renal damage (vehicle 0.6 ± 0.1, vehicle I/R 5.3 ± 1.5, ghrelin 0.5 ± 0.1, ghrelin I/R 2.5 ± 0.8).

The result of these histologic studies was supported by the measurement of renal excretory function. Twenty-four hours

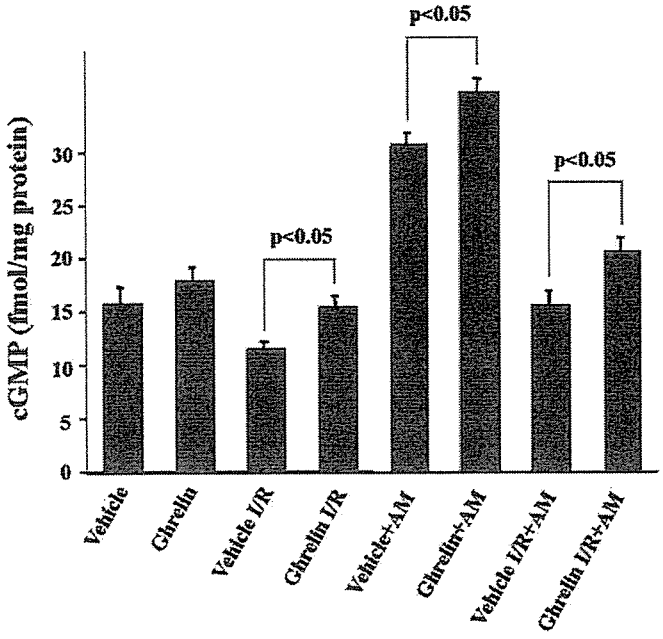

Figure 2. cGMP production in isolated kidneys from mice. The kidneys were stimulated with 10^{-8} M AM, and cGMP extracted from the kidneys was measured by ELISA. Bars indicate means ± SEM; $n = 8$.

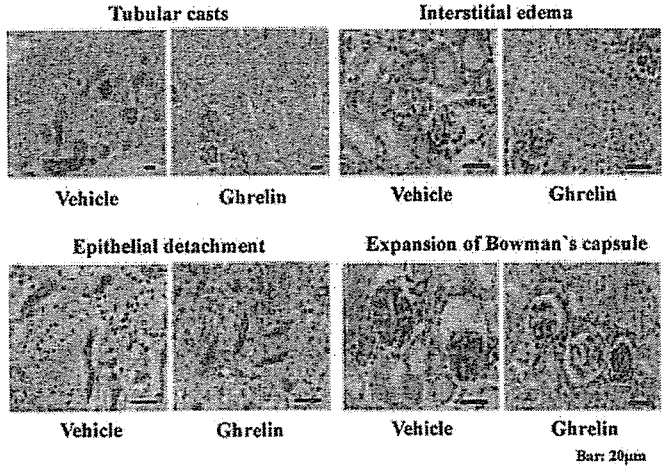

Figure 3. Photographs of renal tissue stained with periodic acid-Schiff reagent. Tubular cell casts, interstitial edema, epithelial detachment, and expansion of Bowman's capsule were observed in the kidneys that were treated with vehicle or ghrelin.

after reperfusion, the concentration of serum BUN and creatinine was markedly elevated in the two I/R groups. The degree of impairment of renal function was significantly smaller in the ghrelin group than in the vehicle group (Figure 5). When we injected ghrelin just before ischemia and 8 h after reperfusion, the serum levels of BUN and creatinine and the renal injury score increased in the two groups, and there were no significant differences between the two groups (BUN 181 ± 21 *versus*

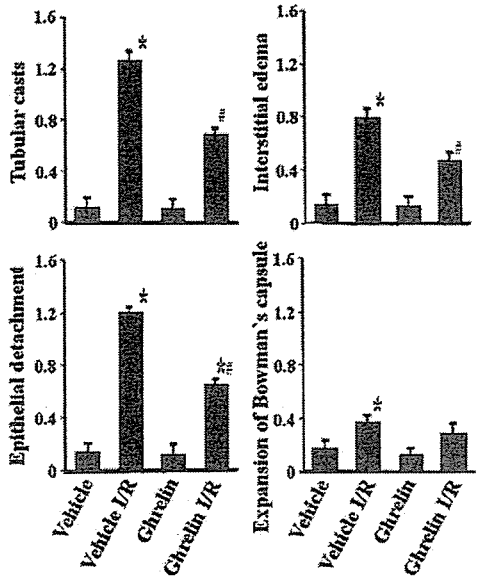

Figure 4. Four types of renal injury scores of vehicle-, ghrelin-, vehicle-I/R–, and ghrelin-I/R–treated mice. *$P < 0.05$ *versus* vehicle; #$P < 0.05$ *versus* vehicle-I/R. Bars indicate means ± SEM; $n = 8$.

176 ± 7, NS; Cr 1.9 ± 0.3 *versus* 2.1 ± 0.1, NS; renal injury score 6.7 ± 0.2 *versus* 7.1 ± 1.3, NS)

Antiapoptotic Effect of Ghrelin

Figure 6 shows apoptosis of renal tubular cells detected by the TUNEL staining method. In both groups with I/R-induced renal injury, apoptosis of proximal tubular cells was particularly prominent. However, administration of ghrelin resulted in a significantly decreased number of apoptotic cells in the kidneys, as compared with vehicle administration.

Survival Rate of Mice with iARF

When the renal arterial clamping period was 45 min, none of the mice died. However, after 60 min of ischemia, most mice that were administered the vehicle solution died by 36 h after reperfusion. Treatment with ghrelin substantially increased the survival of the mice (Figure 7).

Effect of Ghrelin on the IGF-1/IRS Pathway

To explore the mechanism for the renal protective effect of ghrelin, we examined the direct vascular effect of ghrelin. However, ghrelin did not substantially influence the vascular tone in the isolated aorta or isolated perfused kidney. We also examined the effect of ghrelin on apoptosis of cultured human umbilical vein endothelial cells caused by serum deprivation. We did not detect an antiapoptotic action of ghrelin in cultured cells (data not shown).

Next, we examined the indirect effects of ghrelin. Because ghrelin may upregulate IGF-1 *via* stimulation of GH, we measured serum IGF-1 concentration in these mice. Furthermore, to examine the role of the IGF-1/IRS pathway in ghrelin-induced renal protection, we repeated the same experiment using IRS-2

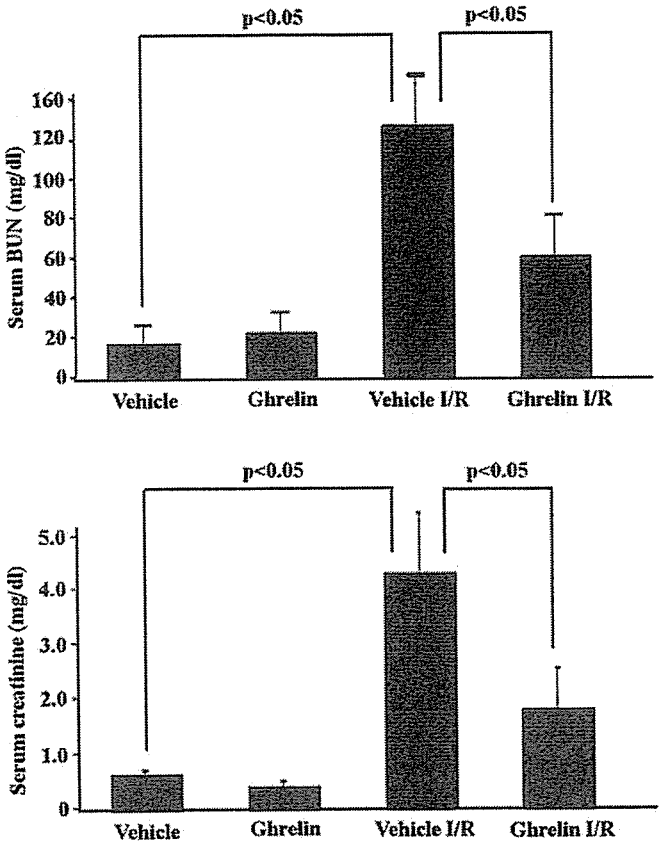

Figure 5. Serum levels of urea nitrogen and creatinine in sham-operated mice and mice that were subjected to renal I/R. Bars indicate means ± SEM; $n = 8$.

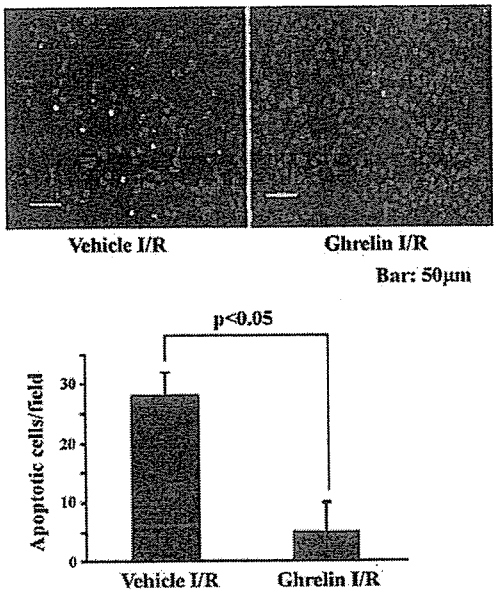

Figure 6. Photographs of apoptotic tubular cells and the numbers determined by terminal deoxynucleotidyl transferase mediated dUTP nick end-labeling (TUNEL) technique. TUNEL-positive cells are shown in yellow. Bars indicate means ± SEM; $n = 8$.

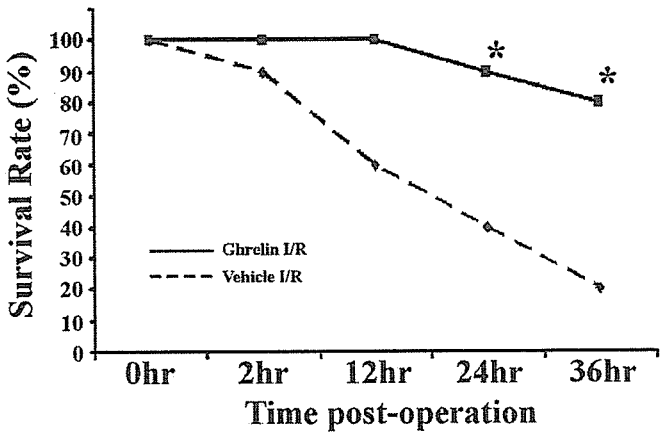

Figure 7. Survival rates of mice after ischemic acute renal failure (iARF) induced by 60 min of clamping of bilateral renal arteries in the vehicle and ghrelin groups. *$P < 0.05$ *versus* vehicle-I/R; $n = 10$.

KO mice. As a result, serum IGF-1 concentration was significantly higher in the ghrelin group than in the vehicle group (Figure 8).

Ischemia for 45 min and reperfusion for 24 h caused iARF also in IRS-2 KO mice. Serum BUN and creatinine levels were markedly high in both treated mice. Their levels were slightly lower in the ghrelin group than in the vehicle group, but the differences were not statistically significant (Figure 9). With regard to histologic analysis, both groups of mice showed marked renal damage. The renal injury scores were almost similar between the two groups. Furthermore, the baseline perfusion pressure of the kidney obtained from IRS-2 KO mice was almost the same between the two groups (vehicle 110.6 ± 5.4 *versus* ghrelin 111.3 ± 7.5 mmHg; NS). There was no significant difference in ACh-induced endothelium-dependent vasorelaxation of isolated perfused kidneys between the vehicle-treated group and the ghrelin-treated group (Figure 9), indicating lack of renal protective effects of ghrelin in IRS-2 KO mice.

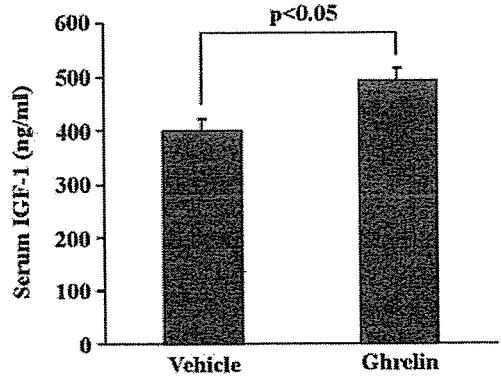

Figure 8. Serum IGF-1 concentrations in vehicle- and ghrelin-treated mice. Bars indicate means ± SEM; $n = 6$.

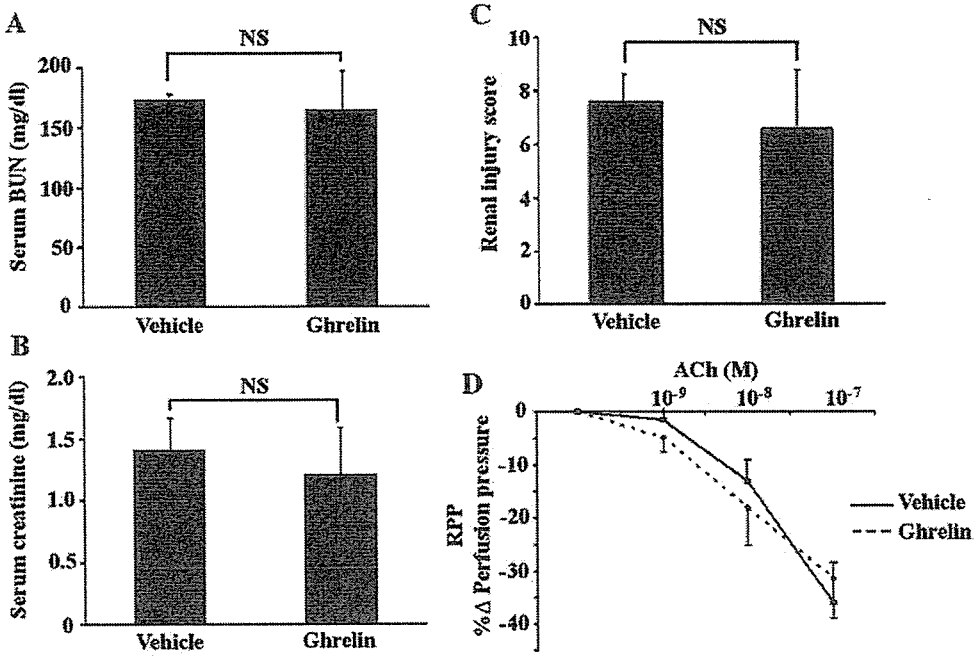

Figure 9. Serum levels of urea nitrogen (A) and creatinine (B), renal injury scores (C), and the effect of ACh on RPP (D) in iARF of IRS-2 knockout mice that were treated with vehicle and ghrelin. Renal injury scores are the sums of four injury scores (expansion of Bowman's space, interstitial edema, epithelial detachment, and tubular cell casts). Bars indicate means ± SEM; $n = 4$.

Discussion

In this study, we showed that ghrelin improved renal tissue damage and renal excretory function in the mice with iARF. These beneficial effects of ghrelin were associated with renal endothelium-dependent vasodilation and increases in NO/cGMP formation, suggesting an improvement of vascular endothelial function in the kidneys. However, no favorable effects of ghrelin were observed in IRS-2 KO mice, although the circulating IGF-1 level was significantly increased by ghrelin administration.

The detailed mechanisms by which ghrelin mitigates iARF are not clear. Previous reports showed that ghrelin treatment increased serum GH and IGF-1 concentrations (2,5) and that GH and IGF-1 exerted a tissue-protective action through endothelial NO formation (11,12). IGF-1 releases NO *via* activation of phosphatidyl inositol-3 kinase (PI3K) and its downstream effector Akt (22–24). Before activation of PI3K, it is necessary that IGF-1 bind to IGF-1 receptor and phosphorylates IRS (25). The IRS proteins are phosphorylated by insulin and IGF-1 stimulation, and four members of this family have been identified (IRS-1, IRS-2, IRS-3, and IRS-4). Through the analysis of IRS KO mice, IRS-1 and IRS-2 have been found to play major roles in the determination of insulin resistance. It has been shown that insulin resistance in IRS-1 and IRS-2 KO mice was related to the skeletal muscle and the liver, respectively (15,26). These IRS proteins are thought to exert a compensatory effect (27). The functions of these IRS proteins in the kidney have not been investigated fully as yet. However, it was reported that the expression level of IRS-2 mRNA in the kidney was more abundant than that of IRS-1 (28). Moreover, it has been suggested that IRS-2 but not IRS-1 may have a vascular protective effect on neointimal formation when the artery is mechanically injured (29). Therefore, we used IRS-2 KO mice to investigate whether the renal protective effect of ghrelin, especially at the vascular level, depended on the IGF-1/IRS-2 signaling pathway. The results showed that ghrelin had no effect on iARF in IRS-2 KO mice. The isolated kidneys of IRS-2 KO mice with iARF showed markedly attenuated responses to ACh. It is possible that insulin resistance in IRS-2 KO mice interferes with the responses to ghrelin independent of its GH stimulation. However, serum levels of BUN and creatinine and the renal injury score were the same in the vehicle and ghrelin treatment groups, suggesting that not only endothelium-dependent but also endothelium-independent actions of ghrelin may be altered in the IRS-2 KO mice. Furthermore, ghrelin improved endothelial function and renal function in iARF mice, which showed marked endothelial dysfunction. Although it is not clear whether IRS-1 has compensatory effects in the kidney, our results suggest that the signaling pathway between IGF-1 and IRS-2 plays a critical role in the renal protective effect of ghrelin. However, a GH/IGF-1–independent cardiovascular effect of ghrelin has also been suggested. Wiley *et al.* (30) reported that ghrelin had a vasodilatory effect on the isolated human internal mammary artery precontracted with endothelin-1 and that its effect was endothelium-independent. Moreover, subcutaneous injection of ghrelin for 3 wk improved ACh-induced vasodilation in GH-deficient rats, indicating a GH-independent action of ghrelin on the vascular endothelium. Physiologic activity of ghrelin is mediated by an interaction between ghrelin and GHSR (1). Recently, several groups reported that GHSR existed

in the pituitary, myocardium, aorta, and kidney and that various tissues, including the kidney, expressed the ghrelin gene (13). Furthermore, Mori *et al.* (31) reported that ghrelin was produced locally in the kidney, suggesting a direct effect of ghrelin on the kidney. However, in this study, we failed to show an improvement of renal function in IRS-2 KO mice by treatment with ghrelin. Thus, it is highly likely that the effect of ghrelin on the kidney is largely mediated by an IGF-1 signaling pathway.

The most rational dosage of ghrelin is still unclear. In this study, to examine whether this therapeutic regimen is rational, we injected ghrelin six times before and three times after ischemia. This injection schedule was based on the report by Nagaya *et al.* (5), in which they examined the effects of ghrelin in rats with heart failure and showed the cardiac-protective effect of ghrelin. Thus, we think that only one injection is not sufficient to protect renal function from iARF and the treatment protocol that was used by our group and others is appropriate to protect ischemic organ damage. It is possible that the continuous effect of ghrelin during the reperfusion period may be essential.

In this study to investigate the beneficial effect of ghrelin on renal endothelium-dependent vasodilation, we stimulated isolated perfused kidneys with ACh and AM. ACh and AM are known to have an endothelium-dependent vasodilating action, and we have already shown that AM induced vasorelaxation in an endothelium-dependent manner *via* the NO-cGMP pathway (16,32). In this study, we showed that treatment with ghrelin improved endothelium-dependent vascular responses to ACh and AM, but we did not observe a direct vasodilatory action of ghrelin in the renal artery of the isolated kidney. It seems well established that improvement of endothelial function is associated with an improvement of I/R injury at least in rodents (33,34). These results indicate that the renal protective effects of ghrelin may be mediated by an improvement of endothelial function through an IGF-1 signaling pathway.

Induction of apoptosis is one of the major causes of tissue damage after I/R injury (35,36). Several reports pointed out the existence of apoptotic cells and upregulation of Fas after I/R injury, particularly apoptosis of renal tubular epithelial cells (37). Inhibition of cellular apoptosis by ghrelin itself has not been investigated. However, the antiapoptotic activity of IGF-1 has been reported in various models, such as the unilateral ureteral obstruction model, ultraviolet radiation model, and I/R injury model (22,36,38). It is known that the tissue-protective effects of GH and IGF-1 are mediated by the PI3K/Akt pathway (22). Activated PI3K/Akt increases the release of NO and shows various effects, including antiapoptotic activity (23,24). Ghrelin binds to GHSR and upregulates the GH concentration in an intracellular calcium–dependent manner, resulting in increases of the serum IGF-1 level. In this study, ghrelin increased the serum level of IGF-1 and decreased the number of apoptotic renal tubular cells after I/R injury. It is possible for ghrelin to act as a tissue survival factor through the IGF-1/IRS-2 signaling pathway such as vascular endothelial growth factor, which also activates PI3K/Akt.

Our assay system is based on the chemiluminescent reaction of organ-derived NO with the luminol-H_2O_2 system, and this chemiluminescence is due to the formation of peroxynitrite from NO and H_2O_2. In previous studies (18,19), to confirm whether the changes of chemiluminescence and RPP were related to endothelium-derived NO, we examined the effect of inhibition of endothelial function using CHAPS, deoxycholic acid, or L-NMMA. After infusion of either agent, ACh-induced NO signal and vasorelaxation were diminished. However, infusion of exogenous NO increased NO chemiluminescence and decreased RPP. To exclude the possibility of superoxide as a precursor of peroxynitrite, we infused superoxide dismutase, but this caused no significant changes in chemiluminescence, denying the possibility of the involvement of organ-derived superoxide. Furthermore, there was a lag time of 5 to 15 s to mix the venous effluent and chemiluminescence agents. This lag time was too long for superoxide or a hydroxyradical but not for NO to be detected. Therefore, this assay system sensitively detected endothelium-derived NO production but not superoxide.

To demonstrate the effect of ghrelin on iARF, we used an I/R model. *In vivo* tissue injury induced by I/R is believed to be mediated by local inflammation and various inflammatory cytokines such as TNF-α and IL-1β. In addition, the production of reactive oxygen species in the kidney during reperfusion is suggested. Very high concentrations of NO, usually derived from inducible NO synthase (iNOS), are also considered to be toxic. The involvement of iNOS expression in iARF is still a matter of controversy. In a previous study, we did not detect iNOS expression in the kidneys with iARF from rats (33). One group investigated the antioxidant effect of ghrelin using an I/R model of the isolated rat heart. In that study, ghrelin suppressed the production of malondialdehyde, one of the markers of oxidative stress, in the myocardium in a dose-dependent manner (39). It has been reported that NO has a renal protective effect against superoxide anion (40,41). AM-induced cGMP production in the kidney with iARF was increased by ghrelin, suggesting an increase in NO availability and a decrease in oxidative stress. Further studies are required to clarify whether ghrelin itself or IGF-1–mediated NO release has an antioxidant activity in the kidney.

In conclusion, 45 min of ischemia and 24 h of reperfusion induced severe iARF in mice. However, administration of ghrelin before and during ischemia improved vascular endothelial function and renal excretory function and decreased the renal tissue damage and apoptosis of the tubular cells. The increment of IGF-1 and the subsequent activation of the IGF-1 signaling pathway play more important roles regarding the renal protective effect of ghrelin than the direct effect of ghrelin. Moreover, ghrelin has an appetite-increasing activity (42) and exerts some other favorable actions on energy metabolism, particularly in the anorexic condition, implicating a clinical application of this peptide in patients with iARF.

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Effect of neuromedin S on feeding regulation in the Japanese quail

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Abstract

Neuromedin S (NMS) was recently isolated from the brains of humans, mice and rats as an endogenous ligand for the orphan G protein-coupled receptors FM-3 and FM-4, which have been identified as neuromedin U (NMU) receptors 1 and 2, respectively. To investigate the role of NMS in avian species, we elucidated the effect of intracerebroventricular (i.c.v.) administration of rat NMS on food intake, body weight, body temperature and gross locomotor activity in adult Japanese quails. NMS significantly decreased food intake (and consequently body weight) in a time-dependent manner during 12-h light period, but increased both body temperature and gross locomotor activity. On the other hand, i.c.v. injection of rat NMU showed the reverse effects of NMS in Japanese quail. These results suggest that NMS may play an important role in regulating food intake and sympathetic nerve activity in the Japanese quail.

Keywords: Food intake; Body temperature; Neuromedin S; Japanese quails

Recently, a novel 36-amino acid residue neuropeptide was isolated from rat brain and was identified as an endogenous ligand for FM-3/GPR66 and FM-4/TGR-1 using a reverse-pharmacological technique [9]. FM-3/GPR66 and FM-4/TGR-1 have been already identified as neuromedin U (NMU) receptor type-1 (NMUR1) and NMU receptor type-2 (NMUR2), respectively [3]. The novel peptide was designated neuromedin S (NMS), because it is specifically expressed in the suprachiasmatic nucleus (SCN) [9]. Although NMS shares a C-terminal core structure (seven amino acid residues) with NMU, and activates recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells, it is not a splice variant of NMU because the genes for NMS and NMU have been mapped to discrete chromosomes [9].

The physiological functions of NMU have recently been clarified. Its most marked effect is on feeding regulation [3,7,10]. Intracerebroventricular (i.c.v.) administration of NMU decreases both the daily food intake during dark period and fasting-induced food intake in rats [5,12]. Conversely, injection of anti-NMU IgG increases dark-phase feeding compared with preimmune serum IgG [7]. Recently, our group has also demonstrated that NMU-knockout mice become obese [2]. These results indicate that NMU is a potent endogenous anorexigenic peptide in rats. In addition to feeding regulation, NMU increases gross locomotor activity, body temperature and heat production in rats, suggesting that it is a catabolic signaling molecule [10]. We previously reported that synthetic Japanese quail NMU decreased food intake and increased both body temperature and gross locomotor activity in Japanese quails [11], thus implying that avian NMU also plays important physiological roles.

As mentioned above, NMS shows homology of the C-terminal core structure with NMU and activates recombinant NMU1R and NMU2R expressed in Chinese hamster ovary cells. It may therefore also play important roles in feeding regulation, locomotor activity and body temperature. With this possibility in mind, we compared the effects of NMS with those of NMU in avian species. In this study, we chose to use adult Japanese quails instead of chickens because the growth curve in chickens is steep and so their body weight and food intake vary widely on a daily basis, whereas in the adult Japanese quail, the growth curve is relatively constant and therefore body weight and food intake are not subject to such great variability. In addition, it is possible to chronically implant an i.c.v. cannula into adult Japanese quails.

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