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A Radioimmunoassay for Rat Ghrelin: Evaluation of Method and Effects of Nonylphenol on Ghrelin Secretion in Force-Fed Young Rats

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Abstract

Antiserum YJC 13-31 against the rat ghrelin conjugated to bovine serum albumin (BSA) was produced in the rabbit and a double antibody radioimmunoassay (RIA) for ghrelin has been developed. Characterization results of this antiserum revealed no cross-reaction with human growth hormone and somatostatin. Weak cross-reactions with insulin (0.1%), rat growth hormone (0.1%) and glucagon (0.3%) were observed, which scarcely interfered the assay system. The sensitivity of this RIA was 5 pg per assay tube. With the rat serum samples, the within-assay precision was 7.1% and the between-assay precision was 12.3%. The RIA was also available to detect the ghrelin in rat tissue extracts with good parallelism to the rat ghrelin standard. In application, the serum ghrelin and corticosterone levels in weaned rats were measured by RIA. Gavage of saline was sufficient to raise serum ghrelin from 2.6 ± 0.18 to 6.7 ± 0.7 ng/ml ($P < 0.01$). Gavage with nonylphenol (NP) suppressed the elevation of serum ghrelin levels in a dose-dependent manner. Besides, gavages of saline elevated the serum levels of corticosterone from 108.8 ± 13.5 to 188.7 ± 23.5 ng/ml ($P < 0.01$) but the elevation effects of corticosterone from gavages were overcome by NP in the low dose of 50 mg/kg. It can be speculated that ingestion of NP is harmful to young animals during growth and environmental adaptation.

Key Words: anti-ghrelin serum, rat ghrelin RIA, serum and tissue ghrelin, corticosterone, nonylphenol

Introduction

Ghrelin is a growth-hormone-releasing acylated peptide isolated from the stomach as an endogenous stimulator for growth hormone secretagogue receptor. An *n*-octanoyl acid is bound to the 28-amino acid-peptide at the serine 3 position (18) (Fig. 1A). This highly conserved peptide is mostly produced by cells in the oxyntic glands of the stomach (7), and it is released into the bloodstream. It has recently been speculated that ghrelin regulates food intake and energy balance (6).

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(A)

Rat:

H - Gly - Ser - [Ser(n - octanoyl)] - Phe - Leu - Ser - Pro - Glu - His - Gln - Lys - Ala - Gln -

Gln - Arg - Lys - Glu - Ser - Lys - Lys - Pro - Pro - Ala - Lys - Leu - Gln - Pro - Arg - OH

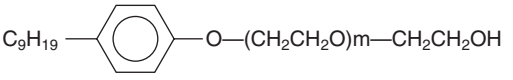
Human:

H - Gly - Ser - [Ser(n - octanoyl)] - Phe - Leu - Ser - Pro - Glu - His - Gln - Arg - Val - Gln -

Gln - Arg - Lys - Glu - Ser - Lys - Lys - Pro - Pro - Ala - Lys - Leu - Gln - Pro - Arg - OH

(B)

Nonylphenol polyethoxylate (NPE):



Nonylphenol (NP):

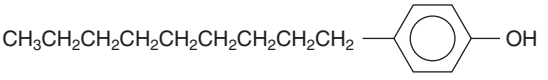


Fig. 1. (A) Sequences of the rat and human ghrelin. The amino acid residues 11 and 12 (framed) are different in these two species. The side chain, *n*-octanyl acid, binds to serine 3 within the 28 amino acid residues. (B) The structure of nonylphenol polyethoxylate (NPE) and nonylphenol (NP) characterized by its phenyl group.

Several mechanisms have been reported to regulate the secretion of ghrelin including fast (4, 8, 31, 37), blood glucose concentration (11), blood insulin concentration (10), other gut peptides such as GIP, GLP-1, glucagon, leptin and gastrin (13, 16, 17, 21, 35), and the autonomic nervous system (13). However, the effects of stress or environmental compounds on ghrelin secretion are still unclear.

Nonylphenol polyethoxylate (NPE) is a widely used non-ionic surfactant in detergents, paints, herbicides and many other synthetic products (15). NPE is graded by microbes in the environment to form its final metabolite, nonylphenol (NP) (Fig. 1B). With development in agricultures and industries, tremendous amounts of NP have been dumped into waters (28). Administration of a high dosage of NP (500 mg/kg/day) to newborn rats may cause disruption of the reproductive system and reproductive performance (25, 29, 30, 38), reduction in the weight of epididymis (14) and epididymal sperm density (26) and testicular abnormalities (19). Otherwise, studies on rats in our laboratory have revealed that the production of corticosterone is increased by NP in isolated rat zona fasciculata-reticularis of the adrenal gland (3), but plasma ghrelin levels were decreased by NP *in vitro* (unpublished data).

Because ghrelin is a small peptide, conjugation of ghrelin and protein to form an immunogen is necessary to generate specific antibodies. In the present study, bis-diazotized-benzidine (BDB) was used as a coupling reagent to induce an antiserum of rat ghrelin for radioimmunoassay (RIA); an assay system was developed for the investigation of serum or tissue-extract ghrelin. We evaluated the assay procedure with regard to its sensitivity, specificity, accuracy and practicality in the measurement of ghrelin levels in sera and tissue extracts. The RIA was then used to investigate the effects of NP on ghrelin secretion in young rats.

Materials and Methods

Reagents

Triton X-100 was obtained from Riedel-deHaen (56029, Germany), and sodium azid was from Kokusan Chemical Works (Tokyo, Japan). Benzidine dihydrochloride (bis-diazofized-benzidine, BDB, B-3383), bovine serum albumin (crystallized, A-4378, and RIA grade, A-7888), potassium chloride (P-4504), sodium chloride, nonylphenol (NP), IGEPAL® CA-630, sodium deoxycholate, sodium dodecyl sulfate (SDS), tris hydrochloride (Tris), protease inhibitor cocktail and boric acid (B-6768) were obtained from Sigma (St. Louis, MO, USA). Unlabeled rat ghrelin (H-Gly-Ser-[Ser(n-octanoyl)]-Phe-Leu-Ser-Pro-Glu-His-Gln-Arg-Val-Gln-Gln-Arg-Lys-Glu-Ser-Lys-Lys-Pro-Pro-Ala-Lys-Leu-Gln-Pro-Arg-OH) was from

AnaSpec (24160, SanJose, CA, USA). Radiolabeled [His(^{125}I)]-human ghrelin was from Perkin-Elmer (Turku, Finland). TiterMax® Gold was obtained from CytRx Corporation (Los Angeles, CA, USA).

Experimental Animals and Design

Female New Zealand white rabbits, weighing from 2.0 to 2.4 kg, were used to induce antibodies against rat ghrelin. For the NP experiment, male and female Sprague Dawley rats aged 3 weeks old, weighing 30 to 45 g, weaned from different mothers, were housed under conditions of controlled temperature (22-26°C), lighting (14 h of light, 10 h of darkness) and humidity. Food and water were provided *ad libitum*. Every morning, oral gavages of NP 50, 100 or 200 mg/kg, or vehicle of the same amount, were performed in these young rats from 19 to 25 days after birth. The young rats were decapitated at 8:30 AM on 26 days of age. Trunk blood samples were collected. Sera were obtained after centrifugation at 1,000 × g for 30 min and stored at -20°C until radioimmunoassay (RIA) for ghrelin and corticosterone.

Conjugation of Rat Ghrelin with Bovine Serum Albumin

Five milligram of the rat ghrelin was dissolved in 5 ml borate-NaCl buffer (0.16 M boric acid, 0.13 M sodium chloride, pH 9.0) and conjugated to 50 mg BSA (Sigma A-4378) using 8 mg benzidine dihydrochloride in 1 ml, 0.2 N HCl solution as previously described by Bassiri and Utiger (1). The reaction mixture was dialyzed at 4°C against 1 L water for 6 days and 0.15 M NaCl for 1 day before determining the protein concentration in the resultant mixture. The protein concentration was determined by the method of Lowry *et al*. (23).

Immunization

Approximately 1 mg conjugant was dissolved in 1 ml 0.15 M NaCl solution and emulsified with 1 ml complete Freund's adjuvant and 0.1 ml TiterMax® Gold before intradermal injections at multiple sites along the backs of rabbits. After the primary injection, booster immunizations were performed once a week in the first month and once a month afterward. Blood samples were collected from an ear vein incision before each immunization and the sera were examined for the presence of antibodies against rat ghrelin at various times of immunization up to 270 days.

Binding Capacity

The highest dose of unlabeled ghrelin precipitated by 0.1 ml of the antiserum against rat ghrelin (anti-r-ghrelin) at a selected concentration was defined as the binding capacity of that antiserum. Radiolabeled ghrelin (^{125}I-ghrelin) at the volume of 0.1 ml (6,000~7,000 cpm) was incubated at 4°C with various doses of unlabeled ghrelin at 0.3 ml adjusted by borate buffer solution and anti-r-ghrelin serum at 1:400 dilution by 0.05 M EDTA in PBS for 24 h. The borate buffer solution was composed of 25 mM boric acid, 25 mM potassium chloride, 0.5% BSA (RIA grade, Sigma A-7888), 0.1% Triton X-100 and 0.05% sodium azide. Tubes containing ^{125}I-ghrelin and the antiserum were assigned as maximum binding. Self-produced goat anti-rabbit gamma globulin (ARGG 6-5) at 0.05 ml, 1:20 dilution, was added with further incubation at 4°C for 48 h. Two milliliter of phosphate buffer saline (PBS; 0.14 M NaCl, 3.4 mM NaH_2PO_4, 6.8 mM Na_2HPO_4, 1.6 mM Na azide, pH 7.0) was added to the solution. Bound ^{125}I-ghrelin and free ^{125}I-ghrelin were separated by centrifugation at 1,000 × g for 30 min. The radioactivity in the precipitates was determined by a gamma counter (PerkinElmer, Wizard, 1470 automatic gamma counter, Turku, Finland). The highest dose of unlabeled ghrelin which failed to significantly decrease the binding of radiolabeled ghrelin to the anti-r-ghrelin serum as compared with the maximum binding was designated as the binding capacity of that antiserum.

Tissue Extracts

The rat medial basal hypothalamus (MBH) block (about 20 mg) was dissected and weighed from the region lying between the rostral borders of the optic chiasma and mammillary bodies with a depth no more than 2 mm. A small piece (about 40 mg) of cerebral cortex was cut and weighed from the frontal lobe. Dissected MBH and cerebral cortex were homogenized with 0.4 ml tissue lysis buffer (150 mM NaCl, 1.0% IGEPAL® CA-630, 0.5% sodium deoxycholate, 0.1% SDS, 50 mM Tris, 1% protease inhibitor cocktail, pH 8.0) and were maintained in ice for 30 min. After centrifugation with 14,000 × g for 5 min, supernatants were obtained. The final volume was adjusted to 4 ml by the assay buffer (borate buffer), and the solution was adjusted to pH 7.0 by HCl.

Fresh rat stomach was diced and boiled for 5 min in 5 volumes of double-distilled water to inactivate intrinsic proteases. Chemicals were added to the suspension to yield final concentrations of 1 M acetic acid and 20 mM HCl. Peptides were extracted by homogenizing with a Polytron mixer. The supernatant of the extracts, obtained with centrifugation at 200,000 × g for 30 min, was subjected to acetone precipitation at a concentration of 66% acetone. After the precipitates had been removed after centrifugation at 200,000 × g for 30 min, the supernatant acetone was

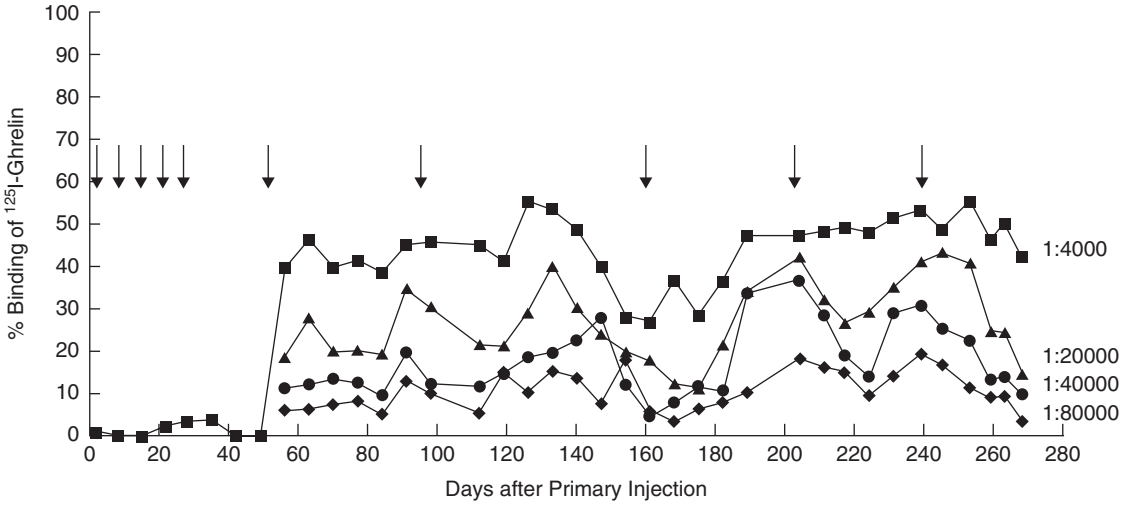


Fig. 2. Time course in days of obtaining antisera and binding percentage to radiolabeled ghrelin of various antisera dilutions in the rabbit No. 13. The arrows represent the booster immunization.

evaporated and stored at -20°C. For ghrelin radioimmunoassay, the dried extracts were restored by PBS and 1:400 diluted by the borate buffer solution.

Radioimmunoassay (RIA) of Ghrelin

A known amount of unlabeled ghrelin, other hormones, or an aliquot of a plasma sample was adjusted to a total volume of 0.3 ml by the borate buffer solution. The mixture was incubated with 0.1 ml antiserum appropriately diluted by 0.25% normal rabbit serum, and 0.1 ml ^{125}I-ghrelin (6,000-8,000 cpm) at 4°C for 24 h. The normal rabbit serum was diluted by 0.05 M EDTA-PBS. Duplicate standard curves with about 12 points ranging from 1 pg to 3,000 pg were included in each assay while unknown samples in an amount of 0.1 ml were assayed in duplicates. An adequate amount of 0.05 ml ARGG at 1:20 dilution was then added with a further incubation at 4°C for 48 h. At the end of incubation, 2 ml cold PBS was added, and the assay tubes were centrifuged at 1,000 × g for 30 min. The supernatants were discarded and radioactivity in the precipitates was counted in a gamma counter. The ghrelin concentrations in unknown samples were calculated from the standard curve using a computer program with a log-logit transformation of the standard curve.

RIA of Corticosterone

The concentrations of corticosterone in 10-fold ether extracted serum were determined by RIA as described elsewhere (2, 22), with anti-corticosterone serum (PSW#4-9, by Dr. P.S. Wang, NYMU, Taipei, Taiwan, ROC); the sensitivity of corticosterone RIA was 5 pg per assay tube. The intra- and interassay coefficients of variation were 4.2% (n = 5) and 7.2% (n = 5), respectively.

Statistical Analysis

All values are given as the means ± SEM. Statistical significance between the mean values was assessed by paired Student's *t*-test. The level of significance chosen was $P < 0.05$. Pearson correlation procedures were used to test the correlation between NP doses and ghrelin plasma levels.

Results

Antiserum to Rat Ghrelin

The highest percent binding of ^{125}I-ghrelin to the immunoreactive antiserum was collected from rabbit no.13 on days 189, 204 and 239 after the initial immunization (Fig. 2). When 0.1 ml of the mixture of anti-r-ghrelin serum collected from these days (YJC 13-31) was used at a dilution of 1:40,000 bound over 30% of the ^{125}I-ghrelin in the absence of unlabeled ghrelin.

Binding capacity. Compared with the maximal binding (56%), the binding percentage sharply declined with 2,000 pg of unlabeled ghrelin. Since the binding of ^{125}I-ghrelin with 0.1 ml of the anti-r-ghrelin serum YJC 13-31 at 1:400 dilution was not significantly reduced by 2,000 pg unlabeled ghrelin (Fig. 3), the binding capacity was estimated as 2,000 (pg ghrelin) × 400 (1:400 dilution) × 10 (1/10 volume of 1 ml) = 8,000,000 pg ghrelin = 8 μg ghrelin for 1 ml of undiluted antiserum.

Sensitivity. The least amount of ghrelin which resulted in 90% maximum binding of ^{125}I-ghrelin

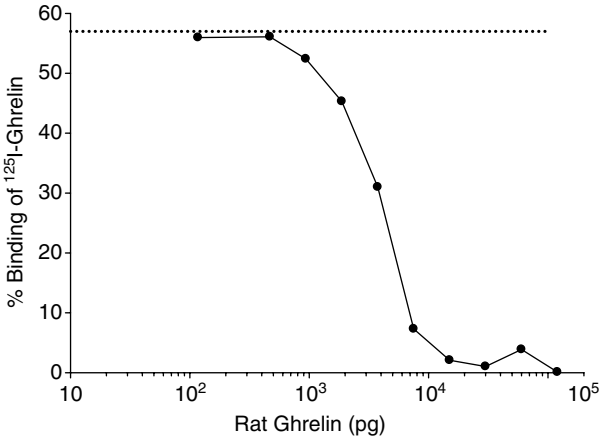


Fig. 3. Displacement of the binding of ^{125}I-labeled ghrelin with 0.1 ml of anti-h-ghrelin serum YJC 13-31 at 1:400 dilution by unlabeled human ghrelin. The dotted line represents maximal binding (56.7%).

with the anti-r-ghrelin serum YJC 13-31 at an initial dilution of 1:60,000 ranged from 5-500 pg in five different assays. The linear range of detectable ghrelin was 5 to 500 pg (Fig. 4A).

Parallelism. The inhibition curves generated by the rat MBH extracts, rat cerebral cortex extracts, rat stomach extracts and fasting rat serum are shown in Fig. 4A. The slopes of the lines that are log-logit-transformed from the inhibition curves did not deviate significantly from the parallelism with the ghrelin standard curves (Fig. 4B).

Specificity. Cross-reaction of the anti-r-ghrelin serum YJC 13-31 with other compounds related to metabolism in the human and the rat is shown in Table 1 and Fig. 4C. Cross-reaction is defined as the amount of rat ghrelin divided by the amount of hormones with 50% replacement of ^{125}I-ghrelin (33). Cross-reaction with other hormones was low, and the RIA system was not interfered by these compounds except human ghrelin that was 79.5% cross-reacted to the anti-r-ghrelin YJC 13-31.

Precision. The within-assay precision was represented by the coefficient of variability (CV) of ghrelin concentration in the plasma of the fasting rat employed as an internal standard at 5 different doses in one assay (10, 20, 40, 60 and 80 µl). The between-assay precision was represented by the CV of ghrelin concentration in a single dose (40 µl) of internal standard in 10 assays. The within-assay CV was 7.1%. The between-assay CV was 12.3%.

Plasma Ghrelin and Corticosterone Response to Nonylphenol in Force-Fed Young Rats

The plasma ghrelin and corticosterone levels in the weaned rats were measured by RIA. Chronic

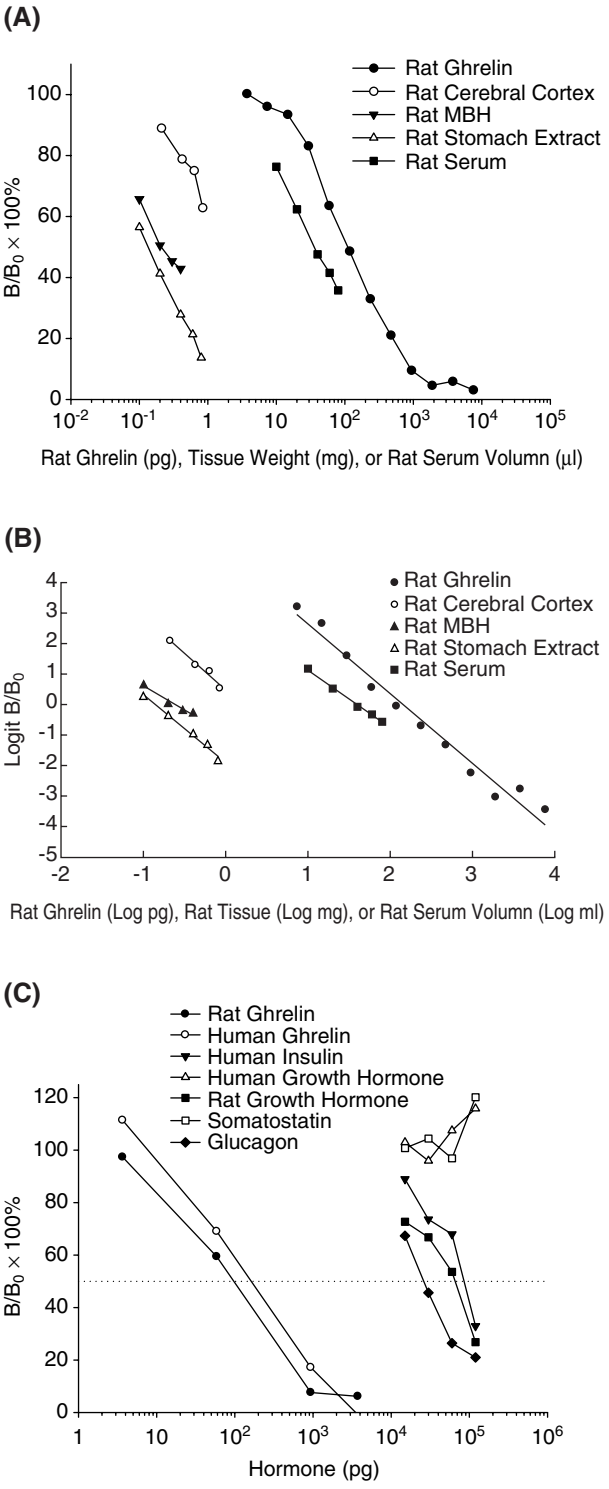


Fig. 4. (A) Dose-response curves for rat standard ghrelin, serum and tissue extracts from the cerebral cortex, MBH and the stomach with the anti-r-ghrelin YJC 13-31 at 1:60,000 dilution. (B) Transformed from Fig. 4A, parallel line analyses of diluted human and rat serum samples to standard human ghrelin. (C) Cross-reaction of YJC 13-31 to human ghrelim, human insulin, human growth hormone, rat growth hormone, somatostatin and glucagon. The dotted line represents 50% binding of ^{125}I-labeled ghrelin.

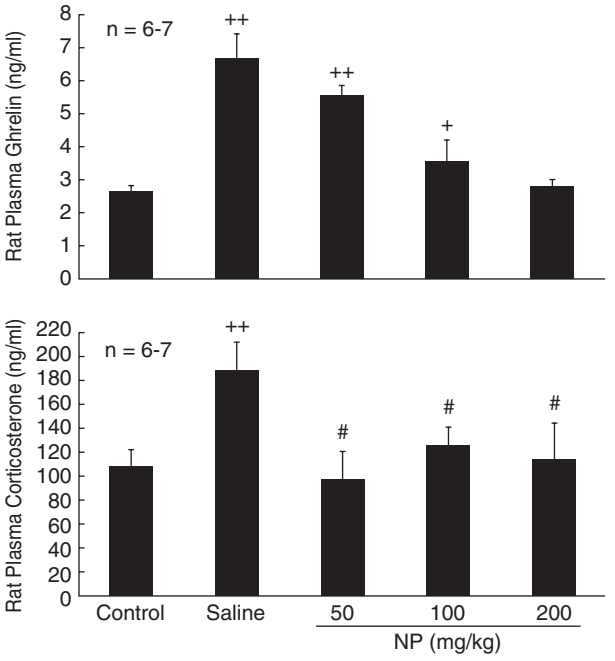


Fig. 5. Plasma concentration of ghrelin and corticosterone in response to gavages and NP in rats. The 19-day-old young rats were force-fed for 7 days and were decapitated on day 8 without gavages; plasma samples for ghrelin and corticosterone RIA were obtained with centrifugation at 1,000 × g for 30 min. The plasma levels of ghrelin were 2.6 ± 0.2 ng/ml in untreated (control) group, elevated to 6.7 ± 0.7 ng/ml in saline force-fed group, and suppressed to 5.5 ± 0.3, 3.5 ± 0.6 and 2.7 ± 0.2 ng/ml with NP dose of 50, 100 and 200 mg/kg, repectively. The 10-fold ether extracted plasma levels of corticosterone were 108.8 ± 13.5 ng/ml in the control group, elevated to 188.7 ± 23.5 ng/ml in the saline force-fed group, and suppressed to 96.9 ± 24.1, 125.1 ± 16.1 and 113.8 ± 31.2 ng/ml with NP dose of 50, 100 and 200 mg/kg, respectively. The data are expressed as means ± SEM. +, $P < 0.05$ *vs.* untreated group; ++, $P < 0.01$ *vs.* untreated group; #, $P < 0.05$ *vs*. the saline gavage group.

force-feeding (gavage) of saline for 7 days was sufficient to raise plasma ghrelin from 2.6 ± 0.2 to 6.7 ± 0.7 ng/ml ($P < 0.01$). Chronic gavage with NP suppressed the elevation of plasma ghrelin concentration in a dose-dependent manner and with a Pearson correlation coefficient of -0.95 and R square of 0.899. NP 200 mg/kg suppressed the ghrelin concentration (2.8 ± 0.2 ng/ml) to the level of the untreated group. Meanwhile, gavages of saline elevated the plasma levels of corticosterone from 108.8 ± 13.5 to 188.7 ± 23.5 ng/ml ($P < 0.01$). However, the elevation effects of corticosterone from gavages were overcome by the lowest dose of NP of 50 mg/kg (Fig. 5). Correlation between plasma ghrelin and corticosterone was poor with a correlation coefficient of + 0.58 and R square of 0.34. This is because the corticosterone level declined to the basal level by the lowest dose of NP. The declining pattern of corticosterone departed from that of ghrelin which was an absolute dose-dependent inhibition.

Table 1. Test for cross reaction of anti-r-ghrelin YJC 13-31[a] serum with various hormones

Hormone[b]	% Cross Reactivity[c]
Rat Ghrelin	100
Human Ghrelin	79.5
Insulin	0.1
Human Growth Hormone	< 0.001
Rat Growth Hormone	0.1
Somatostatin	< 0.001
Glucagon	0.3

[a] Anti-r-ghrelin YJC 13-31 serum was used at 1:60,000 dilution.
[b] The largest dose tested was 120 ng/tube.
[c] Defined as X/Y × 100, where X is the mass of unlabeled ghrelin required to displace 50% of ^{125}I-ghrelin bound to the antibody, and Y is the mass of heterologous compound required to displace 50% of ^{125}I-ghrelin bound to the same antibody.

Discussion

The antiserum YJC 13-31 against rat ghrelin produced in a rabbit has been characterized. The RIA system developed with this antiserum is highly specific, sensitive and reproducible. The RIA is suitable for the measurement of rat ghrelin both in sera or tissue extracts.

Conjugation of small peptides with BSA by BDB is effective and has been widely used (1, 32). In this study, freshly prepared BDB was employed and the production of antisera against BSA conjugated ghrelin was not laborious by a multiple-site immunization procedure.

Rat ghrelin is similar to human ghrelin with only a difference of two amino acids (Fig. 1) (12, 18). Not surprisingly, the anti-r-ghrelin YJC 13-31 that primarily binds to rat ghrelin also has a 79.5% cross reactivity to human ghrelin (Table 1). However, this cross-reaction to human ghrelin is necessary in this RIA system because the only commercially available radiotracer, ^{125}I–labeled ghrelin, is human ghrelin. Otherwise, the antisera revealed slight or no cross-reaction with hormones related to food intake and metabolism such as insulin, rat and human growth hormone, somatostatin and glucagon. Nevertheless, this weak cross-reaction does not significantly disturb the radioimmunoassay.

Ghrelin is produced mainly in gastric mucosal epithelium; however, it is also produced in the hy-

pothalamus where it stimulates the secretion of growth hormone from the anterior pituitary gland (27). Therefore, the ability to detect both the circulatory ghrelin and the tissue harboring ghrelin is important. The fact that the log-logit-transformed inhibition curves with varying amounts of rat plasma as well as the rat tissue extracts from cerebral cortex, MBH and stomach parallel to that of the ghrelin standard suggests that RIA is suitable for the measurement of rat ghrelin in rat samples from sera and tissue extracts.

Studies on exogenous agents that affect the secretion of ghrelin were limited. Stengel *et al*. reported that cold ambient temperature elevated plasma ghrelin levels in both sham and post-laparotomy rats (36). Obviously, the chronic force-feeding procedure for 7 days was a kind of stress to rats and raised plasma corticosterone levels. It has been well documented that growth hormone (GH) and adrenal corticoids are synergistic stress hormones (5, 9, 24, 34). In this study, we found that the up-stream hormone of GH, ghrelin, elevated by the physical distress of one-week gavages. Ghrelin secretion could be speculated as a part of stress response or, even more, a kind of stress hormone to up-regulate the secretion of GH (Fig. 5).

NP is an omnipresent industrial contaminant around us. An environmental investigation reported that the concentrations of NP were 108 to 298 ng/l in bottled water and 1987 ng/l in tap water. Estimated intake of NP was 1410 ng/day in people who drink 2 L of water per day, and the major contact routes are ingestion and inhalation (20). Extensive exposure to NP is especially hazardous to newborn babies whose body weights are lighter than those of adults. Results of this study showed that in young rats, the stress-induced elevation of ghrelin levels was suppressed by NP in a dose-dependent manner. The stress-induced elevation of corticosterone levels was reduced by low doses of NP. Adrenal hormones are necessary for life. Idiopathic or iatrogenic insufficiency of adrenal hormones may cause adrenal crisis or Addison's disease. Without these hormones, blood pressure cannot be maintained during times of physical stress such as illness or injury and may rapidly lead to death. Nursing animals are adapted to harsh circumstances; moreover, they encounter growing up and development. Hence, it is potentially harmful to the pops that NP suppresses stress respondents such as corticosterone and growth initiators such as ghrelin.

In conclusion, a reliable rat ghrelin RIA system based on the antiserum YJC 13-31 has been developed in our lab. Further studies have revealed that serum ghrelin, along with corticosterone, served as a stress hormone that was raised by chronic gavages stimulation. Ingestion of NP inhibited responses of ghrelin and corticosterone that might be harmful to young animals in growth and adaptation.

Acknowledgments

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