nitric oxide synthase (eNOS) through the c-AMP/protein kinase A pathway [19]. Activation of eNOS is known to contribute to bone marrow cell mobilization, leading to neovascularization [20]. These results raise the possibility that BPS may have beneficial effects on the ischemic myocardium through enhancement of bone marrow cell mobilization.

Thus, the purposes of this study were: (1) to examine the effect of BPS on mobilization and recruitment of bone marrow cells after acute myocardial infarction, (2) to investigate whether BPS induces neovascularization in the ischemic myocardium, and (3) to investigate whether treatment with BPS improves cardiac function in rats with myocardial infarction.

Methods

Model of myocardial infarction. We used male Sprague–Dawley rats (Japan SLC Inc., Hamamatsu, Japan) weighing 185–215 g. Myocardial infarction was produced by left coronary ligation, as described previously [21]. Briefly, after rats were anesthetized with sodium pentobarbital (30 mg/kg), they were artificially ventilated with a volume-regulated respirator. The heart was exposed via a left thoracotomy incision. Then, the left coronary artery was ligated 2–3 mm from its origin between the pulmonary artery conus and the left atrium with a 6-0 Prolene suture. Finally, the heart was restored to its normal position, and the chest was closed. Experimental protocols were performed in accordance with the "Guidelines of the Animal Care Ethics Committee of the National Cardiovascular Center Research Institute", which complies NIH Guidelines.

Administration of BPS. Immediately after coronary ligation, BPS (200 μg/kg/day, Astellas Pharma Inc., Tokyo, Japan) was subcutaneously administered to surviving rats using an osmotic mini-pump for 4 weeks (BPS group, $n = 12$). As a control, saline was similarly administered to rats receiving coronary ligation (Control group, $n = 12$).

Echocardiographic studies. Echocardiographic studies were performed 4 weeks after coronary ligation. M-mode tracings were obtained at the level of the papillary muscles using an echocardiographic system equipped with a 7.5-MHz phased-array transducer (HP SONOS 5500; Hewlett Packard Co., Andover, MA). Anterior and posterior end-diastolic and end-systolic wall thickness, LV end-diastolic and end-systolic dimensions, and LV fractional shortening were measured by the American Society for Echocardiography leading-edge method in three consecutive cardiac cycles. LV meridional wall stress was estimated as $0.344 \times$ LV pressure $\times$ {LV dimension/(1 + PWT/LV dimension)}, where PWT is posterior wall thickness [22].

Hemodynamic studies. Hemodynamic studies were performed 4 weeks after coronary ligation, following echocardiography. After anesthesia with pentobarbital sodium, a 1.5F micromanometer-tipped catheter (Millar Instruments Inc., Houston, TX) was advanced into the LV through the right common carotid artery. Hemodynamic variables were measured with a pressure transducer connected to a polygraph. After completion of these measurements, the left and right ventricles and the lungs were excised and weighed. Infarct size was determined as a percentage of the entire LV area ($n = 5$ in each group), as reported previously [23]. Briefly, incisions were made in the posterior LV so that the tissue could be pressed flat. The circumference of the entire flat LV and of the visualized infarcted area, as judged from both the epicardial and endocardial sides, was outlined on a clear plastic sheet. The difference in weight between the two marked areas on the sheet was used to determine infarct size and was expressed as a percentage of LV surface area.

Measurement of plasma ANP level. Blood samples were obtained 4 weeks after coronary ligation. Plasma atrial natriuretic peptide (ANP), a marker for heart failure, was measured by enzyme immunoassay (Peninsula Laboratories Inc., San Carlos, CA).

Mononuclear cell mobilization and FACS analysis. To investigate whether administration of BPS mobilizes bone marrow cells, an additional 12 rats were randomized to receive BPS (200 μg/kg/day, BPS group, $n = 6$) or saline (Control group, $n = 6$). On the third day of BPS or saline treatment, 4 ml of blood was drawn from the inferior vena cava of each rat. Peripheral blood was obtained at the end of infusion. After mononuclear cells were counted, they were incubated for 30 min at 4 °C with fluorescein isothiocyanate (FITC)-conjugated mouse monoclonal antibodies against rat CD34 (clone ICO-115, Santa Cruz) and CD45 (clone OX-1), and FITC-conjugated rabbit anti-rat c-Kit polyclonal antibody (clone C-19, Santa Cruz). Immunofluorescence-labeled cells were analyzed by quantitative flow cytometry with a FACSCalibur flow cytometer (BD Biosciences, Mountain View, CA). Isotype-identical antibodies served as controls.

RT-PCR assay. To investigate whether bone marrow cells express the prostacyclin receptor (IP receptor), we analyzed expression of its mRNA by reverse transcription-polymerase chain reaction (RT-PCR). In brief, total RNA of bone marrow cells was extracted with guanidine isothiocyanate (RNeasy Mini Kit, Qiagen). Then, reverse-transcribed single-stranded cDNA was subjected to PCR (PCR Amplification Kit, Takara) using primer sets for the IP receptor (Hokkaido System Science Co., Ltd., Sapporo, Japan, forward, 5′-GGCACGAGAGGATGAAGTTTACC-3′; reverse, 5′-GTCAGAGGCACAGCAGTCAATGG-3′) and G3PDH (Clonetech Laboratories Inc., Mountain View, CA, forward, 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′; reverse, 5′-CATGTAGGCCATGAGGTCCACCAC-3′).

Creation of bone marrow-chimeric rats. To assess recruitment of bone marrow cells after BPS administration, bone marrow transplantation was performed by using male normal Sprague–Dawley rats as recipients and male Green fluorescent protein (GFP)-transgenic rats (SD-Tg [Act-EGFP] CZ-004Osb, Japan SLC Inc.) as donors, using a previously described method [24]. Briefly, bone marrow was harvested by flushing the cavity of femurs and tibias from GFP-transgenic rats with phosphate-buffered saline. Then, 3×10^7 GFP-positive bone marrow cells were individually administered to 12 lethally irradiated (900c Gray) rats via the tail vein. Four weeks after transplantation, flowcytometric analysis determined that 90% of peripheral blood mononuclear cells from both donors and 8 of 12 chimeric rats were GFP-positive, suggesting the establishment of stable chimerism. These chimeric rats were subjected to left coronary ligation, followed by administration of BPS (200 μg/kg/day, BPS group, $n = 4$) or saline (Control group, $n = 4$) using an osmotic mini-pump for 4 weeks.

Histological examination. To detect fibrosis in the cardiac muscle, the LV myocardium ($n = 5$, each group) was fixed in 10% formalin, cut transversely in three sections, embedded in paraffin, and stained with Masson's trichrome. To detect capillary endothelial cells in the peri-infarct area, we performed DAB staining (LSAB2 System HRP, Dako Cytomation Co., Denmark) using rabbit polyclonal anti-von Willebrand factor (vWF) antibody (Dako). A total of 10 different fields from three different sections were randomly selected, and the number of capillaries was counted in the peri-infarct area using a light microscope at 200× magnification. Capillary density was expressed as the mean number of capillaries per square millimeter. Also, 4 weeks after coronary ligation in bone marrow-chimeric rats ($n = 4$ in each group), the LV myocardium was excised, embedded in OCT compound, snap-frozen in liquid nitrogen, and cut transversely into 6-μm-thick sections from base to apex. Immunofluorescent staining was performed using rabbit polyclonal anti-vWF antibody (Dako), mouse monoclonal anti-cardiac troponin T antibody (Neomarkers, Fremont, CA), and rabbit polyclonal Alexa 488-conjugated anti-GFP antibody (Molecular Probes Inc., Eugene, OR). The nuclei were counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). We measured the number of GFP/vWF-double-positive cells incorporated into vascular structures in 10 randomly selected fields in the peri-infarct area per section in a blinded fashion using a fluorescence microscope.

Statistical analysis. Numerical values are expressed as means ± SEM. Comparisons of parameters between two groups were made by unpaired Student's t test. A value of $p < 0.05$ was considered significant.

Results

Cardiac structure

Body weight at 4 weeks after coronary ligation was significantly greater in the BPS group than in the Control group (Table 1). Right ventricular weight and lung weight in the BPS group were significantly smaller than those in the Control group, although LV weight did not differ between the two groups. Moderate to large infarcts were observed in the Control group (Fig. 1A). However, administration of BPS significantly decreased infarct size in rats with myocardial infarction (Fig. 1A and B). BPS significantly decreased LV end-diastolic dimension (LVDD) (Fig. 1C).

Table 1
Physiological profiles of experimental groups

	Control	BPS
Number	12	12
Body weight (g)		
Baseline	198 ± 3	204 ± 3
After treatment	319 ± 6	352 ± 9*
LV wt/body wt (g/kg)	2.28 ± 0.04	2.27 ± 0.04
RV wt/body wt (g/kg)	0.99 ± 0.05	0.61 ± 0.02**
Lung wt/body wt (g/kg)	6.55 ± 0.62	3.88 ± 0.1**
Plasma AND level (pg/ml)	798 ± 99	498 ± 57*

Control, infarct rats without treatment; BPS, infarct rats treated with BPS administration; AND, atrial natriuretic protein. Data are expressed as means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ vs. Control group.

Cardiac function

Neither heart rate nor mean arterial pressure differed between the BPS and Control groups (Table 2). LV fractional shortening and LV maximum dP/dt in the BPS group were significantly greater than those in the Control group (Fig. 2A and B). LV end-diastolic pressure (LVEDP) in the BPS group was significantly lower than that in the Control group (Fig. 2C). LV minimum dP/dt was also improved by BPS (Fig. 2D). Treatment with BPS attenuated the increase in plasma ANP level after myocardial infarction (Table 1). BPS significantly increased anterior wall thickening, although it did not significantly alter posterior wall thickening (Table 2). Thickness of the anterior and posterior walls tended to be greater in the BPS group, but these changes did not reach statistical significance. LV diastolic wall stress in the BPS group was significantly lower than that in the Control group.

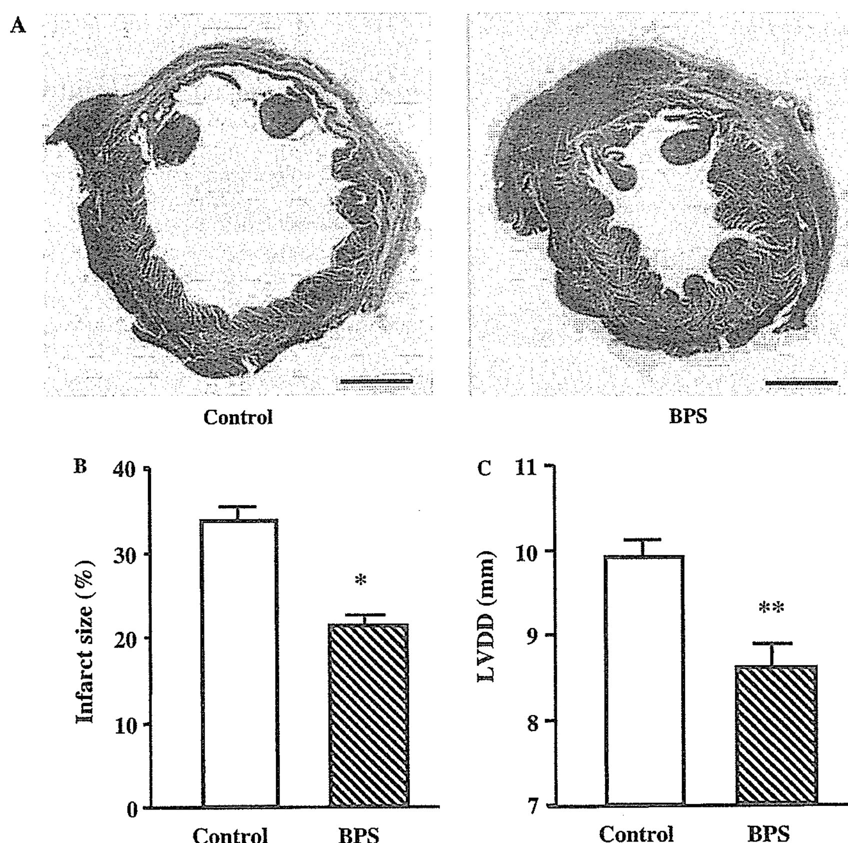

Fig. 1. (A) Representative examples of Masson's trichrome staining of transverse sections of LV myocardium 4 weeks after coronary ligation. Scale bars = 2 mm. (B,C) Quantitative analysis of infarct size and LV end-diastolic dimension (LVDD). Infarcted area and LVDD in the BPS group were significantly smaller than those in the Control group. Data are expressed as means ± SEM. $^*p < 0.05$, $^{**}p < 0.01$ vs. Control group.

Table 2
Echocardiographic and hemodynamic data

	Control	BPS
AWT diastole (mm)	0.62 ± 0.04	0.74 ± 0.05
AW thickening (%)	17 ± 3	34 ± 6*
PWT diastole (mm)	1.55 ± 0.07	1.70 ± 0.04
PW thickening (%)	43 ± 4	49 ± 3
Heart rate (bpm)	458 ± 7	471 ± 10
Mean arterial pressure (mmHg)	103 ± 5	115 ± 4
LV systolic pressure (mmHg)	113 ± 4	127 ± 5*
LV diastolic wall stress (kdyne/cm^2)	24 ± 4	5 ± 1**
LV systolic wall stress (kdyne/cm^2)	267 ± 18	225 ± 14

AWT, anterior wall thickness; AW, anterior wall; PWT, posterior wall thickness; PW, posterior wall. Data are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. Control group.

Mobilization of bone marrow cells

RT-PCR demonstrated that IP receptor mRNA was expressed in bone marrow cells (Fig. 3A), indicating a direct effect of BPS on these cells. Three-day administration of BPS significantly increased the number of peripheral blood mononuclear cells compared to saline administration (Fig. 3B). Administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells (Fig. 3C and D). BPS also increased the number of CD45-positive hematopoietic lineage cells (Fig. 3E).

BPS-induced neovascularization

Chimeric rats with GFP-expressing bone marrow were used to assess recruitment of bone marrow cells. Four weeks after coronary ligation, bone marrow-derived GFP-positive cells were incorporated predominantly into the infarcted region and its border zone (Fig. 4A), while these cells were rarely detected in the noninfarcted myocardium. Some of the GFP-positive cells stained for vWF and formed vascular structures. Semi-quantitative analysis demonstrated that the number of GFP-positive cells in the myocardium was significantly greater in the BPS group than in the Control group (Fig. 4B). The number of GFP-vWF double-positive cells (bone marrow-derived endothelial cells) in the ischemic myocardium was significantly greater in the BPS group than in the Control group (Fig. 4C). In addition, a small number of GFP-troponin T-double-positive cells were observed in the BPS group (Fig. 4D).

Capillary density

In the peri-infarct area, clustering of relatively small vessels was seen in BPS-treated hearts, which is indicative of recent endothelial regeneration (Fig. 5A). Semi-quantitative analysis also demonstrated that administration of BPS significantly increased the capillary density in the peri-infarct area compared to the Control group (Fig. 5B).

Discussion

In the present study, we demonstrated that treatment with BPS (1) decreased infarct size and improved cardiac structure and function in rats with acute myocardial infarction, (2) increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells in rats, and (3) increased the number of bone marrow-derived endothelial cells and the capillary density in the ischemic myocardium. These results suggest that BPS may have beneficial effects on ischemic myocardium at least in part through enhancement of neovascularization by mobilizing bone marrow cells.

Earlier studies have reported that prostacyclin has cardioprotective effects in ischemia–reperfusion injury through inhibition of neutrophil activation and migration [25,26]. BPS is also reported to inhibit chemotaxis and superoxide anion production of neutrophils which contribute to tissue damage by releasing tissue destructive lysosomal enzymes [27]. Infusion of BPS has been shown to reduce infarct size in the dog heart with left coronary occlusion by reducing myocardial oxygen demand and by inhibition of the migration of neutrophils [28]. However, these

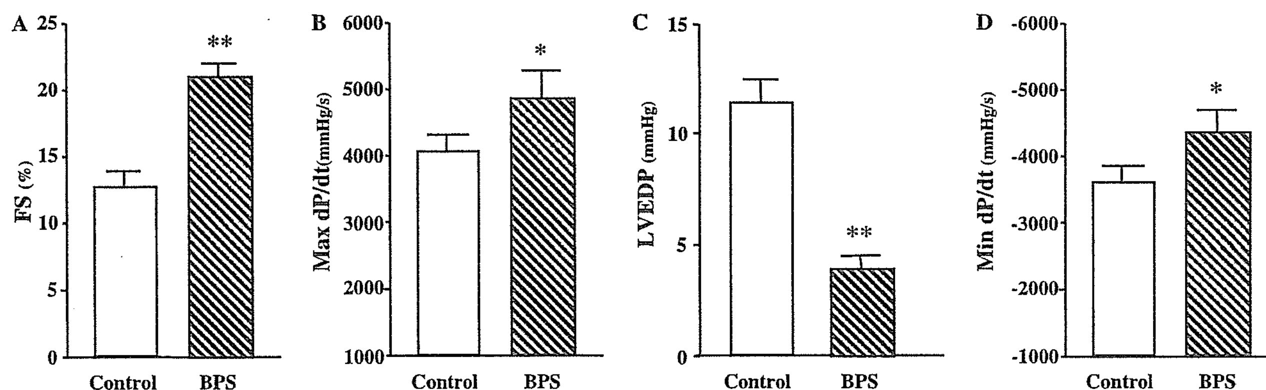

Fig. 2. Cardioprotective effects of BPS on echocardiographic and hemodynamic parameters. FS, fractional shortening; LVEDP, LV end-diastolic pressure; Max and Min dP/dt, maximum and minimum dP/dt. Data are expressed as means ± SEM. *$p < 0.05$, **$p < 0.01$ vs. Control group.

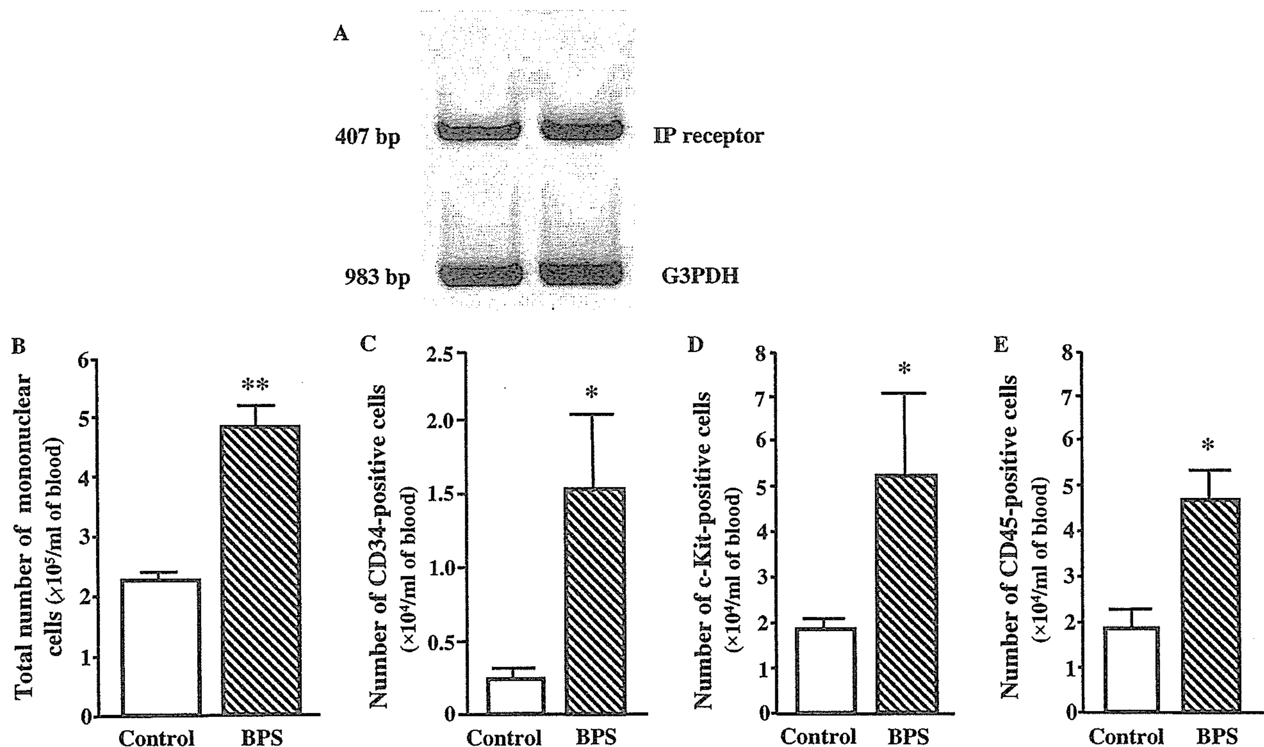

Fig. 3. BPS-induced mobilization of bone marrow cells. (A) Expression of prostacyclin receptor (IP receptor) on bone marrow cells. (B–E) Quantification of BPS-induced MNC mobilization by FACS analysis. Administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells. BPS also increased the number of CD45-positive hematopoietic lineage cells. Data are expressed as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. Control group.

biological activities of BPS appear to be insufficient to explain the decrease in infarct size as well as suppression of LV remodeling.

Recent studies have shown that mobilization of bone marrow cells by cytokines promotes myocardial repair and regeneration after acute myocardial infarction [5,6]. In the present study, three-day administration of BPS markedly increased the number of circulating progenitor cells such as CD34-positive cells and c-kit-positive cells in rats. In addition, treatment with BPS enhanced recruitment of bone marrow cells to the ischemic myocardium and increased capillary density in the peri-infarct area. Earlier studies have shown that CD34-positive cells have angiogenic potential to treat ischemic heart [29–31]. Also, another stem cell fraction, c-kit-positive cells have ability to repair ischemic myocardium by differentiating into vascular endothelial cells [32,33]. These findings suggest that administered BPS induces neovascularization partly via enhancement of bone marrow cell mobilization. RT-PCR demonstrated that IP receptor mRNA was expressed in bone marrow cells, indicating a direct effect of BPS on these cells. A recent study has shown that BPS increases eNOS expression in cultured endothelial cells through activation of c-AMP/Protein kinase A signal transduction [19]. Also, earlier studies have shown that eNOS plays essential role in the recruitment of EPCs to the ischemic myocardium [20]. Taken together, administered BPS may act as a potent stimulator of cell mobilization from bone marrow, although further studies are necessary to examine the underlying mechanisms.

In the present study, treatment with BPS significantly attenuated infarct size after myocardial infarction. BPS improved cardiac function and attenuated the development of LV remodeling after acute myocardial infarction, as indicated by increases in LV fractional shortening and maximum dP/dt, and decreases in LVEDP and LVDD. Taken together, BPS may attenuate myocardial infarction through enhancement of neovascularization via modification of bone marrow kinetics. Interestingly, a small fraction of mobilized bone marrow cells expressed cardiac troponin T in the ischemic myocardium in the BPS group, suggesting that BPS may partially contribute to myocardial regeneration after acute myocardial infarction. Earlier studies have demonstrated that BPS has other beneficial effects for ischemic heart disease including anti-thrombotic activity [34], inhibition of reperfusion injury [35], and prevention of coronary spasm [36], and re-stenosis [37]. These findings suggest that administration of BPS may be a promising therapy for acute myocardial infarction.

Granulocyte colony stimulating factor (G-CSF) is currently used agent for mobilization of bone marrow. Infusion of G-CSF after myocardial infarction improves LV function increasing peripheral stem cell fraction [5,38]. A recent clinical trial, however, claimed the G-CSF therapy

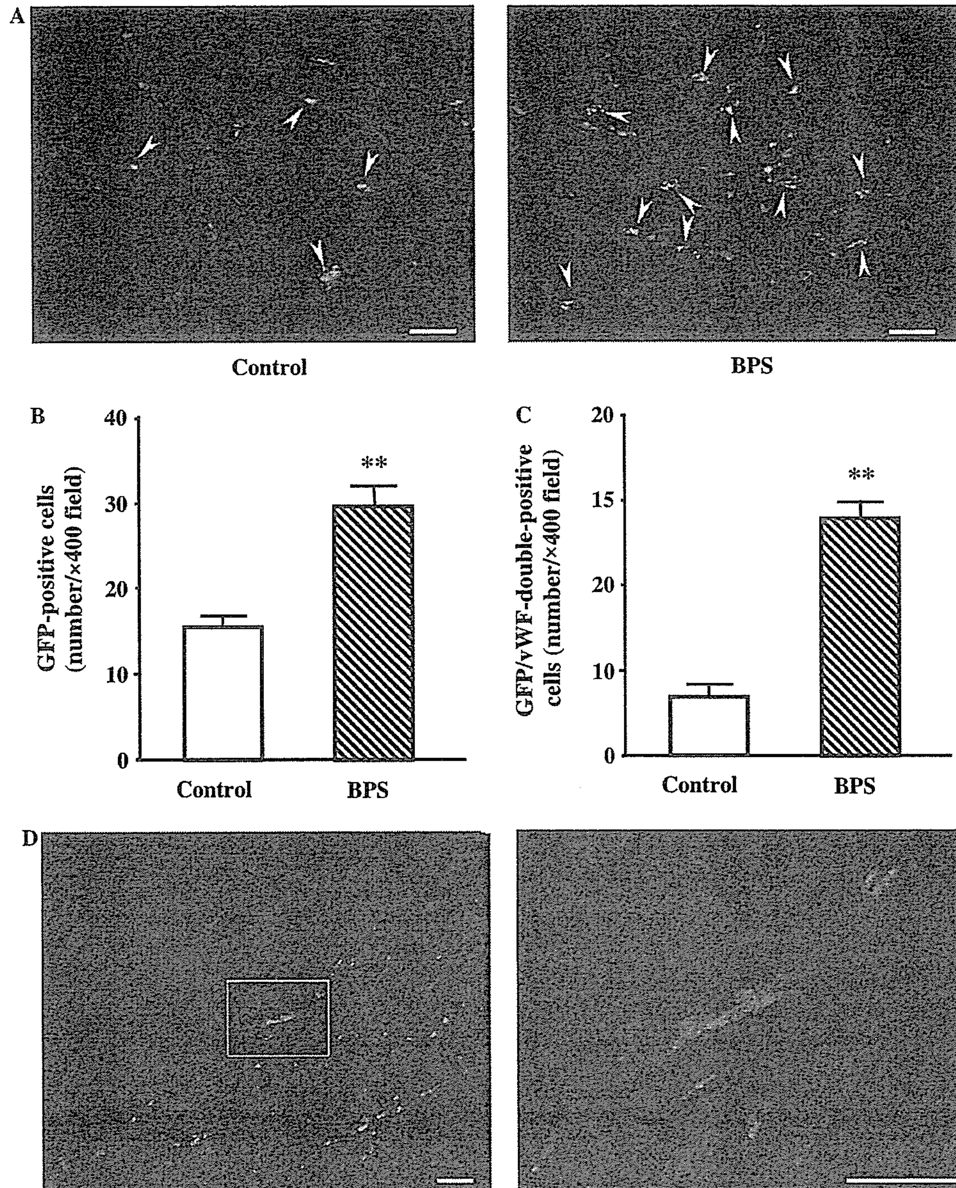

Fig. 4. BPS-induced neovascularization. (A) Representative immunofluorescent images stained with antibodies to von-Willbrand factor (vWF, red) and green fluorescent protein (GFP, green). Nuclei were counterstained with DAPI (blue). (B,C) Semi-quantitative analyses of numbers of GFP-positive cells and GFP-vWF double-positive cells in the peri-infarct area. (D) Representative immunofluorescent image of GFP-positive cells (green) expressing cardiac troponin T (red) observed in the BPS group. Scale bars = 50 μm. Data are expressed as means ± SEM. $^{**}p < 0.01$ vs. Control group.

has serious problem with re-stenosis after recanalization [39]. On the other hand, the safety of BPS has been identified in the treatment of peripheral arterial disease [12,13] and pulmonary arterial hypertension [14,15]. A randomized, controlled clinical trial failed to demonstrate therapeutic potential of prostacyclin for the treatment of severe congestive heart failure [40], which has long discouraged the pursuit of prostacyclin as a therapeutic option for the treatment of acute myocardial infarction. Interestingly, however, double-blinded, randomized, placebo-controlled, large-scale studies showed that treatment with BPS decreased vascular events in patients with peripheral arterial disease [41,42]. Thus, adequate use of BPS for only acute myocardial infarction may have beneficial effects on ischemic myocardium, although further preclinical trials are required to verify the safety and efficacy of BPS.

Conclusion

In summary, administration of BPS improved cardiac structure and function in rats with acute myocardial infarction. This beneficial effect of BPS may be mediated partly by its ability to enhance neovascularization in ischemic myocardium by mobilizing bone marrow cells.

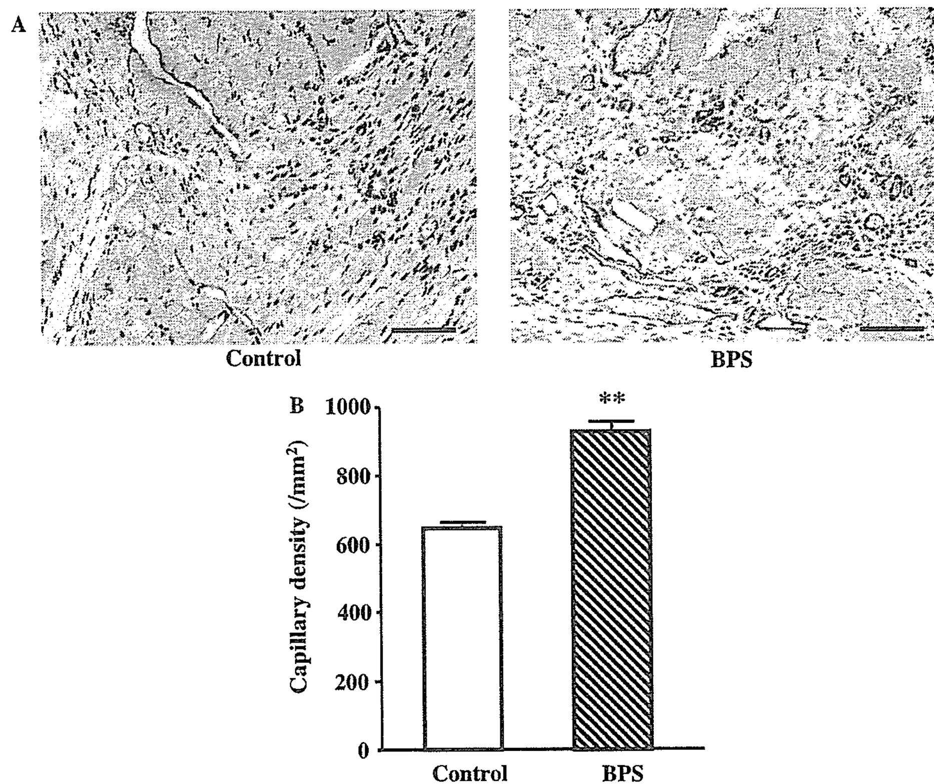

Fig. 5. (A) Representative samples stained with antibody to von Willebrand factor by bright-field DAB. (B) Quantitative analysis of capillary density in peri-infarct area. Administration of BPS increased capillary density by 37%. Scale bars = 50 μm. Data are expressed as means ± SEM. $^{**}p < 0.01$ vs. Control group.

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Central ghrelin acts as an anti-dipsogenic peptide in chicks

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Abstract

The aim of this study was to look at whether ghrelin has an anti-dipsogenic effect, as seen in the eel, when administered centrally in neonatal chicks. Intracerebroventricular (ICV) injection of chicken ghrelin inhibited water intake (WI) in chicks under both *ad libitum* and 17-h water-deprived drinking conditions at doses ranging from 0.01 to 0.1 nmol/chick. This inhibitory effect was observed when 0.1 nmol of rat ghrelin was injected. On the other hand, 0.1 nmol des-acyl rat ghrelin did not reduce WI. To examine the mechanism underlying the effect of ghrelin on WI, chicken B-type (or brain) natriuretic peptide (BNP), an anti-dipsogenic peptide in mammals, was injected at doses ranging from 0.1 to 1 nmol/chick. BNP did not affect WI in chicks under both normal and water-deprived drinking conditions. These findings indicate that ghrelin acts as an anti-dipsogenic peptide through the GHS receptor in the chicken.
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Keywords: BNP; Chick; Ghrelin; Intracerebroventricular injection; Water ingestion

Ghrelin is a peptide which acts as a ligand for the growth hormone secretagogue receptor (GHS-R) [14]. It is unique since the N-terminal third amino acid serine is octanoylated, and the acylation is known to be necessary for ghrelin's activity [14]. Ghrelin is predominantly produced in the stomach, but also found in wide distribution throughout the brain of rats and mice [3,14]. Exogenous ghrelin elicits various physiological functions in the brain such as feeding control, growth hormone (GH) release, and gastric acid secretion [15]. Effects of ghrelin on feeding behavior have been well examined in mammals [9], and it is thought that endogenous ghrelin acts physiologically to increase feeding behavior in mammals [20]. It is known that the orexigenic effects of central ghrelin are mediated by neuropeptides such as neuropeptide Y (NPY), agouti-related protein (AgRP) and orexin [20,29].

In chickens, ghrelin has been purified and identified in various tissues such as the proventriculus, brain, intestine, lung and spleen [12]. The GHS-R is found in the pituitary, brain, liver, intestine, and spleen [8,27]. Chicken ghrelin has relatively low homology to mammalian ghrelin (54% compared with rat and human ghrelin), but the N-terminal seven amino acids are identical to those of mammals [12]. As seen in mammals, ghrelin increases the release of GH after intravenous administration in the chicken [1,12]. ICV injection of ghrelin or a GHS-R agonist, GHRP-2, strongly inhibits food intake in chicks [7,25]; an effect opposite that seen in rats [20]. The anorexic effect has also been observed in another avian species, quail [25], suggesting that the action of ghrelin on food intake is likely to be unique to birds.

Interestingly, Kozaka et al. [16] reported that intracranial administration of ghrelin inhibited water drinking in seawater-acclimated eels. This suggests the possibility that ghrelin, injected into the brain, may affect water intake (WI) in chicks. The purpose of the present study was to investigate whether ICV injection of ghrelin affects WI in chicks. In addition, natriuretic peptide (NP) is known to be a potent anti-dipsogenic factor in mammals [6,11,13,28,33,34]. We examined the effect of chicken B-type NP (BNP), originally isolated from chicken heart [19], on WI in chicks, to investigate the interaction of BNP and the effect of ghrelin.

Day-old male layer chicks were purchased from a local hatchery (Murata Hatchery, Fukuoka, Japan) and kept in a room

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at 30 °C under continuous lighting. The birds were given free access to a commercial diet (Toyohashi Feed and Mills Co. Ltd., Aichi, Japan) and water except where noted elsewhere. Experimental procedures followed the guidance for Animal Experiments in the Faculty of Agriculture and in the Graduate Course of Kyushu University and the Law (No. 105) and Notification (No. 6) of the Japanese Government.

Synthetic octanoylated chicken ghrelin-26 (chicken ghrelin), octanoylated rat ghrelin (rat ghrelin), des-acyl rat ghrelin (des-acyl ghrelin) and chicken BNP were dissolved in a 5% mannitol solution, to prevent the absorption of peptides to the wall of tubes or syringes, with 0.1% Evans Blue. The control group was injected with the mannitol solution. The injected volume was 10 μl in all experiments. ICV injections were performed without anesthesia as reported previously [4]. Briefly, the head of the chick was held with an acrylic device, which positioned a hole in a plate overlying the skull immediately over the left lateral ventricle. Then a microsyringe was inserted into the left lateral ventricle through the hole and the test solution was injected. This injection method is considered to not induce stress the chicks since the ICV injection of 5% mannitol solution, which was used for control injections in the present study, does not alter plasma corticosterone (an indicator for stress response) when compared with intact chicks [22]. At the end of experiment, chicks were sacrificed with an intraperitoneal overdose of sodium pentobarbital. The injection position was confirmed by presence of Evans Blue dye in the lateral ventricle. The results were removed if the Evans Blue dye is not located in the lateral ventricle.

Prior to injection, the birds were assigned to treatment groups in such that the average weights of the groups were the same. WI was determined at 30, 60 and 120 min after peptide injection by measuring the disappearance of water from the pre-weighed water cup with a digital balance with a precision of 0.001 g. Furthermore, evaporation of water during experiment was also measured, and the data was used for correction of WI. In addition, we have uniformly distributed control and experimental groups at the experimental room as the error becomes to be minimum. Food was freely available until the injection, but was removed thereafter.

Experiment 1: Two experiments were performed to examine whether ICV injection of ghrelin affects WI in chicks. In the first, chicks (4-days-old) were injected with 0 (control), 0.01, 0.03 or 0.1 nmol chicken ghrelin under *ad libitum* drinking conditions. In the second, injections were made with the same doses in 5-day-old chicks that were deprived of water for 17 h before the experiment.

Experiment 2: Each chick (3-days-old) was injected with saline (control), 0.1 nmol rat ghrelin or 0.1 nmol des-acyl ghrelin after a 17 h water deprivation.

Experiment 3: Two experiments were performed to investigate the effects of chicken BNP on WI in chicks. In the first, chicks (4-days-old) were injected with 0 (control), 0.1, 0.3 or 1 nmol chicken BNP under *ad libitum* drinking conditions. In the other, injections were made with the same doses in 5-day-old chicks deprived of water for 17 h before the experiment.

Data were expressed as mean ± S.E.M. The data were analyzed with repeated two-way analysis of variance (ANOVA) followed by the Dunnett's test to compare the mean at each time point. A P-value less than 0.05 is considered to be significant.

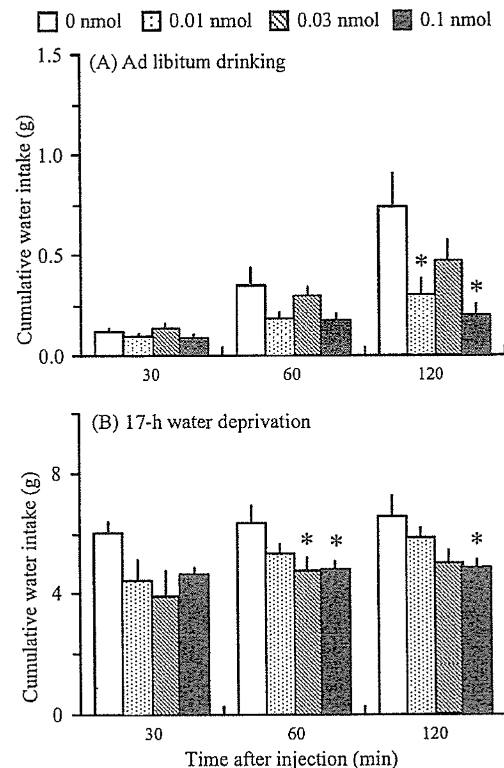

Fig. 1. The effect of ICV injection of chicken ghrelin-26-C8 on WI under (A) *ad libitum* and (B) 17-h water-deprived drinking conditions. In the *ad libitum* drinking study, the number of chicks in each group was as follows: 0 nmol (control), 9; 0.01 nmol, 9; 0.03 nmol, 9; 0.1 nmol, 8. In the dehydration study, the number of chicks was as follows: 0 nmol (control), 6; 0.01 nmol, 8; 0.03 nmol, 6; 0.1 nmol, 7. Data are expressed as mean ± S.E.M. *Significantly different from 0 pmol group ($P < 0.05$) at each time point.

Fig. 1A shows the effect of chicken ghrelin on WI under *ad libitum* drinking conditions. Ghrelin significantly [$F(3,31) = 5.6$, $P < 0.01$] inhibited WI. The inhibitory effect showed a time-dependent interaction [$F(6,62) = 2.9$, $P < 0.05$]. A significant decrease in WI was observed at 120 min after the injection of 0.01 and 0.1 nmol chicken ghrelin.

Fig. 1B shows the effect of chicken ghrelin in 17-h water-deprived chicks. The inhibitory effect was significant [$F(3,23) = 3.3$, $P < 0.05$]. Although 0.01 nmol ghrelin did not affect WI at any time point, higher doses (0.03 and 0.1 nmol) of ghrelin significantly inhibited WI at 60 min after injection. Water intake was still inhibited at 120 min after injection of the highest dose (0.1 nmol) of chicken ghrelin.

Changes in WI after injection of rat ghrelin and des-acyl ghrelin are shown in Fig. 2. Rat ghrelin significantly [$F(3,20) = 5.3$, $P < 0.05$] inhibited WI at 60 min after the injection. In contrast, des-acyl ghrelin did not affect WI at any time point.

The effects of chicken BNP are shown in Fig. 3. Chicken BNP ranging from 0.1 to 1 nmol, which were 10 times higher doses than ghrelin, did not affect WI under *ad libitum* drinking condition [$F(3,29) = 2.1$, $P = 0.1198$] (Fig. 3A) or under

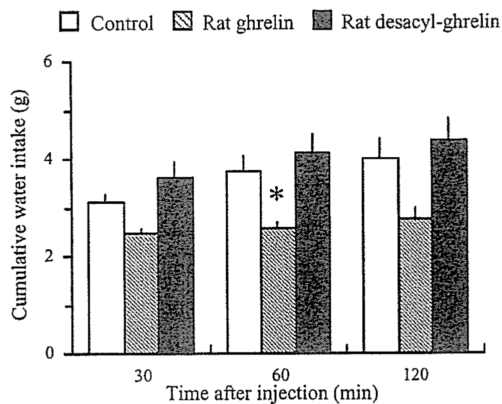

Fig. 2. The effect of ICV injection of rat ghrelin or des-acyl rat ghrelin on WI under 17-h water deprivation. The number of chicks in each group was: saline (control), 9; 0.1 nmol rat ghrelin, 6; 0.1 nmol rat desacyl-ghrelin, 8. Data are expressed as mean ± S.E.M. *Significantly different from the control group ($P < 0.05$) at each time point.

water-deprived condition [$F(3,28) = 0.3$, $P = 0.8089$] (Fig. 3B), respectively.

In this study, we examined the effect of ghrelin on WI in chicks. The ICV injection of ghrelin inhibited WI under both *ad libitum* drinking and 17-h water-deprived conditions. This is the first report to demonstrate an inhibitory effect of ghrelin on WI in birds and is similar to that seen in eels [16]. The injected ghrelin was effective at dose less than 0.1 nmol. This dose is comparable to that which inhibits food intake in chicks [22,23]. This indicates that central ghrelin acts not only as a feeding-inhibitory peptide, but also as an anti-dipsogenic peptide in chicks and would induce both effects at the same time.

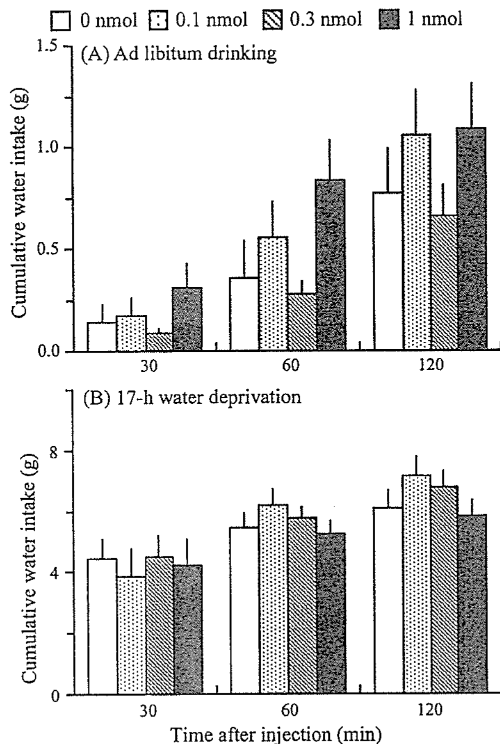

Fig. 3. The effect of ICV injection of chicken BNP on WI under (A) *ad libitum* and (B) 17-h water-deprived drinking conditions. In the *ad libitum* drinking study, the number of chicks in each group was as follows: 0 nmol (control), 6; 0.1 nmol, 9; 0.3 nmol, 10; 1 nmol, 8. In the dehydration study, the number of chicks was as follows: 0 nmol (control), 9; 0.1 nmol, 8; 0.3 nmol, 9; 1 nmol, 6. Data are expressed as mean ± S.E.M. No effect on WI was seen by peptide injection.

The inhibitory effects on WI were not observed during the first 30 min after injection but were evident at 120 min and 60–120 min after injection under *ad libitum* and water-deprived conditions, respectively. Under an *ad libitum* drinking condition, chicks do not drink water if food is not available. The quantity of WI at 30 min after the injection may be too small to detect WI decreased by ghrelin under an *ad libitum* drinking condition. In contrast, dehydrated chicks drank much water after restricted water was replaced. Thus, ghrelin could not inhibit the strong motivation to drink during the first 30 min. However, there was a tendency to reduce WI at 30 min after a ghrelin injection even under dehydration condition, although it is not statistically significant. This suggests the potent anti-dipsogenic effect of ghrelin.

Both chicken ghrelin and rat ghrelin inhibited WI in chicks. In contrast, des-acyl ghrelin had no effect. The N-terminal seven amino acids of ghrelin are identical between chicken and rat [12]. The N-terminal tetrapeptides including octanoylation is known to be the "active core" of ghrelin to bind to the GHS-R and to elicit biological actions [2,17]. Des-acyl ghrelin does not induce intracellular Ca^{2+} influx in cells expressing GHS-R 1a [14,17]. Our results indicate that the conserved N-terminal sequence and octanoylation of chicken and rat ghrelin are essential for this anti-dipsogenic effect through GHS-R1a, and that the observed response is the specific effect of ghrelin.

How ghrelin inhibits WI is still unknown because only one report in the eel is available on this phenomenon [16]. We considered several possibilities on how ghrelin affects drinking behavior. Firstly, a previous report showed that ICV injection of ghrelin (0.15–1.5 nmol) induces sleep-like behavior in chicks [26]. In the present study, we used lower doses of ghrelin (0.01–0.1 nmol) than the previous experiments [26], and did not observe such a behavior in this study although we did not perform any behavioral assessments. This suggested that the ghrelin-induced sleep-like behavior is not related to this anti-dipsogenic effect. Secondary, it is possible that the anti-dipsogenic effect of ghrelin is mediated by some neuronal factors. Neuropeptides and monoamines are possible mediators of this effect. However, possible involvement of monoamines would be excluded because we have reported that ICV ghrelin does not change any monoamine levels in the chick brain [24]. Next, we have reported that the anorexic effect of ICV ghrelin in chicks [7,23], and recently it was revealed that corticotropin-releasing factor (CRF) system mediates the effect [22]. In preliminary experiments we found that ICV ghrelin inhibits both food and water intake (unpublished observation). In addition, injected doses in this study were comparable to that ghrelin-induced inhibition of food intake in chicks [22,23]. These results suggest a possibility that CRF might mediate the central effect

of ghrelin on WI. However, it has been reported that ICV CRF does not inhibit WI in chickens [5,18]. This indicates that CRF is not involved in this anti-dipsogenic effect of ghrelin.

We therefore hypothesized NP as a candidate mediator of the effect of ghrelin. Atrial NP (ANP) has been known to be a potent anti-dipsogenic peptide in various vertebrates such as rats [13], rabbits [28], sheep [34] and eels [16,30]. In contrast, it has been reported that human ANP induces copious drinking when injected ICV in Japanese quail [21]. In chickens, NP was isolated from the chicken heart and designated as chicken alpha-atrial NP (α-ANP) [19]. However, the structure is not similar to ANP but instead belongs to the BNP lineage [10]. BNP also acts as an anti-dipsogen in rats when administered ICV [6,11,33]. However, there is no report about the effect of ICV BNP on drinking in birds. In mammals, ANP at a dose of less than 1 nmol, and BNP at doses from 1.5 to 2 nmol show an anti-dipsogenic effect. Therefore, we examined chicken BNP ranging from 0.1 to 1 nmol with taking mammalian NP studies into consideration. No significant effect on WI was observed following the BNP injections. This is the first report about the effect of BNP on WI in birds. Ghrelin was effective at a dose of only 0.01 nmol in this study. In contrast, a 100 times higher dose of chicken BNP did not show any effect. We cannot rule out an involvement of BNP on ghrelin's effect. However, considering that ANP exhibits a dipsogenic effect in the Japanese quails [21], it is unlikely that BNP mediated an anti-dipsogenic effect by ghrelin in chick brain. Other neuropeptides such as substance P and leu^5-enkephalin exhibit an inhibitory effect on drinking in Japanese quail [31,32]. There is no information about interaction of ghrelin among these neuropeptides in chicken brain. Further study is necessary to clarify the mechanisms of ghrelin-induced anti-dipsogenic effect in chicks.

In conclusion, we found that ghrelin acts as an anti-dipsogenic peptide in the brain of chicks. This suggests a possible involvement of ghrelin on fluid homeostasis.

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C-Type Natriuretic Peptide Is Specifically Augmented by Pituitary Adenylate Cyclase-Activating Polypeptide in Rat Astrocytes

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ABSTRACT: **In rat-cultured astrocytes, pituitary adenylate cyclase-activating polypeptide (PACAP) activates gene expression and secretion of C-type natriuretic peptide (CNP) in a dose- and time-dependent manner. These results suggest that PACAP might be involved in the regulation of CNP biosynthesis in astrocytes.**

KEYWORDS: **PACAP; astrocytes; RIA; RT-PCR**

INTRODUCTION

C-type natriuretic peptide (CNP) belongs to the natriuretic peptide family and has a ring structure formed by an intramolecular disulfide bond, which is conserved among atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP), and CNP. ANP and BNP are mainly secreted from heart as cardiac hormones, whereas CNP is mainly localized in the central nervous system (CNS),[1,2] but the function of CNP in the CNS has not been clarified so far. Receptors for natriuretic peptides are mainly guanylate cyclase-A (GC-A) and guanylate cyclase-B (GC-B).[3] ANP and BNP show high affinity to GC-A, whereas, CNP preferentially binds to GC-B. Both GC-A and GC-B elicit an

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increase in intracellular cGMP that mediates most of the biological actions of natriuretic peptides.[4] It has been reported that the neurotrophic peptide pituitary adenylate cyclase-activating polypeptide (PACAP)[5] stimulates adenylate cyclase activity in cultured astroytes.[6] During the course of a study regarding the effect of PACAP on astrocytes with DNA microarray, we found that CNP gene expression was markedly increased in astrocytes stimulated by PACAP. Then, we tried to clarify how PACAP regulates the biosynthesis and secretion of CNP in the astrocytes.

MATERIALS AND METHODS

Rat astrocytes in primary culture were prepared from the cerebral cortex of newborn rat, and maintained in Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum, penicillin, and streptomycin with Poly–L–Lysin-coated dish. In the second or third passages, after overnight serum starvation, cells were treated with different concentrations of PACAP for 24 h, or with PACAP (10^{-8} M) for different incubation periods.

Total RNA was prepared from astrocytes by TRIZOL LS (Invitrogen, Carlsbad, CA). The first stand cDNA was synthesized from total RNA (2 μg), with random primers and reverse transcriptase (SuperScript™ RT, Invitrogen). The resulting cDNA was amplified with AmpliTaq polymerase, and the PCR products were electrophorezed.

CNP was prepurified using a cartridge column (Sep-pak C_{18}, Waters, Milford, MA) and assessed by means of a specific radioimmunoassay (RIA) for CNP as reported previously.[1,7] CNP-immunoreactivity was characterized by reversed phase high-performance liquid chromatography (RP-HPLC) combined with CNP RIA.

RESULTS AND DISCUSSION

RT-PCR analysis showed that CNP gene expression was observed in the neuronal cells but not in astrocytes under basal conditions (FIG. 1 A). PACAP treatment provoked the expression of the CNP gene in astrocytes in a dose- and time-dependent manner (FIG. 1 B, C). Expression of GC-B, a specific receptor for CNP, was also observed in astrocytes and neuronal cells, but GC-B mRNA levels were not changed by PACAP treatment (FIG. 1 A, C).

Measurement of CNP in the culture medium revealed that PACAP stimulated the secretion of CNP-immunoreactivity in the time- and dose-dependent manner (FIG. 2). Thus, the basal secretion from astrocytes was 15.9 pg/10^6 cells and increased to 796 pg/10^6 cells in the presence of 10 nM PACAP for 24 h. These results suggest that PACAP could be involved in the regulation of CNP biosynthesis and secretion in the CNS.

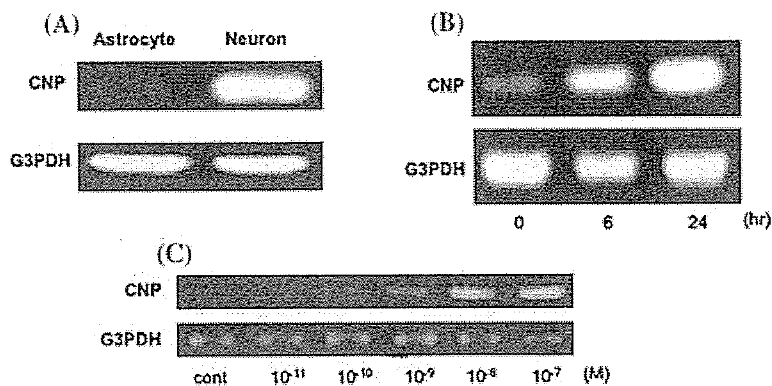

FIGURE 1. The effect of PACAP treatment on CNP gene expression in astrocytes. (**A**) Comparison of CNP expression between neuronal cells and astrocytes under basal condition. (**B**) Time-dependent increase of CNP gene expression in astrocytes by PACAP (10^{-8} M) treatment for various periods. (**C**) Dose-dependent increase of CNP gene expression in astrocytes.

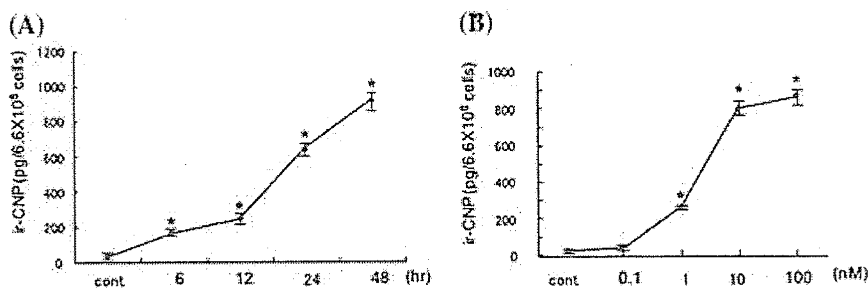

FIGURE 2. The effect of PACAP treatment on CNP secretion from astrocytes. PACAP treatment increased CNP secretion from rat astrocytes in a dose (**A**)- and time (**B**)-dependent manner. $*P < 0.005$ as compared to control.

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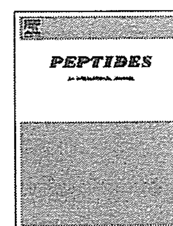

Regulation of food intake by acyl and des-acyl ghrelins in the goldfish

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ABSTRACT

Our recent research has indicated that intracerebroventricular (ICV) and intraperitoneal (IP) administration of *n*-octanoic acid-modified ghrelin (acyl ghrelin) stimulates food intake and locomotor activity in the goldfish. The manner in which peripherally administered acyl ghrelin regulates food intake, however, remains unclear. In contrast to acyl ghrelin, non-acylated ghrelin (des-acyl ghrelin) does not exert an orexigenic action or induce hypermotility. To this extent, the biological role of des-acyl ghrelin in fish is unknown. Given the possible involvement of afferent pathways in mediating the effects of acyl ghrelin, as is known to occur in rodents, we examined the effect of capsaicin, a neurotoxin which destroys primary sensory (vagal and splanchnic) afferents, on the orexigenic activity induced by IP-injected acyl ghrelin. Pretreatment with IP-injected capsaicin (0.16 μmol/g body weight (BW)) cancelled the orexigenic action of IP-injected acyl ghrelin (8 pmol/g BW), although IP-injected capsaicin alone did not affect food intake. The effect of des-acyl ghrelin on the orexigenic action of acyl ghrelin in the goldfish was also investigated. The ICV and IP injection of des-acyl ghrelin at doses 3–10 times higher than that of acyl ghrelin suppressed the orexigenic action of ICV- and IP-injected acyl ghrelin (doses of 1 and 8 pmol/g BW). In contrast, injection of des-acyl ghrelin alone did not show any inhibitory effect on food intake. These results suggest that, as is seen in rodents, circulating acyl ghrelin derived from peripheral tissues acts via primary sensory afferent pathways on feeding centers in the brain. The results also show that des-acyl ghrelin inhibits acyl ghrelin-induced orexigenic activity in goldfish.

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1. Introduction

Ghrelin, a 28 amino acid residue peptide that was originally isolated from rat and human stomachs, acts as an endogenous ligand for the growth hormone secretagogue (GHS) receptor [12]. Although ghrelin is produced most abundantly in the stomach and intestine, it is also expressed at low levels in other organs such as the brain (mainly hypothalamus), pituitary, heart, lungs, pancreas, kidneys and placenta [5,7,9,10,12,19]. It is considered to be a multifunctional peptide that is involved in the regulation of somatic growth, feeding behavior and energy homeostasis in mammals [28]. The GHS receptor is present in the brain, pituitary, gastrointestinal tract, kidneys, pancreas and heart [9,11]. It is known that

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peripheral ghrelin stimulates growth hormone (GH) release and appetite via the vagal afferent pathway, which is sensitive to the neurotoxin capsaicin [6].

Ghrelin possesses an *n*-octanoic acid modification at the third N-terminal serine residue (Ser^3) [12], this form being known as acyl ghrelin. Based on the findings of many studies (see e.g. [12,28]), this fatty acid modification of ghrelin is considered to be essential for ghrelin's biological effect in mammals. In contrast, however, recent studies have indicated that the des-acylated form (where the *n*-octanoic acid modification is absent) of ghrelin also has important physiological activities [2,27].

In the goldfish, ghrelin has been extracted from the intestine [29], and it has been reported that goldfish ghrelin with an *n*-octanoic acid modification at Ser^3 stimulates food intake as well as the release of GH and gonadotropin from the pituitary gland [30]. Recently, we have indicated in the goldfish that intracerebroventricular (ICV) and intraperitoneal (IP) administration of goldfish acyl ghrelin stimulates locomotor activity in addition to food intake, but that des-acyl ghrelin does not the same effects [16]. The explanation underlying these findings is unclear, mainly because the regulatory mechanisms of food intake modification by peripheral ghrelin in the goldfish are not understood. On this basis, we hypothesized an involvement of the vagal afferent pathway in mediating the effects of acyl ghrelin as has been described in rodents [6]. To this extent, the effect of the neurotoxin capsaicin, which destroys the primary sensory (vagal and splanchnic) afferents, on the appetite-stimulating (orexigenic) action of IP-administered acyl ghrelin was studied. Furthermore, we also investigated effect of des-acyl ghrelin on the orexigenic action of acyl ghrelin in the goldfish because the biological role of des-acyl ghrelin in fish is yet to be elucidated.

2. Materials and methods

2.1. *Animals*

Young goldfish (*Carassius auratus*, 3–10 g body weight, BW) of both sexes were obtained from a commercial supplier, and kept for 2 weeks under controlled light/dark conditions (12-h light:12-h dark) in a temperature-regulated fish tank (20–24 °C) before use. The fish were fed with a commercially available granular diet (Tetragold, Tetra GmbH, Herrenteich, Germany, containing 32% protein, 5% dietary fat, 2% dietary fiber, 6% mineral and 8% water) once a day at 10 a.m. All animal experiments were conducted in accordance with the University of Toyama guidelines for the care and use of animals.

2.2. *Effect of pretreatment with capsaicin on orexigenic action of IP-injected acyl ghrelin*

Goldfish acyl ghrelin consisting of 12 amino acid residues deduced from goldfish preproghrelin cDNA [29] was synthesized at the Suntory Institute for Medical Research and Development (Gunma, Japan) as described previously [18]. The peptide was dissolved in saline at 1.0 mM and then stored at −80 °C until use. Capsaicin (Kanto Chemicals Co., Tokyo, Japan) was dissolved in dimethyl sulfoxide at 8.2 mM for storage and diluted with saline before use. Detailed descriptions of the methods used to evaluate the feeding activity of goldfish have been reported [14–17,21]. Briefly, 2 h prior to the start of experiments at 10 a.m., each fish was supplied with a quantity of food equivalent to 1% of its BW. Fish were injected intraperitoneally (IP) with 100 μL of 8 pmol/g BW of acyl ghrelin (an IP injection of this dose is sufficient to induce orexigenic activity, [16]) or saline after pretreatment with an IP injection of capsaicin at 0.16 μmol/g BW or vehicle solution (dimethyl sulfoxide diluted with saline). The capsaicin concentration was determined according to a previous report [13]. Following the IP injection of test substances or saline, fish were placed in individually in a small experimental tank (diameter 24 cm) filled with 4.0 L of tap water, and supplied with food equivalent to 3% BW. Food intake was then measured every 15 min during the 60-min period following treatment.

2.3. *Effect of des-acyl ghrelin on orexigenic action of acyl ghrelin*

Goldfish des-acyl ghrelin consisting of 12 amino acid residues was synthesized at the Suntory Institute for Medical Research and Development, dissolved in saline at 1.0 mM and then stored at −80°C until use. In this experiment, intracerebroventricular (ICV) and IP injections of acyl ghrelin and des-acyl ghrelin were conducted. Briefly, 2 h prior to the start of experiments at 10 a.m., each fish was supplied with food equivalent to 1% of its BW. For ICV administration of acyl and des-acyl ghrelins, each fish was anesthetized with MS-222 (3-aminobenzoic acid ethyl ester, Sigma Chemical Co., St. Louis, MO, USA) and placed in a stereotaxic apparatus. A small part of the parietal bone was carefully removed using a surgical blade (No. 19, Futaba, Tokyo, Japan). One microliter of test solution was injected into the third ventricle of the brain using a small (10 μL volume) Hamilton syringe, and the area of the skull where bone was removed was filled with a surgical bonding agent (Aron Alpha, Sankyo, Tokyo, Japan). Injection into the correct site was confirmed by the appearance in the ventricle of concomitantly injected Evans blue dye. The fish were injected with acyl ghrelin at 1 pmol/g BW (an ICV injection of this dose is sufficient to induce orexigenic activity [16]) or saline after pretreatment with des-acyl ghrelin at 3 or 10 pmol/g BW or saline.

In experiments involving IP administration of ghrelin, fish were injected with acyl ghrelin (100 μL of 8 pmol/g BW) or saline following injection of des-acyl ghrelin (24 or 80 pmol/g BW) or saline. After treatment with test substances or saline, each fish was placed in a small experimental tank (diameter 24 cm) filled with 4.0 L of tap water, and supplied with food equivalent to 3% BW. Food consumption was then measured as described above.

2.4. *Data analysis*

All results are expressed as mean ± S.E.M. Statistical analysis was performed by one-way ANOVA with Bonferroni's method. Statistical significance was accepted at the $P < 5\%$ level.

3. Results

3.1. *Effect of pretreatment with capsaicin on orexigenic action of IP-injected acyl ghrelin*

IP-injected acyl ghrelin elicited orexigenic activity in fish (Fig. 1). In this way, cumulative food intake increased in a statistically significant manner in the first 15 min after IP administration of acyl ghrelin (8 pmol/g BW), and remained significantly increased over a period of 60 min. Pretreatment with capsaicin (0.16 μmol/g BW) inhibited the acyl ghrelin-induced increase of food intake, while IP injection of capsaicin alone did not affect cumulative food intake during an observation period of 60 min.

3.2. *Effect of des-acyl ghrelin on orexigenic action of acyl ghrelin*

Fig. 2 shows the effect of ICV injection of acyl ghrelin and des-acyl ghrelin on food intake. Compared to control (saline), the consumption of food was significantly increased in the first 15 min following ICV injection of acyl ghrelin (1 pmol), and remained significantly increased for a period of 60 min (Fig. 2A). Des-acyl ghrelin injection prior to acyl ghrelin inhibited the acyl ghrelin-induced food intake in a dose dependent manner (Fig. 2A). The administration of des-acyl ghrelin alone at doses of 3 and 10 pmol did not show any effect on food intake (Fig. 2B).

The IP injection of acyl ghrelin (8 pmol) also significantly increased food intake during the first 15 min compared to control, and in a manner that continued for a period of 60 min (Fig. 3A). When des-acyl ghrelin (80 pmol) was injected prior to the acyl ghrelin, the increased food intake observed in the first 15 min following IP injection with 8 pmol acyl ghrelin alone was significantly inhibited. The inhibitory action of des-acyl ghrelin was maintained for the duration of the experiment (60 min). No effect on food intake was seen when des-acyl ghrelin alone was injected IP at doses of 24 and 80 pmol (Fig. 3B).

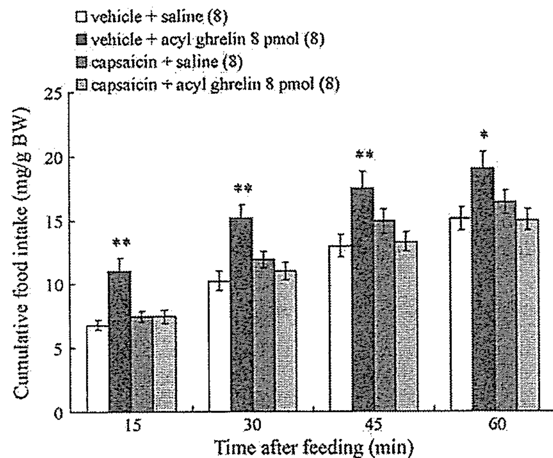
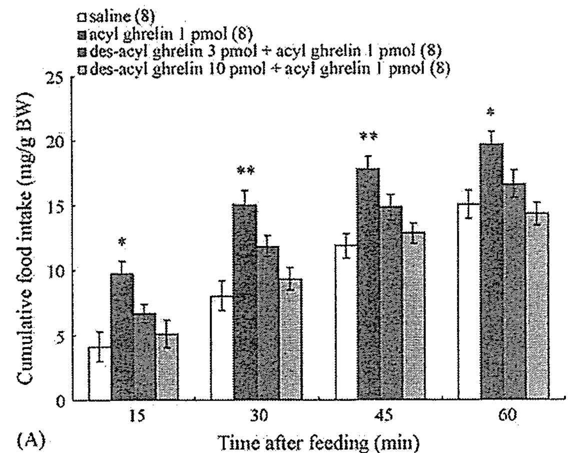


Fig. 1 – Effect of capsaicin pretreatment on the orexigenic activity induced by IP-injected acyl ghrelin. Each column and bar represent the mean and S.E.M., respectively, and the numbers in parentheses in the legend indicate the number of fish used in each group. The significance of differences at each time point, compared to the vehicle + saline-injected group, were evaluated by one-way ANOVA with Bonferroni's method (*P < 0.05; **P < 0.01).

4. Discussion

We investigated the effects of capsaicin and des-acyl ghrelin on the orexigenic action of acyl ghrelin in goldfish. Our recent research indicated that ghrelin is mainly produced in the goldfish intestine, and that the expression of ghrelin mRNA in the intestine changes depending on feeding status. However, the specific mechanism that governs the regulation of food intake by peripheral ghrelin in the goldfish has not been described. In rats, it has been demonstrated that the gastric vagal afferent is the major pathway transmitting stomach-



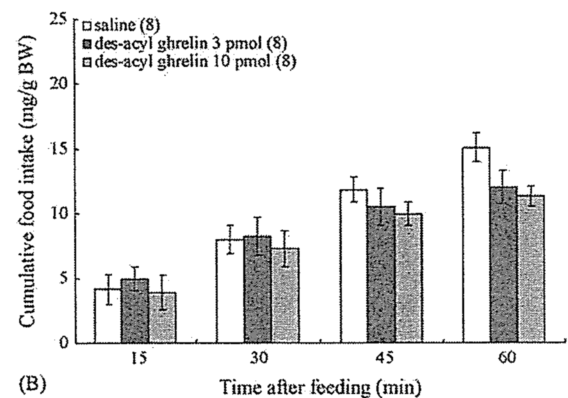


Fig. 2 – Effects of ICV administration of acyl and des-acyl ghrelins. Panel (A) shows the effect of des-acyl ghrelin on the orexigenic activity induced by acyl ghrelin. Panel (B) shows the effect of des-acyl ghrelin alone on food intake. Each column and bar represent the mean and S.E.M., respectively, and the numbers in parentheses in the legends indicate the number of fish used in each group. The significance of differences at each time point, compared to the saline-injected group, were evaluated by one-way ANOVA with Bonferroni's method (*P < 0.05; **P < 0.01).

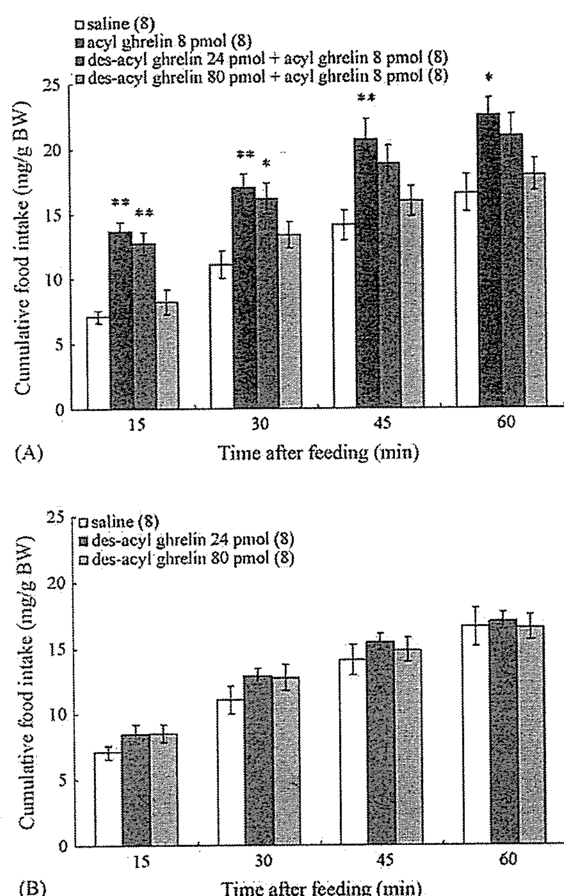


Fig. 3 – Effects of IP administration of acyl and des-acyl ghrelins. Panel (A) shows the effect of des-acyl ghrelin on the acyl ghrelin-induced orexigenic activity of goldfish. Panel (B) shows the effect of des-acyl ghrelin alone on food intake. Each column and bar represent the mean and S.E.M., respectively, and the numbers in parentheses in the legends indicate the number of fish used in each group. The significance of differences at each time point, compared to the saline-injected group, were evaluated by one-way ANOVA with Bonferroni's method (*P < 0.05; **P < 0.01).

derived ghrelin signals related to the regulation of appetite and GH release [6]. There is little information about the capsaicin receptor (vanilloid receptor 1) in the goldfish [31], but both C fibers and polymodal nociceptors have been characterized in the rainbow trout, suggesting that the electrophysiological properties of fish nociceptors are almost identical to those found in mammals [23–25]. In the present study, we demonstrated for the first time that pretreatment with the neurotoxin capsaicin inhibits the orexigenic action of IP-injected acyl ghrelin in the goldfish. This indicates that the stimulatory effect of peripheral ghrelin on food intake in the goldfish is induced via the vagal or splanchnic afferent pathway as demonstrated in rats, suggesting that the pathway mediating for food intake regulation by peripheral ghrelin may be common from fish through to humans.

Until recently, des-acyl ghrelin was considered to be biologically inactive. However, recent studies have indicated that des-acyl ghrelin also exerts important physiological effects, such as stimulating adipogenesis, inotropic action and prevention of cell death in cardiomyocytes, inhibition of glucose output in hepatocytes, and altered food intake [1,2,4,8,20,27]. Recently, we examined the effects of acyl and des-acyl ghrelins on locomotor activity and food intake, and demonstrated that acyl ghrelin induces hypermotility and increases appetite, while des-acyl ghrelin is without effect [16]. As such, the biological role of des-acyl ghrelin in fish remained unknown. In this study, we demonstrated that treatment of goldfish with either ICV or IP injection of des-acyl ghrelin prior to acyl ghrelin injection inhibits acyl ghrelin-induced food intake. This is the first time that the effect of des-acyl ghrelin on feeding regulation has been shown. As des-acyl ghrelin does not bind to the GHS receptor [12], the presence of specific receptor for des-acyl ghrelin has been postulated [26]. GHS receptor in fish has been identified from the pufferfish, black seabream and seabream [3,22], suggesting that the structure of GHS receptor is highly conserved in fish compared to mammals. However, there is no information about the relationship between acyl ghrelin and des-acyl ghrelin for GHS receptor in the goldfish.

In conclusion, we demonstrated that acyl ghrelin regulates food intake via a primary sensory afferent pathway in the goldfish. We also demonstrated that des-acyl ghrelin is capable of inhibiting food intake when it is administered prior to acyl ghrelin. It is noteworthy that the ghrelin gene-derived peptides showed antagonistic effects on the regulation of food intake.

Acknowledgments

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