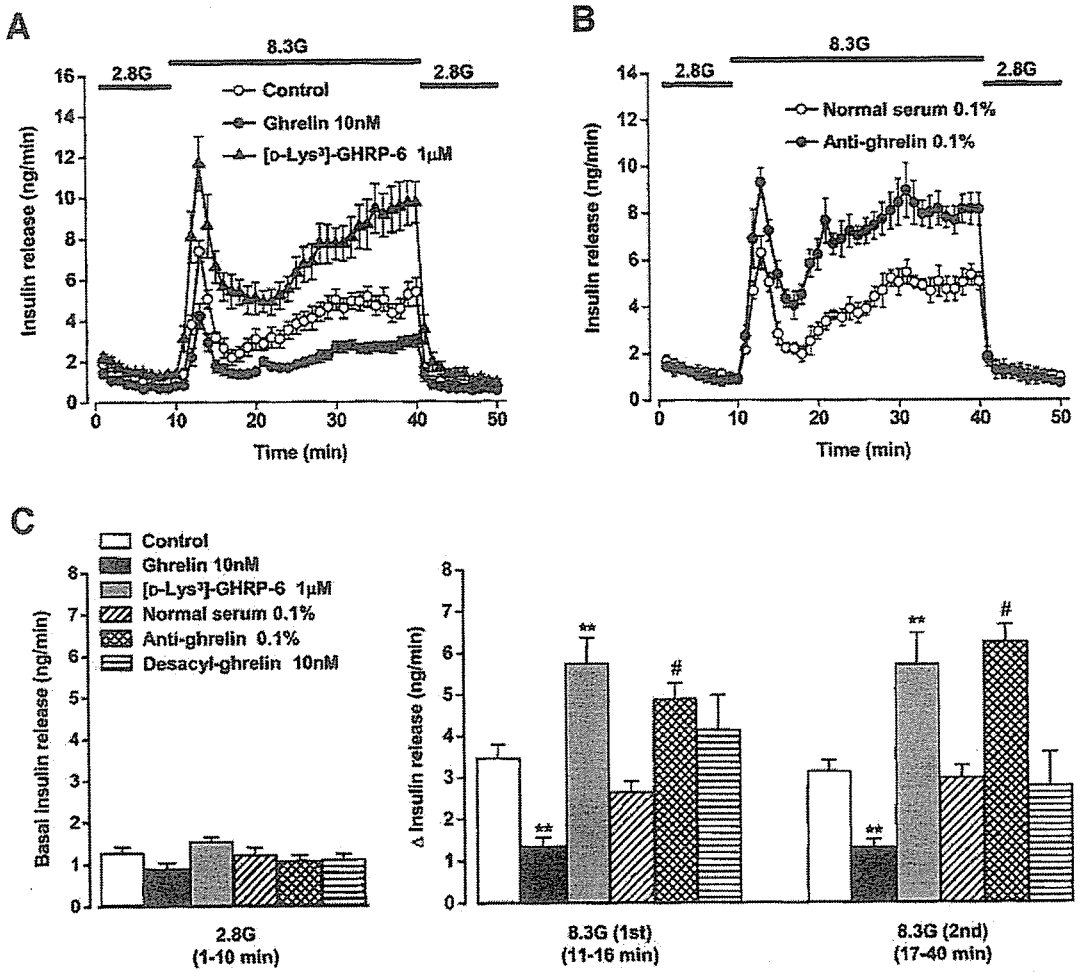

FIG. 2. Insulinostatic effects of endogenous ghrelin in perfused pancreas. *A*: **Blockade of GHSR by [D-Lys^3]GHRP-6 (1 μmol/l) enhanced glucose (8.3 mmol/l)-induced insulin release in perfused rat pancreas, whereas exogenous ghrelin (10 nmol/l) administration inhibited it (n = 6–9).** *B*: **Immunoneutralization of endogenous ghrelin using an anti-ghrelin antiserum (0.1%) enhanced glucose (8.3 mmol/l)-induced insulin release in perfused rat pancreas (n = 3–4).** *C*: **[D-Lys^3]GHRP-6 (1 μmol/l) and anti-ghrelin antiserum (0.1%) increased, whereas exogenous ghrelin (10 nmol/l) inhibited, both the first and second phases of insulin release. Control nonimmune serum (0.1%) had no effect on insulin release. Desacyl-ghrelin (10 nmol/l), an inactive form of ghrelin incapable of activating GHSR, did not alter insulin release (n = 3–9). None of these treatments affected basal levels of insulin release at 2.8 mmol/l glucose. *P < 0.05; **P < 0.01 vs. control; $^{\#}P$ < 0.05 vs. nonimmune normal serum (0.1%). G, glucose.**

insulin release were significantly enhanced both by blockade of GHSR with the GHSR antagonist [D-Lys^3]GHRP-6 (1 μmol/l) (Fig. 2*A* and *C*) and by immunoneutralization of endogenous ghrelin with anti-ghrelin antiserum (0.1%) (Fig. 2*B* and *C*). Conversely, administration of exogenous ghrelin (10 nmol/l) suppressed both phases of glucose-induced insulin release (Fig. 2*A* and *C*). Desacyl-ghrelin, which cannot activate GHSR (1,20), did not significantly alter glucose-induced insulin release (Fig. 2*C*). None of these treatments affected basal levels of insulin release at 2.8 mmol/l glucose.

Endogenous ghrelin downregulates plasma insulin concentrations in both normal and gastrectomized rats. Our findings that glucose-induced insulin release from perfused pancreas was enhanced by ghrelin immunoneutralization and GHSR antagonist suggest that ghrelin originating from pancreatic islets suppresses insulin release. GHSR antagonists also increase systemic insulin responses to GTTs (8), which could be attributable to blockade of ghrelin originated from stomach and/or from other tissues, including islets. To examine the contribution of ghrelin from the stomach and other sources, we produced gastrectomized rats lacking stomach-derived ghrelin and examined the effect of intraperitoneal administration of the GHSR antagonist [D-Lys^3]GHRP-6 (10 μmol/kg) on plasma insulin concentrations in gastrectomized and normal rats fasted overnight. In gastrectomized rats, plasma concentrations of acylated ghrelin were markedly reduced (5.2 ± 0.7 vs. 32.5 ± 9.7 fmol/ml in gastrectomized rats vs. normal rats, respectively; P < 0.01, n = 15) (Fig. 3*C*), indicative of a lack of stomach-derived ghrelin. The remaining levels of acylated ghrelin may be derived substantially from the intestine, the second largest source of ghrelin (12,21). Although the remaining circulating acylated ghrelin was dramatically reduced in gastrectomized rats, intraperitoneal injection of GHSR antagonist increased plasma insulin concentrations at 30 min in gastrectomized rats to a similar extent as that in normal rats (P < 0.05, n = 15) (Fig. 3*B* vs. *A*). These results suggest that the effect of GHSR antagonist is not attributable to antagonism of circulating ghrelin but primarily to blockade of local ghrelin, including that in islets. It was con-

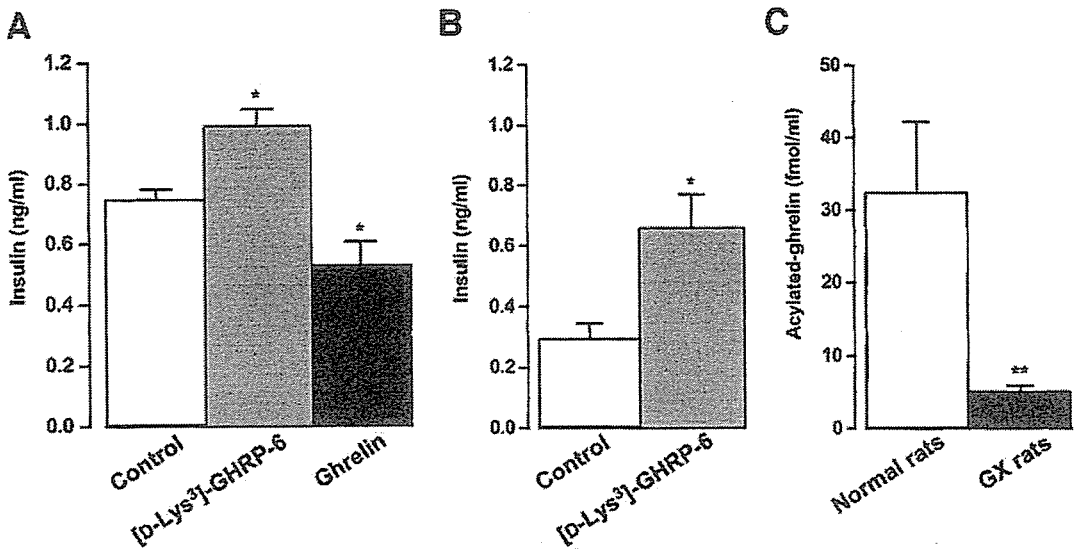

FIG. 3. Endogenous ghrelin decreases plasma insulin concentrations in normal and gastrectomized (GX) rats. *A*: A GHSR antagonist, [D-Lys^3]GHRP-6 (10 μmol/kg i.p.), increased plasma insulin concentrations at 30 min after administration in rats fasted overnight. Conversely, administration of ghrelin (10 nmol/kg i.p.) significantly decreased plasma insulin levels. Data are the means ± SE of 15 rats. *P < 0.05 vs. control. *B*: GHSR blockade increased plasma insulin levels in gastrectomized rats (n = 15). *P < 0.05 vs. control. *C*: Plasma acylated ghrelin levels were reduced in gastrectomized rats (n = 15). **P < 0.01 vs. normal rats.

firmed that application of exogenous ghrelin (10 nmol/kg i.p.) significantly decreased plasma insulin levels in control rats (Fig. 3*A*).

Ghrelin knockout mice display increased insulin and decreased glucose levels. The effects of GHSR antagonist and anti-ghrelin antiserum in the perfused pancreas and in isolated islets may result from counteraction of the action of endogenous ghrelin in islets. This hypothesis was further examined using ghrelin knockout mice. When fed standard chow, no significant differences between male ghrelin knockout and wild-type (C57BL/6J) mice were observed at 8 weeks of age in body weights (23.4 ± 0.7 vs. 23.5 ± 0.3 g in ghrelin knockout vs. wild-type mice, respectively; n = 10), total 24-h food intake (3.51 ± 0.14 vs. 3.54 ± 0.04 g, n = 10), and blood glucose levels in fed states (120 ± 3.1 vs. 127 ± 6.0 mg/dl, n = 10), confirming previous reports in ghrelin knockout mice (22–25). In ghrelin knockout mice, plasma acylated ghrelin levels were undetectable (Fig. 4*A*). Morphological analysis of pancreatic sections showed that the density and average size of islets were not significantly different between wild-type and ghrelin knockout mice (Fig. 4*B* and *C*). Moreover, the number and size of isolated islets obtained by collagenase digestion were not altered in ghrelin knockout mice (islet number: 138.6 ± 14.3, n = 5 mice, vs. 151.2 ± 18.9, n = 5, for ghrelin knockout vs. wild-type mice; islet diameter: 165.2 ± 2.3 μm, n = 714 islets, vs. 164.7 ± 2.3, n = 756). Glucose (8.3 and 16.7 mmol/l)-induced insulin release from isolated islets of ghrelin knockout mice was significantly greater than that of wild-type mice (Fig. 4*D*), whereas basal levels of insulin release at 2.8 mmol/l glucose were not altered. No difference was observed between ghrelin knockout and wild-type mice in insulin content per islet (Fig. 4*E*), mRNA expression of insulin 1, and that of insulin 2 (Fig. 4*F*). These data indicate that the larger amount of insulin release in islets of ghrelin knockout mice results from greater insulin secretory response to glucose, whereas insulin production is unaltered. In GTTs, ghrelin knockout mice exhibited markedly enhanced insulin responses and attenuated glucose responses (Fig. 4*G* and *H*). The profiles of blood glucose levels during ITTs exhibited little differences between ghrelin knockout and wild-type mice (Fig. 4*I*), suggesting that insulin sensitivity was not significantly altered. Thus, the suppressed glycemic responses to GTTs in ghrelin knockout mice may primarily result from enhanced insulin secretion, although possible additional effects of ghrelin on glucose production (26) or insulin sensitivity (27) cannot be disregarded.

High-fat diet–induced glucose intolerance is prevented in ghrelin knockout mice. The enhanced insulin and suppressed glycemic responses to GTTs in ghrelin knockout mice could be beneficial under conditions of increased demand for insulin. We examined this possibility using a model of high-fat diet–induced obesity. Both wild-type and ghrelin knockout mice fed a high-fat diet for 4 weeks displayed moderate increases in body weight (Fig. 5*A*). High-fat diet resulted in moderate increases in blood glucose levels in wild-type mice, whereas this change was not significant in ghrelin knockout mice (Fig. 5*B*). High-fat diet also increased plasma insulin levels, and this change was much greater in ghrelin knockout than wild-type mice (Fig. 5*C*). These results suggest that high-fat diet–induced elevation of blood glucose was corrected by enhanced insulin release in islets of ghrelin knockout mice. A possible impact of this ghrelin knockout mouse islet phenotype on systemic control of glucose and insulin was examined by GTTs. In wild-type mice, increases in blood glucose levels at 15–120 min of the GTT were significantly larger in the high-fat diet group than in the control diet group, exhibiting glucose intolerance (Fig. 5*D*). Insulin response to GTTs at 15 min also tended to be enhanced in the high-fat diet group, although the change was not statistically significant (Fig. 5*E*). In ghrelin knockout mice, by contrast, increases in blood glucose levels at 15–120 min of the GTT in the high-fat diet group were not significantly different from those of the control diet group, and insulin response to GTTs at 15 min was markedly enhanced in the high-fat diet group (Fig. 5*F* and *G*). Thus,

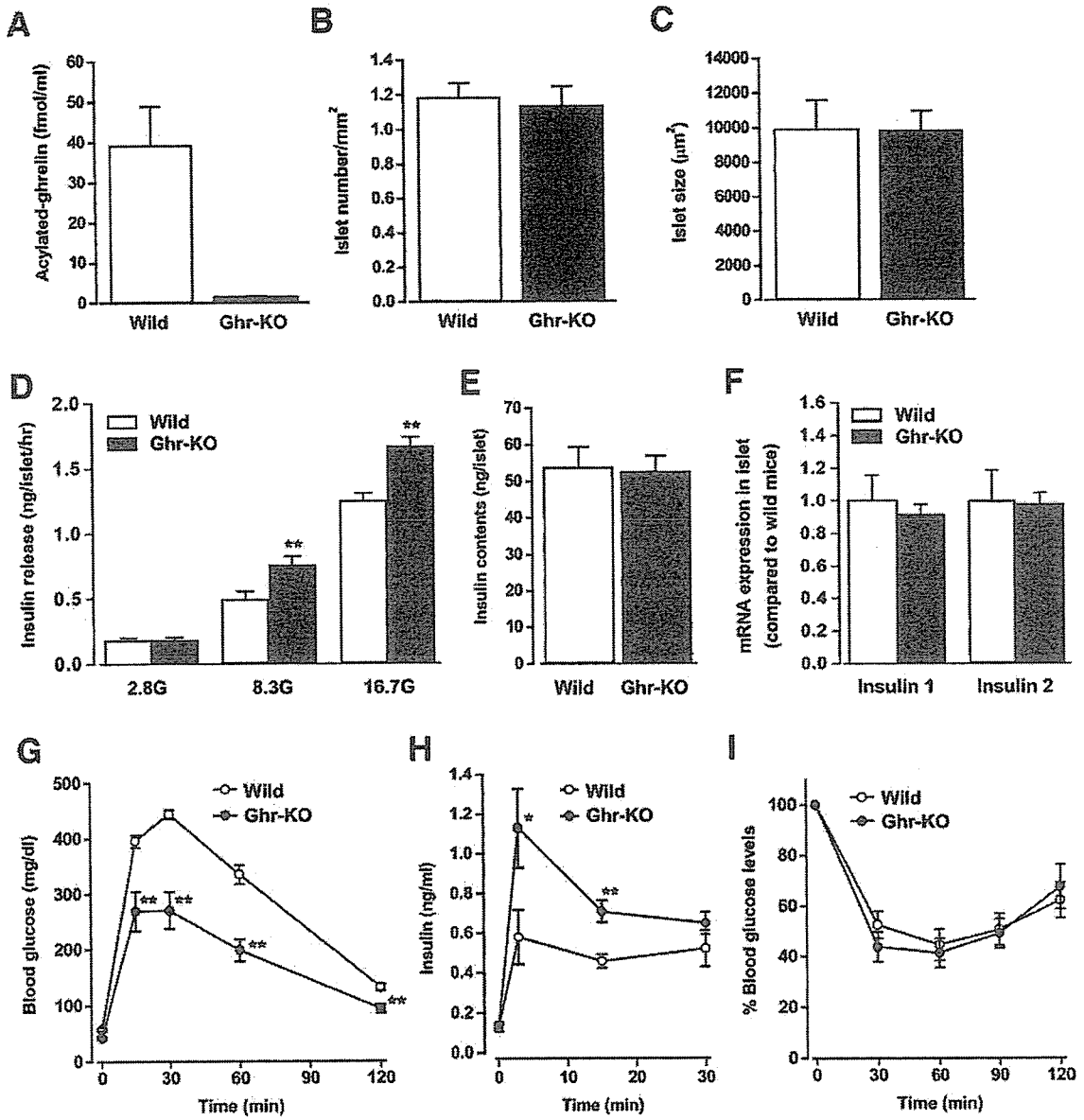

FIG. 4. Ghrelin knockout (Ghr-KO) mice display increased insulin and decreased glucose levels. *A*: Acylated ghrelin was not detected in the plasma of male ghrelin knockout mice (n = 6). *B* and *C*: The number of islets (*B*) in an area unit (1 mm^2, n = 8 slices from three mice) and the size of islets (μm^2, n = 50–51 islets) (*C*) on pancreatic sections were not significantly different between wild-type (Wild) and ghrelin knockout mice at 8 weeks of age. *D*: Glucose (8.3 and 16.7 mmol/l)-induced insulin release was enhanced in ghrelin knockout mouse islets (n = 9–12). **P < 0.01 vs. wild-type mice. *E* and *F*: Islet insulin (*E*) contents (n = 12) and mRNAs expressions (*F*) of insulin 1 and 2 (n = 4–5) were not different between wild-type and ghrelin knockout mice. In GTTs (glucose 2 g/kg i.p.), male ghrelin knockout mice exhibited attenuated elevations of blood glucose (*G*) and enhanced elevations of insulin levels (*H*) in comparison to wild-type mice (n = 9–10). *P < 0.05; **P < 0.01 vs. wild-type mice. *I*: Profiles of blood glucose levels during the ITT (insulin 0.75 units/kg i.p.) did not differ between ghrelin knockout and wild-type mice (n = 12–15). G, glucose.

ghrelin deficiency promoted insulin release and prevented glucose intolerance in a high-fat diet–induced obese model.

DISCUSSION

In this study, we demonstrated that plasma ghrelin concentrations were significantly higher in the pancreatic vein than in the artery in rats and that glucose-induced insulin release from the perfused pancreas was markedly enhanced by blockade of GHSR and immunoneutralization of endogenous ghrelin. Furthermore, GHSR blockade increased plasma insulin concentrations in gastrectomized and normal rats to a similar extent. In addition, in ghrelin-deficient (ghrelin knockout) mice, glucose-induced insulin release from isolated islets was enhanced, systemic insulin response was increased, and glucose response was attenuated in the GTT. Furthermore, ghrelin deficiency promoted insulin release and prevented glucose intolerance in a high-fat diet–induced obese model.

This study demonstrated that ghrelin knockout mice exhibit decreased glucose responses and increased insulin responses in GTTs. Similar results have recently been reported in another line of ghrelin-deficient mice (28). The results of GTTs and ITTs in our ghrelin knockout mice are similar to those previously observed with pharmacological blockade of ghrelin action (8), reinforcing the concept that endogenous ghrelin serves as a downward regulator of

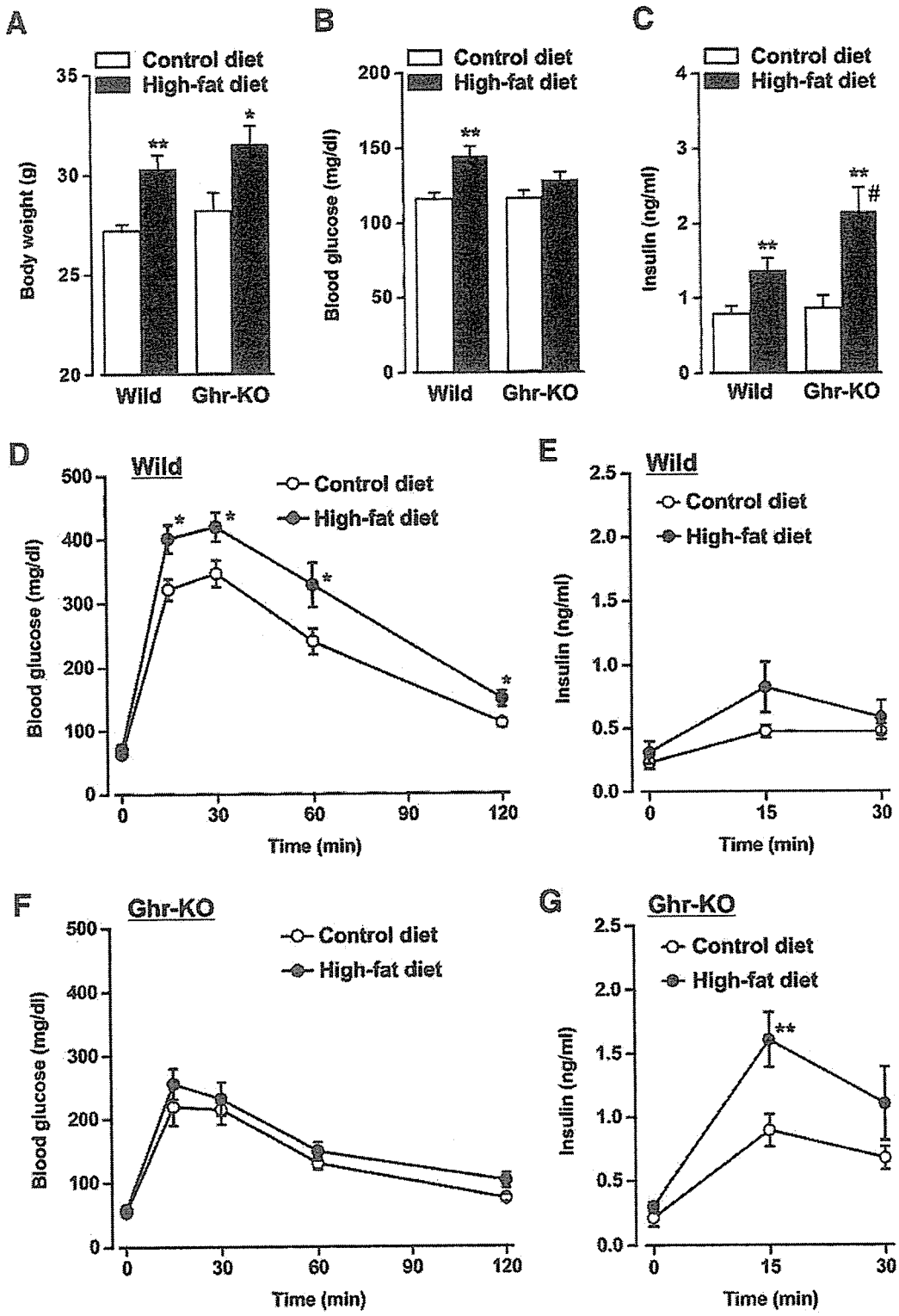

FIG. 5. High-fat diet–induced glucose intolerance is prevented in ghrelin knockout (Ghr-KO) mice. Body weight (*A*), fed blood glucose (*B*), and insulin (*C*) levels in 12-week-old wild-type and ghrelin knockout mice fed a high-fat diet or control diet. The mice were given a high-fat diet or control diet from 8 to 12 weeks old. On a caloric base, high-fat diet consisted of 23% protein, 44.6% carbohydrates, and 32.4% fat (total 17.9 kJ/g), whereas control diet consisted of 29.2% protein, 58.8% carbohydrates, and 12% fat (total 14.5 kJ/g, $n = 9$ per group). In wild-type mice, the high-fat diet group exhibited glucose intolerance (*D*) and slight enhancement of insulin release (*E*) during the GTT (glucose 2 g/kg i.p.). In ghrelin knockout mice, in contrast, glycemic responses to GTTs in the high-fat diet group were not different from those of control diet group (*F*), and insulin response at 15 min was markedly enhanced in the high-fat diet group (*G*). $^{*}P < 0.05$; $^{**}P < 0.01$ vs. control diet mice; $^{\#}P < 0.05$ vs. wild-type high-fat diet mice.

insulin release and consequently upward regulator of glycemia. Furthermore, we found that glucose-induced insulin release from isolated islets of ghrelin knockout mice was greater than that of wild-type mice, whereas insulin content per islet was unaltered in ghrelin knockout mice. Consistent with this observation, glucose-induced insulin release from perfused pancreas in normal rats was augmented by GHSR antagonist and ghrelin immunoneutralization. Thus, pharmacological, immunological, and genetic blockade of ghrelin or ghrelin action in pancreatic islets all markedly enhanced glucose-induced insulin release. These findings reveal the insulinostatic function of endogenous ghrelin within islets. It should be noted that the effect of GHSR antagonist could be partly attributable to blockade of the constitutive activity of GHSR (29,30).

This study demonstrated that the ghrelin level is higher in the pancreatic vein than in the artery. Moreover, blockade of ghrelin action with antagonist and antiserum enhanced glucose-induced insulin release from perfused pancreas, which retains well physiologic circulation. Insulin release from isolated islets was similarly enhanced with ghrelin antagonist and antiserum in our previous report (8). These findings may indicate that ghrelin is released from and acting on pancreatic islets, thus serving as an intraislet regulator of insulin release. The importance of islet-originated ghrelin in the regulation of insulin release is supported by the current finding that GHSR antagonist increased plasma insulin concentrations in gastrectomized and normal rats to a similar extent. Regarding the islet cell species that could release ghrelin, multiple experimental systems have shown ghrelin immunoreactivity in α-cells (8,11), β-cells (13), and islet ghrelin cells (14,31,32), including those named ε-cells (15). It was also reported that ghrelin is expressed together with glucagon or pancreatic polypeptide in immature islet cells in rats (31). mRNAs encoding ghrelin and GHSR are expressed in the pancreas of rats and humans (1,11–13) as well as in β-cell lines (31). Thus, ghrelin appears to be expressed by multiple islet cell types. Further studies are required to identify the cell types that produce ghrelin, which could depend on specific conditions and ages of animals/humans.

We have not yet examined the effect of ghrelin on δ-cells, and therefore a possibility that the observed effects require the participation of δ-cells cannot be excluded. Ghrelin reportedly suppresses the release of somatostatin (6). However, the reduction in this insulinostatic hormone does not appear to mediate the action of ghrelin to inhibit insulin release. Moreover, the direction of microcirculation previously reported was against the physiological role of somatostatin in the regulation of insulin release (33). Because ghrelin directly inhibits β-cells (8), the insulinostatic effect of ghrelin is produced, at least partly, via its direct effect on β-cells. In addition to the regulation of insulin release, ghrelin could also serve as a novel medium of communication between β- and non-β-cells: anterograde versus retrograde perfusion with antisera against ghrelin and conventional islet hormones appears to be a promising approach. However, further studies are definitely needed to address this issue.

Low plasma ghrelin levels are associated with elevated insulin levels (9,10). The inverse relationship between plasma levels of ghrelin and insulin may be explained, at least in part, by the inhibition of insulin release by ghrelin. The current study, by using blockade of ghrelin in pancreas and islets, as well as in vivo in gastrectomized rats, demonstrated that ghrelin originating from pancreatic islets plays an essential role in suppression of insulin release. This study also suggested a pathophysiological role for ghrelin. High-fat diet produced glucose intolerance in wild-type mice. By contrast, ghrelin knockout mice fed a high-fat diet showed close to normal glucose responses and markedly enhanced insulin responses to GTTs compared with control ghrelin knockout mice fed a normal diet. As a possible underlying mechanism, lack of ghrelin and its insulinostatic activity may raise the maximal capacity of glucose-induced insulin release and enable islets to secrete more insulin to meet an increased demand associated with high-fat diet–induced obesity, thereby achieving normoglycemia. It has recently been reported that in *ob/ob* mice, a genetic model of obesity attributable to leptin deficiency, ablation of ghrelin in *ob/ob* mice augmented insulin release and thereby markedly reduced hyperglycemia (28). We hypothesize that the ghrelin system in islets, by altering its insulinostatic efficacy, optimizes the amount of insulin release to meet the systemic demand. In early stages of obesity, antagonization of ghrelin function can prevent glucose intolerance, providing a potential therapeutic application to counteract the progression of type 2 diabetes.

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CNP infusion attenuates cardiac dysfunction and inflammation in myocarditis

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Abstract

Myocarditis is an acute inflammatory disease of the myocardium for which there is currently no specific therapy. We investigated the therapeutic potential of C-type natriuretic peptide (CNP) in acute experimental autoimmune myocarditis. One week after injection of porcine myosin into male Lewis rats, CNP (0.05 μg/kg/min) was continuously administered for 2 weeks. CNP infusion significantly increased maximum dP/dt, decreased left ventricular end-diastolic pressure, and improved fractional shortening compared with vehicle administration. In vehicle-treated hearts, severe necrosis and marked infiltration of CD68-positive inflammatory cells were observed. Myocardial and serum levels of monocyte chemoattractant protein-1 were elevated in myocarditis. However, these changes were attenuated by CNP infusion. In addition, treatment with CNP significantly increased myocardial capillary density. Guanylyl cyclase-B, a receptor for CNP, was expressed in myocarditic heart, and cyclic guanosine monophosphate was elevated by CNP infusion. In conclusion, CNP infusion attenuated cardiac function in acute myocarditis through anti-inflammatory and angiogenic effects.

Keywords: Angiogenesis; Inflammation; MCP-1; Myocarditis; Natriuretic peptides

Acute myocarditis is a non-ischemic heart disease characterized by myocardial inflammation. Acute myocarditis is associated with rapidly progressive heart failure, arrhythmias, and sudden death [1]. Immunomodulatory therapies such as immunoglobulin and interferon are regarded as promising for myocarditis [2,3]; however, the efficacy of those treatments still remains controversial [3,4]. Other treatment options are restricted to supportive care for heart failure or arrhythmias. The lack of specific treatment and the potential severity of the illness underlie the importance of novel and effective therapeutic strategies for myocarditis.

There are three main natriuretic peptides: atrial natriuretic peptide (ANP), brain natriuretic peptide (BNP) and C-type natriuretic peptide (CNP), all of which signal through natriuretic receptors and cyclic guanosine monophosphate (cGMP) signaling pathways. ANP and BNP are predominantly secreted from cardiac myocytes. They have anti-hypertrophic effects on cardiac myocytes in an autocrine manner and also have inhibitory effects on collagen synthesis of cardiac fibroblasts in a paracrine manner, and thus have suppressive effects on cardiac remodeling. Cardioprotective effects of ANP and BNP have already been demonstrated, and they are used clinically for the treatment of heart failure. On the other hand, CNP, originally identified in the porcine brain [5], is predominantly

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expressed in vascular endothelial cells and plays a role in the local regulation of vascular tone and remodeling [6]. However, the potential therapeutic effects of CNP in heart disease are not well understood.

Recently, it has been shown that CNP is also synthesized in cardiac fibroblasts and inhibits collagen synthesis of cardiac fibroblasts more potently than ANP and BNP [7]. In addition, CNP proved to have more potent antihypertrophic effects than ANP in cultured cardiac myocytes [8]. More recently, infusion of CNP has been shown to improve cardiac function after myocardial infarction through anti-fibrotic and anti-hypertrophic effects [9]. These findings indicate the therapeutic potential of CNP in heart disease. However, it remains unknown whether CNP infusion improves acute myocarditis leading to severe heart failure. In the present study, cardiac myosin purified from pig hearts was injected into rats, and autoimmune myocarditis was induced [10].

Thus, the purposes of this study were (1) to investigate whether infusion of CNP improves cardiac function in a rat model of acute myocarditis, and (2) to investigate the mechanisms responsible for the effect of CNP on the myocarditic heart.

Materials and methods

Model of acute myocarditis. We produced a rat model of acute myocarditis by injecting pig cardiac myosin. In brief, purified myosin from the ventricular muscle of pig hearts was prepared according to a procedure described previously [11]. The antigen was dissolved at a concentration of 20 mg/ml in phosphate-buffered saline (PBS) containing 0.3 M KCl, mixed with an equal volume of complete Freund's adjuvant containing 11 mg/ml of Mycobacterium tuberculosis (Difco Laboratories, Detroit, MI, USA). Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (20 mg/kg) and 0.2 ml of the antigen-adjuvant emulsion was injected into the foot pads.

CNP preparation and treatment. CNP was diluted in PBS with 5% glucose and administered via an ALZET mini-osmotic pump (DURECT Corporation, Cupertino, CA, USA) inserted subcutaneously, which discharged CNP at a rate of 0.05 μg/kg/min for the duration of 14 days beginning 1 week after myosin injection.

Experimental groups. Rats with sham operation or those with acute myocarditis were treated with vehicle or CNP. Fifty-four male 10-week-old Lewis rats (Japan SLC, Hamamatsu, Japan) were randomly placed into four groups and received the following treatments: (1) sham rats given vehicle ($n = 12$), (2) sham rats given CNP ($n = 12$), (3) myocarditis rats given vehicle ($n = 15$), and (4) myocarditis rats given CNP ($n = 15$).

Echocardiography. Echocardiography was performed at day 21 post-myosin injection. Rats were anesthetized with sodium pentobarbital. A 12 MHz probe was placed at the left 4th intercostal space for M-mode imaging using 2D echocardiography (Sonos 5500, Philips, Bothell, WA, USA). Left ventricular diastolic dimension (LVDd), left ventricular systolic dimension (LVDs), anterior wall thickness (AWT), and posterior wall thickness (PWT) were measured, and taken as an average of three beats. Fractional shortening (%FS) was calculated as follows;

$$\%FS = (LVDd - LVDs)/LVDd \times 100$$

Hemodynamic study. Hemodynamic measurements were taken at day 21 post-myosin injection. A 1.5F micromanometer-tipped catheter was advanced into the left ventricle through the right carotid artery (Millar Instruments, Houston, TX, USA). Heart rate was also monitored with electrocardiogram. As indexes of hemodynamics, heart rate (HR), mean arterial pressure (MAP), left ventricular systolic pressure (LVSP), left ventricular end-diastolic pressure (LVEDP), maximum dP/dt, and minimum dP/dt were used. Anesthesia was maintained with sodium pentobarbital, and the above mentioned indexes were recorded simultaneously during spontaneous ventilation after an equilibration period of a minimum of 20 min.

Histopathology. The heart was excised above the origin of the great vessels, and the heart and body weights were recorded. A midventricular portion of the heart was fixed with formalin and embedded in paraffin, and 4-μm sections were cut and stained with either hematoxylin and eosin (H&E) or Masson's trichrome stain, or subjected to immunohistochemical staining. H&E-stained sections were graded by a cardiovascular pathologist (H.I-U.) for the characterization of myocardial injury and inflammation, without knowledge of the experimental groups, on the following sc ale: (0) no or questionable presence, (1) limited focal distribution, (2 and 3) intermediate severity, and (4) coalescent and extensive foci throughout the entire transversely sectioned ventricular tissue.

Immunohistochemistry. Paraffin-embedded heart sections were washed in increasing concentrations of ethanol and then in PBS. Sections were incubated with DakoCytomation protein block, then with anti-von Willebrand factor (vWF) (DakoCytomation, Glostrup, Denmark), CD68 (DakoCytomation), or monocyte chemoattractant protein-1 (MCP-1) (BD Biosciences, San Jose, CA, USA) antibodies, followed by sequential incubations with HRP-linked rabbit anti-mouse IgG (DakoCytomation). The reaction products were visualized using 0.5% diaminobenzidine and 0.03% hydrogen peroxide. Sections were counterstained with hematoxylin. The numbers of vWF-stained capillaries and CD68-stained cells were determined in ten randomly selected fields (vWF; 400×, CD68; 200×).

Enzyme-linked immunosorbent assay (ELISA). Serum MCP-1 level on day 21 post-myosin injection was measured using a Rat MCP-1 ELISA Kit (Biosource International, Camarillo, CA, USA).

Reverse transcription-polymerase chain reaction (RT-PCR). Expression of guanylyl cyclase-B (GC-B) mRNA, a receptor for CNP, was examined by RT-PCR. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and myocarditis rats given vehicle ($n = 5$ in each group). Total RNA was extracted from heart with RNeasy Mini Kit (Qiagen, Hilden, Germany) and reverse-transcribed (PCR Amplification Kit, Takara, Shiga, Japan). The complementary DNA was amplified by the PCR using specific primers for GC-B or glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The PCR primers for GC-B were as follows [12]: sense primer 5′-AACGGGCG CATTGTGTATATCTGCGGC-3′ and antisense primer 5′-TTATCA CAGGATGGGTCGTCCAAGTCA-3′. For GAPDH, the primers were as follows: sense primer 5′-TGAAGGTCGGTGTCAACGGATTTGGC-3′ and antisense primer 5′-CATGTAGGCCATGAGGTCCACCAC-3′.

Radioimmunoassay. To investigate whether subcutaneous administration of CNP has a biological activity in heart, we measured myocardial level of cGMP. The hearts were obtained at day 21 post-myosin injection for comparison between sham rats given vehicle and those given CNP ($n = 10$ in each groups). Myocardial level of cGMP was measured with a radioimmunoassay kit (cGMP assay kit; YAMASA Co., Chiba, Japan).

Statistical analysis. Data were presented as means ± SEM. Comparisons of parameters among groups were made by one-way ANOVA, followed by Newman–Keuls' test. Differences were considered significant at $P < 0.05$.

Results

Improvement in cardiac function by CNP treatment

Myocarditis rats given vehicle had two deaths 19 and 21 days after myosin injection, respectively, whereas those treated with CNP showed no mortality. At 3 weeks post-myosin injection, Myocarditis rats given vehicle showed decreased maximum dP/dt and minimum dP/dt, and

increased LVEDP compared with the sham rats (Fig. 1A–C), indicating the presence of acute heart failure in this model. Such parameters subsequently returned to baseline with CNP treatment. On echocardiography, rats with myocarditis showed an increase in LVDd and a significant reduction in %FS (Fig. 1D–F). CNP infusion significantly improved %FS in myocarditis rats. Myocarditic hearts showed significantly increased heart weight to body weight ratio, which was reduced by CNP treatment (Table 1). MAP was significantly decreased in myocarditis rats, and the decrease was significantly attenuated by CNP treatment. CNP did not significantly influence cardiac function in sham rats.

Attenuation of inflammatory cell infiltration by CNP treatment

Histological examination showed that myocardial necrosis and tissue granulation as well as inflammation and edema were markedly increased in our model of acute myocarditis (Fig. 2A and B). CNP administration significantly attenuated necrotic changes observed in myocarditis rats. CNP-treated hearts exhibited a consistent tendency for a reduction of tissue granulation, inflammation and edema, on blinded histological grading by a cardiovascular pathologist (H.I-U.) as compared to vehicle-treated hearts. Although, CNP is known to have potent anti-fibrotic activ-

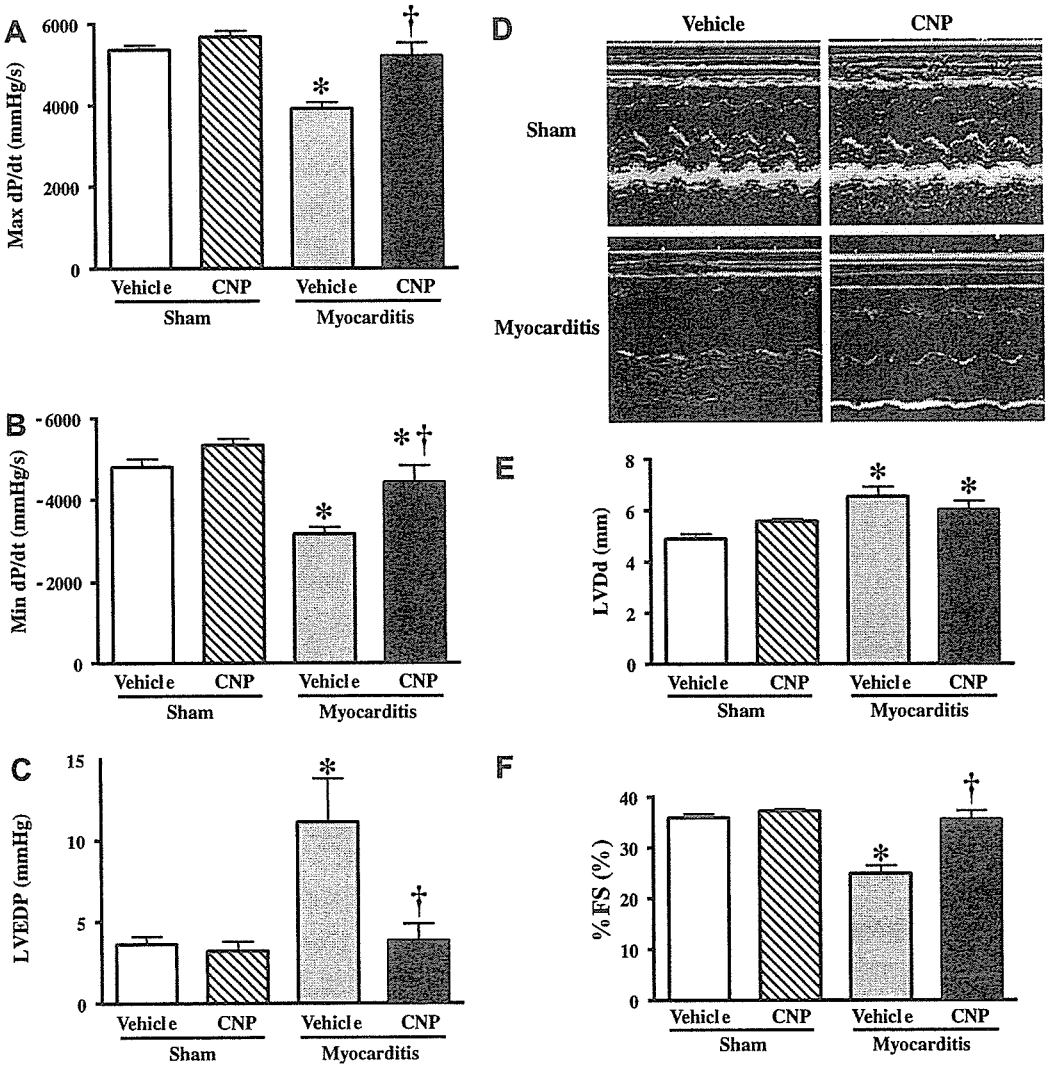

Fig. 1. Effects of CNP administration on hemodynamic parameters in acute myocarditis. (A) Maximum dP/dt (Max dP/dt), (B) minimum dP/dt (Min dP/dt), and (C) left ventricular end-diastolic pressure (LVEDP) were measured in sham rats given vehicle, sham rats given CNP, myocarditis rats given vehicle, and myocarditis rats given CNP. (D) Representative echocardiographic images showing wall thickening and poor myocardial movement in rats with myocarditis and improved cardiac contractility in those treated with CNP. (E,F) CNP administration in myocarditis tended to attenuate the increase in left ventricular diastolic dimension (LVDd) and significantly improved fractional shortening (%FS). Values are means ± SEM. $^{*}P < 0.05$ *vs.* Sham-Vehicle, $^{\dagger}P < 0.05$ *vs.* Myocarditis-Vehicle.

Table 1
Physiological and catheter-based parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 12$)	CNP ($n = 13$)
BW (g)	282 ± 2	282 ± 3	208 ± 4*	224 ± 3*,†
HW/BW (g/kg)	2.86 ± 0.04	2.81 ± 0.03	6.33 ± 0.25*	5.29 ± 0.20*,†
HR (bpm)	428 ± 7	422 ± 5	367 ± 13*	431 ± 13*,†
MAP (mmHg)	111 ± 4	103 ± 4	87 ± 3*	105 ± 5†
LVSP (mm Hg)	124 ± 5	125 ± 4	104 ± 4*	123 ± 6†

BW, body weight; HW/BW, heart weight to body weight ratio; HR, heart rate; MAP, mean arterial pressure; LVSP, left ventricular systolic pressure. Data are means ± SEM.

* $P < 0.05$ *vs.* Sham-Vehicle.

† $P < 0.05$ *vs.* Myocarditis-Vehicle.

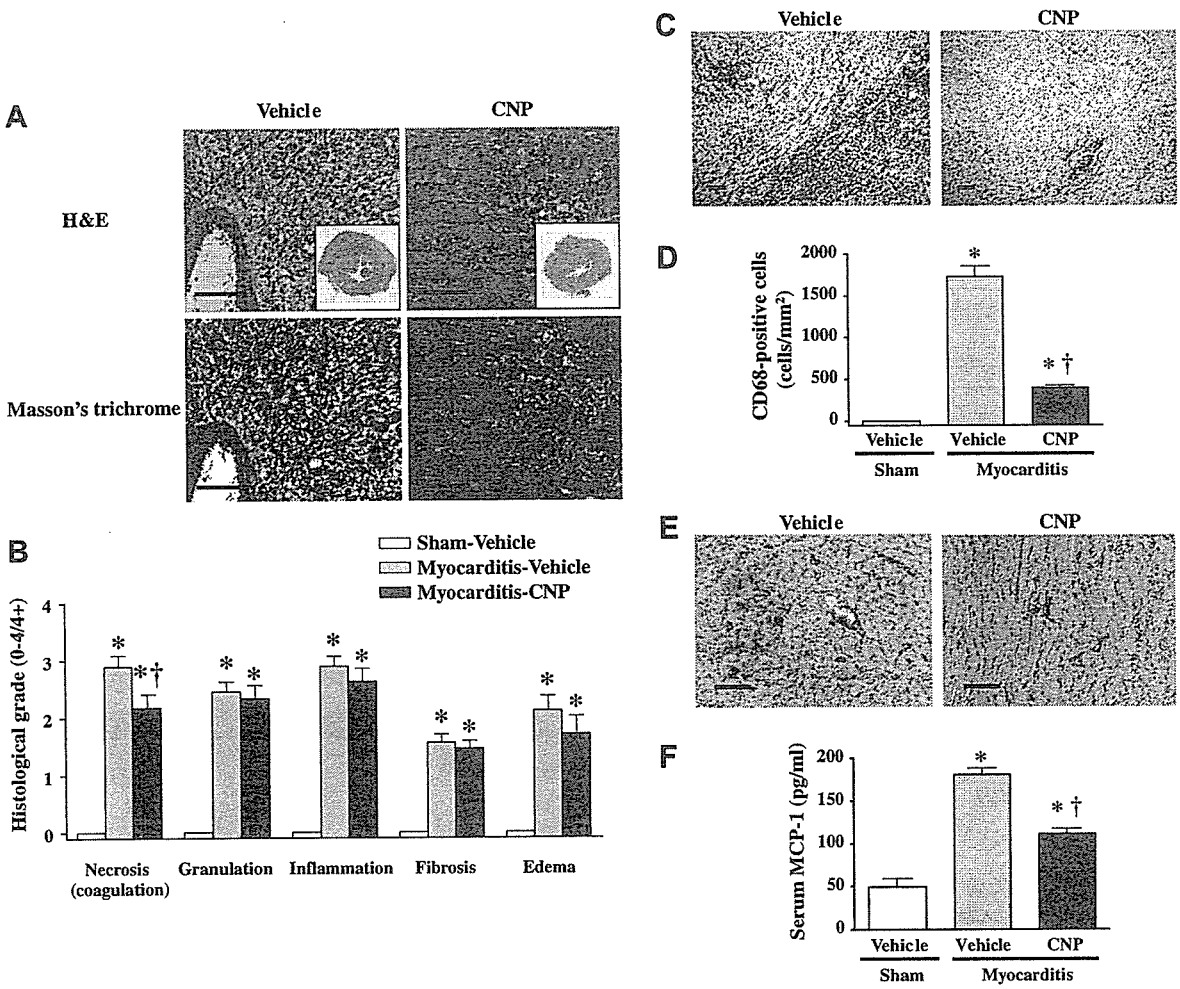

Fig. 2. Histological analysis of the myocardium. (A) Representative myocardial sections showed markedly decreased inflammation and tissue necrosis (H&E) and a comparable degree of early fibrosis (Masson's trichrome) in CNP-treated hearts as compared to myocarditic hearts. Insets are transverse section of the left ventricular section (H&E). (B) Semiquantitative histological grades for necrosis and tissue granulation as well as for inflammation and edema were lower in myocarditis rats treated with CNP as compared to untreated rats. Sham rats exhibited no measurable pathological change. Scale bar is 100 μm. (C) Representative myocardial sections immunohistochemically-stained for CD68 demonstrated a marked decrease in CD68-positive cells, including giant cells, in CNP-treated hearts as compared to vehicle-treated hearts. Scale bar is 100 μm. (D) Semi-quantitative counts of CD68-positive cells demonstrate a significant reduction in CNP-treated hearts. (E) Representative MCP-1-stained myocardial sections from rats with acute myocarditis. Scale bar is 100 μm. (F) Serum level of MCP-1 measured by ELISA. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

ity [9], myocardial fibrosis was not significantly attenuated by CNP infusion (Fig. 2B), probably due to the acute nature of this experiment (Table 2).

Notably, marked histiocytic infiltration was demonstrated by the presence of CD68-positive cells, including multinucleated giant cells, in rats with myocarditis, and this

Table 2
Echocardiographic parameters

	Sham		Myocarditis	
	Vehicle ($n = 12$)	CNP ($n = 12$)	Vehicle ($n = 9$)	CNP ($n = 11$)
LVDd (mm)	5.6 ± 0.1	5.6 ± 0.1	6.5 ± 0.4*	6.0 ± 0.3
LVDs (mm)	3.6 ± 0.1	3.5 ± 0.1	4.9 ± 0.4*	3.9 ± 0.2†
%FS (%)	36 ± 1	37 ± 1	25 ± 2*	36 ± 2†
AWT diastole (mm)	1.9 ± 0.1	1.9 ± 0.1	3.1 ± 0.2*	2.8 ± 0.2*
PWT diastole (mm)	1.9 ± 0.1	1.8 ± 0.1	3.5 ± 0.3*	3.6 ± 0.4*

LVDd, left ventricular diastolic dimension; LVDs, left ventricular systolic dimension; %FS, fractional shortening; AWT, anterior wall thickness; PWT, posterior wall thickness. Data are means ± SEM.
* $P < 0.05$ *vs.* Sham-Vehicle.
† $P < 0.05$ *vs.* Myocarditis-Vehicle.

was significantly attenuated by CNP treatment (Fig. 2C and D). In myocarditis, there was an increase in MCP-1 expression localized to the vascular endothelium and also in myocytes surrounding and adjacent to areas of inflammatory infiltration (Fig. 2E). The hearts in myocarditis rats treated with CNP showed a partial decrease in MCP-1 expression. Serum MCP-1 level was greatly increased in myocarditis rats, whereas it was significantly decreased in those treated with CNP (Fig. 2F).

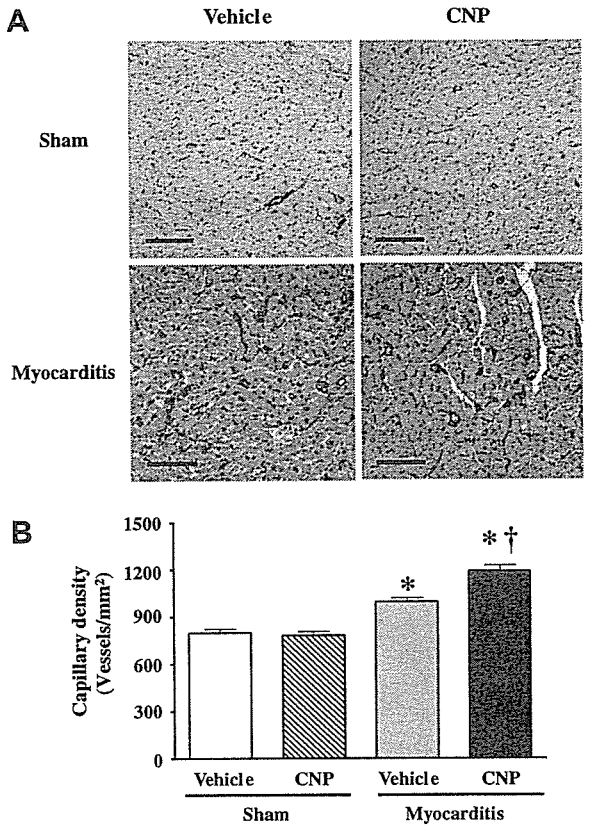

Fig. 3. Angiogenic potential of CNP in acute myocarditis. (A) Representative myocardial sections immunohistochemically-stained for vWF exhibit increased microvasculature in control myocarditic hearts, which was more marked in CNP-treated hearts. (B) Capillary density measured in 10 random representative high powered fields showed a significant increase in rats with acute myocarditis and a further increase in those treated with CNP. Scale bar is 100 μm. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle, †$P < 0.05$ *vs.* Myocarditis-Vehicle.

Effect of CNP on angiogenesis

To determine the angiogenic effect of CNP treatment in the myocardium, immunohistochemical analysis of vWF was performed. Capillary density in the heart was increased in myocarditis, particularly in areas directly adjacent to tissue necrosis (Fig. 3). Notably, capillary density was increased over that in acute myocarditis alone. The clustering of relatively small vessels seen in CNP-treated myocarditic hearts was indicative of recent endothelial regeneration or angiogenesis. On the other hand, CNP did not significantly influence the capillary density in the sham rats.

Expression of GC-B and cGMP in myocardium

RT-PCR demonstrated that GC-B mRNA was expressed in myocarditic heart (Fig. 4A). Myocardial level

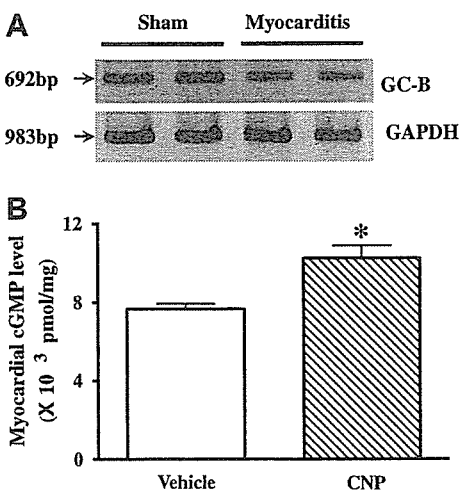

Fig. 4. Expression of GC-B and cGMP in the myocardium. (A) RT-PCR analysis of GC-B mRNA expression in myocarditic heart. (B) Myocardial level of cGMP measured by radioimmunoassay. Values are means ± SEM. *$P < 0.05$ *vs.* Sham-Vehicle.

of cGMP was significantly elevated by the subcutaneous infusion of CNP (Fig. 4B). These results suggest that subcutaneous infusion of CNP (0.05 μg/kg/min) has biological effects on myocaditic heart.

Discussion

In this study, we focused on the therapeutic potential of CNP in the acute phase of autoimmune myocarditis. We showed that CNP treatment 1 week following myosin injection but prior to the development of myocarditis (1) preserved cardiac function after acute myocarditis, (2) significantly decreased tissue necrosis, inflammatory cell infiltration and MCP-1 expression in the heart and serum, and led to a tendency for reduced overall inflammation, granulation and edema, and (3) stimulated angiogenesis in myocarditic hearts beyond the baseline increase seen in myocarditis.

The rat model of myosin-induced experimental autoimmune myocarditis closely resembles human giant cell myocarditis [11]. This disease model is triphasic, consisting of antigen priming phase from days 0–14, an autoimmune response phase from days 14–21, and a reparative phase thereafter, associated chronically with a dilated cardiomyopathy phenotype [13]. In our experiments, CNP was administered 1 week following myosin injection, corresponding to an early time point in the disease process. In the present study, CNP treatment significantly improved cardiac function as determined by increased maximum dP/dt and %FS as well as decreased LVEDP in rats with acute myocarditis. Importantly, earlier studies have shown that the vasodilator effect of CNP is much less potent than that of ANP [5,9,14,15]. ANP and BNP cause vasodilatation and hypotension, thus limiting their use as treatment for patients with severe heart failure. Because the effects of CNP on blood pressure and HR were very small, CNP treatment is considered as a safer alternative for the treatment of those patients [16]. Indeed, administration of CNP did not decrease arterial pressure, but sustained its biological activity.

Our data showed a significant decrease in inflammatory cell infiltration and a consistent tendency for decreased overall inflammation and edema by CNP treatment. In addition, CNP infusion decreased MCP-1 expression in the heart and serum. A previous study has demonstrated that CNP reduces macrophage infiltration by inhibition of MCP-1 expression [17]. These findings suggest that attenuation of inflammatory cell infiltration by CNP may be regulated, at least in part, by suppression of MCP-1 expression.

Recently, it was shown that CNP has anti-fibrotic properties in pulmonary fibrosis and myocardial infarction, through a cGMP-dependent pathway [9,18]. However, since the present experiments were carried out in the acute phase of myocarditis, the anti-fibrotic effect of CNP in the myocarditic heart was not clear. Further studies are necessary to examine the anti-fibrotic effects of CNP in the chronic phase of myocarditis.

We demonstrated that CNP induces endothelial regeneration beyond the increase seen in myocarditis. In rabbit balloon injury, infectious vein graft disease and hindlimb ischemia models, CNP overexpression stimulated reendothelialization via a cGMP-dependent pathway [19]. Endothelial dysfunction including microvascular constriction and microaneurysm formation has previously been reported in myocarditis [20], as well as chronic impairment of endothelial-dependent vasorelaxation of coronary resistance vessels in myocarditis [21]. Thus, the endothelial regenerative effects of CNP are likely to be beneficial in preventing myocardial injury and dysfunction in acute myocarditis. In this study, capillary density in normal heart was not increased by CNP infusion. In inflammatory tissue, it is speculated that CNP does not have an effect on initiation of angiogenesis, but promote angiogenesis at the phase of forming mature blood vessels. However, a further examination is necessary to elucidate the mechanisms of angiogenic effects.

Considering the importance of natriuretic peptides, such as ANP and BNP, in the diagnosis and treatment of cardiovascular diseases, there is currently much interest in the role of CNP. Since, CNP has marked cardioprotective effects including anti-inflammatory and angiogenic effects, and has less vasodilator effects, which enable the use of this peptide in patients with hypotension, this molecule may have great potential for the treatment of patients with acute myocarditis.

In summary, administration of CNP ameliorated cardiac dysfunction in a rat model of acute myocarditis. The beneficial effects may be due, at least in part, to anti-inflammatory and angiogenic effects. This work expands the beneficial effects of CNP to acute myocarditis, and increases our understanding of the role of natriuretic peptides in severe heart failure.

Acknowledgments

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Effects of ghrelin and des-acyl ghrelin on neurogenesis of the rat fetal spinal cord

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Abstract

Expressions of the growth hormone secretagogue receptor (GHS-R) mRNA and its protein were confirmed in rat fetal spinal cord tissues by RT-PCR and immunohistochemistry. *In vitro*, over 3 nM ghrelin and des-acyl ghrelin induced significant proliferation of primary cultured cells from the fetal spinal cord. The proliferating cells were then double-stained using antibodies against the neuronal precursor marker, nestin, and the cell proliferation marker, 5-bromo-2′-deoxyuridine (BrdU), and the nestin-positive cells were also found to be co-stained with antibody against GHS-R. Furthermore, binding studies using [^{125}I]des-acyl ghrelin indicated the presence of a specific binding site for des-acyl ghrelin, and confirmed that the binding was displaced with unlabeled des-acyl ghrelin or ghrelin. These results indicate that ghrelin and des-acyl ghrelin induce proliferation of neuronal precursor cells that is both dependent and independent of GHS-R, suggesting that both ghrelin and des-acyl ghrelin are involved in neurogenesis of the fetal spinal cord.

Keywords: Ghrelin; Des-acyl ghrelin; GHS-R; Neurogenesis; Spinal cord; Fetal development

Ghrelin, a peptide hormone secreted from the stomach, has been identified as the endogenous ligand for the growth hormone secretagogue receptor (GHS-R), through which ghrelin stimulates GH release in the pituitary [1]. Two types of GHS-R, type1a and 1b (GHS-R1a and 1b, respectively), have so far been found, and only the former is able to activate signal transduction of the receptor downstream linking to phospholipase C, resulting in an increase of intracellular calcium [2]. Ghrelin consists of 28 amino acids and is characterized by esterified modification with octanoic acid on serine 3, which is essential for activation of GHSR-1a, although the modification mechanism remains unknown. On the other hand, the level of des-acyl ghrelin, which is inactive on GHS-R1a because of a lack of octanoic acid, is 4 times as high as that of ghrelin in the blood [3].

Many studies have reported that ghrelin has multiple effects other than GH secretion, including regulation of food intake [4] and energy metabolism [5], and gastrointestinal coordination [6,7], as well as facilitation of cell survival, and/or inhibition of apoptosis [8–15]. Although these multiple functions of ghrelin would account for the very wide distribution of GHS-R1a, it is debatable whether GHS-R1a contributes to all of the actions of ghrelin, i.e. that ghrelin may act as a ligand for other types of receptors [16]. So far, however, this possibility remains uninvestigated, and no such alternative receptor has been identified.

We have previously demonstrated that rat fetal growth was increased by treatment of the mother with exogenous ghrelin, and that the effect of ghrelin on fetal growth is diminished by immunization against ghrelin *in vivo* [17]. In addition, we have found that amniotic fluid contains a

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large quantity of des-acyl ghrelin, and that proliferation of cells from rat fetal skin is also stimulated by treatment with both ghrelin and des-acyl ghrelin *in vitro*. On the basis of these findings, we speculate that both ghrelin and des-acyl ghrelin play a crucial role in fetal growth, both peptides acting to facilitate fetal growth not only by enhancement of maternal appetite but also via direct stimulation of fetal cell proliferation by transfer of maternal ghrelin to the fetus.

In the present study, we demonstrated that both ghrelin and des-acyl ghrelin facilitate neural cell proliferation in cultured cells from the fetal spinal cord, which express both the GHS-R gene and its protein, and identified these proliferating cells as neuronal precursor cells. Furthermore, in binding studies using [^{125}I]des-acyl ghrelin, we clarified that des-acyl ghrelin has at least one binding site in the membrane fraction from fetal spinal cord. These results suggest that ghrelin and des-acyl ghrelin can facilitate neurogenesis in the rat fetal spinal cord through both the GHS-R and also an unidentified GHS-R-independent alternative pathway.

Materials and methods

Primary culture of embryonic spinal cord cells. Embryonic spinal cords were obtained from a pregnant rat at day 17. The uterus usually contained 10–14 embryos, 10 of which were utilized for primary culture. The whole spinal cords were mechanically and enzymatically dissociated in papain solution, and the digestion was stopped by addition of culture medium. Cells were passed though a strainer, then centrifuged at 1000 rpm at 4 °C for 10 min and resuspended in DMEM supplemented with $NaHCO_3$, antibiotics (penicillin, streptomycin; Sigma, MO), and 5% fetal calf serum, followed by plating onto laminin-coated 96-well plates at 10^5 cells per well.

Ghrelin and des-acyl ghrelin treatment and cell proliferation assay. Cell proliferation was measured by Cell Proliferation ELISA with BrdU (Roche Diagnostic GmbH, Mannheim, Germany) according to the manufacturer's instructions with some optimization for the present cell conditions as follows. Briefly, after incubation for four days, the cells were treated with ghrelin or des-acyl ghrelin at a final concentration of 0.003–300 nM for 12 h. Subsequently, BrdU was added to the cells to label newly synthesized DNA, followed by further incubation for 6 h. After incubation, the cells were fixed and denatured, and incubated with anti-BrdU antibody for 90 min. Each well was washed out and reacted with substrate solution until color development. The absorbance of the reaction was measured by an immunoreader. Data were expressed as means ± SEM. The significance of differences between the control and treated cells was analyzed by Student's *t* test. Differences at $P < 0.05$ were considered statistically significant.

Immunohistochemistry. Frozen sections of the embryonic spinal cord 14 μm thick were prepared from embryos at embryonic day (ED) 17 and fixed with 4% paraformaldehyde in 0.1 M phosphate buffer for 30 min. After washing with 0.1 M phosphate buffer, the preparations were incubated with 2% normal goat serum in PBS for 30 min at room temperature, washed with PBS three times, and incubated overnight at 4 °C with each of the following primary antibodies: Polyclonal rabbit anti-microtubule-associated protein 2 (Map2; 1:1000, Chemicon International, Inc., CA), anti-neurofilament H (NF-H; 1:1000, Chemicon International), and anti-GHS-R and monoclonal mouse anti-nestin (1:10,000, Chemicon International). Subsequently, all the sections were washed in PBS and incubated with Alexa Fluor 488-conjugated goat anti-rabbit IgG, except the sections that were incubated with the anti-nestin antibody, for which FITC-conjugated goat anti-mouse IgG (1:200, Chemicon International) was used as the secondary antibody. After washing out the residual antibodies and embedding of the sections, they were observed using a light microscope.

Cultured spinal cord cells, which had incorporated BrdU after incubation for 4 days during the ELISA preparation procedure, were fixed with methanol and glacial acetic acid at −20 °C for 20 min. After DNA denaturation with 2 M HCl and blocking with 2% normal goat serum in PBS for 30 min at room temperature, the fixed cells were incubated overnight at 4 °C with either anti-Map2 or anti-nestin as the primary antibody. Afterwards, the cells were incubated at RT for 1 h with the same secondary antibodies as those used for staining the frozen sections. After the washing step, the cells were further incubated with rat anti-BrdU monoclonal antibody (1:1000, Abcam, Cambridge, UK) as a primary antibody for double staining, followed by incubation with Cy^{TM}3-conjugated donkey anti-rat IgG polyclonal antibody (1:1000, Jackson Immuno Research Laboratories, Inc., PA) as the secondary antibody. For double staining of the GHS-R for either Map2 or nestin, cells fixed with 4% paraformaldehyde in 0.1 M phosphate buffer were first incubated with either mouse anti-Map2 or anti-nestin primary antibody, and then with rabbit anti-GHS-R antibody.

RT-PCR. Total RNA was isolated from the spinal cord of embryos at ED 13, 15, 17, 19, and postnatal day (PD) 0 using Trizol Reagent (Life Technologies, Inc., Gaithersburg, MD). Single-strand DNA was generated from 1 μg of total RNA with the use of Superscript 3 preamplification reagent (Life Technologies, Inc., Bethesda, MD) according to the manufacturer's instructions. PCR was carried out using a BD advantage^{TM} 2 PCR Enzyme System (BD Science, CA). The PCR primers specific for GHS-R1a were 5′-GATACCTCTTTTCCAAGTCCTTCGAGCC-3′ for sense and 5′-TTGAACACTGCCACCCGGTACTTCT-3′ for antisense (nucleotides 842–869 and 1001–1025; Accession No. AB001982, GenBank), and those specific for GAPDH were 5′-CGGCAAGTTCA ACGGCACA-3′ for sense and 5′-AGACGCCAGTAGACTCCACGA CA-3′ for antisense (nucleotides 1002–1020 and 1125–1147; Accession No. AF106860, GenBank).

Des-acyl ghrelin binding assay. Binding of des-acyl ghrelin to tissue membranes of fetal spinal cord was studied using [^{125}I]des-acyl ghrelin as a radioligand. Membrane fractions (30,000g pellet) were isolated from fetal spinal cord tissue as described previously [18–20]. Membranes with a protein content of 10 μg, as determined by the Lowry method, were incubated at 4 °C for 1 h with increasing concentrations (0.13–16.64 nM) of [^{125}I]des-acyl ghrelin in a final volume of 0.5 ml assay buffer (50 mM Tris–HCl, 2.5 mM EGTA, 0.1% BSA, and protease inhibitor cocktail (Sigma, MO), pH 7.4). Parallel incubations in the presence of 1.0 μM unlabeled des-acyl ghrelin were used to determine nonspecific binding, which was subtracted from total binding to yield specific binding values. For competition assay, tissue membranes were incubated with 0.1 nM labeled des-acyl ghrelin and either unlabeled des-acyl ghrelin or ghrelin at 4 °C for 1 h. After incubation, the reaction solution was filtered through Whatman GF/B filters, which were then rinsed three times with assay buffer. The radioactivity of the membranes on the filter was measured with a gamma counter. Saturation isotherms were transformed using the method of Scatchard and the maximal number of binding sites (B_{max}) and the dissociation constant (K_d) were calculated using the GraphPAD Prism 4 program (GraphPAD Software, CA).

Results

GHS-R mRNA and protein expression in fetal spinal cord

Using RT-PCR, we examined GHS-R mRNA expression in spinal cords obtained from rat fetuses at ED 13, 15, 17, 19, and PD 0 (Fig. 1a). Abundant levels of GHS-R mRNA were expressed in the spinal cord in fetuses at all ages examined, as we have reported previously [17]. We then investigated GHS-R expression at the protein level by immunohistochemistry. At the same time, we detected neuronal cells by using antibodies against the neuron-specific markers Map2 and NF-H, as well as nestin for

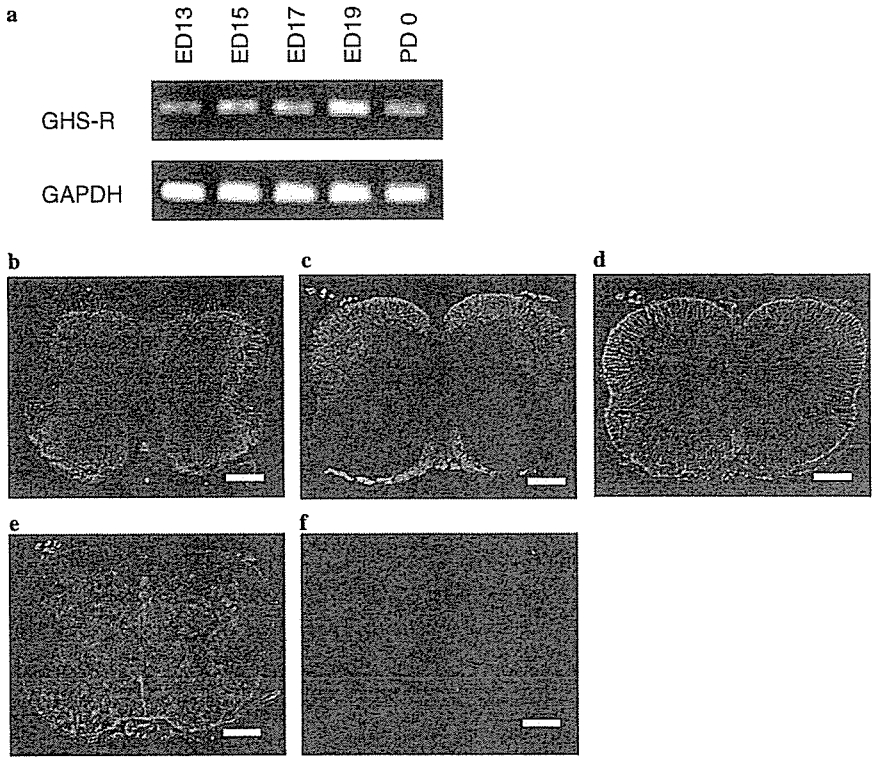

Fig. 1. (a) Detection of GHS-R mRNA in the rat fetal spinal cord by RT-PCR. The PCR product amplified with primers specific for GHS-R1a was detected from embryonic day (ED) 13 to postnatal day (PD) 0. GAPDH mRNA was also detected as an internal control. (b–f) Immunofluorescence staining with antibodies against neuron marker and GHS-R proteins in the rat fetal spinal cord. Map2-positive (b) and Neurofilament H-positive cells (c) were localized in the gray and white matters, respectively. Immunoreactivity of the neuronal precursor cell marker, nestin, was found throughout the spinal cord but with strongest staining in the white matter (d) GHS-R immunoreactivity was localized in the gray matter (e) and was not observed in sections that had been exposed to the preadsorbing antibody (f). Bars, 200 μm.

neuroprogenitor cells. Cells immunoreactive for Map2 were located in the core of the spinal cord, the so-called gray matter (Fig. 1b), while NF-H immunoreactivity was confirmed in the white matter (Fig. 1c). Nestin-positive cells were located in all regions, but the most intense staining was observed in the white matter (Fig. 1d). GHS-R-positive staining was located in the gray matter of the spinal cord (Fig. 1e), and the staining was abolished by pre-absorption of the antibody (Fig. 1f).

Proliferation of spinal cord cells upon treatment with ghrelin and des-acyl ghrelin

Primary culture of spinal cord cells from rat fetus at ED17 was performed. The cells were cultured with BrdU for 6 h after initial incubation for 4 days, and then treated with ghrelin and des-acyl ghrelin for a further 12 h. BrdU is incorporated into DNA when cells synthesize DNA during the S phase of the cell cycle and can be immunodetected using anti-BrdU antibody. BrdU-positive cells were detected under all conditions, irrespective of treatment, although BrdU positivity was more abundant in cells that had been cultured with ghrelin and des-acyl ghrelin than in non-treated cells. To quantify the increase in the number of cells positive for BrdU, we measured cell proliferation by BrdU ELISA. Treatment with both ghrelin (Fig. 2a) and des-acyl ghrelin (Fig. 2b) at over 3 nM significantly increased the incorporation of BrdU.

Identification of the proliferative cell type and cell type expressing GHS-R

Immunofluorescence double staining of cultured cells treated with ghrelin that had incorporated BrdU into their DNA was performed to identify proliferating cells among cultured rat fetal spinal cord cells. Cells with Map2 positivity showed a typical neuron-like shape with extended dendrites and did not show BrdU positivity in their nuclei (Fig. 3a). Cells with nestin positivity were pleomorphic and showed BrdU positivity in their nuclei, i.e., neuronal precursor cells (Fig. 3b). In addition, BrdU positivity was also found in cells that were unstained by antibodies against both Map2 and nestin (data not shown). GHS-R-expressing cells were then examined by immunofluorescence double staining, as was the case for cultured cells without BrdU treatment. GHS-R immunoreactivity was observed in the nestin-positive cells (Fig. 3c).

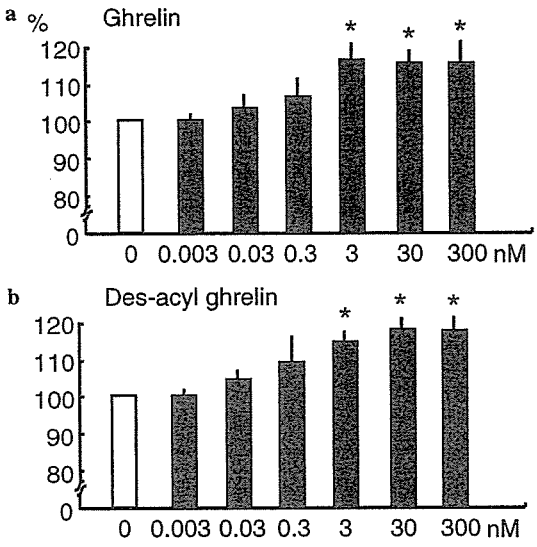

Fig. 2. Cell proliferation effects of ghrelin and des-acyl ghrelin on cultured fetal spinal cells. Proliferative cells were quantified by BrdU ELISA. Significant proliferation was indicated in cells treated with over 3 nM ghrelin (a) and des-acyl ghrelin (b). Values are presented by means + SEM ($^*P < 0.05$).

Binding assay

To identify the presence of the binding site of des-acyl ghrelin, [^{125}I]des-acyl ghrelin binding to membranes from fetal spinal cord was assayed. Specific, high affinity and saturable binding of labeled ghrelin were observed ($K_d = 3.467$, $B_{max} = 1.061$ fmol/mg protein) (Fig. 4a). The binding of labeled des-acyl ghrelin was displaced by unlabeled des-acyl ghrelin and ghrelin (Fig. 4b). The IC_{50} values for des-acyl ghrelin and ghrelin were 23.52 and 41.60 nM, respectively.

Discussion

Our previous study showed that ghrelin, as well as des-acyl ghrelin, play important roles in fetal growth, and that GHS-R mRNA is abundantly expressed in the spinal cord of rat fetus compared with other tissues [17]. Therefore, we reasoned that these ligands and their receptor might exert important actions during neurogenesis of the embryonic spinal cord. In the present study, in fact, ghrelin and des-acyl ghrelin both facilitated the proliferation of cells from fetal spinal cord. In addition, GHS-R mRNA and

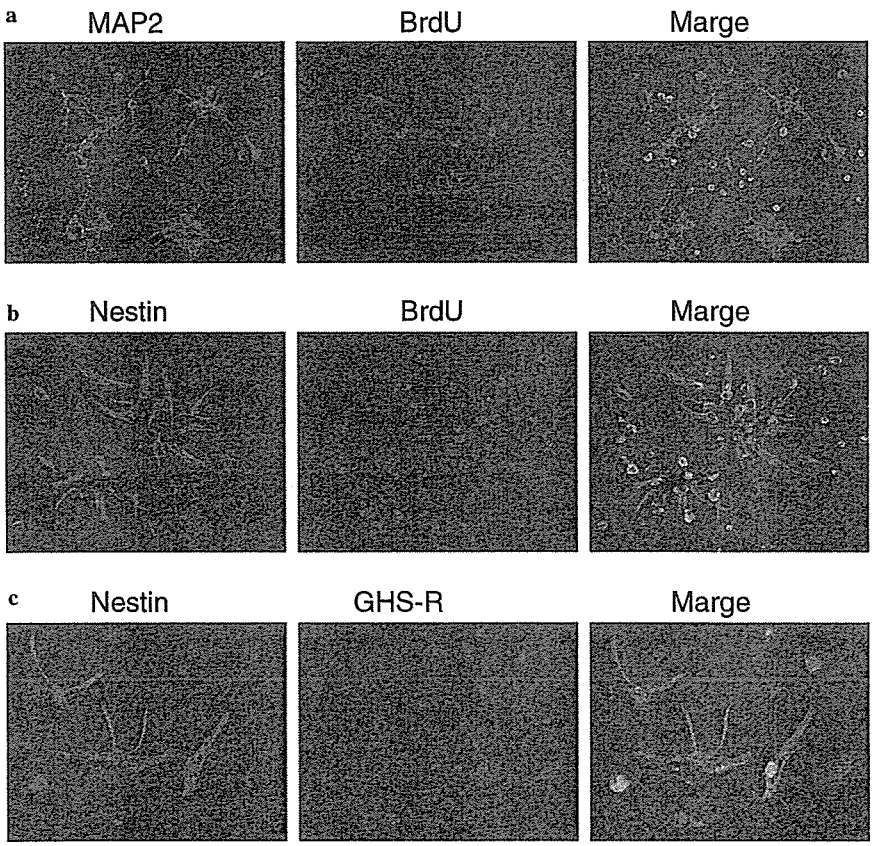

Fig. 3. Identification of proliferative cells in the rat fetal spinal cord. Double immunofluorescence staining demonstrated immunoreactivity for both Map2 and BrdU in distinct cells (a), and co-localization of nestin and BrdU in the same cells (b) Co-localization of nestin and GHS-R was also observed in neuron precursor cells (c).

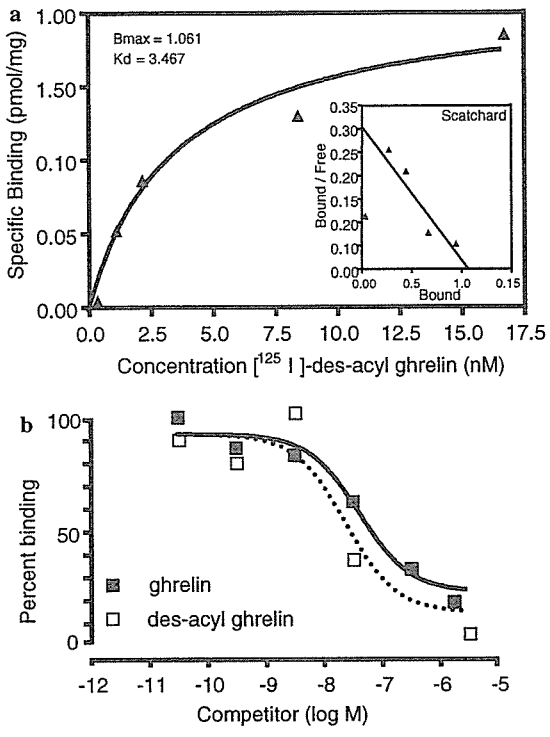

Fig. 4. (a) Representative saturation curve and the Scatchard plot of [^{125}I]des-acyl ghrelin binding to membranes from the fetal spinal cord. Binding assay was performed by incubating a fixed amount of membranes with increasing concentrations (0.13–16.64 nM) of the radioligand. Specific binding values were obtained by subtracting non-specific binding from total binding. (b) Displacement curve of [^{125}I]des-acyl ghrelin binding in the presence of unlabeled des-acyl ghrelin or ghrelin. Binding assay was performed by incubation of fixed amounts of membranes and labeled ligand with increasing concentrations of either of the unlabeled ligands.

GHS-R protein were detected in spinal cord tissue, and neuronal precursor cells in primary culture possessed GHS-R immunoreactivity, indicating that ghrelin stimulated the proliferation of neuronal precursor cells through GHS-R. Although the receptor recognizing des-acyl ghrelin has not yet been characterized, the present binding study indicated that there was at least one binding site specific for des-acyl ghrelin in membranes from fetal spinal cord tissue. Several recent studies have reported that not only ghrelin but also des-acyl ghrelin exert a biological effect even in tissues or cells that do not express GHS-R, suggesting that these reactions would not require octanoic acid modification and could be achieved without GHS-R [11,17,21,22]. Interestingly, many of the effects induced by both peptides at the cellular level are associated with cell fate, such as cell survival and/or apoptosis as well as cell proliferation, although activation or inhibition of the cell survival and proliferation pathways appear to be independent of cell type [11]. Thus, it is assumed that the effect of ghrelin and des-acyl ghrelin on spinal cord cells observed in the present study could be induced through both the GHS-R and another unknown pathway.

Recently, Zhang et al. demonstrated that ghrelin acted directly on dorsal motor nucleus of the vagus neurons to stimulate neurogenesis, and concluded that neuronal proliferation would result from an increase of calcium concentration associated with cellular depolarization through activation of GHSR-1a by ghrelin [15]. In the present study, however, diltiazem, a blocker of L-type voltage-dependent calcium channels, did not inhibit proliferation of spinal cord neuronal cells, inconsistent with dorsal motor nucleus of the vagus neurons, suggesting that the proliferation effect was likely mediated via a pathway other than the calcium increase caused by depolarization of L-type calcium channels (data not shown). Some studies of the molecular mechanism involved in the induction of cell proliferation and adhesion by ghrelin have suggested cascades of intracellular events, such as the MAPK and/or PI3 K/Akt pathways. In hepatoma cells expressing GHS-R, ghrelin has been shown to activate the IRS-1-GRB2-MAPK pathway, downstream from the insulin receptor, but to inhibit Akt activity [23]. Also in cardiomyocytes and endothelial cells, ghrelin induces phosphorylation of tyrosine, and both ghrelin and des-acyl ghrelin activate the MAPK and Akt pathways [9]. In addition, activation of the MAPK pathway by ghrelin has also been reported in a rat pituitary somatotroph cell line [13] and human adrenal zona glomerulosa cells [12]. Nanzer has explained the possible pathways leading to MAPK activation, resulting from stimulation of phospholipase C and PKC, or transactivation of tyrosine kinase receptors via the beta and gamma subunits of the G protein. Similarly, it is assumed that the cell proliferation effect of ghrelin and des-acyl ghrelin in the rat fetal spinal cord might involve activation of MAPK and/or PI3K/AKt.

In this study, not only neuronal precursor cells but also neurons seemed to possess GHS-R protein, because the localization of Map-2-positive cells was consistent with that of GHS-R-positive cells in sections of spinal cord tissue. These results suggested that ghrelin would play an unidentified role via GHS-R in neurons of the rat fetus, for instance during formation of the neuronal network. Although there is no evidence of any abnormality in GHS-R-knockout mouse fetus [24], this function as well as the cell proliferative effect may be concealed by compensating actions of growth factors such as nerve growth factor.

In summary, we have demonstrated that both the GHS-R gene and protein are expressed in the rat fetal spinal cord from ED 13 to PD 0. In primary cultures of fetal spinal cord cells, ghrelin and des-acyl ghrelin induced cell proliferation effects, whereby neuronal precursor cells possessing GHS-R protein were increased. Moreover, a binding study using labeled des-acyl ghrelin showed that specific binding to des-acyl ghrelin could be displaced by unlabeled ghrelin and des-acyl ghrelin in membranes from fetal spinal cord. Taken together, our findings suggest that in the rat fetal spinal cord, ghrelin and des-acyl ghrelin are involved in neurogenesis via both GHS-R and an unidentified receptor

for des-acyl ghrelin. Further examinations to identify this unknown receptor for des-acyl ghrelin are warranted.

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Beraprost sodium enhances neovascularization in ischemic myocardium by mobilizing bone marrow cells in rats

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Abstract

Beraprost sodium, an orally active prostacyclin analogue, has vasoprotective effects such as vasodilation and antiplatelet activities. We investigated the therapeutic potential of beraprost for myocardial ischemia. Immediately after coronary ligation of Sprague–Dawley rats, beraprost (200 μg/kg/day) or saline was subcutaneously administered for 28 days. Four weeks after coronary ligation, administration of beraprost increased capillary density in ischemic myocardium, decreased infarct size, and improved cardiac function in rats with myocardial infarction. Beraprost markedly increased the number of CD34-positive cells and c-kit-positive cells in plasma. Also, four weeks after coronary ligation of chimeric rats with GFP-expressing bone marrow, bone marrow-derived cells were incorporated into the infarcted region and its border zone. Treatment with beraprost increased the number of GFP/von Willebrand factor-double-positive cells in the ischemic myocardium. These results suggest that beraprost has beneficial effects on ischemic myocardium partly by its ability to enhance neovascularization in ischemic myocardium by mobilizing bone marrow cells.

Keywords: Prostacyclin analogue; Myocardial infarction; Neovascularization; Bone marrow mobilization

Interruption of myocardial blood flow leads to rapid death of cardiomyocytes and vascular structures, resulting in the development of heart failure [1]. Stem or progenitor cells are mobilized from bone marrow into the peripheral blood in response to tissue ischemia, migrate to sites of injured tissues, and differentiate into endothelial cells and cardiomyocytes [2–4]. However, the compensatory mechanisms are insufficient to heal infarcted myocardium. Earlier studies have shown that bone marrow cells artificially mobilized by cytokines repair the infarcted heart and improve cardiac function after acute myocardial infarction [5,6]. Therefore, enhancement of bone marrow cell mobilization leading to neovascularization following revascularization would be beneficial for the treatment of acute myocardial infarction.

Beraprost sodium (BPS) is a chemically stable prostacyclin analogue owing to its cyclo-pentabenzofuranyl structure [7]. It has been well established that BPS has vasoprotective effects such as vasodilation and antiplatelet activities [8–11]. Thus, BPS has been used in the treatment of peripheral arterial disease [12,13] and pulmonary arterial hypertension [14,15]. Although a limited number of studies suggest therapeutic potential of prostacyclin for the treatment of myocardial ischemia [16–18], the underlying mechanisms still remain unclear. In addition, little information is available regarding the therapeutic potential of prostacyclin analogues such as BPS for myocardial ischemia. A recent study has shown that BPS activates endothelial

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