Intraperitoneal administration of nesfatin-1 stimulates glucagon-like peptide-1 secretion in fasted mice

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Abstract. Increasing endogenous secretion of glucagon-like peptide (GLP)-1 is considered a promising therapeutic approach for type 2 diabetes because decreased GLP-1 plasma concentrations have been observed in patients with this condition. Nesfatin-1, which is a central and peripheral anorexigenic peptide, has been reported to release GLP-1 from enteroendocrine STC-1 cells, although whether nesfatin-1 stimulates GLP-1 secretion *in vivo* remains to be elucidated. Previous studies have indicated that nesfatin-1 has glucose-lowering and insulinotropic effects in mice and rats; however, the *in vivo* mechanism remains unclear. The present study aimed to investigate whether peripheral administration of nesfatin-1 increased blood concentrations of GLP-1 and insulin in food-deprived mice. Nesfatin-1 was administered intraperitoneally to 18-h fasted mice. Plasma GLP-1 and insulin concentrations in the mice administered 2.5 μmol/kg nesfatin-1 were higher than those in saline-treated mice. Blood glucose concentrations in mice treated with 1.25 and 2.5 μmol/kg nesfatin-1 were lower than those in saline-treated mice. The mRNA expression of preproglucagon in mouse ilea after treatment with 1.25 μmol/kg nesfatin-1 was higher than that in saline-treated mice. The administration of 1.25 μmol/kg nesfatin-1 raised GLP-1 concentrations at 30 and 60 min and insulin concentrations at 30 and 60 min after injection. Furthermore, the higher level of nesfatin-1-induced insulin was diminished by pre-administration of anti-GLP-1 antiserum. Intraperitoneally administered nesfatin-1 increased insulin concentrations by accelerating GLP-1 secretion. The results are the first *in vivo* demonstration of promotion of GLP-1 secretion by nesfatin-1 in the mouse, suggesting the developmental potential of nesfatin-1 for GLP-1 release.

Introduction

Glucose homeostasis is regulated by hormonal and neuronal pathways and is important for the maintenance of energy metabolism (1). Chronic disorders of these regulatory pathways may result in the development of obesity, diabetes and/or arteriosclerotic myocardial infarction (2). Insulin, which is a master regulator of glucose homeostasis, is secreted by pancreatic β cells in response to increased peripheral blood glucose concentrations and activates insulin receptors in various tissues. In muscle and adipocytes, glucose uptake is enhanced *via* the glucose transporter on the cell surface. In liver and muscle, glucose is converted to glycogen and stored and, as a result, blood glucose concentrations quickly return to normal (3). Glucagon-like peptide (GLP)-1 is an incretin that is secreted by ileal L cells in response to nutrient ingestion, enhances insulin secretion through the GLP-1 receptor on β cells and serves a major role in stimulating insulin secretion in healthy subjects.

In patients with type 2 diabetes, decreased GLP-1 plasma concentrations have been observed and GLP-1 function is impaired. However, the reactivity of GLP-1 receptors is preserved and GLP-1 receptor agonists, such as liraglutide, significantly reduce plasma glucose and improve glycemic control in these patients. Due to this pharmacological advantage, the use of GLP-1 receptor agonists is well established for the treatment of type 2 diabetes (4). However, therapeutic agents that promote secretion of endogenous GLP-1 have not yet been developed.

Nesfatin-1 is an 82-amino acid peptide that was originally identified in the hypothalamus as the N-terminal product of the nucleobindin (NUCB)-2 protein (5). Nesfatin-1 is secreted by neurons in the hypothalamus and spinal cord (6) and peripheral tissues (7-11). A previous study reported that centrally administered nesfatin-1 by continuous intracerebroventricular injection decreases food intake and causes body weight loss in rats (5). Nesfatin-1 administered *via* intracerebroventricular infusion increases insulin sensitivity in rats fed a high-fat diet through the activation of the insulin receptor/insulin receptor substrate-1/AMP-dependent protein kinase/AKT kinase target

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of rapamycin complex 2 phosphorylation pathway in the hypothalamus (12). The hypothalamus serves a pivotal role in controlling food intake and energy metabolism (13-16). Peripherally administered nesfatin-1 by intraperitoneal or intravenous injection reduces blood glucose concentrations in *db/db* mice, which is a leptin receptor-deficient model of type 2 diabetes mellitus presenting with hyperglycemia and obesity, and in streptozotocin-induced C57BL/6J mice, which is a model of type 1 diabetes (17). Subcutaneous infusion of nesfatin-1 increases insulin concentrations during oral glucose tolerance tests and decreases glucose concentrations during insulin tolerance tests in rats (18). Li *et al* (19) demonstrate that continuous subcutaneous infusion of nesfatin-1 improves glucose metabolism using the oral glucose tolerance test and insulin sensitivity using the insulin tolerance test in normal and high-fat diet-fed mice. In addition, nesfatin-1 increases insulin-stimulated phosphorylation of AKT in skeletal muscle, adipose tissue and liver and increases glucose transporter 4 membrane translocation in skeletal muscle and adipose tissue in mice fed a normal diet (19). *In vitro* studies demonstrate that nesfatin-1 increases insulin-stimulated glucose uptake in L6 skeletal muscle myoblasts and primary adipocytes (18) and accelerates glucose-dependent insulin release from islets isolated from rats and the rat insulinoma line INS-1 832/13 (20). In a human study, the level of *NUCB2* mRNA expression in islets from patients with type 2 diabetes was lower compared with that of control donors (20). However, plasma nesfatin-1 concentrations in diabetes is a controversial issue. Li *et al* (21) report that plasma nesfatin-1 levels in patients with type 2 diabetes are lower than those in healthy subjects and in patients with type 1 diabetes. Zhang *et al* (22) and Guo *et al* (23) report elevated plasma nesfatin-1 concentrations in patients with newly diagnosed type 2 diabetes.

Nesfatin-1 has been shown to directly stimulate insulin secretion from mouse pancreatic β cells by accelerating Ca^{2+} influx into the β cells through L-type channels (24) and inhibiting voltage-gated K^+ channels that function as a brake on Ca^{2+} influx (25). Furthermore, nesfatin-1 was shown to stimulate GLP-1 secretion from the enteroendocrine STC-1 cell line (26). However, whether peripheral administration of exogenous nesfatin-1 stimulates GLP-1 secretion *in vivo* remains to be elucidated. Therefore, the aim of the present study was to investigate the effects of nesfatin-1 on the secretion of GLP-1 followed by insulin release using fasted mice. It demonstrated for the first time that nesfatin-1 promotes GLP-1 secretion in mice *in vivo*.

Materials and methods

Synthesis of mouse nesfatin-1-related peptides. Mouse nesfatin-1 was synthesized by solid-phase methodology with 9-fluorenylmethoxycarbonyl using an automated peptide synthesizer (Model Pioneer; Thermo Fisher Scientific, Inc.). The crude peptide was purified by reverse-phase high-performance liquid chromatography (HPLC; Delta 600 HPLC system; Waters Corporation) using a Develosil 300 ODS-HG-5 column (2x25 cm; Nomura Chemical Co., Ltd.). Mouse C-terminal nesfatin-1 Cys-(48-82) and mouse N-terminal nesfatin-1 (1-35)-RRC were also synthesized in a manner similar to that described above. The purity of the synthetic

peptides was confirmed by analytical HPLC, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry and amino acid analysis.

Production of antiserum against nesfatin-1. Immunization and antiserum production were performed by Yanaihara Institute Inc. Briefly, three Japanese white rabbits (12 weeks old, male, body weight: 2.0-2.5 kg; SLC Japan Inc.) were individually housed in hutches (W 330 mm x L 480 mm x H 360 mm) placed on an automatic washing machine stand. The rabbits had been bred in an environment with 12-h light/dark cycles at 23±2°C and 55±5% humidity with free access to food and water. Immunization using synthetic mouse C-terminal nesfatin-1 Cys-(48-82) as the immunogen was performed as previously described (27). After the sixth immunization, pentobarbital sodium (45 mg/kg) was administered through the ear vein and then the animals were sacrificed by whole blood collection from the carotid artery under subsequent anesthesia. Mortality of rabbits used for antiserum production was confirmed by cardiac arrest, respiratory arrest and dilated pupils. After separating the serum from collected blood, one of the three rabbits had a high titer antiserum against nesfatin-1 Cys-(48-82). Similarly, a high titer antiserum against mouse N-terminal nesfatin-1 (1-35)-RRC was prepared.

Animals. Male C57BL/6J mice (8 weeks old; weight, 22-26 g) were obtained from SLC Japan Inc. A total of 74 mice were used in the present study (dose-response study: 24 mice, time-course study: 30 mice, blocking study with specific antiserum: 20 mice). Mice were maintained in the pathogen-free animal facility at Kobe Pharmaceutical University under standard conditions at 23±1°C and 55±5% humidity with a 12-h light/dark cycle (light-dark phase reversal: dark phase from 7:00 AM to 7:00 PM) with *ad libitum* access to sterile standard chow (CE-2; CLEA Japan Inc.) and water. Studies were performed in accordance with the Guide for the Care and Use of Laboratory Animals adopted and promulgated by the National Institutes of Health (https://www.ncbi.nlm. nih.gov/books/NBK54050/). All animal protocols for this study were approved by the Kobe Pharmaceutical University Committee for Animal Experiments. The animal study was reviewed and approved by Kobe Pharmaceutical University Committee for Animal Experiments (approval no. 2017-046). Measured values which were far above or below other measurements within the same group were disregarded.

Experimental protocol. After a resting period of 1 week, mice (21-25 g body weight) were randomly assigned to treatment groups for the experiments. In dose-response studies, nesfatin-1 (0.63, 1.25 and 2.5 μmol/kg) or saline (vehicle) was administered to six mice/group and the mice were sacrificed after 30 min. Similarly, in time course studies, nesfatin-1 (0.63 μmol/kg) or vehicle was administered to five mice/group and the mice were sacrificed at 30, 60 or 90 min. Nesfatin-1 was dissolved in 0.1 ml of physiological saline and intraperitoneally administered to mice after 18 h of food deprivation. At 30 min post-injection, peripheral blood samples were collected in tubes that contained 500 KIU/ml aprotinin (cat. no. 010-11834; FUJIFILM Wako Pure Chemical Corporation). In time course experiments, peripheral blood samples were collected at 30, 60 and 90 min

Table I. Primer sequences for the reverse transcription-quantitative PCR analysis.

Gene	NCBI reference sequence	Forward (5'-3') Reverse (5'-3')	Size (base pair)
Insulin 1	NM_008386	AGGACCCACAAGTGGAACAA GCTGGTAGAGGGAGCAGATG	132
Preproglucagon	NM_008100	TGAAGACAAACGCCACTCAC TGACGTTTGGCAATGTTGTT	132
β-actin	NM_007393	AGATCAAGATCATTGCTCCTCCTG ACGCAGCTCAGTAACAGTCC	174
GAPDH	NM_008084	GGTTGTCTCCTGCGACTTCA GCCGTATTCATTGTCATACCAGG	118

after intraperitoneal administration of 1.25 μmol/kg nesfatin-1 or saline from the orbital vein under inhalation anesthesia with 2.5% isoflurane (cat. no. 099-06571; FUJIFILM Wako Pure Chemical Corporation). After blood sampling at experimental time points, the mice were promptly sacrificed by exsanguination under inhalation anesthesia with 2.5% isoflurane and pancreatic tissue and a 1-cm length of terminal ileum were obtained. Using the collected blood samples, plasma was immediately separated and transferred into tubes containing the dipeptidyl peptidase (DPP) IV inhibitor 1c hydrochloride (cat. no. 2783; Tocris Bioscience). The plasma and tissue samples were stored at -80˚C until analysis.

Measurements of blood glucose and plasma GLP-1, insulin, glucagon and nesfatin-1 concentrations. Concentrations of GLP-1, insulin and glucagon were measured using ELISA kits in accordance with the manufacturer's instructions [GLP-1: cat. no. AKMGP-011; Levis GLP-1 (Active); Shibayagi Co., Ltd.; insulin: cat. no. 10-1249-01; Ultrasensitive Mouse Insulin ELISA; Mecrcodia AB; glucagon: cat. no. YK090; Glucagon EIA kit; Yanaihara Institute Inc. or cat. no. 10-1271-01; Glucagon ELISA; Mecrcodia AB]. Glucose concentrations were measured using a Glucocard meter (Arkray, Inc.). Plasma nesfatin-1 concentrations were measured by Kobe pharmaceutical university medical biochemistry laboratory-made two-site sandwich ELISA (measurable range: 0.24-25 ng/ml). The coefficients of variation of within- and between-assays were <3.8 and <7.8%, respectively (see Data S1 for the assay procedure and Fig. S1 for the calibration curve).

Reverse transcription-quantitative (RT-q) PCR. Total RNA was extracted from isolated pancreatic tissues using ISOGEN reagent (Nippon Gene Co., Ltd.) and cDNA was synthesized using a ReverTra Ace qPCR RT Master Mix kit (Toyobo Life Science) in accordance with the manufacturer's protocol. The RT-qPCR analysis was performed with a KOD SYBR qPCR Mix kit (Toyobo Life Science) following the manufacturer's protocol. PCR amplification was performed on PCR machine LightCycler 96 System (Roche Diagnostics GmbH). qPCR was performed using the following thermocycling conditions: Initial denaturation at 98˚C for 120 sec; then 40 cycles were performed at 98˚C for 10 sec, 68˚C for 10 sec and 60˚C for 30 sec; finally, the dissolution process was carried out at 95˚C, 65˚C and 97˚C for 10, 60 and 1 sec, respectively. Expression levels of mRNA were analyzed using the comparative threshold cycle method (28) and normalized to β-actin or

glyceraldehyde 3-phosphate dehydrogenase. The primers used in the RT-qPCR are shown in Table I.

Treatment with antiserum against GLP-1 or nesfatin-1. Rabbit anti-mouse GLP-1 serum and normal rabbit serum were purchased from Yanaihara Institute Inc. Antiserum against GLP-1, nesfatin-1 (RK-IK-10), or normal serum was administered intraperitoneally (0.1 ml/mouse) 30 min before intraperitoneal administration of 1.25 μmol/kg nesfatin-1. Peripheral blood and pancreatic and ileal tissues were collected at 60 min after intraperitoneal administration of nesfatin-1 and stored as described above.

Data analysis. The data are presented as the mean ± standard error of the mean (SEM). Comparisons between two groups were performed using two-sample t-tests. One-way analysis of variance (ANOVA) followed by Tukey's multiple comparison test was used to compare three or more groups. All statistical analyses were performed using StatFlex ver. 6 (Artech Co., Ltd.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Intraperitoneal administration of nesfatin-1 increases GLP-1 and insulin concentrations and decreases glucose concentrations in the blood. To investigate the effect of peripheral nesfatin-1 on GLP-1 and insulin secretion *in vivo,* nesfatin-1 (0, 0.63, 1.25 or 2.5 μmol/kg) was intraperitoneally administered to healthy mice that were fasted overnight. These doses were chosen according to a previous study (29). The 2.5 μmol/kg dose of nesfatin-1 in mice resulted in significantly higher GLP-1 and insulin concentrations than those in vehicle-treated (controls) mice (Fig. 1A; $F_{\text{between-group variation: 3, residual variation: 20}}=9.665$, $P=0.0004$ and B; $F_{3,20}=21.578$, $P<0.0001$; one-way ANOVA). However, the 1.25 and 2.5 μmol/kg nesfatin-1 doses resulted in significantly lower glucose concentrations than those in controls (Fig. 1C; $F_{3,20}=3.765$; $P=0.0272$; one-way ANOVA). Glucagon concentrations were not altered by nesfatin-1 treatment (Fig. 1D; $F_{3,20}=0.117$; $P=0.9492$; one-way ANOVA).

Intraperitoneal administration of nesfatin-1 increases mRNA expression of preproglucagon but not insulin. Nesfatin-1 treatment (1.25 μmol/kg) in mice resulted in significantly
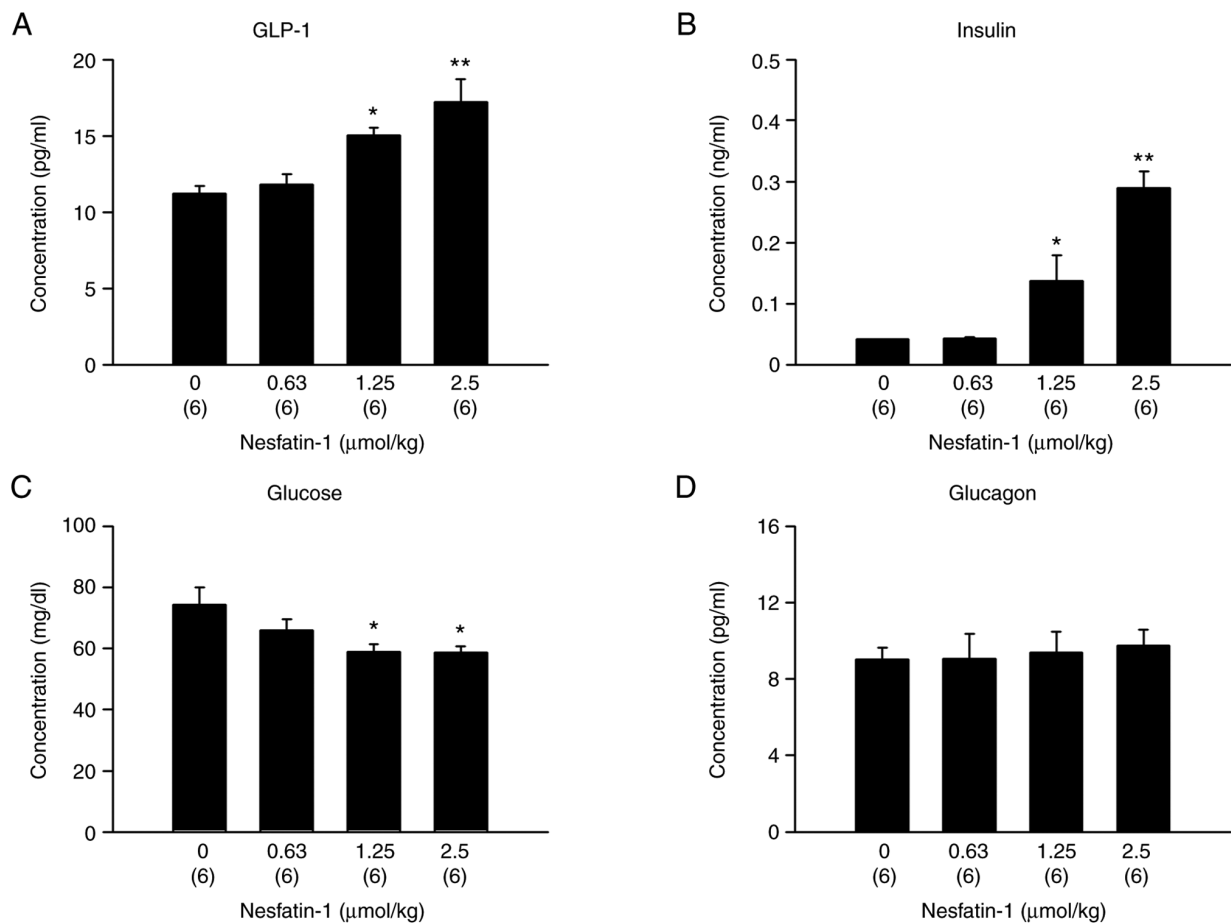
Figure 1. Dose responses to intraperitoneal administration of nesfatin-1. Peripheral blood concentrations of (A) GLP-1, (B) insulin, (C) glucose and (D) glucagon. Blood samples were obtained 30 min following intraperitoneal injection of different doses of synthetic mouse whole nesfatin-1 in overnight food-deprived C57BL/6J mice. Data are presented as the mean ± SEM. The number of mice is shown in parentheses. *$P<0.05$, **$P<0.01$ vs. controls using Tukey's multiple comparison test after one-way ANOVA. GLP, glucagon-like peptide.

higher mRNA expression of preproglucagon compared with vehicle-treated mice in ileal tissue (Fig. 2A; $F_{3,20}=3.800$; $P=0.0263$; one-way ANOVA). However, nesfatin-1 did not alter mRNA expression of insulin in the pancreas (Fig. 2B; $F_{3,20}=0.347$, $P=0.7916$).

Intraperitoneal administration of nesfatin-1 increases blood concentrations of GLP-1 at 30 and 60 min and those of insulin at 30 and 60 min after injection. GLP-1 concentrations in 1.25 μmol/kg nesfatin-1-treated mice were significantly higher than those in vehicle-treated mice at 30 and 60 min after injection (Fig. 3A; $t(8)=-3.3553$; $P=0.0100$ and $t(8)=-4.2537$; $P=0.0028$, respectively). Insulin concentrations in nesfatin-1-treated mice were significantly higher than those in vehicle-treated mice at 30 and 60 min after injection (Fig. 3B; $t(8)=-3.6619$; $P=0.0064$ and $t(8)=-3.1476$; $P=0.0136$, respectively). However, glucose concentrations in nesfatin-1-treated mice were significantly lower than those in vehicle-treated mice at 30 and 90 min after injection (Fig. 3C; $t(8)=2.8669$; $P=0.0209$ and $t(8)=5.9707$; $P=0.0003$, respectively). Control glucose concentrations at 90 min appeared higher than those at 0 min because different mice were killed at each time (Fig. 3C). Nesfatin-1 did not significantly alter glucagon concentrations at any time point (Fig. 3D).

Plasma concentrations of nesfatin-1 following nesfatin-1 injection. Plasma concentrations of nesfatin-1 in nesfatin-1-treated mice during the study period are shown in Fig. 4. At 30 min after administration, the plasma concentration of nesfatin-1 peaked at ~2,800 times that of the non-administered group. It then decreased gradually, but remained ~1,500 times higher than in the non-administered group, even after 90 min. There was no obvious difference in the appearance or behavior of nesfatin-1-treated mice compared with controls throughout the experiment.

Anti-nesfatin-1 and anti-GLP-1 sera block the effects of nesfatin-1. Pre-administration of anti-nesfatin-1 serum blocked nesfatin-1-induced GLP-1 production in peripheral blood (Fig. 5A; $F_{3,16}=32.771$; $P<0.0001$; one-way ANOVA). Anti-GLP-1 serum blocked insulin production induced by nesfatin-1 treatment (Fig. 5B; $F_{3,16}=11.061$; $P=0.0004$; one-way ANOVA). However, neither of these antiserums altered glucagon concentrations (Fig. 5C; $F_{3,16}=0.359$; $P=0.7832$; one-way ANOVA).

Discussion

Blood concentrations of nesfatin-1 in humans (21-23,30) and NUCB2 mRNA expression in human islets (20) suggest that
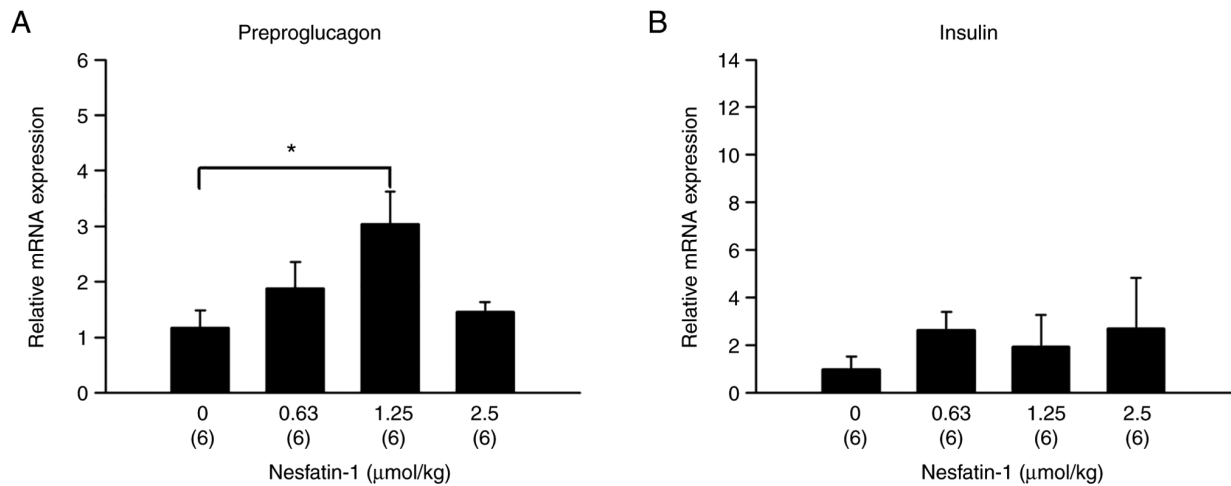
Figure 2. The mRNA expression of preproglucagon and insulin following nesfatin-1 injection. The mRNA levels of preproglucagon in the (A) ileum and (B) insulin in the pancreas. Tissue samples were obtained 30 min following intraperitoneal injection of different doses of synthetic mouse whole nesfatin-1 in overnight food-deprived C57BL/6J mice. Data are presented as the mean ± SEM. The number of mice is shown in parentheses. $^{*}P<0.05$ vs. controls using Tukey's multiple comparison test after one-way ANOVA.
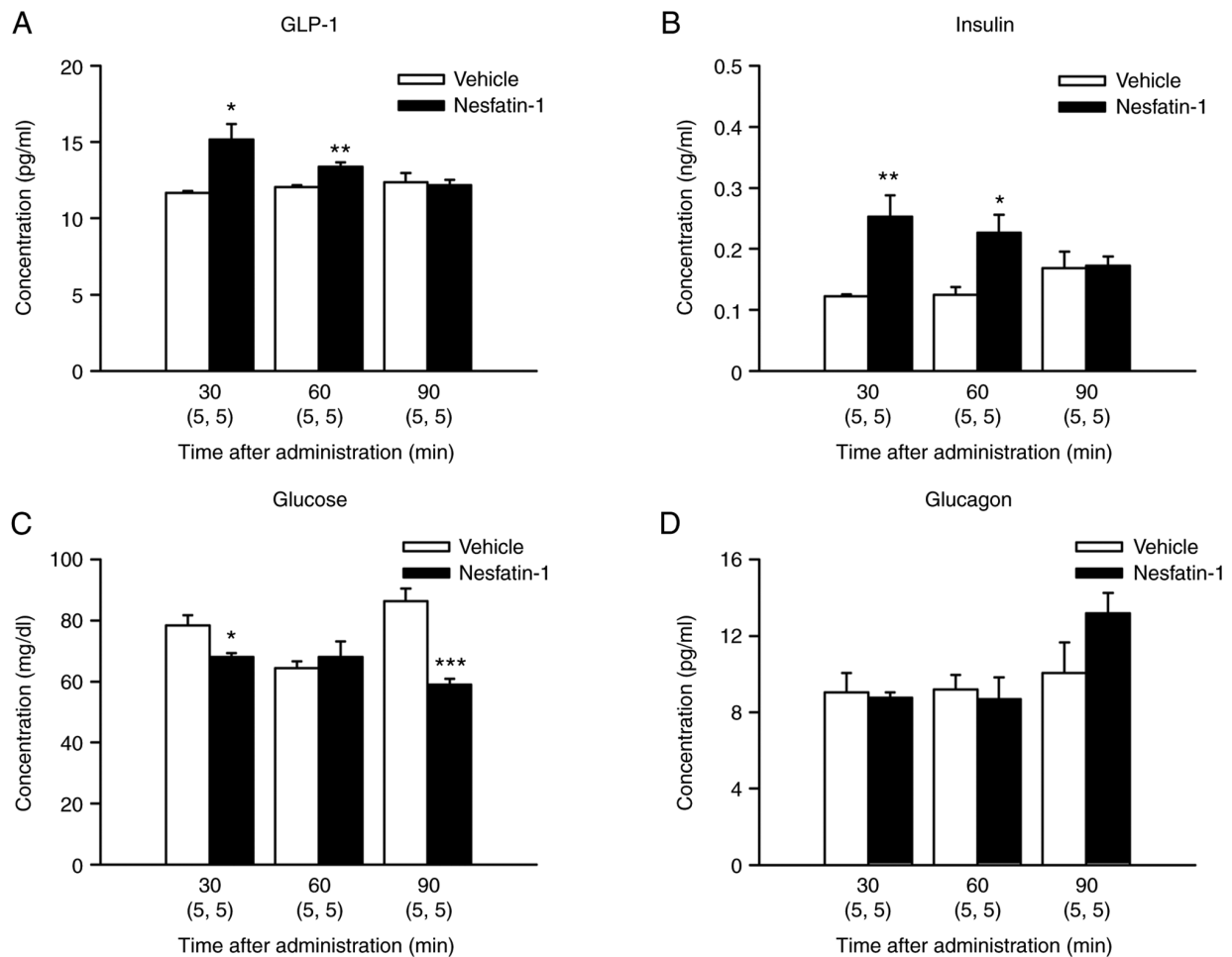

Figure 3. Changes in GLP-1, insulin, glucose and glucagon concentrations after nesfatin-1 injection. Chronological changes in peripheral blood concentrations of (A) GLP-1, (B) insulin, (C) glucose and (D) glucagon following intraperitoneal administration of 1.25 μmol/kg nesfatin-1 to overnight food-deprived C57BL/6J mice. Data are presented as the mean ± SEM. The number of mice is shown in parentheses. $^{*}P<0.05$, $^{**}P<0.01$, $^{***}P<0.01$ vs. controls using two-sample t-tests. GLP, glucagon-like peptide.

nesfatin-1 is associated with metabolic syndrome and type 2 diabetes (30) The results of previous *in vivo* studies (12,17-20)

have indicated that the anti-hyperglycemic effect of peripheral nesfatin-1 is dependent on insulin or glucose. Additionally,
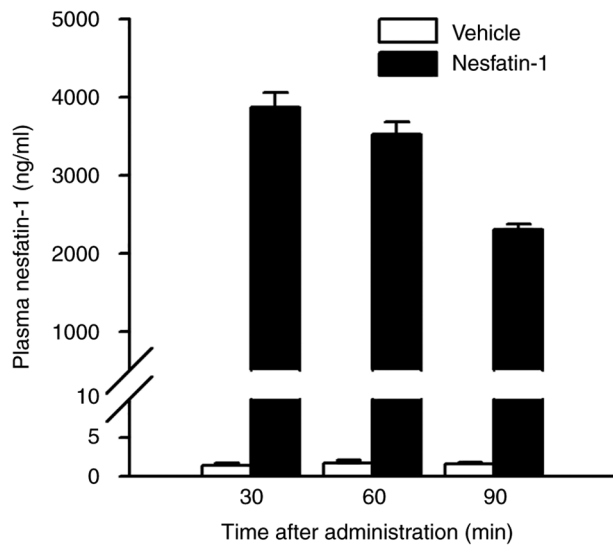
Figure 4. Chronological changes in peripheral blood concentrations of nesfatin-1 following nesfatin-1 injection. intraperitoneal injection of 1.25 μmol/kg nesfatin-1 to overnight food-deprived C57BL/6J mice. Plasma concentrations of nesfatin-1 after nesfatin-1 administration were measured using a mouse nesfatin-1 ELISA. Data are presented as the mean ± SEM. n=5 mice/group.

in vitro studies (24,25) suggest the possibility of direct effects of nesfatin-1 on insulin release from pancreatic islets. However, the underlying mechanism of the release of insulin by nesfatin-1 *in vivo* remains to be elucidated. To clarify the mechanism of the insulinotropic action of nesfatin-1 *in vivo*, the present study examined whether peripheral nesfatin-1 promotes basal insulin secretion from the pancreas by GLP-1 release from the intestine under fasting conditions.

To the to the best of the authors' knowledge, this is the first time that peripheral nesfatin-1 has promoted GLP-1 secretion *in vivo*. The present study found that intraperitoneal administration of nesfatin-1 elevated plasma GLP-1 concentrations, increased plasma insulin concentrations and decreased blood glucose concentrations in overnight-fasted mice. Moreover, the increase in plasma insulin concentrations were diminished by the pre-administration of anti-GLP-1 serum. The results suggested that nesfatin-1 stimulated GLP-1 secretion followed by insulin release and that nesfatin-1 promoted GLP-1 secretion at basal glucose and insulin concentrations. This GLP-1-releasing effect of nesfatin-1 may be glucose- or insulin-independent. However, these findings were obtained from experiments on mice and cannot be directly applied to humans.

Nesfatin-1 increased GLP-1 concentrations in a dose-dependent manner (Fig. 1A) and the changes in preproglucagon mRNA expression showed a bell-shaped dose response (Fig. 2A). Transcriptional activity of preproglucagon mRNA may increase before peptide synthesis and preproglucagon mRNA expression might be affected by plasma GLP-1 concentrations (31,32). However, the biochemical nature and implications of these possibilities remain to be assessed. Nesfatin-1 might affect not only GLP-1-producing L cells in the ileum, but also those in the jejunum and colon. In the present study, changes in preproinsulin mRNA expression (Fig. 2B) were not associated with a significant increase in

insulin concentrations (Fig. 1B). At 30 min following nesfatin-1 administration, insulin released from insulin granules to the extracellular space may be promoted in pancreatic β cells, resulting in a marked increase in blood insulin concentrations. However, preproinsulin transcriptional levels in cells might not be enhanced at this time.

Plasma concentrations of GLP-1 are 5-10 pmol/l in the fasted state and increase rapidly to 15-50 pmol/l in healthy human subjects after eating (33-35). In a previous study on patients with type 2 diabetes, plasma concentrations of GLP-1 after eating were 15-20 pmol/l without incretin treatment and 30-35 pmol/l with incretin treatment, the latter of which resulted in decreased blood glucose concentrations (36). Additionally, GLP-1 concentrations prior to eating were ~5 pmol/l in both patient groups. The GLP-1-insulin system is a therapeutic target for type 2 diabetes (4). GLP-1 secretion from L cells into the circulation is promptly inactivated by DPP-4, an enzyme presents in peripheral blood. GLP-1 receptor agonists and DPP-4 inhibitors have been successful as treatment strategies for type 2 diabetes (37,38). In addition, various substances that accelerate GLP-1 release have been reported, including fats, protein, bile acids, L-arginine, curcumin, glutamine, lipopolysaccharide and berberin (39). G protein-coupled receptor agonists and D-allulose promote GLP-1 secretion in a glucose-independent manner from enteroendocrine cell lines and intestinal L cells, respectively (40,41). In the present study, nesfatin-1 increased plasma GLP-1 concentrations in the fasted state. The mechanism of nesfatin-1 action may be similar to that of G protein-coupled receptor agonists, which promote GLP-1 secretion (40). Mechanistic studies on how nesfatin-1 stimulates GLP-1 secretion *in vivo* are required. Several studies have shown that the effects of nesfatin-1 involve the AKT pathway (42-47). Although various studies have been conducted on nesfatin-1 (48,49), the nesfatin-1 receptor has not yet been identified. To clarify the mechanism by which nesfatin-1 promotes GLP-1 secretion, it is necessary to continue examining the involvement of GPR119 in enteroendocrine cells (40), vagal afferent signaling in animals (41) and Akt/AMP-activated protein kinase/target of rapamycin complex-2 pathways in the brain (12).

The present study selected the doses of nesfatin-1 based on the fact that the intraperitoneal administration of 0.25-1.25 μmol/kg nesfatin-1 previously significantly decreased the 3-h food intake of mice (29). The doses of nesfatin-1 in the present study were higher than those in previous studies of this insulin secretagogue (18-20,50). However, in those studies, nesfatin-1 was administered as a single intravenous injection or continuous subcutaneous infusion in mice or rats. Mouse islet β cells require 10-100 times higher plasma concentrations of nesfatin-1 to potentiate glucose-induced insulin secretion by promoting Ca^{2+} influx through L-type Ca^{2+} channels (24). To maintain high tissue concentrations of target molecules, plasma concentrations of these molecules should be further elevated. For example, if target molecule blood vessel concentrations need to be 10 times higher than those in tissues and if those in tissues need to be maintained at 100 times higher than normal plasma ranges, blood vessel levels will reach 1,000 times higher than normal plasma ranges. In the present study, nesfatin-1 plasma concentrations were ~2,800 times higher than those before its administration.
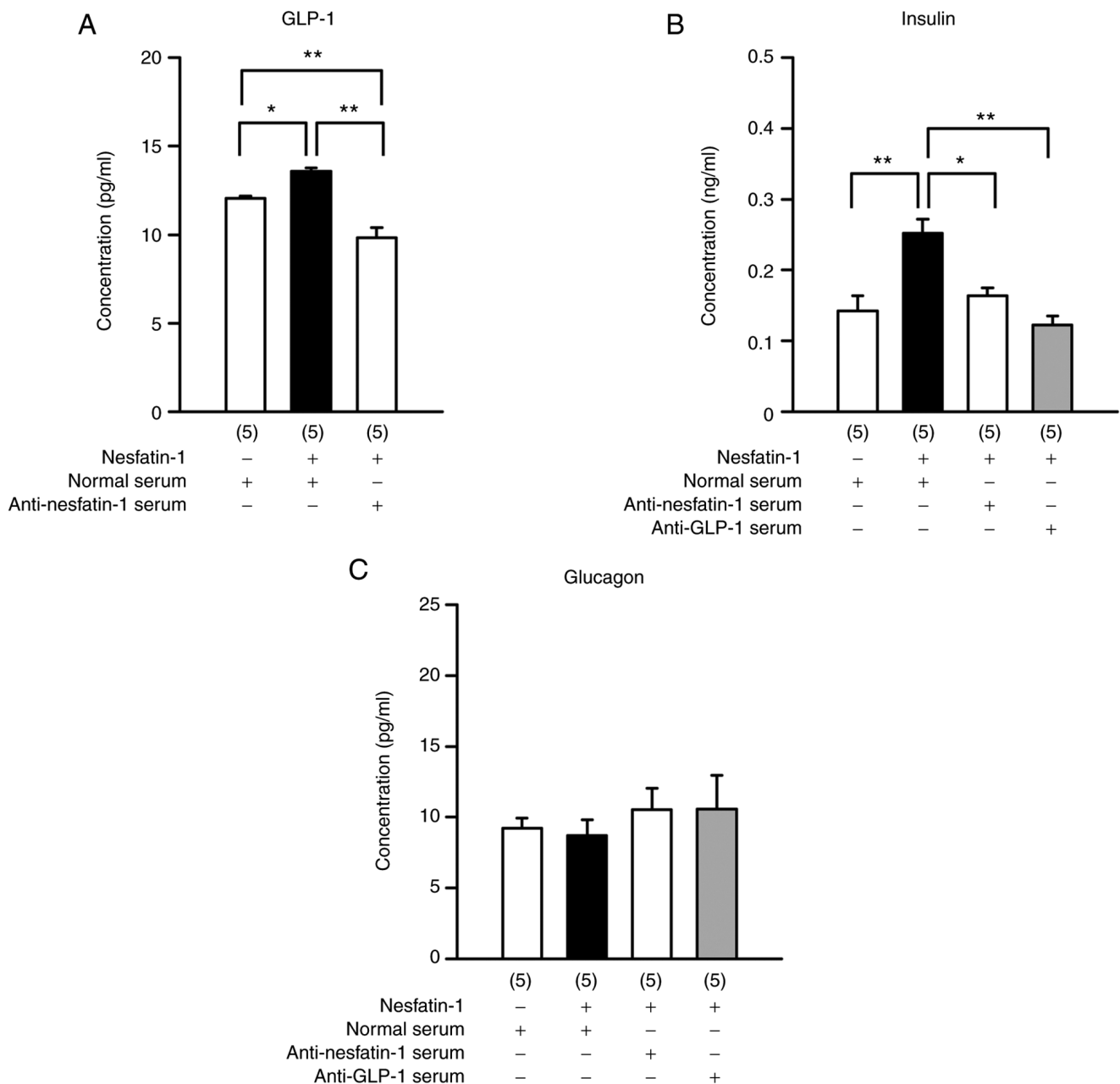
Figure 5. Blocking GLP-1 or nesfatin-1 with specific antiserum decreases nesfatin-1-induced plasma GLP-1 and insulin concentrations. Plasma concentrations of (A) GLP-1, (B) insulin and (C) glucagon. Anti-GLP-1 or anti-nesfatin-1 serum was intraperitoneally administered 30 min before intraperitoneal administration of 1.25 μmol/kg nesfatin-1 to overnight food-deprived C57BL/6J mice and blood samples were obtained 60 min after nesfatin-1 injection. Data are presented as the mean $\pm$ SEM. The number of mice is shown in parentheses. *$P<0.05$, **$P<0.01$ vs. controls using Tukey's multiple comparison test after one-way ANOVA. GLP, glucagon-like peptide.

Therefore, it was hypothesized that 1.25 μmol/kg of nesfatin-1 would be necessary to induce increased GLP-1 secretion.

The present study used normal healthy mice in experiments and showed that nesfatin-1 stimulated GLP-1 secretion *in vivo*. Further studies in hyperglycemic mice to mimic the type 2 diabetes model are required because GLP-1, which is released by nesfatin-1, is considered a promising therapeutic approach for type 2 diabetes. In addition, future studies are necessary to examine the validity of this action of GLP-1 by repeated or continuous administration of nesfatin-1 in the long term.

In recent years, GLP-1 receptor agonists have been used as anti-obesity drugs. By continuously acting on specific nerve cells in the hypothalamus that control appetite and eating behavior, this medication is thought to prevent postprandial hyperglycemia, promote visceral fat burning and improve basal metabolism (51). Therefore, the administration of nesfatin-1 may stimulate the secretion of GLP-1 and cause beneficial effects similar to those of GLP-1 receptor agonists as aforementioned. However, because endogenous GLP-1 has a short half-life in the blood, nesfatin-1 is likely to be less effective than GLP-1 receptor agonists as an antidiabetic agent at present. However, the present study indicated that nesfatin-1 promoted endogenous GLP-1 secretion and it might become a novel antidiabetic drug for stimulating GLP-1 release.

The biological effects of nesfatin-1 need to be evaluated. Previous studies have already reported that nesfatin-1 stimulates

insulin secretion *in vitro* (20,24,25) and *in vivo* (12,17-20). Another study has also reported that nesfatin-1 promotes GLP-1 secretion *in vitro* (26). These studies support the hypothesis of the present study that the significant differences found in this study reflect biological relevance.

In conclusion, the present study showed that intraperitoneal administration of nesfatin-1 stimulated GLP-1 release even under low glucose conditions, such as after fasting. The findings provided the first *in vivo* evidence that peripheral nesfatin-1 increased endogenous GLP-1 secretion. The present study also suggested that nesfatin-1 promoted insulin production via an increase in GLP-1 concentrations. The present study hypothesized that nesfatin-1 is a GLP-1 secretagogue that may be useful as a therapeutic strategy for type 2 diabetes. Further studies are required to clarify the mechanism by which nesfatin-1 promotes GLP-1 secretion and its biological significance in energy homeostasis.

Availability of data and materials

All data generated or analyzed during this study are included in this published article.

Authors' contributions

AA and IK designed the study. NT, HO and HM conducted the experiments. NT, AA and IK wrote the paper. NT, HO and HM analyzed the data. NT and IK confirm the authenticity of all the raw data. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

The animal study was reviewed and approved by Kobe Pharmaceutical University Committee for Animal Experiments (approval no. 2017-046).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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