



Comparison of glucose tolerance between wild-type mice and mice with double knockout of neuromedin U and neuromedin S

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ABSTRACT. Recently, it has been proposed that neuromedin U (NMU) is “decretin”, which suppresses insulin secretion from the pancreas *in vitro*. Here we examined the possible involvement of NMU in insulin secretion *in vivo* by comparing the plasma glucose and insulin levels of wild-type mice with those of double knockout (D-KO) of the NMU and neuromedin S (NMS) genes, as NMS binds to the neuromedin U receptor. If NMU is, in fact, “decretin”, which inhibits insulin secretion from the pancreas, then NMU-deficient mice might result in higher plasma insulin levels than is the case in wild-type mice, or injection of NMU lead to suppression of plasma insulin level. In this study, we found that the fasting plasma level of insulin was not increased in D-KO mice. Glucose tolerance tests revealed no significant difference in plasma insulin levels between wild-type mice and D-KO mice under non-fasting conditions. After peripheral injection of NMU, plasma glucose and insulin levels did not show any significant changes in either wild-type or D-KO mice. Glucose tolerance testing after 3 weeks of high fat feeding revealed no significant difference in plasma insulin levels during 60 min after glucose injection between wild-type and D-KO mice. These results suggest that even if NMU is a decretin candidate, its physiological involvement in suppression of insulin secretion may be very minor *in vivo*.

KEY WORDS: decretin, high fat diet, Insulin, neuromedin S, neuromedin U

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Neuromedin U (NMU), a brain-gut peptide containing 8–25 amino acids, is structurally highly conserved among various animals [15]. In 2000, the orphan G protein-coupled receptors FM-3/GPR66 and FM-4/TGR-1 were identified as NMU receptors and named neuromedin U receptor 1 (NMU-R1) and neuromedin U receptor 2 (NMU-R2), respectively [6, 7, 10, 21, 25]. Later, in 2005, Mori *et al.* isolated another peptide from rat brain that activates both NMU-R1 and NMU-R2 in a manner similar to NMU [16]. The latter peptide comprised 36 amino acids and was designated as neuromedin S (NMS). The C-terminal seven-residue amidated sequence of NMS is identical to that of NMU, and considered essential for NMU receptor binding [16].

It has been clarified that NMU-R1 and R2 are distributed mainly in peripheral organs and the central nervous system, respectively, and various physiological roles for NMU or NMS have been proposed, such as pain reception [3], immune function [5], gastrointestinal motility [2], cardiovascular function [14], energy homeostasis [19], stress responses [26] and secretion of vasopressin, oxytocin [22, 23]. In addition to these physiological roles, some reports have suggested that NMU is involved in glucose homeostasis, although such findings are not unified. Alfa *et al.* has suggested that NMU might be a candidate molecule for “decretin”, which suppresses insulin secretion [1]. Classical studies of human metabolism have led to the suggestion that decretin is an enteroendocrine hormone that is induced by fasting and suppresses insulin production and secretion. Specifically, Ensink *et al.* have proposed that it may suppress the release of insulin to prevent hypoglycemia [4]. The hypothesis of Alfa *et al.* has been supported by the observation that administration of NMU suppresses glucose-induced insulin secretion from human islets [1]. Similarly, it has been shown that NMU suppresses insulin secretion from rat isolated pancreatic islets [8, 9]. In contrast, Peier *et al.* have demonstrated that in the glucose tolerance test, peripheral administration of NMU suppresses the glucose level and induces

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the increase in the level of insulin [20]. More recently, a long-acting NMU1/2 receptor non-selective agonist was shown to inhibit the plasma glucose level during the oral glucose tolerance test [17]. These observations raise the issue of whether NMU has the physiological effect in glucose homeostasis and insulin-releasing. If NMU is, in fact, “decretin”, NMU-deficient mice might result in higher plasma insulin levels than is the case in wild-type mice, or injection of NMU lead to suppression of plasma insulin level.

Therefore, the aim of the present study was to clarify the involvement of NMU in insulin secretion and glucose homeostasis. We did this by comparing plasma insulin levels before and after glucose injection under normal diet and high fat diet conditions between wild-type mice and mice with double deletion of the NMU and NMS genes (double knockout (KO) mice), since NMS may compensate for the action of NMU in NMU-KO mice by also binding to NMU receptors.

MATERIALS AND METHODS

Animals

Male C57black/6J wild-type and NMU/NMS double-KO (D-KO) mice were used in this study at 7 or 10 weeks old age. NMU-KO and NMS-KO mice were originally produced by Dr. M. Kojima (Molecular Genetics, Institute of Life Science, Kurume University, Kurume, Japan) and supplied by the National Cerebral and Cardiovascular Center Research Institute (Osaka, Japan). The D-KO mice were produced in our laboratory [18] and confirmed to lack expression of NMU and NMS mRNA (Fig. 1A). To examine possible effect of NMU on the plasma insulin level under a high fat diet, we used D-KO and wild-type mice supplied a high fat diet (Research Diet #D12492, 60% fat kcal, energy content 5.24 kcal/g) for 3 weeks. All animals were housed individually in Plexiglas cages in an animal room maintained under a constant light–dark cycle (lights on 7:00–19:00 hr) and temperature (22 ± 1°C). Food and water were provided ad libitum. All procedures were performed in accordance with the Japanese Physiological Society’s guidelines for animal care, and the experiments were authorized by the Animal Experiment Committee of the University of Miyazaki (authorization number 2006-053-3).

RT-PCR

The hypothalamus and pancreas were removed from wild-type and D-KO mice, and immediately homogenized in TRIzol reagent (Invitrogen Co., Carlsbad, CA, U.S.A.) to extract the total RNA, which was then purified using a RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany) and used to synthesize first-strand cDNA employing a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA, U.S.A.). The reaction was subjected to 35 cycles on an Applied Biosystems Veriti thermal cycler. The primer sets used for mouse NMU, NMS, NMU-R1, NMU-R2 and GAPDH were: NMU: sense 5'-AAT GTT GTG TCC TCT GTT GTG CAT CC-3', anti-sense 5'-TGA AAC TTG TTG ACC TCT TCC CGT TG-3'; NMS: sense 5'-GAA ACC GAC ACA TCC AGT TAG CG-3', anti-sense 5'-TCG GTG TAT CTT CCA TTC CTA GGC-3'; NMU-R1: sense 5'-CAA GAT AGG GGA CGG AGA CAG GTG AC-3', anti-sense 5'-TGA GCC GAG ATA GAA GAA GAT GCC AGA-3'; NMU-R2: sense 5'-ACC CTC CAG AAA ATC AGT CAC CAA GA-3', anti-sense 5'-GCG CCG AGA CAG GAG GTT ATA TAT AA-3'; GAPDH: sense 5'-TGC CAT CAC TGC CAC TCA GAA GAC TGT-3', anti-sense 5'-TCC ACC ACC CTG TTG CTG TAG CCA TA-3'.

Blood sampling

Fifty μ l of each blood sample was collected by the tail tip incision method into tubes containing EDTA and aprotinin (a protease inhibitor) and then, to collect plasma samples, were centrifuged for 5 min at 10,000 rpm.

Measurement of plasma glucose, insulin, and leptin

Plasma glucose was measured using a DRI-CHEM3500V instrument (Fuji Medical Systems, Tokyo, Japan), and plasma insulin and leptin were measured using a mouse insulin ELISA kit (Shibayagi, Shibukawa, Japan), and a mouse leptin ELISA kit (Morinaga, Yokohama, Japan) respectively, in accordance with the manufacturers’ protocols.

Intraperitoneal administration of NMU

NMU was injected intraperitoneally under non-anesthesia at a dose of 40 and 100 nmol/mouse in the experiment of Figs. 2 and 5, respectively. Control group was injected with the same volume saline. In the glucose tolerance test of Fig. 5C and 5D, NMU was injected at 5 min before glucose administration.

Glucose tolerance test

Glucose tolerance test was performed with intraperitoneal administration at 1.5 (Figs. 3 and 4), 2 (Fig. 5C–F) and 3 mg/g body wt (Fig. 5G and 5H). Blood samples were collected at 0, 10, 30, and 60 min after glucose injection. The conditions for feeding in the experiment were described in the results section.

Statistical analysis

The data (mean ± SEM) were analyzed statistically by Student’s *t*-test. Differences at $P < 0.05$ were considered statistically significant.

RESULTS

Expression of NMU-R1 and NMU-R2 mRNAs in the pancreas

Expression of mRNAs for both NMU-R1 and R2 was clearly observed in the hypothalamus of both wild-type and D-KO mice. In the pancreas, however, no mRNA for either receptor was detected in wild-type and D-KO mice (Fig. 1B).

Fasting blood glucose and insulin levels in wild-type and D-KO mice

Under fasting conditions, there was no significant difference in plasma insulin levels between the two types of mice (Fig. 1C). On the other hand, the plasma glucose level in D-KO mice was significantly lower than that in wild-type and NMU-KO mice (Fig. 1D).

Effect of NMU injection on plasma glucose and insulin levels in wild-type and D-KO mice

We compared the changes of glucose levels and insulin levels between the NMU-injected and saline-injected group without fasting. Forty nmol of NMU-injected did not affect plasma glucose and insulin levels in wild-type mice (Fig. 2A and 2B). Also in D-KO mice, there was no difference in plasma glucose and insulin levels between the NMU-injected and saline-injected group (Fig. 2A and 2B).

Comparison of response to the glucose tolerance test between wild-type and D-KO mice

As the previous experiment had failed to demonstrate any effect of endogenous or exogenous NMU on plasma insulin levels, we subjected wild-type and D-KO mice to the glucose tolerance test without fasting. The plasma glucose and insulin levels increased

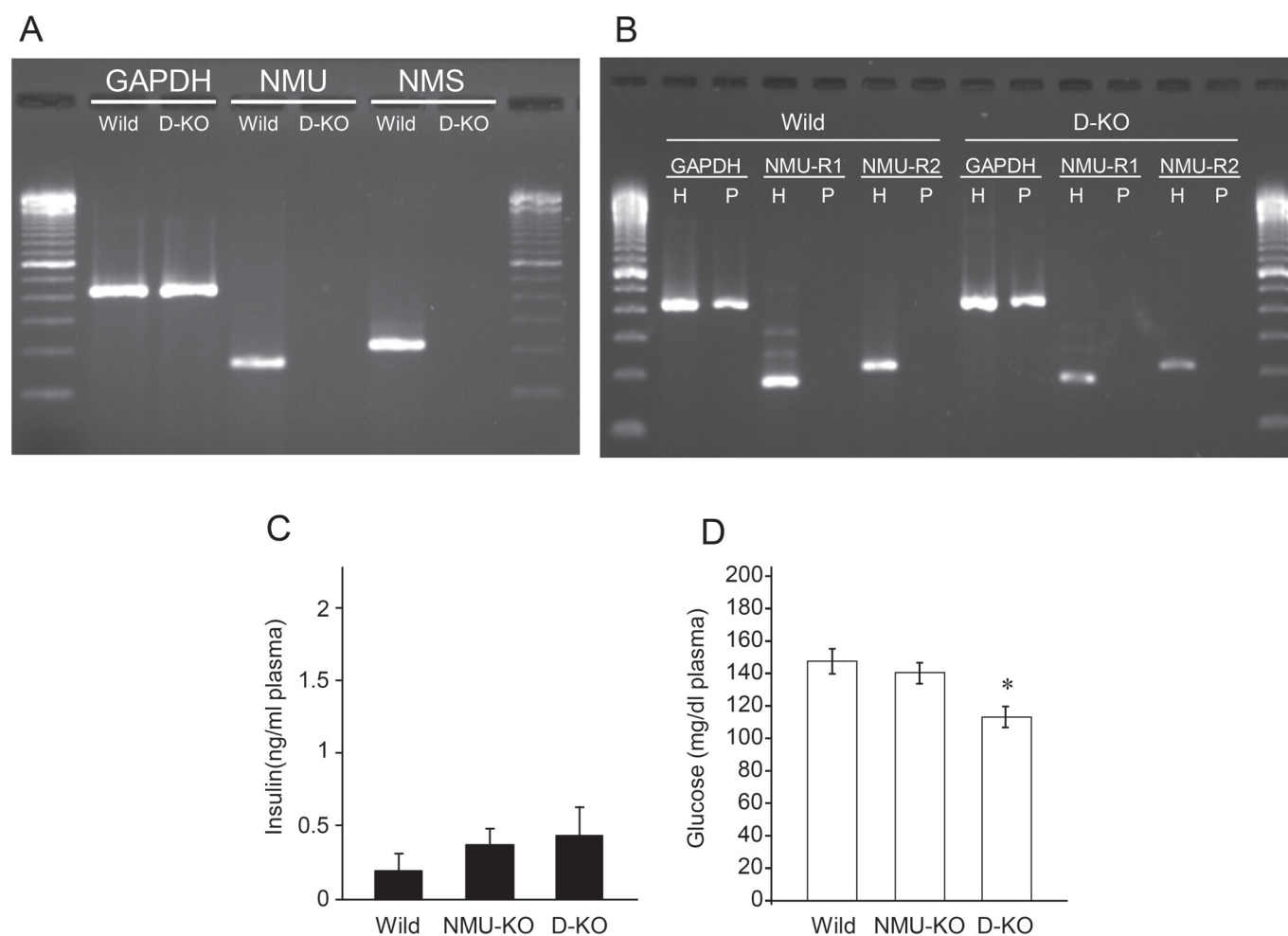


Fig. 1. (A) Confirmation of deletion of neuromedin U (NMU) and/or neuromedin S (NMS) mRNA expression in the hypothalamus of double-KO (D-KO) mice. (B) Expression of neuromedin U receptor 1 (NMU-R1) and neuromedin U receptor 2 (NMU-R2) mRNA in the hypothalamus and pancreas of wild-type and D-KO mice. “H” and “P” represent the hypothalamus and pancreas, respectively. (C), (D) Plasma levels of insulin and glucose in wild-type, NMU-KO and D-KO mice. Bars and vertical lines represent mean \pm SEM (n=5). Asterisks show significant differences (* P <0.05 vs wild-type).

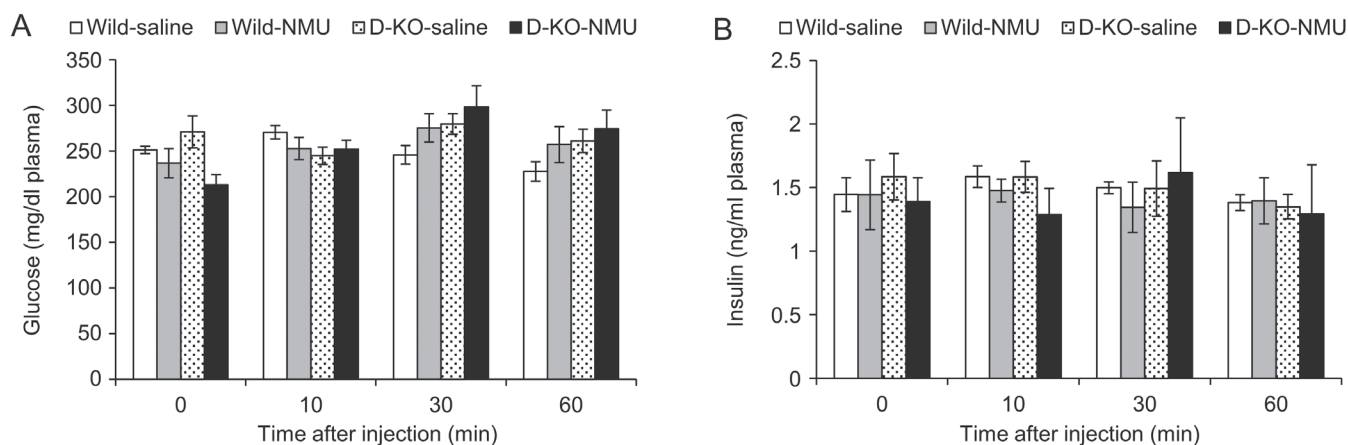


Fig. 2. Effect of peripheral injection of neuromedin U (NMU) on plasma glucose (A) and insulin (B) levels in wild-type and double-KO (D-KO) mice. Blood samples were collected just before, and at 10, 30 and 60 min after i.p. injection of saline or 40 nmol of NMU. Bars and vertical lines represent mean \pm SEM (n=5). There were no significant differences in plasma glucose and insulin levels between the groups.

10 and 30 min after glucose administration in wild-type and D-KO mice, and there was no significant difference in these levels between them (Fig. 3A and 3B).

Possible effect of NMU on the plasma insulin level under a high fat diet

Supply of a high fat diet for three weeks led to a significant increase in body weight. The body weight gain in D-KO mice was larger than that in wild-type mice by the third week (Fig. 4A). Under *ad libitum*-fed condition, the plasma leptin level after 3 weeks of high fat feeding was significantly increased in both wild-type and D-KO mice, and the level in D-KO mice was significantly higher than that in wild-type mice (Fig. 4B). There was no significant change in plasma glucose levels after the 3 weeks of high fat feeding in both wild-type and D-KO mice (Fig. 4C). On the other hand, at the same time point, both groups showed significantly higher levels of plasma insulin under *ad libitum*-fed condition (Fig. 4D).

The glucose tolerance test using high fat diet-fed mice was performed without fasting. In both wild-type and D-KO mice, plasma glucose levels were increased at 10 min after glucose injection, and then gradually declined thereafter (Fig. 4E). There was no significant inter-group difference in plasma glucose levels at 30 and 60 min after injection. Although wild-type mice showed significantly increased plasma insulin levels at 10 min after glucose injection, D-KO mice did not. There was a significant difference between wild-type and D-KO mice in plasma insulin levels before, and at 30 and 60 min after glucose injection (Fig. 4F).

Effect of NMU injection on the results of the glucose tolerance test

The previous experiments had shown that NMU did not participate in insulin secretion and glucose excursion. Therefore, we investigated the influence of a high dose of NMU on blood glucose and insulin levels. A single administration of NMU without fasting did not affect the blood glucose or insulin level (Fig. 5A and 5B), and no abnormal behavior was observed. The blood glucose level after pre-administration of NMU was significantly lower than that after pre-administration of saline (Fig. 5C). On the other hand, the blood insulin level after NMU administration was higher than that after saline administration, but not to a significant degree (Fig. 5D). Considering the short half-life of insulin, decapitation blood was obtained 5 min after glucose administration. As in the previous experiments, the blood glucose level after NMU injection was significantly lower than that after saline injection, whereas the blood insulin level tended to be higher, although not to a significant degree (Fig. 5E and 5F).

Comparison of response to the high-dose glucose tolerance test between wild-type and D-KO mice

The results of previous experiments using D-KO mice had produced no evidence to suggest that NMU is “decretin”. Considering that previous report of Zhang *et al.* [27], there is the possibility that NMU acts as a decretin only under high-glucose condition. Therefore, a high-capacity glucose tolerance test was carried out using D-KO mice that had been fasted for 16 hr. This showed that plasma glucose levels after glucose administration were significantly higher than in wild-type mice (Fig. 5G), whereas there was no significant difference in plasma insulin levels (Fig. 5H).

DISCUSSION

To clarify the involvement of NMU in insulin secretion and glucose homeostasis *in vivo*, we examined the difference in the plasma glucose level and insulin level between wild-type mice and D-KO mice. If NMU is, in fact, “decretin”, deletion of the NMU gene might result in higher plasma insulin levels than is the case in wild-type mice, or injection of NMU lead to suppression of plasma insulin level. However, we found that the fasting plasma level of insulin was not accelerated in D-KO mice. Peripheral

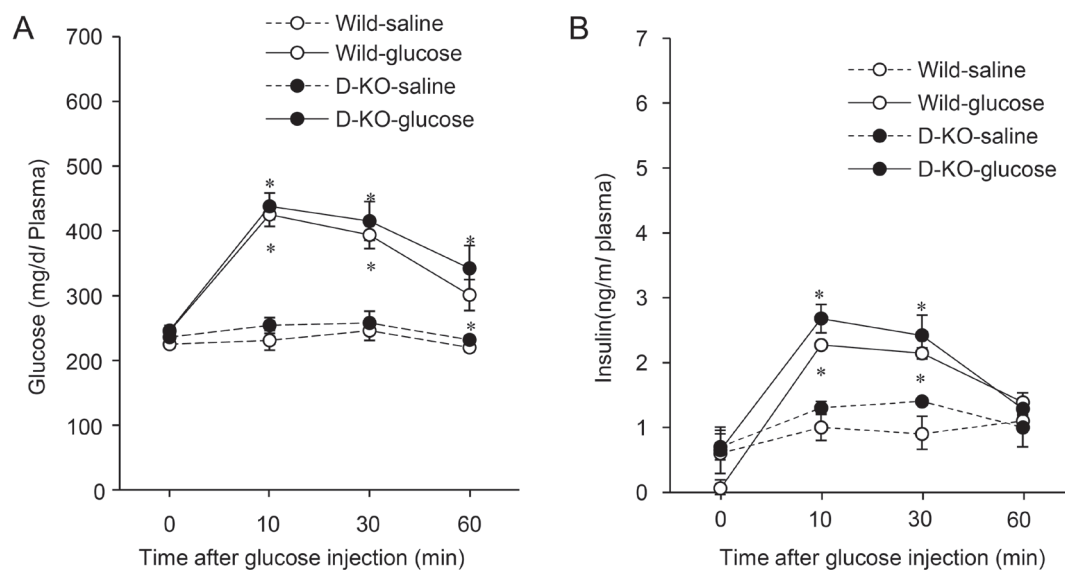


Fig. 3. Effect of peripheral injection of glucose on plasma glucose (A) and insulin (B) levels in wild-type and double-KO (D-KO) mice. The mice in each group received glucose at 1.5 mg/g body weight. Blood samples were collected just before, and at 10, 30 and 60 min after injection. Bars and vertical lines represent mean \pm SEM (n=5). Asterisks indicate significant differences from the levels before administration (* P <0.05). There was no significant difference in plasma glucose and insulin levels between wild-type and D-KO mice administered glucose or insulin.

injection of 40 nmol of NMU did not change the plasma insulin and glucose levels in wild-type and D-KO mice, whereas the previous report has shown that intraperitoneal administration of at least 4 nmol of NMU inhibited food intake sufficiently in mice [20]. Although the dose of NMU was increased to 100 nmol, intraperitoneal administration of high-dose-NMU had no significant effect on the plasma levels of glucose and insulin. There was no significant difference between wild-type and D-KO mice in the results of the glucose tests under normal diet conditions. Also in the glucose tolerance test, we injected 100 nmol of NMU at 5 min before glucose administration. Pre-administration of NMU resulted in a lower plasma glucose level at 10 min after glucose administration than pre-administration of saline. On the other hand, the blood level of insulin after NMU administration was high compared with that after saline administration, but not to a significant degree. These observations show that NMU does not have an inhibitory effect on insulin-releasing and may not be a decretin.

After 3 weeks of high fat feeding, mice fed high fat diet showed higher insulin levels than mice fed normal diet. Remarkably, D-KO/high-fat showed higher insulin levels than wild-type/high-fat. There is the possibility that the lack of NMU might have caused hyperinsulinemia under the high fat diet. It has been known that NMU in CNS stimulates the sympathetic nervous system [18, 24]. Furthermore, the sympathetic nervous system suppresses insulin secretion, whereas the parasympathetic nervous system enhances it [12]. Therefore, the decrease of sympathetic tone in D-KO mice might increase insulin secretion from the pancreas under high fat conditions. If so, then the higher level of plasma insulin in D-KO mice would represent an indirect effect of central NMU, and not a direct effect such as that proposed for a decretin.

It could be proposed that decretin is secreted during starvation, suppressing the secretion of insulin and preventing any excessive decrease in the plasma glucose level. If NMU is "decretin", in NMU-deficient mice after fasting, it should increase the blood level of insulin, leading to hypoglycemia. However, we found that the fasting plasma level of insulin was not increased in D-KO mice. Furthermore, the glucose tolerance test after a 16-hr fast demonstrated no significant difference in plasma insulin levels between wild-type and D-KO mice. Notably, the plasma glucose levels in D-KO mice were higher than those in wild-type mice. We hypothesize that NMU improved glucose tolerance, rather than the possibility of NMU being a decretin. Peier *et al.* reported that peripheral administration of NMU markedly improved glucose tolerance in mice with diet-induced obesity [20], and Kowalski *et al.* found that glucose tolerance was improved in transgenic mice overexpressing NMU after high fat feeding [11]. These previous results of glucose tolerance test were obtained after long-term high fat diet feeding, whereas the present study showed that the single administration of NMU improved glucose tolerance even under a normal diet. NMU and NMS deficiency worsened glucose tolerance after fasting in mice receiving a normal diet. In other words, our results indicate that NMU affects glucose homeostasis after glucose injection under a normal dietary condition, and that endogenous NMU is involved in glucose tolerance after fasting.

It has been reported that administration of NMU suppresses glucose-induced insulin secretion from human islets [1]. Similarly, it has been shown that NMU suppresses insulin secretion from rat isolated pancreatic islets [8, 9]. In addition, Kaczmarek *et al.* reported that NMU-R1 was expressed in rat pancreas islets [8]. These findings strongly support the hypothesis that NMU is indeed a decretin. However, we were unable to detect mRNA for either NMU-R1 or R2 in the pancreas, and peripheral injection of NMU did not decrease the plasma level of insulin. In the newest report of NMU, Kuhre *et al.* claims that, in the human and rat islets, NMU-R1 is not expressed and that NMU cannot stimulate the insulin secretion *in vivo* and *in vitro* [13]. Although we are

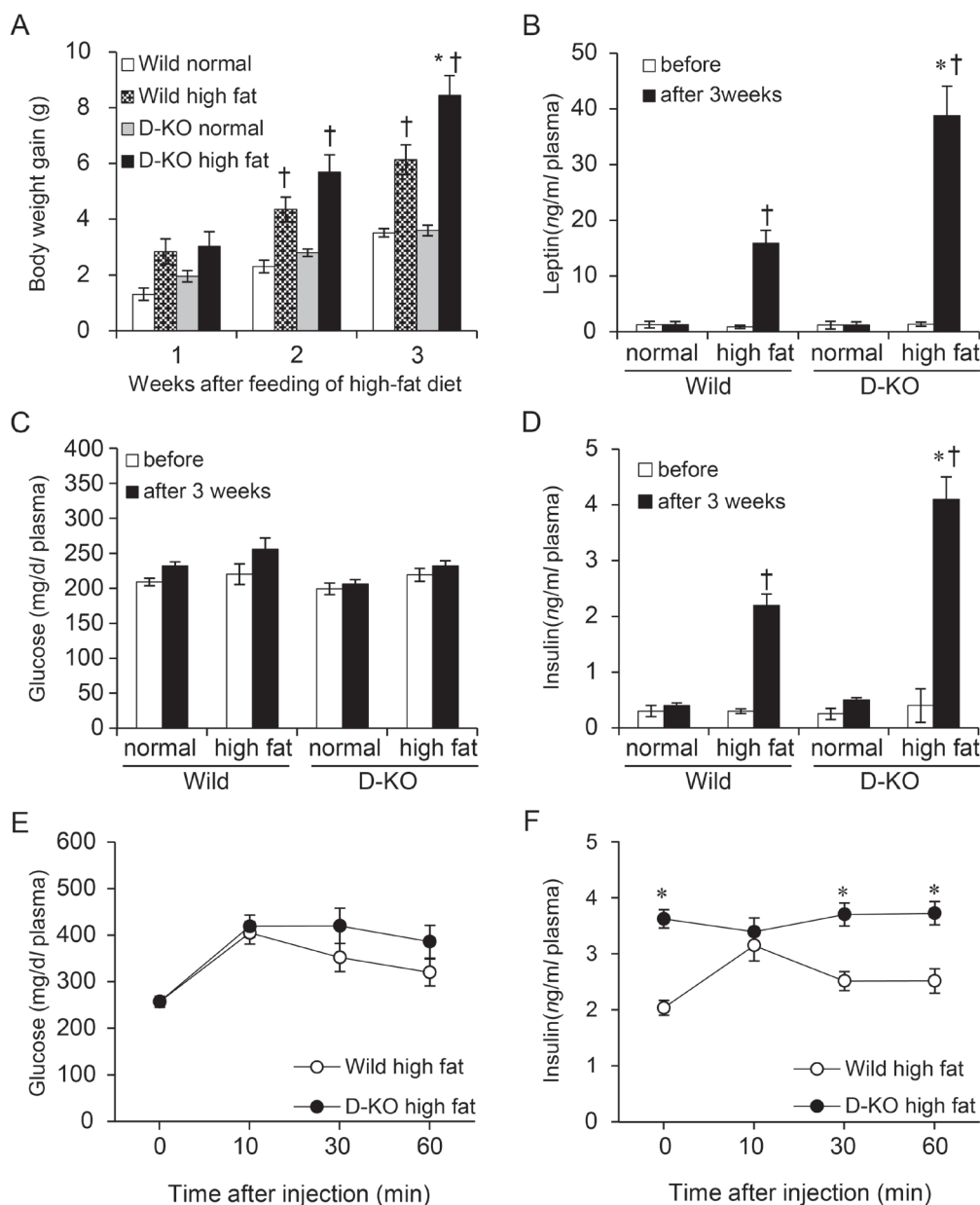


Fig. 4. Effect of a high-fat diet on body weight (A), plasma leptin level (B), plasma glucose level (C), and plasma insulin level (D). The mice were fed a normal or a high-fat diet for three weeks from 7 weeks of age. Plasma glucose, insulin and leptin levels were measured after 3 weeks on each diet. Effect of a peripheral injection of glucose on plasma levels of glucose (1.5 mg/g body weight) (E) and insulin (F) in mice fed the high-fat diet for 3 weeks. Blood samples were collected before, and at 10, 30 and 60 min after glucose injection. Asterisks show significant differences ($\dagger P < 0.05$ vs wild-type or double-KO (D-KO) mice supplied a normal diet, $*P < 0.05$ vs wild-type mice supplied the high-fat diet).

unable to explain the discrepancy between these findings, it is necessary to consider the possibility that NMU might suppress insulin secretion only when the pancreas is exposed to a high glucose concentration. Because administration of NMU has been reported to reduce insulin secretion under high-glucose conditions *in vitro* [1, 8, 9, 27]. If so, then the plasma insulin levels would have been lower in wild-type than in D-KO mice after glucose administration. Also in the glucose tolerance test, administration of NMU would have inhibited plasma insulin level. However, in plasma insulin level during the glucose tolerance test, there was no significant difference between the two types of mice, and between saline-injected and NMU-injected group. Thus, our data supports the report of Kuhre *et al.*, not only in human and rat, but also in mice.

In this study, there was no difference in insulin level between wild-type mice and NMU-, NMS-deficient mice. In high glucose tolerance test, we could not find any difference in insulin level between wild-type and D-KO. In the normal condition and high glucose level condition, peripheral administration of NMU could not suppress plasma insulin level. As a result of this study, we concluded that NMU is not a incretin, at least *in vivo*.

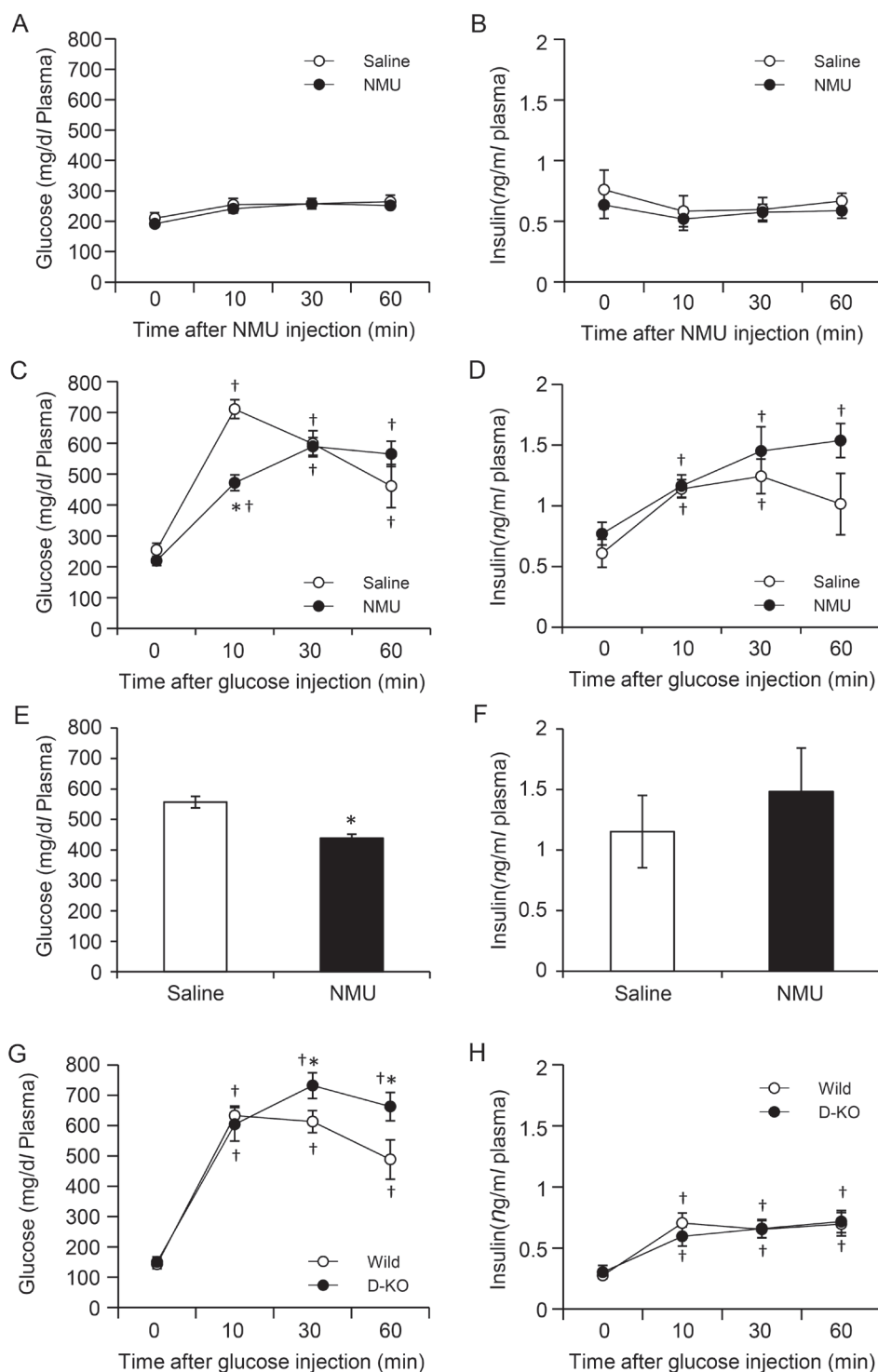


Fig. 5. Effect of peripheral injection of 100 nmol neuromedin U (NMU) on plasma glucose (A) and insulin (B) levels in wild-type mice. Blood samples were collected just before, and at 10, 30 and 60 min after i.p. injection of saline or 100 nmol NMU. Effect of peripheral injection of 100 nmol NMU in the glucose tolerance test (C–F). Blood samples were collected by the tail tip method just before, and at 10, 30 and 60 min after i.p. injection of glucose (C, D) or by decapitation 5 min after i.p. injection of glucose (E, F). After sampling, plasma glucose levels (C, E) and plasma insulin levels were measured. Effect of a peripheral injection of glucose (3 mg/g body wt) on plasma levels of glucose (E) and insulin (F) in mice after fasting for 16 hr. Blood samples were collected before, and at 10, 30 and 60 min after glucose injection. Asterisks show significant differences ($*P < 0.05$ vs saline or wild-type mice, $†P < 0.05$ vs the levels before administration).

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