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The novel function of nesfatin-1: Anti-hyperglycemia

Yijing Su, Jing Zhang, Yanchun Tang, Feng Bi, Jian-Ning Liu *

Institute of Molecular & Experimental Therapeutics, East China Normal University, Shanghai 200062, China

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ABSTRACT

Nesfatin-1 is recently reported as a satiety molecule to suppress food intake via the melanocortin signaling in hypothalamus when injected centrally and peripherally. Here we report that nesfatin-1 is also anti-hyperglycemic. It was found that the intravenous injection of nesfatin-1 significantly reduced blood glucose in hyperglycemic *db/db* mice. This anti-hyperglycemic effect of nesfatin-1 was time-, dose-, insulin-dependent and peripheral.

Introduction

Diabetes is increasing at an alarming rate worldwide even in the developing countries. The hyperglycemia in diabetic patients damages blood vessels, nerves, eyes and the kidneys, which finally causes severe cardiovascular diseases, neuropathy, blindness and renal failure. Therefore, the control of blood glucose is the key battle ground to fight for diabetes.

For the type-1 diabetic patients who lack insulin, the administration of insulin before food intake prevents hyperglycemia. For most diabetic patients classified as type-2 diabetes in which either the body does not produce enough insulin or the cells ignore insulin. Elevated levels of blood glucose are considered to be responsible for excess complications causing morbidity and mortality. Many drugs have been developed to control the blood glucose in type-2 diabetes, and include (1) sulphonylures, which increase insulin release from pancreatic islets; (2) metformin, which acts to reduce hepatic glucose production; (3) glitazones, which are peroxisome proliferator-activated receptor-γ (PPAR-γ) agonists and sensitize the insulin receptor downstream signaling; (4) α-glucosidase inhibitors, which interfere with gut glucose absorption; (5) incretins, which are agonists for GLP-1 receptor and promote insulin secretion; (6) DPP-IV inhibitors, which suppress the degradation of endogenous GLP-1 and enhance insulin secretion; and finally insulin itself, which suppresses glucose production and augments glucose utilization [1]. However, the efficacy of these medicines is yet limited, since the complicated mechanisms of type-2 diabetes are not fully revealed.

Nesfatin-1 has been recently identified as an anorexigenic factor associated with melanocortin signaling in hypothalamus. It is an 82 amino-acid NUCB2 peptide derived based on the putative proteolytic site of pro-hormone convertases [2]. The intracerebroventricular (i.c.v.) [2] or intraperitoneal (i.p.) [3] injection of nesfatin-1 inhibits food intake and thereby reduces body weight. Additionally, it may have a possible role in the response to stress [4]. As a satiety molecule, nesfatin-1 has not been previously reported to have any effects on carbohydrate metabolism. Since NUCB2 mRNA was found to be expressed in pancreatic beta cells [5], we postulated that nesfatin-1 could be anti-hyperglycemic. To test the assumption, recombinant nesfatin-1 was expressed and purified from genetically engineered *Escherichia coli*, and intravenously (i.v.) injected to hyperglycemic *db/db* mice.

Materials and methods

Recombinant nesfatin-1 (rNesfatin-1) was produced in *E. coli* using the pET28a expression vector and further purified using preparative C18 reverse phase HPLC. The purity of rNesfatin-1 was more than 99% as detected by an analytic C18 reverse phase HPLC (Fig. 1).

Animal care. Lepr$^{+/-}$ mice in C57BLKS/J were purchased from Jackson Laboratory (Bar Harbor, ME) and raised in our laboratory. All animals were kept under specific pathogen-free conditions with lab chow available *ad libitum* in a 12-h light/dark cycle. All procedures in animal experiments were in accordance with US National Institutes of Health animal care guidelines and were conducted with the approval from the Animal Study Committee of our institute.

Intracerebroventricular (i.c.v.) injection. The animals were pretreated to install an indwelling needle, and allowed a washout period of at least 1 week before the procedure of i.c.v. injection.

* Corresponding author. Fax: +86 512 65352671.
E-mail address: jianningliu2000@yahoo.com (J.-N. Liu).

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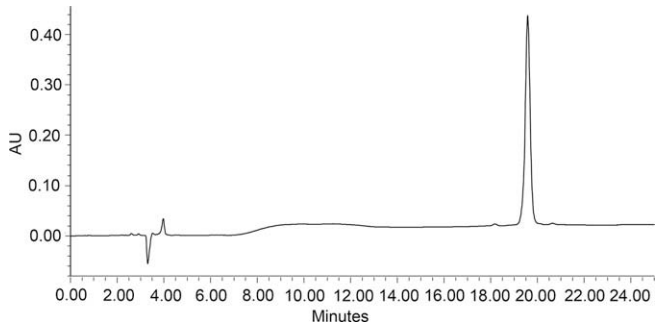

Fig. 1. The purity of rNesfatin-1 was >99% as detected by an analytic C18 reverse phase HPLC.

Without anesthesia, rNesfatin-1 (25 pmol) was infused into the third ventricle of the brain with a total volume of 5 μL over 5 min. The experiments were carried out at the beginning of the dark cycle (18:00 h) with food and water available freely. Food intake was measured at 3 h after the i.c.v. injection.

Intravenous (i.v.) or intraperitoneal (i.p.) injection. Without anesthesia, mice were placed into a restricting tube. rNesfatin-1 was i.v. injected via mice tail vein with a total volume of 150 μL. Mice were then returned to the cages with food and water available freely. In the i.p. injection experiment, mice without anesthesia were injected with rNesfatin-1 directly into the peritoneal with a total volume of 200 μL.

Streptozotocin (STZ) induced type-1 diabetic mice. Male C57BL/6J mice (10 weeks) were i.p. injected with 100 μg STZ per gram per day in 100 mmol/L sodium citrate (pH 4.5) on two consecutive days. Blood glucose was measured by tail vein sampling using the glucose oxidase enzymatic test. When the fasting blood glucose reading was over 16 mmol/L after STZ injection, the mice were considered to be type-1 diabetic. If its blood glucose levels exceeded 30 mmol/L, the diabetic mouse was given 16 ng of porcine insulin (Wangbang, Xuzhou, CN) immediately to prevent the blood glucose being dangerous. rNesfatin-1 (10 nmol per mouse) was i.v. injected either alone or combined with subcutaneous (s.c.) insulin (2 ng/mouse) to STZ-induced type-1 diabetic mice.

Statistical analysis. Data were presented as means ± SEM as indicated in figure legends. All data were representative of at least two different experiments. Comparisons between individual data points were made using a two-tailed Student's *t*-test. Differences were considered statistically significant when *p* value was less than 0.05.

Results

The intravenous administration of rNesfatin-1 time- and dose-dependently reduced blood glucose in hyperglycemic db/db mice

Freely fed hyperglycemic *db/db* mice with blood glucose higher than 25 mmol/L were selected. The i.v. administration of 10 nmol rNesfatin-1 significantly reduced blood glucose in freely fed *db/db* (Fig. 2A). This anti-hyperglycemic effect was shown to be time- and dose-dependent (Fig. 3). It was worthy to be noted that although the half-life of nesfatin-1 was 9–10 min in circulation [6,7], its *in vivo* anti-hyperglycemic effect was found to last more than 6 h, suggesting that the action of nesfatin-1 may involve in an enduring intracellular mechanism.

On the contrary, i.v. administration of rNesfatin-1 did not significantly reduce blood glucose in non-hyperglycemic mice including fasting *db/db* mice and free fed wild-type mice (Fig. 2B). Additionally, i.v. administration of rNesfatin-1 did not significantly affect blood levels of insulin in mice including hyperglycemic *db/db* (data not shown). The long-term effect of i.v. administration of rNesfatin-1 on the blood level of insulin has not been investigated in the current study.

Interestingly, i.p. administration of rNesfatin-1 (up to 1 μmol) was found incapable to affect blood glucose in hyperglycemic *db/db* mice (Fig. 2C).

The anorexigenic effect of rNesfatin-1 was not responsible for the anti-hyperglycemic effect

Since nesfatin-1 was previously identified as an anorexigenic factor, we had to further clarify whether the anorexigenic effect of nesfatin-1 would be responsible for the anti-hyperglycemic effect. The intracerebroventricular (i.c.v.) injection of rNesfatin-1 (25 pmol) to *db/db* mice significantly inhibited food intake (Fig. 2D) without any

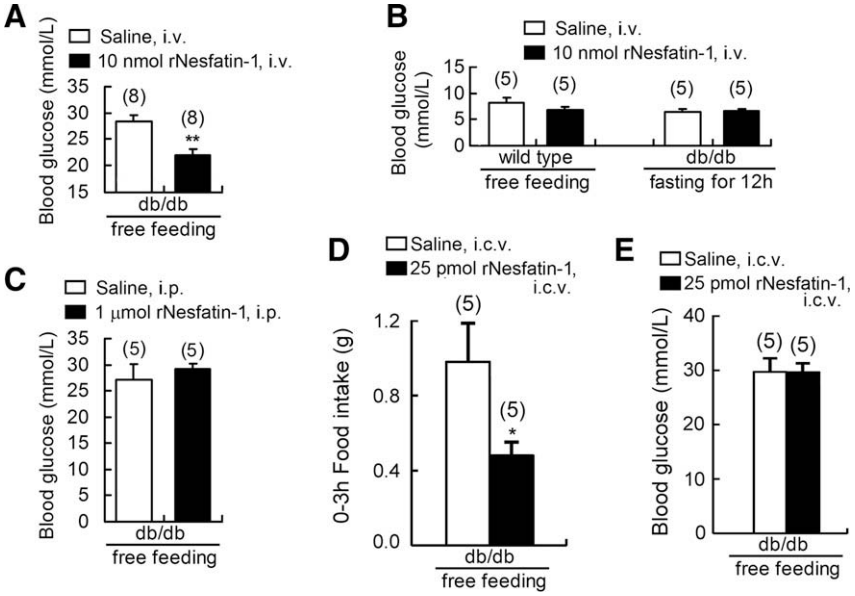

Fig. 2. Blood glucose at 3 h after i.v. injection of rNesfatin-1 in (A) *db/db* mice, (B) wt B6 mice and fasting *db/db* mice. (C) Blood glucose at 3 h after i.p. injection of rNesfatin-1 in *db/db* mice. (D) Food intake within 3 h and (E) blood glucose at 3 h after i.c.v. injection of rNesfatin-1 in *db/db* mice. Data represented the mean ± SEM (*$p < 0.05$; **$p < 0.01$). Number of mice used is showed within parentheses.

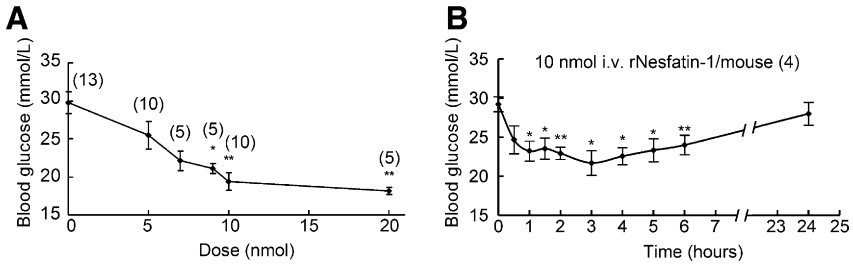

Fig. 3. The anti-hyperglycemic effect of i.v. rNesfatin-1 was dose- (A) and time- (B) dependent in *db/db*. The blood glucose was measured at 3 h after i.v. injection of rNesfatin-1 in the dose-dependent experiment. Data represented the mean ± SEM (*$p < 0.05$; **$p < 0.01$). Number of mice used is showed within parentheses.

effects on the high levels of blood glucose (Fig. 2E). It suggested that the anti-hyperglycemic effect was peripheral rather than neurological, and unrelated to the anorexigenic effect of nesfatin-1.

The anti-hyperglycemic effect of rNesfatin-1 was insulin-dependent

Based on the observation that rNesfatin-1 did not reduce blood glucose in non-hyperglycemic animals, we hypothesized that the anti-hyperglycemic effect of rNesfatin-1 would be insulin-dependent. Indeed, in the streptozotocin-induced type-1 diabetic mice which were unable to produce insulin, blood glucose was not reduced by i.v. injection of rNesfatin-1 (10 nmol per mouse). Conversely, with co-administration of s.c. insulin (2 ng per mouse), i.v. rNesfatin-1 showed a greater and longer anti-hyperglycemic effect than s.c. insulin alone (Fig. 4A).

The anti-hyperglycemic effect of rNesfatin-1 in vivo was abolished by GW9662 or Compound C but not Rosiglitazone

The anti-hyperglycemic effect of rNesfatin-1 in *db/db* mice was abolished by the pre-injection of the PPAR-γ antagonist, GW9662 or the AMPK inhibitor, Compound C, but not the PPAR-γ agonist, Rosiglitazone (Fig. 4B). It suggested that the anti-hyperglycemic effect of rNesfatin-1 was associated with the signaling pathways of insulin.

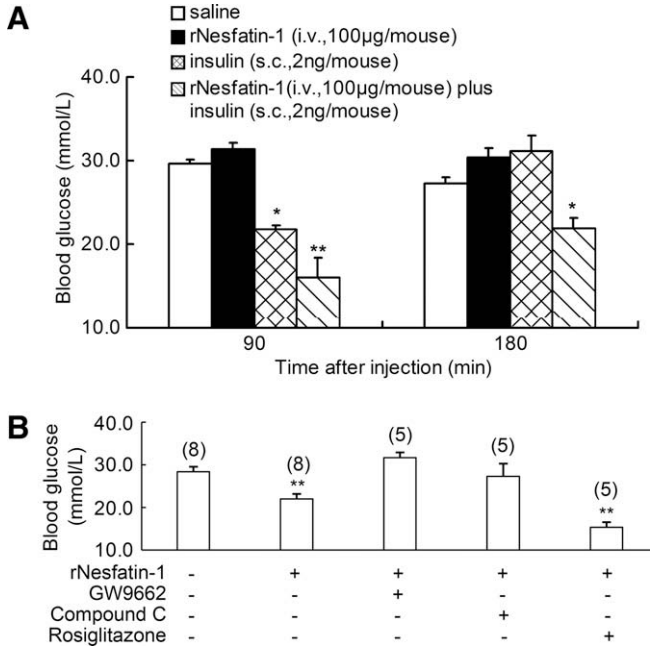

Fig. 4. (A) The effect of rNesfatin-1 on the streptozotocin-induced type-1 diabetic C57BL/6J mice, four males per group. Data represented the mean ± SEM. Number of mice used is showed within parentheses. (B) Blood glucose of *db/db* mice at 3 h after treatment with 10 nmol rNesfatin-1 per mouse, 0.45 μg GW9662, 20 μg Compound C or 10 μg Rosiglitazone per gram of mice. Data represented the mean ± SEM (*$p < 0.05$; **$p < 0.01$). Number of mice used is showed within parentheses.

Discussion

Nesfatin-1 was originally identified as a satiety molecule in the hypothalamus. We now find that it has an anti-hyperglycemic effect when given intravenously to the hyperglycemic animals. This might be overlooked in the previous reports, since i.p. administration and non-hyperglycemic animals were then employed [2,3].

Nesfatin-1 is an 82 amino-acid peptide of NUCB2, a multi-function EF-hand motif containing Ca^{2+}-binding protein [8]. Comparing with all the other anti-hyperglycemic agents, nesfatin-1 probably represents a new class of insulin helpers. Its action was dose-, time- and insulin-dependent. In the hyperglycemic *db/db* mice which mimic type-2 diabetes, a bolus i.v. injection of 10 nmol rNesfatin-1 maintains significantly lower levels of blood glucose for 6 h without any additional administration of insulin. Although nesfatin-1 affects food intake centrally, in the present study we have demonstrated that its anti-hyperglycemic effect is simply peripheral. The i.c.v. injection of rNesfatin-1 to *db/db* mice significantly reduced food intake but not the high levels of blood glucose.

The half-life of nesfatin-1 was reported to be 9–10 min [6,7]. However, we have found that its anti-hyperglycemic effect apparently lasted for hours in the hyperglycemic mice (*db/db*), suggesting that its intracellular effect would be lasting. Although the intracellular mechanism of anti-hyperglycemic nesfatin-1 is yet unknown, it is concluded to interact with the signaling pathways of insulin. In an *in vivo* experiment, we have found that the anti-hyperglycemic effect of rNesfatin-1 was prevented by the PPAR-γ antagonist (GW9662) and the AMPK inhibitor (Compound C), two well-known elements of insulin signaling.

Together, the anorexigenic and anti-hyperglycemic effects of nesfatin-1 importantly affect both food intake and glucose metabolism, implicating its significant roles in the metabolic control of the body. It is warranted that further studies on nesfatin-1 would be valuable to the treatment of metabolic diseases especially for type-2 diabetes and obesity.

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