Nesfatin-1 crosses the blood–brain barrier without saturation

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1. Introduction

Nesfatin-1 is a peptide corresponding to amino acids 1–82 of the larger protein nucleobindin-2 (NUCB2, or DNA binding/EF-hand/acidic protein NEFA) that shows reduced expression in the paraventricular nucleus of the hypothalamus after fasting, and acts through melanocortin signaling to exert satiety effects in rats [4]. After intracerebroventricular injection in rats, nesfatin-1 decreases food intake in a time- and dose-dependent manner and slows weight gain. Although the subtractive cloning strategy used in the initial discovery of NUCB2 and nesfatin-1 in the brain indicates its production by adipocytes, the influence of peripherally produced nesfatin-1 on the neuroendocrine changes of the feeding circuitry is not clear.

Apart from physiological regulation of the production and actions of nesfatin-1, it is not known whether it is feasible to deliver nesfatin-1 by the peripheral route to its hypothalamic targets as a potential therapeutic agent. Thus, the issue focuses around the blood–brain barrier (BBB) which separates the paraventricular nucleus, as well as other parts of the hypothalamus and the whole brain, from the peripheral circulation [2]. The specialized endothelial cells, pericytes, astrocytic endfeet, tight junctions, and continuous basement membranes form a functional unit that limits the nonselective permeation of peptidergic molecules across the BBB.

At this level, peptides and proteins show diverse modes of interaction with the cerebral vasculature based on their physiochemical properties and the level of expression of specific binding sites and transport systems [1,3,6,10,11]. In this report, we show that nesfatin-1 is relatively stable in circulating blood and characterize its unique interactions with the BBB in mice.

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2. Materials and methods

2.1. Animals and iodination

CD1 male mice 5–6 weeks of age were used following the protocol approved by the Institutional Animal Care and Use Committee. The mice were studied after anesthesia induced by intraperitoneal injection of ethyl carbamate. Recombinant rat nesfatin-1 (1–82 aa) was purchased from Phoenix Pharmaceuticals (Burlingame, CA). Bovine serum albumin (BSA) and chloramine-T radioactive labeling (radiolabeling) reagents were obtained from Sigma (St. Louis, MO). Nesfatin-1 was radiolabeled with 125I (Amersham, Piscataway, NJ) by use of chloramine-T. The reaction was stopped at 1 min by addition of sodium metabisulfite, and the mixture was purified on columns of Sephadex G-10. The specific activity of 125I-nesfatin-1 was about 80 Ci/g.

2.2. Degradation assays by acid precipitation and size-exclusion chromatography

For acid precipitation, four groups of mice were studied, with two mice at each time point (10, 20, 30, and 60 min). For size-exclusion chromatography, six mice each were studied, with supernatant from brain homogenates of two mice pooled for each time point (0, 10, and 20 min). The 0 time point was the processing control with 125I-nesfatin-1 added to the homogenizer and blood collecting tubes; this assessed the extent of degradation ex vivo. Each mouse received an intravenous (iv) injection of 3 μCi of 125I-nesfatin-1 in 100 μl of injection at time 0. At designated times (10, 20, 30, or 60 min), arterial blood and brain were obtained and processed on ice. The brain was homogenized in 1 ml of lactated Ringer’s solution containing 1% BSA (LR/BSA) in the presence of Complete Protease Inhibitor Cocktail (Sigma). The brain supernatant was used for acid precipitation. About 30,000 cpm of serum from the 10 and 20 min samples, along with a sample of the stock solution, were precipitated by 15% trichloroacetic acid. For size-exclusion chromatography, a polyacrylamide P10 column (9 cm × 6 cm) was used. The sample volume was 100 μl, with brain supernatant lyophilized to the final volume. Forty-five fractions of 100 μl each were collected, as previously described [12].

2.3. Kinetics of nesfatin-1 transfer from blood to brain

Multiple-time regression analysis was used to measure how fast and how much radiotracer entered the brain from blood [1]. To determine whether there was a saturable transport system, a group of eight mice receiving 1 μg/mouse excess of unlabeled nesfatin-1 were studied along with the control group of nine mice which received only 125I-nesfatin-1. The injection solution was LR/BSA which was prepared fresh each time. An iv injection of 100 μl LR/BSA containing 20,000 cpm/μl 125I-nesfatin-1 was delivered as a bolus into the left jugular vein at time 0. At 1, 2, 3, 5, 7, 10, 15, 17, or 20 min (each mouse representing one time point), blood was collected by dissection of the right carotid artery, and the mouse was decapitated immediately afterwards. The radioactivity in the whole brain and 50 μl of serum was measured, and the brain/serum ratio of 125I-nesfatin-1 in each gram (g) of brain was calculated separately. For comparison and measurement of peripheral tissue uptake, liver and kidney were also sampled.

Based on the exponential decay pattern of serum radioactivity, the exposure time was calculated as the integral of serum radioactivity over time divided by the serum radioactivity at each experimental time. It represents the theoretical value correlated with each experimental time if the blood concentration of 125I-nesfatin-1 was constant from time 0 to time t. The linear regression correlation between the tissue/serum ratio and exposure time was determined with Prism GraphPad Statistical Software (San Diego, CA). The unidirectional influx rate Ki was determined from the slope of the linear regression line, and the initial volume of distribution Vi was determined from the intercept. Differences of the regression lines between the two groups were compared by the least square method with the GraphPad program.

2.4. Capillary depletion

Four groups of mice (n = 4/group) were studied. The groups consisted of mice receiving 125I-nesfatin-1 in LR/BSA buffer with or without cardiac perfusion to wash out residual radioactivity in the cerebral vasculature. Half of the mice also received excess unlabeled nesfatin-1 (2 μg/mouse) in addition to the radiotracers. At the end of the study (10 min after iv injection), blood and brain were collected. The cerebral cortex was homogenized in capillary buffer and mixed thoroughly with 26% dextran to yield a final concentration of about 18% dextran. The mixture was centrifuged at 9000 × g for 15 min at 4°C with a swing bucket rotor to achieve effective separation of brain parenchyma from the capillaries. Three well-formed layers could be observed: a lipid layer on top, a capillary ring at the bottom, and clear solution in the middle. After measurement in a γ-counter, the ratios of tissue/serum radioactivity for 125I-nesfatin-1 were calculated and expressed per g of cerebral cortex. Group means are presented with their standard errors, significant differences being determined by analysis of variance followed by Tukey’s post hoc test.

2.5. In situ brain perfusion

Two groups of CD1 mice (n = 5/group) were anesthetized with ethyl carbamate and the brain was perfused intracardially as described previously [7]. The radiotracer concentrations were 1000 cpm/μl each for 125I-nesfatin-1 and 131I-albumin. The radiotracers were perfused for 5 min, with an additional 2 min of prewash and 1 min of postwash with the same physiological perfusion buffer at 2 ml/min driven by a syringe perfusion pump (Harvard Apparatus, Holliston, MA). At the end of the study, brain and hypothalamus were dissected and weighed. The radioactivity of brain tissue and 50 μl of perfusate was measured for both 125I and 131I, and the ratio of brain/perfusate was calculated for each group. The values of 131I-albumin were subtracted from those of 125I-nesfatin-1 to correct for vascular space. Group means were compared by one-way analysis of variance.
2.6 Efflux transport assay

Two groups of mice (n = 8/group) were studied. One group received radiotracers only and the other group received an additional 1000-fold excess of unlabeled nesfatin-1. 125I-nesfatin-1 and 131I-albumin (20,000 cpm each) were delivered to the right lateral ventricle by a perfusion pump with a 5 μl Hamilton syringe connected to the PE10 cannula. The coordinates were 2.5 mm lateral and 0.2 mm posterior to the bregma and 2.5 mm below the skull. The perfusion rate was 12 μl/min and the duration of delivery was 5 s; the cannula remained in place for another 5 s with slow withdrawal. The mice were sacrificed at 2, 5, 10, 20, 30, 45, and 60 min afterwards. A control time point (time 0) was also included to assess diffusion of radiotracers independent of cerebrospinal fluid dynamics, and this involved euthanasia of the mice by an overdose of ethyl carbamate before the radioisotope injection. The mice were decapitated immediately afterwards. The half-time disappearance of 125I-nesfatin-1 and 131I-albumin was determined from the linear regression correlation between brain radioactivity and time. Significant changes between the two groups were determined with the Prism GraphPad program.

3. Results

3.1 Stability of 125I-nesfatin-1 after iv injection into mouse

There was no significant decrease of the acid precipitable radioactivity within 10 min after iv injection of 125I-nesfatin-1. At this time, more than 96% of the total radioactivity in both serum and the supernatant of brain homogenate was intact. There was a significant decrease of the percent precipitated at 20 min (Fig. 1). Size-exclusion chromatography further showed that the reduction of acid precipitable radioactivity was gradual and that the majority of 125I-nesfatin-1 remained intact in both serum and the supernatant of brain homogenate. The results indicate that a significant amount of nesfatin-1 can reach the brain after a single bolus injection. With the dose used to test the potential saturability (1 mg/mouse, about 67-fold excess), excess unlabeled nesfatin-1 did not significantly inhibit the influx of 125I-nesfatin-1 (Fig. 3A).

Fig. 1 – Degradation of 125I-nesfatin-1 in blood and brain at different time points 0–20 min after iv injection. Significant reduction of acid precipitability was only seen at 20 min, but not earlier time points. ***p < 0.005 compared with the stock solution and processing controls.

Fig. 2 – Intact 125I-nesfatin-1 was shown by polyacrylamide size-exclusion chromatography in serum (A) and brain (B) samples. Most of the radioactivity in samples obtained from mice that received 125I-nesfatin-1 10 or 20 min earlier eluted at the same fraction as the intact control.

3.2 Kinetics of BBB influx of 125I-nesfatin-1 in mice

The blood-to-brain influx of 125I-nesfatin-1 was linear during the study period (1–20 min after iv injection in mice). As shown in Fig. 3, the influx rate was 0.27 ± 0.11 μl/g-min and the initial volume of distribution was 14.22 μl/g. Of the total amount injected iv, the percent of brain uptake was about 0.3%/g of brain at 20 min. Given the specific activity of 80 Ci/g of 125I-nesfatin-1 and the injection amount of 1.2 μCi, about 45 pg of 125I-nesfatin-1 was present in a g of brain tissue. The results indicate that a significant amount of nesfatin-1 can reach the brain after a single bolus injection. With the dose used to test the potential saturability (1 μg/mouse, about 67-fold excess), excess unlabeled nesfatin-1 did not significantly inhibit the influx of 125I-nesfatin-1 (Fig. 3A).
3.3. Penetration of nesfatin-1 across the BBB by in situ brain perfusion

To avoid interference by serum binding proteins and degradative enzymes, nesfatin-1 was delivered intracardially by in situ brain perfusion. After 5 min of perfusion, the uptake of $^{125}$I-nesfatin-1 by the brain was higher than that of $^{131}$I-albumin. However, the uptake was not inhibited by inclusion of excess unlabeled nesfatin-1. Fig. 4 shows the uptake of nesfatin-1 by the total brain and hypothalamus after correction of vascular space by subtraction of the corresponding albumin values.

3.4. Uptake of $^{125}$I-nesfatin-1 by peripheral organs

$^{125}$I-Nesfatin-1 also showed rapid influx into liver and kidney (Fig. 5A and B). The values were significantly higher than those of co-injected $^{131}$I-albumin. There was no effect of excess unlabeled nesfatin-1 on the influx rate in either organ.
3.5. Brain parenchymal uptake of $^{125}$I-nesfatin-1

Capillary depletion studies were performed on mice 10 min after iv injection of $^{125}$I-nesfatin-1. At this time, the total amount of $^{125}$I-nesfatin-1 in the brain compartments was $8.74 \pm 0.80 \, \mu g/g$. Of this, more than 68% ($5.98 \pm 0.71 \, \mu g/g$) was already present in brain parenchyma. The capillary uptake was $1.20 \pm 0.30 \, \mu g/g$. This was about 14% of the total apparent uptake by the brain. The reversible binding, representing the amount of $^{125}$I-nesfatin-1 trapped in the vascular space, was negligible (less than 2%) (Fig. 6).

3.6. Efflux of $^{125}$I-nesfatin-1 from brain to blood

The efflux rates of $^{125}$I-nesfatin-1 and $^{131}$I-albumin were not significantly different. The half-time disappearance of $^{125}$I-nesfatin-1 and $^{131}$I-albumin was 23.8 and 22.9 min, respectively. Inclusion of excess unlabeled nesfatin-1 did not cause significant changes for either substance (Fig. 7). Thus, nesfatin-1 exits the brain by bulk flow and reabsorption of cerebrospinal fluid.

4. Discussion

We showed that nesfatin-1 was relatively stable in the circulation and had a moderate influx from blood to the brain compared with other peptides of similar size [5,8,9,13]. The influx rate into liver was relatively slow in comparison with other substances we have studied previously (unpublished observations), indicating that hepatic degradation does not constitute the main pathway. There was, however, a significant influx of nesfatin-1 into the kidney where it is probably excreted. In all three organs studied (brain, liver, and kidney), excess unlabeled nesfatin-1 failed to compete with $^{125}$I-nesfatin-1 and reduce its influx rate. This was shown not only by iv delivery and multiple-time regression analyses, but also by in situ brain perfusion.

The uptake of nesfatin-1 by the hypothalamus was greater than that by the total brain, suggesting that binding sites for nesfatin-1 are in the hypothalamus. Nonetheless, the permeation of nesfatin-1 from blood to brain was not mediated by a saturable transport system. The influx of nesfatin-1 by simple diffusion obviates the necessity for pharmacological manipulation at the BBB level to enhance its delivery. The lack of saturability indicates that nesfatin-1 can be delivered by the peripheral route in large quantities, assuming the absence of systemic toxicity.

When delivered by intracerebroventricular injection, nesfatin-1 had a relatively long half-life in exiting the brain. Its efflux was mediated by bulk flow and reabsorption of cerebrospinal fluid, without accelerated transport. In this sense, the BBB serves as a restrictive permeability barrier to nesfatin-1 in both brain-to-blood and blood-to-brain directions.

Thus, nesfatin-1 is relatively stable in blood for the first 20 min after injection, and crosses the BBB by simple diffusion. There was no saturable efflux or influx transport system. Delivery of nesfatin-1 from the peripheral route, therefore, is feasible within the limits imposed by any possible systemic toxicity.

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References


