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Review

Nesfatin-1: An Overview and Future Clinical Application

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Abstract. Nesfatin/nucleobindin 2 (NUCB2) is expressed in the appetite-control hypothalamic nuclei and brainstem nuclei. Nesfatin/NUCB2 expression in the paraventricular nucleus of the hypothalamus was modulated by starvation and refeeding. Intracerebroventricular administration of nesfatin-1 dose-dependently inhibited food intake for 6 hours in male Wistar and leptin resistant, Zucker fatty rats. Intraperitoneal administration of nesfatin-1 and its mid-segment (M30) dose-dependently inhibited food intake for 3 hours in male ICR mice. Intraperitoneal administration of M30 also decreased food intake in leptin-resistant, genetically obese (*ob/ob*), diabetic (*db/db*) mice and mice fed a 45% high fat diet for 28 days. Intraperitoneal administration of M30 increased proopiomelanocortin and cocaine- and amphetamine- related peptide mRNA expression in the nucleus of the solitary tract of mice. In addition, intranasal administration of nesfatin-1 significantly inhibited food intake for 6 hours in male Wistar rats. We summarize recent observations about nesfatin-1, and attempt to present future direction of nesfatin-1 research for developing a new anti-obesity treatment.

Key words: Nesfatin-1, Leptin, Food intake, Body weight, Obesity

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WE have proposed the concept of brain-adipose axis that endogenous molecule, expressed in both hypothalamus and adipose tissue, exists in the general circulation, and is involved in the regulation of both feeding behavior, and the determination of size and/or number of adipocytes [1]. Troglitazone, peroxisome proliferator-activated receptor-γ (PPARγ) agonist, might modify the satiety in type 2 diabetic patients [2], and we attempted to identify a new protein regulated by PPARγ, which modulates feeding behavior. By using a subtraction-cloning assay, we found a new anorexigenic protein increased by troglitazone, and named it NEFA/nucleobindin 2 encoded satiety- and fat-influencing protein (nesfatin), corresponding to NEFA/nucleobindin 2 (NUCB2), which had been reported to be a secreted protein of unknown function [3]. Nesfatin/NUCB2 is composed of a signal peptide of 24 amino acids and a protein structure containing 396 amino acids (Fig. 1). The homology of the amino acid sequence of nesfatin/NUCB2 is highly conserved in humans, mice and rats. In this review, we summarize recent observation about the physiological role of nesfatin-1, and discuss future direction for the research of this anorexigenic protein.

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Anorexigenic effect of nesfatin and its fragments

Nesfatin/NUCB2 is expressed in the appetite-control hypothalamic nuclei such as paraventricular nucleus (PVN), arcuate nucleus (ARC), supraoptic nucleus (SON) of hypothalamus, lateral hypothalamic area (LHA), and zona incerta in rats [3]. Nesfatin-1-immunoreactivity was also found in the brainstem nuclei such as nucleus of the solitary tract (NTS) and dorsal nucleus of vagus [3, 4]. Selective reduction of nesfatin/NUCB2 mRNA expression in the PVN of rat was found under starved conditions, and refeeding restored its reduction, while no significant changes by starvation were observed in other hypothalamic nuclei. Intracerebroventricular (i.c.v.) injection of nesfatin/NUCB2 in male Wistar rats dose-dependently reduces food consumption for 6 hours

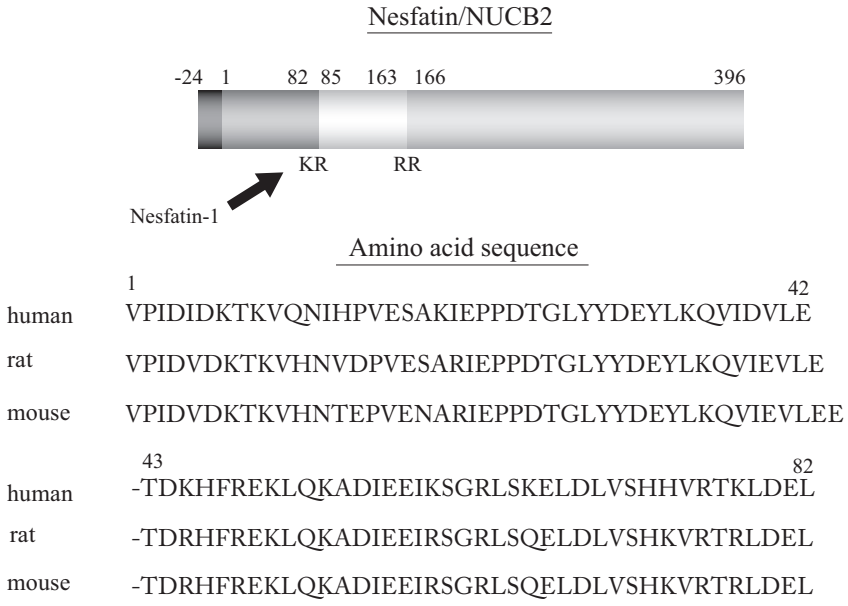


Fig. 1. Molecular structure of nesfatin/NUCB2 and comparison of amino acid sequence of human, rat, and mouse nesfatin-1.

after the injection. Nesfatin/NUCB2 has potential cleavage sites by prohormone convertase (PC) (Fig. 1). Imunohistochemical analysis showed that nesfatin/NUCB2 is present in the cytoplasm of neuronal cell, but not the nucleus, where it was co-localized with PC-1/3 and -2. I.c.v. infusion of nesfatin-1, an amino-terminal fragment derived from NUCB2, dose-dependently decreased food intake by 6 hours. In contrast, i.c.v. infusion of other possible fragments processed from nesfatin/NUCB2, nesfatin-2, nesfatin-3, and nesfatin2/3, does not promote satiety at all. Furthermore, i.c.v. infusion of the antibody neutralizing nesfatin-1, but not nesfatin-3, stimulates appetite. It was also demonstrated that rat cerebrospinal fluid contains nesfatin-1 indicating that nesfatin/NUCB2 is physiologically cleaved in the brain. Conversion of nesfatin/NUCB2 to nesfatin-1 by PC should be necessary to suppress feeding behavior.

Chronic infusion of nesfatin-1 into the rat third ventricle consistently reduced body weight gain, and decreased white adipose tissue weight on sacrifice. On the other hand, rats gained body weight after the start of chronic i.c.v. administration of antisense morpholino oligonucleotide against the gene encoding nesfatin/NUCB2. These data indicated that nesfatin/NUCB2 is involved in the physiological regulation of feeding behavior in rats.

In addition to its role as a satiety peptide, it has been reported that nesfatin-1 may mediate anxiety- and/or fear-related responses in the brain (5). I.c.v. injection of nesfatin-1 dose-dependently decreased the percentage of time spent on the open arms of the elevated plus maze, increased latency to approach, and increased the fear-potentiated startle response and the time spent freezing to both context and conditioned cues in a conditioned emotional response test. However, influences of peripherally administered nesfatin-1 or its analogues have not been examined.

It was demonstrated by two different groups that nesfatin-1 can cross the blood-brain barrier without saturation [6, 7]. They raised a possibility that both endogenous and peripherally administered exogenous nesfatin-1 can reach the brain, and inhibit feeding behavior. In non-starved male ICR mice, intraperitoneal injection of nesfatin-1 dose-dependently suppressed 3-hour food intake during the dark cycle (IC_{50}=0.35 nmoles/g body weight) [8]. Subcutaneous administration of nesfatin-1 also inhibited food intake, and its anorexigenic action continued for 14 hours after the injection, and the anorexigenic action of subcutaneous administration was longer than intraperitoenal administration. For determining chronic effects of nesfatin-1, 10 nmoles of nesfatin-1 was intraperitoneally administered to male ICR mice twice a day in our preliminary study. Repeated, intraperitoneal injection of nesfatin-1 significantly inhibited body weight gain for

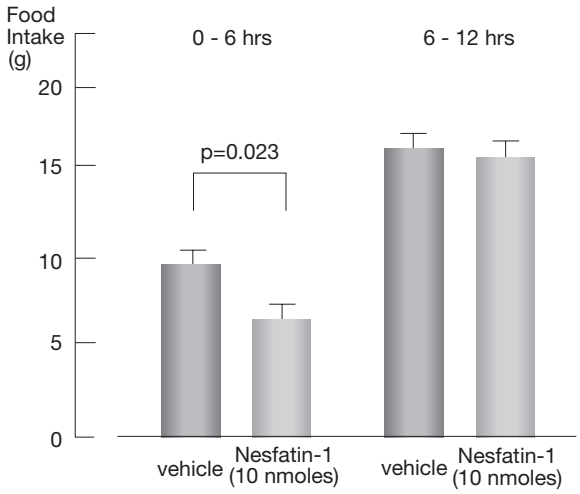
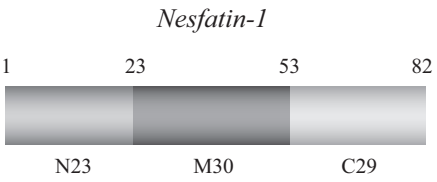


Fig. 2. Change of food intake for 12 hours after intranasal administration of nesfatin-1 in male Wistar rats. Nesfatin-1 (10 nmoles/rat) was infused into the nasal cavity (5 nmoles in each side of nasal cavity).



N23 VPIDVDKTKVHNTEPVENARIEP

N30 PDTGLYYDEYLKQVIEVLEETDPHFREKLQK

C29 ADIEEIRSGRLSQELDLVSHKVRTRLDEL

Fig. 3. Amino acid sequence of three distinct fragments of nesfatin-1.

6 days. These results suggested that peripherally administered nesfatin-1 could reduce food intake, and subcutaneous administration route appears to be most possible for the development of anti-obesity drugs by nesfatin-1.

In addition, we examined the possibility of nasal administration of nesfatin-1 in male Wistar rats, because intranasal route is easy to access to the brain, independent of altered blood-brain barrier function such as "leptin resistance" [8]. Nesfatin-1 (10 nmoles/rat) was administered into the bilateral nasal spaces of rats. Intranasal administration of nesfatin-1 significantly inhibited food intake for 6 hours (Fig. 2). Anorexigenic action of nesfatin-1 disappeared from 6 hours after the administration. From those observations, it is supposed that the route from nasal cavity to the brain may be one of possible candidates of nesfatin-1 administration in the treatment of human obesity.

Next, we sought to identify the active segment of nesfatin-1 for the future development of anti-obesity drugs using nesfatin-1 analogues. Since nesfatin-1 has three distinct segments (Fig. 3), we examined the effect of each segment on food intake in mice. Among those three segments of nesfatin-1, only the mid-segment (M30), composed of 30 amino acids, was effective on the appetite [9]. The anorexigenic action of intraperitoneally administered M30 was dose-dependent (IC_{50}=0.36 nmoles/g body weight), similarly with nesfatin-1. However, single injection of M30 into the peritoneal spaces did not affect circulating glucose and lipids concentrations at 3 hours after the injection, in spite of the significant reduction of food intake. Nesfatin-1 may not have direct action on glucose and lipid metabolism at this dosage. It is supposed that a region with amino acid sequence similarity to the active site of AgRP was indispensable for anorexigenic induction. This observation may be valuable for the future development of nesfation-1 analogues.

Mechanism of anorexigenic effect of nesfatin-1

Nesfatin-1-induced anorexia occurred in Zucker fatty rats with a leptin receptor mutation, and pretreatment with anti-nesfatin-1 antibody did not block leptin-induced anorexia [3]. Intraperitoneal injection of M30 decreased food intake under leptin-resistant conditions such as genetically obese (*ob/ob*), genetically diabetic (*db/db*) mice and mice fed a 45% high fat diet for 28 days [9]. Those data indicate that hypothalamic leptin signaling pathway dose not exist at the downstream of the pathway by which nesfatin-1 causes the anorexia. In contrast, central injection of alpha-melanocyte-stimulating hormone (MSH) elevated nesfatin/NUCB2 gene expression in the PVN, and the reduction of food intake by nesfatin-1 was abolished by administration of the melanocortin-3/4 receptor antagonist, SHU9119. However, i.c.v. administration of nesfatin-1 failed to show a significant change in POMC gene expression in the ARC. From those observations, it is supposed that nesfatin-1 signaling pathway might be associated with melanocortin sig-

naling pathway in the hypothalamus.

Next, which part of the brain is important in the suppression of feeding behavior by nesfatin-1. Nesfatin/NUCB2 expression is modulated by starvation and refeeding in the PVN and SON [3, 10]. Nesfatin-1 influences the excitability of a large proportion of different subpopulations of neurons located in the PVN [11]. Nesfatin-1 neurons in the PVN and SON co-expressed oxytocin and vasopressin [10]. Double-labeling immunohistochemistry revealed co-localization of nesfatin/NUCB2 with vasopressin and oxytocin in magnocellular neuroendocrine neurons, thyrotropin-releasing hormone (TRH), corticotropin-releasing hormone (CRH), somatostatin, neurotensin, and growth hormone-releasing hormone (GRH) in parvocellular neuroendorine neurons of the PVN [12]. Magnocellular oxytocin neurons are activated by feeding, and i.c.v. administration of oxytocin antagonist increases food intake [12], indicating a possible role of oxytocin in the regulation of feeding behavior. These data raised a possibility that oxytocin may be involved in the nesfatin-1-induced anorexia in the hypothalamus.

Nesfatin/NUCB2-immunopositive neurons are also located in the ARC, and are co-localized with proopiomelanocortin (POMC)/cocaine- and amphetamine-regulated transcript (CART)-neurons, but not in neuropeptide Y (NPY)/agouti gene-related protein (AgRP)-neurons [3, 4]. However, NPY neurons in the ARC are hyperpolarized by nesfatin-1, and glibenclamide, an ATP-sensitive potassium conductance antagonist, prevents nesfatin-1-induced hyperpolarization, indicating that hyperpolarization of NPY neurons are important in nesfatin-1-induced anorexia [13]. Nesfatin-1, released from POMC/CART neurons, may directly affect NPY/AgRP neuronal activities. In addition, Inhoff *et al*. indicated that nesfatin-1 may be involved in the desacyl ghrelin-induced inhibition of the orexigenic effect of peripherally administered ghrelin in freely fed rat, since nesfatin/NUCB2-immunoreactive neurons in the ARC are activated by simultaneous injection of ghrelin and desacyl ghrelin [14]. They proposed a new pathway that peripherally administered desacyl ghrelin might block ghrelin-activated NPY/AgRP-neurons in the ARC via nesfatin-1 positive neurons.

Nesfatin/NUCB2 also co-expressed with melanin concentrating hormone (MCH) in tuberal hypothalamic neurons of the rat, and approximately 80% of nesfatin-1-immunoreactive neurons were labeled for MCH [15]. These data suggest that nesfatin/NUCB2, co-expressed in MCH neurons, may play a complex role not only in the regulation of food intake, but also in other essential integrative brain functions involving MCH signaling, ranging from autonomic regulation, stress, mood, cognition to sleep.

Nesfatin/NUCB2-immunoreactive cells were choline acetyltransferase positive in the Edinger-Westphal nucleus and dorsal motor nucleus of vagus; tyrosine hydroxylase positive in the NTS, and 5-hydroxytryptamine positive in the causal raphe nucleus [4]. After intraperitoneal injection of M30, expression of *c-fos* was significantly activated in the brainstem, NTS, accompanied by a significant inhibition of 3-hour food intake, but not in the ARC. Mid-segment injection significantly increased expression of POMC and CART genes in the nucleus of the NTS, but not in the ARC. Nesfatin/NUCB2 was co-localized with CART in almost of NUCB2-expressing brain regions including brainstem nuclei [16]. Our findings indicate that intraperitoneal injection of M30 causes anorexia, possibly by activating POMC and CART neurons in the NTS via a leptin-independent mechanism.

Peripheral nesfatin/NUCB2 expression

Reverse transcriptase-polymerase chain reaction study demonstrated that nesfatin/NUCB2 mRNA was expressed in various tissues of the body including the brain and the adipose tissue. Nesfatin/NUCB2 was also detected in the rat serum and cerebrospinal fluid by Western blot analysis. The origin of circulating nesfatin-1 in the serum has not been still determined, because PC is also distributed in various tissues of the body. There is a possibility that nesfatin-1 may be processed and released from various tissues expressing PC-1/3 or PC-2 in the whole body.

Recently, nesfatin/NUCB2-immunoreactivity was identified in the rat gastric mucosa [17]. It was supposed that nesfatin/NUCB2-related peptides may be involved in the physiological regulation of gastrointestinal function, although it has not been shown whether nesfatin/NICB2 is processed to nesfatin-1 in the gastric mucosa. There is a possibility that nesfatin-1 may be produced in gastric mucosa, since the mRNAs for PC-1/3 and PC-2 has been reported to be abundant in gastric mucosa [18]. They raised the possibility that nesfatin/NUCB2 gene expression may be regulated by

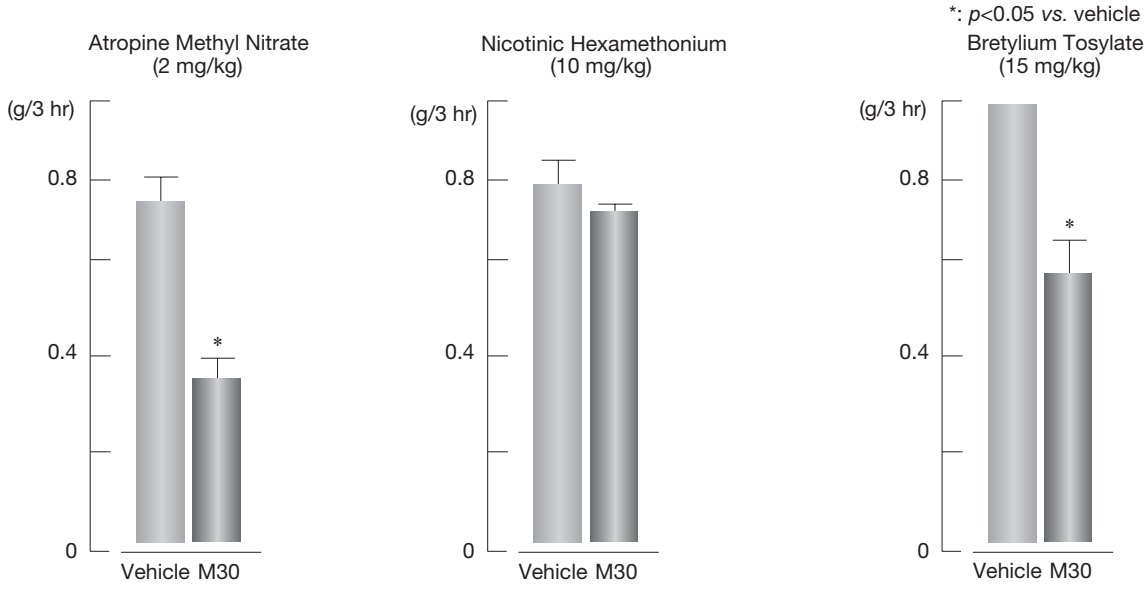


Fig. 4. Effects of ganglion blockers on the anorexia by intraperitoneal administration of 250 pmoles/g body weight of M30 in male ICR mice.

nutritional status, suggesting a regulatory role of peripheral nesfatin-1 in energy homeostasis.

In addition, the gastric vagal afferent is the major pathway conveying stomach-derived ghrelin's signal for starvation [19]. Lots of nesfatin/NUCB2-immunoreactive cells in the rat gastric mucosa co-express ghrelin [17]. It is possible that anorexigenic signal of peripherally administered nesfatin-1 and its active mid-segment, M30, may be transported to the brainstem via vagal nerve, similarly with ghrelin. We tested anorexigenic effects of M30 administered intraperitoneally in the mice pretreated with systemic capsaicin injection to examine possible involvement of vagal nerve in the anorexia. Intraperitoneal administration of M30 failed to show any anorexigenic action in capsaicin-pretreated mice [20]. It is, therefore, suggested that vagal nerve should be an important role in the induction of anorexia by M30 administered intraperitoneally. Furthermore, atropine methyl nitrate, an antagonist for the muscarinic cholinoceptor, and bretylium tosylate, an adrenergic ganglion blocker, did not affect suppression of food intake by itraperitoneally administered M30, and the nicotinic cholinergic blocker, hexamethonium, abolished the suppression of food intake by M30 (Fig. 4). It is supposed that nicotinic cholinergic receptor system should be also involved in M30-induced anorexia.

Nesfatin/NUCB2-immunoreactive cells are co-localized with insulin in pancreatic islets of both CD1 mice and Fischer 344 rats [21]. Since it is well known that pancreatic β-cells express both PC-1/3 and PC-2, it is conceivable that nesfatin/NUCB2 must be processed to nesfatin-1 in pancreatic β-cells. They raised the possibility that the abundant presence of nesfatin/NUCB2-immunoreactivity and its co-localization with insulin suggests a potential role for nesfatin/NUCB2-derived peptides in islet biology and glucose homeostasis.

Possible clinical application in the future

Based upon those observations above, we believe that nesfatin-1 should be clinically useful from two different points. First, it may be available for the diagnosis of various diseases. Second, systemic or local administration of nesfatin-1-related drugs may improve metabolic disorders by reducing body weight of the patients with obesity and metabolic syndrome.

Diagnosis

We had already found that nesfatin/NUCB2 exists in human serum by Western blot analysis. Now we are trying to establish the sensitive and selective ELISA assay system for nesfatin-1, and our preliminary observation confirmed the existence of nesfatin-1 in human serum. By using this system, future accumulation of clinical data will clarify the significance of the deter-

mination of circulating nesfatin-1 levels in various disorders including obesity and metabolic syndrome.

On the other hand, it was recently demonstrated that serum nesfatin-1 levels, measured by using commercially available assay system, are remarkably higher in patients who are newly diagnosed with primary generalized epilepsy [22]. They suggested that elevation of circulating nesfatin-1 levels might be useful as a biomarker for the diagnosis of epilepsy and for monitoring the response to anti-epileptic treatment. Although there remains the issue whether this commercially available assay system may detect not only nesfatin-1, but also whole nesfatin/NUCB2 in human serum, a remarkable increase of serum nesfatin-1 levels may be related to the existence of epilepsy or epileptic seizure.

Treatment

Lots of clinical studies have shown that most obese people demonstrate marked leptin resistance. A randomized, controlled, dose-escalation trial about clinical leptin treatment already demonstrated that huge dose of leptin for a long period should be necessary to significantly decrease body weight in obese adults [23]. In contrast, our data obtained from basic experiments indicated that intraperitoneally administered nesfatin-1 significantly inhibited food intake even in leptin-resistant animal models. Chronic administration of nesfatin-1 in the periphery significantly decreased body weight gain for 6 days in non-obese mice. Nesfatin-1 and its analogue are possible candidates of anti-obesity drug in leptin-resistant obese people. Possible methods for the future treatment of leptin-resistant obese people by using nesfatin-1-related drugs are presented in Fig. 5. On the data obtained from peripheral administration of nesfatin-1 and M30 in mice, a most possible administration route should be subcutaneous, similarly with daily subcutaneous insulin administration for diabetic patients. However, needle phobia to daily, repeated subcutaneous injection may exist

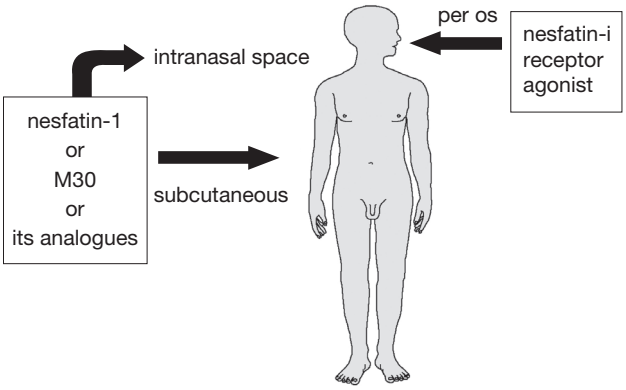


Fig. 5. Clinical possibility of anti-obesity treatment by nesfatin-1 -related drugs in the future.

in obese people only for the weight reduction, and long acting nesfatin-1 analogue has to be developed to make a daily injection once a day. Since nesfatin-1 can be effective on the reduction of food intake after intranasal administration in rats, intranasal route may be another possibility for the treatment. Nasal route is easy for frequent administration before each meal in obese people. Although a selective receptor for nesfatin-1 has not been identified, nesfatin-1 receptor agonist can be developed and administered *per os* in the future when a specific receptor will be found.

Summary

Recent observations about nesfatin-1 were summarized. The data obtained in basic experiments of nesfatin-1 and M30 should be useful for the development of a new anti-obesity drug effective for leptin-resistant obese people. However, the details of endocrine and metabolic effects of nesfatin-1 have not been known well by now, and the influences of nesfatin-1, M30 and its analogues administered peripherally should be much more clarified *in vivo* before starting clinical trials in the future.

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