Expression of Nesfatin-1/NUCB2 and Its Binding Site in Mouse Testis and Epididymis

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ABSTRACT : Nesfatin-1/NUCB2, which is secreted from the brain, is known to control appetite and energy metabolism. Recent studies have been shown that nesfatin-1/NUCB2 was expressed not only in the brain, but it was also expressed in the gastric organs and adipose tissue. However, little is known about the expression of nesfatin-1/NUCB2 in the male reproductive system. Therefore, we examined whether the nesfatin-1/NUCB2 and its binding site exists in the male reproductive organs. Nesfatin-1/NUCB2 mRNA and protein were detected in the mouse testis and epididymis by PCR and Western blot analysis. As a result of the immunohistochemistry staining, the nesfatin-1 protein was localized at the interstitial cells and Leydig cells in the testis. Nesfatin-1 binding sites were also displayed at boundary cells in the tunica albuginea. Furthermore, in order to examine if the expression of nesfatin-1/NUCB2 mRNA in the testis and epididymis were affected by gonadotropin, its mRNA expression was analyzed after PMSG administration into mice. NUCB2 mRNA expression levels were increased in both of the testis and epididymis after PMSG administration. These results demonstrated for the first time that nesfatin-1 and its binding site were expressed in the mouse testis and epididymis. In addition, nesfatin-1/NUCB2 mRNA expression was controlled by gonadotropin, suggesting a possible role of nesfatin-1 in the male reproductive organs as a local regulator. Due to this, further study is needed to elucidate the functions of nesfatin-1 on the male reproductive system.

Key words : Epididymis, nesfatin-1, NUCB2, PMSG, Testis

INTRODUCTION

Nucleobindin protein, which was identified for the first time in the human and mouse cell lines, is known to have two isotypes, nucleobindin 1 (NUCB1) and nucleobindin 2 (NUCB2) (Miura et al., 1992; Kanai & Tanuma et al., 1992; Barnikol-Watanabe et al., 1994). However, NUCB2 only functions physiologically in humans and rodents (Miura et al., 1992). NUCB2 produces nesfatin-1, nesfatin-2 and nesfatin-3 by enzyme pro-hormone convertase (PC)-1/3 after translational processing. Until now, a physiological activity has only been demonstrated for nesfatin-1. The intracerebroventricular infusion of nesfatin-1 decreases food intake, whereas the infusion of the antibody neutralizing nesfatin-1 stimulates appetite (Oh-I et al., 2006).

Nesfatin-1 is initially known to express in the hypothalamic nuclei for appetite control such as arcuate nucleus (ARC), paraventricular nucleus (PVN), supraoptic nucleus (SO), lateral hypothalamic area (LHA), and zona incerta in rats (Brailoiu et al., 2007; Foo et al., 2008; Fort et al., 2008; Kohno et al., 2008) and exists in the cerebrospinal fluid and brain stem. Recent study demonstrated that nesfatin-1 immunoreactivity was identified in the rat gastric organs (Gonzalez et al., 2009; Stengel et al., 2009a). A large amount of the nesfatin-1 expression was detected in gastric endocrine cells and the duodenal Brunner's glands of the rodents

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(Zhang et al., 2010). X/A-like cells secreted ghrelin, which stimulates the appetite, in the gastric mucosal layer was also demonstrated to express nesfatin-1 (Stengel et al., 2009b). These results raise the possibility that nesfatin-1/NUCB2 gene expression may be regulated by nutritional status, suggesting a regulatory role of peripheral nesfatin-1 in energy homeostasis. Nesfatin-1 immunoreactive cells are also co-localized with insulin in pancreatic beta-cells of mouse and rat (Gonzalez et al., 2009), suggesting a potential role for nesfatin-1 in pancreatic islet and glucose homeostasis (Foo et al., 2010).

Recently, nesfatin-1 was detected in the fat of humans and rodents showing that it was expressed more in the subcutaneous than in the visceral fat (Ramanjaneya et al., 2010). In experiments with fat cell lines, nesfatin-1 expression was observed to increase exponentially in the process of the fat progenitor cells differentiate into the mature fat cells (Ramanjaneya et al., 2010). The fact that high nesfatin-1 concentration in the blood of people with high BMI index demonstrates that nesfatin-1 secreted from fat may regulate food intake independently, rather than relying on leptin (Shimizu et al., 2009, Ramanjaneya et al., 2010).

Until now, only one report demonstrated the expression of nesfatin-1 protein in the reproductive system. In the report, they have been shown that nesfatin-1 was localized within the interstitial cells including the Leydig cells in the mouse testis using immunohistochemical staining (Garcia-Galiano et al., 2010). However, it is not clear yet whether nesfatin-1/NUCB2 mRNA is expressed in the male reproductive organs and if the nesfatin-1 binding site exists in the testis. Therefore, we first investigated whether nesfatin-1/NUCB2 mRNA and protein are expressed and its binding site exists in male reproductive organs. Next, we examined the change of NUCB2 mRNA expression levels in the male reproductive system after PMSG administration.

MATERIALS AND METHODS

1. Animals

Six-week-old male ICR mice were purchased from Samtako Bio Korea (Korea) and housed in groups of five per cage under controlled illumination (12:12 h light/dark cycle, lights on/off: 6 h/18 h) and temperature (22±2℃). Animals were fed a standard rodent diet and tap water *ad libitum*. Animal care and experimental procedures were approved by the Institutional Animal care and the use committee at the Seoul Women's University in accordance with guidelines established by the Korea Food and Drug Administration.

2. RNA Extraction and cDNA Synthesis

Mice were euthanized by CO_2 anesthesia followed by cervical dislocation. Cerebrum, hypothalamus, pituitary, testis, and epididymis were quickly removed and homogenized with 300 ㎕ ice cold RNA isoplus (TaKaRa Bio, Shiga, Japan). After chloroform extraction and isopropyl alcohol precipitation, RNA was dissolved in 20 ㎕ RNase-free DEPC (TaKaRa Bio, Shiga, Japan) solution. The RNA concentrations were measured with the Nano-drop (Thermo Fisher Scientific Inc., Waltham, MA). First strand cDNA synthesis was performed using 2 ㎍ RNA, 10 pmol oligo dT and RNase-free DEPC solution at 70℃ for 5 min, followed by double-strand synthesis in 5X RT buffer (Invitrogen, Carlsbad, CA) with 8 mM dNTP (BIO BASIC INC., Ontario, Canada), 200 unit/㎕ RTase (Invitrogen, Carlsbad, CA) and RNase-free DEPC solution at 37℃ for 60 min and at 72℃ for 15 min.

3. Conventional PCR

Conventional PCR was performed in buffer solution containing 3 ㎕ of template cDNA, 5 unit/㎕ of Taq polymerase (BIONICS, Korea), 0.25 mM dNTPs (BIO BASIC INC., Ontario, Canada) and 10 pmol of each primer. Primers were designed for NUCB2 and β-actin on the basis of the mouse cDNA sequences. The following primer pairs were used: NUCB2 forward 5'-TTTGAACACCTGAACCACCA-3'; reverse 5'-TGGTCTTCGTGCTTCCTCTT-3' and β-actin forward 5'-CTCTTTGATGTCACGCACGATTTC-3'; reverse 5'-ATCGTGGGCCGCTCTAGGCACC-3' primers (BIONICS,

Korea). The optimum temperature cycling protocol was used as 95℃ for 15s, 60℃ for 30 s and 72℃ for 30 min, using the GenePro thermal cycler (Bioer, China). The reaction products were run on a 2% agarose gel and visualized with ethidium bromide to check the length of the amplified cDNA.

4. Real-Time PCR

Real-time PCR was performed in a total volume of 20 ㎕ buffer solution containing 2 ㎕ of template cDNA, 10 ㎕ of SYBR Green (Roche, Manheim, Germany), and 10 pmol of each primer. Primer pairs were as follows: NUCB2 forward 5'-AAAACCTTGGCCTGTCTGAA-3'; reverse 5'-CATCGATAGGAACAGCTTCCA-3' and GAPDH forward 5'-TTGATGGCAACAATCTCCAC-3'; reverse 5'-CGTCC CGTAGACAAAATGGT-3' (BIONICS, Korea). The optimum temperature cycling protocol was determined to be 95℃ for 5 min followed by 45 reaction cycles at 95℃ for 10 s, 60℃ for 10 s and 72℃ for 10 s using the LightCycler® 480 Real-time PCR System (Roche, Manheim, Germany).

5. Western Blotting

The cerebrum, pituitary, testis, and epididymis were removed and kept on ice. All tissues were homogenized with 300 ㎕ ice cold EDTA homogenization buffer (0.606 g Tris-Base, 0.876 g NaCl, 0.036 g EDTA, 100 ㎕ Tween 20, 100 mM PMSF in 100 ㎖ DW). Protein fractions were obtained by centrifugation at 14,000 rpm for 20 min at 4℃. Final protein concentrations were determined using a BCA protein assay according to the SpectraMax M3 Multi-Mode Microplate Reader (Molecular Devices, Sunnyvale, CA). Protein samples (20 ㎍) were mixed with EDTA homogenization buffer, 1 ㎕ 5% mercaptoethanol, and 4 ㎕ 5X SDS sample loading buffer, and then boiled at 95℃ for 5 min. The samples were loaded on a 12% SDS-PAGE gel and run in 1X Tris-Glycine SDS Running buffer (KOMABIOTECH, Korea). After gel running, proteins were transferred by electrophoresis to PVDF membrane (Amersham; GE Healthcare, Buckinghamshire, England) for 24 h at 4℃. The membrane was stained in Ponceau-S and washed in distilled water, and then incubated in 3% Casein/PBS Blocking Solution (KOMABIOTECH, Korea) for 2 h at room temperature. The membrane was incubated with rabbit anti-rat nesfatin-1 polyclonal antibody (Phoenix Pharmaceuticals, INC., Burlingame, CA) and anti-mouse β-actin antibody (Santa Cruz Biotechnology, Inc., Paso Robles, CA) for 1 h at room temperature. The membrane was washed three times with washing buffer and incubated with goat anti-rabbit IgG-HRP (Santa Cruz Biotechnology, Inc., Paso Robles, CA) and donkey anti-mouse IgG-HRP (Santa Cruz Biotechnology, Inc., Paso Robles, CA) for 1 h, respectively. After washing three times, the membrane was detected by ECL Plus Western Blotting Detection Reagents (Amersham; GE Healthcare, Buckinghamshire, England). The relative protein levels were analyzed by Scion Image (National Institutes of Health, Bethesda, MD).

6. Immunohistochemistry Staining

The tissues were fixed in 4% paraformaldehyde buffer saline for 2 h or immersed in 20% sucrose solution before freezing. The tissues were rinsed in ethanol series to remove fixative residues, embedded in paraffin block. The tissues blocks were cut 10 ㎛ sections using a microtome, deparaffinized, and rehydrated with graded xylene-alcohol series, and then washed with PBS before immunostaining. The sections were incubated with rabbit anti-rat nesfatin-1 polyclonal antibody (Phoenix Pharmaceuticals, INC., Burlingame, CA) at 4℃ for overnight followed by incubation with Alexa fluor 594 conjugated goat anti-rabbit IgG (Jackson immuno research laboratory, West grove, PA). The tissues for cryosection were placed in holders with O.C.T embedding medium (Sakura, Belgium) and immediately immersed in liquid nitrogen. The tissues were cut 10 ㎛ sections using a cryostat. The sections were incubated in biotin conjugated nesfatin-1 (Phoenix Pharmaceuticals, INC., Burlingame, CA) at 4℃ overnight and then incubated with Alexa fluor 594 conjugated streptavidin (Invitrogen, Carlsbad, CA) for 30 min followed by staining with DAPI (4',6-diamidino-

2-phenylindole; Sigma, St. Louis, MO) for 10 min. The sections were mounted on the slides with mounting medium (Vector laboratories, INC., Burlingame, CA) and observed under fluorescence microscopy (Axioskop2, Carl Zeiss, Germany).

7. PMSG Treatment

Five unit of PMSG (Pregnant mare serum gonadotropin, Sigma, St. Louis, MO) was injected in the abdomen of the mouse and saline was injected in the control group. The testis and epididymis were obtained on 24 h after PMSG injection. NUCB2 mRNA expression was measured by real-time PCR.

8. Statistical Analysis

The results were presented as the mean and the standard error of the mean (SEM). Data were analyzed by student *t*-test. Values of $p<0.05$ were considered significant.

RESULTS

1. Expression of Nesfatin-1/NUCB2 in Male Reproductive Organs

We investigated NUCB2 mRNA expression by conventional PCR and real-time PCR to confirm whether the NUCB2 gene is expressed in the testis and epididymis. NUCB2 mRNA was detected in the testis and epididymis as much as the brain. Interestingly, the level of NUCB2 mRNA expression was higher in the pituitary than in the hypothalamus. We also confirmed a large amount of NUCB2 mRNA in the testis and epididymis (Fig. 1). In addition, the expression of nesfatin-1 protein in the reproductive organs was investigated by western blotting. We found that nesfatin-1 protein was detected in the testis and epididymis similar to the nesfatin-1/NUCB2 mRNA expression (Fig. 2).

2. Localization of Nesfatin-1 and Its Binding Site in Testis

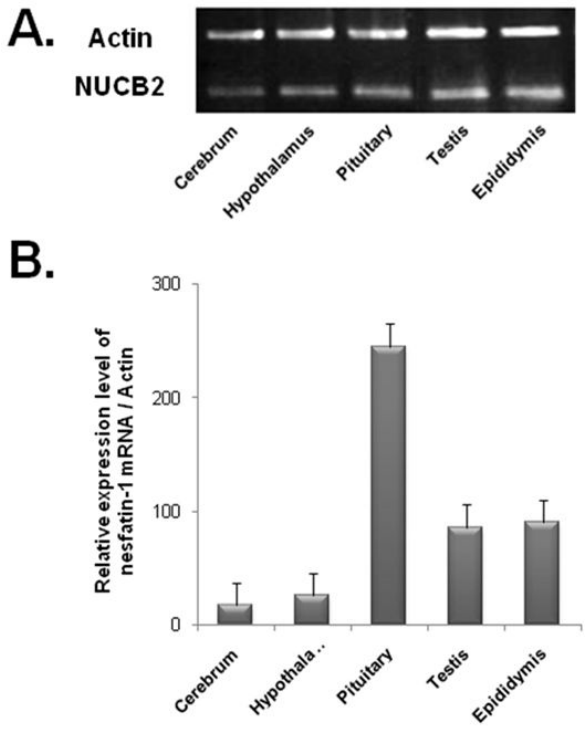

Fig. 1. Levels of NUCB2 mRNA expression in various tissues of male mice. (A) Nesfatin-1 mRNA expression in various tissues included testis and epididymis was confirmed by conventional PCR. (B) The levels of nesfatin-1 mRNA expression in various tissues were analyzed by real-time PCR. The expression levels of NUCB2 mRNA in the testis and epididymis were as much as the expression level of hypothalamus.

Nesfatin-1 protein expression site in the testis was investigated by immunohistochemistry staining using testis sections. As a result, nesfatin-1 was localized in the Leydig cells and the interstitial cells between the seminiferous tubules of testis (Fig. 3A). Nesfatin-1 binding sites were displayed at boundary cells in tunica albuginea and in part of Leydig cells (Fig. 3B).

3. Increase of NUCB2 mRNA Expression after PMSG Treatment

In order to examine if the expression of NUCB2 mRNA in the testis and epididymis was affected by gonadotropin, the expression of NUCB2 mRNA was analyzed after

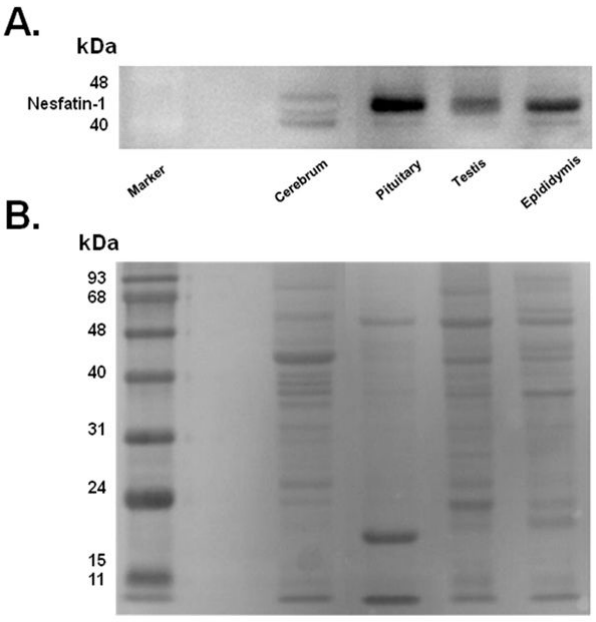

Fig. 2. Expression of nesfatin-1 protien in in the testis and epididymis. (A) Nesfatin-1 protein expression in male reproductive system was investigated by western blotting. The expression of nesfatin-1 protein was confirmed in testis and epididymis showing high levels. (B) Picture of the PVDF membrane stained with Ponceau-S after transferring proteins.

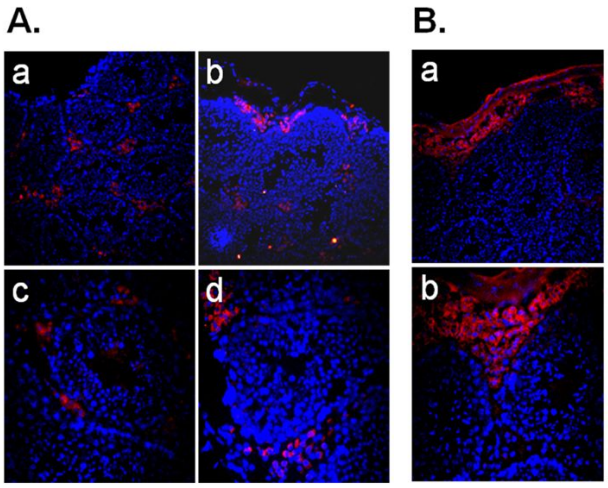

Fig. 3. Expression of nesfatin-1 and its binding site in testis. (A) The localization of nesfatin-1 in the mouse testis. Testis section was stained with nesfatin-1 antibody to localize nesfatin-1 protein. Nesfatin-1 protein was expressed at interstitial cells and Leydig cells among seminiferous tubules. Original magnification, a, b; 200×., c, d; 400×. (B) The localization of nesfatin-1 binding sites in the mouse testis. Testis section was stained with biotin conjugated nesfatin-1 to observe the nesfatin-1 binding sites. Nesfatin-1 protein binding sites were displayed at boundary cells in tunica albuginea. Original magnification, a; 200×, b; 400×.

PMSG treatment by real-time PCR. Level of NUCB2 mRNA expression in the testis and epididymis was significantly increased in PMSG administrated mice compared with the control (Fig. 4).

DISCUSSION

Nesfatin-1 protein produced by neurons in the hypothalamus is known to be secreted into cerebrospinal fluid, be controlling appetite and energy metabolism (Oh-I et al., 2006). Recently, it has been reported that nesfatin-1/NUCB2 mRNA is expressed in gastric organs such as stomach and pancreas (Stengel et al., 2009b) and in adipose tissues of humans and rodents (Garcia-Galiano et al., 2010). However, little is known about the expression of nesfatin-1/NUCB2 in male reproductive system. Therefore,

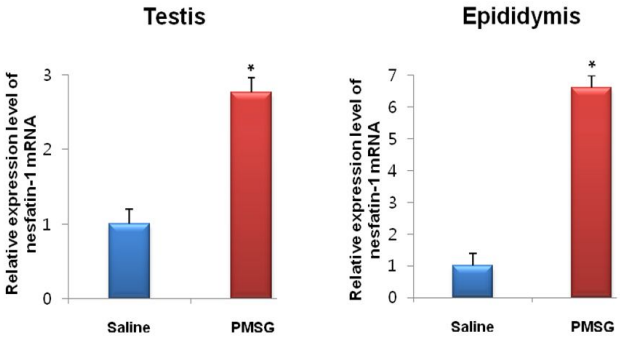

Fig. 4. The expression levels of nesfatin-1 mRNA in the testis and epididymis after PMSG administration. The amounts of nesfatin-1 mRNA expression were analyzed by real-time PCR. The amount of nesfatin-1 mRNA expression was increased about 2.8 times in the testis and about 6.6 times in the epididymis after PMSG administration. * indicates a significant difference between saline and PMSG ($p<0.05$).

in the present study we investigated whether nesfatin-1/NUCB2 mRNA and protein are expressed and if its binding site exists in the male reproductive organs to explore the possibility that nesfatin-1 can influence the male reproductive function.

To examine the expression of nesfatin-1/NUCB2 mRNA in the male reproductive organs, we performed conventional PCR using total RNA extracted from the testis and epididymis. The conventional PCR analysis showed that nesfatin-1/NUCB2 mRNA expression was detected in the testis and epididymis similar to the hypothalamus and pituitary. Next, we performed real-time quantitative PCR to evaluate the levels of nesfatin-1/NUCB2 expressed in the organs. It is well known that nesfatin-1/NUCB2 mRNA is abundantly expressed in the hypothalamus (Oh-I et al., 2006). Furthermore, recent reports have been shown that nesfatin-1 protein and nesfatin-1/NUCB2 mRNA is also expressed in the anterior pituitary (Foo et al., 2008; Goebel et al., 2009). Interestingly, our real-time PCR data showed that the level of nesfatin-1/NUCB2 mRNA expression was higher in the pituitary than in the hypothalamus. These results raise the possibility that nesfatin-1 produced in the brain may control the metabolic homeostasis not only through the hypothalamus, but also through the pituitary. In other words, nesfatin-1 protein produced by the pituitary may be transported to target cells in the various organs in the body along the bloodstream and work as a hormone like other ones produced by the pituitary.

The present study demonstrated that nesfatin-1/NUCB2 was expressed in the testis and epididymis as much as in the hypothalamus that was already known to express nesfatin-1/NUCB2. As a result of real-time quantitative PCR, nesfatin-1/NUCB2 expression level was higher in the testis and epididymis more in the hypothalamus and cerebrum. An amount of nesfatin-1 protein expressed in the testis and epididymis was also higher than in the brain. These results suggest that nesfatin-1 may play an important role in the testis which produces sperm and testosterone. Therefore, we next investigated which cells in the testis express nesfatin-1 protein using immunohistochemistry staining. Nesfatin-1 was localized in the interstitial cells and Leydig cells among the seminiferous tubules, but not in germ cells including sperm. Currently, it is not clear the function of nesfatin-1 on testicular cells, but our immunohistochemistry result raises the possibility that nesfatin-1 is involved in steroidogenesis to produce testosterone in the Leydig cells.

Our data showed that nesfatin-1/NUCB2 mRNA and protein were expressed in the testis. However, the nesfatin-1 protein should bind to its receptor to function in the testis. Although a lot of study on the nesfatin-1 is going on, the structure and function of nesfatin-1 receptor are still unknown (Stengel & Taché, 2011). Consequently, it is not possible to produce an antibody against nesfatin-1 receptor until now and to stain the nesfatin-1 receptor in tissue by immunohistochemistry staining using its antibody. Therefore, we used the biotin-conjugated nesfatin-1 protein instead of nesfatin-1 receptor antibody to detect nesfatin-1 binding sites. The staining data showed that the nesfatin-1 protein bound to in the interstitial cells including Leydig cells and the boundary cells in tunica albuginea. These results suggest that autocrine signaling by nesfatin-1 as a local regulator in the testis can occur in the Leydig cells to regulate the steroidogenesis and sperm production.

Leydig cells produce and secrete testosterone, androstenedione, and dehydroepiandrosterone (DHEA) by cholesterol desmolase by which cholesterol changes to pregnenolone after stimulation of LH secreted from pituitary (Akingbemi et al., 2004). LH receptors on Leydig cell membrane play a critical role in the steroidogenesis and the number of them is increased by prolactin resulting increasing LH-mediated response (Dufau, 1988). Leydig cells are also known to produce oxytocin and vitamin C (Kukucka et al., 1992), suggesting that they may play as local regulators to control the function of Leydig cells. The fact that Leydig cells express both nesfatin-1 protein and its binding sites proposes that nesfatin-1 also has an important role in testicular function as one of local regulators similar to other proteins present in the testis. However, we do not

know yet the exact mechanism by which nesfatin-1 can regulate the steroidogenesis in Leydig cells. Recently reported data demonstrated that protein kinase A in neuron was activated by nesfatin-1 injected in hypothalamus of rats and Ca^{2+} ions were released from them (Brailoiu et al., 2007), suggesting that nesfatin-1 receptor is associated with G-protein coupled proteins to transduce intracellular signals. The data raises the possibility that nesfatin-1 receptors present in Leydig cells also transduce signals produced from nesfatin-1 binding through the G-protein coupled proteins.

We next investigated whether nesfatin-1/NUCB2 expression in the testis is affected by gonadotropins such as LH and FSH. As for the real-time PCR results, nesfatin-1/NUCB2 mRNA expression levels were significantly increased in the testis and epididymis after administration of PMSG. The data suggest that nesfatin-1/NUCB2 expression in male reproductive system is able to be regulated by gonadotropins secreted by pituitary like other proteins produced in the testis under gonadotropin control.

The present study demonstrated for the first time that nesfatin-1 and its binding site were expressed in the mouse testis and epididymis. In addition, nesfatin-1/NUCB2 mRNA expression was regulated by gonadotropin, These results suggest that nesfatin-1 may have a possible role as a local regulator in the male reproductive organs. However, further study is needed to elucidate the exact function of nesfatin-1 in the male reproductive system.

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