potently inhibited GOAT activity. Specifically, GOAT activity was completely blocked over 5 m*M* Fe^{3+} and 0.5 m*M* Cu^{2+}. EDTA and EGTA did not affect GOAT activity, indicating that the enzyme had no absolute requirement for cations.

5. ALTERATIONS IN GOAT MRNA EXPRESSION IN THE STOMACH UNDER FASTING CONDITIONS

The most important factor regulating ghrelin expression in the stomach is how recently one has eaten. Ghrelin mRNA expression levels increased during fasting and decreased after refeeding. To examine the relationship of GOAT production to fasting/fed conditions, we investigated changes in the expression of GOAT mRNAs in the rat stomach after fasting and refeeding (Takahashi et al., 2009).

1. To measure GOAT mRNA expression levels in the rat stomach, real-time PCR was performed using a PRISM 7000 Sequence Detection system (PE Applied Biosystems, Foster City, CA, USA).
2. cDNA amplification was performed using SYBR Green PCR Core Reagents (PE Applied Biosystems). All samples were amplified on a single MicroAmp Optional 96-well reaction plate (PE Applied Biosystems). Results reflect duplicate values from at least two independent experiments.
3. Primer sequences used for PCR analysis were as follows: rat ghrelin, sense 5′-GAAGCCACCAGCTAAACTGC-3′ and antisense 5′-GCTGCTGGTACTGAGCTCCT-3′; rat GOAT, sense 5′-TTTGTATCCCAGTATCTCTTTCTGG-3′ and antisense 5′-CCAGTGGGAGTAGTAGGTGAGTTTA-3′.
4. After an initial 15 min at 95 °C to activate the HotStarTaq DNA polymerase, PCR fragments were amplified by 40 cycles as follows: 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 30 s. Standard wells contained a TOPO vector (Invitrogen) bearing the standard cDNA fragment. The concentration of standards covered at least six orders of magnitude.
5. We also included no-template controls on each plate. Experimental samples with a threshold cycle value within 2 SD of the mean threshold cycle value of the no-template controls were considered to be below the limits of detection.
6. The relative mRNA levels were standardized to a housekeeping gene, namely, glyceraldehyde-3-phosphate dehydrogenase, to correct for any bias among the samples caused by RNA isolation, RNA degradation, or efficiencies of the reverse transcriptase.

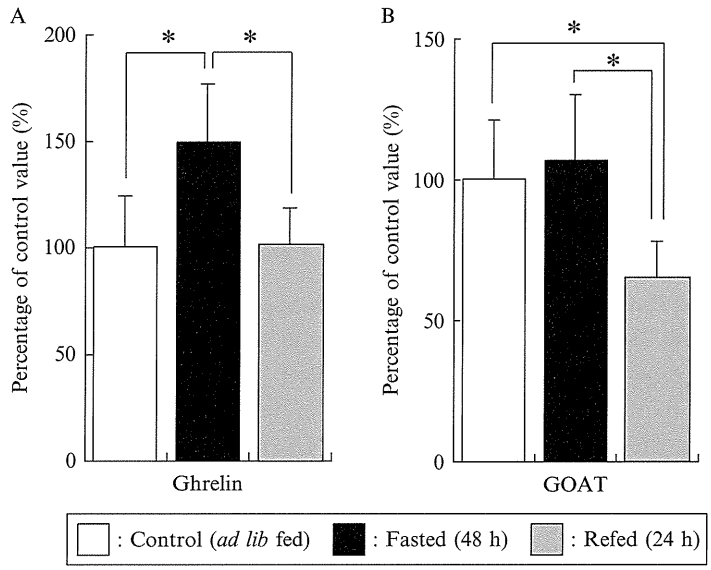

Figure 10.10 Real-time PCR analysis for mRNA levels in the stomach of rats fed *ad libitum* (control), 48-h fasted rats, or rats fasted for 48 h and refed. GAPDH was used as the internal control. Control values (*ad lib fed*) were normalized to 100%. (A) Ghrelin; (B) GOAT mRNA levels. Results are expressed as mean ± SD ($n = 12$). Asterisks indicate the differences between each group ($P < 0.05$).

7. After amplification, PCR products were analyzed according to their melting curve to confirm amplification specificity. Amplicon size and reaction specificity were confirmed by agarose gel electrophoresis. Ghrelin mRNA expression in the stomach after fasting for 48 h was significantly increased (by 49%) as compared to the *ad libitum* fed control (Fig. 10.10A). GOAT expression levels after fasting were not significantly changed as compared to the control (Fig. 10.10B). Although ghrelin mRNA expression returned to control levels after refeeding, the expression levels of GOAT were significantly decreased.

6. CONCLUSION

GOAT is a member of an acyltransferase family that comprises at least 16 enzymes (Hofmann, 2000). Among them, only GOAT shows the ability to acyl-modify ghrelin. Just as ghrelin is observed in all vertebrate species, GOAT is also found in mammals, birds, and fish (Yang et al., 2008). A thorough characterization of GOAT is an important step in understanding the molecular mechanisms underlying the acyl modification of ghrelin.

Recently, a GOAT-specific inhibitor was developed and demonstrated to improve glucose tolerance and suppress weight gain in wild-type mice but not in ghrelin-KO mice (Barnett et al., 2010). These results suggested that GOAT could be a clinical target for metabolic diseases.

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CHAPTER SEVEN

High-Performance Liquid Chromatography Analysis of Hypothalamic Ghrelin

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Contents

Abstract

Ghrelin, first identified in the stomach, is a ligand of an orphan G-protein coupled receptor. Early studies indicated that the growth hormone secretagogue receptor (GHS-R; ghrelin receptor) is ubiquitously distributed in the brain. In addition, centrally administered ghrelin and ghrelin receptor agonist have effects on central neurons in many regions, including the hypothalamus, caudal brain stem, and spinal cord. These effects are due to ghrelin secreted from the brain, rather than from the stomach; ghrelin does not cross efficiently through the blood–brain barrier. Identification of ghrelin in the hypothalamus demonstrated that, as with stomach ghrelin, hypothalamic ghrelin also has two molecular forms, namely, octanoyl ghrelin and des-acyl ghrelin. Hypothalamic ghrelin plays diverse roles in processes including feeding regulation and thermoregulation. Thus, the analysis of hypothalamic ghrelin will provide new information about the action of ghrelin in the central nervous system. In this chapter, we outline high-performance liquid chromatography and real-time PCR analysis of hypothalamic ghrelin.

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1. INTRODUCTION

In early work, Bowers et al. (1980) observed that some opioid peptide derivatives had weak growth hormone (GH)-releasing activity. They referred to these compounds as growth hormone secretagogues (GHSs). Thereafter, many types of GHSs were identified, such as GHRP-6 and L-163,191 (MK-0677), and the action of the GHSs was gradually elucidated (Bowers et al., 1984; Cheng et al., 1993; Patchett et al., 1995). Growth hormone-releasing hormone (GHRH), which promotes GH secretion from GH-secreting cells in the anterior pituitary, acts on the GHRH receptor to increase intracellular cAMP, which serves as a second messenger (Akman et al., 1993; Blake and Smith, 1991; Cheng et al., 1989, 1991; Popovic et al., 1996). GHSs also act on a different receptor expressed by GH-secreting cells in the anterior pituitary, increasing intracellular Ca^{2+} concentration via an inositol 1,4,5-trisphosphate (IP_3) signal transduction pathway. These results led us to predict the existence of an endogenous ligand of GHS.

Growth hormone secretagogue receptor (GHS-R) was identified as a receptor with which GHSs stimulate phospholipase C, resulting in an increase in IP_3 and intracellular Ca^{2+} (Howard et al., 1996). GHS-R is expressed in the pituitary, hypothalamus, and hippocampus. Therefore, a search for its endogenous ligand was actively undertaken using the orphan receptor strategy, focusing especially on the hypothalamus. However, working with rats, Kojima et al. (1999) discovered the endogenous ligand, the 28-amino acid peptide they named ghrelinin, in an unexpected organ: the stomach.

Subsequently, through high-performance liquid chromatography (HPLC) analysis, we determined that *n*-octanoyl ghrelin is present in the hypothalamus, where it is synthesized (Sato et al., 2005). We also showed that, as in the stomach, *n*-octanoyl and des-acyl ghrelin are the two major molecular forms of ghrelin in the hypothalamus.

2. IDENTIFICATION OF HYPOTHALAMIC GHRELIN

2.1. Sample preparation

To identify hypothalamic ghrelin, it is essential to boil the samples in order to inactivate the intrinsic protease (Hosoda et al., 2000). In practice, we use a water bath over a strong flame (e.g., a stove burner) in order to prevent a decrease in water temperature after the addition of samples (Fig. 7.1). The temperature of water should remain above 95 °C during the boiling of samples. In order

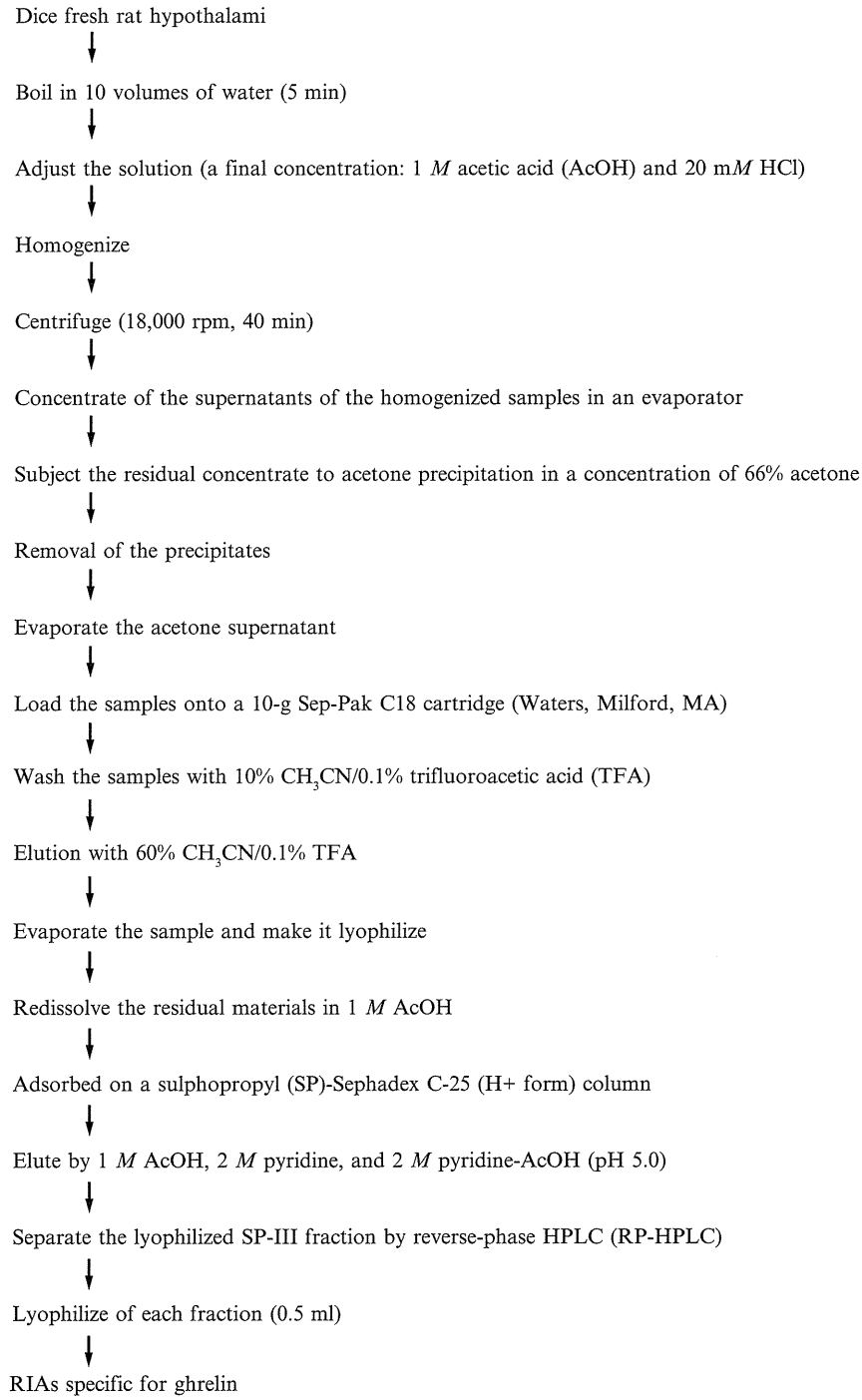

Figure 7.1 Flowchart of the preparation of hypothalamic samples.

to obtain sufficient quantities of the peptide sample, it is necessary to sacrifice ten or more rats. To date, we have been unable to successfully identify hypothalamic ghrelin in mice.

Procedure

1. Remove rat brains, dissect out the hypothalami, and mince them (not less than 20 g).
2. Boil for 5 min in 10 volumes of water to inactivate intrinsic proteases.
3. Adjust the solution to a final concentration of 1 *M* acetic acid (AcOH) and 20 m*M* HCl.
4. Homogenize the boiled hypothalami using a Polytron mixer.
5. Centrifuge the homogenized sample at 15,000 rpm (18,000 × *g*) for 40 min.
6. Concentrate the supernatants from homogenized samples to ~20 ml in an evaporator.
7. Subject the residual concentrate to acetone precipitation in a concentration of 66% acetone.
8. After removal of the precipitates, evaporate the acetone supernatant.
9. Load the sample onto a 10-g Sep-Pak C18 cartridge (Waters, Milford, MA) and wash with 10% CH_3CN/0.1% trifluoroacetic acid (TFA).
10. Elute with 60% CH_3CN/0.1% TFA.
11. Lyophilize the sample.
12. Redissolve the residual materials in 1 *M* AcOH.
13. Adsorb on a sulfopropyl (SP)-Sephadex C-25 (H^+ form) column, pre-equilibrated in 1 *M* AcOH.
14. Elute successively with 1 *M* AcOH, 2 *M* pyridine, and 2 *M* pyridine–AcOH (pH 5.0) and designate these three fractions as SP-I, SP-II, and SP-III.
15. Separate the lyophilized SP-III fraction by reverse-phase HPLC (RP-HPLC) using a μBondasphere C18 column (3.9 × 150 mm; Waters). A linear gradient of CH_3CN from 10% to 60% in 0.1% TFA for 40 min serves as the solvent system, at a flow rate of 1 ml/min (Fig. 7.2A).
16. Lyophilize each fraction (0.5 ml) and subject to RIAs specific for ghrelin.

2.2. RIAs for rat ghrelin

To characterize the molecular forms of immunoreactive ghrelin, we recommend RIA analysis using two polyclonal antibodies: no. 6-6 for amino-terminal RIA (N-RIA) and no. 1-7 for carboxyl-terminal RIA (C-RIA), raised against the amino terminal (Gly^1-Lys^{11} with *O*-*n*-octanoylation at Ser^3) and C-terminal (Gln^{13}-Arg^{28}) fragments of rat ghrelin, respectively.

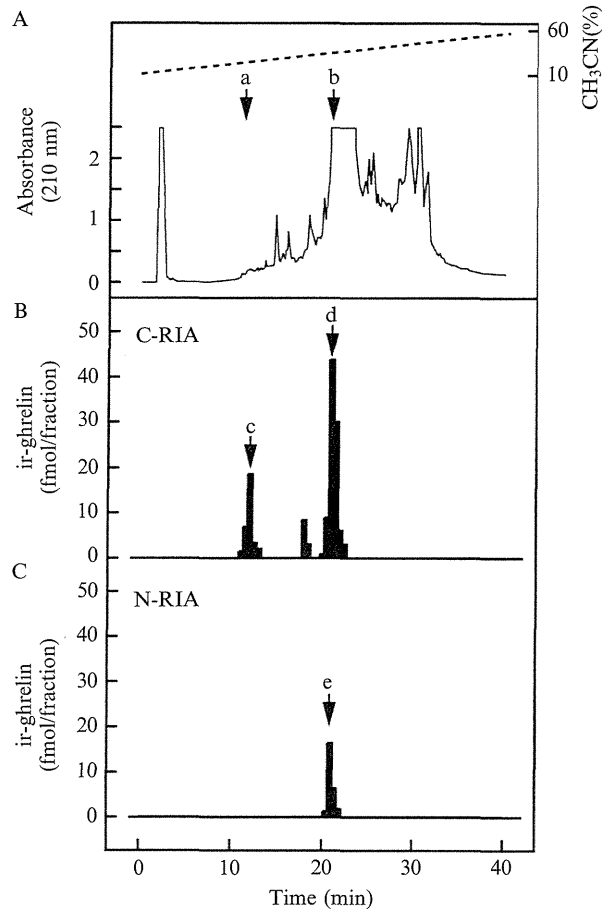

Figure 7.2 Representative RP-HPLC profiles of ghrelin immunoreactivity in the rat hypothalamus. A linear gradient of 10–60% CH_3CN containing 0.1% TFA was run for 40 min at 1.0 ml/min. (A) Chromatograph of rat hypothalamic extract. RP-HPLC of rat hypothalamus was monitored by C-RIA (B) and N-RIA (C) for ghrelin, using fraction volumes of 0.5 ml. The arrows indicate the elution points of des-acyl rat ghrelin-(1–28) (arrow a) and *n*-octanoylated rat ghrelin-(1–28) (arrow b). The two major peaks observed were consistent with the elution points of des-acyl rat ghrelin-(1–28) (arrow c) and *n*-octanoylated rat ghrelin-(1–28) (arrows d and e) [Sato et al., 2005].

2.2.1 Preparation for RIA

1. RIA buffer:

   To make the RIA buffer, mix the following reagents in distilled water: 50 m*M* PBS (pH 7.4), 80 m*M* NaCl, 25 m*M* EDTA-2Na, 0.05% NaN_3, and 0.5% Triton X-100. Add 4 N NaOH to adjust the solution to pH 7.4. To prevent nonspecific binding (NSB) of peptides to RIA

tubes, bovine serum albumin (BSA) should be added to the RIA buffer. Add 29.23 ml of 8.55% BSA into 400 ml of the RIA buffer and then adjust the volume of the RIA buffer to 500 ml.

2. Standard preparation:

   For ghrelin RIA, use rat or human ghrelin peptide with the *n*-octanoyl modification. Prepare 1 nmol/ml of peptide standard solution by RIA buffer. One nanomole of rat ghrelin (MW = 3,315) is 3.315 μg; 1 nmol of human ghrelin (MW = 3,371) is 3.371 μg. Dilute the standard peptide for duplicate assays by serial dilution as follows: 8196, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5, 0.25, and 0.125 fmol/100 μl.

2.2.2 RIA

1. Day 1
   - **a.** Add RIA buffer to the lyophilized samples. Vortex well.
   - **b.** Centrifuge the samples to remove insoluble materials (4 °C, 3,000 rpm, 10 min).
   - **c.** Pipet 100 μl of standard peptides and unknown samples into appropriately labeled tubes, in duplicate.
   - **d.** Pipet 300 μl of the RIA buffer into the NSB tubes. Add 10 μl per tube of NRS (1/10 dilution).
   - **e.** Pipet 200 μl antiserum solution into all tubes except the NSB tubes and record the total counts (TCs). The antiserum solution is constituted as follows: ghrelin serum, x μl; NRS (1/10 dilution), 10 μl; and RIA buffer, [200 $(x+10)$] μl. For N-terminal RIA, dilute no. 6-6 serum 1:2,500,000 (final); for C-terminal RIA, dilute no. 1-7 serum 1:12,000 (final).
   - **f.** Vortex well.
   - **g.** Incubate at 4 °C for 12 h.
2. Day 2
   - **a.** Dilute ^{125}I-labeled ghrelin to 20,000 cpm/100 μl RIA buffer in each tube. Pipet 100 μl tracer into all tubes.
   - **b.** Incubate at 4 °C for 36 h.
3. Day 4
   - **a.** Dilute secondary antibody (goat anti-rabbit IgG) 1:35 with the RIA buffer. Pipet 100 μl of the diluted secondary antibody into all tubes.
   - **b.** Incubate at 4 °C for 24 h.
4. Day 5
   - **a.** Centrifuge the tubes at 4 °C for 30 min at 3,000 rpm.

b. After centrifugation, place the tubes on ice and then aspirate the supernatant.
c. Quantitate the radioactivity in the pellet with a γ counter using an apparatus such as the ARC-1000M (Aloka, Tokyo, Japan) (Fig. 7.2B and C).

3. QUANTIFICATION OF IMMUNOREACTIVE GHRELIN IN RATS

3.1. Preparation of tissue samples

Broadly, this protocol is similar to the one described in Section 2.1.

1. Sacrifice rats and quickly remove the whole hypothalamus from each brain.
2. Mince the hypothalami and boil for 5 min in 10 volumes of water to inactivate the intrinsic proteases.
3. After cooling, adjust the solutions to a final concentration of 1 *M* AcOH and 20 m*M* HCl.
4. Homogenize tissues with a Polytron mixer and centrifuge at 15,000 rpm for 10 min; preserve the supernatants.
5. Load the supernatants onto Sep-Pak C18 cartridges (Waters). Wash the cartridges in 0.9% NaCl and 10% CH_3CN/0.1% TFA. Elute the bound protein with 60% CH_3CN/0.1% TFA. Lyophilize and subject the eluate to ghrelin-specific ELISA.

3.2. ELISA for rat ghrelin

Hypothalamus ghrelin levels can be easily measured using an Active Ghrelin ELISA Kit (Mitsubishi Kagaku Iatron, Inc., Tokyo, Japan) to assess the *n*-octanoyl-modified ghrelin and a Desacyl-Ghrelin ELISA Kit (Mitsubishi Kagaku Iatron, Inc.) to measure des-acyl ghrelin. To measure ghrelin levels by ELISA, samples must be obtained from whole rat hypothalami. The protocols should follow the manufacturer's instructions. In the case of plasma samples, it is important to calculate a corrected volume because of the addition of 1 N HCl to plasma.

4. QUANTIFICATION OF RAT GHRELIN MRNA IN HYPOTHALAMIC GHRELIN IN RATS

To understand the process of ghrelin secretion, it is necessary to investigate the synthesis of ghrelin. Real-time PCR is an useful tool for the analysis of ghrelin mRNA levels. Because the level of hypothalamic ghrelin

mRNA is very low, purification of poly(A)$^+$ RNA is necessary. In addition, in order to obtain accurate readings, it is essential to carefully compare samples with a "no template" control.

4.1. Synthesis of cDNA

1. Extract the total RNA from frozen hypothalami using TRIzol (Invitrogen, Tokyo, Japan).
2. Purify poly(A)$^+$ RNA from 75 μg or more total hypothalamic RNA using Oligotex-dT30 <Super> (Roche, Tokyo, Japan), according to the manufacturer's instructions.
3. Synthesize cDNA from the poly(A) $^+$ RNA (0.4 μg per animal or more).
4. Incubate the reaction mixtures at 37 °C for 60 min.
5. Stop the reaction by incubation at 70 °C for 15 min.

4.2. Real-time PCR

1. cDNA amplification is performed using SYBR Green PCR Core Reagents (PE Applied Biosystems) and uracil-*N*-glycosylase (Invitrogen), to prevent contamination by carried-over PCR products, as suggested by the manufacturer. Samples are amplified in a single MicroAmp Optical 96-well reaction plate (PE Applied Biosystems). The results reflect duplicate runs of at least two independent experiments. Primer pairs for ghrelin gene are designed using Primer3 software as follows:

   sense primer 5′-GAAGCCACCAGCTAAACTGC-3′;

   antisense primer 5′-GCTGCTGGTACTGAGCTCCT-3′.

   Researchers can also purchase the primer-pair-optimized real-time PCR as a regent product from a company such as Takara Bio, Inc.
2. Each standard well contains a pGEM-T Easy vector carrying the standard cDNA fragment. The concentrations of the standards cover at least six orders of magnitude. We also include no template controls on each plate.
3. PCR cycling conditions are initiated by a 2-min incubation at 50 °C to eliminate any deoxyuridine triphosphate-containing PCR products resulting from carry-over contamination. After a 15-min period at 95 °C to activate HotStarTaq DNA polymerase, PCR fragments are amplified by 40 cycles of 95 °C for 30 s, 60 °C for 30 s, and 1 min at 72 °C.
4. Experimental samples with a threshold cycle value within 2 SD of the mean threshold cycle value for the "no template" controls are considered to be below the limits of detection. The relative levels of mRNA are

standardized to a housekeeping gene, such as glyceraldehyde-3-phosphate dehydrogenase or ribosomal protein S18, to correct for any bias among the samples caused by RNA isolation, RNA degradation, or efficiencies of RT. After amplification, PCR products are analyzed by a melting curve to confirm amplification specificity. Amplicon size and reaction specificity are confirmed by agarose gel electrophoresis.

5. IDENTIFICATION OF GHRELIN-PRODUCING NEURONS IN RATS

Hypothalamus is central to the regulation of autonomic functions. Therefore, in order to investigate the projections of ghrelin-producing neurons, it is useful to obtain information regarding the neuronal circuit of autonomic function. Ghrelin immunohistochemical staining is performed using the avidin–biotin–peroxidase complex (ABC) system, for example, the VECTASTAIN ABC-PO kit (Vector Laboratories Inc., Burlingame, CA). In this section, we briefly introduce an immunohistochemical method for use in porcine hypothalamus (Fig. 7.3). Other researchers have demonstrated the presence of ghrelin-producing neurons in the hypothalami of rats treated with colchicine (Canpolat et al., 2006; Kojima et al., 1999; Mondal et al., 2005).

1. Immerse the porcine hypothalamus in 4% paraformaldehyde solution overnight.
2. Immerse the sample in a series of 10%, 20%, and 30% sucrose solutions with 10% alabia gum every 24 h.
3. Embed the tissues in OCT compound (Tissue-Tek Miles, Elkhart, IN).
4. Cut sections to a thickness of 20 μm using a cryostat (CM 3050S; Leica Microscopy and Scientific Instruments Group, Heerbrugg, Switzerland) and mount them on Matsunami adhesive-coated slides (Matsunami, Osaka, Japan).
5. Dry the sections at 37 °C for 30 min.
6. Wash the sections in 10 m*M* PBS (pH 7.4).
7. Pretreat the sections with 3% hydrogen peroxide in methanol for 10 min to block endogenous peroxidase activity.
8. Treat the sections with 0.01% saponin in PBS for 20 min.
9. After rinsing with PBS, treat the sections with 3% normal goat serum for 1 h.
10. Incubate the sections in polyclonal rabbit anti-ghrelin antibody (no. 6-6; dilute 1:80,000) for 16 h at 4 °C.
11. Rinse the sections with PBS.

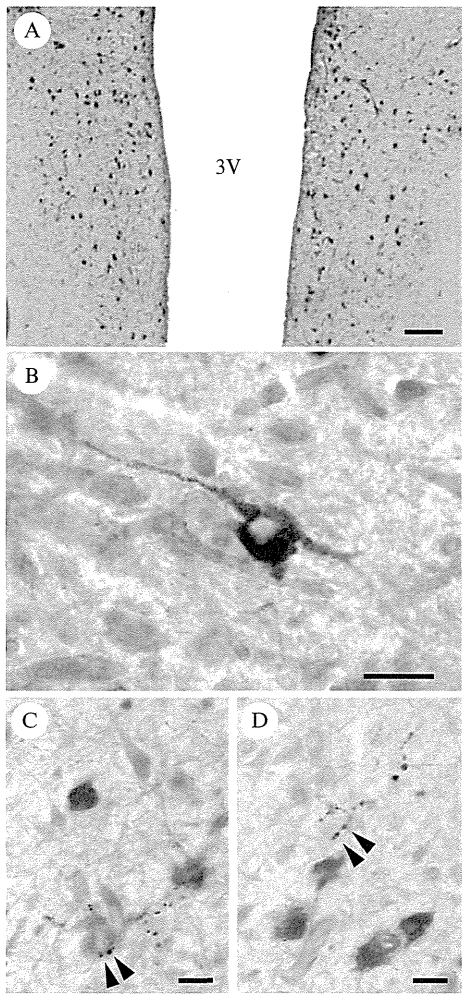

Figure 7.3 Localization of ghrelin-immunopositive neurons in the porcine hypothalamus. (A) Ghrelin neuron distribution in the paraventricular nucleus. (B) A ghrelin-producing neuron in paraventricular nucleus. A subset of ghrelin-positive neurons projected to cell bodies of either additional ghrelin-positive neurons (C, arrowheads) or ghrelin-negative neurons (D, arrowheads). 3V, Third ventricle. Bar, 200 μm (A), 20 μm (B–D) [Sato et al., 2005].

12. Incubate the sections with biotinylated anti-rabbit IgG for 40 min.
13. Rinse the sections with PBS.
14. Incubate the sections with VECTASTAIN ABC Reagent for 1 h.
15. Rinse the sections with PBS.

16. Develop the samples in 3,3′-diaminobenzidine using the Dako liquid diethylaminobenzidine substrate-chromogen system (Dako, Kyoto, Japan).
17. Enclose the samples with cover glass, and image.

6. SUMMARY

Analysis of hypothalamic ghrelin secretion is important to our understanding of ghrelin function. The wide distribution of GHS-R led us to propose a range of functions for ghrelin in the central nervous system. HPLC analysis is useful for the detection of hypothalamic ghrelin. However, the analysis of hypothalamic ghrelin of rats requires meticulous attention to details, because the concentration of hypothalamic ghrelin is low. Therefore, we recommend the combined use of HPLC analysis and real-time PCR analysis to accurately measure hypothalamic ghrelin synthesis and secretion.

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Research report

Apelin-13 microinjection into the paraventricular nucleus increased sympathetic nerve activity innervating brown adipose tissue in rats

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ABSTRACT

The aim of present study is to clarify the role of apelin in regulating energy homeostasis in brown adipose tissue (BAT). We examined the central effects of apelin-13 on the brain c-fos like immunoreactivity (c-FLI), BAT temperature and the activity of the sympathetic nerve activity innervating BAT in rats. In the hypothalamus, central infusion into the third cerebral ventricle (i3vt) of apelin-13 caused induction of c-FLI in the paraventricular nucleus (PVN) compared with the controls (PBS-treated) group. In addition, microinjection of apelin-13 into the PVN produced significant increases in BAT temperature. Furthermore, microinjection of apelin-13 treatment increased BAT sympathetic nerve activity compared with controls.

We conclude that apelin-13 microinjection into PVN increases sympathetic nerve activity innervating BAT.

1. Introduction

Apelin is a peptide ligand of the previously described orphan G protein coupled receptor APJ [12,27,29]. This receptor is expressed in the central nervous system, including the paraventricular nucleus (PVN) of the hypothalamus and supraoptic nucleus [12,27,29] and the recent reports reveal a widespread brain distribution of apelin synthesizing neurons involved in energy homeostasis [20,21]. Little is known of the physiological role of apelin although some studies indicate a role in blood pressure regulation [27] and the central control of body fluid homeostasis [20,21,26]. Apelin-13 also released the appetite inhibitory peptide cholecystokinin from dispersed intestinal endocrine cells, and central infusion of apelin-13 reduced food intake [25] and involved in obesity and related metabolic disorder [3,8,11]. Brown adipose tissue (BAT) plays a major role in energy expenditure and thermogenesis via the function of uncoupling protein 1. The regulation of energy expenditure in BAT is under the control of the several factors [14,15]. Discrete hypothalamic nuclei and various neuropeptides regulate it by affecting efferent sympathetic nerve activity [30–32].

Therefore, it is high probable that apelin-13 regulates adiposity by influencing both energy intake and energy expenditure. However, very little is known of the central function of apelin-13 that is one of the activated forms of apelin, in modulating energy expenditure. To address this, this study examined the effects of the apelin microinjection on electrophysiological sympathetic nerve activity innervating BAT.

2. Materials and methods

Mature male Sprague-Dawley rats (8–10 weeks old; Seac Yoshitomi, Fukuoka, Japan) were maintained in a 12:12 h light:dark photoperiod (lights on at 0700 h) in a temperature (21 ± 1 °C) and humidity (55 ± 5%) controlled room. They were allowed free access to food (rodent chow No. CE-2, Clea Japan, Tokyo, Japan) and water. All studies were conducted in accordance with Oita Medical University Guidelines, which are based on the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

Apelin-13 (Peptide Institute, Osaka, Japan) was dissolved in phosphate-buffered saline (PBS) to a concentration of 1×10^{-4} M and 1×10^{-5} M (adjusted pH 6.4–7.2.). CRH (Sigma Chemical Co., St. Louis, MO) was used as a positive control. Solutions were prepared on the day of administration. The dose of apelin-13 was based on our preliminary study and previous studies [13,26]. Under pentobarbital anesthesia, a stainless steel guide cannula was implanted into the third ventricle or unilaterally into a discrete hypothalamic region. The tip of the cannula was located 1 mm above the hypothalamic region being studied. The stereotaxic coordinates of the injection sites (Ant, anterior; Lat, lateral) were as follows. PVN: Ant = 2.0; Lat = 0.4; Height = 7.8. Details of the surgical procedures are described elsewhere [18,22,31].

To prevent stress-induced c-fos expression on the test day, rats were regularly handled during recovery from surgery. On the test day, rats were given i3vt infusion of apelin-13 (1 nmol) or PBS ($n = 4$). Then 1.5 h after injection, rats were anesthetized with Nembutal (3.3 ml/kg ip) and transcardially perfused with isotonic PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and post-fixed for 24 h and then processed for c-FLI. Forty-micrometer slices were cut from the brain with a microtome. Forebrain slices were made in the coronal plane to allow visualization of the central nucleus of the various nuclei of the hypothalamus [the PVN, ventromedial nucleus (VMH), arcuate nucleus (ARC), and lateral hypothalamic area (LHA)]. Tissues were rinsed (3× PBS), incubated for 1 h in 0.3% H_2O_2 in

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absolute methanol to quench endogenous peroxidase, and rinsed (3× PBS). Slices were then transferred without rinsing to the primary antibody solution, consisting of 0.005 g/ml polyclonal rabbit anti-serum (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), which recognizes residues 3–16 of the c-Fos protein. After 24 h of incubation on ice, slices were rinsed (3× PBS) and processed with the ABC method (Vector Laboratories, Burlingame, CA). Slices were transferred to biotinylated goat antirabbit antibody for 1 h, rinsed, transferred to avidin-biotinylated peroxidase for 1 h, rinsed, and developed with diaminobenzidine substrate (6 min). Slices were rinsed, mounted on slides, and coverslipped with Permount. Camera lucida drawings of c-Fos-positive brain structures were prepared by an experimenter naive to group treatments. Care was taken so that structures were scored in approximately the same plane. Drawings were scored by blinded raters who recorded the number and location of c-Fos-positive nuclei (Olympus Corp. Optical Co. Ltd., Tokyo, Japan). Scores across raters were averaged for statistical analyses. Microinjection of apelin-13 (0.1 nmol) was infused via a 30-gauge infusion cannula that was lowered to 1 mm below the tip of the guide cannula. Apelin-13 was infused into the PVN (0.1 nmol) over 30 s. Controls were infused with PBS alone.

A plastic-coated thermocouple was inserted into the interscapular BAT of 8 additional rats, each with a central cannula, under anesthesia (urethane, 0.8 g/kg; α-chloralose, 80 mg/kg). The temperature was measured at 10-min intervals for 60 min after apelin-13 or PBS was infused.

BAT was dissected 1.5 h after central apelin-13 treatment. BAT UCP1 mRNA was amplified by PCR and quantified using real-time quantitative PCR as follows. Total cellular RNA was prepared from BAT tissues using TRIzol (Lifetech, Tokyo, Japan) according to the manufacturer's protocol. Total RNA (20 μg) was electrophoresed on 1.2% formaldehyde agarose gels. RNA quality and quantity were assessed using EtBr agarose gel electrophoresis and by measuring the absorbance at 260 nm relative to that at 280 nm. cDNA was synthesized from total RNA (150 ng) in a volume of 20 μl using a ReverTra-Dash reverse transcriptase kit (Toyobo, Tokyo, Japan) with random hexamer primers. The reactions were diluted to 50 μl with sterile distilled water and stored at −20 C. Primers were designed, synthesized, optimized, and obtained as preoptimized kits: UCP1 (catalog no. Mm00494069m1). Primers for ribosomal RNA for use as an internal control was also obtained as a preoptimized kit (catalog no. Hs99999901). These preoptimized kits were purchased from Applied Biosystems (Foster City, CA). Using an ABI PRISM 7000 sequence detector (Applied Biosystems), PCR amplification was performed in 50-μl volumes containing 100 ng cDNA template in PCR Master Mix (Roche, Nutley, NJ), according to the following protocol: 50 d for 2 min, 95 C for 10 min, and 40 cycles at 95 C for 15 s and 60 C for 1 min. Samples were analyzed in duplicate. Target mRNA amounts were normalized to ribosomal RNA. In brief, target genes and ribosomal RNA values were calculated from standard curves obtained by amplification of 2-fold serial dilutions of cDNA from the tissue. We verified that the cDNAs and ribosomal RNA were amplified at approximately the same efficiency. Results are expressed as the percent of ribosomal RNA-normalized target mRNA in experimental groups vs. control groups. The results were analyzed using sequence detection software (Applied Biosystems), as outlined in PerkinElmer's user bulletin no. 2 (PerkinElmer, Wellesley, MA).

After being allowed 10 days to recover from surgery, experiments were carried out under anesthesia (urethane, 0.8 g/kg; α-chloralose, 80 mg/kg). After dissection of the fine branches of the intercostal nerves that supply the interscapular BAT, the nerves were transected where they entered the IBAT. Electrical discharges were recorded from fine filaments placed proximal to the transaction site using bipolar tungsten wire electrodes immersed in heavy white mineral oil to prevent dehydration of the nerves. Discharges were amplified through a condenser-coupled differential input preamplifier and fed into a window discriminator to differentiate signals from background noise. The number of pulses was integrated over 5 s. During stable periods of neuronal activity, the method used to record nerve has been described elsewhere [33].

Differences among groups were assessed using ANOVA with repeated measures and the Dunnett test for multiple comparisons. A two-sided p value of less than 0.05 was considered statistically significant.

3. Results

Data of the number of c-FLI-positive cell in hypothalamic nuclei are shown in Fig. 1 and representative photomicrographs of PVN are presented in Fig. 1A. In the hypothalamus, administration of apelin-13 to rats caused induction of c-FLI after 1.5 h in the PVN (Fig. 1A; $p < 0.01$) but not the ARC, VMH and lateral hypothalamic area (Fig. 1B; $p > 0.1$) compared with the controls group.

Administration of apelin-13 into the PVN caused a rapid, significant elevation in BAT temperature compared with the controls (Fig. 2; $p < 0.05$ or $p < 0.01$). It reached a nadir about 30 min after the infusion and gradually recovered. In addition, BAT UCP1 expression was increased after apelin-13 treatment compared with the controls (Fig. 2B; $p < 0.05$).

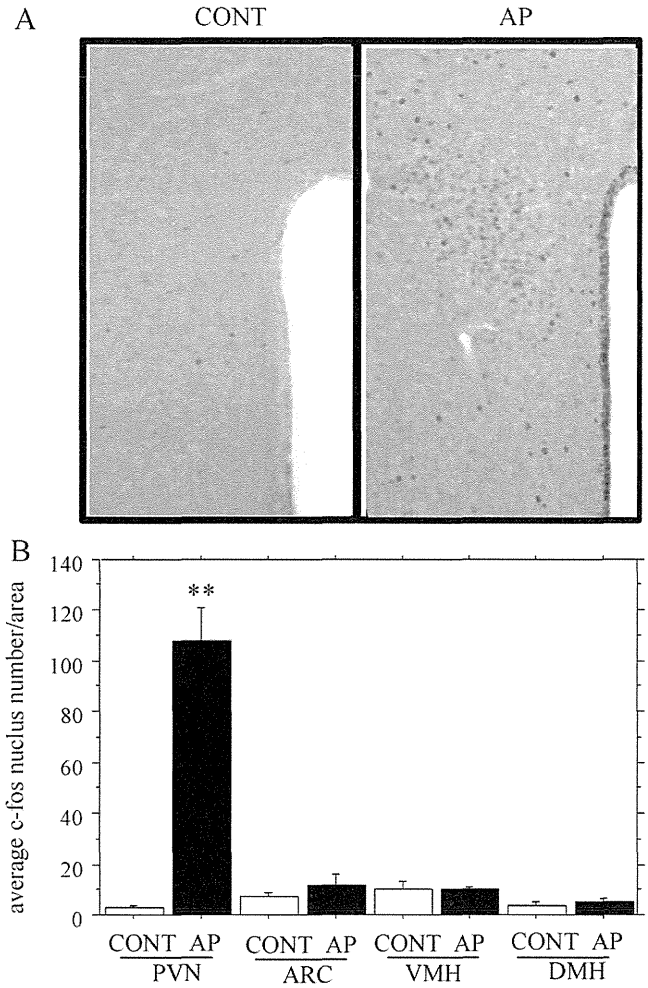

Fig. 1. Effects of central infusion of apelin-13 (AP) or PBS (CONT). Representative photomicrographs of c-FLI in PVN (A). Effects of central infusion of AP or CONT on c-FLI in the hypothalamus (B). The columns represent the mean ± SEM. All data are the mean ± SEM ($n = 4$ per group). $^{**}p < 0.01$ vs. control.

As a positive control, central infusion of CRH caused a significant elevation in BAT sympathetic nerve activity (Fig. 3A). Fig. 3B demonstrated the responses of BAT sympathetic nerve activity in response to the administration of apelin-13 into the PVN. Sympathetic nerve activity significantly increased after the administration of apelin-13 into the PVN. This response reached a nadir about 30 min after the infusion and gradually recovered. The mean changes in sympathetic nerve activity in response to apelin-13 treatment were statistically different to those in the control groups (Fig. 3C; $p < 0.05$ or $p < 0.01$).

4. Discussion

In this study, we firstly investigated the effect of centrally administered apelin-13 on c-FLI in the hypothalamus. The results showed that apelin-13 treatment caused induction of c-FLI in the PVN. Previous studies also demonstrated that expression of apelin receptor mRNA was found in the hypothalamic paraventricular nucleus (PVN) [16,17]. BAT is densely innervated by the sympathetic nervous system from the hypothalamus. Among the aforementioned hypothalamic nuclei, a neuroanatomical study using a trans-synaptic retrograde tracer identified the PVN as the origins of the sympathetic nerves that innervate BAT [2]. The PVN projects sympathetic preganglionic neurons directly to the intermediolateral cell column of the spinal cord [23]. Several studies

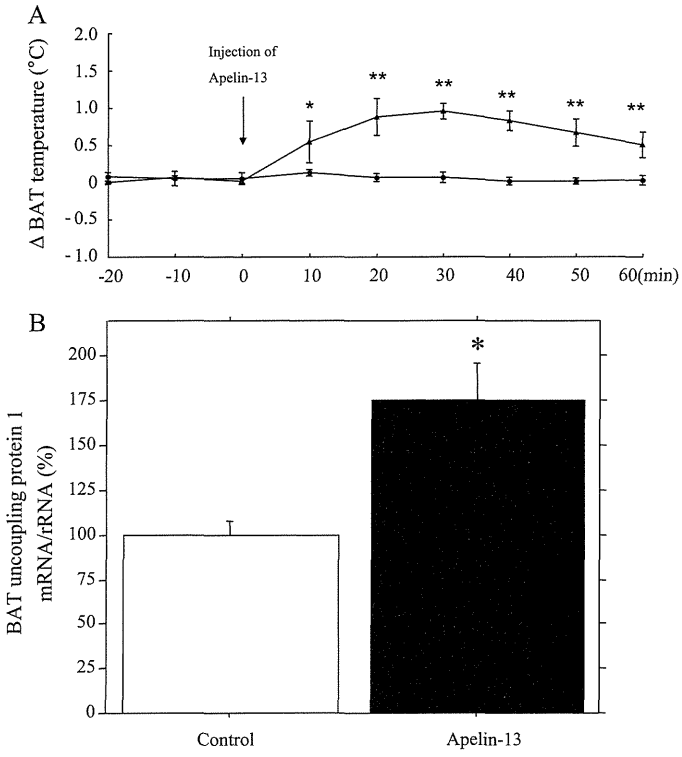

Fig. 2. Effects of into the paraventricular nucleus (PVN) infusion of apelin-13 (0.1 nmol) or PBS (control) on BAT temperatures (A). Effects of central infusion of apelin-13 on uncoupling protein 1 mRNA/rRNA (B). All data are the mean ± SEM ($n = 4$ per group). ▲ = apelin-13-treated animals. ● = control. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

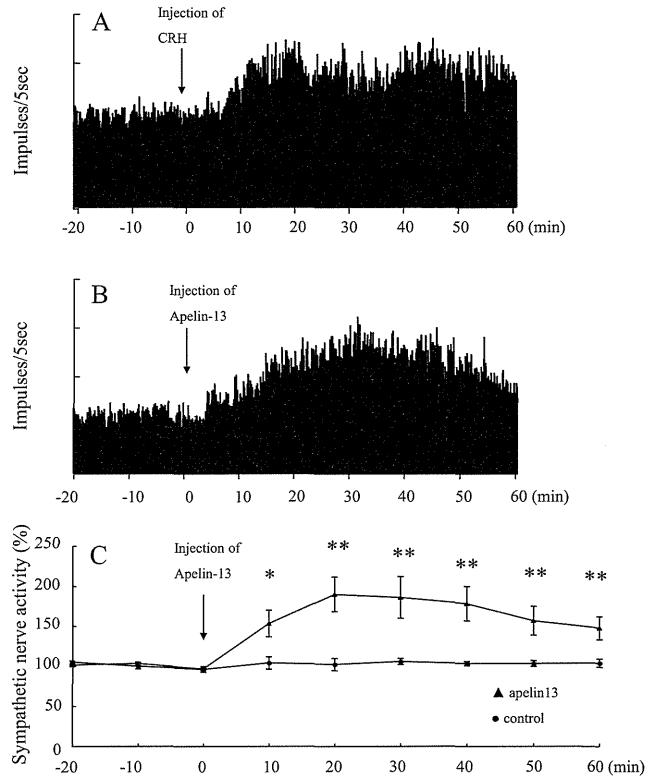

Fig. 3. Rate meter plots of BAT sympathetic nerve activity following central infusion of CRH (A). Rate meter plots of BAT sympathetic nerve activity following administration into the paraventricular nucleus (PVN) infusion of apelin-13 (A). Vertical axis: nerve impulses per 5 s. Horizontal bars: 10-min time scale. Percentage differences in sympathetic nerve activity from baseline (100%) after infusion of apelin-13 or PBS (control) (C). All data are the mean ± SEM ($n = 4$ per group). ▲ = apelin-13-treated animals. ● = control. $^{*}p < 0.05$, $^{**}p < 0.01$ vs. control.

demonstrated that the PVN a key area in the brain involved in influencing endocrine function and inflammation [7,19]. In addition, the PVN is the most suitable hypothalamic nuclei to examine the effects of central infusion on BAT sympathetic nerve activity.

Next, it is important to note whether the administration of apelin-13 into the PVN actually affects energy expenditure or non-shivering thermogenesis in BAT. BAT uncoupling protein 1 (UCP1) is under the control of the sympathetic nervous system. The administration of a β_3 agonist increases UCP1 mRNA expression in BAT [34]. In fact, we found that microinjection of apelin-13 treatment increased BAT temperature and BAT UCP1 mRNA expression similar to that seen in the sympathetic nerve activity.

It has previously been reported that central administration of apelin-13 reduced food intake [25] and increased drinking [26]. Sympathetic nerve activity and food intake are reciprocally related in a number of experimental settings, including hypothalamic lesions, injection of peptides, or treatment with several drugs. Neuropeptide Y (NPY) increases food intake when injected into the third cerebroventricle [6] and decreases BAT sympathetic nerve activity [9]. The effects of corticotropin-releasing hormone (CRH) are opposite to those of NPY. Injection of CRH into the third cerebroventricle decreases food intake [1] and increases BAT sympathetic nerve activity [10]. In this study, apelin-13 increases BAT sympathetic nerve activity. It is consist with previous studies suggested that increase food intake are usually associated with low levels of sympathetic activity and vice versa [4,5].

Recent studies shown that apelin-13 microinjected into the nucleus tractus solitus and the rostral ventrolateral medulla increased arterial pressure [24]. On the other hand, it was found that mean arterial pressure after the administration of apelin-13 in anesthetized rats was reduced [28]. It is unknown which factor induces the different response among these arterial pressure. We speculated that there was the possibility that some type of receptors modulate it. In the present study, central infusion of apelin-13 caused induction of c-fos only in the PVN. It is possibility that PVN is a most sensitive area for induction of c-fos by apelin-13 treatment. However, further studies are needed to clarify the point.

In conclusion, we demonstrated that administration of apelin-13 into the PVN increased BAT sympathetic nerve activity. The reciprocal effects of apelin-13 in the regulation of energy balance may contribute to the homeostatic control of energy metabolism.

Conflict of interests

All authors have no conflict of interest.

Acknowledgments

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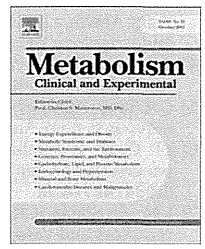

Association between hippocampal volume and serum adiponectin in patients with type 2 diabetes mellitus

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ABSTRACT

Type 2 diabetes mellitus (DM) is associated with cognitive dysfunction and hippocampus volume. The aim of the present study was to test the hypothesis that the level of the adipocytokine adiponectin correlates with hippocampus volume and insulin resistance in patients with type 2 DM. A total of 45 patients with type 2 DM were divided into two groups: a low adiponectin group and a normal adiponectin group. Hippocampus volume was measured by computer-assisted analysis using a magnetic resonance imaging (MRI) voxel-based specific regional analysis system developed for the study of Alzheimer's disease as the end point for assessment of hippocampus volume. Mean hippocampus volume was lower in the low adiponectin group than in the normal adiponectin group ($P < .0001$). Fasting serum concentrations of glucose ($P < .05$) and insulin ($P < .0001$), and homeostasis model assessment index ($P < .0001$), were all higher in the low adiponectin group than in the normal adiponectin group. Multiple regression analysis showed that hippocampus volume independently predicted serum adiponectin level. These results suggest that circulating levels of adiponectin are related to hippocampus volume in patients with type 2 DM.

1. Introduction

Memory impairment and cognitive dysfunction have been linked to type 2 diabetes mellitus (DM) [1,2]. In addition, the pathogenesis of type 2 DM is related to dementia and Alzheimer's disease (AD) [3]. Hippocampus volume has been measured by computer-assisted analysis using a magnetic resonance imaging (MRI) voxel-based specific regional analysis system developed for the study of Alzheimer's disease (VSRAD), which yields a Z-score as a measure of hippocampal volume [4,5].

The adipocyte-derived protein adiponectin appears to be strongly associated with insulin sensitivity, risk of diabetic complications, liver disease and coronary artery disease [6,7]. Accumulating evidence shows that adiponectin has a number of vasculoprotective qualities such as insulin sensing and anti-inflammatory and anti-atherogenic effects [6-12]. In addition, it plays roles in the pathogenesis of multiple sclerosis and Alzheimer's disease [13,14].

The results of these studies suggest the existence of a relationship between adiponectin and hippocampus volume. However, no studies have examined such a relationship in

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