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ORIGINAL ARTICLE

GnRH secretion is inhibited by adiponectin through activation of AMP-activated protein kinase and extracellular signal-regulated kinase

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Abstract Adipokines produced from adipose tissues participate in regulation of reproduction, energy homeostasis, food intake, and neuroendocrine function in the hypothalamus. We have previously reported that adiponectin significantly reduced GnRH secretion from GT1-7 hypothalamic GnRH neuron cells. In this study, we further investigated the inhibition of GnRH secretion by adiponectin in vivo and found that extracellular signal-regulated kinase (ERK) was inhibited and AMPK activated. Furthermore, we found that activated AMPK by adiponectin reduced ERK phosphorylation, which possibly impaired GnRH secretion in GT1-7 cells.

Keywords Adiponectin · GnRH · AMPK · MAPK

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Introduction

Obesity is associated with a diverse set of metabolic disorders and reproductive disorders which poorly understood. Adipose tissues participate in regulation of feeding, thermogenesis, and reproduction through adipokines [1]. Leptin deficient (ob/ob) mice show obese and infertile. It is also noted that serum adiponectin levels are reduced in obesity. However, it is rarely investigated whether adiponectin participates in the regulation of reproduction. Adiponectin (also known as Acrp30) is specifically secreted from adipose tissues into the circulation [2]. It participates in the regulation of food intake [3], and glucose and lipid homeostasis [4]. Adiponectin consists of an N-terminal collagenous domain and a C-terminal globular domain [5]. Adiponectins circulate in the blood in distinctly stable forms including trimers, low-molecular-weight hexamers and high-molecular-weight multimeric complexes [5].

Recently, two adiponectin receptors, adiponectin receptor 1 (AdipR1) and adiponectin receptor 2 (AdipR2) that contain seven-transmembrane domains, were identified [6]. Initial studies showed that AdipRs were widely expressed in skeletal muscles, the heart, the liver, and the brain[6], including paraventricular nucleus (PVN) [7], amygdale, area postrema (Ap) [8], and even diffusely distributed in the periventricular areas and the pituitary [6, 9]. AdipR1 and AdipR2 were also expressed in GT1-7 cells [10] and in POMC and NPY neurons [11]. Adiponectin is increased in cerebrospinal fluid (CSF) after intravenous injection, indicating a blood–brain transport [12].

The hypothalamic/pituitary/gonadal axis is central to mammalian reproductive system. Pulsatile secretion of gonadotropin-releasing hormone (GnRH) from hypothalamus neurons stimulates the release of luteinizing hormone

(LH) and follicle-stimulating hormone from gonadotropes in the anterior pituitary. Several studies showed that adiponectin inhibited the release of LH in short-term-treated rat pituitary cells [9] and mouse LβT2-immortalized gonadotropic cells [13]. Other studies showed that serum adiponectin concentrations alter in men from childhood to adulthood in correlation with serum androgen levels [14] and were elevated in patients with hypogonadotropic hypogonadism (IHH) [15] and anorexia nervosa [16], in which GnRH pulses are out of order. In this study, we investigated the molecular mechanism of adiponectin inhibition of GnRH secretion.

Materials and methods

Animals and surgery

Male Sprague–Dawley rats (200–250 g) were purchased from Shanghai Slac Laboratory Animal Co. Ltd (Shanghai, China). The rats were acclimated for 1 week to a 12-h light and dark cycle at 22°C before experimentation. Rats were housed individually and fed with standard chow ad libitum. Left and right jugular veins were cannulated with SILASTIC tubing (Dow Corning, Midland, MI, USA) under general anesthesia with 2.5% pentobarbitone. The tubing was coiled under the skin, and exteriorized at the back of the neck. Each tube was sealed with polyvinyl pyrolidon (6 g in 5 ml of 1,000 IU/ml of heparinized saline). In 2–3 days after jugular veins cannulation, a L-shaped cannula (ALZET brain infusion kit 2, Alza, Palo Alto, CA, USA) was stereotactically implanted into the right lateral ventricle (0.8 mm posterior, 1.5 mm lateral to bregma, and 4.0 mm below the dura). The cannula was adhered to the skull with dental cement which was used to secure the cannula. A 5-cm length of catheter tubing was used to attach the cannula to allow infusion with a calibrated 10 µl Hamilton syringe. The animals were allowed to recover for 7 days at maximum before experimentation. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the Ruijin Hospital.

LH pulse measurements

The rats were fitted with a 30-cm length silica gel catheter jointed to each indwelling cannula via the dual jugular veins and the distal end of the cannula was attached to a fluid swivel, which allowed the rats to freely move around the enclosure. About 200 µl of blood samples was taken every 5 min from one catheter using 1-ml syringe for 5 h. About 100 µl plasma was collected after centrifugation and red blood cells resuspended with an equal volume of heparinzed saline (1 ml saline with 1,000 IU heparin) were replenished into the rats via the other catheter. Plasma samples were frozen at −20°C for late LH assays. After 2 h of sampling, the rats were injected i.c.v. with 5 µg adiponectin (R&D systems, Minneapolis, MN, USA) ($n = 4$) or PBS vehicle ($n = 3$) in a total volume of 6 µl using a calibrated 10 µl Hamilton syringe without disturbing the rats during the experimentation. Blood sampling continued for another 3 h after i.c.v. infusion.

GT1-7 cell culture and GnRH secretion assay

The hypothalamic GT1-7 cells were kindly provided as a gift from Dr. Ernst Knobil at University of Texas-Houston Medical School (Houston, TX, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (GIBCO, Laboratories, Grand Island, NY, USA), 100 µU/ml penicillin, and 100 µg/ml streptomycin at 37°C in an atmosphere of 7% CO_2. GT1-7 cells were seeded in six-well plates at a density of 2×10^5 cells/well. Compound C (20 µM, an inhibitor of AMPK) and U 0126 (10 µM, an inhibitor of extracellular signal-regulated kinase (ERK)) were added to GT1-7 cells 1 h before adiponectin treatment. The cells were harvested for immunoblotting assay 30 min later after adiponectin or AICAR (an activator of AMPK) treatment.

For GnRH secretion assay, GT1-7 cells were placed in 24-well plates at a density of 2×10^5 cells/well. In 2–3 days, the complete medium was replaced by DMEM supplemented with 0.2% BSA (serum-free medium) for 12–16 h before the experimentation. The medium was discarded and replaced with 200 µl of Locke's medium, including 154 mM NaCl, 5.6 mM KCl, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 6 mM $NaHCO_3$, 10 mM glucose, 2 mM Hepes, and 20 µM bacitracin (Sigma, Fluka Chemie, USA). The supernatants were harvested in 30 min after adiponectin treatment at concentrations of 1.0 µg/ml and frozen at −80°C for GnRH assay.

AMPKα1 siRNA

In order to investigate AMPK and ERK signaling in GT1-7 cells, small interfering RNA (siRNA) duplex oligonucleotides for *AMPK α1* were adopted to treat GT1-7 cells at a final concentration of 100 nM using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Cell lysates were extracted for AMPK and ERK immunoblotting at 72 h after transfection.

Western blotting

Adiponectin (5 µg) or AICAR (6 µg) were injected into lateral ventricle using a calibrated 10 µl Hamilton syringe.

The hypothalamus was dissected in 2 h after injection and stored in liquid nitrogen for late immunoblotting assay. The hypothalamus tissues and GnRH cells were lysated at 4°C in radioimmunoprecipitation buffer containing 1 mM phenylmethylsulfonyl fluoride, 1:1,000 phosphatase inhibitor and 1:2,000 protease inhibitor, then centrifuged at 15,000×g for 15 min. Protein concentrations were determined by BCA method (Pierce, Rockford, IL, USA). 50 μg of proteins were boiled in Laemmli sample buffer for 10 min, loaded on 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked in TBST (TBS, PH7.4 with 0.05% Tween) with 10% non fat milk for 2 h and then incubated with specific primary antibody against phosphorylated-AMPK, total-AMPK, phosphorylated-ERK1/2, and total-ERK1/2 (all for 1:1,000 dilution) (Cell Signaling Technology, Beverly, MA, USA). The blots were washed in TBST and then incubated with HRP-conjugated secondary antibodies. The immunoblotting was visualized with ECL-plus kit (Amersham Pharmacia Biotech, USA).

LH and GnRH measurement

Plasma LH concentrations were determined in a singleton using competitive ELISA kit (Adlitteram Diagnostic Laboratories, San Diego, CA, USA). The average intra-assay and inter-assay coefficients of variation were 4.7 and 6.8%, respectively. The sensitivity of measurement is 0.1 ng/ml. GnRH concentrations in the supernatants were measured using ELISA kit (BACHAM, AG, and USA) according to the manufacture's protocol. The sensitivity of the assay is 8 pg/ml. Intra-assay and inter-assay coefficients of variation were 6.4 and 11.2%, respectively.

Statistical analysis

The quantitative values were represented as mean ± SEM. The parameters of LH secretion analyzed in rat pulse experiment were the mean plasma concentrations of LH; the amplitude of LH pulses, calculated as the difference between the peak and preceding nadir of a LH pulse; the frequency of LH pulses, expressed as the number of LH pulses per hour; and total LH output, which was the product of the amplitude multiplied by the number of pulses per hour and expressed as ng/ml/h. Pulses of LH were identified according to an accepted definition [17, 18]. Unless otherwise noted, data were analyzed by ANOVA followed by Tukey post hoc tests. Individual pair-wise comparisons were performed using paired t test. $P < 0.05$ was considered statistically significant.

Results

Adiponectin suppressed LH pulses in male rats

The LH pulse profiles showed that the means of plasma LH concentrations were significantly reduced after i.c.v. infusion of adiponectin (5 μg) as compared with either the PBS control or the baseline levels (Fig. 1). However, the mean number of LH pulses per hour was not significantly different between adiponectin or PBS treated rats (Fig. 1d).

Adiponectin activates AMPK and inhibits ERK in hypothalamus and GT1-7 cells

Phosphorylated AMPK was markedly increased in hypothalamus after i.c.v. injection of either adiponectin or AICAR, whereas phosphorylated ERK was greatly reduced (Fig. 2). Phosphorylated ERK was also markedly reduced in GT1-7 cells treated with adiponectin in a time course (Fig. 3a). Whereas, phosphorylated ERK was dramatically increased by compound C (20 μM), an AMPK inhibitor (Fig. 3b, c). ERK phosphorylation was also dramatically increased while treated with AMPK α1 siRNA (Fig. 3d).

Adiponectin reduces GnRH secretion from GT1-7 cells via activation of AMPK

GnRH secretion was markedly reduced in GT1-7 cells treated with adiponectin (1.0 μg/ml) for 30 min (Fig. 4). However, GnRH secretion was dramatically increased by AMPK inhibitor comp C (20 μM) (Fig. 4).

Discussion

Adipose tissues play a crucial role in energy homeostasis, not only in storing triglycerides, but also in response to nutrients, neural, and hormonal signals through adipokines secretion that control feeding, thermogenesis, immunity, neuroendocrine function, and reproduction [1]. It is well known that leptin plays a role in regulation of energy homeostasis and reproduction as a mediator in the cross-talk between adipose tissue and the brain [1]. Recently, several studies demonstrate that adiponectin also participates in the regulation of energy expenditure, thermogenesis [12], and food intake in the hypothalamus [3], even in the pituitary function [9]. It has been reported that serum adiponectin levels are inversely correlated with androgen in humans [14] and adiponectin inhibits both basal and GnRH-stimulated LH secretion in short-term treated rat pituitary cells [9]. Those studies suggest a potential role of adiponectin participating in the regulation of neuroendocrine reproductive processes [9, 13].

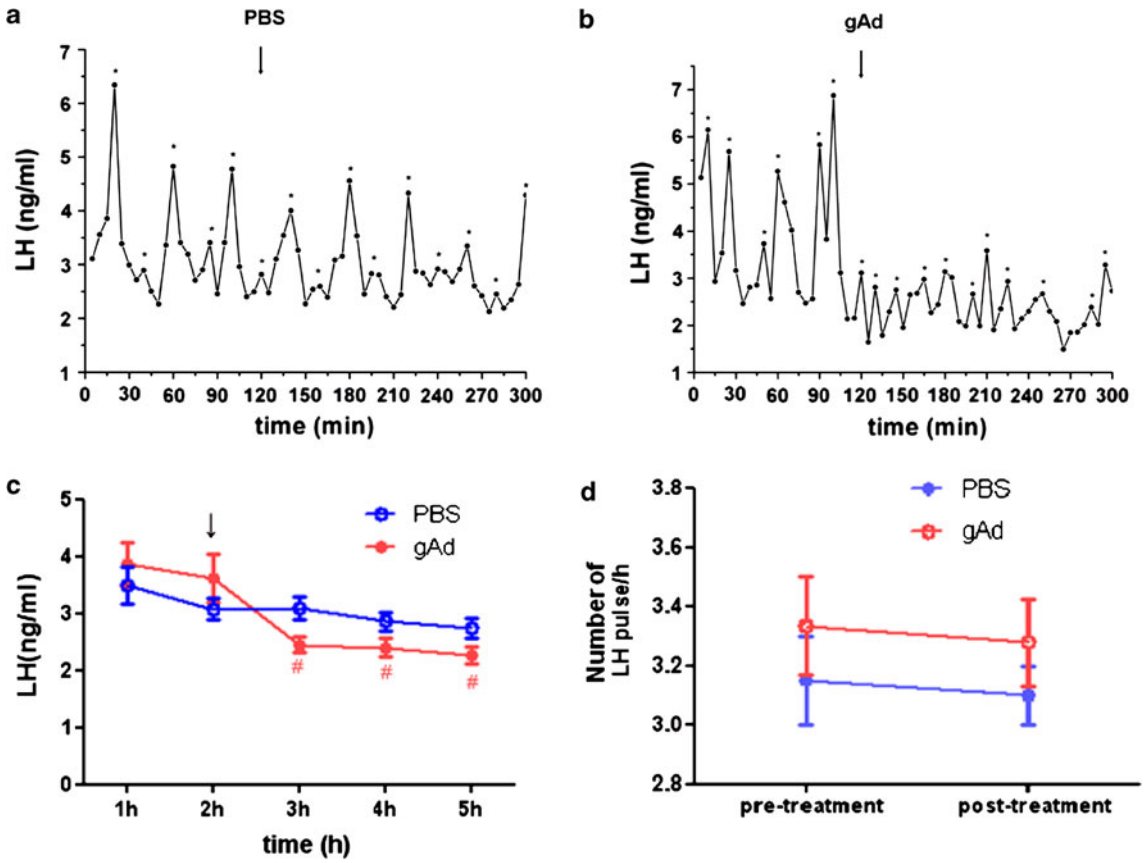

Fig. 1 Pulsatile LH secretion profiles. Plasma LH levels were recorded in male rats with i.c.v. administration of *PBS* (**a**) or adiponectin (*gAd*) (**b**). The data represents one of 4 (*gAd*) or 3 (*PBS*) treated rats. Mean plasma LH concentrations(**c**) and pulse numbers (**d**) were shown. *Arrows* indicate the time of injections. Symbol # indicates $P < 0.01$

Adiponectin receptors are expressed widely in peripheral tissues and the brain [6], particularly in the PVN, amygdale, area postrema [6–8]. GnRH neurons and fibers are located in the preoptic area–anterior hypothalamus, amygdale, and median eminence of rodents. These areas or nucleus above all surround the third ventricle in microscopic anatomy [19, 20]. It was reported that adiponectin can be detected in CSF even though it is 1,000-fold lower than in serum [7, 21]. GT1-7 cells, a subclone of GT1 cell lines developed by targeting expression of the potent oncogene, SV40 T-antigen, with the regulatory region of GnRH gene, have been shown to faithfully exhibit a mouse-derived clone of immortalized GnRH-secreting neurons [22]. We have previously reported the presence of leptin, orexin, glucagons-like peptide 1 (GLP1), melanin-concentrating hormone 1 receptors and AdipR1 and AdipR2 in GT1-7 cells [10, 23]. Kos et al. [7] also reported adiponetin receptor expression in the human hypothalamus, especially in the PVN; Guillod-Maximin et al. [11] found that AdipoR1 and AdipoR2 were expressed in POMC and NPY neurons in the arcuate nucleus. Those results are supportive of an adiponectin action in the brain. We have investigated whether adiponectin, like leptin, regulates hypothalamus GnRH productions.

In this study, we revealed that total and peak LH secretion was reduced in i.c.v. injected male rats (Fig. 1). We further found that phosphorylated AMPK was increased in hypothalamus with adiponectin treatment. AMPK is a master regulator of cellular and systemic energy homeostasis [24]. Adiponectin activates AMP kinase by increasing phosphorylation in skeletal muscle [25], primary adipocytes [26], and the liver [27], subsequently accelerating fatty-acid oxidation, glucose uptake in myocytes and adipocytes, and the reduction of gluconeogenesis in the liver [27]. Recently, Kubota and Guillod-Maximin have reported that adiponectin activates AMP kinase in the hypothalamus and leads to the increase of food intake [3, 11]. Coyral-Catel and colleagues [28] reported that metformin and AICAR could inhibit GnRH release from GT1-7 cells through AMPK activation and compound C completely eliminated this effect. And we have also reported that adiponectin activated AMP kinase in GT1-7 cells and led to the reduction of GnRH secretion [10]. Based on those results, it is speculated that adiponectin regulates energy balance via AMP kinase activation in response to nutritional states of whole-body and subsequently determines the release of GnRH or gonadotropin to influence reproduction.

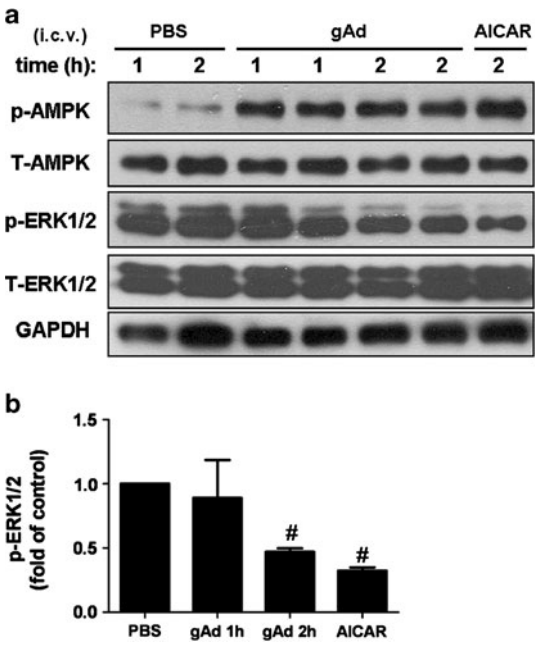

Fig. 2 Immunoblotting of AMPK and ERK in hypothalamus. **a** The rat hypothalamus was removed and dissected in 1 and 2 h, respectively, after i.c.v. administration of gAd (5 μg) or AICAR (6 μg). **b** Arbitrary densitometry of the above interest proteins was shown. The quantitation of immunoblots was indicated as the folds of the control. $n = 3$, $^{*}P < 0.05$, $^{**}P < 0.01$

Although most identified substrates of AMP kinase are metabolic enzymes which directly regulate the metabolic pathways, some studies showed that AMP kinase is also involved in ERK phosphorylation [29, 30]. The signaling pathway Ras, Raf, MEK, ERK has been known to play a significant role in the regulation of gene expression, particularly in the processes of proliferation, differentiation, and apoptosis [31]. However, some studies demonstrated that GnRH secretion was also regulated by ERK MAPK pathway [32]. Recently, Kim et al. reported that AMP kinase down-regulated ERK cascades by inhibiting Ras activation or activating Ras-independent pathway in response to varying energy states in NIH-3T3 cells [29]. Whereas Sprenkle et al. [33] found that AMP kinase could phosphorylate Raf-1 by Ser^{621}, further phosphorylate and activate ERK MAPK kinase 1 (MEK1), and ERK MAPKs in *Escherichia coli* or *Sf9* insect cells. Our study showed that AICAR-activated AMP kinase and suppressed ERK, whereas comp C (an inhibitor of AMPK) worked in an opposite way (Fig. 2a, b, c). In order to further investigate the relationship between AMP kinase and ERK pathways, we adopted AMPK α1 specific siRNA duplexs to knock down AMP kinase expression. ERK phosphorylation is markedly increased by AMPK α1

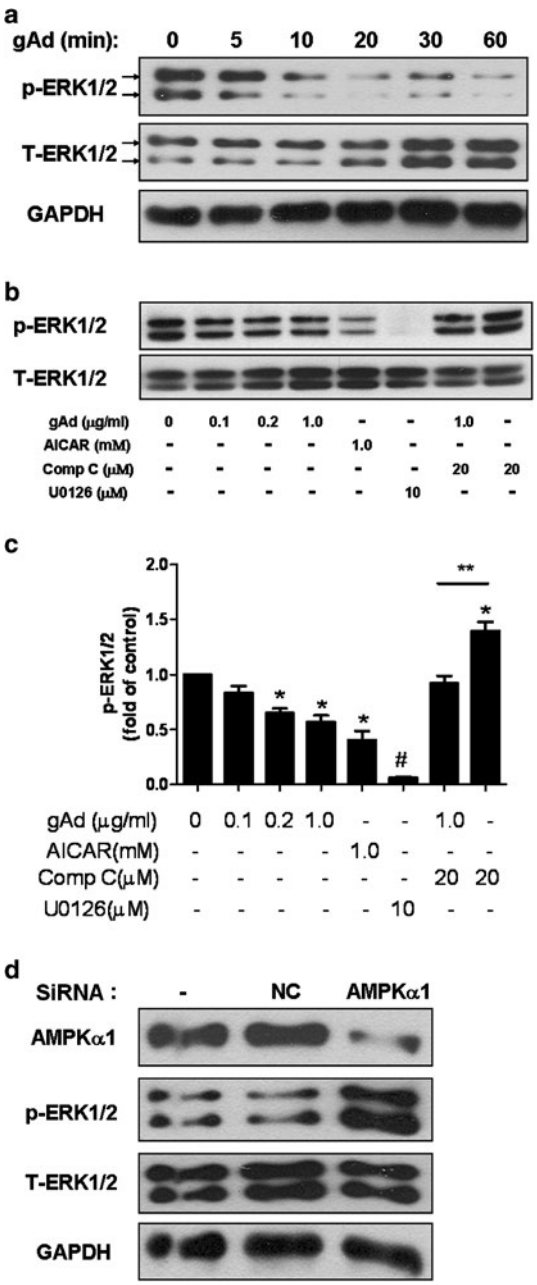

Fig. 3 Immunoblotting of phosphorylated ERK. **a** GT1-7 cells were treated with globular adiponectin (gAd) at 1.0 μg/ml for 5, 10, 20, 30, and 60 min. The phosphorylated ERK was correspondingly reduced at 20 min and thereafter. **b** Phosphorylated ERK was reduced by gAd, AICAR, and U0126 (a MEK inhibitor). Whereas, phosphorylated ERK was increased by AMPK inhibitor comp C which also reverted the inhibition induced by gAd. **c** The quantitation for ERK immunoblots was shown as the folds of the vehicle control. Arbitrary densitometric units of the interest protein were normalized for the total ERK. Quantity One Quantitation Software (Bio-Rad, Hercules, CA, USA) was used. $^{*}P < 0.05$, $^{**}0.01 < P < 0.05$, $^{\#}P < 0.01$. **d** Phosphorylated ERK was increased with AMPK α1 siRNA treatment in GT1-7 cells

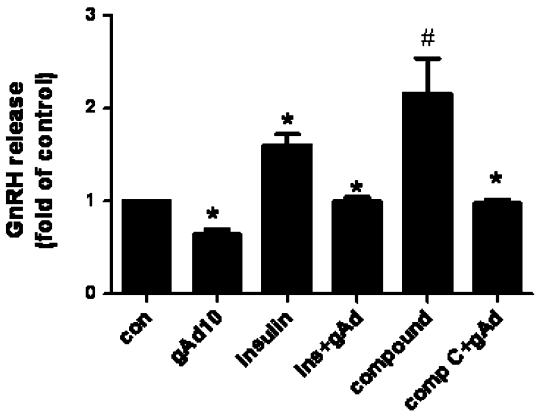

Fig. 4 GnRH secretion from GT1-7 cells. GT1-7 cells were incubated with globular adiponectin at 1.0 μg/ml, and compound C at 20 μmol/l for 30 min. GnRH concentrations in the supernatants were measured from three wells. The data are shown as mean ± SEM. The results represent one of the three experiments. $^{*}P < 0.01$, $^{\#}P < 0.05$ as compared with the vehicle

siRNA. Thus, we postulated that adiponectin inhibited GnRH secretion through activation of AMPK and inhibition of ERK pathway.

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Conflict of interest statement None.

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