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Adiponectin regulates ACTH secretion and the HPAA in an AMPK-dependent manner in pituitary corticotroph cells

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ABSTRACT

It is known that adipokines can regulate the hypothalamic–pituitary–adrenal axis (HPAA). In this study, we confirmed that adiponectin regulates the HPAA by affecting pituitary corticotroph cells. Using RT-PCR and immunofluorescence, we determined that adiponectin receptors were expressed in pituitary corticotroph tumour cells (AtT-20 cells and human corticotroph tumours). Adiponectin stimulated calcium influx and increased basal ACTH secretion without affecting corticotrophin-releasing hormone (CRH)-stimulated ACTH secretion, which was most likely due to the expression of adiponectin repressing CRH receptor 1 (CRHR1). Adiponectin also acutely stimulated ACTH release in primary culture pituitary cells. Lastly, adiponectin directly phosphorylated 5′ AMP-activated protein kinase (AMPK) in AtT-20 cells. The effects of adiponectin were mimicked by AICAR, which was blocked by compound C. Taken together, our results suggested that adiponectin stimulated ACTH secretion and down-regulated CRHR1, possibly via an AMPK-dependent mechanism in pituitary corticotroph cells.

1. Introduction

Adiponectin is one of the most abundantly secreted adipokines and is also considered an insulin-sensitising factor (Kadowaki et al., 2006; Berg et al., 2001). Adiponectin levels are low in subjects with obesity, diabetes and metabolic syndrome and are elevated in insulin-sensitive subjects (Arita et al., 1999; Weyer et al., 2001). Adiponectin is also present in the cerebrospinal fluid (CSF) and acts on the central nervous system to modulate feeding and energy expenditure (Kos et al., 2007; Neumeier et al., 2007; Qi et al., 2004). Adiponectin can exist in full-length form (fAd) or as a smaller, globular fragment (gAd). Moreover, gAd is a proteolytic cleavage product offAd and has been demonstrated to display independent biological activities compared to the properties of fAd (Palanivel et al., 2007; Bobbert et al., 2008).

Two receptors for adiponectin have been identified: AdipoR1 and AdipoR2. AdipoR1 is a high-affinity receptor for gAd, but possesses only marginal binding-affinity for fAd. In contrast, AdipoR2 is an intermediate affinity receptor for both gAd and fAd (Yamauchi et al., 2003; Kadowaki and Yamauchi, 2005). However, both AdipoR1 and AdipoR2 receptors can increase the activation of 5′ AMP-activated protein kinase (AMPK) and the activity of the peroxisome proliferator-activated receptor alpha (PPARα) Yamauchi et al., 2007; Yamauchi et al., 2002.

Pituitary corticotroph cells, a vital component of the hypothalamic–pituitary–adrenal axis (HPAA), mediate the response to stress by secreting adrenocorticotropic hormone (ACTH). In addition to positive regulation by corticotropin-releasing hormone (CRH) and vasopressin (AVP) of the hypothalamus and negative regulation by glucocorticoid (GC) from the cortex of the adrenal gland (Engler et al., 1999; Papadimitriou and Priftis, 2009), ACTH is regulated by locally produced pituitary proteins or extracellular matrix factors (Kuchenbauer et al., 2001; Katahira et al., 1998). Furthermore, adipokines modulate ACTH and HPAA in vivo and in vitro; for example, leptin inhibits HPAA by regulating hypothalamic CRH (Heiman et al., 1997; Spinedi and Gaillard, 1998). Moreover, adiponectin has been reported to regulate the HPAA in the hypothalamus and adrenal gland (Hoyda et al., 2009; Li et al., 2009). Adiponectin and its receptors are expressed in the pituitary gland and modulate the endocrine axis to control both somatotrophs and gonadotrophs (Rodriguez-Pacheco et al., 2007; Lu et al., 2008); however, little is known regarding the effects of adiponectin on pituitary corticotroph cells.

In the present study, we demonstrated that adiponectin receptors were expressed in pituitary corticotroph cells and that adiponectin increased basal ACTH secretion to regulate the HPAA by binding to adiponectin receptors and activating the AMPK signalling pathway.

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2. Materials and methods

2.1. Materials

Unless otherwise stated, all reagents were purchased from Sigma (Sigma, St. Louis, MO, USA), e.g., corticotropin-releasing hormone (CRH), AICAR.

2.2. Cell culture

The mouse AtT-20 pituitary corticotroph cell line was obtained from the American Tissue Type Collection (ATCC, Manassas, VA, USA). The cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% foetal bovine serum (FBS) and 2 mM L-glutamine and 1X penicillin/streptomycin (GIBCO, Carlsbad, CA, USA) in a humidified incubator with 5% CO_2 at 37 °C. The cell cultures were routinely passaged when the cells reached 90–95% confluence. Before the experimental treatments, the cells were grown in serum-free medium for 14 h. The cells were then treated with recombinant murine gAd and fAd (R&D, Minneapolis, MN, USA) for the indicated time periods. The culture media was collected, and the cells were harvested for the experiments as indicated. Human pituitary adenoma samples were collected according to the guidelines of the local committee on human research. Tissue fragments were immediately frozen in liquid nitrogen or fixed using 4% paraformaldehyde.

2.3. Primary cell culture

The pituitary tissues of 30 rats were pooled, and the cells were dissociated using collagenase (0.2% w/v) as previously described (Ma et al., 2010). Briefly, pituitary glands were obtained from adult male Sprague Dawley rats (180–250 g) after decapitation. The tissue was washed with HBSS, and the sliced fragments were dispersed in a preparation buffer containing 2-g/l collagenase. The dispersed cells were centrifuged and resuspended in DMEM supplemented with 10% FBS and 2 mM l-glutamine, streptomycin, and penicillin. The cells were quantified and examined using light microscopy to evaluate the effectiveness of the dispersion. Cell viability was assessed using the trypan blue exclusion test. The cells were then distributed in 6-well plates and incubated at 37 °C under 5% CO_2 for 12 h until further use.

2.4. Measurement of intracellular calcium concentrations ($[Ca^{2+}]_i$)

AtT-20 cells were grown on coverslips and loaded with 5 μM Fura-2/AM for 30 min at 37 °C in a solution containing the following compounds (in mM): 119 NaCl, 4.75 KCl, 5 $NaHCO_3$, 1.2 $MgSO_4$, 1.18 KH_2PO_4, 2.54 $CaCl_2$, 3 glucose and 20 HEPES (pH7.4). The coverslip was placed in a superfusion chamber under an IX71 inverted microscope (Olympus, Tokyo, Japan). The $[Ca^{2+}]_i$ was measured using the Video Imaging System (Till Photonics, Munich, Germany). Cells were illuminated using 340-nm and 380-nm wavelength alternative excitation light, which was produced by a monochromator (Till Photonics, Munich, Germany). The images were captured with a510-nm wavelength emission light using an image-intensifying CCD camera (SensiCam, PCO, Kelheim, Germany) and processed with an image processing system (TillVision, Till Photonics, Munich, Germany). The ratio images were captured at 10-s intervals. Calcium concentrations were indicated as the ratio of F340/F380. Cells were continuously superfused with the solution as previously described throughout the experiment. All drugs were applied through superfusion.

2.5. Western blotting analysis

AtT-20 cells were harvested and lysed in RIPA buffer containing phenylmethylsulfonylfluoride. After the protein concentration was measured by the Bradford assay, 50μgof total protein was separated on a 12% polyacrylamide gel and transferred onto a nitrocellulose membrane. After blocking with 2% (w/v) bovine serum albumin (BSA), the membranes were incubated overnight at 4 °C with rabbit anti-CRHR1 antibody (1:200, Santa Cruz, Santa Cruz, CA, USA) or rabbit anti-AMPK antibody (1:500, Cell Signaling, Danvers, MA, USA) and then incubated for at least 1 h at room temperature with HRP-conjugated secondary antibody (1:1000, Dako, DK-2600 Glostrup). The signal was detected using enhanced chemiluminescence (PerkinElmer, Waltham, MA, USA). Densitometry was performed directly on the blotted membranes using a CCD camera system (LAS-4000, Fujifilm, Tokyo, Japan). The bands were quantified by densitometry using the Quantity One system. Statistical analyses of the data were performed using arbitrary units for the band quantified by the scanner.

2.6. RT-PCR and real-time PCR

Total RNA was isolated from AtT-20 cells using TRIzol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions, and 2 μg RNA was reverse-transcribed to cDNA using the anchor primer oligonucleotide (dT)15 and Superscript II Reverse Transcriptase (Invitrogen). The reverse-transcribed cDNA was used as a template for PCR amplification. PCR analysis was performed to measure the expression of adiponectin and its two receptors (AdipoR1 and AdipoR2) in AtT-20 cells using specific primers (Li et al., 2009). As an internal control, amplification of a 600-bp fragment of GAPDH was performed in parallel in each sample. The PCR products were electrophoresed in a 2% agarose gel containing ethidium bromide. Real-time PCR was performed with an ABI Prism 7300 instrument (Applied Biosystems, Carlsbad, CA, USA) in 96-well plates using the SYBR Premix Ex Taq kit (Takara, Otsu, Shiga, Japan) according to the manufacturer's instructions. The cycle parameters were 95 °C for 10 s followed by 40 cycles of 95 °C for 5 s and 60 °C for 31 s. Gene expression was analysed by relative quantification using the $2^{-\triangle\triangle}$Ct method. Quantification was performed in quadruplicate, and the experiments were repeated independently three times. The sequences of the primers were as follows: GAPDH forward primer: 5′-TTCACCACCATGGAGAAGGC-3′, reverse primer: 5′-CACACCCATCACAAACATG GG-3′; corticotropin-releasing hormone receptor 1 (CRHR1) forward primer: 5′-CGCATCCT CATGACC AAACTC-3′, reverse primer: 5′-AACATGTAGGTGATGCCCAGG-3′; *POMC* forward primer: 5′-CCACTGAACATCTTTGTCCCCA-3′, reverse primer: 5′-GCATCTTC CACGTGTCAGGC-3′. The target gene levels were normalised against GAPDH, and the results were expressed as the fold-changes in the threshold cycle (Ct) values relative to the controls. All primer sets were designed to span at least one intron to avoid genomic DNA-derived amplification.

2.7. Hormone determination

After the cells were incubated for the specified time interval, the culture media from each well was collected for further measurement. The ACTH in the culture media was measured using ELISA (Phoenix, Milpitas, CA, USA). The samples in each experimental group were analysed in triplicate or quadruplicate.

2.8. Immunofluorescence

The expressions of AdipR1 and AdipR2 were examined using immunofluorescence. Paraffin-embedded pituitary corticotroph