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Brain pericyte-derived soluble factors enhance insulin sensitivity in GT1-7 hypothalamic neurons

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ABSTRACT

Insulin signaling in the hypothalamus plays an important role in food intake and glucose homeostasis. Hypothalamic neuronal functions are modulated by glial cells; these form an extensive network connecting the neurons and cerebral vasculature, known as the neurovascular unit (NVU). Brain pericytes are periendothelial accessory structures of the blood–brain barrier and integral members of the NVU. However, the interaction between pericytes and neurons is largely unexplored. Here, we investigate whether brain pericytes could affect hypothalamic neuronal insulin signaling. Our immunohistochemical observations demonstrated the existence of pericytes in the mouse hypothalamus, exhibiting immunoreactivity of platelet-derived growth factor receptor β (a pericyte marker), and laminin, a basal lamina marker. We then exposed a murine hypothalamic neuronal cell line, GT1-7, to conditioned medium obtained from primary cultures of rat brain pericytes. Pericyte-conditioned medium (PCM), but not astrocyte- or aortic smooth muscle cell-conditioned medium, increased the insulin-stimulated phosphorylation of Akt in GT1-7 cells in a concentration-dependent manner. PCM also enhanced insulin-stimulated tyrosine phosphorylation of insulin receptor β without changing its expression or localization in cytosolic or plasma membrane fractions. These results suggest that pericytes, rather than astrocytes, increase insulin sensitivity in hypothalamic neurons by releasing soluble factors under physiological conditions in the NVU.

1. Introduction

The central actions of insulin are critical for the maintenance of glucose and energy homeostasis. Insulin crosses the blood–brain barrier (BBB) by a saturable transport mechanism [1] and acts on the hypothalamus to regulate feeding and blood glucose [2]. Insulin receptors (IR) are expressed throughout the brain, in particular in the hypothalamus [3]. Mice with a neuron-specific disruption of the IR gene show increased food intake and develop diet-sensitive obesity with increases in body fat [4]. These findings suggest that hypothalamic insulin is essential for the regulation of food intake [5]. In addition, previous studies have indicated that neuronal insulin resistance, associated with inflammation in the hypothalamus, plays a key role in the mechanism underlying increased weight gain in diet-induced obesity [6,7].

Many neurological diseases are known to be caused by communicational or functional defects in cells constituting the neurovascular unit (NVU) [8]. The NVU comprises brain microvascular endothelial cells (BMECs) and pericytes at the capillary level, vascular smooth muscle cells at the arterial level, astrocytes, microglia and neurons. This integrated network of neurons and vasculature supports neuronal development, maintenance, and regeneration in the brain [9]. Many studies on the interaction between neurons and other NVU cells have focused on glia–neuron communication for neuronal proliferation, metabolism, and synapse formation [10]. In the hypothalamus, astrocytes control neuronal synaptic input onto hypothalamic neurons, regulating feeding [11]. Brain pericytes cover capillaries, sharing a common basement membrane with BMECs and making direct contact with astrocyte endfeet [12]. BMECs, astrocytes, and pericytes constitute

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the BBB. Pericytes communicate with BMECs by the release of soluble factors, leading to upregulation of BBB functions [13–15]. Bell et al. recently reported that pericyte-deficient mice show age-dependent cerebrovascular damage that precedes neurodegenerative changes, impairments in learning and memory, and neuroinflammatory responses [16]. These findings suggest that brain pericytes communicate with neurons by direct contact and/or diffusible molecules. However, it remains unclear how pericytes interact with neurons in the mediation of neuronal functions such as insulin sensitivity in the hypothalamus. In the present study, we investigate the hypothesis that brain pericytes elevate the sensitivity of hypothalamic neurons to insulin by producing soluble substances.

2. Materials and methods

2.1. Immunohistochemistry

All procedures involving experimental animals adhered to the law (No. 105) and notification (No. 6) of the Japanese Government, and were approved by the Laboratory Animal Care and Use Committee of Fukuoka University. Animal experiments were carried out in accordance with EU Directive 2010/63/EU for animal experiments.

Male ICR mice aged 8 weeks (Kyudo, Tosu, Japan) were housed at 22 ± 2 °C under a 12 h light/dark schedule (lights on at 07:00) and given water and chow ad libitum. Mice were anesthetized with urethane and transcardially perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4). The brains were removed, post-fixed, and coronal sections (10 μm thick) of the hypothalamus were prepared as described previously [17]. In brief, sections were washed in Tris-buffered saline (pH 7.4) containing 0.2% Triton X-100, blocked with Blocking One Histo (Nacalai Tesque, Kyoto, Japan) and incubated with goat anti-platelet-derived growth factor receptor β (PDGFRβ) antibody (R&D Systems, Minneapolis, MN, USA) and rabbit anti-laminin antibody (Sigma, St. Louis, MO, USA), followed by Cy3-conjugated anti-goat IgG (1:200 dilution, Jackson ImmunoResearch, West Grove, PA, USA) and fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (1:200 dilution, Jackson ImmunoResearch). The sections were mounted in anti-fade Vectashield mounting medium with DAPI (Vector Laboratories, Burlingame, CA, USA) and examined under a fluorescence microscope (Keyence BZ-X710, Keyence Corporation, Osaka, Japan). Control sections were prepared by omitting primary antibodies.

2.2. Cell culture

The GT1-7 mouse cell line was originally derived from an immortalized fetal hypothalamic neuron. GT1-7 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Wako, Osaka, Japan) supplemented with 10% fetal bovine serum (FBS; Biowest, Nuaillé, France), 100 units/mL penicillin and 100 μg/mL streptomycin (Nacalai Tesque) at 37 °C in a humidified atmosphere of 5% CO_2/95% air.

Primary cultures of rat brain pericytes were prepared from 3-week-old Wistar rats, as described previously [13]. In brief, the meninges were carefully removed from the forebrain, and gray matter was minced in ice-cold DMEM and digested with collagenase II (CLS2; Worthington, Freehold, NJ, USA). The cell pellets were separated by centrifugation in 20% bovine serum albumin-DMEM and digested with collagenase/dispase (Roche, Mannheim, Germany). Microvessel clusters were separated on a 33% continuous gradient of Percoll (GE Healthcare, Buckinghamshire, UK), collected, and plated in non-coated flasks. Brain pericyte cultures were maintained in DMEM supplemented with 20% FBS and 50 μg/mL gentamycin at 37 °C in a humidified atmosphere of 5% CO_2/95% air. After 7 days in culture, pericytes at 80–90% confluence were used for experiments.

Primary astrocyte cultures were prepared from the cerebral cortex of 1–3-day-old Wistar rats, as described previously [18]. Briefly, after removing the meninges and blood vessels, the forebrains were minced and gently dissociated by repeated pipetting in DMEM containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin, and filtered through a 70 μm cell strainer. Cells were cultured on 75 cm^2 flasks (Nunc, Roskilde, Denmark) in a humidified atmosphere of 5% CO_2/95% air, at 37 °C. After 10–14 days in culture, the floating and weakly attached cells were removed by vigorous shaking of the flask. Astrocytes on the bottom of the culture flask were then trypsinized and seeded into new culture flasks. The primary cultured astrocytes were maintained in 10% FBS DMEM in a humidified atmosphere of 5% CO_2/95% air at 37 °C. Second- or third-passage cells were used for experiments.

Rat aortic vascular smooth muscle cells (RASMs) were isolated from adult Wistar rats as described previously [19]. Cells were grown in DMEM supplemented with 2 mM L-glutamine, 100 units/mL penicillin, 100 μg/mL streptomycin, and 10% FBS, at 37 °C in 5% CO_2.

2.3. Preparation of cell-conditioned media

Pericytes, astrocytes and RASMs were cultured in 75 cm^2 culture flasks to reach confluence. The culture medium was then replaced with 5 mL of serum-free DMEM and conditioned by incubation for 24 h with each cell type, and stored at −80 °C until use. The conditioned media were diluted with fresh serum-free DMEM to obtain the indicated dilutions.

2.4. Preparation of membrane and cytosolic fractions

Fractionated proteins were obtained using cytosolic lysis buffer (20 mM Tris–HCl, 150 mM NaCl, 2 mM EDTA, 1 mM PMSF, 1 mM $NaVO_4$) and phosphoprotein lysis buffer (10 mM Tris–HCl, pH 6.8, 100 mM NaCl, 1 mM EDTA, 10% glycerol, 1% Triton X-100, 0.1% SDS, 0.5% sodium deoxycholate, 2 mM Na_3VO_4, 50 mM NaF, 10 mM $Na_4O_7P_2 \cdot 10H_2O$, 50 μg/mL phenyl-methylsulfonyl fluoride, phosphatase inhibitor cocktails 1 and 2 (Sigma), and protease inhibitor cocktail (Sigma)). The cells were washed, harvested in ice-cold PBS, and pelleted by centrifugation (1000 × g, 5 min at 4 °C). The pellet was resuspended in cytosolic lysis buffer, and sonicated on ice. The samples were centrifuged (20,000 × g, 40 min at 4 °C) and the supernatant (i.e., cytosolic fraction) was collected. Pellets were resuspended in phosphoprotein lysis buffer and shaken for 60 min at 4 °C. The membrane extract was centrifuged (15,000 × g, 40 min at 4 °C), and the supernatant was collected.

2.5. Western blot

Whole cell lysates were obtained from GT1-7 cells, untreated or treated with human recombinant insulin (Life Technologies, Grand Island, NY, USA), by scraping in phosphoprotein lysis buffer. The total protein concentration was determined using a BCA Protein Assay Kit (Pierce, Rockford, IL, USA). Whole cell lysates and fractionated proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were incubated with antibodies to Akt, phospho-Akt (Thr308), insulin receptor (IR) β, IR substrate (IRS) 1 and 2, and flotillin-1 (all from Cell Signaling, Danvers, MA, USA), phospho-IRβ (Tyr1162/1163) (sc-25103-R; Santa Cruz Biotechnology, Santa Cruz, CA), and β-actin (ab8227; Abcam, Cambridge, UK). After washing, membranes were