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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

incubated with an appropriate horseradish peroxidase-conjugated secondary antibody. The results were visualized using ImmunoStar LD (Wako). Images of the bands were captured digitally with a FluorChem SP imaging system (Alpha Innotech, San Leandro, CA, USA) and the band intensities were quantified using the public domain software Image J (NIH Image, Bethesda, MD, USA).

2.6. Statistical analysis

Data are expressed as means ± SEM. Statistical analysis was carried out using GraphPad Prism 5.0 (GraphPad, San Diego, CA, USA). Student's t-test was used for comparisons between two groups. One-way or two-way analyses of variance followed by Dunnett's or Tukey–Kramer's tests were used for multiple comparisons. $p < 0.05$ was considered statistically significant.

3. Results

3.1. Distribution of PDGFRβ immunoreactivity in the hypothalamus

A large number of cells in the hypothalamus were immunoreactive for PDGFRβ (a pericyte marker) and laminin (a basal lamina and vascular marker) (Fig. 1A and B, respectively). Pericytes, showing both PDGFRβ and laminin immunoreactivity, were observed in the dorsomedial and arcuate nucleus of the hypothalamus and were localized in the hypothalamic microvasculature (Fig. 1C and D).

3.2. Pericyte conditioned medium (PCM) increased insulin-induced Akt phosphorylation in GT1-7 cells

To test whether brain pericyte-derived soluble factors affect insulin signaling in hypothalamic neurons, GT1-7 cells were incubated in the presence or absence of PCM for 2, 4, or 8 h, followed by exposure to 1 μg/mL insulin for 10 min. Total and phosphorylated Akt (p-Akt) levels were measured by western blotting. Cells treated with PCM had 1.7-fold greater levels of insulin-induced p-Akt expression than control cells at 2 h after PCM exposure (Fig. 2A). GT1-7 cells were also treated with PCM diluted 2- or 4-fold with serum-free DMEM (normal medium), followed by insulin (1 μg/mL, 10 min). This revealed that the elevation of insulin-induced Akt phosphorylation by PCM was concentration-dependent (Fig. 2B).

3.3. Astrocyte- and RASM-conditioned media did not influence insulin-induced phosphorylation of Akt in GT1-7 cells

GT1-7 cells were incubated with or without astrocyte- or RASM-conditioned medium for 2 h, followed by insulin (1 μg/mL, 10 min). Neither medium produced significant changes in insulin-stimulated phosphorylation of Akt (Fig. 2C and D).

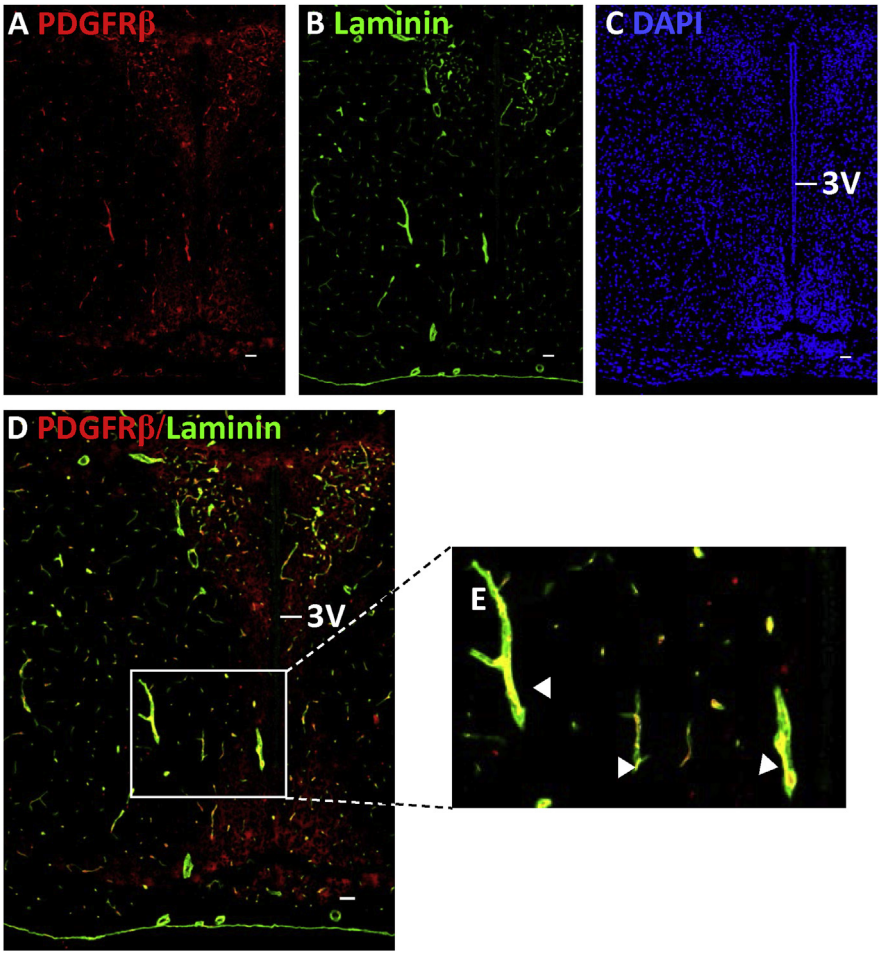

Fig. 1. Distribution of PDGFRβ-immunoreactive cells and laminin-immunoreactive basal lamina in the hypothalamus. Double immunofluorescence staining of PDGFRβ (red) (A) and laminin (green) (B) in the hypothalamus. Nuclei are stained with DAPI (blue) (C). Merged images (D) show that PDGFRβ-immunoreactive cells covered with laminin-immunoreactive basal lamina (arrowheads) are pericytes. The enlarged photograph (E) represents the area indicated in the merged image (D). 3V, third ventricle. Scale bars = 50 μm.

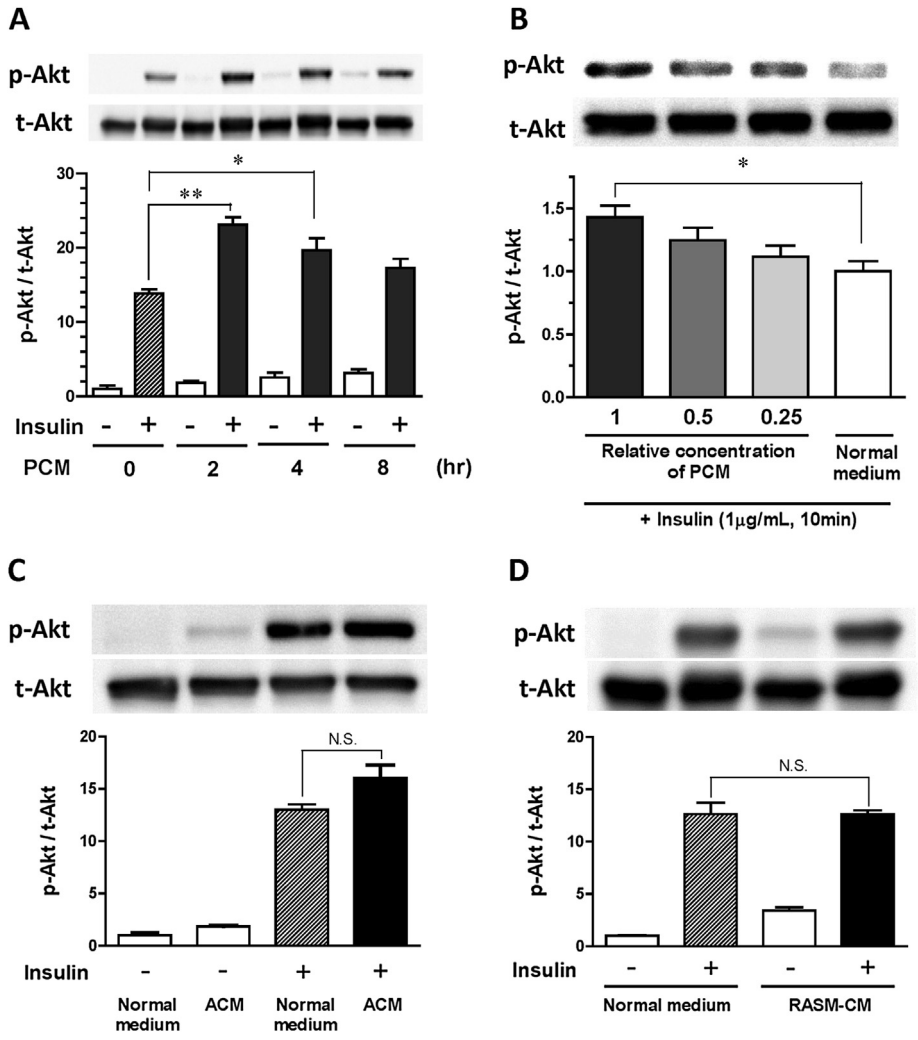

Fig. 2. Effects of conditioned media obtained from primary cultures of pericytes, astrocytes and RASMs on the insulin-induced phosphorylation of Akt at Thr308 in GT1-7 cells. Representative western blots of phosphorylated Akt (p-Akt) and total Akt (t-Akt) (upper) and quantitative analysis of western blots (lower). (A) GT1-7 cells treated with PCM for the indicated time periods, followed by insulin (1 μg/mL) for 10 min. (B) GT1-7 cells were treated with insulin (1 μg/mL) for 10 min following a 2 h exposure to PCM prepared at the indicated relative concentrations (0.25–1). (C, D) GT1-7 cells were treated with astrocyte- (C) or RASM- (D) conditioned medium for 2 h followed by insulin treatment (1 μg/mL) for 10 min. Band intensities were quantified by scanning densitometry. Expression levels of p-Akt were normalized to total levels and results are expressed relative to the corresponding control levels (set to 1.0). Values are means ± SEM ($n = 3$). $^{*}p < 0.05$, $^{**}p < 0.01$.

3.4. Effects of PCM on insulin signaling molecules in GT1-7 cells

To determine whether PCM acts upstream of Akt in the insulin signaling pathway, we examined its effects on the total protein and localization of IRβ. GT1-7 cells exposed to PCM for 2 h were fractionated. Expression of flotillin 1, a marker of membrane microdomains, was observed in the membrane fraction but not in the cytosolic fraction. Exposure of GT1-7 cells to PCM did not alter the expression or localization of IRβ in the cytosolic or plasma membrane fractions (Fig. 3A). However, insulin-stimulated phosphorylation of IRβ at Tyr1162/1163 was significantly greater in PCM-treated GT1-7 cells than in cells in normal medium (Fig. 3B). PCM had no effect on IRS-1 or IRS-2 total protein in GT1-7 cells (Fig. 3C and D).

4. Discussion

In the present study, we have demonstrated that brain pericyte-derived soluble factors enhance insulin-induced phosphorylation of IRβ and Akt in immortalized hypothalamic GT1-7 neurons without affecting the total protein expression levels of IRβ, IRS-1, IRS-2 and Akt. We have also shown that humoral factors derived from aortic smooth muscle cells or astrocytes do not affect insulin sensitivity. These results suggest that brain pericytes contribute to the regulation of insulin sensitivity in the hypothalamus.

A previous immunohistochemical study of the rat hypothalamus indicated that pericytes were immunoreactive for NG2 [20]. Cells expressing NG2 are found in the developing and adult CNS and are known to generate oligodendrocytes and astrocytes [21]. Recently, Winkler et al. showed that PDGFRβ was expressed specifically in brain pericytes and not in other CNS cells [22], suggesting that it is more specific pericyte marker than NG2 in vivo. We previously demonstrated that pericytes are covered with basal lamina in hippocampal microvessels [17]. Therefore, in the present study, we used both PDGFRβ and laminin to identify pericytes localized in the hypothalamus, suggesting a possible interaction of pericytes and neurons in the hypothalamic NVU.

Nutrient homeostasis is regulated by insulin acting on neurons in the hypothalamus [23], where the IR is highly expressed [24]. Activation of the IR with insulin stimulates receptor tyrosine kinases to

phosphorylate tyrosine residues of the IRS; this activates a downstream signal transduction pathway coupled to phosphatidylinositol-3-kinase (PI3K) and Akt. Although multiple intracellular signaling pathways are activated by insulin, the principal pathway in the CNS is PI3K–Akt. In the present study, we demonstrated that PCM enhances insulin-stimulated Akt phosphorylation in GT1-7 cells in a concentration-dependent manner, but that astrocyte- and RASM-derived soluble factors have no effect on insulin signaling. Astrocytes are known to regulate synaptic plasticity by interacting with neurons and RASMs; the latters express the contractile protein α-smooth muscle actin, similarly to pericytes. The lack of effect of astrocyte- or RASM-derived soluble factors on insulin signaling in GT1-7 cells suggests that pericytes produce specific substances to enhance insulin signaling. We previously demonstrated that the baseline levels of monocyte chemotactic protein 1, macrophage inflammatory protein 1α and interleukin-6 released from pericytes were higher than those released from other cell types of the BBB [25]. Insulin sensitivity is increased by interleukin-6 in a concentration-dependent manner in the hypothalamus of exercised rats [26]. Further work is required to identify pericyte-derived substances that enhance insulin sensitivity in GT1-7 cells.

We also studied IRβ and IRS to elucidate the mechanism by which PCM enhances insulin-stimulated Akt phosphorylation in GT1-7 cells. IR and functional tyrosine kinases are concentrated in lipid rafts obtained from neuronal plasma membranes [27]. Reduced IR activity in SH-SY5Y neuroblastoma cells may be attributed to a disruption of lipid rafts [28]. Here, exposure of GT1-7 cells to PCM facilitated insulin-induced phosphorylation of IRβ at Tyr1162/1163, but did not change the expression or localization of IRβ in either the cytosolic or plasma membrane fractions. Furthermore, PCM had no effect on the expression of flotillin 1 which belongs to a family of lipid raft-associated integral membrane proteins. Finally, we examined whether pericyte-derived soluble factors affect IRS family members recruited by tyrosine phosphorylation of the IRβ. Treatment with PCM had no effect on IRS-1 or IRS-2 total protein in GT1-7 cells. Therefore, PCM likely enhances insulin-induced Akt phosphorylation by increasing IRβ tyrosine phosphorylation. Alternatively, these findings raise the possibility that PCM inhibits insulin clearance via insulin-degrading enzyme and/or IRβ dephosphorylation by protein tyrosine phosphatase (PTP) 1B. Elevated levels of insulin-degrading enzyme and PTP1B are responsible for hypothalamic neuronal insulin resistance in obese rodents [29,30]. We cannot exclude the possibility that PCM has a direct effect on PI3K and phosphatidylinositol (3,4,5)-trisphosphate downstream of IRS. Further work is needed to elucidate the target molecules via which PCM facilitates the insulin-induced phosphorylation of IRβ in hypothalamic neuronal cells.

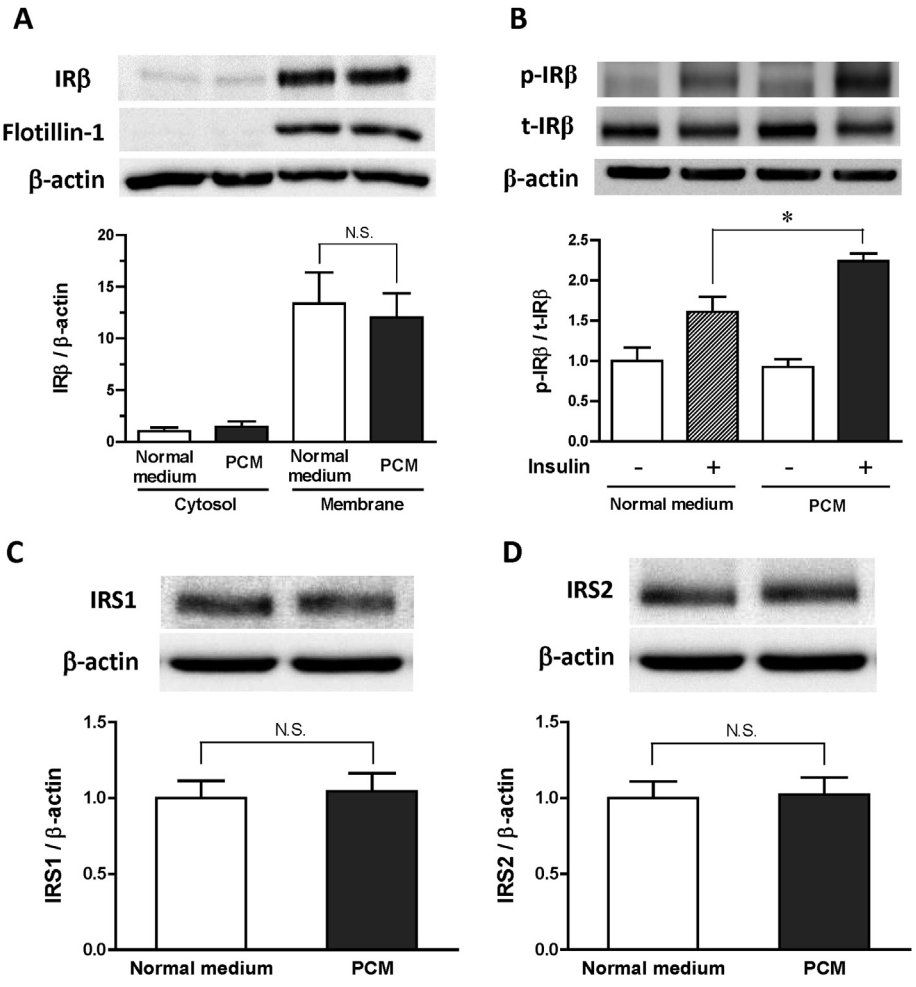

Fig. 3. Effects of PCM on insulin signaling molecules in GT1-7 cells. Western blot images and quantitative analysis of GT1-7 cells with or without 2 h PCM treatment. (A) Expression and localization of IRβ in GT1-7 cells. (B) Insulin-induced phosphorylation of IRβ at Tyr1162/1163 in GT1-7 cells. GT1-7 cells were treated with insulin (1 μg/mL) for 10 min following 2 h exposure to PCM. (C, D) Expression levels of IRS-1 (C) and IRS-2 (D) in GT1-7 cells. Band intensities were quantified by scanning densitometry and results were expressed relative to the corresponding control levels (set to 1.0). Expression level of p-IRβ was normalized to total IRβ level. IRβ, IRS-1 and IRS-2 were normalized to β-actin. Values are means ± SEM (n = 3–4). $^{*}p < 0.05$.

In addition to the hypothalamus, the IR is also highly expressed in the hippocampus, cerebral cortex, and cerebellum. Neuronal IR-mediated insulin signaling is related to neuronal survival and functions, such as synaptic transmission, learning and memory, rather than glucose uptake and metabolism in the brain. Therefore, neuronal insulin resistance is considered to be involved in the pathogenesis of cognitive decline and neurodegenerative diseases. Together with our findings that brain pericytes enhance neuronal insulin sensitivity, these suggest pericytes regulate and maintain neuronal functions in the NVU. Pericyte dysfunction or loss under pathological conditions would reduce insulin signaling in neurons, leading to neurodegeneration.

In conclusion, pericytes potentiate insulin sensitivity in hypothalamic neurons by releasing substances which enhance insulin-induced tyrosine phosphorylation of IRβ. Our findings contribute to the understanding of the interaction between pericytes and neurons in the NVU.

Conflict of interest

The authors declare no conflict of interest.

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