Substance P enhances the activation of AMPK and cellular lipid accumulation in 3T3-L1 cells in response to high levels of glucose

MARIA JOSE DUBON[1], YEJI BYEON[1] and KI-SOOK PARK[2,3]

[1]Department of Genetic Engineering, Graduate School of Biotechnology, Kyung Hee University, Yongin, Gyeonggi 17104;
[2]East-West Medical Research Institute; [3]College of Medicine, Kyung Hee University, Seoul 02447, Republic of Korea

Abstract. The rescue of glucose tolerance and insulin-sensitivity in peripheral tissues, including adipose tissue, is essential in therapeutic strategies for diabetes. The present study demonstrated that substance P (SP) increases the accumulation of lipids in 3T3-L1 cells during their differentiation into adipocytes in response to a high concentration of glucose. SP reciprocally regulated the activities of AMP-activated protein kinase (AMPK) and Akt: SP enhanced the activation of AMPK, although the activity of Akt was downregulated. Notably, SP induced an increase in the expression level of glucose transporter 4 in the 3T3-L1 adipocytes. Therefore, it is possible that SP leads to an increase in glucose uptake and the accumulation of lipids in adipocytes, and may contribute towards the rescue of insulin-sensitivity in diabetes.

Introduction

White adipose tissue is innervated by sensory nerves (1-5), however, the roles they exert in this tissue remain to be fully elucidated (1,2,5). It may be possible that these sensory innervations inform the central nervous system of the correct size of fat stored in the peripheral white adipose tissue. It was previously demonstrated that substance P (SP) and calcitonin gene-related peptide are expressed in sensory neurons (4,5).

SP is a conserved 11-amino-acid peptide (6) and is a member of the tachykinin family of neurotransmitters (7). Previous studies have defined the role of SP as a pain transmitter, and it was demonstrated that SP and its specific receptor, neurokinin 1 receptor (NK-1R), are expressed in nervous tissues (8,9). However, a burgeoning body of evidence has revealed that NK-1R is also expressed in a variety of non-neuronal cell types including endothelial cells (10), monocytes (11), macrophages (11) and adipocytes (12). Therefore,

novel roles identified for SP in non-neuronal cells have been reported, including immune modulation (13), mobilization of bone-marrow-derived stem cells (14), wound healing (15,16), and the regulation of insulin signaling (17).

Adipocyte dysfunction following the onset of insulin resistance is associated with type 2 diabetes (18). These dysfunctions may contribute to insulin resistance in the peripheral tissues, including adipose tissue, through mechanisms including the release of non-esterified fatty acids, glycerol, proinflammatory cytokines and proteins, which induce the development of insulin resistance (19-21). Notably, insulin resistance leads to a decrease in the uptake of glucose and in the expression level of glucose transporter 4 (GLUT4) in adipose tissue (22,23).

Signaling pathways, which regulate energy homeostasis, are associated with the development of insulin resistance. The AMP-activated protein kinase (AMPK) is a key protein associated with these signaling pathways (24). Indeed, AMPK is dysregulated in animals and humans with type 2 diabetes, and its pharmacological activation is one of the therapeutic targets which has been identified for the treatment of this condition (25). The activation of AMPK following its phosphorylation on residue Thr-172 occurs when intracellular ATP levels decrease (26,27). Notably, AMPK promotes the translocation of GLUT4 to the plasma membrane (28) and also increases the expression of GLUT4 (29,30).

The level of SP varies under pathological conditions, including type 2 diabetes. Previous studies demonstrated that the level of SP in the serum from patients with type 2 diabetes (31), in skin biopsies from patients with types 1 and 2 diabetes (32), and in heart tissue from patients with type 1 diabetes (33), is markedly lower compared with the controls, although a previously published study contradicted this evidence (34). Therefore, it is possible that the decreased expression of SP in various different tissues from patients with type 2 diabetes may be associated with pathological features, including insulin resistance, through the regulation of cellular functions in the peripheral tissues, including adipose tissue. Although a previous study suggested that SP decreases the insulin-mediated uptake of fatty acids in 3T3-L1 cells (12), the aim of the present study was to investigate the role of SP in the process of lipid accumulation in 3T3-L1 cells during their differentiation into adipocytes in response to a high concentration of glucose, under different medium conditions and using different concentrations of SP.

Correspondence to: Professor Ki-Sook Park, College of Medicine, Kyung Hee University, 26 Kyungheedae-ro, Dongdaemun-gu, Seoul 02447, Republic of Korea
E-mail: kisookpark@khu.ac.kr

Materials and methods

Cells and cell culture. The 3T3-L1 preadipocytes were purchased from American Type Culture Collection (Manassas, VA, USA; cat. no. CL-173) and the cells were maintained in Dulbecco's modified Eagle's medium (DMEM; GE Healthcare Life Sciences, Little Chalfont, UK) with 25 mM glucose, supplemented with 10% fetal bovine calf serum (FBS; Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA), 100 U/ml penicillin/100 μg/ml streptomycin (Gibco; Thermo Fisher Scientific, Inc.). The cells were incubated at 37°C in a humidified atmosphere, containing 5% CO_2, and their subcultures were performed at <70% confluence. The cells in passage numbers P10 to P20 were used for subsequent experiments.

Differentiation of 3T3-L1 preadipocytes into adipocytes. The 3T3-L1 preadipocytes were cultured until they reached 100% confluence under normal culture conditions. At 48 h following the attainment of confluence, the cells were cultured with DMEM containing 25 mM glucose and supplemented with 10% heat-inactivated FBS, 5 μg/ml insulin, 5 μM dexamethasone and 5 μM rosiglitazone (all from Sigma-Aldrich, St. Louis, MO, USA) for 48 h. Subsequently, the cells were incubated for 48 h with DMEM (25 mM glucose), supplemented with 10% FBS and 5 μg/ml insulin. The medium was subsequently exchanged with DMEM (25 mM glucose), supplemented with 10% FBS on every other day for 4 days. If necessary, DMEM containing a different concentration of glucose (5.5 mM) was used for the differentiation of 3T3-L1 preadipocytes into adipocytes.

Use of SP in experiments. SP was purchased from EMD Millipore (San Diego, CA, USA; cat. no. 05-23-0600), and was prepared with 5% acetic acid (Sigma-Aldrich). When required, SP was added to the 3T3-L1 cells at various concentrations whenever the medium was exchanged.

5-Bromo-2'-deoxyuridine (BrdU) incorporation assay. The 3T3-L1 cells were seeded onto fibronectin-coated coverslips (1 μg/ml) in 24-well plates at a density of 4x10^3 cells/well. The cells were initially incubated for 24 h with normal culture medium, prior to an incubation of 18-24 h duration under conditions of serum starvation. The cells were subsequently treated with SP for 48 h. For the final 6 h of the incubation period, 20 μM BrdU (Sigma-Aldrich) was added to the cells. The cells were subsequently prepared for the immunocytochemical analysis using BrdU by fixation in 4% paraformaldehyde (Electron Microscopy Sciences, Hatfield, PA, USA) in phosphate-buffered saline (PBS) for 10 min on ice. The fixed cells were incubated with 2 N HCl for 15 min at room temperature and washed with PBS vigorously. Following permeabilization with 0.2% Triton X-100 (Affymetrix, Inc., Santa Clara, CA, USA), the cells were treated with blocking solution (5% non-fat milk in PBS with 0.1% Triton X-100) for 30 min at room temperature. Subsequently, the cells were incubated with primary mouse monoclonal anti-BrdU antibody (1:20, cat. no. #11-170-376-001; Roche Diagnostics GmbH, Mannheim, Germany) for 1.5 h at room temperature. Following three washes with 1% non-fat milk in PBS with 0.1% Triton X-100, the secondary antibody, Invitrogen Alexa-488 anti-mouse

immunoglobulin G1 (Thermo Fisher Scientific, Inc.), was added, and the cells were incubated for a further 45 min at room temperature. Finally, the samples were mounted using Invitrogen ProLong® Gold Antifade mounting solution with 4',6-diamidino-2-phenylindole (Thermo Fisher Scientific, Inc.), and left to dry overnight prior to observation. Images were captured using a fluorescence microscope (DMI4000; Leica, Solms, Germany), and the total number of cells and BrdU-positive cells were counted.

RNA isolation and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). SP (0, 10, 100 or 300 nM) was added to the 3T3-L1 cells on the initial day of differentiation, and the total RNA was extracted from the cells on day 2 following the induction of adipogenesis using the Invitrogen TRIzol™ reagent (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. Aliquots of 5 μg total RNA were used for single-strand cDNA synthesis using the Invitrogen Superscript First-Strand cDNA Synthesis system (Thermo Fisher Scientific, Inc.), according to the manufacturer's protocol. RT-qPCR was performed using the Invitrogen Power SYBR Green PCR Master mix (Thermo Fisher Scientific, Inc.). The ribosomal protein 36B4 gene from the mouse was used as an endogenous control. The following primers were used to detect the expression of peroxisome proliferator-activated receptor-γ (PPAR-γ), adipocyte protein 2 (aP2) and 36B4 protein: PPAR-γ, sense: 5'-CGCTGA TGCACTGCCTATGA-3' and antisense: 5'-AGACCTCCA CAGAGCTGATTCC-3'; aP2, sense: 5'-CATGGCCAAGCC CAACAT-3' and antisense: 5'-CGCCAAGTTTGAAGGAAA TC-3'; 36B4, sense: 5'-GAACATCTCCCCCTTCTCCTT-3' and antisense: 5'-GCAGGGCCTGCTCTGTGAT-3'.

Oil Red O staining. To assess adipogenesis in the 3T3-L1 cells, Oil Red O staining was performed on differentiated cells. Oil Red O solution (0.3%; Sigma-Aldrich) was prepared by dissolving Oil Red O in 60% isopropanol (Daejung Chemicals & Metals, Co., Ltd., Shiheung, Korea). The differentiated cells were fixed in 4% paraformaldehyde (Electron Microscopy Sciences) in PBS for 10 min at room temperature, and subsequently washed with PBS. The fixed cells were incubated with Oil Red O solution for 30 min at room temperature. Images of the stained cells were captured using a light microscope (DMI4000; Leica, Solms, Germany). To further quantify the Oil-Red O-stained lipid drops, the stained cells were rinsed twice with 60% isopropanol and subsequently dried. The stains were eluted with 1 ml isopropanol for 10 min at room temperature and the optical density was measured at 490 nm using an absorbance plate reader (Spectramax190; Molecular Devices; Thermo Fisher Scientific, Inc.).

Western blotting. The cells were rinsed twice with ice-cold PBS and lysed with 2X SDS loading buffer [100 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% glycerol and 200 mM β-mercaptoethanol]. Subsequently, the cell lysates were denatured at 92°C for 10 min. The denatured protein samples were separated using 10% SDS polyacrylamide gel electrophoresis and transferred onto nitrocellulose membranes (Whatman, Dassel, Germany. Following blocking with 5% non-fat milk

in 20 mM Tris buffer, containing 0.1% Tween-20 (TBS-T), the membranes were incubated with primary antibody diluted with TBS-T buffer, including 5% non-fat milk, overnight at 4°C. The following primary antibodies were used: Rabbit monoclonal anti-phosphorylated AMPK (1:1,000, cat. no. #2535; Cell Signaling Technology, Inc., Danvers, MA, USA), rabbit monoclonal anti-phosphorylated Akt (1:4,000, cat. no. #4060; Cell Signaling Technology, Inc.,), rabbit polyclonal anti-GLUT4 (1:500, cat. no. ab65976; Abcam, Cambridge, UK) and mouse monoclonal anti-α-tubulin antibody (1:5,000, cat. no. T5618; Sigma-Aldrich). Subsequently, the membranes were incubated with goat anti-rabbit (1,5,000; cat. no. 7074; Cell Signaling Technology, Inc.) or goat anti-mouse IgG (1:10,000; cat. no. 170-6516; Bio-Rad Laboratories, Inc., Hercules, CA, USA) horseradish peroxidase-conjugated secondary antibodies at room temperature for 30 min. The target proteins were visualized using an enhanced chemiluminescence detection kit (EMD Millipore). The band densities were measured using ImageJ software (NIH, Bethesda, MD, USA). If required, the stripping of the membranes was performed using Restore™ Western Blot Stripping buffer (Thermo Fisher Scientific, Inc.) for 15 min at room temperature. The membranes were reblotted using an antibody raised against α-tubulin.

Statistical analysis. The data are expressed as the mean ± standard deviation or the mean ± standard error of the mean. An unpaired Student's t-test was used to evaluate differences between the two groups. All statistical analyses were performed using GraphPad Prism version 5.01 software (GraphPad Software, Inc., San Diego, CA, USA; http://www.graphpad.com). $P<0.05$ was considered to indicate a statistically significant difference.

Results

SP causes no effect on the proliferation of the 3T3-L1 preadipocytes. A previous study demonstrated that SP increases the cellular proliferation of mesenteric preadipocytes (35). Therefore, whether or not SP affected the proliferation of the 3T3-L1 preadipocytes was examined using a BrdU incorporation assay. The 3T3-L1 preadipocytes were revealed to express NK-1R, which is a receptor of SP (data not shown) (12). As shown in Fig. 1, SP caused no effect on the cellular proliferation of 3T3-L1 cells, irrespective of the concentration of SP.

SP causes no affect on the differentiation of the 3T3-L1 preadipocytes. Whether SP regulated the differentiation of 3T3-L1 preadipocytes into adipocytes was subsequently examined. The 3T3-L1 cells were incubated with the differentiation medium, which included 10% FBS, 5 µg/ml insulin, 5 µM dexamethasone and 5 µM rosiglitazone (36), for 2 days. SP failed to affect the expression level of PPAR-γ or aP2 (Fig. 2), which were previously used as markers for differentiated adipocytes (37). Therefore, it is possible that SP does not promote the differentiation of 3T3-L1 cells into adipocytes.

SP upregulates the accumulation of lipids in the 3T3-L1 preadipocytes. Although SP failed to promote the differentiation of the 3T3-L1 cells, it was hypothesized that SP may affect the
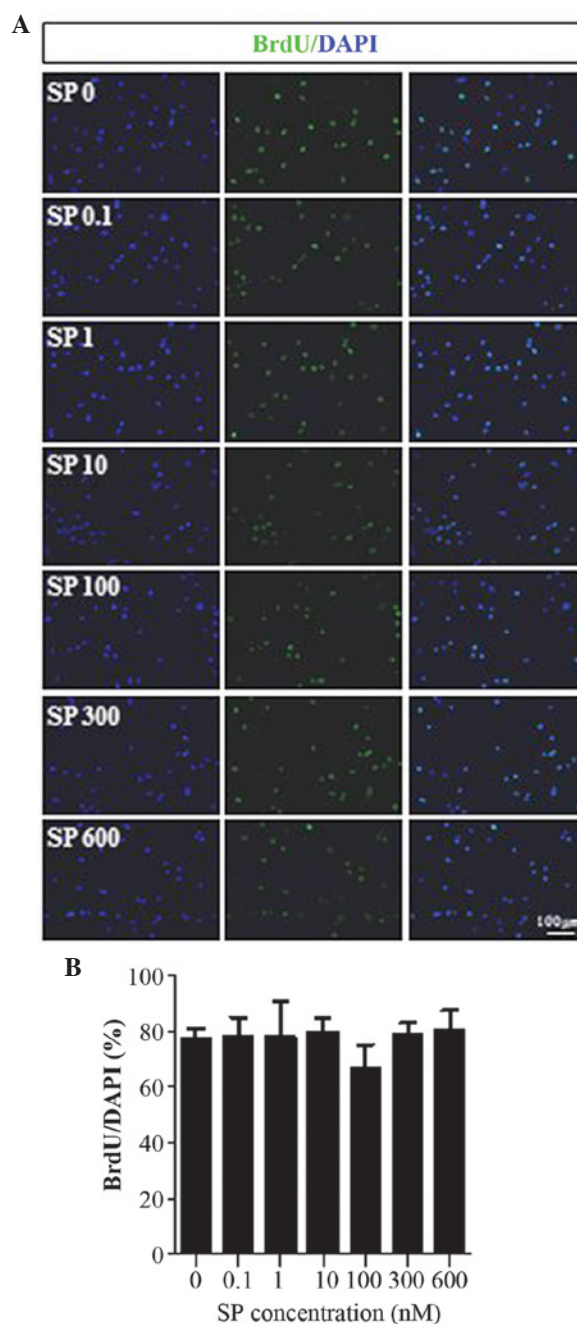
Figure 1. Cellular proliferation of 3T3-L1 preadipocytes treated with SP. (A) BrdU incorporation assays were performed to examine whether SP affected the proliferation of 3T3-L1 preadipocytes. The 3T3-L1 preadipocytes were treated with various concentrations of SP (0, 0.1, 1, 10, 100, 300 or 600 nM; denoted in Fig. 1A as SP 0, SP 0.1, SP 1, SP 10, SP 100, SP 300 and SP 600, respectively). The BrdU staining is in green and nuclei are illustrated by the staining in blue. (B) Graph illustrating the quantitative analysis of the data (expressed as the mean ± standard deviation). SP, substance P; BrdU, bromodeoxyuridine; DAPI, 4',6-diamidino-2-phenylindole.

accumulation of lipids in these cells. Therefore, the effects of SP on the accumulation of lipids in the 3T3-L1 adipocytes were analyzed using Oil Red O staining. SP was added to the 3T3-L1 cells every other day during the differentiation of the cells into adipocytes. Compared with the undifferentiated control cells, the accumulation of lipids increased markedly in the differentiated cells without SP treatment (Figs. 3A and B). Notably, the treatment with SP increased the accumulation of
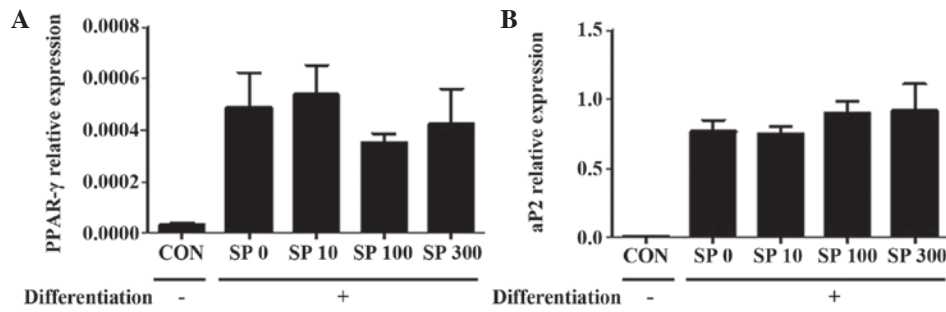
Figure 2. Effect of SP on the expression levels of PPAR-γ and aP2 in the 3T3-L1 cells. Reverse transcription-quantitative polymerase chain reaction was performed to observe the expression levels of (A) PPAR-γ and (B) aP2 in 3T3-L1 cells, which were treated with different concentrations of SP (0, 10, 100 or 300 nM; illustrated by the labels SP 0, SP 10, SP 100 and SP 300, respectively) under the differentiation medium conditions for 2 days. Ribosomal protein 36B4 gene was used as an internal control. Two independent experiments were performed and the quantitative results are shown as the mean ± standard error of the mean. aP2, adipocyte protein 2; CON, control; PPAR-γ, peroxisome proliferator-activated receptor-γ; SP, substance P.
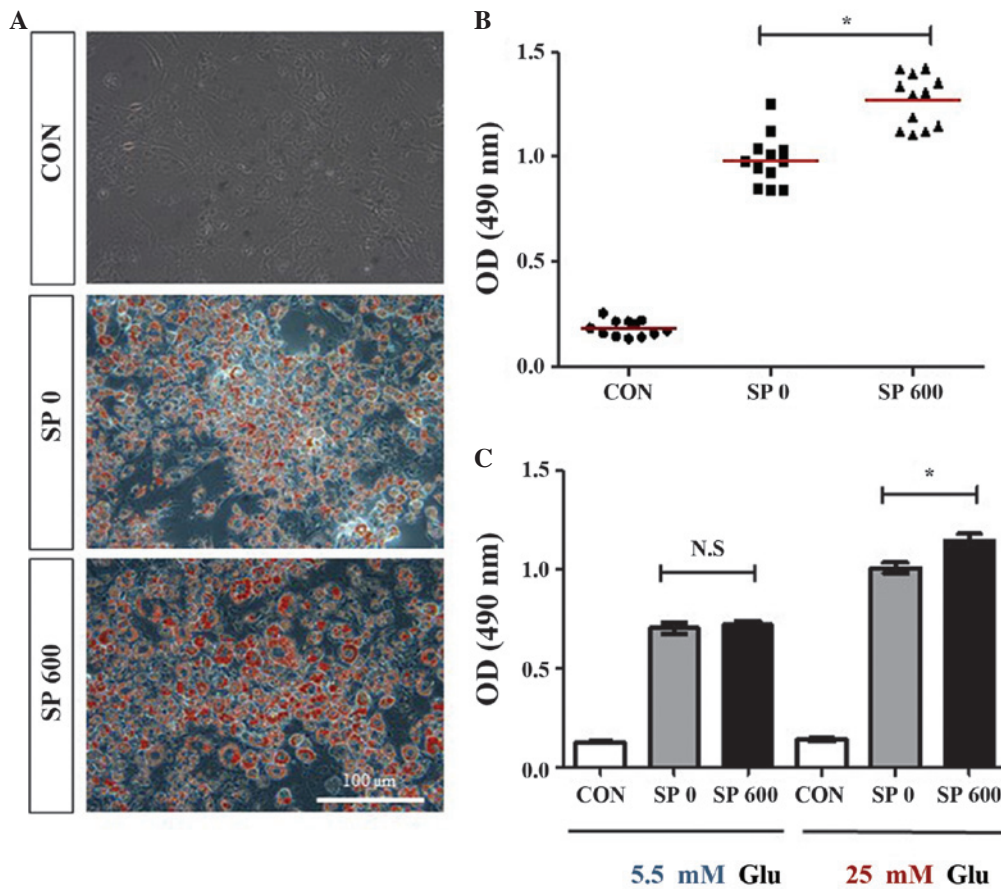

Figure 3. Effect of SP on lipid accumulation in the 3T3-L1 adipocytes. (A and B) The 3T3-L1 cells were cultured in the absence or presence of 600 nM SP (SP 0 or SP 600) under the differentiation medium conditions for 8 days. The accumulation of lipids was analyzed using Oil Red O staining of the 3T3-L1 adipocytes maintained in the presence of a high concentration of glucose (25 mM). (A) Representative images are shown (scale bar, 100 μm), and (B) illustrates the quantification of the results (the mean values are indicated by the red horizontal bars; *P<0.0001, compared with CON). (C) The accumulation of lipids was analyzed by Oil Red O staining of the 3T3-L1 adipocytes, which had differentiated in the absence or presence of 600 nM SP (SP 0 or SP 600) under the differentiation medium conditions, including the presence of either 5 or 25 mM Glu, for 8 days. Three independent experiments were performed, and the quantitative results are shown as the mean ± standard error of mean [*P<0.003, compared with SP 0; N.S, not significant (P>0.6)]. Undifferentiated 3T3-L1 cells were used as the control in all the experiments. CON, control; SP, substance P; OD, optical density; Glu, glucose.

lipids in the 3T3-L1 adipocytes by ~0.3-fold compared with the untreated differentiated 3T3-L1 cells (Figs. 3A and B), even though SP failed to promote the differentiation of 3T3-L1 cells into adipocytes (Fig. 2). In addition, an SP-mediated increase in the accumulation of lipids was observed in the differentiated 3T3-L1 adipocytes, which were cultured with medium, containing a high concentration of glucose (25 mM), however, not in the cells which were cultured in medium containing a normal concentration of glucose (5 mM; Fig. 3C). These results suggested that SP may increase the accumulation of lipids in adipocytes in a manner which is dependent on the concentration of glucose.
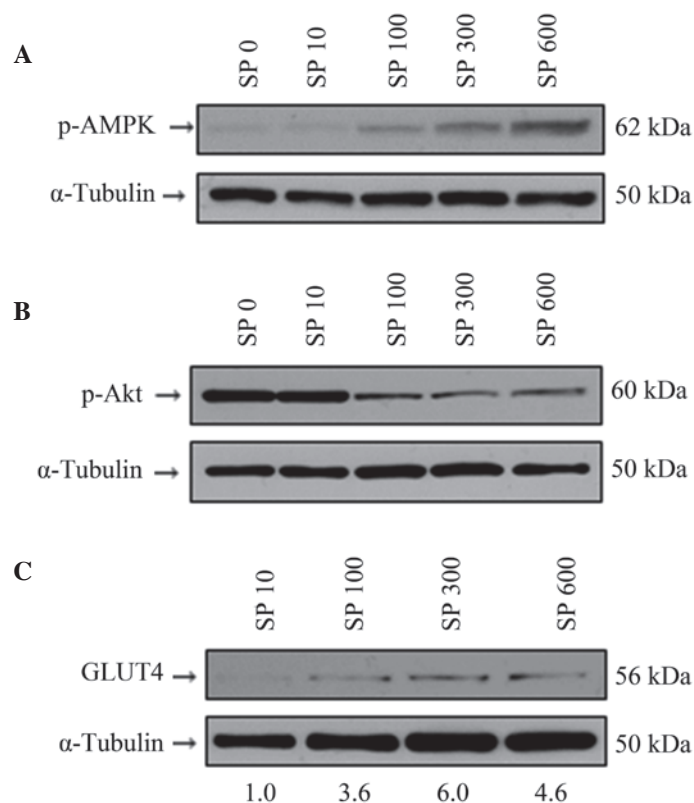
Figure 4. Effect of SP on the protein expression levels of p-AMPK, p-Akt and GLUT4 in the 3T3-L1 adipocytes. (A-C) The 3T3-L1 adipocytes were treated with various concentrations of SP (0, 100, 300 or 600 nM; denoted by the labels SP 0, SP 100, SP 300 and SP 600, respectively). Western blotting was performed to observe the phosphorylation levels of (A) p-AMPK and (B) p-Akt, and (C) the protein expression level of GLUT4. α-Tubulin was used as an internal control. The numbers underlying the gel in (C) indicate the protein expression values relative to the control (SP 10). p-, phosphorylated; AMPK, AMP-activated protein kinase; GLUT4, glucose transporter 4; SP, substance P.

SP regulates the activity of AMPK and Akt, and the expression levels of GLUT4. It is well known that AMPK is a key regulator in the metabolism of glucose and fatty acids in various cell types, including adipocytes (27,38). Therefore, whether SP regulated the activity of AMPK in the 3T3-L1 cells was examined. Indeed, SP was revealed to induce the activation of AMPK in a dose-dependent manner (Fig. 4A). Notably, however, the activity of Akt was downregulated by SP (Fig. 4B). These results are in very good agreement with a previous report, which demonstrated that the regulation of AMPK activity is associated with the regulation of Akt activity (39). It is also known that AMPK promotes the translocalization of GLUT4 to the plasma membrane (28), and furthermore, that AMPK increases the expression level of GLUT4 (29,30). It is noteworthy that SP increased the expression level of GLUT4 in 3T3-L1 cells (Fig. 4C). Therefore, these results suggested that SP may modulate glucose uptake in 3T3-L1 adipocytes, and that this is associated with the activity of AMPK.

Discussion

The present study has demonstrated the ability of the neurotransmitter SP to increase the accumulation of lipids in 3T3-L1 preadipocytes in the presence of a high concentration of glucose, although not under normal glucose conditions. This increase in the accumulation of lipids was associated with an SP-mediated increase in the expression level of GLUT4 following the activation of AMPK.

AMPK exerts an essential role in the regulation of glucose uptake by adipocytes and muscle cells (30,40,41). A previous report demonstrated that the activation of AMPK increases glucose uptake, upregulates the expression of GLUT4 in 3T3-L1 adipocytes, and that the activation of AMPK is independent of the insulin receptor-mediated signaling pathway (41). The present study also suggested that the SP-mediated activation of AMPK increased glucose uptake by means of an increased expression of GLUT4 in the 3T3-L1 adipocytes. Although it remains to be fully elucidated whether insulin signaling functions in association with SP in order to increase the accumulation of lipids in 3T3-L1 adipocytes (the differentiation medium used in this study contained insulin), SP was able to induce this effect only under high glucose conditions. Notably, SP decreased the activation of Akt in the adipocytes. Akt activity is required for the differentiation of mouse embryonic fibroblasts and 3T3-L1 cells into adipocytes (42,43), and the presence of the constitutively active mutation of Akt is sufficient to induce the differentiation of the 3T3-L1 cells (44). However, it is also known that the insulin-mediated Akt signaling pathway is not a major pathway for the induction of glucose uptake by adipocytes, according to a previous report (41). Therefore, Akt was not be expected to be involved in the SP-mediated accumulation of lipids following glucose uptake. In the present study, the SP-mediated increases observed in the accumulation of lipids and in the expression level of GLUT4 may occur via AMPK activation, as the exercise-mediated activation of AMPK was

revealed to increase glucose uptake, following the intracellular translocation of GLUT4 to the plasma membrane (45,46).

Defects in the innervation of adipose tissues impair the formation of blood vessels, damage the architecture of adipose tissues and reduce the insulin-sensitivity, and the metabolism of adipocytes (47). The defects in innervation also include defects in the sensory neurons that express SP, which may be involved in the regulation of cellular functions in the adipose tissues. The present study suggested that SP may rescue insulin-sensitivity and glucose tolerance in the adipose tissue of patients with diabetes through the AMPK signaling pathway. In addition, SP may be useful for the therapeutic treatment of diabetes in order to control insulin resistance.

Acknowledgements

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