Visfatin, a novel adipokine, stimulates glucose uptake through the Ca^{2+} -dependent AMPK-p38 MAPK pathway in C2C12 skeletal muscle cells

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Abstract

Visfatin is a novel adipocytokine produced by visceral fat. In the present study, visfatin increased AMP-activated protein kinase (AMPK) phosphorylation in mouse C2C12 skeletal muscle cells. It also increased phosphorylation of the insulin receptor, whose knockdown blocked visfatin-induced AMPK phosphorylation and glucose uptake. Visfatin stimulated glucose uptake in differentiated skeletal muscle cells. However, inhibition of AMPK α 2 with an inhibitor or with knockdown of AMPK α 2 using siRNA blocked visfatin-induced glucose uptake, which indicates that visfatin stimulates glucose uptake through the AMPK α 2 pathway. Visfatin increased the intracellular Ca^{2+} concentration. STO-609, a calmodulin-dependent protein kinase kinase inhibitor, blocked visfatin-induced AMPK phosphorylation and glucose uptake. Visfatin-mediated activation of p38 MAPK was AMPK α 2-dependent. Furthermore, both inhibition and knockdown of p38 MAPK blocked visfatin-induced glucose uptake. Visfatin increased glucose transporter type 4 (GLUT4) mRNA and protein levels. In addition, visfatin stimulated the translocation of GLUT4 to the plasma membrane, and this effect was suppressed by AMPK α 2 inhibition. The present results indicate that visfatin plays an important role in glucose metabolism via the Ca^{2+} -mediated AMPK-p38 MAPK pathway.

Key Words

- ▶ adipokine
- ▶ AMPK
- ▶ calcium
- ▶ diabetes
- ▶ glucose transport

Journal of Molecular Endocrinology
(2015) **54**, 251–262

Introduction

Visfatin was originally cloned from human peripheral blood lymphocytes and characterized as a nicotinamide phosphoribosyltransferase, an enzyme that synthesizes nicotinamide mononucleotide from nicotinamide (Samal *et al.* 1994, Rongvaux *et al.* 2002). Visfatin is also a rate-limiting enzyme for the biosynthesis of NAD, an electron carrier that enables the body to produce ATP. Visfatin

functions in various biological processes, such as obesity and metabolism (Sethi & Vidal-Puig 2005, Revollo *et al.* 2007, Hallschmid *et al.* 2009, Laudes *et al.* 2010), immunology and inflammation (Moschen *et al.* 2007, Li *et al.* 2008, Rongvaux *et al.* 2008), and survival and longevity (Sinclair 2005, Yang *et al.* 2007, Benigni *et al.* 2009). Some of its functions, such as the regulation of the

circadian clock (Yang *et al.* 2007, Nakahata *et al.* 2009, Ramsey *et al.* 2009), are related to NAD production. Interestingly, as its name implies, visfatin has been characterized as a new adipokine that is mainly expressed in and secreted from visceral fat rather than subcutaneous fat. Visfatin exerts insulin-mimetic effects in stimulating muscle and adipocyte glucose transport and in inhibiting hepatocyte glucose production (Adeghate 2008). Visfatin binds and activates the insulin receptor, thereby inducing receptor phosphorylation and the activation of downstream signaling molecules. However, visfatin and insulin do not compete for binding to the insulin receptor, which indicates that the two proteins recognize different regions of the receptor (Adeghate 2008). Thus, visfatin might play a role in glucose homeostasis and in the pathogenesis of diabetes. However, the precise mechanism by which visfatin achieves its hypoglycemic effect has not been elucidated.

AMP-activated protein kinase (AMPK) belongs to a family of serine/threonine kinases. AMPK is a cellular fuel sensor that monitors the AMP:ATP ratio to maintain cellular homeostasis. AMPK is activated when cellular energy is depleted (Hardie & Carling 1998). Upon phosphorylation at Thr¹⁷² of the catalytic subunit, activation of AMPK accelerates catabolic pathways, including glycolysis and fatty acid oxidation (Makinde *et al.* 1997, Zong *et al.* 2002) and simultaneously reduces energy-consuming anabolic pathways, such as the synthesis of cholesterol, fatty acids, and triacylglycerol (Henin *et al.* 1995). AMPK α 2 is also activated by physiological stimulation, such as muscle contraction, and by a pharmacological activator, 5-amino-1- β -D-ribofuranosyl-imidazole-4-carboxamide (AICAR), which results in a significant increase in glucose uptake mediated by the translocation of glucose transporter type 4 (GLUT4) (Hayashi *et al.* 1998, Mu *et al.* 2001). GLUT4, which is highly expressed in adipose tissue and skeletal muscle (Tsao *et al.* 1996, 2001), mediates the removal of circulating glucose. Thus, GLUT4 is a key regulator of systemic glucose homeostasis (Rossetti *et al.* 1997, Stenbit *et al.* 1997, Li *et al.* 2000). The expression of GLUT4 is controlled by a complex mechanism (Huang & Czech 2007). Translocation of GLUT4 to the plasma membrane is a prerequisite for the stimulation of glucose uptake by insulin in muscle and fat tissues (Klip & Marette 1992, Kahn 1996). The glucose transport in skeletal muscle is regulated by two distinct pathways. One is stimulated by insulin through insulin receptor substrate 1 (IRS-1)/PI3-kinase; the other is stimulated by muscle contraction/exercise through the activation of AMPK

(Krook *et al.* 2004). Both pathways also increase the phosphorylation and activity of MAPK family components (Goodyear *et al.* 1996, Widegren *et al.* 1998); for example, p38 MAPK participates in the full activation of GLUT4 (Konrad *et al.* 2001, Michelle Furtado *et al.* 2003). In the present study, the effects of visfatin on AMPK α 2 and glucose uptake were examined in order to determine the role of visfatin in hypoglycemia. Visfatin stimulates glucose uptake in skeletal muscle cells through a mechanism that involves the Ca²⁺-mediated AMPK-signals pathway. These results provide novel insights into the contribution of visfatin to glucose metabolism in skeletal muscle cells.

Materials and methods

Reagents

Antibodies against the following proteins were used in the present study: AMPK α 2, phospho-AMPK α 2 (Thr¹⁷²), acetyl-CoA carboxylase (ACC), phospho-ACC (Ser⁷⁹), anti-AS160, and phospho-AS160 (Thr⁶⁴²) (Millipore-Upstate, Billerica, MA, USA); p38 and phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²) (Epitomics, Inc., Burlingame, CA, USA); GLUT4, insulin receptor, and phospho-insulin receptor (Tyr¹¹⁵⁸) (Abcam, Cambridge, UK); and β -actin (Sigma). Insulin and *o*-phenylenediamine dihydrochloride (OPD) reagent were also obtained from Sigma. HRP-conjugated secondary antibodies were obtained from Enzo Life Sciences (Farmingdale, NY, USA). Compound C, SB202190, and STO-609 were obtained from Calbiochem (San Diego, CA, USA). Visfatin was purchased from Phoenix Pharmaceuticals (Burlingame, CA, USA). The fluorescent Ca²⁺ indicator fluo-3 AM was obtained from Invitrogen.

Cell culture

L6 rat myoblasts and C2C12 mouse myoblasts were maintained in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (FBS) and antibiotics (37 °C; 5% CO₂). For differentiation into myotubes, L6-GLUT4myc rat myoblasts were reseeded into 12-well plates (for glucose uptake) at a density of 2 × 10⁴ cells/ml. After 48 h (more than 80% confluence), the medium was switched to DMEM with 2% (v/v) FBS and was replaced after 2, 4, and 6 days of culture. Experiments were initiated on day 7 when myotube differentiation was complete. Cells were deprived of serum for 3–5 h before any experimental manipulation.

Immunoblot analysis

L6 and C2C12 cells were grown on six-well plates and were subjected to serum-starvation for 24 h before treatment. Following the experimental manipulations, the medium was removed. The cells were washed twice with ice-cold PBS and lysed with 60 μ l of lysis buffer (50 mM Tris-HCl (pH 7.4), 1% Triton X-100, 0.25% sodium deoxycholate, 150 mM EDTA, 1 mM sodium orthovanadate, 1 mM NaF, and 1 mM Na_3VO_4). The samples were sonicated, and the resulting supernatants were collected for immunoblot analysis. The protein concentration of the cell lysates was measured using Bio-Rad Protein Assay Reagent (Bio-Rad). The supernatants were resolved on 10% SDS-PAGE gels and then transferred to nitrocellulose membranes, which were incubated overnight at 4 °C with primary antibodies. After six washes in TBS with 0.1% Tween 20, the membranes were incubated for 1 h with HRP-conjugated secondary antibodies at room temperature. The blots were washed and visualized with chemiluminescence using the Amersham Biosciences ECL Western Blotting Detection System (Amersham International PLC). Densitometric analysis was performed with ImageJ Software (National Institutes of Health, Bethesda, MD, USA).

AMPK α 2, insulin receptor, and p38 MAPK silencing

Cells were seeded in six-well plates and grown to 70% confluence over 24 h. siRNA for AMPK α 2 (NM_001013367; Dharmacon, Lafayette, CO, USA), insulin receptor (Santa Cruz Biotechnology, Inc.), p38 MAPK (NM_053842; Dharmacon), and a non-targeted control siRNA (Dharmacon) were also purchased. Transient transfections were performed with Lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol. Briefly, 5 μ l of siRNA and 5 μ l of Lipofectamine 2000 were each diluted with 95 μ l of reduced serum medium (Opti-MEM; Invitrogen) and then combined. The mixtures were incubated for 20 min at room temperature and then added dropwise to a culture well containing 800 μ l of Opti-MEM, for a final siRNA concentration of 100 nM. After 4 h, the medium was replaced with fresh DMEM. The cells were cultivated for 48 h and then harvested for immunoblot analysis.

RT-PCR for first-strand cDNA synthesis

One microgram of total RNA was extracted from C2C12 cells. The reaction was performed at 55 °C for 20 min using the ThermoScript II One-Step RT-PCR Kit (Invitrogen).

cDNA was amplified using the Gene Amp System 9700 thermocycler (Applied Biosystems). The reverse transcriptase was heat-inactivated in the first step of the PCR (94 °C for 5 min). The following primers were used for amplification: GLUT4 forward: 5'-CAGCCTACGCCACCATAG-TAC-3' and reverse: 5'-TTCCAGCAGCAGCAGAG-3'; β -actin forward: 5'-ATTTGGTCGTATTGGGCGCCTGGT-CACC-3' and reverse: 5'-GAAGATGGTGATGGGATTTC-3'. The amplification steps were as follows: 27 cycles of 94 °C for 30 s, 56 °C for 30 s, and 72 °C for 30 s, followed by 7 min at 72 °C. For each RT-PCR, 10 μ l of product was analyzed by agarose gel electrophoresis. Bands were stained with ethidium bromide, and the band intensity was quantified using a gel documentation system (Gene Genius; Syngene, Cambridge, UK).

Immunodetection of GLUT4myc

Cell surface expression of GLUT4myc was quantified using an antibody-coupled colorimetric absorbance assay. Briefly, following stimulation, cells that stably expressed L6-GLUT4myc were incubated with polyclonal anti-myc antibody (1:1000) for 60 min, fixed with 4% paraformaldehyde in PBS for 10 min, and incubated with HRP-conjugated goat anti-rabbit IgG (1:1000) for 1 h. Cells were washed six times in PBS and incubated in 1 ml of OPD reagent (0.4 mg/ml OPD and 0.4 mg/ml urea hydrogen peroxide) for 30 min at room temperature. The reaction was stopped with 0.25 ml of 3 M HCl. The optical absorbance of the supernatant was measured at 492 nm. Background levels as measured in samples that had been incubated in peroxidase-conjugated anti-rabbit IgG alone (without primary antibody), were subtracted from all values.

2-Deoxyglucose uptake

Glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D-[^3H] glucose in differentiated L6 myotubes. Cells were rinsed twice with warm PBS (37 °C) and then starved in serum-free DMEM for 3 h. After treatment with the indicated agents, the cells were incubated in KRH buffer (20 mM HEPES (pH 7.4), 130 mM NaCl, 1.4 mM KCl, 1 mM CaCl_2 , 1.2 mM MgSO_4 , and 1.2 mM KH_2PO_4) containing 0.5 μ Ci of 2-deoxy-D-[^3H]glucose for 15 min at 37 °C. The reaction was terminated by placing the plates on ice and washing them twice with ice-cold PBS. The cells were lysed in 0.5 M NaOH, and 400 μ l of the lysate was mixed with 3.5 ml of scintillation cocktail. Radioactivity was determined by scintillation counting.

Ca²⁺ measurement with fluo-3 AM

Ca²⁺ concentration was determined by detecting fluorescence in cells treated with the Ca²⁺-sensitive indicator fluo-3 AM using confocal microscopy (Zeiss LSM 510 Meta; Zeiss, Oberkochen, Germany). C2C12 cells were loaded with 5 mM fluo-3 AM in regular culture medium for 45 min at room temperature. Following washes with medium, the cells were incubated for 15 min in the absence of fluo-3 AM in order to completely de-esterify the dye. The cells were treated with visfatin. The culture plates were placed on a temperature-controlled microscope stage and observed using the 20× objective. The excitation and emission wavelengths for signal detection were 488 and 515 nm respectively.

Statistical analysis

Data were expressed as the mean ± S.E.M. One-way ANOVA, followed by the Holm–Sidak multiple-range test, was used for comparisons between groups. *P* values of <0.05 were considered statistically significant.

Results

Visfatin activates AMPKα2 and its downstream target ACC in skeletal muscle cells

To assess whether visfatin plays a role in AMPKα2 regulation, AMPKα2 phosphorylation in visfatin-treated C2C12 mouse skeletal muscle cells was assessed. The levels of phospho-AMPKα2 (Thr¹⁷²) and its downstream target phospho-ACC (Ser⁷⁹) were higher in visfatin-treated cells than they were in control cells, and the effect was dose-dependent (Fig. 1A). In addition, visfatin induced a time-dependent increase in AMPKα2 phosphorylation (Fig. 1B). To determine whether visfatin is involved in the regulation of AMPKα2 in other skeletal muscle cells, the level of AMPKα2 phosphorylation in visfatin-treated rat myoblast L6 cells was examined. Visfatin increased the phosphorylation of AMPKα2 and ACC in a time-dependent manner (Fig. 1C). These results indicate that visfatin activates AMPKα2 in skeletal muscle cells.

Visfatin activates the AMPKα2 signaling pathway through the insulin receptor in skeletal muscle cells

It has been reported that visfatin regulates insulin secretion and insulin receptor signaling (Brown *et al.*

2010). In an effort to understand the signaling pathways involved in visfatin-mediated glucose uptake, we investigated the effects of visfatin on the phosphorylation of the insulin receptor. Upon visfatin treatment, the insulin receptor was activated in C2C12 cells in a time-dependent manner (Fig. 2A). To determine whether insulin receptor expression is required for the phosphorylation of AMPKα2, the levels of phospho-AMPKα2 (Thr¹⁷²) were measured in cells treated with the insulin receptor siRNA in the presence of visfatin. Visfatin-induced AMPKα2 phosphorylation was not observed upon knockdown of the insulin receptor, moreover, non-target siRNA did not produce such an effect (Fig. 2B), which indicates that visfatin activates AMPKα2 through the insulin receptor. Among established skeletal muscle cells, L6 myotubes exhibit greater glucose uptake than C2C12 cells (Sarabia *et al.* 1990), which indicates that L6 cells could be a promising model for investigating glucose uptake. To examine whether visfatin has insulin-sensitizing potential, differentiated L6 cells were treated with 100 nM insulin in the presence or absence of visfatin. When L6 cells were co-treated with visfatin and insulin, glucose uptake increased further (Fig. 2C). To provide direct evidence of the role of the insulin receptor in visfatin-mediated glucose uptake, we knocked down the insulin receptor by using insulin receptor siRNA. As shown in Fig. 2D, visfatin-mediated glucose uptake was not observed in cells transfected with insulin receptor siRNA, which indicates that visfatin stimulates glucose uptake through the insulin receptor. To test the involvement of AKT and ERK during visfatin-mediated glucose uptake, inhibitors of PI3-kinase and ERK, LY-294002, and PD98059 were used. Visfatin-mediated glucose uptake was not blocked by treatment with these inhibitors (Fig. 2E and F), which indicates that AKT and ERK are not involved in visfatin-induced glucose uptake. To further characterize the signal pathway of visfatin, we examined the effects of visfatin on the activity of Akt substrate 160 (AS160), a downstream target of AMPK and a protein with a Rab-GTPase activating protein (Rab-GAP) domain that is involved in the regulation of GLUT4 translocation (Treebak *et al.* 2006). In addition, it has been reported that AS160 integrates AMPKα2 signals that regulate GLUT4 traffic (Thong *et al.* 2007). We observed that visfatin increased the phosphorylation of AS160 (Thr⁶⁴²) when the former was used at a concentration of 300 ng/ml in a time-dependent manner (Fig. 2G). Taken together, these findings indicate that the insulin receptor plays a crucial role in visfatin-mediated glucose uptake.

Figure 1

Visfatin activates AMPKα2 and its downstream target ACC in skeletal muscle cells. (A) C2C12 cells were stimulated for 60 min with several concentrations of visfatin. The cell lysates (30 μg) were analyzed by western blotting using antibodies against phospho-AMPKα2 (Thr¹⁷²) and phospho-ACC (Ser⁷⁹), with AMPKα2 and ACC as controls. The results shown are representative of three independent experiments. **P* < 0.05 versus control. (B) C2C12 cells were incubated with visfatin (300 ng/ml) for the indicated times. Cell lysates were analyzed by western blotting using antibodies against phospho-

AMPKα2 (Thr¹⁷²) and phospho-ACC (Ser⁷⁹), with AMPKα2 and ACC as controls. The results shown are representative of three independent experiments. **P* < 0.05 versus control. (C) L6 cells were incubated with visfatin (300 ng/ml) for the indicated times. Cell lysates were analyzed by western blotting using antibodies against phospho-AMPKα2 (Thr¹⁷²) and phospho-ACC (Ser⁷⁹), with AMPKα2 and ACC as controls. The results shown are representative of three independent experiments. **P* < 0.05 versus control. Cropped images of full-length blots are shown.

Visfatin stimulates glucose uptake through activation of the AMPKα2 pathway in skeletal muscle cells

It has been reported that visfatin has a glucose-lowering effect in diabetic mice (Naz *et al.* 2011). To confirm this effect in a cell culture system, we analyzed the glucose uptake using differentiated skeletal muscle cells. The presence of visfatin resulted in dose- and time-dependent increases in glucose uptake in L6 cells (Fig. 3A and B). Maximum glucose uptake was observed after 3 h at a concentration of 300 ng/ml. To verify the role of AMPKα2 in visfatin-mediated glucose uptake, compound C, an AMPKα2 inhibitor, was used. First, we showed that compound C inhibited the visfatin-mediated phosphorylation of AMPKα2 (Fig. 3C). Furthermore, the pretreatment of cells with compound C (5 μM) in the presence of visfatin prevented visfatin-mediated glucose uptake (Fig. 3D),

which indicates that visfatin stimulates glucose uptake by acting on AMPKα2. Next, to confirm that visfatin-mediated glucose uptake was the result of AMPKα2 activation, AMPKα2 expression was knocked down using siRNA. Visfatin did not increase the phosphorylation of AMPKα2 in AMPK knockdown cells (Fig. 3E). In addition, visfatin-mediated glucose uptake was not observed in AMPKα2 siRNA-transfected cells (Fig. 3F). These results indicate that visfatin mediates glucose uptake through the AMPKα2 pathway.

Intracellular Ca²⁺ is involved in visfatin-induced AMPKα2 phosphorylation

Ca²⁺/calmodulin-dependent protein kinase kinase (CaMKK) is involved in the regulation of contraction-induced

Figure 2

Visfatin activates the AMPK α 2 signaling pathway through the insulin receptor in C2C12 cells. (A) C2C12 cells were treated with visfatin (300 ng/ml) for the indicated times. Cell lysates (30 μ g) were analyzed by western blotting using an antibody against phospho-insulin receptor. Insulin receptor was used as a loading control. The results shown are representative of three independent experiments. * P < 0.05 versus control. (B) C2C12 cells were transiently transfected with insulin receptor siRNA (100 nM) for 48 h and then incubated with visfatin (300 ng/ml) for 1 h. The cell lysates (30 μ g) were analyzed by western blotting using antibodies against phospho-AMPK α 2 (Thr¹⁷²) and insulin receptor. AMPK α 2 and β -actin were used as loading controls. The results shown are representative of three independent experiments. ** P < 0.05 versus visfatin-treated cells. (C) L6 myotube cells were treated with visfatin (300 ng/ml, 3 h) in the presence of insulin (100 nM, 30 min) and then assayed for glucose uptake.

** P < 0.05 versus visfatin-treated cells. (D) L6 myotube cells were transiently transfected with rat insulin receptor siRNA (100 nM) for 48 h and then incubated with visfatin (300 ng/ml) for 3 h before assaying for glucose uptake. ** P < 0.05 versus visfatin-treated cells. (E and F) Pharmacological inhibition of PI3K and ERK has no effect on visfatin-induced glucose uptake. After treatment with the inhibitor of PI3K (LY-294002 (5 μ M)) and ERK (PD98059 (20 μ M)) for 30 min, L6 myotube cells were incubated for 3 h with visfatin (300 ng/ml) and then assayed for glucose uptake. # P < 0.05 versus co-treatment with visfatin and inhibitor. (G) C2C12 cells were incubated with visfatin (300 ng/ml), and lysates were analyzed by western blotting using an antibody against phospho-AS160 (Thr⁶⁴²), with AS160 as a control. The results shown are representative of three independent experiments. * P < 0.05 versus control. Cropped images of full-length blots are shown.

substrate metabolism (Abbott *et al.* 2009). CaMKK activates AMPK α 2 in yeast and *in vitro* in cells that lack the known AMPK kinase LKB1 (Wright *et al.* 2004, Hawley *et al.* 2005, Hong *et al.* 2005, Hurley *et al.* 2005). In addition, it has been reported that CaMKK acts an upstream effector of AMPK α 2 in the activation of glucose uptake (Jensen *et al.* 2007). CaMKK is activated by elevated intracellular calcium. To corroborate the role of CaMKK in

visfatin-mediated glucose uptake, intracellular Ca²⁺ levels were measured in the presence of visfatin. Visfatin treatment increased the intracellular Ca²⁺ concentration (Fig. 4A). To determine the hierarchy between AMPK α 2 and CaMKK in visfatin-mediated signaling pathways, C2C12 cells were pretreated with STO-609, a CaMKK inhibitor, before the addition of visfatin. Levels of phospho-AMPK α 2 (Thr¹⁷²) were used as a measure of

Figure 3

Visfatin stimulates glucose uptake through activation of the AMPK pathway in differentiated muscle cells. (A) L6 myotube cells were incubated with several concentrations of visfatin for 3 h and then assayed for glucose uptake. **P* < 0.05 versus control. (B) L6 myotube cells were incubated with visfatin (300 ng/ml) for the indicated times and then assayed for glucose uptake. **P* < 0.05 versus control. (C) After treatment with compound C, C2C12 cells were incubated with visfatin (300 ng/ml) for 1 h, and the cell lysates (30 μg) were analyzed by western blotting using an antibody against phospho-AMPKα2. The results shown are representative of three independent experiments. **P* < 0.05 versus visfatin-treated cells. (D) L6 myotube cells were incubated for 3 h with visfatin (300 ng/ml) in the

presence of the AMPK inhibitor compound C (5 μM) and then assayed for glucose uptake. **P* < 0.05 versus visfatin-treated cells. (E) C2C12 cells were transiently transfected with AMPK siRNA (100 nM) for 48 h and then incubated with visfatin (300 ng/ml) for 1 h. The cell lysates (30 μg) were analyzed by western blotting using antibodies against phospho-AMPKα2 (Thr¹⁷²) and AMPKα2. β-actin was used as a loading control. The results shown are representative of three independent experiments. **P* < 0.05 versus visfatin-treated cells. Cropped images of full-length blots are shown. (F) L6 myotube cells were transiently transfected with rat AMPKα2 siRNA (100 nM) for 48 h and then incubated with visfatin (300 ng/ml) for 3 h before assaying for glucose uptake. **P* < 0.05 versus visfatin-treated cells.

AMPKα2 activation. STO-609 blocked visfatin-induced AMPKα2 phosphorylation (Fig. 4B), which indicates that visfatin increases the activity of AMPKα2 via CaMKK. Furthermore, pretreatment with STO-609 blocked visfatin-induced glucose uptake (Fig. 4C). These results indicate that visfatin induces glucose uptake through the Ca²⁺-mediated CaMKK/AMPKα2 pathway.

The p38 MAPK pathway is involved in visfatin-mediated glucose uptake

It has been reported that p38 MAPK is involved in glucose uptake (Somwar *et al.* 2001, Cheng *et al.* 2006). To verify the role of p38 MAPK in visfatin-mediated glucose uptake, the effect of visfatin on the activation of p38 MAPK was examined. Immunodetection of phosphorylated p38

MAPK was used to determine the levels of activated p38 MAPK. Visfatin treatment (300 ng/ml) increased p38 MAPK activation after 10 min, and this increase was sustained for at least 180 min in C2C12 cells (Fig. 5A). The presence of the AMPKα2 inhibitor compound C abrogated the visfatin-induced increase in p38 MAPK activation (Fig. 5B), which indicates that p38 MAPK is a downstream target of AMPKα2. In addition, the visfatin-induced uptake of glucose was abolished upon treatment with SB202190, a p38 MAPK inhibitor (Fig. 5C). To confirm the role of p38 MAPK in glucose uptake, we examined glucose uptake in p38 MAPK knockdown cells. Visfatin-mediated glucose uptake was not observed in cells transfected with p38 MAPK siRNA (Fig. 5D). Taken together, these results indicate that visfatin stimulates glucose uptake via the p38 MAPK pathway.

Figure 4

Intracellular Ca^{2+} is involved in visfatin-induced AMPK phosphorylation. (A) For Ca^{2+} detection, cells were pre-incubated in fluo-3 AM (10 μM) for 45 min. The Ca^{2+} response was measured after cells that had been pretreated with EDTA for 30 min were incubated with visfatin (300 ng/ml). The Ca^{2+} concentration correlates with the fluorescence intensity. (B) C2C12 cells were pretreated with the CaMKK inhibitor STO-609 (2 μM) and then incubated with visfatin (300 ng/ml) for 60 min. Cell lysates (30 μg)

were analyzed by western blotting using an antibody against phospho-p38 MAPK (Thr¹⁸⁰/Tyr¹⁸²), with AMPKα2 as a control. The results shown are representative of three independent experiments. * $P < 0.05$ versus visfatin-treated cells. (C) L6 myotube cells were incubated with visfatin (300 ng/ml) for 3 h in the presence of STO-609 (2 μM) before being assayed for glucose uptake. * $P < 0.05$ versus visfatin-treated cells. Cropped images of full-length blots are shown.

Visfatin induces GLUT4 expression and also stimulates GLUT4 translocation

Glucose uptake in skeletal muscle is accompanied by an increase in GLUT4 translocation. Furthermore, a close correlation between the GLUT4 concentration in muscle and maximally stimulated glucose transport activity has been reported (Henriksen *et al.* 1990, Ren *et al.* 1994). To determine the effect of visfatin on the expression of GLUT4, we evaluated GLUT4 expression after treatment with visfatin. Visfatin treatment induced a

time-dependent increase in the levels of GLUT4 mRNA (Fig. 6A) and protein (Fig. 6B) in C2C12 cells. According to a report that described the enhancement of GLUT4 translocation by AMPKα2 (Yamaguchi *et al.* 2005), AMPK regulates GLUT4 translocation. Therefore, we assessed the effect of AMPK and p38 MAPK on visfatin-mediated GLUT4 translocation. A decrease in plasma membrane GLUT4myc was observed in the presence of visfatin when cells were pretreated with compound C, an AMPKα2 inhibitor, and SB202190, a p38 MAPK inhibitor

Figure 5

The p38 MAPK pathway is involved in visfatin-mediated glucose uptake. (A) C2C12 cells were incubated with visfatin (300 ng/ml), and lysates were analyzed by western blotting using an antibody against phospho-p38 MAPK, with p38 MAPK as a control. The results shown are representative of three independent experiments. * $P < 0.05$ versus control. (B) C2C12 cells were pretreated with compound C (5 μM) and then incubated with visfatin (300 ng/ml) for 60 min. Cell lysates (30 μg) were analyzed by western blotting using an antibody against phospho-p38 MAPK, with p38 MAPK as a control.

The results shown are representative of three independent experiments. * $P < 0.05$ versus visfatin-treated cells. (C) L6 myotube cells were incubated with visfatin (300 ng/ml) for 3 h in the presence of the p38 MAPK inhibitor SB202190 (2 μM) before assaying for glucose uptake. * $P < 0.05$ versus visfatin-treated cells. (D) L6 myotube cells were transiently transfected with rat p38 MAPK siRNA (100 nM) for 48 h and then incubated with visfatin (300 ng/ml) for 3 h before assaying for glucose uptake. * $P < 0.05$ versus visfatin-treated condition. Cropped images of full-length blots are shown.

Figure 6

Visfatin induces GLUT4 expression and also stimulates GLUT4 translocation. (A) Total RNA from C2C12 cells was prepared after visfatin treatment, and RT-PCR was performed using GLUT4-specific primers. β -actin mRNA served as a positive control. The results shown are representative of three independent experiments. $*P < 0.05$ versus visfatin-treated cells. (B) C2C12 cells were incubated with visfatin (300 ng/ml) for the indicated times. Cell lysates (30 μ g) were analyzed by western blotting using an antibody against GLUT4, with β -actin as a control. The results shown are from three independent experiments. $*P < 0.05$ versus visfatin-treated cells. (C and D) Confluent monolayers of myotubes were pretreated with compound C

(5 μ M) and a p38 MAPK inhibitor, SB202190 (20 μ M), respectively for 30 min and then incubated with visfatin (300 ng/ml) for 3 h. Cell surface expression of GLUT4myc was detected using an antibody-coupled colorimetric absorbance assay. $*P < 0.05$ versus visfatin-treated cells. (E) L6-GLUT4myc-tagged cells were transiently transfected with rat p38 MAPK siRNA (100 nM) for 48 h and then incubated with visfatin (300 ng/ml) for 3 h. Cell-surface expression of GLUT4myc was detected using an antibody-coupled colorimetric absorbance assay. $*P < 0.05$ versus visfatin-treated cells. Cropped images of full-length blots are shown.

(Fig. 6C and D). Furthermore, visfatin-induced GLUT4 translocation was blocked under p38 MAPK knockdown conditions (Fig. 6E). These results indicate that visfatin-induced GLUT4 translocation is mediated by the AMPK α 2–p38 MAPK signaling pathways.

Discussion

The key finding of the present study is that visfatin stimulates glucose uptake via Ca^{2+} -mediated AMPK α 2 activation in C2C12 cells. This was determined directly by observing glucose uptake in visfatin-treated cells (Fig. 2) and indirectly through knockdown of the insulin receptor and examination of GLUT4 localization at the plasma membrane in the presence of visfatin (Figs 3 and 6). Taken together, these results indicate that the hypoglycemic effect of visfatin involves the regulation of the AMPK pathway in C2C12 cells.

Visfatin is an adipokine secreted by visceral fat in humans and mice. Like insulin, it increases glucose transport and lipogenesis by adipocytes and myocytes and decreases glucose production by hepatocytes (Berndt *et al.* 2005, Hammarstedt *et al.* 2006). Visfatin binds the insulin receptor and stimulates phosphorylation of the insulin receptor, phosphorylation of IRS-1 and IRS-2, binding of PI3K to IRS-1 and IRS-2, and phosphorylation of the downstream kinases Akt and MAPK. However, visfatin and insulin do not compete for binding to the insulin receptor, which indicates that the two proteins recognize different regions of the receptor (Adeghate

2008). The circulating levels of visfatin are not affected by feeding (Kang *et al.* 2011). Taken together, these findings indicate that visfatin regulates glucose metabolism in a manner that is distinct from that of insulin.

Recent studies have provided evidence for a glucose-lowering effect of visfatin in insulin-resistant and insulin-dependent diabetic mice (Naz *et al.* 2011). Visfatin levels increased in obese mice, and administration of recombinant histidine-tagged soluble murine visfatin significantly decreased the blood glucose levels of obese and insulin diabetic mice. Obesity induces the release of visfatin from adipocytes, and visfatin is expressed at higher levels in visceral fat than it is in subcutaneous fat. It has been suggested that the increase in visceral fat in the obese mice resulted in substantial increases in visfatin levels, whereas the hyperglycemia was the result of insulin-resistant diabetes mellitus (Berndt *et al.* 2005). Furthermore, the results of studies on adipocytes have indicated that visfatin release is regulated by hormones and cytokines that influence glucose homeostasis (Arner 2006, Haider *et al.* 2006). Apart from its hypoglycemic action, visfatin induces proliferation and the production of type 1 collagen, similarly to the insulin receptor transduction pathway (Xie *et al.* 2007). Given that visfatin mimics the effects of insulin through the insulin receptor in cell cultures of adipocytes, myocytes, and hepatocytes, osteoblasts may be future targets for studies of visfatin action (Xie *et al.* 2007).

p38 MAPK plays a pivotal role in glucose uptake in skeletal muscles (Lemieux *et al.* 2003) and adipocytes (Bazuine *et al.* 2003). In the present study, visfatin-induced

glucose uptake disappeared in the presence of inhibitors of AMPK or p38 MAPK. Thus, these findings indicate that visfatin stimulates glucose uptake by activating the AMPK–p38 MAPK signaling pathways. On the other hand, p38 MAPK might not mediate the inhibition of insulin-stimulated glucose transport by SB203580 (Antonescu *et al.* 2005). Novel targets of SB203580, such as cyclin-G-associated kinase, protein kinase N β , and JNK1/2 have been identified (Godl *et al.* 2003). Thus, insulin might independently stimulate glucose uptake via p38 MAPK. At the same time, the activity of p38 MAPK in AMPK-mediated downstream signaling may be critical for glucose uptake (Lee *et al.* 2008). These results indicate that p38 MAPK plays an important role in glucose uptake, at least insofar as it is part of AMPK downstream signaling.

Some controversy surrounds the exact mechanism of visfatin function. Specifically, it is not clear whether visfatin has insulin-mimetic activity or whether visfatin is a modulator of insulin action. An insulin-like effect has been reported in osteoblasts (Xie *et al.* 2007), and *in vitro* exposure to visfatin has been shown to increase skeletal muscle GLUT (Harasim *et al.* 2011), but these effects have not been demonstrated in human adipocytes (Wanecq *et al.* 2009). Given the known interaction of visfatin with the insulin receptor, these observations could be attributed to the variety of insulin and visfatin receptors. Visfatin is expressed not only by adipocytes but also in tissues involved in energy homeostasis, such as the liver, heart, and skeletal muscle (Sommer *et al.* 2008). Although the results of the present study support a role for visfatin in glucose metabolism in skeletal muscle cells, the results from previous studies make it difficult to draw definite conclusions about the *in vivo* function of visfatin. Future work should address the relationship between visfatin and insulin receptors in order to elucidate the molecular mechanisms that underlie visfatin function.

In summary, visfatin stimulates glucose uptake via AMPK α 2 activation in muscle cells through a mechanism that probably involves the CaMKK-mediated phosphorylation of AMPK α 2. These findings provide insight into the hypoglycemic functions of visfatin, which could become a focus of future research into the treatment of diabetes.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the National Research Foundation of Korea, which is funded by the Korean government (grant number NRF-2013R1A2A2A05004796).

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Received in final form 25 March 2015

Accepted 14 April 2015