MAPK pathway mediates the induction of visfatin in neonatal SD rat cardiomyocytes pretreated with glucose

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Abstract. The protein visfatin is an insulin mimetic that has been shown to reduce plasma glucose levels, increase cytokine production and induce angiogenesis. However, few studies have focused on visfatin expression in cardiomyocytes at the cellular level. Therefore, the aim of the present study was to investigate visfatin expression and its potential mechanisms in cultured neonatal rat cardiomyocytes exposed to high-glucose concentrations. Primary cultures of 2-to 3-day-old Sprague Dawley (SD) rat cardiomyocytes were pretreated with increasing concentrations of glucose. P38 mitogen-activated protein kinase (MAPK) pathway inhibitor SB203580, extra cellular signal-regulated kinase (ERK1/2) pathway inhibitor PD098059 and c-Jun NH 2-terminal kinase (JNK) pathway inhibitor SP600125 were used to block the effect of glucose on visfatin expression. Cell viability following each glucose treatment was determined using the MTT assay. Expression of visfatin was detected using RT-PCR and western blot analysis. Increased glucose concentration directly correlated with an increased expression of visfatin mRNA and protein in neonatal rat cardiomyocytes. Following high doses of glucose, visfatin mRNA and protein expression peaked after 24 h with no significant change thereafter. Increased visfatin expression was blocked by the P38 MAPK inhibitor SB203580, suggesting a potential mechanism not yet identified. Expression of visfatin in cardiomyocytes was increased through the P38 MAPK pathway in the presence of high-glucose concentrations.

Introduction

The primary purpose of adipose tissue is energy storage; however, it has been recently identified (1) as an active endocrine organ producing a number of hormones and cytokines (adipocytokines) that are crucial in energy regulation and metabolism in the body. The abnormal secretion of hormones and adipocytokines has been attributed to a variety of metabolic disorders including insulin resistance (IR), obesity, diabetes and cardiovascular disease.

Visfatin is a newly identified adipocytokine derived from visceral adipose tissue that has been shown to have insulin-mimetic actions (1). It binds to the insulin receptor at a site distinct from that of insulin, enhancing glucose utilization in adipocytes, muscle cells and myocytes while simultaneously inhibiting hepatic gluconeogenesis. Clinical studies using the glucose clamp test in normoglycemic patients have shown, that acute increases in the glucose concentration produced increased plasma levels of visfatin. This physiologic effect was blocked by insulin or somatotropin release-inhibiting hormone (2). It has also been suggested that elevated plasma levels and visfatin mRNA expression in type II diabetics correlate with the development and progression of IR (3). Additionally, this protein has been identified in skeletal muscle, bone marrow, stromal and liver cells and has demonstrated a role in the formation of vulnerable plaque, vascular proliferation, endothelial dysfunction, inflammation and coronary heart disease.

Results of previous studies have shown that an increased visfatin expression may be cardioprotective following myocardial ischemia related to induction by HIF-1α (4-6). Lim *et al* (7) demonstrated that visfatin reduced infarct size by 50% following ischemia reperfusion injury in a murine model. This cardioprotective effect was considered to be the result of phosphatidylinositol-3-kinase (PI3K) and MEK 1/2 activation as evidenced by the lack of protection in the presence of kinase inhibitors wortmannin and UO126 (7). At present, there are limited systematic studies evaluating these mechanisms under controlled, *in vitro* conditions in various disease models.

Therefore, the aim of this study was to determine the expression and potential role of visfatin in neonatal rat cardiomyocytes pretreated with increasing concentrations of glucose. The findings are likely to elucidate the function of visfatin in the pathogenesis of diabetes and provide new theories for the clinical diagnosis and treatment of cardiovascular disease associated with diabetes mellitus.

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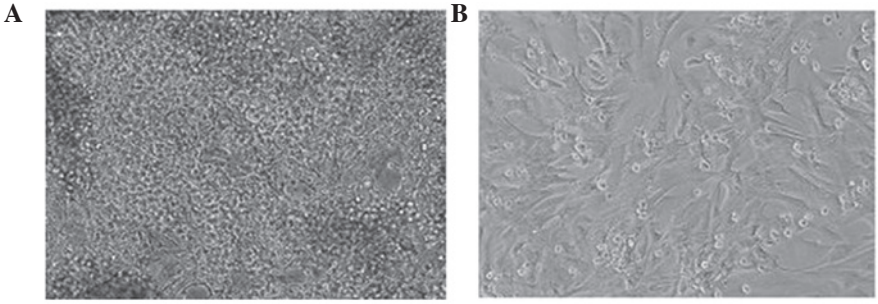

Figure 1. Cardiomyocytes of neonatal rats at (A) 0 h and (B) 72 h after primary culture.

Materials and methods

Experimental procedures were in accordance with the Experimental Animal Center of Hebei Province. Rats were housed in a temperature- and humidity-controlled room with a 12-h light/dark cycle prior to the beginning of experiments. No anesthetics were administered to avoid interferences with biochemical values.

Preparation of ventricular cardiomyocytes and cell culture. Neonatal left ventricular myocytes were enzymatically isolated from 2-to 3-day-old Sprague-Dawley (SD) rat hearts, pre-plated in two steps and plated onto 6-well microplates (Corning Costar, Corning, NY, USA) to yield confluent cardiomyocytes (8). 5-BrdU was applied to the cardiomyocytes to inhibit cardiac fibroblast growth for 48 h (9) followed by glucose treatment in serum-free medium for 24 h.

Analysis of cardiomyocyte viability. Cell viability of glucose-treated cardiomyocytes was detected using the MTT assay. Cardiomyocytes were plated at a density of $1x10^5$/ml in a 96-well plate for 48 h and then serum-starved for 24 h prior to treatment with glucose (Sigma-Aldrich, St. Louis, MO, USA). The cardiomyocytes were incubated with 20 μl of 5 mg/ml MTT solution (HyClone, Logan, UT, USA) for 4 h at 37˚C. Then, 150 μl of DMSO (HyClone) was added to each well to dissolve the dye crystal formazan, and the plate was agitated for 10 min until all the crystals were dissolved. The amount of MTT formazan was quantified by determining the absorbance at 490 nm using a microplate reader (Fig. l).

RNA isolation and RT-PCR analysis. Total RNA was isolated from cardiomyocytes using TRIzol reagent (SBS Genetech Co., Ltd., Shanghai, China). Purity was determined by absorbance of light at 260 nm. A specific 338-bp fragment was amplified using specific primers for the detection of visfatin gene expression (forward: 5'-ACTTTGAATGCCGTGAA-3'; reverse: 5'-AAT CCAGT =TGGTGAGCC-3'). GAPDH expression was used as an internal control (forward: 5'-GAGGCTCTCTTCCAGCC TTC-3'; reverse: 5'-AGGGTGTAAAAGCAGCTCA-3'). RT-PCR was run for 30 cycles under the following conditions: DNA was denatured at 94˚C for 60 sec, specific annealing occurred at 52˚C for 60 sec and 72˚C for 60 sec and a final extension step at 72˚C for 10 min. Amplification was linear under these conditions and carried out in a Biometra T-gradient Thermoblock PCR System (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). RT-PCR reactions for each gene were performed at the same time with the same batch of *Taq* polymerase to reduce variations in RT-PCR efficiency. Band densities were measured using a scanning densitometer with the scanning software Gel-Pro Analysizer 3.1.

Western blot analysis. Visfatin protein expression in cardiomyocytes was evaluated using western blot analysis. Cells were lysed during centrifugation, quantified and boiled in RIPA buffer. Samples (80 μg) were loaded and separated using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis and then electro-blotted onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA). The PVDF membrane was then blocked by incubating with 5% skim milk (Becton-Dickinson and Co., Franklin Lakes, NJ, USA) in 20 mM Tris, 150 mM NaCl, 0.05% Tween-20 tris-buffered solution (TBS) plus Tween-20 (TBS-T), at pH 7.4 for 1 h at room temperature. Membranes were incubated with the primary antibody anti-PBEF rabbit polyclonal (1:2,007) diluted in 5% TBS-T (Santa Cruz Biotechnology, Inc.) overnight at 4˚C and then washed thoroughly for 30 min at room temperature with TBST (0.1%), followed by incubation with secondary antibodies IRDye700DX and IRDye800CW (1:5,000; Rockland, Gilbertsville, PA, USA) for 1 h at room temperature. Antibody complexes were visualized using chemiluminescence (Odyssey9120; LI-COR Corporation, Lincoln, NE, USA). Band densities were measured using a scanning densitometer with the scanning software Gel-Pro Analysizer 3.1. In addition to the quantitative loading of gels, the membranes were also re-probed with GAPDH (Santa Cruz Biotechnology, Inc.) to use as a comparison for equal protein loading.

Statistics. Data were normalized for the housekeeping gene GAPDH and expressed as a mean ± standard deviation for the three experiments. Data were analyzed by t-test and ANOVA using SPSS 10.0 software where appropriate. $P<0.05$ was considered to indicate a statistically significant difference.

Results

Cell morphology. Newly inoculated cardiomyocytes (72 h) suspended in culture medium were morphologically bright and round (Fig. lA). After 8-12 h, a limited number of cardiac myocytes adhered to the wall and had a small nucleus, dense cytoplasm and were spontaneously beating (30-40 beats/min). After 24 h, all adherent cardiac myocytes were spindle-,

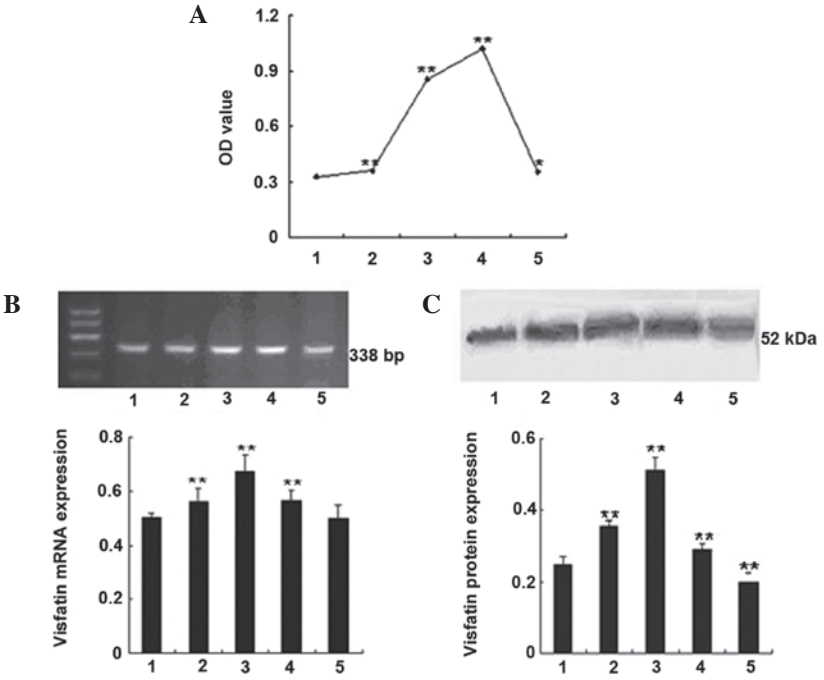

Figure 2. Cell viability and visfatin expression in cardiomyocytes pretreated with increasing concentrations of glucose over 48 h. Analysis of cardiomyocyte viability with (A) MTT, (B) visfatin mRNA and (C) protein expression in cardiomyocytes: 1, glucose 5.5 mmol/l; 2, glucose 10 mmol/l; 3, glucose 30 mmol/l; 4, glucose 50 mmol/l; 5, mannitol 50 mmol/l; *P<0.05 compared to glucose 5.5 mmol/l and **P<0.01 compared to glucose 5.5 mmol/l.

diamond- or polygonal-shaped and spontaneously beating at 60-100 beats/min. The cells migrated across the surface and developed pseudopodia, forming irregular stars and weaving into a network of mutual contacts. After 72 h, cultured cells were arranged in radial clusters (Fig. 1B) beating synchronously and autonomously at a rate of 100 beats/min.

Expression of visfatin in cardiomyocytes treated with different concentrations of glucose for 48 h. The optsical density (OD) value of the MTT products of cardiomyocytes pretreated with increasing concentrations of glucose (5.5, 10, 30 and 50 mmol/l) for 48 h increased in a dose-dependent manner peaking at the glucose concentration of 50 mmo1/l (Fig. 2A). Additionally, cardiomyocyte visfatin mRNA and protein expression increased with increasing glucose concentrations. The highest level of expression coincided with treatment with 30 mmol/l of glucose (Fig. 2B and C). There was no significant difference in visfatin mRNA expression between glucose 5.5 mmol/l and mannitol 50 mmol/l groups. Visfatin protein expression was lower in the mannitol 50 mmol/l group compared to the glucose 5.5 mmol/l group. The effect of high glucose on the increased visfatin expression in cardiomyocytes is the result of the glucose concentration and not the osmotic pressure. The OD value of MTT products of cardiomyocytes treated with 30 mmol/l glucose for 0, 6, 12, 24 and 48 h increased in a time-dependent manner peaking at 24 h followed by a notable decrease after 48 h (Fig. 3A). Visfatin mRNA and protein expression increased in a time-dependent manner, peaking after 24 h with no significant changes between 24 and 48 h (Fig. 3B and C).

The mechanism of visfatin expression increased in glucose-induced neonatal rat cardiomyocytes. We targeted the mitogen activated protein kinase (MAPK) pathway to investigate potential mechanisms contributing to changes in visfatin expression in glucose-treated cardiomyocytes. This was carried out using the inhibitors: SP600125 [c-Jun NH 2-terminal kinase (JNK); 10 μmol/l], PD098059 [extra cellular signal-regulated kinase (ERK1/2); 10 μmol/l] and SB203580 (P38 MAPK; 10 μmol/l) (10). Cells were exposed to inhibitors for 30 min prior to the addition of 30 mmol/l glucose and incubated for 24 h. Visfatin mRNA and protein expression were significantly decreased in cells exposed to the SB203580 group at 30 mmol/l, with no significant differences in the expression of myocytes treated with SP600125 or PD098059 (Fig. 4A and B).

Discussion

Cardiomyocytes in embryonic or neonatal rats are more sensitive and responsive to external stimuli than mature cardiomyocytes, allowing direct cell manipulation and control of environmental parameters without interference from compensatory feedback mechanisms that exist *in vivo*. This permits the study of developmental, physiological and pharmacological changes in cardiac tissue. Thus, we used these cells to identify unique intracellular signal transduction pathways present in a diabetic murine model.

Visfatin has been shown to preserve islet function and regulate insulin secretion while haplo-deficiency and the chemical inhibition of visfatin produces defects in glucose-stimulated insulin secretion in pancreatic islets (11). This protein activates target cells by binding to the IR and producing insulin-mimetic effects. It also plays a role in the development of obesity-associated IR and diabetes (12-14). Through the use of RT-PCR and western blot analysis, we were able to show a correlation

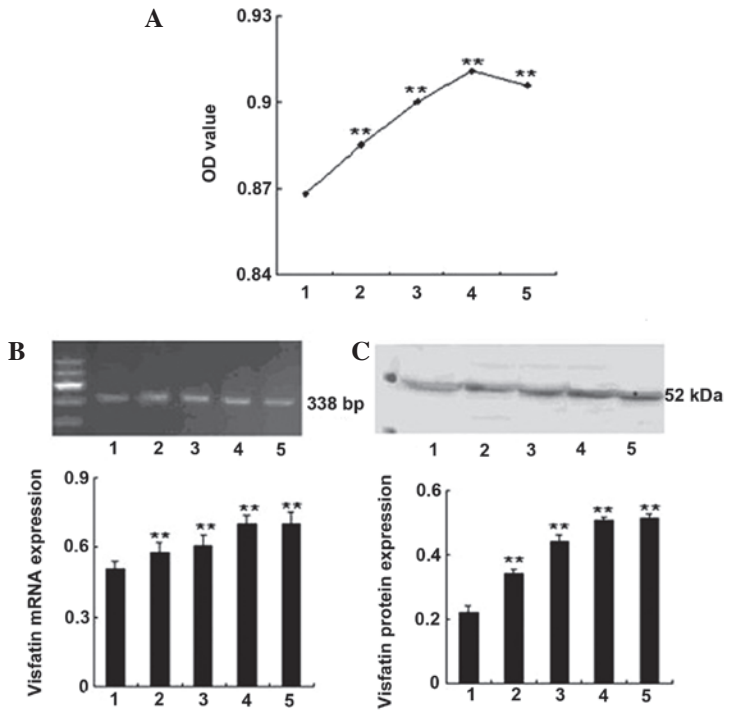

Figure 3. Cell viability and visfatin expression in cardiomyocytes treated with glucose 30 mmol/l for 0, 6, 12, 24 and 48 h. Analysis of cardiomyocytes viability with MTT (A). (B) Visfatin mRNA and (C) protein expression in cardiomyocytes: 1, control group; 2, 6 h; 3, 12 h; 4, 24 h and 5, 48 h. **P<0.01; ANOVA was used for comparison against the glucose 5.5 mmol/l group.

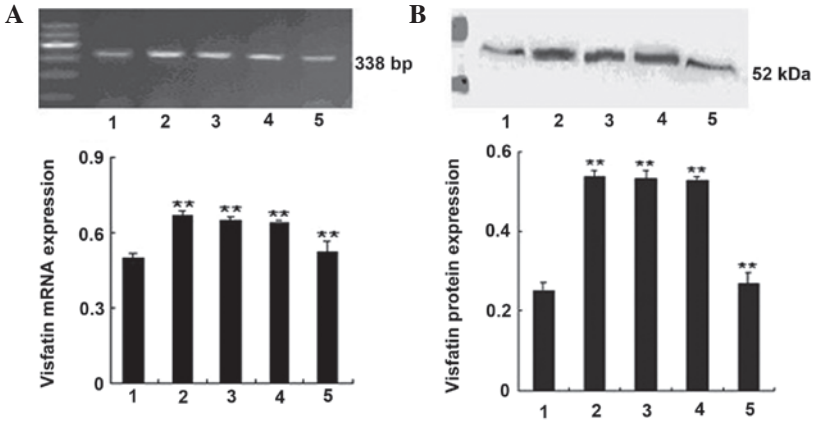

Figure 4. Visfatin (A) mRNA and (B) protein expression in cardiomyocytes treated with glucose at 30 mmol/l for 24 h in the presence of SB203580 (10 μmol/l), PD098059 (10 μmol/l) or SP600125 (10 μmol/l): 1, glucose 5.5 mmol/l; 2, glucose 30 mmol/l; 3, glucose 30 mmol/l+PD098059; 4, glucose 30 mmol/l+SP600125; 5, glucose 30 mmol/l+SB203580 **P<0.01 comparison against the glucose 5.5 mmol/l group.

between elevated glucose concentrations and visfatin mRNA and protein expression in cultured cardiomyocytes, suggesting these changes in visfatin expression may play a role in IR. Therefore we assessed the possible mechanisms of visfatin expression in glucose-treated cardiomyocytes and investigated the association of visfatin with diabetes and diabetic cardiomyopathy.

Haider *et al* (2) have previously shown a relationship between elevated blood sugar concentrations and compensatory increases of visfatin. There is evidence supporting that visfatin activities are the result of the phosphorylation of tyrosine residues on insulin receptor substrate IRS-1 and IRS-2, promoting the binding of phosphatidylinositol-3-kinase (PI3K) to IRS-1, IRS-2. This activity results in activation of the signal transduction pathway between protein kinase B and MAPK, suggesting an alternative mechanism in regulating blood sugar concentrations independent from that associated with insulin activity. Consequently, the relationship between visfatin and insulin is non-competitive. However, under normal physiologic conditions, visfatin may only play a secondary role in glucose metabolism.

To evaluate the effects of high glucose on visfatin expression in myocardial cells, we exposed myocytes to 10, 30 and 50 mmol/l of glucose. A total of 5.5 mmol/l exposure correlated to the average glucose concentration in our diabetic rat model. The results showed that cardiomyocytes express

visfatin. Myocardial cell visfatin mRNA and protein expression in the 5.5 mmol/l group was significantly lower than that of the 30 mmol/l high-glucose group. Visfatin expression in the cardiomyocytes increased with increasing concentrations of glucose. Although the expression of visfatin in the hypertonic group was >5.5 mmol/l group, it was not statistically different, indicating that the activities of high glucose on visfatin expression in myocardial cells are independent of osmotic pressure.

Myocardial cells exposed to high-glucose concentrations increased the expression of visfatin in a time-dependent manner, peaking at 24 h. Visfatin expression significantly increased at 6, 12 and 24 h of exposure compared to baseline with no statistical difference after 48 h. These data confirm changes in visfatin expression are a time- and -glucose concentration phenomenon, suggesting intracellular regulation of glucose homeostasis is a feedback mechanism.

Current evidence indicates visfatin expression is associated with inflammation and immune function. The level of visfatin is upregulated when inflammation occurs. Moschen *et al* (15) demonstrated that recombinant visfatin induces human peripheral blood CD14+ mononuclear cells to synthesize IL-1B, TNF-α and IL-6, supporting the theory that fat cells have endocrine properties capable of secreting inflammatory cytokines. These adipokines have been shown to alter the body's energy intake, storage and metabolism of insulin (16). MAPK is the important signaling system between extracellular signaling and intracellular reaction. Numerous factors activate the MAPK signaling transduction pathway when suffering from diabetes mellitus. It has been reported that high glucose regulates the biochemical events of renal cells by the PKC/MAPK signal pathway in diabetic nephropathy, while the PKC pathway activates the MAPK signaling transduction pathway (17). The P38 MAPK signal transduction pathway, which is activated by high sugar levels, mediates the stimulating processes of physiological stress, endotoxin, osmotic stress and ultraviolet light. The activated P38 is involved in regulating cell inflammatory response, apoptosis and microtubule function by phosphorylating different substrates relevant to cell metabolism and function, and then generating a unique biological effect. The activated P38 also plays a role in the secretion of fibronectin in glomeruli mesangial cells, inducing the formation of fibronectin and type I collagen, leading to glomerulosclerosis and tubulointerstitial fibrosis. Our data indicate that pretreatment with P38 MAPK inhibitor SB203580 in myocytes 30 min prior to exposure of 30 mmol/l glucose significantly reduced visfatin expression; however, pretreatment with ERK1/2 signaling pathway inhibitor PD098059 and inhibitor JNK SP600125 had no effect compared to control.

In conclusion, results of the present study have demonstrated that cardiomyocytes are capable of expressing visfatin, and are time- and glucose concentration-dependent. Visfatin mRNA and protein expression peaked at 24 h following elevated glucose exposure. This effect was blocked by a P38 MAPK pathway inhibitor, indicating P38 MAPK is a vital intracellular pathway in the process of high glucose-induced visfatin expression in cardiomyocytes.

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