

Visfatin inhibits apoptosis of pancreatic β -cell line, MIN6, via the mitogen-activated protein kinase/phosphoinositide 3-kinase pathway

Qun Cheng^{1,2}, Weipin Dong¹, Lei Qian¹, Jingcheng Wu¹ and Yongde Peng¹

¹Diabetes Research Laboratory, Department of Endocrinology and Metabolism, Shanghai Jiaotong University Affiliated First People's Hospital, 100 Hainan Road, Shanghai 200080, People's Republic of China

²Shanghai Geriatric Institute, Fudan University Affiliated Huadong Hospital, Shanghai 200040, People's Republic of China

(Correspondence should be addressed to Y Peng; Email: pengyongdetx@medmail.com.cn)

Abstract

Visfatin is an adipocytokine that plays an important role in attenuating insulin resistance by binding to insulin receptor. It has been suggested that visfatin plays a role in the regulation of cell apoptosis and inflammation by an as yet unidentified mechanism. This study investigated the protective effects of visfatin on palmitate-induced islet β -cell apoptosis in the clonal mouse pancreatic β -cell line MIN6. The cells were treated with palmitate and/or recombinant visfatin. An 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan assay was used to detect cell proliferation, V-FITC/propidium iodide staining was used to measure cell apoptosis and necrosis, and western blot analysis was used to detect the expression of proapoptotic proteins. The incubation of the cells with visfatin led to a concentration-dependent increase of cell proliferation (1.55-fold at 10^{-7} M and 24 h compared with control, $P < 0.05$). Visfatin significantly reduced the cell apoptosis induced by palmitate and caused a significant change in the expression of several proapoptotic proteins, including upregulation of Bcl-2 and a marked downregulation of cytochrome *c* and caspase 3. Visfatin also activated the ERK1/2 and the phosphoinositide 3-kinase (PI3K)/AKT signaling pathways in a time- and concentration-dependent manner, and the effect of visfatin on apoptosis was blocked by the specific ERK1/2 and PI3K/AKT inhibitors, PD098059 and LY294002. We conclude that visfatin can increase β -cell proliferation and prevent apoptosis, activate intracellular signaling, and regulate the expression of proapoptotic proteins. The antiapoptotic action of visfatin is mediated by activation of mitogen-activated protein kinase-dependent and PI3K-dependent signaling pathways.

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Introduction

Visfatin was originally identified as a secreted growth factor for early lymphocytes (pre-B-cell colony-enhancing factor, PBEF) by Samal *et al.* It is strongly induced by pokeweed mitogen and cycloheximide, and it enhances the effect of IL7 and stem cell factor on pre-B-cell colony formation (Samal *et al.* 1994). Rongvaux *et al.* (2002) demonstrated the involvement of PBEF in the synthesis of NAD, which is an essential cofactor in cell metabolism, and because it has intra- and extracellular nicotinamide phosphoribosyltransferase (NAMPTase) activity, this protein was also called Nampt. Fukuhara *et al.* (2005) isolated cytokine PBEF from visceral fat, and renamed it as visfatin, since the adipokine is highly expressed in visceral fat cells. Therefore, the terms 'visfatin', 'PBEF', and 'Nampt' refer to the same protein.

Visfatin was shown to activate its target cells by binding to insulin receptor (IR) at a site distinct from insulin, and exert a variety of insulin-mimetic effects, and play a role in the development of obesity-associated insulin resistance and diabetes (Fukuhara *et al.* 2005, Xie *et al.* 2007, Brown *et al.* 2010).

Visfatin is evolutionarily conserved and functions in an endocrine, autocrine, and paracrine manner. Visfatin exhibits important effects on the sensitivity of liver cells to insulin action through its effects on NAD biosynthesis (Skop *et al.* 2010). In response to a variety of inflammatory stimuli, visfatin is upregulated in neutrophils by IL1 β with functions as an inhibitor of apoptosis (Jia *et al.* 2004), and upregulated in colorectal cancer associated with regulation of the cell cycle (Nakajima *et al.* 2010). Its Nampt activity is shown to be important for vascular smooth muscle cell (VSMC) maturation (Van der Veer *et al.* 2005). A recent study reported that visfatin-deficient heterozygous (visfatin +/–) female mice showed a moderately impaired glucose tolerance and a reduced glucose-stimulated insulin secretion (Revollo *et al.* 2007), which strongly suggests that visfatin plays a critical role in the regulation of β -cell function.

Adipocytes have a unique capacity to store excess free fatty acids (FFAs) in the form of triglycerides in lipid droplets. The accumulation of excess lipids in adipose tissues leads to cell dysfunction and/or cell death, a phenomenon known as lipotoxicity (Unger *et al.* 2010).

Palmitate is the most abundant saturated FFA in the circulation of diabetic patients and has been found to inhibit the action of insulin and to cause apoptosis in a variety of cell types. Specifically, it plays key roles in obesity-associated islet cell apoptosis and thus has a substantial impact on the progress of diabetes. Moreover, it has been reported that circulating visfatin levels are increased with progressive β -cell deterioration and that plasma levels of visfatin are also increased in obesity (López *et al.* 2006). Therefore, we hypothesize that there is a relationship between visfatin and β -cell apoptosis in obesity.

Several studies have revealed that visfatin inhibits apoptosis in cardiomyocytes (Lim *et al.* 2008), endothelial cells (Borradaile & Pickering 2009), lymphocytes (Rongvaux *et al.* 2008), hepatocytes (Dahi *et al.* 2010), and many other cell types, but the effect of visfatin on pancreatic β -cells has not yet been clarified. In this paper, we investigated whether visfatin could impact proliferation and apoptosis in islet cells subjected to palmitate damage, which is associated with obesity and obesity-related diseases, and we evaluated the possible mechanisms of visfatin's action on β -cells.

Materials and methods

The mouse insulinoma cell line, MIN6, was a gift from Dr Xiaoying Li (Shanghai Institute of Endocrine and Metabolic Disease, Ruijin Hospital, Shanghai Jiaotong University, China). Fetal bovine serum (FBS), PBS, penicillin–streptomycin, and cell culture media were obtained from Gibco Life Technologies. The anti-Bcl-2, anti-Bax, anti-cytochrome *c*, anti-caspase 3, ERK1/2, p-ERK1/2, AKT, p-AKT, and anti- β -actin antibodies were obtained from Cell Signaling Technology (Beverly, MA, USA). Collagenase type XI, palmitate, FFA-free BSA, LY294002, and PD098059 were purchased from Sigma. Recombinant visfatin was purchased from Peprotech (Rocky Hill, NJ, USA). The Annexin V-FITC kit was purchased from Bender (San Diego, CA, USA).

Cell culture

The MIN6 cells (passage 25–35) were cultured in DMEM with 25 mmol/l glucose, 15% (w/v) FBS, 100 U/ml penicillin G, 100 μ g/ml streptomycin sulfate, and 5 μ l/l β -mercaptoethanol at 37 °C and 5% (v/v) CO₂. At 80% confluence, the cultures were washed twice with DMEM (without FBS) and kept in a serum-free medium for 14 h before induction of cell apoptosis. Apoptosis was induced by culturing cells with 0.5 mM palmitate for 24 h in the presence or absence of the indicated peptides or drugs for the specified time period.

Recombinant visfatin was diluted in PBS (pH 7.4), and LY294002 and PD098059 were dissolved in

dimethyl sulfoxide. The control cultures were grown under the same conditions as treated cells but in the absence of the drugs. The final concentration of dimethyl sulfoxide was identical in every culture, regardless of the particular treatment group. Fresh aliquots of 100 ng/ml visfatin were added every 8 h to the culture medium in all experiments.

Palmitate/BSA complex solution preparation

The fatty acid-supplemented medium was prepared as described previously (Cousin *et al.* 2001). A 100 mM palmitate stock solution was prepared in 0.1 M NaOH along with heating at 70 °C in a shaking water bath. In an adjacent water bath, at 55 °C, a 10% (w/v) FFA-free BSA solution was prepared in ddH₂O. Various concentrations of palmitate complexed to BSA were made by mixing the two solutions together at different proportions. For example, a 5 mM palmitate/10% (w/v) BSA stock solution was prepared by adding 50 μ l of the 100 mM palmitate solution dropwise to 950 μ l of the 10% (w/v) BSA solution at 55 °C in the shaking water bath, followed by vortexing for 10 s and a 10-min incubation at 55 °C. The palmitate/BSA complex solution was cooled to room temperature and sterile filtered (0.45- μ m pore size membrane filter). The complex solution was stored at –20 °C, where it is stable for 3–4 weeks. The stored 5 mM palmitate/10% (w/v) BSA stock solutions were heated for 15 min at 55 °C and then cooled to room temperature before use.

1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan assay

Cellular metabolic activity was measured using an 1-(4,5-dimethylthiazol-2-yl)-3,5-diphenylformazan (MTT) assay, which is now widely used to quantify cellular proliferation and cytotoxicity. The MIN6 cells were plated at a concentration of 2×10^5 cells/100 μ l into a 96-well microtiter plate and incubated with 0–10^{–7} M visfatin. To synchronize the cells, the MIN6 cells were incubated in a serum-free medium overnight. The experiments were done in quadruplicate using separate cell cultures. After 24 h, the cells were centrifuged, the supernatant was carefully removed, and the cells were resuspended in cell culture medium (DMEM containing 0.5% BSA) containing visfatin in concentrations ranging from 0 to 10^{–7} M. The cultures were incubated for 24–72 h. After the incubation period, 25 μ l MTT from a 5 mg/ml stock solution prepared in PBS was added to each well. The plates were transferred to an incubator. After 4 h incubation period, 100 μ l DMSO was added. The MTT reaction was measured using a microplate reader (BioTek ELx800) to read the absorbance at 570 nm. The cell proliferation or

metabolism was expressed as the percentage of the level for control cells. Raw data were statistically analyzed to compare cellular metabolic activity in visfatin-treated cells with that in control cells; Tukey's *post hoc* test was performed to compare each concentration with the control. Significant differences for each concentration were examined using a one-way ANOVA.

Annexin V assay

The quantitative evaluation of apoptosis was performed by flow cytometry after double staining with an Annexin V-FITC apoptosis detection kit. This kit distinguishes among early apoptotic (single Annexin V-positive), late apoptotic (double Annexin V/propidium iodide (PI)-positive), and necrotic cells (single PI positive); PI red staining of the nuclei allows for the differentiation of cells that have lost membrane integrity (necrotic cells) from living cells.

The MIN6 cells were collected from the culture flasks and washed twice with PBS by centrifugation at 200 *g* for 5 min. The cell pellet was then suspended in 100 μ l staining solution and incubated for 15 min at room temperature. The apoptosis level was determined by measuring the fluorescence of the cells by flow cytometric analysis.

Subcellular fractionation

The lysis buffer for isolating total cellular fractions consisted of 50 mM Tris (pH 7.4), 150 mM NaCl, 1% (v/v) Nonidet P-40, 10% (v/v) glycerol, and 5 μ l/ml of a mixture of protease inhibitors (Sigma). Protein concentrations were determined using the Bio-Rad protein dye microassay.

The cytosolic protein fractions were isolated from one 100-mm dish of MIN6 cells. After incubation, cells were scraped from the dishes, collected by centrifugation at 800 *g* for 4 min at 4 °C, washed twice with ice-cold PBS, and centrifuged at 800 *g* for 4 min. The cell pellets were resuspended in 50 μ l of extraction buffer containing 220 mM mannitol, 68 mM sucrose, 50 mM HEPES (pH 7.5), 50 mM KCl, 5 mM EGTA, 2 mM MgCl₂, 1 mM dithiothreitol, 10 μ g/ml phenylmethylsulfonyl fluoride, 10 μ g/ml pepstatin A, and 10 μ g/ml leupeptin. After incubation on ice for 30 min, the cells were homogenized by 50 strokes of a polypropylene homogenizer and spun at 12 000 *g* for 15 min. The supernatants (cytosolic fraction) were then recovered and collected.

SDS-PAGE and western blot analysis

At the end of the incubation, after wash of the cell layer with ice-cold PBS, the cell extract was prepared by treating cells with 50 μ l of Triton X 1% (v/v) and 5 μ l of a mixture of protease inhibitors on ice for 20 min.

Adherent cells were scraped off and spun at 12 000 *g* for 30 min. The pellets were either stored at -70 °C or immediately used for the experiment. The protein concentration was determined according to the Bradford method using BSA as the standard. The cell lysates (40–50 μ g per sample) were then separated by 12% (v/v) SDS-PAGE and transferred onto a nitrocellulose membrane using standard procedures. The membranes were incubated overnight at 4 °C with TBST (20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.2% (v/v) Tween-20). The primary antibodies were used at the following dilutions: Bcl-2 (1:1000), Bax (1:1000), cytochrome *c* (1:1000), caspase 3 (1:1000), ERK1/2 (1:2000), p-ERK1/2 (1:2000), AKT (1:1000), p-AKT (1:1000), and anti β -actin (1:5000).

Statistical analysis

The data were expressed as the mean \pm S.E.M. Statistical analysis for western blot was performed using a Mann-Whitney *U* test. For MTT and Annexin V experiments, ANOVA followed by Tukey's *post hoc* test was used. $P \leq 0.05$ was taken as a point of significance.

Results

Visfatin induces MIN6 cell proliferation

The effect of visfatin on MIN6 cell proliferation was time dependent (24–72 h) with the maximal response noted at 24 h. Treatment with visfatin (10^{-11} – 10^{-8} M) at 24 h

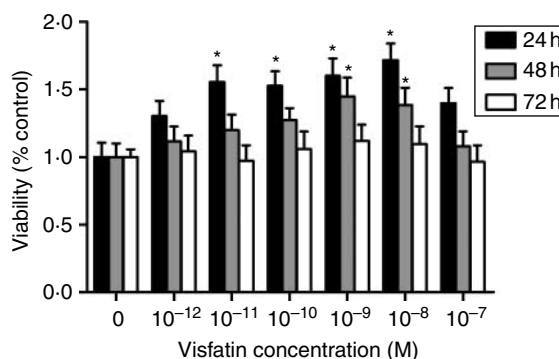


Figure 1 Visfatin induces MIN6 cell proliferation. The effect of visfatin on MIN6 cell proliferation was time dependent (24–72 h) with the maximal response noted at 24 h. Treatment with visfatin at 24 h led to a concentration-dependent proliferative effect compared with the control. MIN6 cells treated with visfatin at 10^{-11} – 10^{-8} M showed a significant difference compared with the control. * P was 0.037, 0.031, 0.024, and 0.020 versus control at 10^{-11} , 10^{-10} , 10^{-9} , and 10^{-8} M respectively for 24 h, and was 0.027 and 0.031 versus control at 10^{-9} and 10^{-8} M respectively for 48 h. The other pancreatic β -cell line INS-1 also showed similar results (Supplementary Materials and Methods and Supplementary Figures 1–6, see section on supplementary data given at the end of this article).

led to a concentration-dependent increase of proliferation to 1.55-fold over the control with maximal concentration of visfatin (Fig. 1; * $P < 0.05$; $n = 6$ experiments).

Visfatin protects insulin-secreting MIN6 cells from palmitate-induced apoptosis

To quantify the degree of apoptosis in the various culture conditions, we performed double staining with Annexin V-FITC/PI. We found that exposure of the cells to palmitate (0.05 mM) induced apoptosis in a significant percentage of the cells (Fig. 2; $P = 0.007$

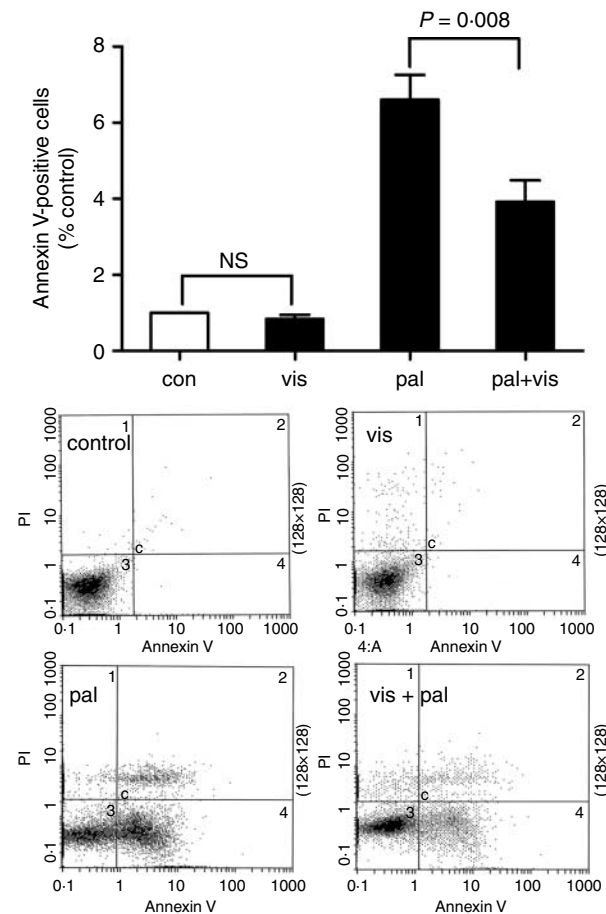


Figure 2 Visfatin prevents the early events associated with cell apoptosis. Analysis of FITC-labeled Annexin V. con, which comprises MIN6 cells cultured in regular medium; vis, the cells cultured in medium containing 100 nM visfatin for 24 h; pal, the cells exposed to palmitate (0.5 mM) for 24 h; vis + pal, the MIN6 cells that were co-incubated with visfatin (100 nM) and palmitate (0.5 mM) for 24 h. The top part of the figure represents the average of four independent experiments with different culture conditions, whereas the bottom part shows a representative FITC-staining flow cytometry analysis for the four different culture conditions studied. For each FITC report, the lower left quadrant represents the detection of living cells, the upper right quadrant represents necrotic cells, and the lower right quadrant represents apoptotic cells. NS, not significant. P was 0.008.

versus control). Visfatin (100 nM) treatment did not influence apoptosis in the MIN6 cells ($P > 0.05$ versus control) but counteracted the effects of palmitate. The simultaneous administration of visfatin and palmitate significantly reduced the occurrence of apoptosis (Fig. 2; $P = 0.008$ versus palmitate).

A characteristic biochemical change that is associated with cell apoptosis is the translocation of Annexin V from the inner leaflet to the outer leaflet of the plasma membrane. We stained the MIN6 cells with Annexin V and then subjected the cells to FITC analysis. The fluorescence intensity of Annexin V-FITC-stained cells is directly proportional to the amount of Annexin V on the outer leaflet of the plasma membrane. The results indicated that 10.4 ± 3.6 , 8.1 ± 3.2 , 39.6 ± 9.5 , and $15.2 \pm 5.8\%$ of the MIN6 cells were Annexin V-positive when grown in regular medium for 24 h, cultured in the presence of visfatin (100 nM) for 24 h, treated with palmitate (0.5 mM) for 24 h, and co-treated with visfatin (100 nM) and palmitate (0.5 mM) for 24 h respectively (Fig. 2).

Visfatin exerts an antiapoptotic effect in palmitate-induced MIN6 cell apoptosis through mitochondrial pathways

In a biological system, apoptosis involves the disruption of mitochondrial function through abnormal expression of Bcl-2 and/or Bax, which induces the release of cytochrome *c* from mitochondria into the cytosol. Cytosolic cytochrome *c* can lead to the activation of caspases in the apoptosome and ultimately to the activation of caspase 3. The activation of caspase 3 subsequently leads to apoptosis. Palmitate-induced apoptosis typically involves the mitochondrial pathway. Therefore, we investigated whether the antiapoptotic effect of visfatin in MIN6 cells undergoing palmitate-induced apoptosis is associated with mitochondrial function and the caspase pathway. By western blot analysis, we found that the treatment of cells with palmitate resulted in an increase in cytochrome *c* release from mitochondria, activation of caspase 3, and expression of Bax, and additionally, a decrease in the expression of Bcl-2. We also found that visfatin treatment in palmitate-induced MIN6 cells resulted in a significant decrease in cytochrome *c* release from mitochondria, a decrease of caspase 3, and a significant increase of Bcl-2; however, the expression of Bax was not significantly changed (Fig. 3).

Visfatin-induced activation of the ERK1/2 signaling pathway

The mitogen-activated protein kinase (MAPK) signaling pathway is involved in cell proliferation and

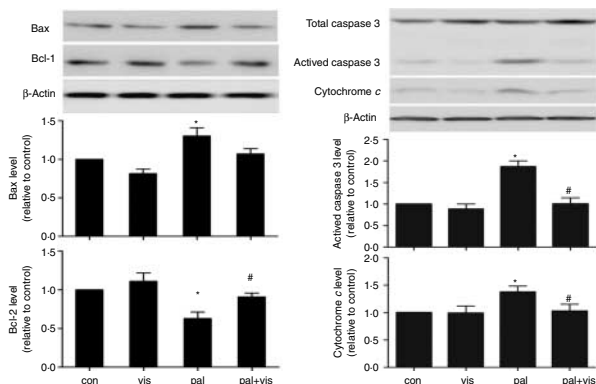


Figure 3 Visfatin increases the expression of the antiapoptotic protein, Bcl-2, decreases the expression of the proapoptotic protein, Bax, and inhibits cytochrome *c* and caspase 3 activity in response to palmitate. (Left) Western blot analysis for Bcl-2, Bax, and β -actin in MIN6 cells. (Right) Western blot analysis for cytochrome *c*, caspase 3, and β -actin. Con, MIN6 cells were cultured in regular medium; vis, the MIN6 cells exposed to visfatin (100 nM) for 24 h; pal, the MIN6 cells treated with palmitate (0.5 mM) for 24 h; pal + vis, the MIN6 cells were co-incubated with visfatin (100 nM) and palmitate (0.5 mM) for 24 h. The blot presented is representative of four independent experiments. The graphs on the bottom represent the average of four independent western blotting experiments for Bcl-2 and Bax, cytochrome *c*, and caspase 3. Protein levels were normalized against the β -actin level and are shown in the graphs as a fold difference compared with the control. **P* was 0.029 versus control of Bax expression; **P* was 0.030 versus control and #*P* was 0.043 versus palmitate alone of Bcl-2 expression; **P* was 0.045 versus control and #*P* was 0.018 versus palmitate alone of activated caspase 3 expression; **P* was 0.037 versus control and #*P* was 0.022 versus palmitate alone of cytochrome *c* expression.

apoptosis. Interestingly, 100 nM visfatin significantly phosphorylated both isoforms of ERK in a time-dependent manner maximally at 30 min (Fig. 4 left: 5–8-fold compared with controls; $P < 0.05$) and decreased thereafter. More importantly, visfatin incubation for 30 min also phosphorylated ERK1/2 in a concentration-dependent manner with maximal phosphorylation occurring at 100 nM (Fig. 4, right: 7.5-fold compared with controls; $P < 0.05$).

Visfatin-induced activation of the phosphoinositide 3-kinase/AKT signaling pathway

The phosphoinositide 3-kinase (PI3K)/AKT pathway is known to regulate cellular apoptosis. To determine whether visfatin acts via this pathway in MIN6 cells, we treated the cells with visfatin. At a concentration of 100 nM visfatin significantly phosphorylated AKT in a time-dependent manner maximally at 15 min (Fig. 5, left: 2.1-fold increase compared with basal levels; $P < 0.05$) and decreased thereafter. Visfatin incubation for 15 min also phosphorylated AKT in a

concentration-dependent manner with maximal phosphorylation occurring at 100 nM (Fig. 5, right: 2.3-fold increase compared with basal levels; $P < 0.05$).

The antiapoptotic action of visfatin is mediated by the activation of a MAPK-dependent and a PI3K-dependent signaling pathway

Visfatin has also been demonstrated to operate via a kinase-dependent signaling pathway in the regulation of gene transcription in endothelial cells. For this reason, we investigated the role of protein kinases PI3K and MAPK in visfatin-induced antiapoptosis and also observed the regulation of antiapoptotic proteins in the MIN6 cells. Annexin V staining revealed that the antiapoptotic action of visfatin was significantly reduced by the inhibitors PD098059 and LY294002, which were capable of selectively blocking MAPK and PI3K respectively. As shown in Table 1, the antiapoptotic action of visfatin was reversed by exposure of the cells to inhibitors of the MAPK or PI3K signaling pathways or both. PD098059 and LY294002 not only increased apoptosis reversed by visfatin but also led to significant necrosis, which showed that the two signaling pathways had a cooperative mechanism of activation. Application of PD098059 or LY294002 resulted in significantly increased cell apoptosis; when both inhibitors were co-administered, there was a synergistic effect that resulted in a greater extent of apoptosis, and secondary necrosis occurred when massive apoptosis overwhelmed the available apoptotic scavenging capacity.

Western blot analysis of the apoptotic proteins Bcl-2, Bax, caspase 3, and cytochrome *c* demonstrated that

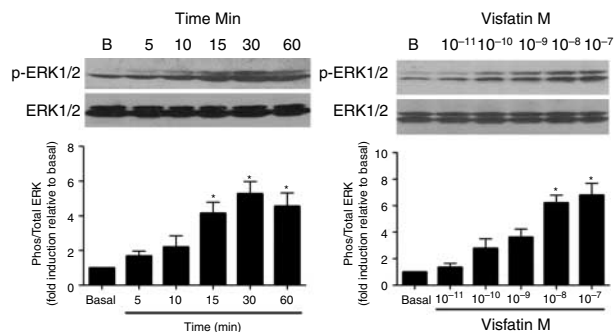


Figure 4 Visfatin-induced activation of the ERK1/2 signaling pathway. Visfatin significantly phosphorylated ERK1/2 in a time- and concentration-dependent manner. (Left) Time-dependent activation was seen maximally at 30 min when visfatin concentration was 100 nM. **P* was 0.032, 0.019, and 0.026 versus basal at 15, 30, and 60 min respectively. (Right) Concentration-dependent activation was seen maximally at 100 nM visfatin incubation for 30 min. **P* was 0.022 and 0.010 versus basal at 10 and 100 nM respectively.

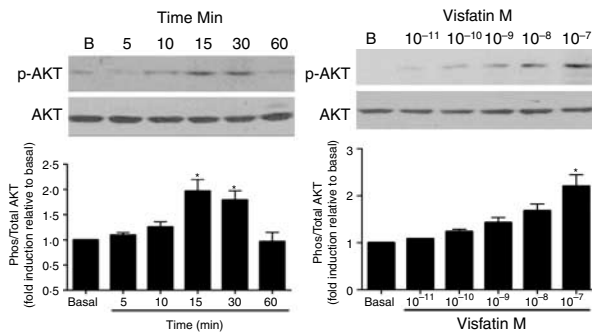


Figure 5 Visfatin-induced activation of the PI3K/AKT signaling pathway. Visfatin significantly phosphorylated PI3K/AKT in a time- and concentration-dependent manner. (Left) Time-dependent activation was seen maximally at 15 min when visfatin concentration was 100 nM. **P* was 0.029 and 0.037 versus basal at 15 and 30 min respectively. (Right) Concentration-dependent activation was seen maximally at 100 nM visfatin incubation for 15 min. **P* was 0.021 versus basal at 100 nM.

LY294002 (50 μ M) or PD098059 (30 μ M) greatly changed the visfatin-dependent regulation of these proteins (Fig. 6). These data suggest that visfatin inhibits β -cell apoptosis by activating at least two pre-survival signaling pathways: ERK1/2 and PI3K/AKT.

Discussion

This study investigated whether visfatin had protective effects against FFA-induced cell death in the insulin-producing cell line. In addition, we revealed the mechanism underlying the visfatin-induced improvement of the function and viability of pancreatic β -cells. We demonstrated that visfatin was able to inhibit apoptosis and prevent metabolic dysfunction induced by cellular exposure to FFAs. Finally, we show that visfatin regulates the expression of the antiapoptotic protein, Bcl-2, as well as the proapoptotic proteins, cytochrome *c* and caspase 3; this regulation occurs likely via ERK1/2- and PI3K/AKT-dependent pathways.

Many studies have shown that saturated long-chain fatty acids are the major contributor to lipotoxicity (Kusminski *et al.* 2009). Palmitate, a long-chain (C16:0)

saturated fatty acid, has been reported to induce apoptosis in many cell types, including cardiomyocytes (Wang *et al.* 2009a,b), hepatocytes (Cazanave *et al.* 2009), myoblasts (Peterson *et al.* 2008), and pancreatic B cells (Hovsepian *et al.* 2010). Although the mechanism of palmitate-induced damage and death in these cell types has not been completely determined, it is generally accepted that cytochrome *c* is a major intracellular mediator of the effects of palmitate (Rachek *et al.* 2007). Cell susceptibility to apoptosis is regulated by a balanced expression of apoptosis-suppressing and -inducing proteins. In human and rodent islets, FFA-induced apoptosis is associated with a decrease in the Bcl-2/Bax ratio (Santangelo *et al.* 2007). It has also been reported that the overexpression of Bcl-2 proteins enhances islet viability.

In this study, MIN6 cells cultured in the presence of 0.5 mM palmitate showed apoptosis and the activation of caspase 3. We also observed that palmitate decreased the expression of the antiapoptotic protein Bcl-2 and increased the expression of the proapoptotic protein Bax. On the basis of these data, we used palmitate as a model to study β -cell apoptosis.

Our study demonstrates that visfatin prevents apoptosis in MIN6 cells subjected to damage by the FFA, palmitate. Visfatin was capable of maintaining the Bcl-2/Bax ratio by counteracting the downregulation of Bcl-2 by FFA; additionally, it inhibited cytochrome *c* and caspase 3 released from the mitochondria. This observation led to the hypothesis that visfatin acts by inducing or enhancing the expression or activity of cellular antiapoptotic proteins. We further showed the molecular mechanism/signaling pathway through which visfatin exerts its antiapoptotic effects.

The MAPK signal transduction pathway and the PI3K pathway are activated by various growth factors; these pathways are thought to mediate cell proliferation, differentiation, and apoptosis. It has been shown that visfatin can activate eNOS and improve endothelial cell function and angiogenesis through activation of the MAPK and PI3K/AKT signaling pathways (Lovren *et al.* 2009). However, it was not previously known whether visfatin induced ERK1/2 and AKT activation

Table 1 Necrotic, living, and apoptotic cells under different treatment conditions. The data are expressed as the mean \pm s.e.m. of five independent experiments. The statistical analysis of differences was performed by comparing each treatment with control (untreated) cells. The significance was determined by ANOVA and confirmed by *post hoc* analysis

| Group and treatment | Living (%) | Necrotic (%) | Apoptotic (%) |
|---------------------------|-----------------------|-----------------------|------------------------------------|
| con | 83.5250 \pm 3.55188 | 3.8225 \pm 0.67293 | 10.4350 \pm 3.63667 |
| pal | 50.6500 \pm 4.51258 | 11.6450 \pm 1.66634 | 37.4500 \pm 4.12351* |
| pal+vis | 78.2500 \pm 3.93743 | 5.4100 \pm 0.8255 | 15.8400 \pm 3.55667 |
| pal+vis+LY294002 | 50.8500 \pm 8.39861 | 12.2300 \pm 1.53162 | 37.1750 \pm 6.80312* |
| pal+vis+PD098059 | 45.8500 \pm 7.94544 | 12.5900 \pm 1.28546 | 36.5050 \pm 3.69057* |
| pal+vis+LY294002+PD098059 | 18.1550 \pm 5.37039 | 37.2500 \pm 2.36714 | 47.0500 \pm 6.53606 [†] |

**P* < 0.05 versus con, [†]*P* < 0.01 versus con. con, control; pal, palmitate; vis, visfatin.

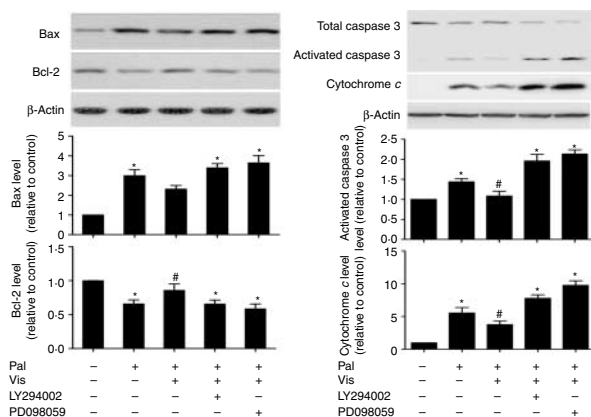


Figure 6 The antiapoptotic action of visfatin requires the activation of MAPK-dependent and PI3K-dependent signaling pathways. The cells were exposed to palmitate (0.5 mM for 24 h) in the presence or absence of visfatin (100 nM). The antiapoptotic activity of visfatin was tested in the absence or presence of the PI3K inhibitor LY294002 or the MAPK inhibitor PD098059.

Western blot of Bcl-2, Bax, cytochrome c, caspase 3, and β -actin expression were shown. Lane 1, control MIN6 cells cultured in regular medium; lane 2, cells exposed to palmitate alone; lane 3, cells exposed to visfatin and palmitate; lane 4, cells exposed to visfatin, palmitate, and PI3K inhibitor LY294002; lane 5, cells exposed to visfatin, palmitate, and MAPK inhibitor PD098059. The blot presented was representative of four independent experiments. The graphs on the bottom represented the average of four independent western blotting experiments for Bcl-2, Bax, cytochrome c, and caspase 3. The protein levels were normalized to β -actin levels and were shown in the graphs as a fold difference compared with the control. * P was 0.029, 0.015, and 0.014 respectively versus control of Bax expression; * P was 0.025, 0.017, and 0.010 respectively versus control, and # P was 0.015 versus palmitate alone of Bcl-2 expression; * P was 0.041, 0.019, and 0.014 respectively versus control, and # P was 0.045 versus palmitate alone of activated caspase 3 expression; * P was 0.037, 0.022, and 0.011 respectively versus control, and # P was 0.035 versus palmitate alone of cytochrome c expression.

and affected the apoptosis and gene expression in the pancreatic β -cell line. The present study showed that visfatin had significant protective effects on palmitate-induced apoptosis that involved MAPK/ERK1/2 signaling. This observation is similar to those studies conducted to characterize the angiogenic action of visfatin, i.e. the induction of endothelial VEGF and MMP via the MAPK and PI3K/AKT signaling pathways (Adya *et al.* 2008). It is reported that in some cells, there is a close correlation between the PI3K/AKT and the MAPK/ERK signaling pathways, suggesting occurrence of crosstalk (Chiu *et al.* 2005). These two pathways regulate different downstream events involved in cell survival. For example, the MAPK/ERK pathway is usually associated with proliferation, whereas the PI3K/AKT pathway is associated with apoptosis. Furthermore, the MAPK/ERK and the PI3K/AKT pathways promote cell-cycle events that cause interactions with the p53 pathway. Some of these interactions control the activity and

subcellular localization of Bim, Bak, Bax, and Bcl-2 (McCubrey *et al.* 2007).

The results do not rule out the involvement of other pathways in the antiapoptotic action of visfatin, and focusing studies need to be carried out to further elucidate whether other signaling molecules are mediating visfatin-dependent protection from cell death.

The above results provide evidence for PI3K and ERK1/2 as essential mediators through which visfatin inhibits FFA-induced oxidative stress and apoptosis in pancreatic β -cells by potential regulation of the Bcl-2/Bax ratio. Because of its strong prosurvival and antiapoptotic actions, visfatin treatment appears to be a useful approach to preserving β -cell function and survival in a lipotoxicity setting.

Similar to the present study results, previous reports have demonstrated that visfatin is a survival factor for various cell types. For example, perivascular adipose tissue-derived visfatin is a VSMC growth factor. Visfatin stimulates VSMC proliferation via NMN-mediated ERK1/2 and p38 signaling (Wang *et al.* 2009a,b). Visfatin protects macrophages from ER stress-induced apoptosis by activating an IL6/STAT3 signaling pathway beyond phosphoribosyltransferase (Namt) and enzymatic mechanism (Li *et al.* 2008). Visfatin also participates in cellular resistance to genotoxic/oxidative stress, and enhances immune cell survival during stressful situations such as inflammation (Rongvaux *et al.* 2008). In normal pregnancy, increased SIRT1 and decreased p53 expression in amniotic epithelial cells paralleled the changes induced by visfatin, which contributed to cell survival; moreover, the knockdown of visfatin with antisense probes abrogated this protective effect (Kendal *et al.* 2008). FK866, a potent Namt catalytic inhibitor, prevented visfatin-mediated cell protection, indicating that Namt activity of visfatin is required for cell protection (Yang *et al.* 2007). Visfatin can exert direct cardioprotective effects to reduce myocardial injury when administered at the time of myocardial reperfusion in both *in situ* murine hearts and isolated murine cardiomyocytes (Lim *et al.* 2008). Van der Veer *et al.* (2007) reported that visfatin is a longevity protein that extends the lifespan of human smooth muscle cells by activating SIRT1 and restricting the accumulation of p53. Visfatin also has an essential role in the maintenance of normal glucose-dependent insulin secretion, and systemic NAD biosynthesis is critical for β -cell function (Revollo *et al.* 2007). More importantly, it has also been shown that visfatin is a useful tool to preserve islet function and regulate insulin secretion when haplo-deficiency and chemical inhibition of visfatin cause a defect in glucose-stimulated insulin secretion in pancreatic islets *in vivo* and *in vitro* (Tanaka & Nabeshima 2007). The results also showed that visfatin is able to improve the function

and viability of the pancreatic β -cells in accordance with a recent report (Brown *et al.* 2010) which shows that visfatin can significantly regulate insulin secretion, IR phosphorylation and intracellular signaling and reveals the expression of several β -cell function-associated genes in mouse pancreatic β -cells. Thus, visfatin has multifunctional activities and exerts its pro-survival action by different mechanisms depending on the apoptotic protection.

In this study, we show for the first time that visfatin has anti-apoptotic properties in addition to its effect on the proliferation of islet cells. Visfatin protects islet cells from palmitate-induced apoptosis by increasing the Bcl-2/Bax ratio and inhibiting cytochrome *c* and caspase 3 via the ERK1/2 and PI3K/AKT signaling pathways. These data suggest a novel mechanism of action of visfatin that affects the balance of islet cell survival and death in the setting of obesity. We suggest that the increase of visfatin seen in obesity may be a compensatory mechanism of the body to protect islet β -cells against lipotoxicity and that this process plays an important role in obesity-associated diseases.

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/JME-10-0106>.

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.

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