

Original Article



Visfatin Protects Rat Pancreatic β -cells against IFN- γ -Induced Apoptosis through AMPK and ERK1/2 Signaling Pathways*

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Abstract

Objective Interferon- γ (IFN- γ) plays an important role in apoptosis and was shown to increase the risk of diabetes. Visfatin, an adipokine, has anti-diabetic, anti-tumor, and regulating inflammatory properties. In this study we investigated the effect of visfatin on IFN- γ -induced apoptosis in rat pancreatic β -cells.

Methods The RINm5F (rat insulinoma cell line) cells exposed to IFN- γ were treated with or without visfatin. The viability and apoptosis of the cells were assessed by using MTT and flow cytometry. The expressions of mRNA and protein were detected by using real-time PCR and western blot analysis.

Results The exposure of RINm5F cells to IFN- γ for 48 h led to increased apoptosis percentage of the cells. Visfatin pretreatment significantly increased the cell viability and reduced the cell apoptosis induced by IFN- γ . IFN- γ -induced increase in expression of p53 mRNA and cytochrome c protein, decrease in mRNA and protein levels of anti-apoptotic protein Bcl-2 were attenuated by visfatin pretreatment. Visfatin also increased AMPK and ERK1/2 phosphorylation and the anti-apoptotic action of visfatin was attenuated by the AMPK and ERK1/2 inhibitor.

Conclusion These results suggested that visfatin protected pancreatic islet cells against IFN- γ -induced apoptosis via mitochondria-dependent apoptotic pathway. The anti-apoptotic action of visfatin is mediated by activation of AMPK and ERK1/2 signaling molecules.

Key words: Visfatin; IFN- γ ; Pancreatic β -cell; Apoptosis; AMPK; ERK1/2

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INTRODUCTION

Apoptosis of pancreatic β -cells is postulated to be a common feature of diabetes^[1]. The cytokines implicated in pancreatic islets autoimmune damage participate in the initial destruction of β -cell by inducing apoptosis,

resulting in the development of diabetes^[2]. The treatments of pancreatic β -cells with interleukin-1 β and tumor necrosis factor- α , alone or in combination, result in significant inhibition of insulin secretion in the absence or presence of stimulatory glucose concentration^[3]. It had been well established that T-lymphocyte-derived interferon- γ (IFN- γ) plays

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Biographical note of the first author XIANG Ruo Lan, female, born in 1975, PhD, lecturer, majoring in biochemistry and molecular biology.

significant roles in diabetes onset by inducing inflammation and apoptosis, which disrupts the normal β -cell function. Recent studies have shown that a prolonged pancreatic β -cells inflammatory reaction, combined with hypersecretion of IFN- γ , disturbs the β -cell balance of pro- and anti-apoptotic factors *in vivo*, causing an accelerated development of diabetes^[4]. Therefore, IFN- γ plays a central role in inflammatory reaction and was shown to increase the risk of diabetes. Anti-inflammatory pharmacological approaches aimed at blocking cytokine signaling pathways are highly advisable to alleviate the progression of diabetes.

Visfatin, also known as pre-B-cell colony-enhancing factor, is originally isolated from peripheral blood lymphocytes and characterized as an adipokine that exerts insulin-mimetic effects on different insulin-sensitive tissues^[5-6]. It is secreted mainly by adipocytes and macrophages, but also by human pancreatic β -cells where its secretion is upregulated by glucose^[7-8]. Several studies have shown that visfatin plays a role in the development of obesity-associated insulin resistance and diabetes. Yilmaz et al. found that plasma level of visfatin is associated with obesity, insulin resistance, and the level of albuminuria in patients with type 2 diabetes^[9]. Visfatin expression is upregulated in circulating blood monocytes from obese patients with type 2 diabetes compared with obese patients without diabetes^[10]. Visfatin not only increases insulin secretion from mice pancreatic β -cells, but also directly activates insulin receptors by increasing their phosphorylation^[11]. These results suggested that enhanced visfatin expression might be related to diabetes. However, the exact role of visfatin in pathogenesis of diabetes has not been fully understood.

Recent reports have provided evidence that visfatin may mitigate cell injury by affecting the inflammatory response and apoptosis^[12-13]. Visfatin significantly reduces the cell apoptosis induced by palmitate and improves cell viability and protects against H₂O₂-induced apoptotic damage in H9c2 cardiomyocytes^[14-15]. However, the inhibition of visfatin by FK-866 attenuates acute lung injury induced by intestinal ischemia-reperfusion in mice by inhibiting cellular apoptosis and nuclear factor- κ B (NF- κ B) activation^[16]. These results suggested that visfatin might be involved in the regulation of apoptosis. However, the effect and mechanism of visfatin on the modulation of apoptosis in pancreatic β -cells remains unclear.

In the present study, a series of experiments was designed to explore the effects of visfatin on IFN- γ -induced pancreatic β -cells apoptosis by using RINm5F cell line. The possible mechanism of visfatin regulating apoptosis was also evaluated.

METHODS

Materials

Rat insulin-secreting RINm5F cells were from American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI1640 complete medium and fetal bovine serum were from Gibco Life Technologies. Recombinant visfatin and IFN- γ were from Peprotech (Rocky Hill, NJ, USA). TRIzol were from Invitrogen (Carlsbad, CA, USA). Caspase-3 activity kit was from Promega (Madison, Wis, USA). AnnexinV-FITC apoptosis kit was from Sigma Chemical (USA). RevertAid First Strand cDNA Synthesis Kit was from Fermentas (Burlington, Maryland, USA). The SYBR green PCR kit was from Qiagen (Hilden, Germany). Antibodies were from the following sources: anti-extracellular signal-regulated kinase 1/2 (ERK1/2), anti-phospho-ERK1/2 (Santa Cruz Biotech, CA, USA), anti-AMP-activated protein kinase (AMPK), anti-phospho-AMPK, anti-p38 mitogen-activated protein kinase (p38MAPK), anti-phospho-p38MAPK, anti-c-Jun N-terminal kinase (JNK), anti-phospho-JNK (Cell Signaling Technology, Beverly, MA, USA).

Cell Culture

The RINm5F cells were cultured at 37 °C with 5% CO₂ in 1640 supplemented with 10% fetal bovine serum, 10 mmol/L HEPES, 2 mmol/L L-glutamine, 100 U/mL penicillin, and 100 μ g/mL streptomycin. The cells between passages 10-20 of the original RINm5F cells were used in the experiments. The RINm5F cells were pretreated with visfatin (100 ng/mL) for 1 h and then exposed to IFN- γ (1000 U/mL) for 48 h. U0126 (10 μ mol/L), an ERK1/2 upstream kinase inhibitor, compound C (10 μ mol/L), an AMPK inhibitor, SB203580 (10 μ mol/L), a p38 kinase inhibitor, SP600125 (10 μ mol/L), a JNK kinase inhibitor, were added and incubated for 30 min before visfatin treatment.

RNA Extraction and Quantitative Real-time PCR

Total RNA was extracted by using TRIzol. Total cDNA was produced by using the RevertAid First Strand cDNA Synthesis Kit according to the manufacturer's protocol. Real-time PCR was

performed by using miScript SYBR green PCR kit according to the manufacturer's instructions.

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

The cell viability was measured by using a 1-(4, 5-dimethylthiazol-2-yl)-3, 5-diphenylformazan (MTT) assay. Briefly, The RINm5F cells were plated at a concentration of 2×10^5 cells/mL into a 96-well microtiter plate. After a specific treatment, 20 μ L MTT solutions was added at a concentration of 5 mg/mL and the mixture was incubated at 37 °C for 4 h to allow MTT to be metabolized. After the incubation, 100 μ L DMSO was added. The MTT reaction was measured by using a microplate reader to measure the absorbance at 570 nm.

Flow-cytometric Analysis for Apoptosis

The percentage of cells undergoing apoptosis was evaluated by using simultaneous Annexin V-FITC and propidium iodide (PI) staining. Briefly, 2×10^5 cells/mL was cultured in 24 well plates as described above. After treatment, the cells were stained with Annexin V-FITC and PI according to the manufacturer's instructions. The apoptotic cells were analyzed by flow cytometry on a FACScan (Beckton-Dickson, USA).

Measurement of Caspase-3 Activity

Caspase-3 enzyme activity in cells was assayed by using a caspase-3 activity kit with fluorimetric detection CaspACE according to the manufacturer's instructions.

Western Blot Analysis

The RINm5F cells were collected and lysed with lysis buffer (20 mmol/L Tris, pH 8.0, 150 mmol/L NaCl, 2 mmol/L EDTA, 100 mmol/L NaF, 1% NP40, 1 μ g/mL leupeptin, 1 μ g/mL antipain, 1 mmol/L PMSF). The homogenate was centrifuged at 1000 g for 10 min at 4 °C, and the protein concentration of the supernatant was measured with BCA protein assay. Equal amounts of proteins (40 μ g) were separated by using 12% SDS-PAGE and transferred onto a nitrocellulose membrane by using standard procedure. The membranes were blocked with 5% non-fat milk for 1 h at room temperature, probed with primary antibodies at 4 °C overnight, and then incubated with horseradish peroxidase-conjugated secondary antibodies. The target proteins were detected by enhanced chemiluminescence reagent.

The densities of bands were quantified with the Image J Software (National Institutes of Health, MD, USA). The levels of proteins were normalized to β -actin.

Statistical Analysis

Data are presented as means \pm SEM for the indicated number of distinct experiments. Statistical analysis for multiple groups was performed with one-way ANOVA followed by Bonferroni's tests using GraphPad software (GraphPad Prism, CA, USA). Values of $P < 0.05$ were considered statistically significant.

RESULTS

Visfatin Protects RINm5F Cells Against IFN- γ Induced Apoptosis

To explore the cytotoxic effect of IFN- γ on RINm5F cells, MTT assays and FACS assays were performed after exposure to IFN- γ for 48 h. As shown in Figure 1A, 1000 U/mL IFN- γ exposure for 48 h significantly reduced the cell viability of the RINm5F cells rather than 500 U/mL and 2000 U/mL IFN- γ exposure. Annexin V/PI staining revealed that maximal increasing of apoptosis induced by IFN- γ occurred at the final concentration of 1000 U/mL (Figure 1B).

Then, the effect of visfatin on RINm5F cell viability was quantified by MTT assay. As shown in Figure 1C, the exposure of RINm5F cells to 1000 U/mL IFN- γ for 48 h markedly decreased the cell viability, and this effect was attenuated by 1 h of pretreatment with 100 ng/mL visfatin ($P < 0.05$) rather than pretreatment with 1 ng/mL, 10 ng/mL, and 1000 ng/mL. On the other hand, the pretreatment with visfatin for 24 h showed no obvious effect. Visfatin alone did not influence the viability of RINm5F cells. In our next experiments, the RINm5F cells were exposed to IFN- γ (1000 U/mL) 48 h with/without 100 ng/mL visfatin pretreatment for 1 h.

The activation of caspase-3 is an important to mediate cell apoptosis. The activity of caspase-3 was significantly elevated by IFN- γ and visfatin pretreatment inhibited IFN- γ -induced increase in caspase-3 activity (Figure 1B). To further quantify the degree of the apoptosis, we performed double staining with Annexin V-FITC/PI. The apoptosis percentage of the cells increased with the exposure of RINm5F cells to IFN- γ for 48 h ($P < 0.01$), while this effect was significantly attenuated by visfatin

pretreatment ($P<0.01$, Figure 1C). Visfatin alone did not influence apoptosis of the cells. These data indicated that visfatin reduced the cell injury and prevented the apoptosis of RINm5F cells induced by IFN- γ .

Visfatin Inhibites Mitochondria-dependent Apoptotic Pathway

Then we explored the possible anti-apoptotic pathway of visfatin. Real-time PCR indicated showed that IFN- γ -induced increase in mRNA level of p53, a stressor of the mitochondria-dependent pathway, was inhibited by visfatin pretreatment (Figure 2A). IFN- γ significantly reduced the level of Bcl-2 mRNA, while elevated the expression of Bax mRNA. Visfatin pretreatment restored IFN- γ -decreased Bcl-2 mRNA to near control level (Figure 2B), but did not affect IFN- γ -elevated Bax mRNA expression (Figure 2C). The ratio of Bcl-2/Bax mRNA was significantly decreased by IFN- γ , while it was markedly restored after visfatin pretreatment (Figure 2D). Additionally, IFN- γ -induced decrease in Bcl-2 protein was restored, whereas IFN- γ -induced increase in Bax protein was not obvious after visfatin pretreatment (Figure 2E, 2F, and 2G). The change of Bcl-2/Bax protein ratio was consistent with the gene expression (Figure 2H). Moreover, compared with the IFN- γ -exposed cells, IFN- γ -induced increase in the level of cytochrome c

was significantly inhibited in the visfatin-treated cells (Figure 2E and 2I). However, either IFN- γ or visfatin did not change the levels of Fas (CD95) and tumor necrosis factor receptor-1 (TNFR1) mRNA, the two best-characterized death receptors (Figure 2J and 2K). The results suggested that IFN- γ -induced apoptosis of RINm5F cells was mainly mediated through the mitochondrial pathway rather than the death receptor-dependent apoptotic pathway. Visfatin protected RINm5F cells against apoptosis by inhibiting IFN- γ -induced activation of mitochondrial-dependent apoptotic pathway.

Visfatin Induces Activation of AMPK and ERK1/2 Signaling Pathway

AMPK and MAPK signaling pathways are known to regulate cellular apoptosis^[17]. As expected, visfatin significantly increased the phosphorylation level of AMPK at 10 and 30 min. The amount of total AMPK was not changed by visfatin treatment (Figure 3A). Moreover, visfatin increased the phosphorylation level of ERK1/2 at 10 and 30 min (Figure 3B), but did not change the levels of p-p38MAPK and p-JNK (Figure 3C and 3D). The amounts of total ERK1/2, p38MAPK, and JNK were not changed by visfatin. These results confirmed that AMPK and ERK1/2 were the downstream signaling molecules selectively regulated by visfatin in RINm5F cells.

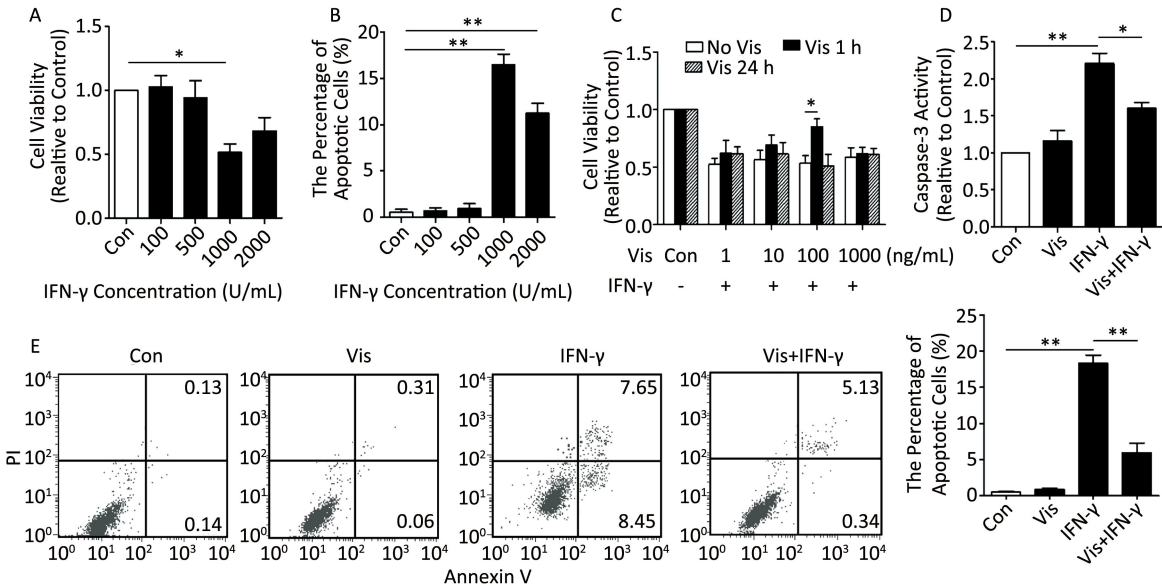


Figure 1. Visfatin protects RINm5F cells against IFN- γ -induced apoptosis. RINm5F cells were treated with IFN- γ 100 U/mL, 500 U/mL, 1000 U/mL, 2000 U/mL for 48 h. (A, C) Cell viability was measured by MTT assay. (D) Caspase-3 activity was measured by fluorometric detection. (B, E) Cells were stained with Annexin V-FITC and PI. The degree of apoptosis was analysis by flow cytometry. The results are presented as means \pm SEM. Data are obtained from 5 independent experiments. * $P<0.05$, ** $P<0.01$.

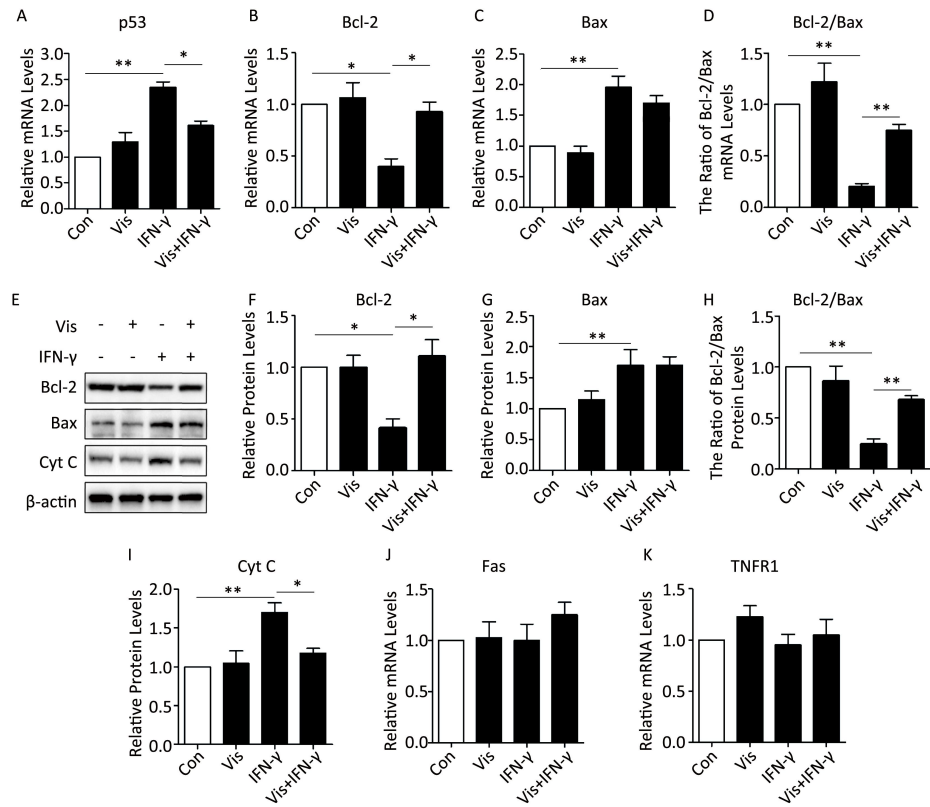


Figure 2. Visfatin mainly regulates the mitochondria related apoptotic proteins in RINm5F cells. The mRNA levels of p53 (A), Bcl-2 (B), Bax (C), and Bcl-2/Bax ratio (D) were determined by real-time PCR. The protein levels of Bcl-2 (E, F), Bax (E, G), Bcl-2/Bax ratio (E, H), and cytochrome c (Cyt C) (E, I) were evaluated by Western blot analysis. β -actin was used as a loading control. The mRNA levels of Fas (J) and TNFR1 (K) were determined by real-time PCR. The results are presented as means \pm SEM. Data are obtained from 5 independent experiments. $P<0.05$, $P<0.01$.

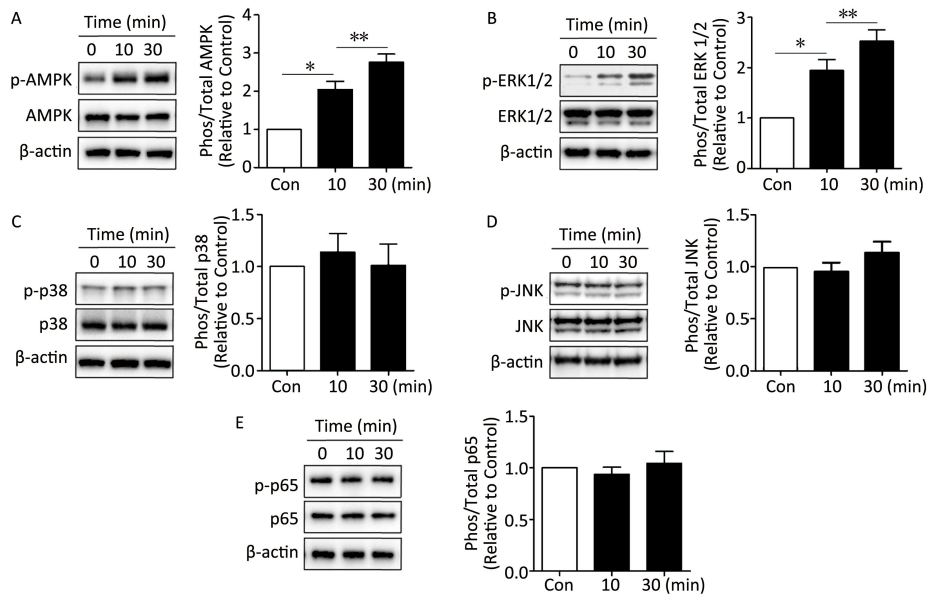


Figure 3. Visfatin increases AMPK and ERK1/2 phosphorylation. The levels of phosphorylated and total AMPK (A), ERK1/2 (B), p38 (C), JNK (D), and p65 (E) were detected by Western blot analysis. β -actin was used as a loading control. $P<0.05$, $P<0.01$.

NF- κ B is the other major regulatory signaling molecule for cellular apoptosis^[18]. We studied whether visfatin could activate NF- κ B in RINm5F cells. As shown in Figure 3E, the phosphorylation level of p65 and amount of total p65 protein had no significant change after visfatin treatment.

Anti-apoptotic Action of Visfatin Requires Activation of AMPK and ERK1/2-mediated Signaling Pathways

To further explore the possible intracellular signaling molecule involved in anti-apoptosis effect of visfatin, we used compound C (an AMPK inhibitor), U0126 (an ERK1/2 inhibitor), SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), or PDTC (a NF- κ B inhibitor) to preincubate RINm5F cells. Annexin V-FITC/PI double staining analysis revealed that the anti-apoptotic effect of visfatin was significantly attenuated by compound C or U0126 (Figure 4A). But the pretreatment with SB203580, SP600125, or PDTC showed no obvious effect. Moreover, the effect of visfatin on protecting cell viability against IFN- γ -induced injury was inhibited in the compound C- or U0126-treated cells (Figure 4B). IFN- γ -induced increase in activity of caspase-3 was also decreased by compound C or U0126 pretreatment (Figure 4C). Western blot analysis of the apoptotic proteins showed that both compound C and U0126 abolished the effects of visfatin on IFN- γ -induced increase in cytochrome c and decrease in Bcl-2 expression (Figure 4D, 4E, and 4F). These data suggested that visfatin inhibited IFN- γ -induced β -cell apoptosis by activating at least two signaling molecules AMPK and ERK1/2.

DISCUSSION

In the present study, we demonstrated that visfatin had protective effect against IFN- γ -induced apoptosis in rat pancreatic β -cells. We found that the anti-apoptotic effect of visfatin in RINm5F cells is associated with the mitochondrial dependent pathway. Furthermore, we revealed that AMPK and ERK1/2-mediated signaling pathways were involved in the protective effect of visfatin. These data suggested that the increase of visfatin observed in obese patients might be a compensatory mechanism of the body to protect pancreatic β -cells against apoptosis.

Visfatin is preferentially secreted by visceral fat cells and increased in obese and type 2 diabetes patients^[19]. It has been shown that visfatin activates

its target cells by binding to insulin receptor at a site distinct from insulin, and exerts a variety of insulin-mimetic effects, and plays a role in the development of obesity-associated insulin resistance and diabetes^[20-21]. These findings imply that visfatin may play a role in the development of diabetes. However, the biological mechanisms of visfatin in the pathogenesis of diabetes have not been well understood.

It is well known that the increase of inflammatory factors plays an important role in the development of diabetes^[22]. It is further believed that apoptosis is the main form of cytokine-induced pancreatic β -cell death^[23]. It has been observed that the knocking out of the IFN- γ gene, IFN- γ neutralisation, IFN- γ blockade, or deletion of IFN- γ -R positive cells in NOD mice and BB rats all led to delayed or decreased incidence of type 1 diabetes^[24]. STZ administration led to a significant increase in the expression of IFN- γ , lower insulin content, and enhanced apoptosis in the mice pancreatic β -cells^[25]. Moreover, a significant increase was observed in the IFN- γ level in plasma in both the only high-fat diet rat and fed high-fat diet rat in which type 2 diabetes was generated groups^[26]. Therefore, we have used higher concentration IFN- γ alone to induce apoptosis on RINm5F cells.

Visfatin has recently been described as an adipokine with potentially important effects on apoptosis. Visfatin protects macrophages from endoplasmic reticulum stress-induced apoptosis by activating an IL-6 and signal transducer and activator of transcription 3 signaling pathway via a nonenzymatic mechanism^[27]. Visfatin also exerts anti-apoptotic effects in liver cells through enzymatic synthesis of nicotinamide adenine dinucleotide^[28]. However, the inhibition of visfatin attenuated acute lung injury^[16] and visfatin silencing with siRNA inhibited the expression of some inflammatory cytokines, including IL-6, IL-8, TNF- α , and IP-10, and decreased the apoptosis induced by Fas ligand in pandemic influenza A virus-infected human pulmonary microvascular endothelial cells^[29]. Therefore, the exact role of visfatin on apoptosis may vary with the stimuli and the cells investigated. In the present study, we demonstrated that IFN- γ significantly promoted apoptosis in RINm5F cells and visfatin increased the cell viability and prevents apoptosis induced by the IFN- γ .

Mitochondria-dependent apoptotic pathway is now recognized to be involved in the development of diabetes^[30]. Bax and Bcl-2 are major proteins that

have been shown to regulate mitochondriadependent apoptosis. Bcl-2 is an anti-apoptotic protein whose expression protects cell against death, while

Bax is a pro-apoptotic protein during apoptosis. This pathway is regulated by a balanced expression of Bcl-2 and Bax proteins^[31]. In human and rodent pancreatic

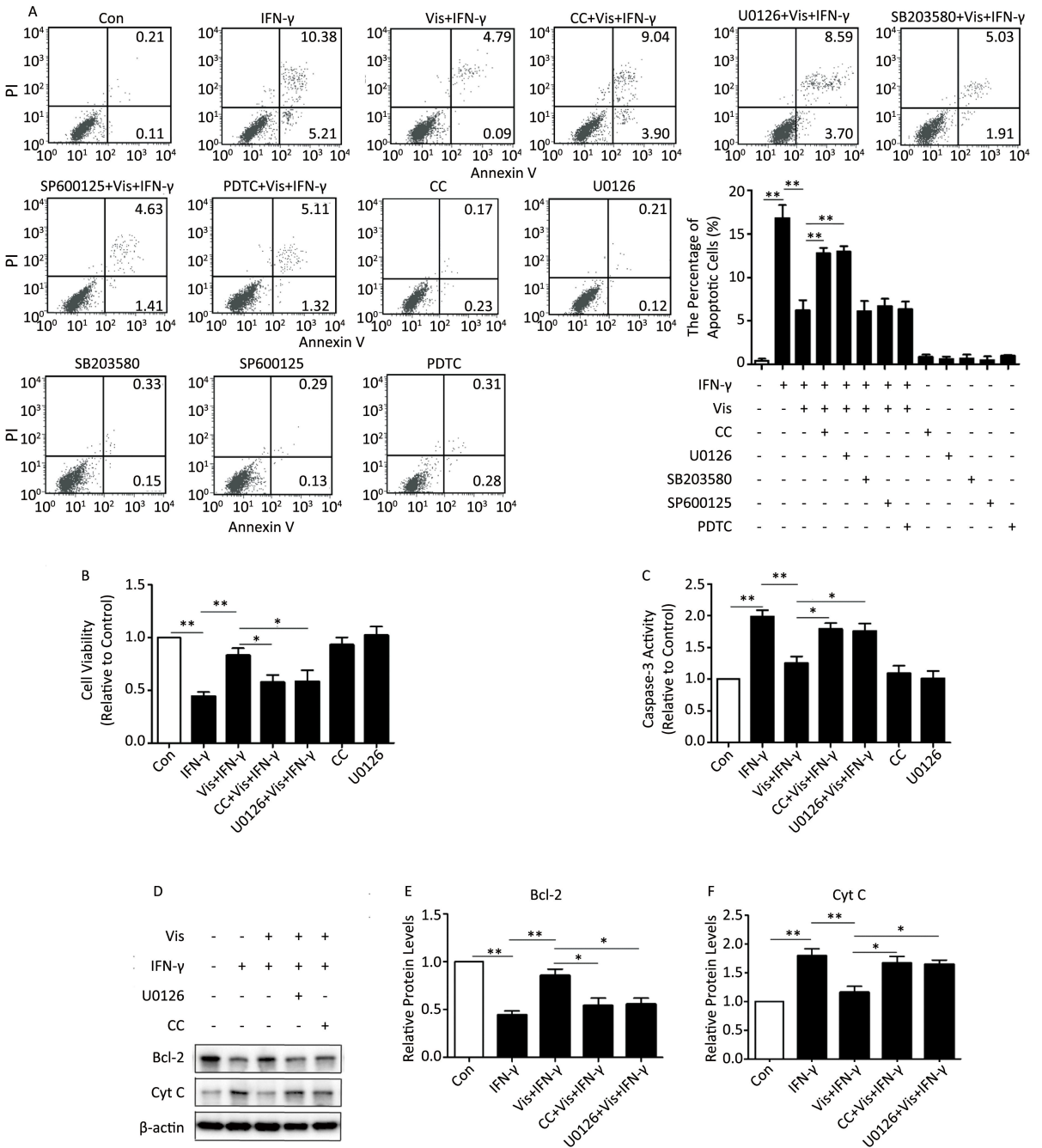


Figure 4. AMPK and ERK1/2 are involved in the anti-apoptotic effect of visfatin. RINm5F cells were pretreated with the pharmacological inhibitors compound C (an AMPK inhibitor), U0126 (an ERK1/2 inhibitor), SB203580 (a p38 inhibitor), SP600125 (a JNK inhibitor), or PDTC (a NF-κB inhibitor). (A) Cell apoptosis was analysis by flow cytometry. (B) Cell viability was measured by MTT assay. (C) Caspase-3 activity was measured by fluorometric detection. The protein levels of Bcl-2 (D, E) and Cyt C (D, F) were determined by Western blot analysis. β-actin was used as a loading control. The results are presented as means±SEM. Data are obtained from 3 independent experiments. *P<0.05, **P<0.01.

β -cells, free fatty acids (FFAs)-induced apoptosis is associated with a decrease in the Bcl-2/Bax ratio. FFAs significantly decrease the expression of Bcl-2, but has no significant effect on Bax expression^[32]. It has also been reported that overexpression of Bcl-2 proteins enhances pancreatic β -cells viability. Over-expression of Bcl-2 or deletion of Bax provides a partial protection to pancreatic islets from glucotoxicity in pancreatic β -cells^[33-34]. In this study, we found that the pretreatment with visfatin ameliorated IFN- γ -induced downregulation of Bcl-2 protein and restored decreased Bcl-2/Bax ratio. Visfatin pretreatment also inhibited cytochrome c released from mitochondria. Death receptor dependent apoptotic pathway also plays an important role in the development of diabetes^[35-36]. Exogenous administration of IFN- γ increases Fas (CD95) and caspase-8 expression and activity in dry eye-induced conjunctival epithelial apoptosis using C57BL/6 mice^[37]. In the present study, no significant change was observed in the expression of two death receptors, Fas and TNFR1. These results suggested that the anti-apoptotic effect of visfatin in RINm5F cells was mainly associated with the mitochondrial-mediated apoptotic pathway rather than the death receptor-mediated pathway.

Then we explored the signaling pathway which mediated the anti-apoptotic effect of visfatin. AMPK and MAPK signal transduction pathways are activated by different growth factors and thought to mediate cell proliferation, differentiation, and apoptosis^[38-40]. Previous studies have shown that visfatin increases insulin-like growth factor-1-induced steroidogenesis and cell proliferation, and metformin regulates visfatin expression through the AMPK signaling pathway in human granulosa cells^[41]. Visfatin can activate eNOS and improve endothelial cell function and angiogenesis through activation of MAPK signaling pathway^[42]. Phosphorylation of ERK1/2 induced by visfatin is closely associated with angiogenesis in endothelial cells. Inhibition of ERK1/2 activation markedly decreases visfatin-induced tube formation in human umbilical vein endothelial cells and visfatin-stimulated endothelial cell sprouting from rat aortic rings^[43]. These data indicated that AMPK and MAPK family pathways might contribute to the anti-apoptosis process triggered by visfatin. In this study, we found that the levels of p-AMPK and p-ERK1/2 were significantly increased by visfatin rather than p-p38MAPK and p-JNK. NF- κ B is another

major regulatory signaling molecule for cellular apoptosis. But the level of p-p65 subunit of the NF- κ B complex was not changed by visfatin. By using pharmacological inhibitors of ERK1/2 and AMPK, the anti-apoptotic action of visfatin was significantly attenuated. Moreover, the effects of visfatin on decrease in Bcl-2 expression and increase in Bcl-2/Bax ratio were also inhibited by compound C and U0126. These results indicated that AMPK and ERK1/2 were the downstream signal molecules to mediate the anti-apoptotic effect of visfatin in RINm5F cells rather than NF- κ B.

In summary, we demonstrated that visfatin protected pancreatic β -cells against IFN- γ -induced apoptosis via the inhibition of mitochondria-dependent apoptotic pathway. AMPK and ERK1/2 signaling molecules were required in the anti-apoptosis effect of visfatin. These findings deepen our understanding of the physiological and pathophysiological roles of visfatin in rat pancreatic β -cells and provide new insights into future a potential therapeutic target for diabetes.

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