

Visfatin is regulated by interleukin-6 and affected by the PPAR- γ pathway in BeWo cells

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Abstract. Visfatin, an adipocytokine and cytosolic enzyme with nicotinamide phosphoribosyltransferase (Nampt) activity, is involved in the pathogenesis of numerous metabolic disorders. In addition, the nuclear receptor peroxisome proliferator-activated receptor- γ (PPAR- γ) serves important roles in anti-inflammatory reactions and regulates glucose and lipid metabolism. The aim of the present study was to investigate the effect of interleukin-6 (IL-6) on the expression and secretion of visfatin in BeWo cells, and to determine whether the PPAR- γ pathway is involved in the regulation of visfatin by IL-6. Therefore, BeWo cells were stimulated with serial concentrations of IL-6 or pioglitazone, and the expression levels of visfatin and PPAR- γ were determined by reverse transcription-quantitative polymerase chain reaction and western blotting. The results of the present study demonstrated that IL-6 downregulated the mRNA levels of visfatin and PPAR- γ , which were strongly associated. Activation of PPAR- γ by pioglitazone resulted in significantly increased expression of visfatin, which abrogated the inhibitory effect of IL-6 on visfatin in BeWo cells. Furthermore, treatment using pioglitazone alone increased the expression and secretion of the visfatin protein, compared with the control or IL-6 alone group. In summary, the findings of the present study suggested that IL-6 inhibited the expression of visfatin and PPAR- γ at the transcriptional level; in addition, activation of PPAR- γ upregulated visfatin at the mRNA and protein expression levels. Therefore, the PPAR- γ signaling pathway may be involved in the regulation of visfatin by IL-6 in BeWo cells. These results

may provide novel insight into the roles of visfatin in trophoblastic cells. Furthermore, thiazolidinedione pioglitazone, by upregulating visfatin expression, may promote the energy metabolism of trophoblastic cells, maintain the function of the placenta and improve the outcome of pregnancy.

Introduction

Visfatin, additionally termed pre-B-cell colony-enhancing factor or nicotinamide phosphoribosyltransferase (Nampt), is an adipocytokine that is predominantly produced in visceral adipose tissue (1-3); however, during pregnancy, visfatin is additionally expressed and secreted by the placental tissue (4,5). Visfatin is involved in the pathogenesis of various metabolic disorders; increased plasma concentrations of visfatin have been reported in individual with obesity, as well as in patients with gestational diabetes mellitus (GDM) or metabolic syndromes (6-8).

Visfatin functions as an immunomodulatory cytokine involved in the inflammatory responses, and is a factor associated with obesity, inflammation and insulin resistance (3,6). Upregulation of inflammatory cytokines, including tumor necrosis factor- α (TNF- α) and interleukin 6 (IL-6), serves important roles in the induction of insulin resistance (9). Numerous previous studies indicated that visfatin was associated with inflammation (10-12); however, the underlying molecular mechanisms remain unknown. Furthermore, visfatin functions as the rate-limiting enzyme of NAD⁺ biosynthesis and converts nicotinamide into nicotinamide mononucleotide, a key NAD⁺ intermediate (13). In addition, visfatin-mediated NAD⁺ biosynthesis in adipocytes is an important physiological regulator of the metabolic function of adipose tissue and whole-body (13). Although still controversial, visfatin was reported to exert insulin mimetic or sensitizing effects; similar to insulin, visfatin increases lipogenesis, enhances glucose uptake of myocytes and adipocytes, and inhibits hepatocyte glucose release, consequently contributing to glucose and lipid metabolism (12,14,15). Previous studies identified the upregulation of IL-6 in the placenta of patients with GDM (16,17). In addition, it was indicated that the expression of visfatin in placenta was unchanged in patients with GDM (18). IL-6 (16,17) and visfatin (8,9) are associated with GDM. Kralisch *et al* (19) observed that IL-6 suppressed the mRNA synthesis of visfatin in 3T3-L1 adipocytes; however, in human

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Abbreviations: GDM, gestational diabetes mellitus; Nampt, nicotinamide phosphoribosyltransferase; PPAR- γ , peroxisome proliferator-activated receptor γ ; IL-6, interleukin 6; TNF- α , tumor necrosis factor- α

Key words: visfatin, Nampt, PPAR- γ , IL-6, inflammation

adipocytes and amniotic epithelial cells, IL-6 significantly promoted visfatin protein expression (20,21). Therefore, the present study hypothesized that the modulation of visfatin expression by IL-6 may be tissue specific; however, how pro-inflammatory IL-6 regulates the expression of visfatin in placenta remains unknown.

Peroxisome proliferator-activated receptor- γ (PPAR- γ) is a nuclear receptor that is abundantly expressed in adipocytes, where it regulates adipogenesis and lipid homeostasis (22). PPAR- γ functions in other tissues by promoting anti-immune responses and lipid metabolism in macrophages, increasing lipid storage in the liver and enhancing glucose-stimulated insulin secretion in pancreatic β -cells (22,23). PPAR- γ is involved in the pathophysiology of GDM and serves key roles in the regulation of glucose metabolism, lipid homeostasis and anti-inflammatory processes (22,23). Previous studies identified the downregulation of PPAR- γ in the trophoblast cells of patients with GDM (24-26). Mayi *et al.* (12) observed that synthetic PPAR- γ ligands upregulated the expression of visfatin gene in a PPAR- γ -dependent manner in resting primary human macrophages and adipose tissue macrophages, but not in adipocytes; however, whether PPAR- γ ligands induce the gene expression of visfatin in placenta remains unknown.

BeWo cells possess characteristics of the syncytiotrophoblast and extravillous trophoblast, and serve as an *in vitro* model to investigate trophoblast fusion (27,28). BeWo cells are frequently used to investigate the regulation of transplacental transport and cellular activities and uptake mechanisms (28). The aim of the present study was to determine the effect of IL-6 on the expression and secretion of visfatin in BeWo cells, and to determine whether the PPAR- γ pathway is involved in the regulation of visfatin by IL-6.

Materials and methods

Cell culture and treatment. All materials were purchased from Gibco (Thermo Fisher Scientific, Inc., Waltham, MA, USA) unless otherwise stated. BeWo cells were obtained from the National Infrastructure of Cell Line Resource (Beijing, China). The cells were cultured in Ham's F12 medium supplemented with 10% fetal bovine serum (ScienCell Research Laboratories, Inc., San Diego, CA, USA), 1% L-glutamine, 1% penicillin and 1% streptomycin (Beijing Solarbio Science & Technology Co., Ltd., Beijing, China) at 37°C in a humidified atmosphere of 5% CO₂. BeWo cells were seeded at a density of 2x10⁶ cells/ml in six-well plates or 25 cm² culture flasks (Corning Incorporated, Corning, NY, USA). Cells in the logarithmic growth phase were incubated at 37°C for 12, 24, 48 and 72 h with recombinant human IL-6 (cat. no. 200-06; PeproTech, Inc.) at serial concentrations (0, 1, 5 and 10 ng/ml). Additionally, BeWo cells were treated with the PPAR- γ ligand pioglitazone (cat. no. HY-14601; MedChem Express Co., Ltd., Monmouth Junction, NJ, USA) at serial concentrations (0.1, 1 and 10 μ M) or dimethyl sulfoxide as a control at 37°C for 48 h. Subsequently, BeWo cells were incubated with recombinant human IL-6 (5 ng/ml) and/or the PPAR- γ agonist pioglitazone (10 μ M), or dimethyl sulfoxide as a control at 37°C for 48 h. The supernatants were collected, and levels of secreted visfatin were determined using an ELISA kit (cat. no. KE1720; ImmunoWay Biotechnology Company, Plano, TX, USA) according to the

manufacturer's protocols. Additionally, BeWo cells were treated with recombinant human visfatin (cat. no. 130-09; PeproTech, Inc., Rocky Hill, NJ, USA) at serial concentrations (0, 10, 50 and 100 ng/ml) at 37°C for 48 h. The secretion levels of IL-6 and TNF- α were determined using an IL-6 ELISA kit (cat. no. EHC007; Xinbosheng Biotechnology Co., Ltd., Beijing, China) and TNF- α ELISA kit (cat. no. EHC103a; Xinbosheng Biotechnology Co., Ltd.) respectively, according to the manufacturer's protocol.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from BeWo cells using TRIzol[®] reagent (Invitrogen; Thermo Fisher Scientific, Inc.). RNA was reverse transcribed into cDNA using the Easy Script First Strand cDNA Synthesis Super Mix kit (Beijing Transgen Biotech Co., Ltd., Beijing, China) according to the manufacturer's protocol. RT was performed with a total of 20 μ l reaction mixture at 42°C for 15 min and 95°C for 3 min. Specific primers designed for the amplification of visfatin, PPAR- γ , and GAPDH were verified by NCBI BLAST (<http://blast.ncbi.nlm.gov/Blast.cgi>). The sequences were as follows: Human visfatin, forward, 5'-GCCAGCAGGGAATTTTGTTA-3' and reverse, 5'-TGATGTGCTGCTTCCAGTTC-3'; human PPAR- γ , forward, 5'-GCCCTTCACTACTGTTGACTTCT-3' and reverse, 5'-CAGGCTCCACTTTGATTGC-3'; and human GAPDH, forward, 5'-TGAACGGGAAGCTCACTG-3' and reverse, 5'-GCTTACCACCTTCTTGATG-3'. Reactions were performed on an ABI PRISM 7300 PCR system (Applied Biosystems; Thermo Fisher Scientific, Inc.) using PowerUp[™] SYBR Green Master Mix (Applied Biosystems; Thermo Fisher Scientific, Inc.). qPCR was performed with a total of 20 μ l reaction mixture at 50°C for 2 min and 95°C for 2 min, followed by 40 cycles of 95°C for 15 sec and 60°C for 1 min. Gene expression levels were analyzed in triplicate and normalized to GAPDH using the 2^{- $\Delta\Delta$ C_q} method (29).

Western blot analysis. Treated BeWo cells were washed twice using ice-cold PBS, and incubated using lysis buffer [1% NP-40, 150 mM NaCl, 50 mM Tris (pH 8.0), 0.1% aprotinin, 0.1% leupeptin, 0.035% pepstatin A and 100 μ g/ml PMSF] supplemented with protease and phosphatase inhibitor cocktail (Beijing Solarbio Science & Technology Co., Ltd.) at 4°C for 30 min. The samples were centrifuged at 11,600 x g at 4°C for 30 min, and protein concentrations were determined using a bicinchoninic acid assay kit (Thermo Fisher Scientific, Inc.). The protein samples were subsequently denatured in SDS sample buffer (125 mM Tris-HCl, pH 6.8, 50% glycerol, 2% SDS, 5% β -mercaptoethanol and 0.01% bromophenol blue) at 100°C for 10 min. Equal amounts of protein (30 μ g/lane) were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (EMD Millipore, Billerica, MA, USA). Subsequently, the membranes were blocked in Tris-buffered saline with 5% non-fat milk at room temperature for 4 h. The membranes were incubated with rabbit anti-human primary antibodies raised against visfatin (1:250; cat. no. ab45890; Abcam, Cambridge, MA, USA) and GAPDH (1:5,000; cat. no. AP0063; Bioworld Technology, Inc., Louis Park, MN, USA) overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated anti-rabbit immunoglobulin G secondary antibody (1:5,000; cat. no. L3012-2;

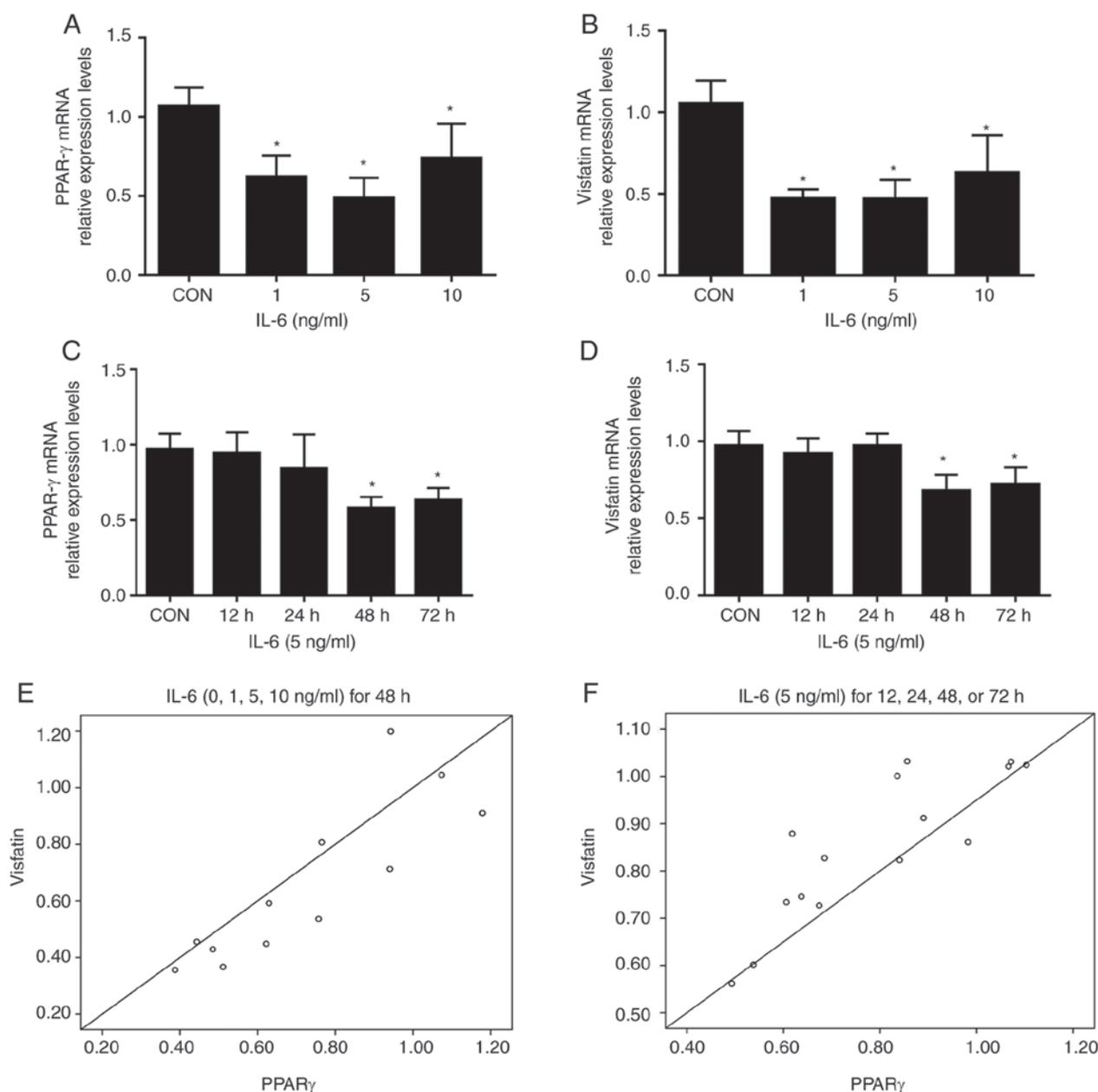


Figure 1. IL-6 regulates the expression levels of PPAR- γ and visfatin in BeWo cells. BeWo cells were incubated with IL-6 (0-10 ng/ml) for 48 h, and in the presence or absence of IL-6 (5 ng/ml) for 12, 24, 48 and 72 h. (A and B) IL-6 reduced the mRNA levels of PPAR- γ and visfatin. (C and D) Time course experiments indicated the inhibitory effects of IL-6 (5 ng/ml) on visfatin and PPAR- γ mRNA, which peaked at 48 h. (E and F) Expression levels of visfatin were correlated with the expression of PPAR- γ . * $P < 0.05$ vs. CON. CON, control; IL-6, interleukin 6; PPAR- γ , peroxisome proliferator-activated receptor- γ .

Signalway Antibody, LLC, College Park, MD, USA) at room temperature for 2 h. The bands were visualized using an enhanced chemiluminescence detection kit (GE Healthcare, Chicago, IL, USA). Band densities were quantified using Bio-Rad ChemiDoc™ XRS+System with Image Lab™ software version 4.1 (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The experiments were repeated at least three times.

Statistical analysis. The experiments were repeated at least three times. Data were presented as the mean \pm standard deviation and analyzed using SPSS 21.0 (IBM Corp., Armonk, NY, USA). Groups were compared by one-way analysis of variance and a Student-Newman-Keuls post-hoc test for normally distributed data. Correlation analysis was performed

using Spearman's or Pearson's correlation coefficients. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

IL-6 reduces the expression of visfatin gene in BeWo cells. Treatment of BeWo cells with increasing concentrations of IL-6 (1, 5, and 10 ng/ml) resulted in significantly reduced levels of PPAR- γ and visfatin mRNA compared with the control; no further decrease was detected with 10 ng/ml IL-6 (Fig. 1A and B). Time course experiments demonstrated that the inhibitory effects of IL-6 (5 ng/ml) on visfatin and PPAR- γ mRNA peaked at 48 h; no further reductions were observed at 72 h (Fig. 1C and D). Pearson correlation analysis

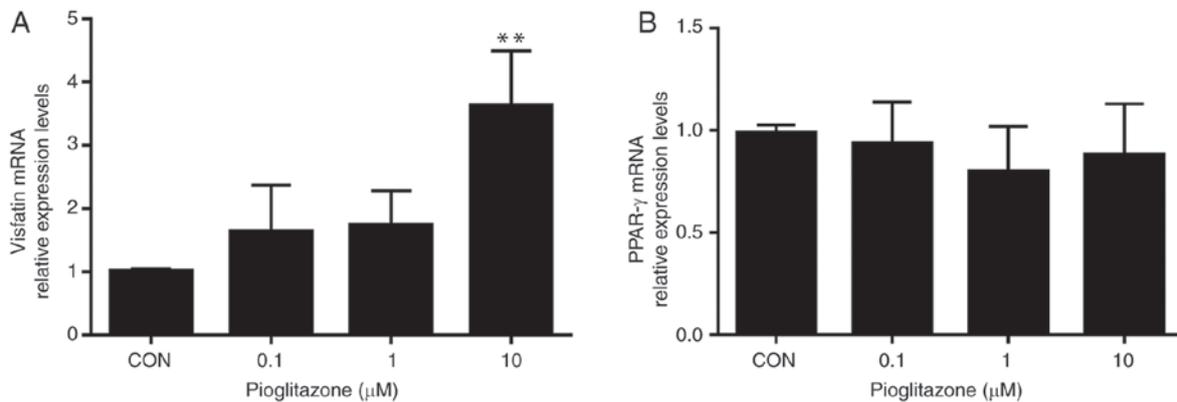


Figure 2. Effects of PPAR- γ agonist pioglitazone on the expression levels of visfatin and PPAR- γ in BeWo cells. The mRNA levels of visfatin and PPAR- γ were analyzed by reverse transcription-quantitative polymerase chain reaction. (A) PPAR- γ agonist pioglitazone induced the expression of visfatin in BeWo cells in a dose-dependent manner. (B) No significant differences were observed in the mRNA expression levels of PPAR- γ among the treatment groups. The results were representative of at least three independent experiments. Data were presented as the mean \pm standard deviation. ** $P < 0.01$ vs. CON, and 0.1 and 1 μ M pioglitazone. CON, control; PPAR- γ , peroxisome proliferator-activated receptor- γ .

demonstrated that the mRNA levels of visfatin were positively correlated with the expression of PPAR- γ ($r = 0.857$, $P < 0.05$; $r = 0.854$, $P < 0.001$, Fig. 1E and F, respectively). In summary, the data of the present study demonstrated that IL-6 inhibited the expression of visfatin in BeWo cells, which was associated with PPAR- γ .

PPAR- γ agonist pioglitazone induces the expression of visfatin gene of BeWo cells. Treatment with increasing concentrations of pioglitazone (0.1, 1 and 10 μ M) upregulated the mRNA expression levels of visfatin in a dose-dependent manner, whereas, no significant differences were observed in the mRNA levels of PPAR- γ among the treatment groups (Fig. 2). Furthermore, activation of PPAR- γ by exposure to pioglitazone (10 μ M) for 48 h resulted in significantly increased expression of visfatin compared with the control, which abrogated the inhibitory effects of IL-6 on visfatin in BeWo cells (Fig. 3A). In summary, the results of the present study demonstrated that visfatin may be a target gene of PPAR- γ in BeWo cells and that the PPAR- γ pathway may be involved in the regulation of visfatin by IL-6.

PPAR- γ activation regulates the expression and secretion of visfatin protein of BeWo cells. To investigate whether pioglitazone-induced expression of visfatin resulted in increased protein levels, BeWo cells treated with IL-6 (5 ng/ml) and/or pioglitazone (10 μ M) for 48 h were subjected to western blot analysis. IL-6 exhibited no significant effect on the protein expression of visfatin, while activation of PPAR- γ resulted in significantly increased protein expression levels of visfatin compared with the control, IL-6 treatment alone or in combination with pioglitazone (Fig. 3B); however, the expression levels of PPAR- γ protein were not affected by IL-6 or pioglitazone (data not shown). To examine whether induction by pioglitazone promoted the secretion of visfatin secretion, the ability of PPAR- γ to stimulate the release of visfatin was evaluated. As presented in Fig. 3C, treatment with pioglitazone (10 μ M) significantly increased the levels of visfatin in BeWo cell compared with IL-6 alone (5 ng/ml) or in combination with pioglitazone. The findings suggested that pioglitazone upregulated the expression level of visfatin by activating the

PPAR- γ pathway and the PPAR- γ pathway may be involved in the regulation of visfatin by IL-6.

Discussion

In the present study, the expression of visfatin in BeWo cells was evaluated in the presence of IL-6. Downregulation of visfatin was observed in BeWo cells treated with IL-6, which was consistent with the findings in 3T3-L1 adipocytes by Kralisch *et al* (19). The present study additionally identified that IL-6 reduced the mRNA expression level of PPAR- γ in BeWo cells and was associated with the expression of visfatin. The PPAR- γ agonist pioglitazone induced the expression of visfatin in BeWo cells, possibly in a PPAR- γ -dependent manner, which was consistent with the findings by Mayi *et al* (12) in resting primary human macrophages and adipose tissue macrophages. To the best of our knowledge, the present study is the first to demonstrate that visfatin may be a target gene of PPAR- γ in BeWo cells. In addition, the PPAR- γ pathway could be involved in the regulation of visfatin by IL-6; however, Lv *et al* (15) reported that pioglitazone ameliorates insulin resistance by downregulating visfatin expression in visceral adipose tissue and reducing circulating visfatin in high-fat-fed rats, whereas, no effect of pioglitazone was observed during the differentiation of 3T3-L1 adipocytes. This discrepancy may be due to the different sources of cells and tissues, which indicates that the effects of pioglitazone on visfatin expression could be cell- or tissue-specific.

PPARs are a member of the nuclear hormone receptor superfamily consisting of three isoforms: PPAR- α , PPAR- δ and PPAR- γ . All three PPAR isoforms are expressed in the placenta (30). The expression levels of PPAR- γ were downregulated in the placenta of females with GDM; however, that of PPAR- δ remained unchanged. A previous study demonstrated that PPAR- α agonist fenofibrate stimulated the expression of PPAR- α and induced the mRNA expression of visfatin in visceral fat deposits of Otsuka Long-Evans Tokushima fatty rats (31). The protein expression levels of PPAR- α in placenta were reduced, whereas, the mRNA levels were unchanged in females with GDM (24). Therefore, PPAR- γ was selected as

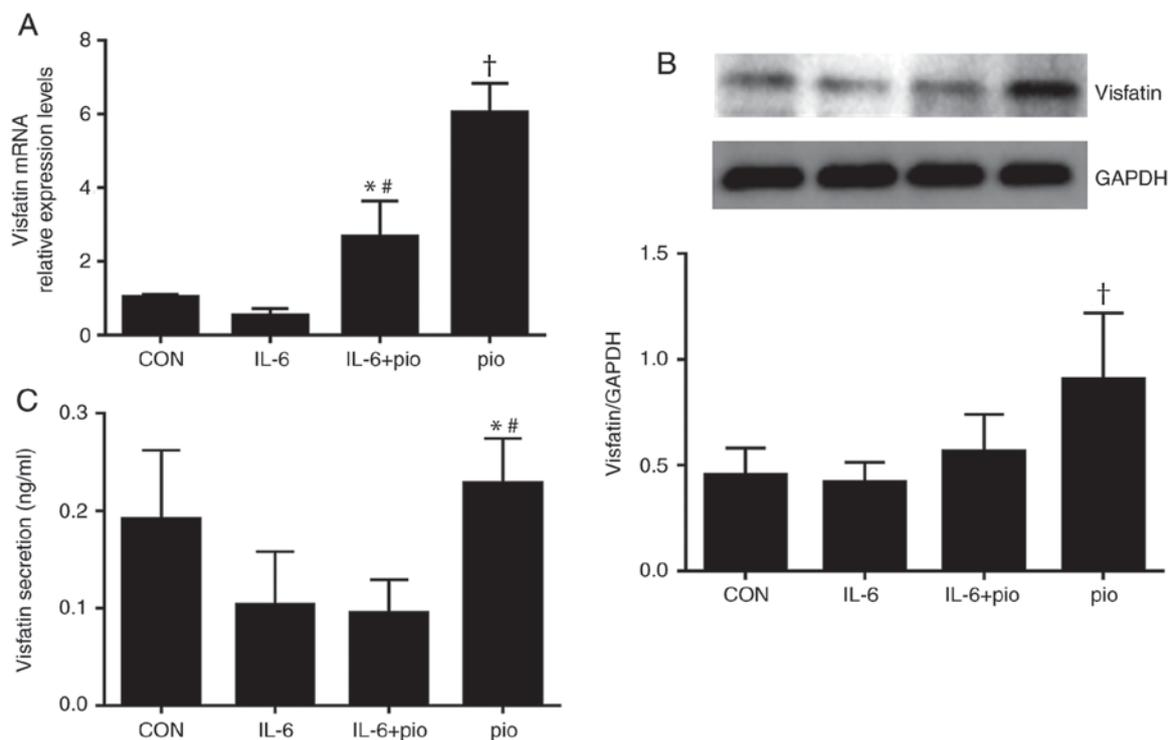


Figure 3. Effects of pio and IL-6 on the expression of visfatin in BeWo cells. BeWo cells were treated with IL-6 (5 ng/ml) and/or pio (10 μ M) for 48 h. (A and B) Pio significantly upregulated the expression levels of visfatin, which abrogated the inhibitory effects of IL-6 on visfatin in BeWo cells. ^{*}P<0.05 vs. IL-6; [#]P<0.05 vs. CON; [†]P<0.05 vs. CON, IL-6 and IL-6 + pio. (C) Treatment with pio significantly increased the secretion levels of visfatin in BeWo cell compared with IL-6 alone or in combination with pio. ^{*}P<0.05 vs. IL-6; [#]P<0.05 vs. IL-6 + pio. Data were presented as the mean \pm standard deviation. CON, control; IL-6, interleukin 6; pio, pioglitazone.

the potential target in the present study. A chemical agonist of PPARs was easier to obtain compared with overexpression vectors and/or PPAR small interfering RNAs, thus, pioglitazone was used in the present study.

The results of the present study demonstrated that IL-6 downregulated PPAR- γ and visfatin at the transcriptional level; the expression levels of visfatin and PPAR- γ were strongly correlated in BeWo cells. In addition, visfatin and PPAR- γ expression was reduced as the concentration of IL-6 increased between 0 and 5 ng/ml in BeWo cells, but their expression levels increased as the concentration of IL-6 reached 10 ng/ml; however, the expression levels of visfatin and PPAR- γ were significantly decreased compared with control group. The results of the present study suggested that the inhibitory effect of IL-6 on visfatin and PPAR- γ did not occur in a dose-dependent manner. The underlying mechanisms require further investigation.

Following treatment with pioglitazone, the mRNA and protein expression levels of visfatin were significantly increased in a dose-dependent manner in the present study. Therefore, visfatin may serve within trophoblastic cells as a protective factor, and may be involved in the regulation of energy metabolism and inflammatory reactions. It is well-established that visfatin/Nampt promotes intracellular NAD⁺ synthesis and induces the activation of sirtuin1 (12,32-34). Furthermore, placental visfatin/Nampt may additionally serve as the Nampt enzyme within trophoblasts (34). The overexpression of visfatin/Nampt may increase intracellular NAD⁺ and stimulate sirtuin1, consequently affecting DNA repair, protecting telomeres and reducing inflammation (34). NAD⁺

is an important coenzyme in numerous oxidation-reduction reactions (34). NAD⁺ accepts and donates electrons in reactions, thus leading to the production of ATP, which is required by cells for most energy-consuming processes (34). Sirtuin1, additionally termed NAD⁺-dependent deacetylase, is a negative regulator of pro-inflammatory cytokines (35-37). In summary, visfatin/Nampt may be the regulatory factor underlying the survival of trophoblastic cells and maintenance of placental function in a hostile environment, including the inflammation response or hyperglycemia. Upon inflammation, reduced expression of visfatin may lead to disrupted placental function and increased risk of abortion, preterm birth, and/or fetal distress (33).

However, treatment with the PPAR- γ agonist pioglitazone induced the mRNA and protein expression of visfatin by increasing the secretion of visfatin and offsetting the inhibitory effect of IL-6 at the mRNA level. The findings of the present study suggested that visfatin was a potential target gene of PPAR- γ in BeWo cells, and pioglitazone upregulated the expression level of visfatin by activating the PPAR- γ pathway. Additionally, the mRNA expression levels of PPAR- γ remained unchanged following the treatment of pioglitazone. Subsequent to being activated by its ligands, PPAR- γ forms a heterodimer with the retinoic X receptor, which transactivates the PPAR-response elements of target genes involved in insulin sensitivity, glucose metabolism and immune responses (12,38,39). Therefore, upon activation, PPAR- γ upregulates its target genes, including visfatin, rather than promoting its own transcription. Furthermore, treatment with pioglitazone alone for 48 h resulted in increased secretion of visfatin compared with IL-6 alone or in combination with

pioglitazone; however, the expression levels of visfatin in all groups remained low, indicating that visfatin may serve as an autocrine/paracrine factor rather than a typical endocrine agent in trophoblastic cells. Therefore, the present study hypothesized that visfatin was able to exert local effects in the placenta. Additionally, the activation of PPAR- γ suppressed the activity of nuclear factor- κ B, consequently regulating the balance between cytokine production and lipid metabolism (40); however, the present study indicated that treatment with visfatin for 48 h did not alter the secretion levels of inflammatory cytokines, including IL-6 and TNF- α (data not shown). In summary, the results of the present study provide novel insight into the roles of visfatin in trophoblastic cells. Placental visfatin may serve as the Nampt enzyme by increasing NAD⁺ biosynthesis and activating sirtuin1, consequently functioning as an anti-inflammatory factor rather than a pro-inflammatory factor, and serving an important role in maintaining the energy metabolism of trophoblastic cells and the function of the placenta.

In conclusion, the findings of the present study suggested that IL-6 downregulated visfatin and PPAR- γ at the transcriptional level; in addition, activation of PPAR- γ induced the expression of visfatin in BeWo cells. The thiazolidinedione pioglitazone may promote the energy metabolism of trophoblastic cells, maintain the function of the placenta and improve the outcome of pregnancy; however, further study is required to confirm whether visfatin may be a target gene of PPAR- γ in BeWo cells. The roles of visfatin as a Nampt enzyme, as well as its downstream signaling pathway and underlying mechanisms in trophoblastic cells or placenta, require further investigation.

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Availability of data and materials

The datasets used and/or analyzed in the current study are available from the corresponding author upon reasonable request.

Authors' contributions

YZ and SL conducted the experiments, were involved in data collection and drafted the manuscript. YH and WH performed the statistical analyses and contributed to the study design. HL and LL helped with the literature review and collected the data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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