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# Involvement of visfatin in palmitate-induced upregulation of inflammatory cytokines in hepatocytes

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ABSTRACT

Free fatty acids (FFAs) lead to the activation of inflammatory pathways related to the induction of insulin resistance. Visfatin is known to play a role in obesity-related metabolic diseases and inflammatory conditions. Here, the role of visfatin in FFA-induced inflammation was investigated in hepatocytes. The following factors were examined: (1) the protein and messenger RNA (mRNA) expression of visfatin in the liver tissue of insulin-resistant rats and in (2) in HepG2 cells treated with palmitate, (3) the palmitate-induced mRNA expression and protein synthesis of interleukin-6 and tumor necrosis factor–α in HepG2 cells transfected with visfatin-specific small interfering RNA, and (4) the expression of visfatin in HepG2 cells treated with a nuclear factor–κB (NF-κB) inhibitor (SN50) and infected with Ad-IκBα. The protein and mRNA levels of visfatin were significantly higher in insulin-resistant rat liver tissue compared with the control group. Visfatin expression and protein synthesis significantly increased in HepG2 cells treated with palmitate in a time- and concentration-dependent manner. Visfatin-specific small interfering RNA significantly decreased the palmitate-induced mRNA expression and protein synthesis of interleukin-6 and tumor necrosis factor–α. A NF-κB inhibitor induced the downregulation of visfatin in HepG2 cells following treatment with palmitate. HepG2 cells infected with Ad-IκBα showed decreased expression of visfatin following treatment with palmitate. The expression of visfatin is closely associated with the expression of proinflammatory cytokines in FFA-induced inflammation and is significantly decreased by NF-κB inhibition in HepG2 cells. Visfatin may play a role in FFA-induced inflammation in hepatocytes through the NF-κB pathway.



## 1. Introduction

Obesity is closely associated with insulin resistance, type 2 diabetes mellitus, and metabolic syndrome [1]. It has been suggested that free fatty acids (FFAs) are the major link between obesity and insulin resistance [2]. An overload of FFAs leads to the activation of inflammatory pathways related to the induction of insulin resistance [3]. Recently, Boden et al [4] reported that FFAs activate the proinflammatory nuclear factor–κB (NF-κB) pathway in the rat liver and proposed an

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association between FFA-induced inflammation and hepatic insulin resistance.

Visfatin was recently identified as an adipocytokine preferentially expressed in visceral adipose tissue [5]. It is also present in skeletal muscle, bone marrow, hepatocytes, and lymphocytes, where it was initially identified as pre–B-cell colony-enhancing factor [6,7]. Visfatin has been described as a highly expressed protein with insulin-mimetic biological and physiological effects [5]; however, these properties have not been confirmed using in vivo and in vitro studies. It has been shown that visfatin has nicotinamide adenine dinucleotide (NAD) biosynthetic activity; and thus, visfatin is also known as nicotinamide phosphoribosyltransferase, the rate-limiting enzyme that converts nicotinamide to nicotinamide mononucleotide, a NAD precursor [8]. Recent studies have demonstrated that visfatin acts as a proinflammatory cytokine and that its expression is increased in inflammatory conditions such as acute lung injury, sepsis, and rheumatoid arthritis [9-11]. Visfatin is associated with obesity and obesity-related insulin resistance, type 2 diabetes mellitus, and metabolic syndrome [12-14].

Thus, it is hypothesized that visfatin plays a role in the association between FFA-induced hepatic inflammation and insulin resistance. The association between the NF-κB pathway and visfatin is also examined in HepG2 cells treated with FFAs.

## 2. Materials and methods

### 2.1. Materials

Bovine serum albumin (BSA), palmitate (PA), and the IκB kinase (IKK) inhibitor sodium salicylate were purchased from Sigma-Aldrich (Sigma Korea, Seoul, Korea). The NF-κB inhibitor SN50 was purchased from Merck Bioscience (Korea Merck, Seoul, Korea). Human recombinant tumor necrosis factor (TNF)–α and interleukin (IL)-6 were purchased from R&D Systems (Minneapolis, MN); anti-NF-κB and anti-phospho-NF-κB antibodies were purchased from Cell Signaling Technology (Beverly, MA); and anti-visfatin antibody was obtained from Bethyl Laboratories (Montgomery, AL). Culture media, culture supplements, and fetal bovine serum (FBS) were obtained from Gibco-BRL (Grand Island, NY).

Chemicals were dissolved in dimethyl sulfoxide or other appropriate vehicle and used for treatment at the required dilution and dose. Palmitate/BSA conjugates were prepared as described previously [15]. Briefly, 20 mmol/L PA in 0.01 mol/L NaOH was incubated at 70°C for 30 minutes; and then the fatty acid soaps were complexed with 5% BSA in phosphate-buffered saline at an 8:1 molar ratio of fatty acid to BSA. The PA/BSA conjugates were administered to cultured cells.

### 2.2. Animals and plasma assays

Male Zucker diabetic fatty (ZDF) and Zucker lean (ZL) rats, 5 weeks of age, were purchased from Charles River Laboratories (Wilmington, MA). The animals were housed, 2 per cage, in a temperature-controlled (22°C) room with a 12-hour light/dark cycle and free access to a standard diet (60% carbohydrate, 10% fat, and 30% protein) and water.

At 6 weeks of age, the animals were fasted overnight; and blood samples were collected from the heart. The levels of triglycerides, total cholesterol, FFAs, glucose, insulin, visfatin, TNF-α, and IL-6 were, respectively, determined using a Pureauto S TG-N kit (Daiichi, Tokyo, Japan), a Pureauto S CHO-N kit (Daiichi), a nonesterified fatty acid kit (Eiken, Tokyo, Japan), an Accu-Chek active blood glucose monitoring system (Roche Applied Science, Indianapolis, IN), a rat insulin radioimmunoassay kit (RI-13K; Linco Research, St Charles, MO), a rat nicotinamide phosphoribosyltransferase (visfatin/pre–B-cell colony-enhancing factor) enzyme immunoassay (EIA) kit (Adipogen, Incheon, Korea), a rat TNF-α EIA kit (ALPCO Diagnostics, Salem, NH), and a rat IL-6 EIA kit (ALPCO Diagnostics).

### 2.3. Cells and culturing

The human hepatoma cell line HepG2 was cultured in Dulbecco modified Eagle medium (DMEM; Gibco-BRL) containing 10% FBS, streptomycin (100 mg/mL), and penicillin (100 U/mL) at 37°C in a humidified atmosphere of 5% $CO_2$/air.

### 2.4. Semiquantitative real-time polymerase chain reaction analysis

The expression levels of the respective mRNAs were compared by semiquantitative real-time polymerase chain reaction (PCR) using a Takara RNA PCR kit (version 3.0; Takara, Shiga, Japan). Briefly, HepG2 cell complementary DNA (cDNA) was synthesized with avian myeloblastosis virus reverse transcriptase and random 9-mers, and was used as a template for PCR amplification with specific primer sets.

The human PCR primers were as follows (5′→3′): visfatin, TGACAAAGCCCTCAGGAACA and TCCACCCAACACAAGCAAAG; GAPDH, TGGTATCGTGGAAGGACTCATG and TCCTTGAGAGCCATGTGGGCCAT; IL-6, TGAACTCCTTCTCCACAAGCG and TCTGAAGAGGTGAGTGGCTGTC; and TNF-α, CCCAGATAGATGGGCTCAT and CAGGGACCTCTCTCTAATCA. The PCR reaction conditions for visfatin were 25 to 30 cycles of denaturation at 95°C for 30 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR conditions for GAPDH were 25 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds. For IL-6, the PCR conditions were 29 cycles of 95°C for 30 seconds, 57°C for 30 seconds, and 72°C for 30 seconds. The PCR conditions for TNF-α were 30 cycles of 95°C for 30 seconds, 55°C for 30 seconds, and 72°C for 30 seconds.

The rat PCR primers were as follows (5′→3′): Visfatin, AGTCATTCAAGGGGACGGAG and AAGGGTGACAAAGGTCCC; GAPDH, GGCCAAAAGGGTCATCATCT and GTGATGGCATGGACTGTGGT; IL-6, AGCGATGATGCACTGTCAGAA and TGGAAGTTGGGGTAGGAAGG; and TNF-α, GACACCATGAGCACGGAAAG and TTTGCTACGACGTGGGCTAC. The PCR conditions for visfatin and GAPDH were 28 cycles of denaturation at 95°C for 30 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The PCR conditions for IL-6 were 30 cycles of 95°C for 30 seconds, 60°C for 30 seconds, and

72°C for 30 seconds. For TNF-α, the PCR conditions were 30 cycles of 95°C for 30 seconds, 56°C for 30 seconds, and 72°C for 30 seconds.

The amplified PCR products were separated by electrophoresis in a 1.5% agarose gel and stained with ethidium bromide. The levels of amplified DNA were quantitatively determined by densitometric analysis of the stained bands. The relative quantities of amplified DNA were compared on the basis of amplified GAPDH DNA.

### 2.5. Immunoblotting

Cells or liver tissues were suspended in radioimmunoprecipitation assay buffer (150 mmol/L NaCl, 1% NP-40, 0.5% DOC, 0.1% sodium dodecyl sulfate [SDS], 50 mmol/L Tris-HCl, pH 7.5) containing protease inhibitor cocktail (Roche Applied Science) and were incubated on ice for 15 minutes. Total protein was extracted by differential centrifugation (10 000*g* for 10 minutes). Protein concentrations were determined using a protein assay kit (BioRad, Hercules, CA). An equal volume of 2× SDS sample buffer (125 mmol/L Tris-HCl, pH 6.8, 4% SDS, 4% 2-mercaptoethanol, 20% glycerol) was added to the cell lysates. Equivalent amounts of protein (30 mg) were separated by electrophoresis in 10% to 15% polyacrylamide gels and transferred to polyvinylidene fluoride membranes (Millipore, Bedford, MA). The membranes were blocked with 5% skim milk for 1 hour, incubated with primary antibodies, and washed with phosphate-buffered saline. Following incubation with horseradish peroxidase–conjugated anti-mouse immunoglobulin G or anti-rabbit immunoglobulin G as secondary antibody, the immunoreactive bands were detected by enhanced chemiluminescence (Amersham Pharmacia Biotech, Piscataway, NJ, USA).

### 2.6. Enzyme-linked immunosorbent assay

Cells were plated in 24-well cell culture plates at a density of $2 \times 10^5$ cells per well and allowed to grow for 24 hours. Afterward, the cells were treated with 500 μmol/L PA for 24 and 48 hours. The PA-induced IL-6 or TNF-α in the culture media was quantified by using enzyme-linked immunosorbent assay (ELISA) kit (IL-6 [human] EIA kit, Assay Designs, Ann Arbor, MI; TNF-α [human] EIA kit, Enzo Life Sciences International, Plymouth Meeting, PA).

### 2.7. Small interfering RNAs

The 21-nucleotide small interfering RNA (siRNA) duplexes for green fluorescent protein (GFP) (5′-GUUCAGCGUGUCCGGCGAGTT-3′) and visfatin (5′-AGUCUUCUGUAAUCCAUGATT-3′) were designed and created at Genolution Pharmaceuticals (Seoul, South Korea). HepG2 cells were transfected with the siRNA oligonucleotides and cDNA using a pipet-type electroporator (microporator-mini, Digital Biotechnology, Seoul, South Korea) per the manufacturer's instructions. Briefly, $5 \times 10^5$ HepG2 cells were transfected with 100 pg of each siRNA cDNA and 3 μg of pcDNA3 in 100 μL of R buffer using a microporator at 990 V with a 50-millisecond interval. After transfection, the cells were seeded in 6-well plates ($5 \times 10^5$ cells per well) and treated with and without 500 μmol/L PA.

### 2.8. Preparation of recombinant adenoviral stock and adenovirus infection

HEK293 cells ($2 \times 10^6$) were grown to 80% confluence in 100-mm culture plates and then infected with a replication-defective adenovirus expressing IκBα or β-galactosidase at a multiplicity of infection (MOI) of 10 in 2 mL of DMEM. The cells were incubated with adenovirus in 5% $CO_2$ at 37°C for 1 hour, and then 8 mL of DMEM containing 5% FBS was added. After the cytopathic effect was observed, virus-containing extracts were clarified by centrifugation at 600*g* for 5 minutes; and virus particles were precipitated from the supernatant by the addition of 1 mL of DMEM. After 3 freeze-thaw cycles, the virus was collected by centrifugation at 12 000*g* for 10 minutes and stored at –70°C. The titers of the recombinant adenovirals were determined by plaque assay and tissue culture infectious dose 50 assay.

HepG2 cells were seeded in 6-well plates ($5 \times 10^5$ cells per well) and infected with Ad-IκBα and Ad-β-galactosidase at an MOI of 25 and 50 (respectively) by incubation with the recombinant adenovirals in serum-free medium at 37°C. After reaching approximately 80% confluence, the cells were serum-starved for 24 hours in medium containing 0.5% FBS and then treated with 500 μmol/L PA for 24 hours.

### 2.9. Statistical analysis

Data are presented as means ± SE of at least 3 independent experiments. Statistically significant differences between groups were determined using analysis of variance with Turkey post hoc tests or Student *t* test (as appropriate), with *P* values < .05 indicating statistical significance.

## 3. Results

### 3.1. Increased hepatic expression of visfatin in an insulin-resistant rat model

The biochemical data of the ZL and ZDF rats are summarized in Table 1. Free fatty acid and insulin levels were significantly higher in the ZDF rats. Interleukin-6 and TNF-α levels were higher in the ZDF rats, but the difference was not statistically significant. The visfatin level in the ZDF rats was about 10-fold that in the ZL rats. The mRNA and protein levels of visfatin

**Table 1 – Biochemical data of the ZL and ZDF rats**

| Characteristic | ZL (6 wk) (n = 4) | ZDF (6 wk) (n = 6) | *P* value |
|---|---|---|---|
| Body weight (g) | 181.7 ± 7.8 | 190.3 ± 3.9 | .317 |
| Total cholesterol (mg/dL) | 80.4 ± 3.0 | 103.6 ± 1.7 | <.001 |
| TG (mg/dL) | 54.2 ± 7.5 | 90.4 ± 25.5 | .211 |
| FFA (μEq/L) | 1092.8 ± 113.7 | 1724.6 ± 115.7 | .005 |
| Glucose (mg/dL) | 93.0 ± 0.4 | 112.7 ± 6.6 | .032 |
| Insulin (ng/mL) | 0.9 ± 0.1 | 4.7 ± 0.4 | <.001 |
| IL-6 (pg/mL) | 2.3 ± 0.3 | 8.5 ± 2.8 | .154 |
| TNF-α (pg/mL) | 9.1 ± 1.1 | 15.6 ± 3.0 | .123 |
| Visfatin (ng/mL) | 2.2 ± 0.8 | 20.2 ± 3.7 | .001 |

Data are means ± SE. TG indicates triglycerides.

(Fig. 1A, B) and the IL-6 and TNF-α protein levels (Fig. 1C) in liver tissues were significantly higher in the ZDF rats.

### 3.2. *Effects of PA on mRNA expression and protein synthesis of visfatin in HepG2 cells*

To determine whether higher levels of FFAs increased hepatic visfatin expression, HepG2 cells were treated with PA and assayed for visfatin by real-time PCR. Treatment with 250 and 500 μmol/L PA dose-dependently increased visfatin expression ($P < .01$, Fig. 2A), and treatment with 500 μmol/L PA for 24 and 48 hours time-dependently increased visfatin expression ($P < .01$, Fig. 2B). To further confirm that FFA increased inflammatory cytokines and visfatin production in HepG2 cells, the effects of FFA on visfatin (by Western blot analysis), IL-6, and TNF-α (by ELISA) were also evaluated. Treatment with 500 μmol/L PA for 24 and 48 hours time-dependently increased visfatin, IL-6, and TNF-α protein synthesis ($P < .05$ and $P < .01$, respectively, Fig. 2C).

### 3.3. *Effects of visfatin on inflammation in HepG2 cells*

To examine the effects of visfatin on inflammation in HepG2 cells, visfatin was knocked down with siRNA. Visfatin expression did not increase after treatment with 500 μmol/L PA in the visfatin knockdown cells compared with control cells transfected with GFP siRNA (Fig. 3A). The expression of IL-6 and TNF-α mRNA significantly decreased in visfatin knockdown cells treated with 500 μmol/L PA (Fig. 3B, C). The protein synthesis of visfatin, IL-6, and TNF-α also significantly decreased in the visfatin knockdown cells treated with 500 μmol/L PA using ELISA and Western blot analysis (Fig. 3D, E, F).

### 3.4. *Effects of IKK and NF-κB inhibitors on PA-induced visfatin expression in HepG2 cells*

To determine whether the NF-κB pathway contributes to visfatin expression in hepatocyte, IKK and NF-κB were inhibited in HepG2 cells in the presence and absence of PA; and visfatin expression was examined. Visfatin expression was almost completely suppressed by 1 and 2 mmol/L NaS, an IKK inhibitor, in the presence of PA ($P < .01$; Fig. 4A); and inhibition of NF-κB by 5 and 10 μmol/L SN50 dose-dependently suppressed PA-induced expression of visfatin ($P < .05$ and $P < .01$, respectively; Fig. 4B).

To confirm the effect of NF-κB on the expression of visfatin, IκBα was added to HepG2 cells using adenovirus (Fig. 4C). Visfatin levels significantly decreased as the expression of IκBα increased with PA. On the other hand, there was no change in visfatin levels as IκBα expression increased without PA.

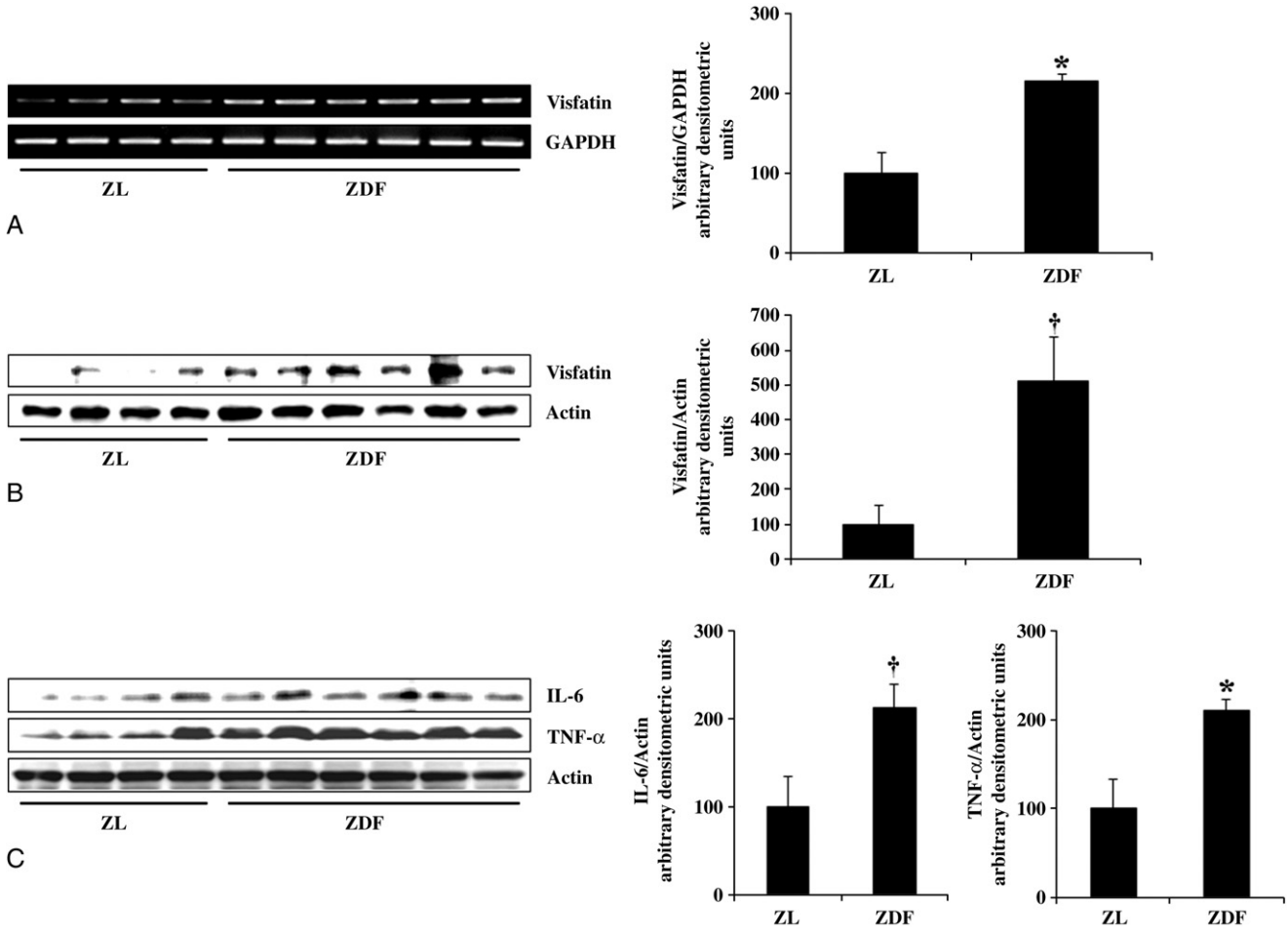


**Fig. 1 – Hepatic levels of visfatin (A) mRNA and (B) protein, and (C) TNF-α and IL-6 protein in liver tissues of ZDF rats and ZL control rats (*$P < .01$, †$P < .05$ vs ZL control rats).**

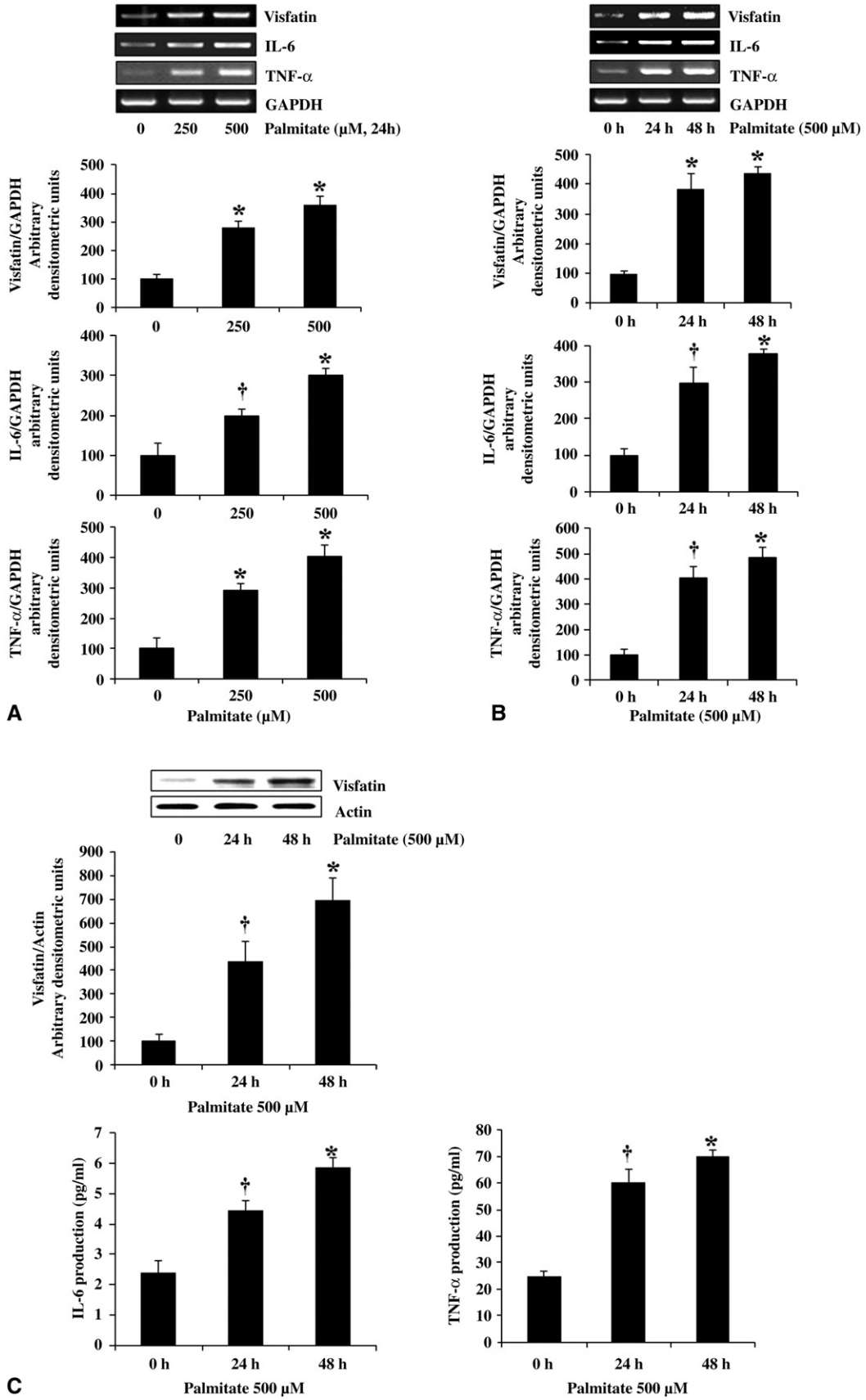


**Fig. 2 – Effects of PA on mRNA expression of visfatin, IL-6 and TNF-α in HepG2 cells according to (A) PA concentration (*P < .01, †P < .05 vs without PA) and (B) exposure time (*P < .01, †P < .05 vs at zero time). C, Effects of PA on protein synthesis of visfatin, IL-6, and TNF-α according to exposure time (*P < .01, †P < .05 vs at zero time).**

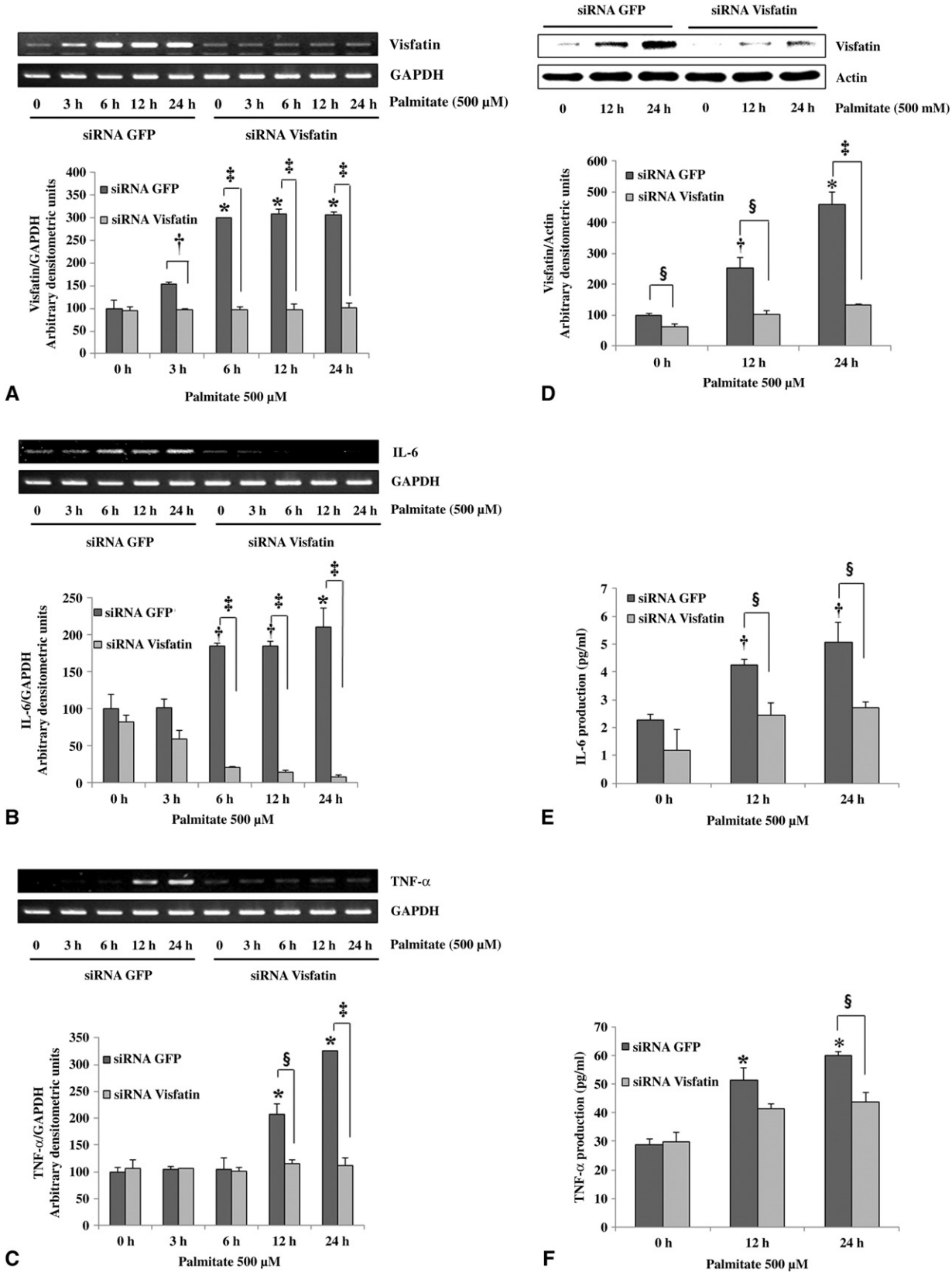


**Fig. 3 – Following knockdown of visfatin with siRNA, the cells were treated with 500 μmol/L PA. In response, (A) visfatin expression did not increase; and (B) IL-6 and (C) TNF-α mRNA expression significantly decreased. D, The protein synthesis of visfatin significantly decreased by using Western blot analysis. The protein synthesis of (E) IL-6 and (F) TNF-α also significantly decreased by using ELISA (*$P < .01$, †$P < .05$ vs at zero time, the comparison between transfection of siRNA GFP and siRNA visfatin of the same time frame: ‡$P < .01$, §$P < .05$).**

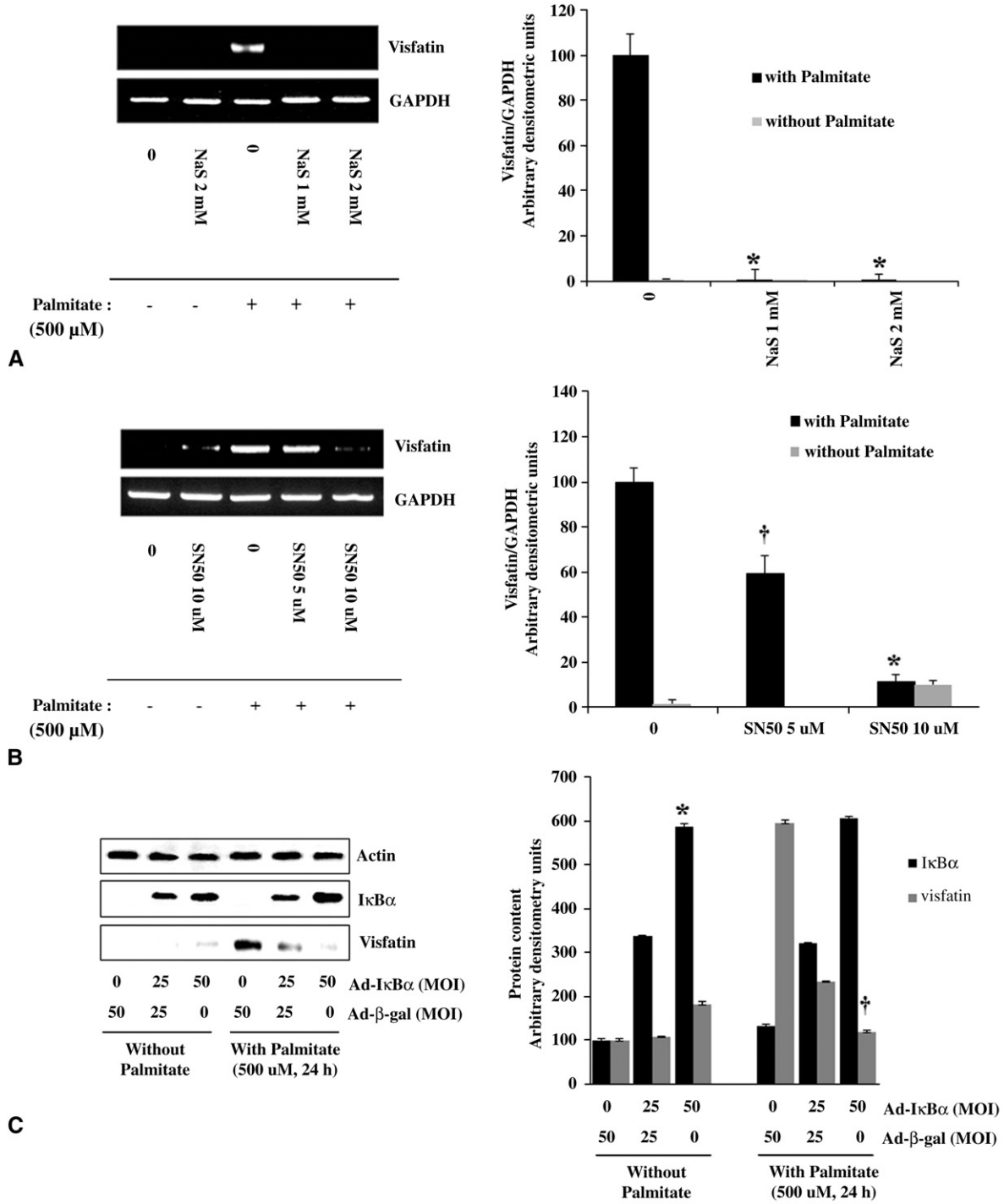


**Fig. 4 – Effect of (A) IKK inhibition by sodium salicylate (NaS) (*P < .01 vs visfatin expression at NaS = 0 with PA) and (B) NF-κB inhibition by SN50 (*P < .01, †P < .05 vs visfatin expression at SN50 = 0 with PA) on visfatin expression in HepG2 cells. C, Effect of infection with Ad-IκBα on visfatin expression in HepG2 cells, with or without PA (*P < .01 vs IκBα expression at Ad-IκBα [MOI] = 0 without PA, †P < .01 vs visfatin expression at Ad-IκBα [MOI] = 0 with PA).**

## 4. Discussion

These results demonstrate that FFAs and the hepatic expression of visfatin are increased in insulin-resistant rats. Moreover, the expression of visfatin is significantly associated with the expression of proinflammatory cytokines in FFA-induced hepatic inflammation in hepatocytes. This study also suggests that the expression of visfatin may be regulated by the NF-κB pathway in hepatocytes.

In the current in vivo study, 6-week-old male ZDF rats were used. It was reported that the ZDF male rats are characterized with hyperinsulinemia and hyperglycemia at 6 to 7 weeks of age [16]. Moreover, they are less obese but more

insulin resistant than Zucker fatty rats [17]. We also ascertained insulin resistance state using an insulin tolerance test on 6-week-old male ZDF rats and Zucker lean rats (Supplementary Fig. 1). The insulin-resistant ZDF rats also exhibited higher FFA, IL-6, TNF-α, and visfatin levels in serum; and visfatin mRNA expression was increased in liver tissue. Serum IL-6 and TNF-α levels were higher in ZDF rats, and their mRNA expression in liver tissue was significantly increased. Some reports have suggested that both tissue and circulating visfatin levels are increased in inflammatory disorders [9-11]. It has been shown that FFAs play a significant role in hepatic inflammation and insulin resistance [4] and that the liver secretes visfatin [6,7]. Thus, it was hypothesized that visfatin is associated with hepatic inflammation in an insulin-resistant state with high FFA levels. In the present study, PA treatment increased the levels of the proinflammatory cytokines IL-6 and TNF-α and significantly enhanced visfatin mRNA expression and protein synthesis in HepG2 cells. In visfatin knockdown HepG2 cells, PA did not increase IL-6 or TNF-α expression and protein synthesis. Thus, visfatin may play an important role in FFA-induced hepatic inflammation.

A number of reports concerning the proinflammatory effects of visfatin have shown that it dose-dependently upregulates the production of IL-1α, IL-6, and TNF-α in human monocytes [18,19]. When administered to mice, visfatin had similar effects on proinflammatory cytokine levels, especially increasing circulating IL-6 [18,19]. Furthermore, Li et al [20] demonstrated that visfatin affects macrophage function and IL-6 production. These studies examined extracellular effects of visfatin as a proinflammatory cytokine. We also found that ZDF rats showed increased amounts of visceral fat and that mRNA expression of visfatin in ZDF rats was higher in visceral fat (Supplementary Fig. 2A, B), although the difference of the expression of visfatin from visceral fat in both groups was less than that from the liver. Based on these findings, it could be thought that increased visfatin from visceral fat induced the expression of inflammatory cytokines in hepatocytes. However, in the current study, we knocked down visfatin to examine its intracellular effects on the inflammation process and found that the expression of inflammatory cytokines significantly decreased in visfatin knockdown hepatocytes. Intracellular visfatin has been investigated primarily as a mediator of energy metabolism and longevity via NAD. It is well known that visfatin is the rate-limiting component in the biosynthesis of NAD [7], which is an important cofactor in regulating various cellular metabolic pathways as a substrate of NAD-dependent enzymes including sirtuin, a NAD-dependent deacetylase, and poly-ADP ribosyltransferase [21]. These enzymes are thought to be related to the inflammation process. Poly-ADP ribosyltransferase–1 was reported as a transcriptional coactivator of NF-κB, an important transcription factor in inflammation [22]. Recently, Van Gool et al [23] reported that intracellular NAD levels positively regulate TNF-α biosynthesis through sirtuin-6; the study was performed using monocytes and showed effects at the posttranscriptional level. The current findings suggest that visfatin may act as an extracellular cytokine similar to proinflammatory mediators and may also control inflammation through the regulation of intracellular transcription factors.

Several studies have reported that extracellular visfatin activates the NF-κB pathway in myocytes and endothelial cells [24,25]. These studies describe the effects of extracellular visfatin on the activation of the NF-κB pathway. In the present study, the NF-κB pathway was inhibited; and we examined visfatin expression to determine whether the NF-κB pathway contributes to visfatin expression in hepatocyte. Visfatin expression was almost completely suppressed by the inhibition of the NF-κB pathway. The current findings suggest that visfatin not only may activate the NF-κB pathway but also be produced via the NF-κB pathway.

High concentrations of FFAs result in insulin resistance and activate the NF-κB pathway in rat liver [4]. In addition, FFAs increase levels of reactive oxygen species [26] and activate IKK-β, which produces IκBα distinct from the NF-κB complex [27], allowing NF-κB to enter the nucleus [28]. Based on previous findings and the current results, it is possible that FFAs activate the NF-κB pathway via reactive oxygen species production and that the activated NF-κB pathway then enhances the expression of visfatin, which induces IL-6 and TNF-α expression and augments the inflammation process. Although hepatic steatosis was not identified in the ZDF rats (data not shown), these results suggest that visfatin may play a role in the link between obesity-induced hepatic insulin resistance and nonalcoholic fatty liver disease/nonalcoholic steatohepatitis.

In summary, the present study shows that visfatin increases along with FFAs and proinflammatory cytokines in an insulin-resistant rat model. This study also demonstrates that visfatin expression is closely associated with the expression of proinflammatory cytokines in FFA-induced inflammation in hepatocytes and that the expression of visfatin is significantly decreased by NF-κB inhibition.

Supplementary materials related to this article can be found online at doi:10.1016/j.metabol.2011.05.003.

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## Conflict of Interest

The authors have no conflicts of interest.

## REFERENCES

[1] Mokdad AN, Bowman BA, Ford ES. The continuing epidemic of obesity and diabetes in the United States. JAMA 2001;286:1195-200.

[2] Boden G. Free fatty acids as targets for therapy. Curr Opin Endocrinol Diabetes 2004;11:258-63.

[3] Maury E, Brichard SM. Adipokine dysregulation, adipose tissue inflammation and metabolic syndrome. Mol Cell Endocrinol 2010;314:1-16.
[4] Boden G, She P, Mozzoli M, et al. Free fatty acids produce insulin resistance and activate the proinflammatory nuclear factor–κB pathway in rat liver. Diabetes 2005;54:3458-65.
[5] Fukuhara A, Matsuda M, Nishizawa M, et al. Visfatin: a protein secreted by visceral fat that mimics the effects of insulin. Science 2005;307:426-30 Retracted.
[6] Aller R, de Luis DA, Izaola O, et al. Influence of visfatin on histopathological changes of non-alcoholic fatty liver disease. Dig Dis Sci 2009;54:1772-7.
[7] Garten A, Petzold S, Barnikol-Oettler A, et al. Nicotinamide phosphoribosyltransferase (NAMPT/PBEF/visfatin) is constitutively released from human hepatocytes. Biochem Biophys Res Commun 2010;391:376-81.
[8] Revollo JR, Korner A, Mills KF, et al. Nampt/PBEF/visfatin regulates insulin secretion in beta cells as a systemic NAD biosynthetic enzyme. Cell Metab 2007;6:363-75.
[9] Jia SH, Li Y, Parodo J, et al. Pre–B-cell colony-enhancing factor inhibits neutrophil apoptosis in experimental inflammation and clinical sepsis. J Clin Invest 2004;113:1318-27.
[10] Brentano F, Schorr O, Ospelt C, et al. Pre–B-cell colony-enhancing factor/visfatin, a new marker of inflammation in rheumatoid arthritis with proinflammatory and matrix-degrading activities. Arthritis Rheum 2007;56: 2829-39.
[11] Garcia JG, Moreno Vinasco L. Genomic insights into acute inflammatory lung injury. Am J Physiol Lung Cell Mol Physiol 2006;291:L1113-7.
[12] Zhong M, Tan HW, Gong H, et al. Increased serum visfatin in patients with metabolic syndrome and carotid atherosclerosis. Clin Endocrinol(Oxf) 2008;69:878-84.
[13] Retnakaran R, Youn BS, Liu Y, et al. Correlation of circulating full-length visfatin (PBEF/NAMPT) with metabolic parameters in subjects with and without diabetes: a cross-sectional study. Clin Endocrinol (Oxf) 2008;69:885-93.
[14] Haus JM, Solomon TP, Marchetti CM, et al. Decreased visfatin after exercise training correlates with improved glucose tolerance. Med Sci Sports Exerc 2009;41:1255-60.
[15] Listenberger LL, Ory DS, Schaffer JE. Palmitate-induced apoptosis can occur through a ceramide-independent pathway. J Bio Chem 2001;276:14890-5.
[16] Shafrir E, Ziv E. A useful list of spontaneously arising animal models of obesity and diabetes. Am J Physiol Endocrinol Metab 2009;296:E1450-2.
[17] Shafrir E. Animal models of non–insulin dependent diabetes. Diabetes Metab Rev 1992;8:179-208.
[18] Moschen AR, Kaser A, Enrich B, et al. Visfatin, an adipocytokine with proinflammatory and immunomodulating properties. J Immunol 2007;178:1748-58.
[19] Tilg H, Moschen AR. Role of adiponectin and PBEF/visfatin as regulators of inflammation: involvement in obesity-associated diseases. Clin Sci (Lond) 2008;114:275-88.
[20] Li Y, Zhang Y, Dorweiler B, et al. Extracellular Nampt promotes macrophage survival via a non-enzymatic interleukin-6/STAT 3 signaling mechanism. J Biol Chem 2008;283:34833-43.
[21] Moschen AR, Gerner RR, Tilg H. Pre–B-cell colony enhancing factor/NAMPT/visfatin in inflammation and obesity related disorders. Curr Pharm Des 2010;16:1913-20.
[22] Hassa PO, Hottiger MO. The functional role of poly (ADP-ribose) polymerase 1 as novel coactivator of NF-kappaB in inflammatory disorders. Cell Mol Life Sci 2002;59:1534-53.
[23] Van Gool F, Galli M, Gueydan C, et al. Intracellular NAD levels regulate tumor necrosis factor protein synthesis in a sirtuin-dependent manner. Nat Med 2009;15:206-10.
[24] Oita RC, Ferdinando D, Wilson S, et al. Visfatin induces oxidative stress in differentiated C2C12 myotubes in an Akt- and MAPK-independent, NFkB-dependent manner. Pflugers Arch 2010;459:619-30.
[25] Kim SR, Bae YH, Bae SK, et al. Visfatin enhances ICAM-1 and VCAM-1 expression through ROS-dependent NF-kappaB activation in endothelial cells. Biochim Biophys Acta 2008;1783:886-95.
[26] Inoguchi T, Li P, Umeda F, et al. High glucose level and free fatty acid stimulate reactive oxygen species production through protein kinase C–dependent activation of NAD(P)H oxidase in cultured vascular cells. Diabetes 2000;49:1939-45.
[27] Karin M, Yamamoto Y, Wang QM. The IKK NFκB system: a treasure trove for drug development. Nat Rev 2004;3:17-26.
[28] Barnes PJ, Karin M. Nuclear factor–kappa B: a pivotal transcription factor in chronic inflammatory diseases. N Engl J Med 1997;336:1066-71.