

Visfatin/PBEF/Nampt induces EMMPRIN and MMP-9 production in macrophages via the NAMPT-MAPK (p38, ERK1/2)-NF- κ B signaling pathway

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Abstract. The adipocytokine visfatin is closely associated with metabolic disorders. This study explored the effects of visfatin on macrophage-induced inflammation in atheroma. The ability of visfatin to enhance extracellular matrix metalloproteinase inducer (EMMPRIN) expression, matrix metalloproteinase-9 (MMP-9) production and enzymatic activity in THP-1 derived macrophages as well as the mechanisms involved were investigated. EMMPRIN and MMP-9 mRNA levels were investigated by RT-PCR. EMMPRIN and MMP-9 protein levels, nuclear factor (NF)- κ B -p65 protein levels, peroxisome proliferator-activated receptor γ (PPAR γ) protein levels, and mitogen-activated protein kinase (MAPK) phosphorylation were determined by Western blotting. MMP-9 enzymatic activity was assayed by gelatin zymography. Visfatin (50-400 ng/ml) induced EMMPRIN and MMP-9 depending on the dosage used. Visfatin elicited the activation of NF- κ B and MAPK (p38, ERK1/2). Exogenous nicotinamide mononucleotide (NMN), the product of nicotinamide phosphoribosyltransferase (NAMPT) activity, mimicked the effects of visfatin on MAPK (p38, ERK1/2)-NF- κ B activation and EMMPRIN/MMP-9 induction. Using the p38 inhibitor, SB203580, the ERK1/2 inhibitor PD98059, the NF- κ B inhibitor, pyrrolidine dithiocarbamate and the NAMPT inhibitor FK866, we demonstrated that the visfatin pro-inflammatory action was through the NAMPT-MAPK (p38, ERK1/2)-NF- κ B pathway. Furthermore, the visfatin pro-inflammatory action was not prevented by insulin receptor blockade or by a

PPAR γ agonist. Visfatin did not modulate PPAR γ expression. Retinoid X receptor (RXR) agonist suppressed the effects of visfatin on EMMPRIN/MMP-9, NF- κ B, but not on MAPK activation. In conclusion, we have demonstrated that visfatin enhances atheroma inflammation through the NAMPT-MAPK (p38, ERK1/2)-NF- κ B-EMMPRIN/MMP-9 pathway, a key feature of atherosclerotic diseases linked to metabolic disorders.

Introduction

Visfatin, initially described as pre-B-cell colony-enhancing factor (PBEF) or nicotinamide phosphoribosyltransferase (NAMPT), is a novel identified adipocytokine. It is preferentially released by visceral fat (1). Enhanced circulating visfatin/PBEF/Nampt (for concision, mentioned as visfatin in the following text) levels have been reported in patients affected by metabolic disorders, such as diabetes mellitus, obesity, or the metabolic syndrome (2,3). The pathophysiological relevance of visfatin remains poorly understood, especially in obesity-associated cardiovascular diseases. Recently, it is hypothesized that visfatin might directly promote atheroma inflammation, therefore representing a link between metabolic disorders and acute coronary syndrome (ACS, including acute myocardial infarction and unstable angina). It has previously been reported that enhanced circulating levels of visfatin are positively associated not only with increased plasma levels of inflammatory markers (2), but also with vascular damage and endothelial dysfunction (4,5).

However, the ability of visfatin to exert direct pro-inflammatory effects on macrophages, which is the main source of inflammatory factors in atheroma, remains largely unknown. To address this question, THP-1 cells were cultured and differentiated into macrophages to investigate the ability of visfatin to up-regulate extracellular matrix metalloproteinase inducer (EMMPRIN) and matrix metalloproteinase-9 (MMP-9) expression, which play the most important roles in atherosclerotic plaque destabilization (6,7). The study further aimed to identify the mechanism mediating the pro-inflammatory action of visfatin in macrophages.

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Materials and methods

Reagents. RPMI-1640 medium and fetal bovine serum (FBS) were from Gibco. Phorbol-12-myristate 13-acetate (PMA) was from Calbiochem (San Diego, CA). Human recombinant visfatin was from Axxora (Nottingham, UK). The reagents 9-cis retinoic acid [9-cis, a natural ligand of retinoid X receptor (RXR)], dimethyl sulfoxide, nicotinamide mononucleotide (NMN), and pyrrolidine dithiocarbamate (PDTC, an NF- κ B inhibitor) were from Sigma-Aldrich (St. Louis, MO). SB203580 (SB, a specific p38 inhibitor), PD98059 (PD, a specific MEK/ERK inhibitor), and SP600125 (SP, a specific JNK inhibitor) were from Tocris Bioscience (Bristol, UK). Hydroxy-2-naphthalenylmethylphosphonic acid tris-acetoxymethyl ester (HNMPA, an insulin receptor inhibitor) was from Calbiochem (La Jolla, CA, USA). The reagents 15-deoxy-12, 14-prostaglandin J2 [PGJ2, a natural ligand for peroxisome proliferator-activated receptor γ (PPAR γ)], pioglitazone (piog, a synthetic ligand for PPAR γ), and FK866 (FK, an NAMPT activity inhibitor) were from Cayman Chemical (Ann Arbor, MI). The anti-p38, ERK1/2, and JNK (both phosphorylated and non-phosphorylated) antibodies were from Cell Signaling Technologies (Danvers, MA). The EMMPRIN monoclonal antibody was from Zymed (San Francisco, CA). The MMP-9 and the β -actin monoclonal antibodies were from Abcam (Cambridge, UK). The PPAR γ monoclonal and the NF- κ B p65 polyclonal antibody were from Santa Cruz (Santa Cruz, CA). The goat anti-rabbit secondary antibody (No. A-21109) and the goat anti-mouse secondary antibody (No. A-21058) were from Invitrogen (Carlsbad, CA). All other chemicals were of the highest purity available commercially.

Cell culture and treatment. The human monocyte cell line, THP-1 (American Type Culture Collection, Rockville, MD), was used. THP-1 cells can be differentiated into macrophages that mimic human macrophage activity and function, particularly in atherosclerotic lesions. Thus, this cell line has been widely used in studies of macrophage function in atherosclerosis (8,9). Cells were maintained at a density of 10^6 cells/ml in RPMI-1640 medium supplemented with 10% FBS and 10 mM HEPES (Sigma) under 5% CO₂ at 37°C in an incubator. Cells were then cultured in PMA (100 nM) in 6-well plates for 48 h to promote differentiation into macrophages. After three washes with sterile PBS, cells were sub-cultured in fresh medium.

After being starved with 0.3% FBS for 12 h, macrophage cells were stimulated with different concentrations of visfatin (0, 50, 100, 200, 400 ng/ml) or NMN (100 μ mol/l) (10). Cells were also treated with visfatin (200 ng/ml) for different times (0, 5, 10, 15, 30, 60 min). Inhibitors (SB, PD, JNK, PDTC, HNMPA, and FK866) or ligands (piog, PGJ2, and 9-cis) were added 1 h prior to incubation with visfatin (200 ng/ml) for 15 min or 24 h.

RNA isolation and RT-PCR. Total RNA was extracted from visfatin-treated cells using the Trizol reagent. Total RNA (2 μ g) was then reverse-transcribed into cDNA using a random primer and the resultant cDNA was amplified by PCR with the following primer: EMMPRIN (163 bp), 5'-TCCTGGGCATCGTGGCT-3' (sense), and 5'-CCTCTGGCGGACGTTCTTG-3' (antisense);

MMP-9 (115 bp), 5'-ACTACTGTGCCTTTGAGTCC-3' (sense), and 5'-AGAATCGCCAGTACTTCCC-3' (antisense); β -actin (249 bp), 5'-CATGTACGTTGCTATCCAGGC-3' (sense), and 5'-CTCCTTAATGTACGCACGAT-3' (antisense). All gels were detected by the Tanon-4100 digital Gis image system (Beijing, China). Densitometric analysis was performed with Quantity One (Bio-Rad) to scan the signals.

Preparation of cytoplasmic and nuclear extracts. Macrophages were rinsed in cold PBS and lysed in a solution containing 0.6% Nonidet P-40, 10 mM KCl, 10 mM HEPES, 0.1 mM EDTA (all products from Sigma Aldrich), and Complete™ Mini-EDTA-free protease inhibitor cocktail (Roche Diagnostics). After centrifugation (30 sec, 2,000 \times g), supernatants were incubated on ice for 5 min. Nuclei were precipitated by centrifugation (4°C, 3 min, 15,000 \times g), supernatants collected as cytosolic extracts, and the nuclei resuspended in a solution of 10% glycerol, 20 mM HEPES, 400 mM NaCl, 1 mM EDTA and Complete Mini-EDTA-free protease inhibitor cocktail. The mixture was incubated on ice for 1 h, the supernatant was collected after centrifugation for 5 min at 15,000 \times g, and saved as nuclear extracts.

Western blotting. Protein concentrations were measured by the BCA Protein Assay (Pierce, Rockford). The protein extracts were then denatured and the solubilized proteins (20 μ g) were electrophoresed on 10% polyacrylamide SDS gels. The proteins were subsequently transferred onto polyvinylidene difluoride membranes (Millipore, MA) and then blocked with TBS with 0.05% Tween-20 and 5% skim milk for 1 h at room temperature, followed by probing with the primary antibody overnight, and then with the secondary antibody labeled with the far-red-fluorescent dye, Alexa Fluor 680. All signals were detected by Odyssey (Li-Cor, USA). Densitometric analysis was performed with the Quantity One software.

Zymography. MMP activity was determined by SDS-polyacrylamide gel zymography. Briefly, the culture supernatant was collected, loaded onto a 10% polyacrylamide gel containing 0.1% gelatin, which was run at 125 V for 90 min. The gel was renatured in renaturing buffer for 30 min. After equilibration in developing buffer for 30 min, fresh developing buffer was added, and the gelatin-containing gel was allowed to develop overnight at 37°C. The gelatin gel was stained with 0.5% Coomassie blue in a buffer consisting of 10% acetic acid, 50% methanol, and 40% distilled water before being destained in the same buffer for 30 min to visualize the zymogen bands. An image of each gel (after drying) was scanned and the zymogen bands were quantified by using densitometry. Results are expressed in arbitrary densitometry units.

Statistical analysis. Data comparisons were performed by the Student's t-test with the SPSS 13.0 software. Results are expressed as the mean \pm SD. P-values <0.05 were considered significant. All experiments were repeated at least 3 times.

Results

Visfatin enhances EMMPRIN and MMP-9 expression as well as MMP-9 enzymatic activity in macrophages. In macrophages

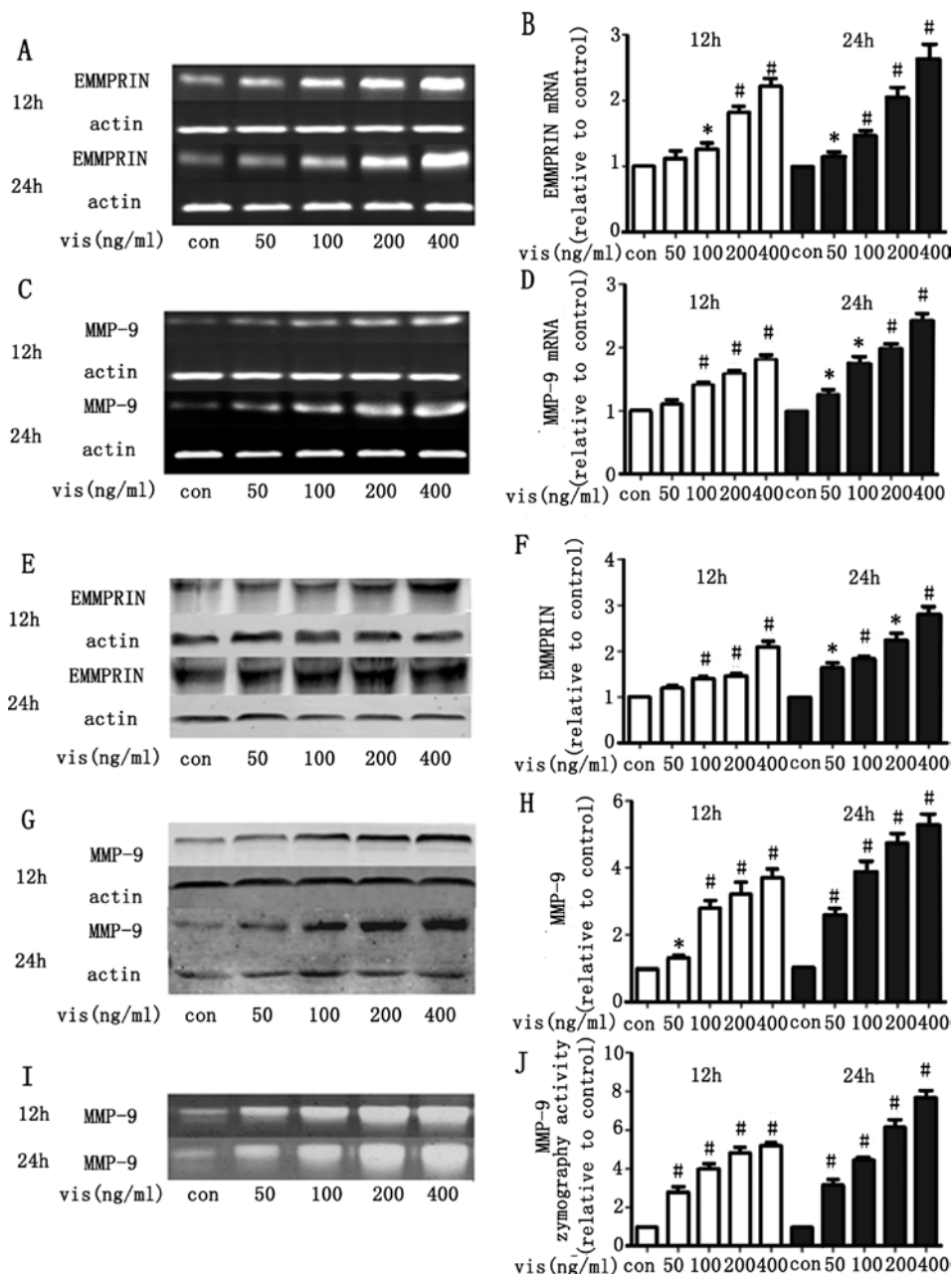


Figure 1. Visfatin (vis) induces EMMPRIN and MMP-9 expression in macrophages. Effects of visfatin on (A) EMMPRIN mRNA, (C) MMP-9 mRNA, (E) EMMPRIN protein production, (G) MMP-9 protein production and (I) on MMP-9 enzymatic activity. (B, D, F, H and J) Respective densitometric analysis measurements. Values are the mean \pm SD (n=3). Band density of untreated macrophages were defined as control and set to 1. *P<0.05; #P<0.01.

exposed to visfatin (0, 50, 100, 200, and 400 ng/ml) for 12 or 24 h, a concentration-dependent increase in the mRNA and protein levels of EMMPRIN and MMP-9 was observed over time.

With a threshold concentration of 100 ng/ml (after 12 h) or 50 ng/ml (after 24 h), visfatin induced a significant dose-dependent increase in the mRNA expression of EMMPRIN and its downstream inflammatory factor MMP-9, with a maximum response at 400 ng/ml (Fig. 1A-D). There was a peak increase of 2.63-fold in EMMPRIN and a peak increase of 2.46-fold in MMP-9 after 24 h.

The protein expression of EMMPRIN and MMP-9 following 12 or 24 h visfatin treatments (0, 50, 100, 200, and 400 ng/ml) were measured by Western blot analysis. The results were similar to those observed at the mRNA level

(Fig. 1E-H). Peak increases of 2.79-fold in EMMPRIN and of 5.29-fold in MMP-9 were observed after 24 h.

Furthermore, the enzymatic activity of MMP-9 was assessed by gelatin zymography. Visfatin dose-dependently up-regulated MMP-9 enzymatic activity over time (Fig. 1I and J). There was a peak increase of 7.7-fold after 24 h.

Visfatin pro-inflammatory action requires the MAPK (p38, ERK1/2)-NF- κ B pathway. The MAPK-NF- κ B pathway plays an important role in atheroma destabilization as it regulates the production of inflammatory factors. To ascertain whether visfatin signals via this pathway in macrophages, macrophages were treated with visfatin and compared with the control subjects (untreated). In macrophages, visfatin (200 ng/ml) elicited a gradual increase in nuclear NF- κ B p65

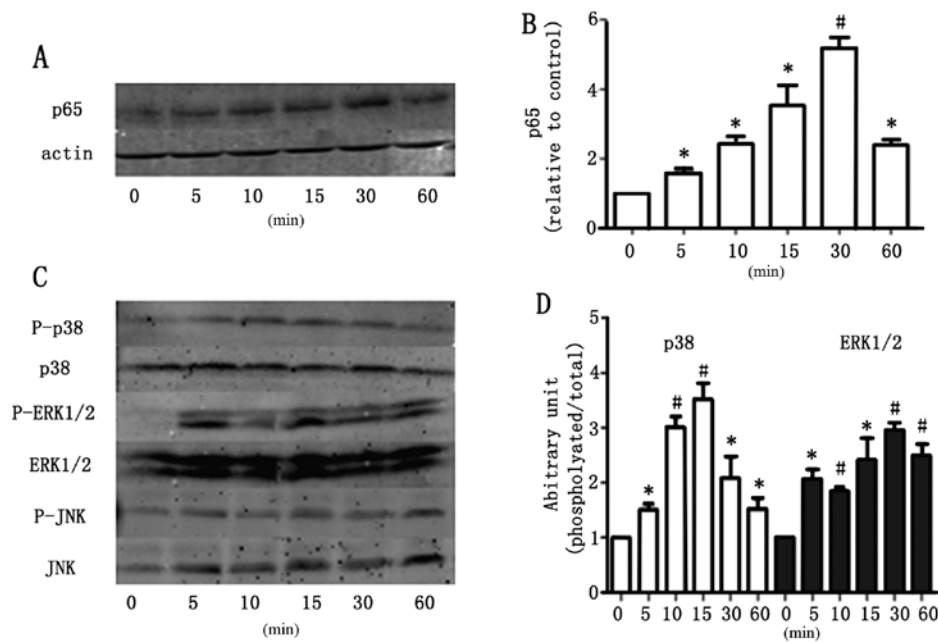


Figure 2. Visfatin activates MAPK (p38, ERK1/2) and NF- κ B. Effect of visfatin on (A) nuclear NF- κ B p65 levels and (C) MAPK activation. (B and D) Respective densitometric analysis measurements. Values are the mean \pm SD (n=3). The band density of untreated macrophages were defined as control and set to 1. *P<0.05 and #P<0.01, compared to control.

from 5-30 min, when the maximal activation was attained. At 60 min, the NF- κ B p65 level was about 50% of that achieved at 30 min, although it remained enhanced compared to the basal levels (Fig. 2A and B). In addition, visfatin significantly activated ERK1/2 and p38 MAPK (Fig. 2C and D). Visfatin increased the phosphorylation of both p42 and p44 (ERK1/2). It attained a maximum at 30 min and decreased thereafter. The adipocytokine also increased p38 phosphorylation. It attained a maximum response at 15 min and then gradually decreased. Visfatin had no effect on JNK phosphorylation (Fig. 2C and D).

The implications of both NF- κ B and MAPK (p38, ERK1/2) in visfatin-mediated EMMPRIN and MMP-9 inductions in macrophages were then assessed. Macrophages were pre-treated with inhibitors of p38 (SB, 10 μ M), ERK1/2 (PD, 50 μ M), JNK (SP, 10 μ M), or of NF- κ B (PDTC, 100 μ M) for 1 h, prior to visfatin treatment (200 ng/ml) for 24 h. The co-incubation of visfatin (200 ng/ml) with SB or PD significantly suppressed the inductions of EMMPRIN and MMP-9 by the adipocytokine. PDTC completely blocked visfatin pro-inflammatory action (Fig. 3A-F). Furthermore, SB and PD attenuated NF- κ B activation triggered by visfatin (Fig. 3G and H), highlighting the role of MAPK (p38, ERK1/2) in NF- κ B activation by visfatin. Based on these findings, it can be concluded that visfatin exerts pro-inflammatory effects on macrophage via the MAPK (p38, ERK1/2)-NF- κ B pathway.

NAMPT enzymatic activity mediates the pro-inflammatory action of visfatin in macrophages. Since it has been reported that visfatin may exert its cellular actions through its intrinsic NAMPT enzymatic activity (11), we explored whether NMN, the product of NAMPT activity and a precursor of NAD (12), could mimic the pro-inflammatory effect of visfatin in macrophages. Exogenous NMN (100 μ mol/l), activated

MAPK (p38, ERK1/2)-NF- κ B to a similar extent with visfatin (200 ng/ml) (Fig. 4A and B). NMN (100 μ mol/l) also triggered EMMPRIN and MMP-9 induction in macrophages, which was not significantly different from that achieved by 200 ng/ml visfatin (Fig. 4C and D).

The impact of the inhibition of the NAMPT activity on the pro-inflammatory action of visfatin was next assessed. The activation of MAPK (p38, ERK1/2)-NF- κ B elicited by visfatin (200 ng/ml) in macrophages was completely abolished by co-incubation with the NAMPT activity inhibitor FK866 (10 nmol/l) (13-15) (Fig. 4E and F). Similarly, FK866 (10 nmol/l) blocked the induction of EMMPRIN and MMP-9 by 200 ng/ml visfatin (Fig. 4G and H). Based on these results, it can be concluded that NAMPT enzymatic activity mediates the pro-inflammatory action of visfatin in macrophages. It is worth noting that the NAMPT substrate nicotinamide is contained in the culture medium of macrophages (RPMI-1640).

The pro-inflammatory action of visfatin in macrophages is not mediated by the insulin receptor. Visfatin was initially proposed as an insulin mimetic (1). Therefore, whether the pro-inflammatory action of visfatin in macrophages is mediated by the insulin receptor was explored. For this purpose, macrophages were pre-treated with HNMPA (100 μ mol/l), an inhibitor of insulin receptor signaling (16), for 1 h prior to co-stimulation with visfatin (200 ng/ml). EMMPRIN induction was then examined. Pre-incubation of macrophages for 1 h with HNMPA did not affect the NF- κ B activation by visfatin (200 ng/ml) after 15 min (Fig. 4I and J).

A ligand of RXR, not PPAR γ , attenuates visfatin inductions of EMMPRIN and MMP-9 by modulating NF- κ B activation. To gain further insight into the mechanisms of the visfatin pro-inflammatory actions in macrophages, the role of the nuclear

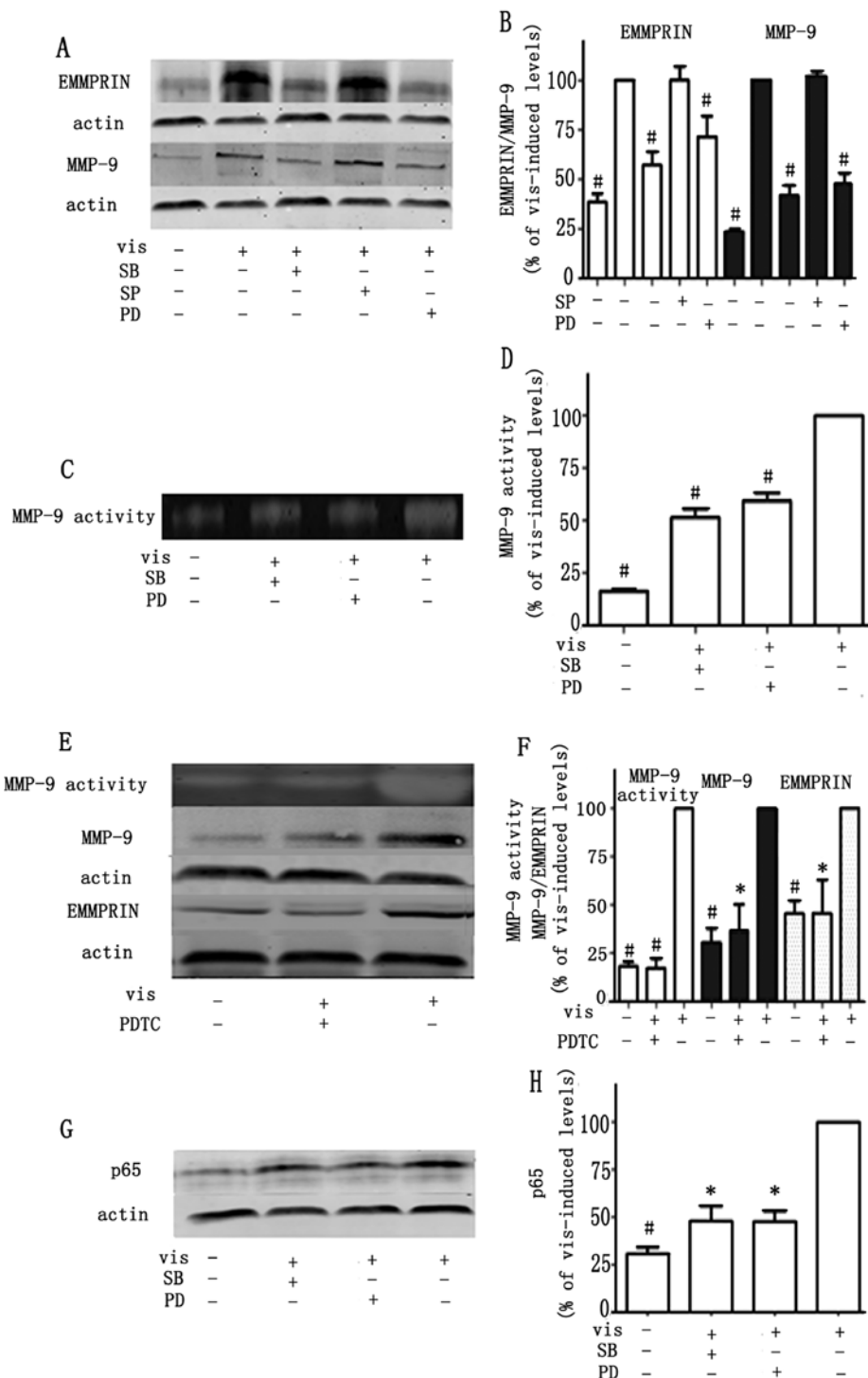


Figure 3. Inhibitors of MAPK (p38, ERK1/2) and NF- κ B block the pro-inflammatory action of visfatin (vis). Inhibitors of MAPK (p38, ERK1/2) also suppress NF- κ B activation by visfatin stimulation. Cells were pre-treated with the p38 MAPK inhibitor, SB, the ERK1/2 MAPK inhibitor PD, the JNK MAPK inhibitor, SP, or the NF- κ B inhibitor, PDTC for 1 h, prior to incubation with visfatin for 24 h or 15 min. (A) Effect of SB, PD, or SP on the expression of EMMPRIN and MMP-9 by visfatin stimulation. (C) Effect of SB and PD on MMP-9 activity by visfatin stimulation. (E) Effect of PDTC on visfatin pro-inflammatory action. (G) Effect of SB and PD on nuclear NF- κ B p65 levels. Respective densitometric analysis measurements (B, D, F and H). Values are the mean \pm SD (n=3). The band density of macrophages treated with visfatin (200 ng/ml) alone was set to 1. *P<0.05 and #P<0.01, compared to macrophages treated with visfatin alone.

receptors, RXR and PPAR γ , were analyzed. For this purpose, the expression of PPAR γ in macrophage stimulated with visfatin for 24 h was assayed by Western blotting. Visfatin did not modulate PPAR γ expression (Fig. 5A and B). Cells were then pre-treated with the PPAR γ natural ligand PGJ2 (10 μ M), the synthetic ligand piog (20 μ M), or the RXR ligand 9-cis

(10 $^{-7}$ M) for 1 h, prior to visfatin treatment (200 ng/ml) for 24 h. It is notable that 9-cis attenuated visfatin inductions of EMMPRIN and MMP-9, but PGJ2 or piog did not (Fig. 5C-F). Furthermore, 9-cis attenuated the visfatin-induced activation of NF- κ B, but not of MAPK (p38, ERK1/2) (Fig. 5G-J). Based on these results, it can be inferred that RXR is involved in the

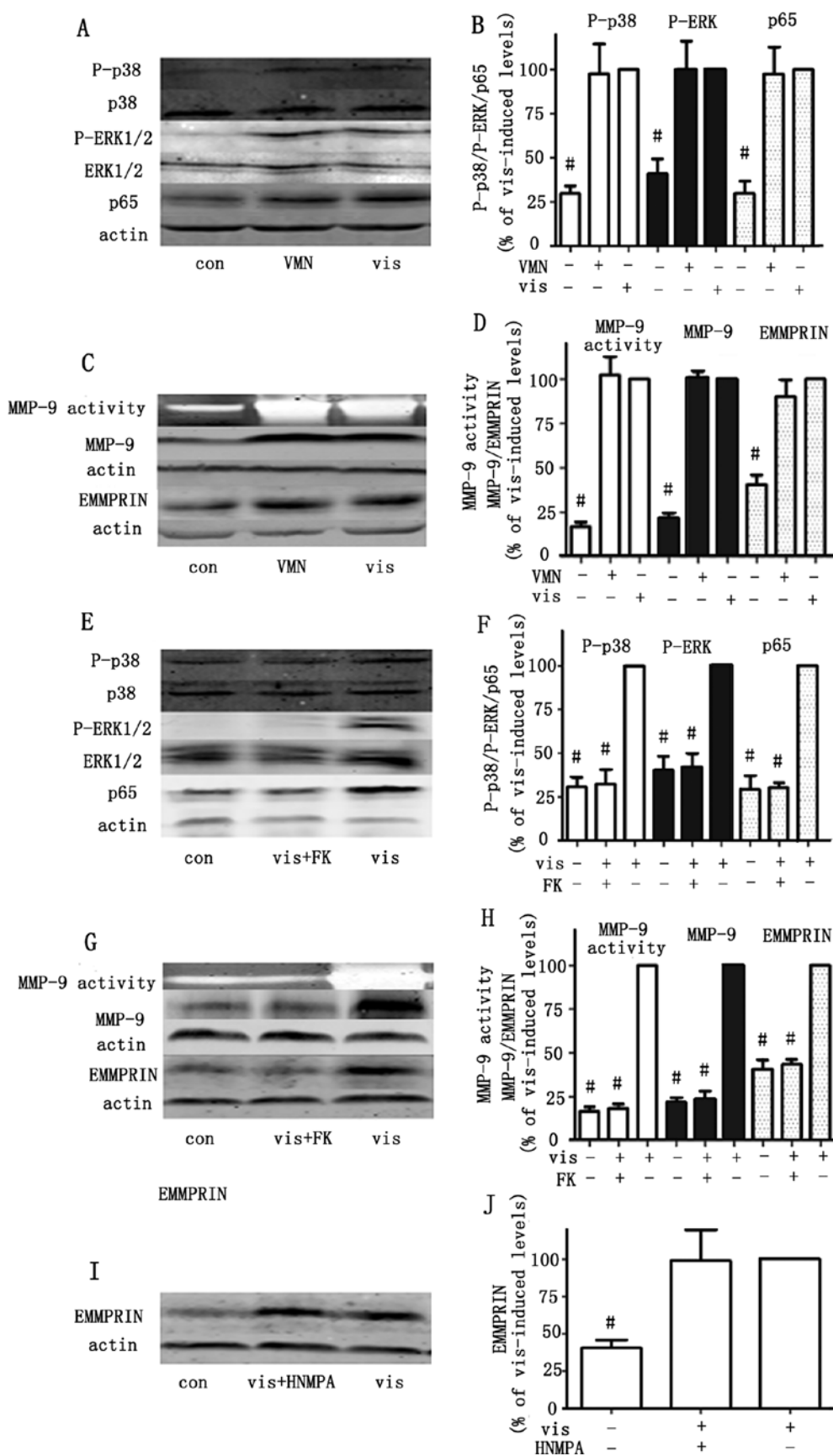


Figure 4. NAMPT activity, but not insulin receptor, is required for the visfatin pro-inflammatory action. (A) Effect of NMN on the activation of MAPK (p38, ERK1/2) and NF- κ B. Cells were treated with NMN for 15 min. (C) Effect of NMN on EMMPRIN and MMP-9. Cells were treated with NMN for 24 h. (E) Effect of the NAMPT inhibitor, FK, on the activation of MAPK (p38, ERK1/2) and NF- κ B by visfatin stimulation. Cells were pre-treated with FK for 1 h, prior to incubation with visfatin for 15 min. (G) Effect of the NAMPT inhibitor, FK, on the induction of EMMPRIN and MMP-9 by visfatin stimulation. Cells were pre-treated with FK for 1 h, prior to incubation with visfatin for 24 h. (I) Effect of HNMPA on NF- κ B activation by visfatin stimulation. (B, D, F, H and J) Respective densitometric analysis results. Values are the mean \pm SD (n=3). Band density of macrophages treated with visfatin (200 ng/ml) alone was set to 1. *P<0.05, and #P<0.01, compared with macrophages treated with visfatin alone.

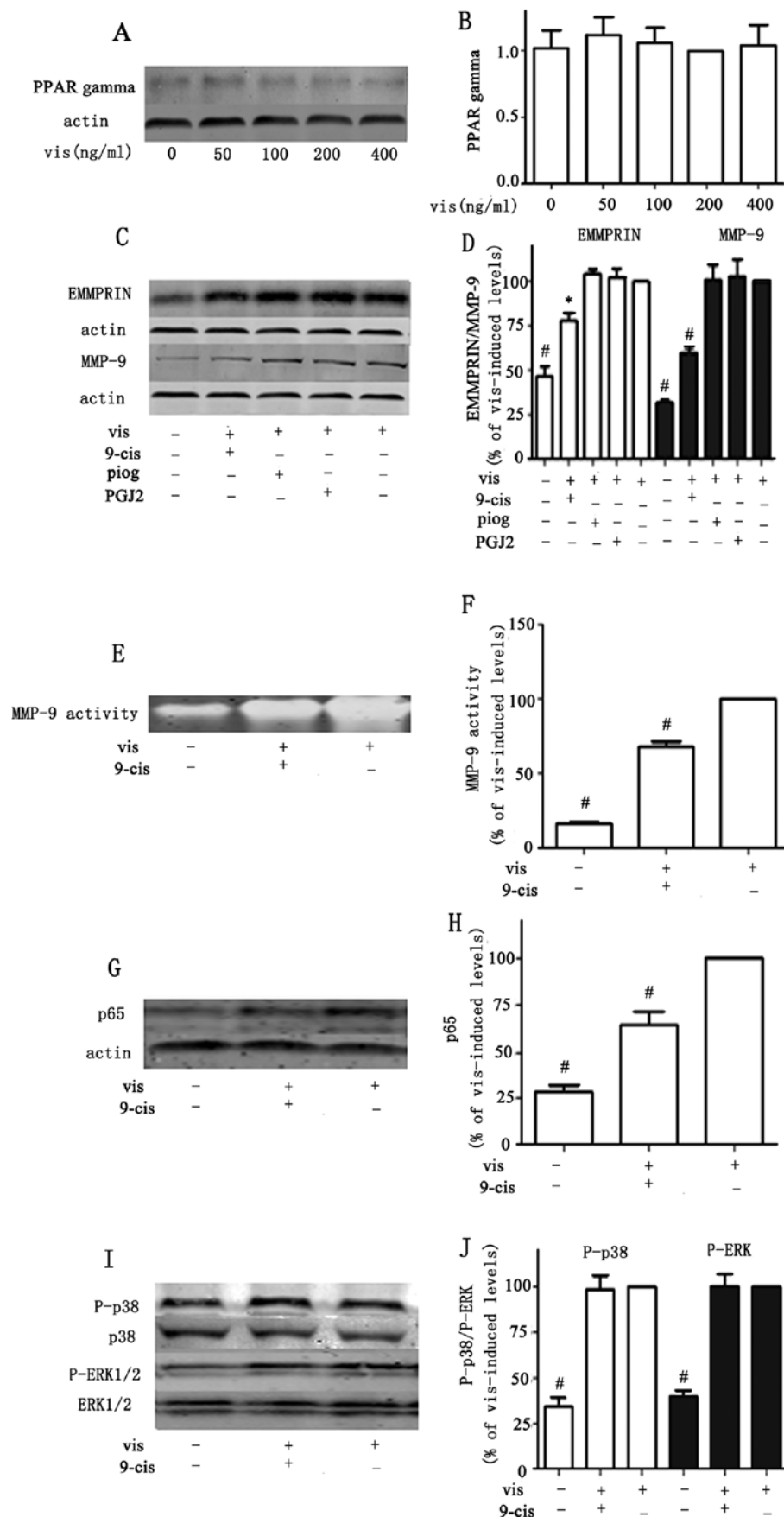


Figure 5. A ligand of RXR, but not of PPAR γ , attenuates the pro-inflammatory action of visfatin (vis) by modulating NF- κ B activation. (A) Effect of visfatin on PPAR γ expression. (C) Effect of 9-cis, piog, or PGJ2 on EMMPRIN and MMP-9 production by visfatin stimulation. (E) Effect of 9-cis on MMP-9 activity. (G) Effect of 9-cis on nuclear NF- κ B p65 levels. Macrophages were pre-treated with 9-cis for 1 h, prior to incubation with visfatin for 15 min. (I) Effect of 9-cis on MAPK (p38, ERK1/2) activation. Macrophages were pre-treated with 9-cis for 1 h, prior to incubation with visfatin for 15 min. (B, D, F, H and J) Respective densitometric analysis results. Values are mean \pm SD (n=3). Band density of macrophages treated with visfatin (200 ng/ml) alone was set to 1. *P<0.05 and #P<0.01, compared with macrophages treated with visfatin (200 ng/ml) alone.

pro-inflammatory action of visfatin by modulating NF- κ B activation.

Discussion

The ability of visfatin to exert direct pro-inflammatory effects on macrophages was investigated. This study reports for the first time that visfatin dose-dependently increased EMMPRIN and MMP-9 production as well as MMP-9 enzymatic activity in THP-1-derived macrophages. This pro-inflammatory action was through the NAMPT-MAPK (p38, ERK1/2)-NF- κ B pathway. The nuclear receptor RXR is believed to be involved in visfatin's pro-inflammatory action by modulating NF- κ B activation.

The inflammatory status plays key roles in undermining atherosclerotic plaque stability (7,17-19), which leads to life-threatening ACS. Metabolic disorders are closely associated with atherosclerosis inflammation (20). The pro-inflammatory effect of visfatin, a novel identified adipocytokine, has been suggested. Enhanced visfatin content has been reported in human unstable carotid and coronary atherosclerotic plaques (16) as well as in the adipose tissue of patients with coronary artery disease compared with the individuals in the control group (21). In monocytes, visfatin has been reported to enhance MMP-9, tumor necrosis factor- α , and interleukin-8 expression (16). In endothelial cells, visfatin has been shown to up-regulate the monocyte chemoattractant protein-1 and MMP-9 production (22-24). In smooth muscle cells, visfatin has been found to induce production of the pro-inflammatory factor, iNOS (10). This study demonstrates herein that visfatin induces EMMPRIN and MMP-9 in macrophages. Both macrophage mRNA expression and protein production of EMMPRIN and MMP were enhanced by visfatin, demonstrating visfatin-induced up-regulation at both the transcriptional and translational level. Furthermore, visfatin also increased MMP-9 enzymatic activity. It is worth noting that the pro-inflammatory action of visfatin in macrophages was observed beginning at a concentration of 50 ng/ml, a level that was reported in obese or type 2 diabetic patients, whereas the corresponding healthy subjects showed lower levels (2,3). Thus, our findings highlight visfatin's pro-inflammatory action as a link between atherosclerotic disease and metabolic disorders.

The MAPK-NF- κ B pathway has been proven to be associated with visfatin's action. It has been demonstrated that visfatin up-regulates iNOS via the ERK1/2-NF- κ B pathway in smooth muscle cells (10). Liu *et al* have reported that p38 and ERK1/2 phosphorylation is required in the monocyte chemoattractant protein-1 and interleukin-6 inductions in endothelial cells (25). One of the aims of this study was to elucidate the role of the MAPK-NF- κ B pathway in visfatin inductions of EMMPRIN and MMP-9 in macrophages. Visfatin was found to phosphorylate both ERK1/2 and p38 MAPK. Furthermore, p38 and ERK1/2 inhibitor, respectively, blocked visfatin inductions of EMMPRIN and MMP-9. These results demonstrate the role of MAPK (p38, ERK1/2) in the action of visfatin. Next, the role of NF- κ B was investigated. In this study, the nuclear p65 level was chosen to reflect the degree of NF- κ B activation. In the inactivated status, dimeric complexes of NF- κ B p50/p65 are held in the cytoplasm. When NF- κ B

is activated, these complexes translocate into the nucleus and regulate gene transcription (26). Therefore, nuclear NF- κ B p65 levels have been widely accepted to represent the degree of NF- κ B activation. This study found that nuclear p65 levels were up-regulated by visfatin over time. Moreover, inhibitors of p38 and ERK1/2 suppressed the visfatin-induced activation of NF- κ B. The NF- κ B inhibitor significantly blocked visfatin induction of inflammatory factors. Thus, consistent with aforementioned studies, it can be concluded that the MAPK (p38, ERK1/2)-NF- κ B pathway is required for the pro-inflammatory action of visfatin.

One of the major findings in this study is that NAMPT action is required for visfatin's pro-inflammatory action. NAMPT converts nicotinamide to NMN, which is then transformed into NAD by nicotinamide/nicotinic acid mononucleotide adenylyltransferase (12). NAMPT enzymatic activity plays an important role in visfatin's action. Revollo *et al* (11) have demonstrated that visfatin regulates insulin secretion in pancreatic β -cells through its intrinsic NAMPT enzymatic activity. Wang *et al* (27) have reported that visfatin stimulates vascular smooth muscle cell proliferation via the NMN-mediated ERK1/2 and p38 signaling. In a recent study, Romacho *et al* (10) have shown that NAMPT activity is involved in visfatin's pro-inflammatory action in smooth muscle cells. Our findings are in line with these reports. In this study, exogenous NMN mimicked the effects of visfatin on EMMPRIN, MMP-9, and MAPK (p38, ERK1/2)-NF- κ B activation. This stimulatory action of visfatin was abolished upon co-incubation with the NAMPT activity inhibitor, FK866. Taken together, these results strongly suggest that extracellular NMN generation through NAMPT activity is on the basis of the pro-inflammatory activity of visfatin in macrophages.

Visfatin was initially claimed to be an insulin mimetic acting through the insulin receptor by modulating PPAR γ levels (1,16,28,29). Such a statement was later retracted and remains highly controversial (30-32). The results of the present study do not support this statement. Indeed, blocking the activation of the insulin receptor did not interfere with NF- κ B activation by visfatin in macrophages. Furthermore, the PPAR γ agonist PGJ2 failed to block visfatin's pro-inflammatory activity, and PPAR γ expression was not modulated by visfatin. This discrepancy could be due to different cell types, cell differentiation stages, visfatin concentrations, and PPAR γ agonists.

We suggest that RXR is involved in visfatin activity by regulating NF- κ B. RXR is a novel identified nuclear receptor associated with atherosclerosis (33). We found that the RXR agonist significantly suppressed not only visfatin's action on EMMPRIN and MMP-9, but NF- κ B activation. This is in line with other reports. Razny *et al* have demonstrated that NF- κ B levels are enhanced in hepatocytes of RXR α -deficient mice compared to wild-type mice (33). Na *et al* have reported that I κ B β interacts with the RXR and inhibits retinoid-dependent transactivation in lipopolysaccharide-treated cells (34). Despite modulating the NF- κ B activation by visfatin, the RXR ligand did not attenuate p38 and ERK1/2 phosphorylation. This implies that there is a much more complicated mechanism for the RXR-mediated effects on the pro-inflammatory action of visfatin.

In conclusion, visfatin is an adipocytokine that promotes macrophage inflammation through the NAMPT-MAPK

(p38, ERK1/2)-NF- κ B pathway. RXR is believed to be involved in this process by modulating NF- κ B activation. Our study highlights the pro-inflammatory action of visfatin in macrophages as a link between atherosclerotic disease and metabolic disorders.

Acknowledgements

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