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Perivascular adipose tissue-derived visfatin is a vascular smooth muscle cell growth factor: role of nicotinamide mononucleotide

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Aims Perivascular adipose tissue (PVAT) inhibits vascular smooth muscle cell (VSMC) contraction and stimulates VSMC proliferation by releasing protein factors. The present study was to determine whether visfatin is involved in these paracrine actions of PVAT, and if so, to explore the underlying mechanisms.
Methods and results Visfatin was preferentially expressed in Sprague–Dawley rat and monkey aortic PVAT, compared with subcutaneous and visceral adipose tissues. The PVAT-derived visfatin was found to be a VSMC growth factor rather than a VSMC relaxing factor, which was proved by visfatin-specific antibody/inhibitor and direct observation of recombinant visfatin. Exogenous visfatin stimulated VSMC proliferation in a dose- and time-dependent manner via extracellular signal-regulated kinase (ERK 1/2) and p38 signalling pathways. This proliferative effect was further confirmed by enhancement of DNA synthesis and upregulation of proliferative marker Ki-67. Visfatin had no anti-apoptotic effect on normal cultured VSMCs, and it exerted an anti-apoptotic effect only during cell apoptosis induced by H_2O_2, excluding a role of anti-apoptosis in the visfatin-induced VSMC proliferation. Insulin receptor knockdown did not show any action on the visfatin effect. However, visfatin acted as a nicotinamide phosphoribosyltransferase to biosynthesize nicotinamide mononucleotide (NMN), which mediated proliferative signalling pathways and cell proliferation similar to the visfatin effect.
Conclusion Visfatin stimulates VSMC proliferation via NMN-mediated ERK1/2 and p38 signalling. The present study provides a molecular link of visfatin to the paracrine action of PVAT, demonstrates a novel function of visfatin in promoting VSMC proliferation, and reveals NMN as a novel signalling molecule that triggers the proliferative process.

1. Introduction

Adipose tissue has now been recognized as the largest endocrine organ. It weighs $\sim$15 kg in an average adult human[1] and releases a large number of adipokines, such as leptin, adiponectin, resistin, interleukin-6, and tumor necrosis factor-α.[1,2] This concept has emerged from the increasing evidence obtained in subcutaneous and visceral adipose tissue. However, few studies have focused on the perivascular adipose tissue (PVAT), an adipose depot found in close proximity to the vascular wall and present in virtually all blood vessels. The function of PVAT is usually ignored in vascular biology, since PVAT is routinely removed in isolated blood vessel experiments. Recently, several reports suggested that PVAT may inhibit vascular smooth muscle cell (VSMC) contraction by releasing a relaxing factor(s),[3] and may stimulate VSMC proliferation by releasing a growth factor(s).[4] These factors are thought to be proteins and remain to be identified.[3,4]

Visfatin is a protein of interest. It was originally cloned from a cDNA library of activated human peripheral blood lymphocytes, and is known as pre-B cell colony-enhancing factor according to its function.[5] Later, it was identified as nicotinamide phosphoribosyltransferase (Nampt), biosynthesizing nicotinamide mononucleotide (NMN) from nicotinamide.[6] Interestingly and strikingly, it was found to be a new adipokine, as its name visfatin implies, mainly expressed in and secreted from visceral fat as opposed to subcutaneous fat. In this regard, visfatin exerted insulin-mimetic effects in cultured adipocytes, myocytes, and

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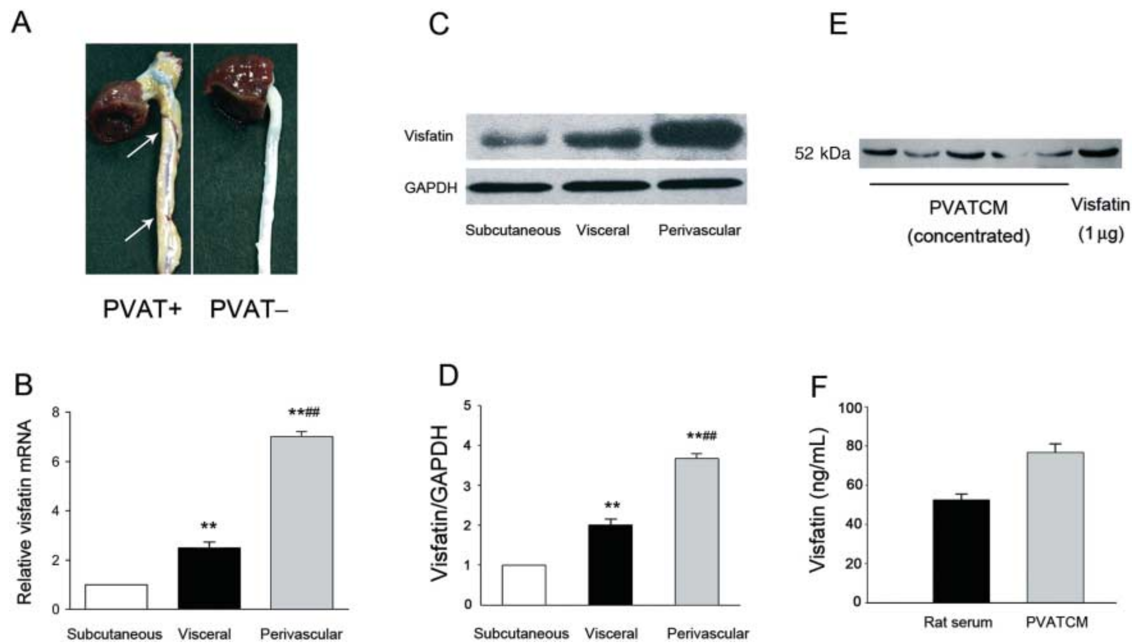

Figure 1 Expression and secretion of visfatin in perivascular, subcutaneous, and visceral adipose tissue. (*A*) Photographs of rat thoracic aortas with and without perivascular adipose tissue (PVAT). (*B*) Real-time RT–PCR showing the highest expression of visfatin mRNA in perivascular adipose tissue. $^{**}P < 0.01$ vs. Subcutaneous, $^{\#\#}P < 0.01$ vs. Visceral. $n = 8$. (*C* and *D*) Western blotting showing the highest expression of visfatin in perivascular adipose tissue. $^{**}P < 0.01$ vs. Subcutaneous, $^{\#\#}P < 0.01$ vs. Visceral. $n = 3$. (*E*) Western blotting showing the presence of visfatin in concentrated perivascular adipose tissue-conditioned medium (PVATCM). Standard recombinant visfatin (1 μg) is as a reference. (*F*) Visfatin levels in rat serum and perivascular adipose tissue-conditioned medium determined by enzyme immunoassay.

hepatocytes and lowered plasma glucose levels in mice by binding to and activating the insulin receptor (IR). However, the relationship between visfatin and IR was unable to be reproduced in a recent study,[7] which reported a new function of visfatin in regulating insulin secretion in β-cells as systemic Nampt. It was also proposed that visfatin may act on its own unidentified receptor to exert certain biological functions.[8]

Intracellular visfatin has been reported to be implicated in VSMC differentiation and survival.[9,10] Both studies focused on the function of intracellular visfatin and demonstrated it as an intracellular Nampt. However, the role of extracellular visfatin in VSMC proliferation, as opposed to VSMC differentiation, has not been investigated. Furthermore, no attention has been given to the potential role of visfatin in the paracrine effects of PVAT on VSMCs.

In the present study, we sought to determine whether visfatin acts as a relaxing factor and/or a growth factor secreted from PVAT and is involved in the regulation of VSMC function, and if so, to explore the underlying mechanisms. Here, we provide a molecular link of visfatin to the paracrine action of PVAT and demonstrate a novel function of visfatin in stimulating VSMC proliferation via extracellular signal-regulated kinase (ERK) 1/2 and p38 signalling pathways, in which NMN acts as a key mediator.

2. Materials and methods

A supplementary methods section is available at *Cardiovascular Research* online.

2.1 Reagents

NMN, bromodeoxyuridine (BrdU), and 5-hydroxytryptamine (5-HT) were obtained from Sigma. Visfatin inhibitor FK866 was donated by Apoxis SA, Switzerland. LipofectAMINE 2000, Dulbecco modified Eagle medium (DMEM), Ham's F-12 medium, and fetal bovine serum (FBS) were purchased from Gibco. Anti-BrdU monoclonal antibody was obtained from Chemicon. Ki-67 rabbit-monoclonal antibody and IR β-subunit monoclonal antibody were obtained from LabVision. U0126, SB203580, LY294002, SP600125, and antibodies of Phospho-ERK1/2 (thr202/thr204), total ERK1/2, phosphor-p38 (thy180/thy182), total p38, phospho-JNK (Thr183/Tyr185), total JNK, phospho-Akt (ser 473), and total Akt were purchased from Cell Signaling Technology. Smooth myosin heavy chain and caldesmon antibodies were purchased from Santa-Cruz. Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories, Japan. Cy3-conjugated goat anti-rabbit, Cy3-conjugated rabbit anti-mouse IgG, and FITC-conjugated goat anti-mouse IgG were purchased from Jackson ImmunoResearch. Terminal-deoxynucleotidyl-transferase-mediated nick end labelling (TUNEL) staining kit was purchased from Roche. Visfatin polyclonal antibody, visfatin C-terminal enzyme immunoassay kit, recombinant visfatin, and recombinant leptin were obtained from Phoenix, Belmont, CA, USA.

2.2 Tissue and blood sampling

Male Sprague–Dawley rats, monkeys, and ICR mice were used in accordance with our institutional guidelines for animal care and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996). Subcutaneous, visceral, and perivascular adipose tissues, and blood were taken from the animals as described previously.[11,12] *Figure 1A* showed the rat descending thoracic aorta before and after dissection of PVAT.

2.3 Preparation of perivascular adipose tissue-conditioned medium

PVAT-conditioned medium (PVATCM) was prepared and concentrated as described previously.[4]

2.4 Visfatin detection by RNA quantification, western blotting, and enzyme immunoassay

Visfatin mRNA was quantified by real-time RT–PCR. Visfatin protein was determined by western blotting and enzyme immunoassay.

2.5 Vasomotor study and cell proliferation study

Vascular tension was measured in rat aortic rings in which PVAT or endothelium was either removed or left intact.[12,13] The effects of visfatin antibody, visfatin inhibitor FK866, and recombinant human visfatin were observed.

Rat VSMCs were isolated from rat descending thoracic aortas and cultured as reported previously.[14] Cells were cultured in DMEM with 10% FBS. Experiments were performed using cells between passages 3 and 8. Cells were grown to 60% confluence, and switched to serum-starved medium (0.2% FBS) 24 h before further treatments. The effects of PVATCM, recombinant human visfatin, visfatin antibody, signal protein inhibitors, and NMN were observed. Cell viability was evaluated by a non-radioactive CCK-8 assay[15] and direct cell counting with haemocytometer. Absorbance of the dye with a wavelength of 450 nm was obtained in CCK-8 assay, which was proportional to the number of live cells. The phosphorylation of signal proteins was detected by western blotting.

The recombinant human visfatin was produced in *E. coli*. Amino acid sequence similarity between human and rat visfatin exceeded 95%. Endotoxin (lipopolysaccharide) levels in all visfatin-treated experiments were <50 pg/mL (limulus amebocyte lysate test), similar to the previous studies.[8,16]

2.6 Immunofluorescence

Immunofluorescent assay was used to examine the nuclear BrdU incorporation and Ki-67 expression for further evaluating cell proliferation, and to examine TUNEL staining for evaluating cell apoptosis.

2.7 RNA interference to insulin receptor

Cultured VSMCs were transfected with small interfering RNA (siRNA) using LipofectAMINE 2000. Knockdown of IR was accomplished by transfecting a pool of three different siRNAs targeted to rat IR (IR-siRNAs 100 nM).

2.8 Effect of visfatin on mouse serum glucose level

Male ICR mice were divided into three groups and fasted overnight. Blood samples were taken from the retrobulbar venous plexus before and after drug treatment under transient anaesthesia with ether. Vehicle, insulin (100 pmol), or visfatin (100 pmol) were injected intravenously through tail vein. Serum was separated for determination of glucose by the hexokinase method with a commercial kit.

2.9 Nicotinamide mononucleotide detection by high-performance liquid chromatography and ion-trap tandem mass spectrometry

NMN was detected by high-performance liquid chromatography (HPLC) and the NMN peak fraction was confirmed by ion-trap tandem mass spectrometry.[7]

2.10 Statistical analysis

Data are expressed as mean $\pm$ SEM. Comparisons were made by Student's t-test or the analysis of variance followed by Turkey HSD test. Statistical significance was set at $P < 0.05$.

3. Results

3.1 Expression and secretion of visfatin in perivascular adipose tissue

Real-time RT–PCR demonstrated that visfatin mRNA was more abundantly expressed in rat descending thoracic aortic PVAT, 7.0-fold and 2.8-fold higher than that in inguinal subcutaneous adipose tissue and mesenteric visceral adipose tissue, respectively (*Figure 1B*). The specific expression of visfatin was further confirmed by western blotting; visfatin protein expression was the highest in PVAT, 3.7-fold and 1.8-fold higher than that in subcutaneous and visceral adipose tissue, respectively (*Figure 1C* and *D*). Similar results were obtained in monkey adipose tissues (see Supplementary material online, *Figure S1*).

Western blotting showed the existence of visfatin in concentrated PVATCM (*Figure 1E*). Enzyme immunoassay demonstrated that the visfatin level averaged 76.6 ng/mL (1.42 nM) in PVATCM and 52.4 ng/mL (0.97 nM) in rat serum (*Figure 1F*). Visfatin was not detected in control medium without PVAT.

3.2 Visfatin is not involved in the regulation of vascular tone by perivascular adipose tissue but contributes to perivascular adipose tissue-induced vascular smooth muscle cell proliferation

In vasomotor study, concentration–response curve of 5-hydroxytryptamine (5-HT)-induced contraction was right shifted in aortas with PVAT, compared with that in aortas without PVAT, indicating PVAT has an anticontractile effect on vascular tone (*Figure 2A* and *B*). The anticontractile effect of PVAT was not affected by visfatin-specific antibody or visfatin-specific inhibitor FK866 (1, 100, and 10 000 nM). Furthermore, visfatin in cumulative concentrations (0.0001–10 nM) had neither relaxation nor contraction in aortas with and without endothelium, in which PVAT was removed ($n = 6$). A higher concentration of visfatin (50 nM) did not produce aortic relaxation and contraction ($n = 2$). These results exclude the possibility of the PVAT-secreted visfatin as a VSMC relaxing factor.

However, visfatin was found to contribute to PVAT-induced VSMC proliferation. Both CCK-8 cell viability assay and direct cell counting showed that PVATCM stimulated VSMC proliferation and this proliferative effect was significantly inhibited by visfatin specific antibody (*Figure 2C* and *D*).

3.3 Visfatin stimulates vascular smooth muscle cell proliferation

In CCK-8 cell viability assay, the cultured VSMCs were treated with increasing concentrations (0.01–10 nM) of recombinant visfatin for 24 h. It was found that visfatin stimulated VSMC proliferation in a dose-dependent manner (*Figure 2E*). The visfatin (1 nM)-induced VSMC proliferation was 1.3–1.8-fold higher than the corresponding controls in several independent experiments (*Figures 2E*, *3C*, *4A*, and *5E*). This proliferative effect was totally blocked by visfatin antibody (*Figure 2E*). Similar results for visfatin-induced VSMC proliferation and its blockade by visfatin antibody were observed using another method of direct cell counting (*Figure 2F*). We tested the specificity of visfatin antibody and found that visfatin antibody itself did not affect VSMC

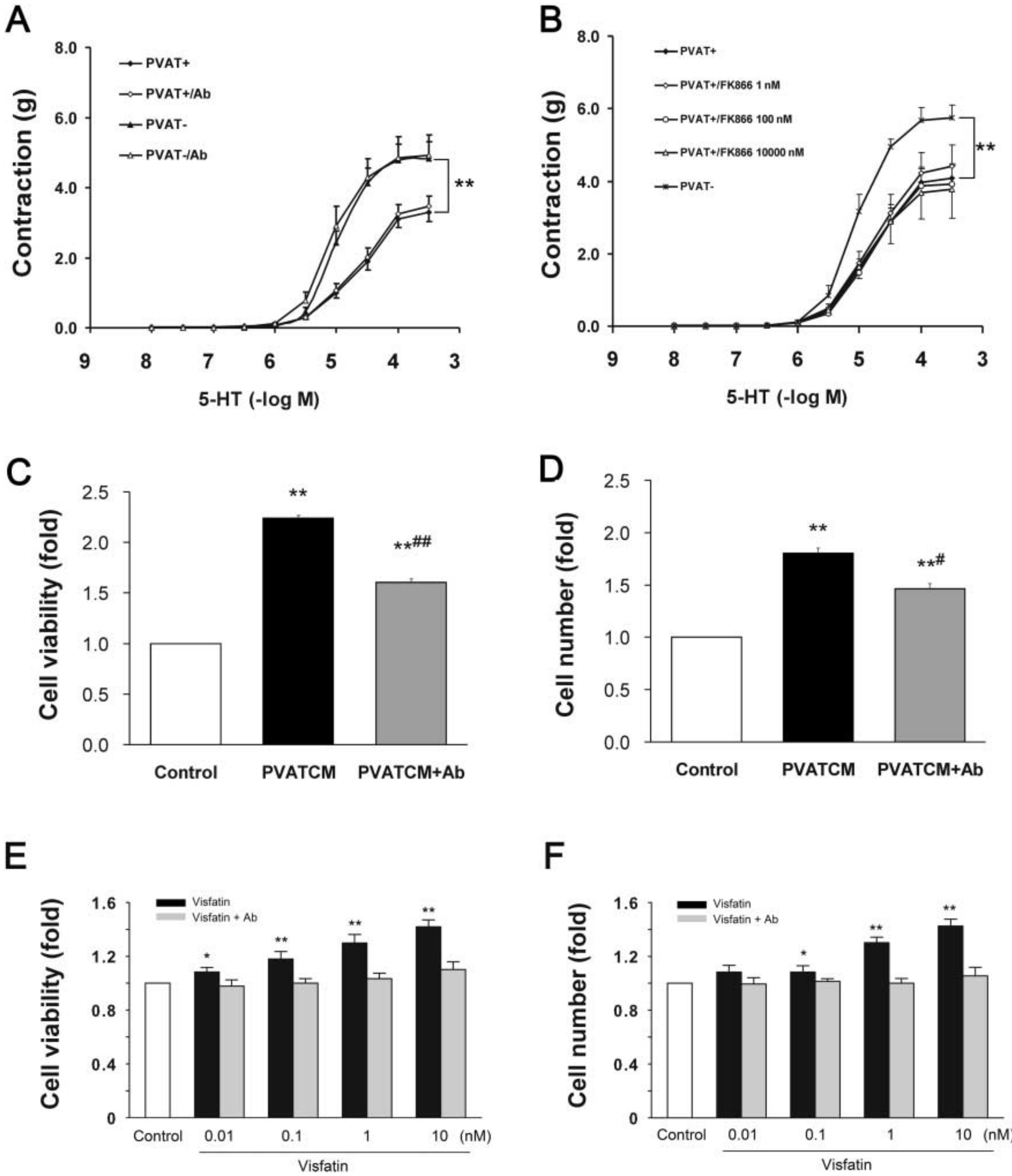

Figure 2 Role of visfatin in the paracrine action of perivascular adipose tissue. (*A* and *B*) 5-Hydroxytryptamine (5-HT)-induced aortic contractions are smaller in aortas with perivascular adipose tissue than without perivascular adipose tissue, indicating perivascular adipose tissue has an anticontractile effect. This anticontractile effect of perivascular adipose tissue is not affected by visfatin antibody (Ab) and inhibitor FK866 (1, 100, and 10 000 nM). $^{**}P < 0.01$. $n = 6$–7. (*C* and *D*) CCK-8 cell viability assay and direct cell counting showing perivascular adipose tissue-conditioned medium-induced vascular smooth muscle cell proliferation. This proliferative effect is inhibited by visfatin antibody. $^{**}P < 0.01$ vs. Control, $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ vs. PVATCM. $n = 6$. (*E* and *F*) Visfatin stimulates vascular smooth muscle cell proliferation in a dose-dependent manner. This proliferative effect is blocked by visfatin antibody. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control. $n = 6$.

proliferation (see Supplementary material online, *Figure S2A* and *B*), and visfatin antibody did not inhibit VSMC proliferation induced by another adipokine, leptin (see Supplementary material online, *Figure S2C* and *D*). These indicate that visfatin antibody blocks extracellular visfatin specifically. We also used FK866, a specific enzymatic inhibitor of visfatin, to study the effect of visfatin on VSMC proliferation. It was found that FK866 itself inhibited VSMC proliferation and exogenous visfatin did not show any proliferative effect on VSMCs under co-incubation with FK866 (see Supplementary material online, *Figure S3A* and *B*). Next, we chose a single concentration of visfatin 1 nM, which was close to the visfatin levels in both PVATCM and serum, for time course study. Visfatin stimulated VSMC proliferation in a time-dependent manner at 6, 24, and 48 h after treatment (*Figure 3C*).

Furthermore, the nuclear incorporation of BrdU was examined at three time points (6, 24 and, 48 h after visfatin treatment) to directly evaluate the DNA synthesis in VSMCs. In visfatin-treated cells, the fractions of BrdU-positive cells were significantly higher than those in control cells (*Figure 3A* and *B*).

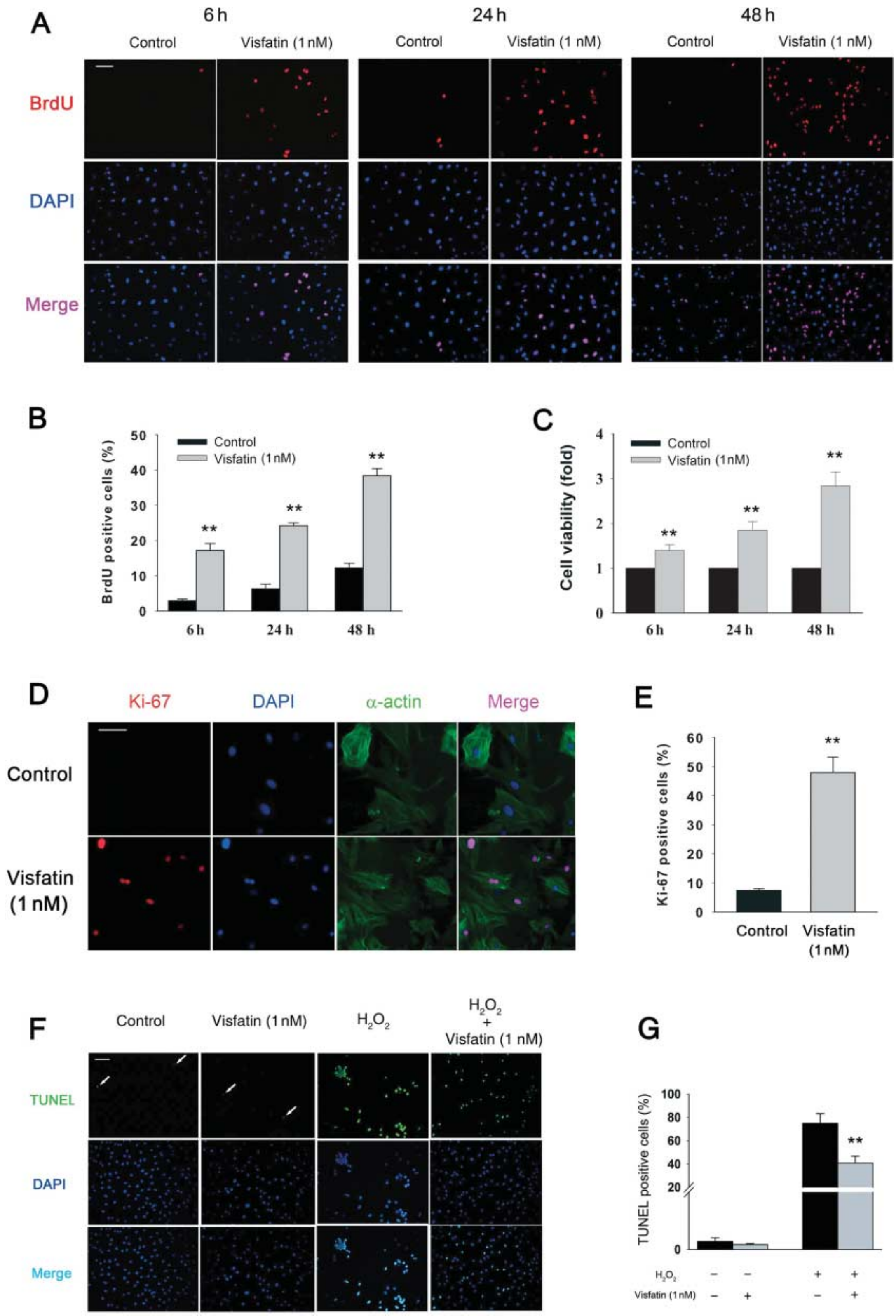

Figure 3 Effect of visfatin on BrdU incorporation, Ki-67 expression, and cell apoptosis in cultured vascular smooth muscle cells. (*A* and *B*) BrdU incorporation analysis showing visfatin (1 nM) enhances DNA synthesis of vascular smooth muscle cells in a time-dependent manner. Vascular smooth muscle cells were immunochemically stained with anti-BrdU monoclonal antibody and then Cy3-conjugated goat anti-mouse IgG (red). Nuclei were counterstained with DAPI (blue). $^{**}P < 0.01$ vs. Control. (*C*) CCK-8 cell viability assay showing visfatin (1 nM) stimulates vascular smooth muscle cell proliferation in a time-dependent manner. $^{**}P < 0.01$ vs. Control. (*D* and *E*) Expression of Ki-67, a proliferative marker, is upregulated by incubation with visfatin (1 nM) for 24 h. Vascular smooth muscle cells were immunochemically stained with anti-Ki-67 rabbit-monoclonal antibody and then Cy3-conjugated goat anti-rabbit IgG (red). Nuclei were counterstained with DAPI (blue). Vascular smooth muscle cells were also stained with anti-α-actin monoclonal antibody and then FITC-conjugated goat anti-mouse IgG (green). $^{**}P < 0.01$ vs. Control. (*F* and *G*) The fractions of TUNEL positive cells (green) are very low in both control and visfatin-treated vascular smooth muscle cells, having no difference between these two groups. The fraction of TUNEL positive cells is markedly increased in vascular smooth muscle cell apoptosis induced by H_2O_2 (150 μM), which can be reduced by co-incubation with visfatin. ** $P < 0.01$ vs. H_2O_2. Scale bars, 20 μm.

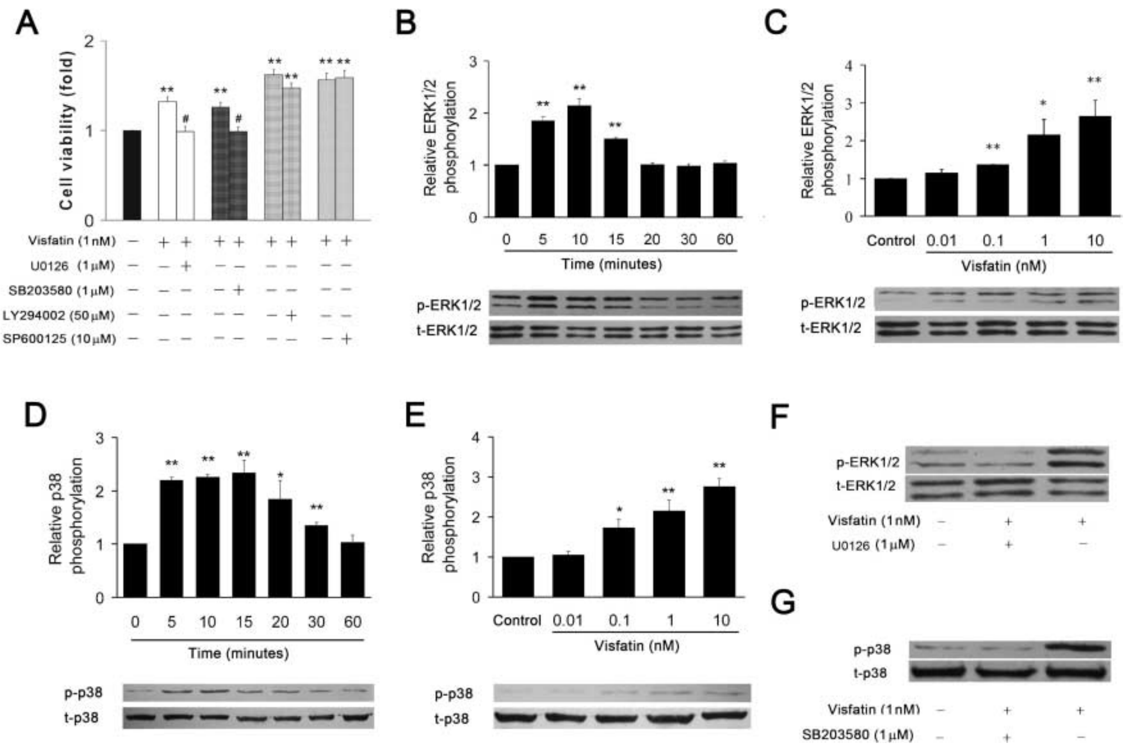

Figure 4 Signalling pathways involved in visfatin-induced vascular smooth muscle cell proliferation. (*A*) Visfatin-induced vascular smooth muscle cell proliferation is inhibited by ERK1/2 inhibitor U0126 and p38 inhibitor SB203580, but not by PI3K inhibitor LY294002 and JNK inhibitor SP600125. $^{**}P < 0.01$ vs. Control (no treatment), $^{\#}P < 0.05$ with U0126 vs. without U0126, or with SB203580 vs. without SB203580. $n = 6$. (*B* and *D*) Time course for visfatin (1 nM)-induced phosphorylation of ERK1/2 and p38. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control. $n = 3$. (*C* and *E*) Dose-dependent response for phosphorylation of ERK1/2 and p38 after incubation with visfatin for 10 min. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. Control. $n = 3$. (*F* and *G*) Visfatin-induced phosphorylation of ERK1/2 and p38 is inhibited by their respective inhibitors, U0126 and SB203580.

Also, the cell proliferation was evaluated by measuring protein expression of Ki-67, a proliferative marker, at 24 h after visfatin incubation. Visfatin markedly increased the Ki-67 expression in nuclei of VSMCs (*Figure 3D* and *E*), indicating that visfatin activates the proliferative state of VSMCs. However, exogenous visfatin (0.1, 1, and 10 nM) did not increase the expressions of two different VSMC differentiation markers, smooth myosin heavy chain and h-caldesmon (see Supplementary material online, *Figure S4A* and *B*).

To exclude a possibility that exogenous visfatin inhibits apoptotic response of VSMCs and contributes to VSMC proliferation, we examined the effect of visfatin on cell apoptosis using TUNEL staining (*Figure 3F* and *G*). The fractions of TUNEL positive cells were very low in both control and visfatin-treated VSMCs under normal cell culture, and no difference was found. However, visfatin exerted anti-apoptotic effect under cell apoptotic state induced by H_2O_2.

3.4 Role of ERK1/2 and p38 in visfatin-induced vascular smooth muscle cell proliferation

To determine whether ERK1/2, p38, c-Jun N-terminal kinase (JNK) and phosphatidylinositol 3 kinase (PI3K)/Akt signalling pathways are involved in visfatin-induced VSMC proliferation, their inhibitors were used for the study. Pretreatment of cells with ERK1/2 inhibitor U0126 and p38 inhibitor SB203580 blocked visfatin-induced VSMC proliferation (*Figure 4A*). In contrast, PI3K inhibitor LY294002 and JNK inhibitor SP600125 did not affect the visfatin-induced VSMC proliferation.

To further study the molecular mechanisms involved in visfatin-induced proliferation, the phosphorylation of ERK1/2, p38, JNK, and Akt was evaluated by western blotting. In time course experiments, visfatin (1 nM) activated the ERK1/2 phosphorylation at 5, 10, and 15 min (*Figure 4B*), and the p38 phosphorylation at 5, 10, 15, 20, and 30 min (*Figure 4D*), but failed to increase the JNK and Akt phosphorylation within 60 min (see Supplementary material online, *Figure S5A* and *C*). The effects at 10 min seemed maximal for visfatin-induced phosphorylation of both ERK1/2 and p38. Thus, incubation for 10 min was used in dose–response experiments. The results showed increasing concentrations of visfatin (0.01–10 nM) activated the ERK1/2 and p38 phosphorylation in a dose-dependent manner (*Figure 4C* and *E*), but did not enhance the JNK and Akt phosphorylation (see Supplementary material online, *Figure S5B* and *D*). Additional experiments demonstrated that visfatin-induced phosphorylation of ERK1/2 and p38 could be abolished by their respective inhibitors, U0126 and SB203580 (*Figure 4F* and *G*). All abovementioned results indicate visfatin-induced VSMC proliferation is through ERK1/2 and p38 signalling pathways, rather than JNK and PI3K/Akt signalling pathways.

3.5 Visfatin-induced vascular smooth muscle cell proliferation is not mediated by insulin receptor

To investigate whether visfatin-induced VSMC proliferation is mediated by IR, RNA interference was used. The transfection

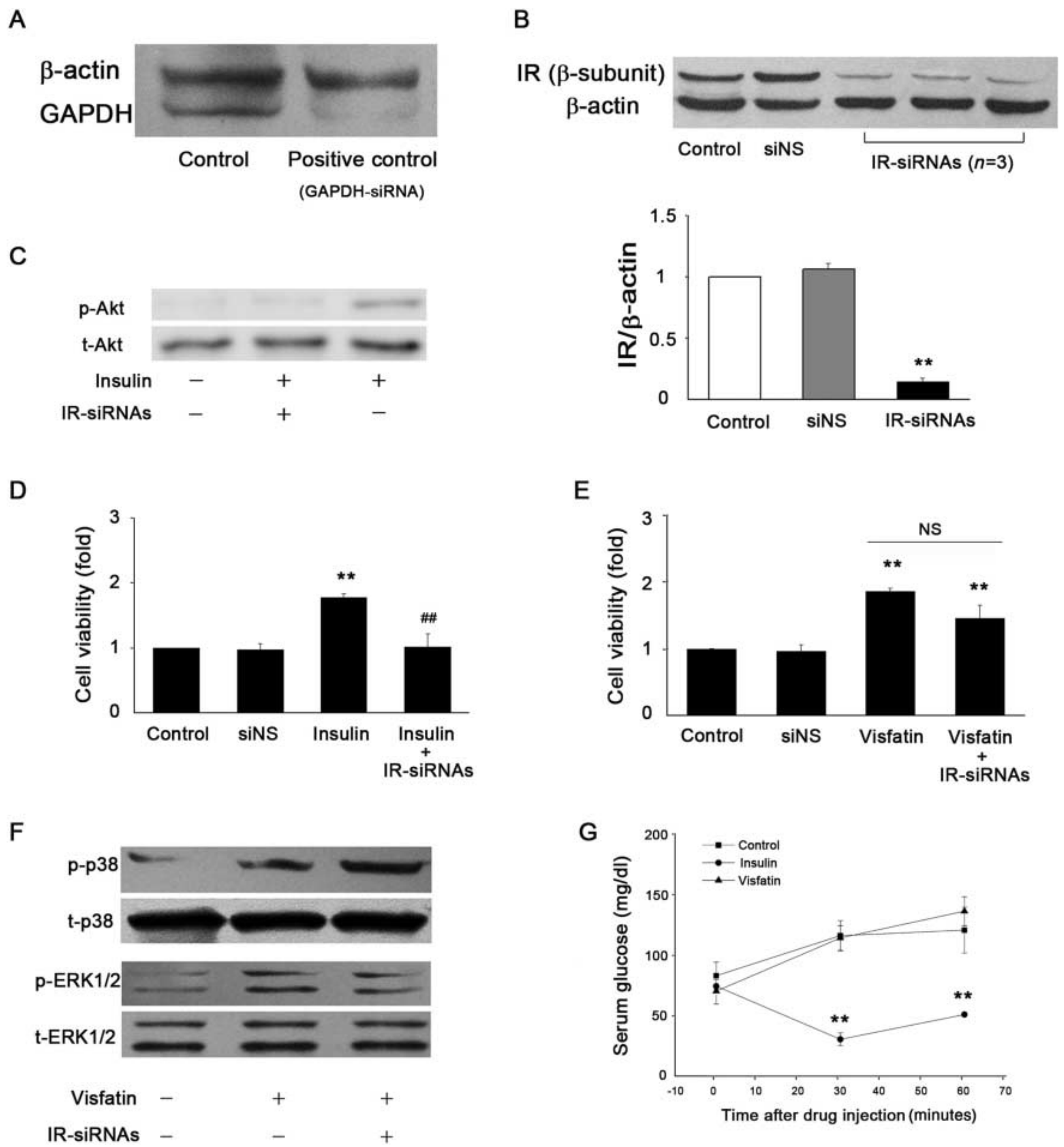

Figure 5 Knockdown of insulin receptor (IR) in vascular smooth muscle cells. (*A*) Western blotting showing successful transfection of siRNA. (*B*) Western blotting showing a pool of three different siRNAs targeted to rat insulin receptor (IR-siRNAs) reduces markedly the insulin receptor expression. $^{**}P < 0.01$ vs. Control and siNS (non-silencing siRNA). $n = 3$. (*C*) Western blotting showing insulin (10 nM)-induced Akt phosphorylation is inhibited by insulin receptor-siRNAs. (*D*) CCK-8 cell viability assay showing insulin (10 nM)-induced vascular smooth muscle cell proliferation is abolished by insulin receptor-siRNAs. $^{**}P < 0.01$ vs. Control and siNS. $^{\#\#}P < 0.01$ vs. Insulin. $n = 6$. (*E*) CCK-8 cell viability assay showing insulin receptor-siRNAs has no significant effect on visfatin (1 nM)-induced vascular smooth muscle cell proliferation. $^{**}P < 0.01$ vs. Control and siNS. NS, no significance. $n = 6$. (*F*) Western blotting showing visfatin (1 nM)-induced phosphorylation of ERK1/2 and p38 is not affected by insulin receptor-siRNAs. (*G*) The effect of intravenous injection of vehicle (PBS as vehicle control), insulin (100 pmol, as positive control), and visfatin (100 pmol) on serum glucose levels in mice. $^{**}P < 0.01$ vs. Control. $n = 5$.

of siRNA in VSMCs was successful, as demonstrated by positive control GAPDH-siRNA (*Figure 5A*). IR-siRNAs reduced IR protein expression to $14 \pm 2\%$ (*Figure 5B*). The knockdown of IR abolished insulin-induced Akt phosphorylation and VSMC proliferation (*Figure 5C* and *D*). However, it did not significantly affect visfatin-induced VSMC proliferation (*Figure 5E*). Furthermore, Akt, the downstream signalling kinase of IR, was not activated by visfatin (see Supplementary material online, *Figure S5C* and *D*), and the visfatin-induced phosphorylation of ERK1/2 and p38 was not inhibited by the IR knockdown (*Figure 5F*). Thus, in contrast with insulin, visfatin-induced VSMC proliferation is not mediated by IR. Further support is from *in vivo* study. Visfatin, unlike insulin, failed to decrease the serum glucose level in mice (*Figure 5G*).

3.6 Visfatin acts as Nampt to synthesize nicotinamide mononucleotide that promotes vascular smooth muscle cell proliferation

We checked the composition of the culture medium, and found the Nampt substrate nicotinamide in it. To test whether visfatin acts as Nampt to exert the proliferative effect in cultured VSMCs, the enzymatic product NMN was measured in cultured medium and VSMCs. NMN was detected by HPLC and confirmed by ion-trap tandem mass spectrometry (see Supplementary material online, *Figure S6*). NMN levels were significantly increased in both cultured medium and cultured VSMCs 24 h after visfatin incubation (*Figure 6A* and *B*).

We further investigated the effect of exogenous NMN on VSMC proliferation. Surprisingly, NMN itself could promote VSMC proliferation (*Figure 6C*), enhance the nuclear incorporation of BrdU (*Figure 6D* and *E*), and activate ERK1/2 and p38 but not JNK and Akt (*Figure 6F* and *G*). In addition, NMN had no anti-apoptotic effect under normal cell culture (data not shown), whereas it exerted anti-apoptotic effect under cell apoptotic state induced by H_2O_2 (see Supplementary material online, *Figure S7*). All these effects of NMN are mimetic to those of visfatin, suggesting that visfatin may act as Nampt to produce NMN which stimulates VSMC proliferation through ERK1/2 and p38 signalling pathways.

4. Discussion

4.1 Visfatin is a secreted protein highly expressed in perivascular adipose tissue

We demonstrated for the first time the adipokine expression and secretion in aortic PVAT. Visfatin expression was compared in three different adipose tissues using real-time RT–PCR and western blotting. To our surprise, the highest expression was found in PVAT, far more than the expression in subcutaneous and visceral adipose tissues. The similar results were confirmed in rodent rat and primate monkey. Meanwhile, both western blotting and enzyme immunoassay showed the presence of visfatin in PVAT-conditioned medium, suggesting visfatin can be released from PVAT. The high expression and secretory property of visfatin in PVAT indicate that visfatin may have a potential role in local regulation of blood vessels.

A recent study reported that visfatin was expressed more abundantly in brown adipose tissue than in white adipose tissue in mice.[7] In the present study, rat aortic PVAT is brown adipose tissue as demonstrated in *Figure 1A* and described in a previous study,[17] while rat subcutaneous and visceral adipose tissues are white adipose tissue. Therefore, it is reasonable that the highest expression of visfatin is in rat PVAT. Besides adipocytes (the most abundant cell type), adipose tissue also contains pre-adipocytes, endothelial cells, fibroblasts, leukocytes and, most importantly, macrophages. Visfatin is expressed not only in adipocytes but also in non-adipocytes, especially in macrophages.[18,19] So the detected visfatin in PVAT-conditioned medium is perhaps released from both adipocytes and non-adipocytes, e.g. macrophages.

4.2 Visfatin is not involved in the regulation of vascular tone by perivascular adipose tissue

PVAT inhibits vascular contraction and produces vascular relaxation in rat and human arteries.[3,17] The effects are caused by a relaxing factor(s) released from PVAT and recognized as a protein(s). To determine whether visfatin plays a role in this paracrine action of PVAT, visfatin antibody and visfatin inhibitor FK866 were used in aortas with and without PVAT. Our data did not show any effect on the anticontractile action of PVAT after blockade of visfatin with antibody or inhibitor FK866 (1, 100, and 10 000 nM). Furthermore, no relaxation was produced by the cumulative concentrations of visfatin (0.0001–10 nM) or a single higher concentration of visfatin (50 nM). In addition, visfatin (0.0001–10 nM) did not produce vasoconstriction. These negative results were not due to insufficient concentrations of the reagents used, since the IC_{50} of FK866 is between 1 and 3 nM,[20] and the plasma concentration of visfatin is between 0.005 and 0.25 nM in human and mouse. In the present study, visfatin levels in rat serum and PVAT-conditioned medium are around 1 nM. Taken together, these results suggest that PVAT-secreted visfatin does not act as a relaxing factor.

4.3 Visfatin plays a role in the perivascular adipose tissue-induced vascular smooth muscle cell proliferation

PVAT stimulates VSMC proliferation. The adipocyte-derived growth factor(s) has been proposed and considered as a protein(s).[4] To determine whether visfatin plays a role in this paracrine action of PVAT, visfatin antibody was used in cultured VSMCs stimulated with PVAT-conditioned medium. The results demonstrated PVAT-conditioned medium induced VSMC proliferation, which is consistent with the previous data.[4] Interestingly, this proliferative action of PVAT-conditioned medium was significantly attenuated by visfatin antibody. Furthermore, direct observation revealed that exogenous visfatin itself stimulated VSMC proliferation, which could be totally blocked by visfatin antibody. And, visfatin did not show any proliferative effect on VSMCs during co-incubation with FK866, a visfatin-specific inhibitor. These proliferative effects were demonstrated by the two different approaches of CCK-8 assay and direct cell counting. The dose–response and time-course of the visfatic effect were studied in detail. The visfatin concentrations used in the present study overlap with the physiological visfatin levels in human, mouse, and rat. This visfatin-induced VSMC proliferation was further confirmed by enhancement of DNA synthesis (nuclear incorporation of BrdU) and by upregulation of a proliferative marker (Ki-67 protein). All these results suggest that PVAT-secreted visfatin may act as a growth factor involved in PVAT-induced VSMC proliferation.

It was reported that intracellular visfatin, acting as Nampt, converted VSMCs from a proliferative state to a non-proliferative, contractile state, and promoted the expression of several differentiation proteins in VSMCs.[9] Differently from the previous report that implicated intracellular visfatin in VSMC differentiation,[9] the current study focused on the function of extracellular visfatin, i.e. the circulating plasma visfatin and local visfatin secreted from PVAT, and implicated extracellular visfatin in VSMC proliferation. In addition, it is impossible for an anti-apoptotic effect to have contributed to the visfatin-induced VSMC proliferation, since visfatin had no anti-apoptotic effect under normal cell culture, and it exerted an anti-apoptotic effect only during apoptosis induced by H_2O_2.

4.4 Visfatin-induced vascular smooth muscle cell proliferation is through ERK1/2 and p38 signalling pathways, rather than JNK and PI3K/Akt signalling pathways

VSMC proliferation is regulated by several signalling pathways, especially PI3K/Akt and mitogen-activated protein

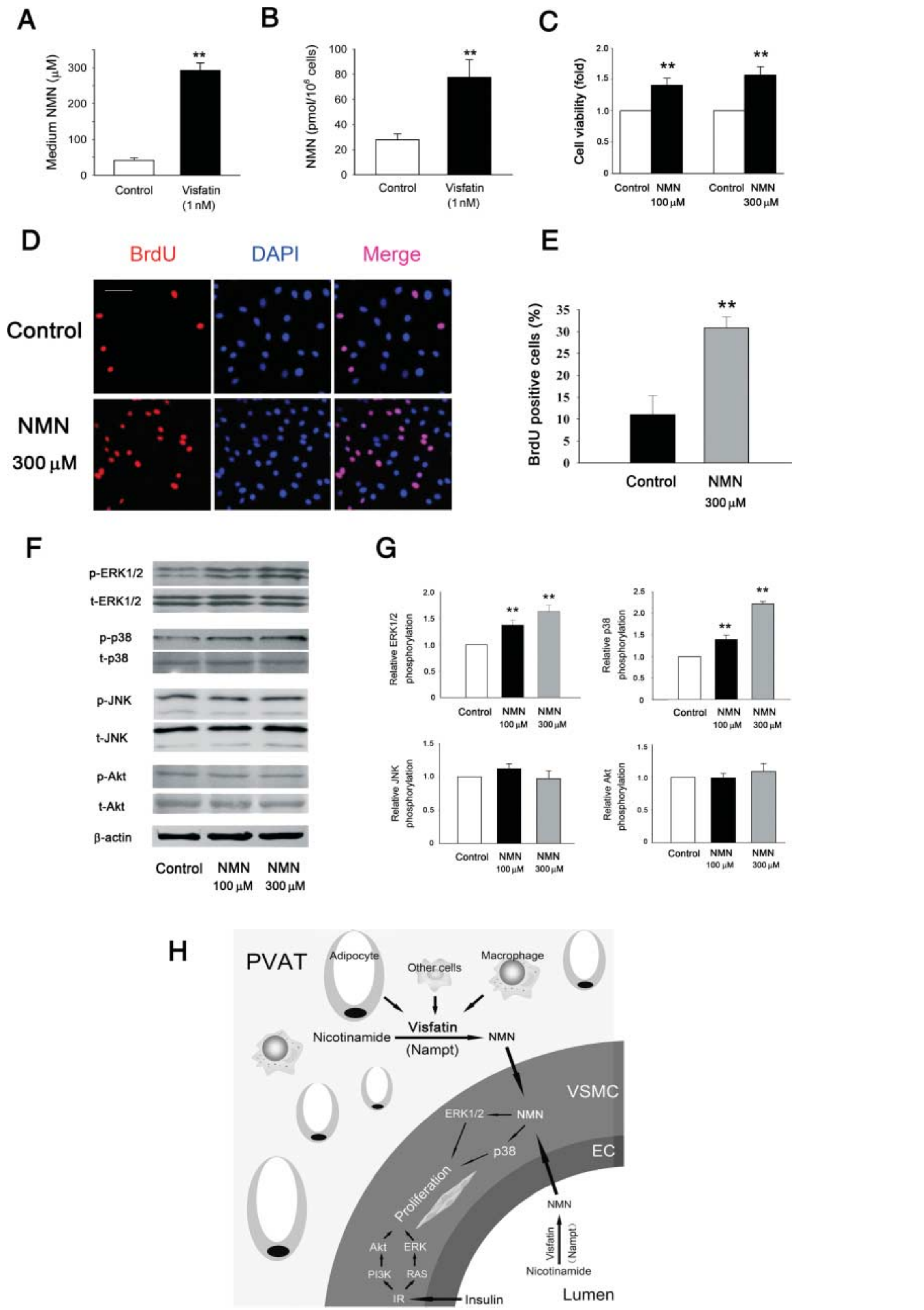

Figure 6 Identification of nicotinamide mononucleotide as a mediator involved in visfatin-induced vascular smooth muscle cell proliferation. (*A*) Nicotinamide mononucleotide concentrations in medium of cultured vascular smooth muscle cells. $^{**}P < 0.01$ vs. Control. $n = 6$. (*B*) Intracellular nicotinamide mononucleotide concentrations in cultured vascular smooth muscle cells. $^{**}P < 0.01$ vs. Control. $n = 6$. (*C*) CCK-8 cell viability assay showing nicotinamide mononucleotide-induced vascular smooth muscle cell proliferation. $^{**}P < 0.01$ vs. Control. $n = 6$. (*D* and *E*) BrdU incorporation experiment showing nicotinamide mononucleotide (300 μM) increases DNA synthesis in vascular smooth muscle cells. $^{**}P < 0.01$ vs. Control. $n = 6$. Scale bar, 20 μm. (*F* and *G*) Western blotting showing nicotinamide mononucleotide activates phosphorylation of ERK1/2 and p38, but not JNK and Akt. $^{**}P < 0.01$ vs. Control. $n = 4$. (*H*) A mechanism framework for the paracrine and endocrine actions of visfatin on vascular smooth muscle cells. See text for details.

kinase pathways.[21] Mitogen-activated protein kinase pathways mainly include ERK1/2, p38, and JNK pathways. Previous studies have demonstrated visfatin promotes angiogenesis by activation of ERK1/2 and PI3K/Akt in endothelial cells,[22] and induces cytokine production via p38 pathway in osteoblasts.[23] In the present study, we found visfatin-induced VSMC proliferation could be blocked by ERK1/2 and p38 inhibitors, but not by JNK and PI3K inhibitors. In accordance with these data, visfatin activated ERK1/2 and p38 in a dose- and time-dependent manner, but failed to enhance the phosphorylation of JNK and Akt. In addition, visfatin-induced phosphorylation of ERK1/2 and p38 could be abolished by their respective inhibitors. These results suggest that visfatin-induced VSMC proliferation is through ERK1/2 and p38 signalling pathways, rather than JNK and PI3K/Akt signalling pathways.

The proliferation of VSMCs and the activation of ERK1/2 and p38 are not due to the very low levels of lipopolysaccharide contained in recombinant visfatin. In our visfatin-treated experiments, the lipopolysaccharide levels were <50 pg/mL, similar to the previous studies.[8,16] However, the lipopolysaccharide levels for proliferation of VSMCs and activation of ERK1/2 and p38 were, respectively, 25 and 100 ng/mL as described in the previous studies,[24,25] above 500-fold higher than that in the present study.

4.5 Unlike insulin, visfatin-induced vascular smooth muscle cell proliferation is not mediated by insulin receptor

Fukuhara *et al*. have reported that visfatin exerts insulin-mimetic actions in cultured cells and lowers plasma glucose levels in mice by binding to and activating IR. Two studies in monocytes and in osteoblasts have also indicated that IR may be involved in visfatin function; both studies demonstrated that visfatin effects could be blocked by IR tyrosine kinase inhibitor, HNMPA-$(AM)_3$.[16,23] However, recent studies indicated that visfatin cannot activate IR,[7] and proposed that visfatin may act on its own unidentified receptor.[8] To determine whether visfatin-induced VSMC proliferation is mediated by IR, we first used more specific method of IR knockdown to elucidate this question, with insulin as a comparison. A pool of siRNAs was used to obtain the efficient knockdown, according to a previous report.[26] The IR protein expression was reduced by $\sim$86% with IR-siRNAs. The knockdown of IR abolished insulin-induced VSMC proliferation and insulin-induced Akt phosphorylation. However, it did not significantly affect the visfatin-induced VSMC proliferation and visfatin-induced phosphorylation of ERK1/2 and p38. These results suggest that unlike insulin, visfatin-induced VSMC proliferation is not mediated by IR. Further supporting evidence is that in contrast to insulin, visfatin-induced VSMC proliferation was not through PI3K/Akt pathway, and visfatin did not reduce the blood glucose levels in mice. Therefore, we propose that visfatin stimulates VSMC proliferation via a non-IR signalling pathway.

4.6 Nicotinamide mononucleotide is a key mediator for visfatin-induced vascular smooth muscle cell proliferation

The other receptor or non-receptor mechanism needs to be explored for visfatin-induced VSMC proliferation. We checked the composition of the cultured medium, and found nicotinamide, the substrate of Nampt for synthesizing NMN, in it. Thus, we tested whether visfatin-induced VSMC proliferation is mediated by its Nampt enzymatic activity.

Our observation demonstrated that recombinant visfatin was enzymatically active and synthesized NMN in the cultured medium. The synthesized NMN in the cultured medium could enter into the cultured cells, since the intracellular NMN level increased with the extracellular NMN biosynthesis under visfatin stimulation. Exogenous NMN itself could produce visfatin-mimetic effects in VSMCs, including activating ERK1/2 and p38, enhancing the nuclear BrdU incorporation and stimulating cell proliferation. Therefore, we propose a mechanism framework for visfatin-induced VSMC proliferation; visfatin acts as Nampt to promote VSMC proliferation through NMN-mediated activation of ERK1/2 and p38 signalling pathways. Our data, in combination with the reported evidence that adipocyte-secreted visfatin has Nampt enzymatic activity,[7] indicate that PVAT-derived visfatin stimulates VSMC proliferation via NMN-mediated ERK1/2 and p38 signalling pathways (*Figure 6H*). This is also the first demonstration for NMN as a signal molecule to activate cell proliferative process.

The possibility of visfatin receptor-mediated mechanism were not further studied in the present study, since the extent of the proliferative effect (1.3–1.8-fold, *Figures 2E*, *3C*, *4A*, and *5E*) produced by visfatin (1 nM) was similar to that (1.4–1.6-fold, *Figure 6C*) produced by NMN 100 and 300 μM (close to the extracelluar NMN levels 292 ± 20 μM produced by visfatin 1 nM, *Figure 6A*). These indicate that the cytokine-like effect of visfatin-induced VSMC proliferation is due to its enzyme activity rather than unknown receptor. Perhaps, the so-called unidentified visfatin receptor does not exist.

Recently, it was reported that visfatin was correlated with atherosclerosis in patients.[16,27] Local visfatin was increased in macrophages of human unstable carotid and coronary atherosclerosis, indicating a possible role in plaque destabilization.[16] Serum visfatin was increased in patients with metabolic syndrome, especially in those with carotid plaques.[27] It is reasonable to suppose that like serum visfatin, PVAT-derived visfatin may also contribute to VSMC proliferation in progression of atherosclerosis. This hypothesis remains to be tested in the future study.

In summary, the present study demonstrates for the first time that visfatin is highly and preferentially expressed in aortic PVAT, and may act as a VSMC growth factor rather than a VSMC relaxing factor involved in the paracrine action of PVAT (*Figure 6H*). Unlike insulin, visfatin stimulates VSMC proliferation via its Nampt enzymatic activity. The biosynthesized NMN seems to be a signalling molecule leading to activation of the proliferative ERK1/2 and p38 signalling pathways. Both sources of visfatin from PVAT and the bloodstream may act on VSMCs. This paracrine and endocrine action of visfatin provides a novel insight into the molecular links between adipose tissue and blood vessels, and may be of particular importance in certain vascular diseases with altered visfatin levels in local PVAT and/or the systemic circulation.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.

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