Notch1 mediates visfatin-induced FGF-2 up-regulation and endothelial angiogenesis

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Keywords

Visfatin • Notch1 • FGF-2 • Angiogenesis • Endothelial cells

Aims

Our aims were to determine the role of Notch1 in mediating visfatin-induced angiogenesis and to explore potential target genes involved.

Methods

Inhibition of Notch signalling attenuated visfatin-induced angiogenesis in vitro, ex vivo, and in vivo. Visfatin increased γ-secretase activity, Notch1 cleavage and activation, and Hes1 gene induction. Visfatin also stimulated fibroblast growth factor-2 (FGF-2) gene expression in a Notch1-dependent manner. Enforced expression of active Notch1 intracellular domain increased FGF-2 protein levels and stimulated endothelial tube formation, whereas blocking Notch1 signalling or knockdown of Notch1 by small interfering RNA suppressed visfatin-induced FGF-2 up-regulation and angiogenesis. Reporter analysis of FGF-2 promoter revealed the presence of CSL (CBF-1, suppressor of hairless, LAG-1)-binding site, and chromatin immunoprecipitation analysis demonstrated the binding of Notch1–CSL complex to this site in response to visfatin.

Conclusion

Our data provide the first example of Notch1-dependent endothelial FGF-2 induction by visfatin and of Notch1 activation in visfatin-stimulated endothelial angiogenesis, suggesting that the signalling axis of visfatin/Notch1/angiogenic factors like FGF-2 might be a valuable target for pathological angiogenesis.

1. Introduction

Visfatin is a novel adipokine that is predominantly produced in visceral adipose tissue.1 Visfatin was first reported as a nicotinamide phosphoribosyltransferase, an enzyme involved in nicotinamide adenine dinucleotide biosynthesis; it was later rediscovered as a pre-B cell colony-enhancing factor that enhances the effects of interleukin-7 and stem cell factor on pre-B-cell colony formation.2,3 In addition to these activities, visfatin has been reported as a pro-inflammatory cytokine.4,5 Moreover, we, and others, have shown that visfatin induces angiogenesis, which requires the up-regulation of angiogenic factors such as vascular endothelial growth factor (VEGF), IL-6, and fibroblast growth factor-2 (FGF-2) in endothelial cells.5–9 To date, several signalling pathways such as PI3K, p38 MAPK, ERK1/2, NF-κB, and STAT3 have been reported to contribute to visfatin-induced activation of gene transcription.5–9

Notch signalling is an evolutionarily conserved intercellular signalling mechanism, affecting fate determination, growth, survival, and differentiation of various cell types in diverse tissues during embryonic and post-natal development.10 Notch family members (Notch1 to Notch4) are large single-pass type I transmembrane receptors. Notch interacts with a membrane-bound ligand (Delta or Jagged family members) expressed on neighbouring cells, which induces sequential cleavage of Notch by metalloprotease and γ-secretase.
leading to the formation and release of the Notch intracellular domain (NICD). The released NICD translocates into the nucleus where it associates with the DNA-binding protein CSL [CBF-1/RBP-jk, Su(H), LAG-1] and coactivators, such as MAML (Mastermind-like) to activate the transcription of the primary target genes of Notch signalling, such as members of the Hes and Hey families. Recent reports have shown the important role of Notch signalling in vascular development, including proliferation and migration of endothelial cells, smooth muscle differentiation, arterial-venous differentiation, and embryonic angiogenesis. In addition, Notch signalling is known to be essential for post-natal neovascularization including angiogenesis, arteriogenesis, and vasculosaturation. In contrast to what is known about the significance of Notch signalling in the vascular system, relatively little is known about the molecular mechanisms underlying angiogenic regulation of Notch signalling in endothelial cells. Here, we attempted to investigate whether Notch signalling plays a role in visfatin stimulation of angiogenesis and to characterize the mechanism involved. We report for the first time that visfatin activates endothelial Notch1 signalling, leading to FGF-2 induction and angiogenesis.

2. Methods

2.1 Materials
Reagents and recombinant proteins are described in detail in the Supplementary material online.

2.2 Human endothelial cells
Human microvascular endothelial cells (HMECs) were obtained from the Center for Disease Control (CDC) in Atlanta, GA, USA. Primary human umbilical vein endothelial cells (HUVECs) (passages 5–8) were purchased from CLONTECH.

2.3 In vitro tube formation assay
Briefly, ice-cold 24-well plates were coated with growth factor-reduced Matrigel (GFR-Matrigel, BD Biosciences) and allowed to solidify for 30 min at 37°C. Cells were trypsinized, pre-treated with or without DAPT for 1 h, seeded onto GFR-Matrigel-coated wells, and then were stimulated with visfatin (500 ng/mL). After the indicated hours, tube-like structures were photographed. The branch point number was counted.

2.4 Ex vivo rat aortic ring assay
The aortas dissected from male Sprague–Dawley rats (6 weeks of age) were sectioned into 1 mm-thick slices and the aortic rings were placed on Matrigel-coated wells, covered with additional 50 µL Matrigel, and allowed to gel formation at 37°C. The aortic rings were cultured with visfatin (5 µg/mL) in the presence or absence of DAPT. After 3 days, the outgrowth of microvessels was photographed. Quantitative analysis showed that the number of microvessel outgrowth was counted.

2.5 In vivo chorioallantoic membrane assay
Sterile filter disks absorbed with visfatin (500 ng/disk) dissolved in PBS were placed on the growing chorioallantoic membranes (CAMs) of a fertilized 10-day-old chicken embryo. Then, DAPT or vehicle control (PBS) was added directly to CAMs topically. After 72 h, the digital images of CAM sections exposed to filters were captured. Quantification of new branches formed from the main vessel as well as existing blood vessels of the whole field (disk area) was performed.

2.6 In vivo Matrigel plug assay
In brief, Matrigel (0.5 mL), containing visfatin (5 µg/mL) and 50 U/mL of heparin in the presence or absence of DAPT, was injected subcutaneously into both flanks of 7-week-old male C57BL/6 mice. After 7 days, the Matrigel plugs were recovered and photographed. All animal studies were conducted in accordance with our institutional guidelines for animal care and with the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication No. 85-23 revised 1996) and approved by the Institutional Animal Care and Use Committee at Pusan National University, Korea.

2.7 In vitro peptide cleavage assay for γ-secretase activity
Briefly, solubilized membrane fraction was incubated with 10 µM of a fluorescence-conjugated peptide substrate (Calbiochem). The degree of substrate cleavage was measured by the emitted fluorescence using a fluorescent microplate reader (Tecan, Salzburg, Austria) with an excitation wavelength at 355 nm and an emission wavelength at 440 nm.

2.8 Transfection and luciferase assay
For the luciferase assays, luciferase reporter plasmids were transfected into HMECs using the polyethylenglymine transfection reagent (Sigma). After 24 h, the cells were incubated with or without visfatin (500 ng/mL) for the indicated times. Then, the cell extracts were prepared and analysed for luciferase activity using luciferase assay kit (Promega) and a luminometer (Tuner Biosystems).

2.9 Human FGF-2 reporter plasmids and site-directed mutagenesis
From genomic DNA isolated from HMECs, human FGF-2 promoter fragments were generated by PCR and subcloned into the pGL3 basic vector (Promega). Site-directed mutagenesis at the CSL-binding site in the FGF-2 promoter was performed according to the manufacturer’s instructions using the QuickChange mutagenesis kit (Stratagene) and checked by automatic DNA sequencing (Cosmo, Korea).

2.10 Gene knockdown by small interfering RNA
The small interfering RNA (siRNA) duplexes for human Notch1 and a negative control siRNA were obtained from Santa Cruz Biotechnology (sc-36095) and Bioneer Co. (SN-1022), respectively. Transfection of HMECs and HUVECs was carried out using oligofectamine and Amaxa nucleofector, respectively, according to the manufacturer’s instructions.

2.11 Chromatin immunoprecipitation assay
Chromatin immunoprecipitation (ChIP) assay was performed on HMECs using the ChIP assay kit following the manufacturer’s protocol (Upstate Biotechnology). Immunoprecipitation was performed with anti-CSL or anti-cleaved Notch1 (c-Notch1) antibodies. The putative CSL-binding site located at −1456 to −1450 bp in the human FGF-2 promoter region was amplified by PCR with specific primers (forward, 5′-GTG TCTTTTGTACAGGGGCTTCAG-3′; reverse, 5′-CTCCTACTTCAG CCTACCGAATAG-3′).

2.12 Quantitative real-time RT–PCR and RT–PCR
Methods for quantitative real-time RT–PCR (qRT–PCR), RT–PCR, and the sequences of oligonucleotide primers for the PCR are described in the Supplementary material online.
2.13 Western blot analysis, immunocytochemistry, and histological analysis

Western blot analysis, immunocytochemistry, and immunohistochemistry were performed as described in the Supplementary material online.

2.14 Statistical analysis

Data are the mean ± standard deviation of at least three independent experiments. Statistical comparisons between groups were performed by the one-way analysis of variance followed by the Student t-test.

3. Results

3.1 Notch1 signalling is involved in visfatin-induced angiogenesis in vitro and in vivo

To investigate the molecular basis underlying visfatin-induced angiogenesis, we tested the efficacy of several pharmacological inhibitors in suppressing the angiogenic activity of visfatin and found that DAPT, which potently inhibits Notch cleavage and signalling,19 suppressed visfatin-induced angiogenesis in an in vitro endothelial tube formation assay (Figure 1A; see Supplementary material online, Figure S1A). Next, we examined the effect of DAPT on visfatin-induced angiogenesis using a CAM assay, an in vivo model of angiogenesis.4 Visfatin increased the number of newly formed blood vessels, which was significantly suppressed by the treatment of DAPT (Figure 1B; see Supplementary material online, Figure S1B). Similar results were obtained in in vivo Matrigel plug assay (Figure 1C). The histological analysis of plugs by haematoxylin–eosin (H&E) staining revealed that treatment with DAPT strongly decreased visfatin-induced cellular invasion of Matrigel and the formation of vessels (Figure 1C). To quantify the functional vasculature in the Matrigel plugs, the haemoglobin content was determined. Visfatin increased the haemoglobin content of the Matrigel plugs in mice and DAPT blocked this increase (see Supplementary material online, Figure S1C). To examine whether visfatin activates Notch signalling in vivo, we performed immunohistochemistry on the sections of Matrigel plugs with antibody against c-Notch1, a major vascular-specific Notch receptor.14,18 Visfatin treatment increased the number of c-Notch1-positive cells, which was blocked by DAPT treatment (Figure 1C; see Supplementary material online, Figure S1E). Double confocal immunofluorescence staining for c-Notch1 and PECAM-1 revealed that almost all of the c-Notch1 signals were detected in the nucleus of endothelial cells displaying immunoreactivity for PECAM-1 (Figure 1D). Overall, these results suggest that Notch1 signalling mediates visfatin-induced angiogenesis in vitro and in vivo.

3.2 Notch1 is activated by visfatin in human endothelial cells

To determine whether Notch1 activity is affected by visfatin, HMECs were incubated with visfatin and then the levels of Notch1 protein were analysed by western blot analysis. Visfatin stimulated the cleavage of Notch1 protein in a time-dependent manner in the HMECs with a peak at 6 h (~6.3-fold) (Figure 2A; see Supplementary material online, Figure S2A). Then, the levels of c-Notch1 slightly declined by 12 h, but were sustained for at least 48 h following visfatin treatment (Figure 2A; see Supplementary material online, Figure S2B). The levels of full-length total Notch1 (t-Notch1) were not significantly affected by visfatin (Figure 2A; see Supplementary material online, Figure S2B). Similar results were obtained in HUVECs (see Supplementary material online, Figure S2C). We next examined the localization of c-Notch1 in visfatin-treated HMECs. As determined by immunocytochemistry using antibody against the cleaved form of Notch1, visfatin treatment produced more c-Notch1-positive nuclei than the control (Figure 2B). We subsequently examined whether visfatin-induced Notch1 activation was accompanied by the up-regulation of Hes1, a well-known target gene of Notch1 signalling.10,11,20 at the mRNA and protein levels in visfatin-treated cells. Visfatin significantly increased the levels of Hes1 protein (Figure 2A; see Supplementary material online, Figure S2B) and mRNA (Figure 2C; see Supplementary material online, Figure S3A) in a time-dependent manner. To further examine whether visfatin-induced Hes1 up-regulation was a consequence of increased transcriptional activity of the Hes1 promoter, we performed a reporter assay using a mouse Hes1 reporter construct. Visfatin time-dependently increased the transcription levels of the luciferase gene driven by mouse Hes1 promoter (pHes1-Luc)21 (see Supplementary material online, Figure S2D). Furthermore, the activation of the CSL-dependent reporter gene (4XCSL-Luc)22 was also induced by visfatin in the HMECs (see Supplementary material online, Figure S2E).

The γ-secretase is an intramembrane-cleaving protease that is required for the cleavage and activation of the Notch family receptors.10,11 Thus, to determine whether visfatin affects γ-secretase activity, we measured γ-secretase activity using a fluorogenic γ-secretase substrate in visfatin-treated HMECs. VEGF is known to increase γ-secretase activity.19 Thus, we used VEGF as a positive control for in vitro peptide cleavage assay for γ-secretase activity. Visfatin significantly enhanced the activity of γ-secretase with a maximal 3.5-fold effect after 3 h of visfatin stimulation (Figure 2D). In addition, visfatin-induced γ-secretase activation was inhibited in a dose-dependent manner by DAPT (Figure 2E). Taken together, these results indicate that visfatin increases Notch1 signalling activity, which is mediated by γ-secretase activation in the human endothelial cells.

3.3 Notch1 signalling is involved in visfatin-induced FGF-2 production in endothelial cells

We investigated whether visfatin-induced Notch1 cleavage and signalling are blocked by DAPT in human endothelial cells. HMECs were incubated with DAPT followed by visfatin treatment and then the levels of c-Notch1 protein and Hes1 mRNA were analysed. Visfatin-induced Notch1 cleavage (Figure 3A) and Hes1 induction (Figure 3B; see Supplementary material online, Figure S3B) were efficiently blocked by DAPT. Next, to examine which angiogenic factors are induced by Notch1 signalling for mediating visfatin-induced angiogenesis, we performed an ex vivo rat aortic ring assay and then examined mRNA levels of several angiogenic factors by RT–PCR in rat aortic rings treated with visfatin in the presence or absence of DAPT. DAPT significantly suppressed visfatin-induced microvessel sprouting from the adventitia of cultured rat aortic rings (Figure 3C; see Supplementary material online, Figure S1F). We found that FGF-2 mRNA levels were increased in visfatin-treated rat aortic...
rings by \( \sim 2.2 \)-fold compared with the control, which was almost completely inhibited in the presence of DAPT (Figure 3D). To confirm this effect in human endothelial cells, we incubated the HMECs with DAPT followed by visfatin treatment, and then the levels of human FGF-2 mRNA and protein were analysed. DAPT significantly attenuated the levels of visfatin-induced FGF-2 mRNA (Figure 3E; see Supplementary material online, Figure S3O) and protein (Figure 3F) in HMECs. Therefore, these results suggest that FGF-2 up-regulation by visfatin may be attributable to Notch1 activation in endothelial cells.

### 3.4. Notch1 is required for visfatin-induced FGF-2 production and endothelial angiogenesis

To more specifically verify the significance of Notch1 in mediating visfatin-induced FGF-2 gene activation and endothelial angiogenesis, we used siRNA targeting for Notch1 to knockdown Notch1 expression and pME-FNIC, a mammalian expression vector encoding mouse Notch1 intracellular domain (NICD), to overexpress NICD in human endothelial cells. The transfection of HMECs with Notch1 siRNA resulted in the reduction of both t-Notch1 and c-Notch1 protein levels, which led to a decrease in FGF-2 protein levels below basal levels and blocked visfatin-induced FGF-2 up-regulation (Figure 4A). Moreover, a Matrigel-based in vitro angiogenesis assay showed that tube formation was significantly attenuated in Notch1 siRNA-transfected HUVECs, when compared with control siRNA-transfected cells (Figure 4B). Conversely, when the HMECs were transfected with pME-FNIC, the levels of endogenous FGF-2 proteins were increased in accordance with the enforced overexpression of NICD (Figure 4C). The findings from western blot analysis were further corroborated by immunocytochemistry. The FGF-2 signals were stronger in the NICD-overexpressed cells than in control cells.
Moreover, the increased levels of NICD protein in NICD-transfected HUVECs were concomitant with an increase in tube formation of these cells (Figure 4E). DAPT treatment had no effects on enhanced angiogenesis by overexpression of NICD (Figure 4E). Overexpression of NICD proteins induced the activation of both pHes1-Luc and 4XCSL-Luc in the HMECs (see Supplementary material online, Figure S4A and B), confirming that these mouse NICD proteins were active in the HMECs. Taken together, these results show that Notch1 plays an important role in mediating visfatin-induced FGF-2 production and endothelial angiogenesis.

3.5 FGF-2 promoter contains a functional CSL-binding site to which the c-Notch1/CSL complex binds in response to visfatin

To investigate the mechanism by which Notch1 activates FGF-2 gene expression, we constructed a human FGF-2 reporter plasmid, F2Pwt(1979)-Luc, by subcloning a 1979 bp fragment (−1818 to +161 bp) of human FGF-2 gene promoter into the pGL3 basic vector (Figure 5A and C) and performed a reporter assay after transfection of the plasmid into the HMECs. Visfatin elicited an increase in FGF-2 promoter-dependent luciferase activity [F2Pwt(1979)-Luc] by at least two- to three-fold compared with the control (Figure 5B and D); this effect was eliminated by inhibition of the Notch signalling with DAPT in a dose-dependent manner (Figure 5B). These results confirmed that the cloned promoter region was sufficient for responding to visfatin and/or DAPT. We evaluated the sequence of the cloned 1979 bp FGF-2 promoter region and found that there is one putative CSL-binding site (ATGGGAG) spanning from −1456 to −1450 bp from the transcription start site in the human FGF-2 promoter (Figure 5A and C). To examine the involvement of this putative CSL-binding site in the activation of the FGF-2 promoter by visfatin, we constructed a deletion vector that lacks the CSL-binding site, named F2P(1042)-Luc (Figure 5C). In addition, we introduced nucleic acid substitutions at the CSL-binding site (A TGGGAG → AACCGAG) of F2Pwt(1979)-Luc to construct F2Pmut(1979)-Luc (Figure 5C). Both the deletion [F2P(1042)-Luc] and mutation [F2Pmut(1979)-Luc] constructs failed to elicit a response to visfatin (Figure 5D).

To further examine whether c-Notch1 and CSL complex directly binds to the putative CSL-binding site on the FGF-2 promoter, we performed ChIP assay in the HMECs. Visfatin treatment resulted in a dramatic increase in the recruitment of both c-Notch1 and CSL proteins to the CSL-binding site (Figure 5E), indicating that the effects of c-Notch1 on FGF-2 gene transactivation were CSL-dependent. Therefore, these results indicate that the putative CSL-binding site in the FGF-2 promoter functions as a Notch1-response element.
Adipokines such as leptin, resistin, IL-6, VEGF, and FGF-2 have been reported to play an important role in promoting angiogenesis, thus suggesting obesity is a risk factor for cardiovascular diseases such as atherosclerosis and other angiogenesis-associated disorders including diabetes, arthritis, and cancer. We have recently demonstrated that a new adipokine, visfatin, which expression is up-regulated by hypoxia-inducible factor-1 under hypoxic conditions, acts as a proangiogenic factor by increasing FGF-2 production in human endothelial cells. In this study, we have shown for the first time that visfatin stimulates γ-secretase and Notch1 activation in human endothelial cells and furthermore that Notch1 plays an important role in visfatin-induced FGF-2 up-regulation and endothelial angiogenesis.

Growing evidence indicates that Notch signalling acts as a key regulator of angiogenesis. The loss of Notch or its most important ligand DLL4 or DLL1 in the endothelium leads to excessive angiogenesis, whereas its overexpression reduces sprouting angiogenesis. In addition, Notch is activated secondary to VEGF induction to regulate VEGF signalling by down-regulation of VEGF receptor expression. Thus, these results indicate the role of Notch signalling as a regulator/modulator in angiogenesis but not as an inducer. In contrast, other studies also suggest that Notch signalling
positively mediates endothelial angiogenesis. For instance, ethanol enhances endothelial angiogenesis by stimulating a novel Notch/CBF-1/RBP-JK–Ang1/Tie2-dependent pathway. In addition, activation of Notch signalling by ectopic overexpression of Notch IC results in enhanced network formation of endothelial cells. Furthermore, a recent report has shown that Notch1 signal activation is required for FGF-4-induced tumour angiogenesis. Therefore, these different results indicate that Notch signalling acts in a context-dependent manner which makes interpretation of data very complicated. In this study, we found that overexpression of NICD stimulated endothelial network formation, whereas inhibition of Notch signalling by DAPT or knockdown of Notch1 protein levels by siRNA led to blockade of visfatin-induced angiogenesis, indicating that visfatin-activated Notch1 signalling is positively involved in endothelial angiogenesis.

Our present findings indicate that γ-secretase-mediated Notch1 activation plays a critical role during visfatin-induced angiogenesis. Considering that γ-secretase is also able to release β-amyloid (Aβ) peptide with the proteolytic cleavage of amyloid precursor protein and that the Aβ peptides play key roles in stimulating angiogenesis as well as in the pathogenesis of Alzheimer’s disease, it would be of interest to investigate the involvement of Aβ in the process of visfatin-induced angiogenesis.

Although the role of Notch signalling has been extensively studied in the vascular system, less attention has been paid to the molecular mechanisms whereby Notch signalling regulates angiogenesis. Thus, the identification of downstream targets of Notch signalling during endothelial angiogenesis is of significant interest. Recently, both angioigenin-1 and Tie2 genes have been identified as direct target genes of ethanol-induced Notch signalling activation, leading to angiogenesis. Our results provide evidence that FGF-2, one of the first identified potent angiogenic growth factors, acts as a downstream target gene of Notch1 signalling in endothelial cells. Besides on FGF-2, the stimulatory effects of visfatin on angiogenic factors such as

Figure 4 Visfatin-induced FGF-2 production and endothelial network formation is Notch1-dependent. (A and B) HMECs and HUVECs were transiently transfected with control siRNA or with Notch1 siRNA. (A) After 16 h of transfection, HMECs were stimulated with visfatin (500 ng/mL) and incubated for 24 h. Expression levels of t-Notch1, c-Notch1, and FGF-2 proteins were detected by western blot analysis; representative western blot (A, left), cumulative densitometric data (A, right). n = 3, *P < 0.05, **P < 0.01. (B) Following transfection and incubation for 16 h, HUVECs were plated onto the surface of the GFR-Matrigel and treated with or without visfatin. Capillary-like tube formation was observed and photographs were taken after 5 h (B, left). The branch point number was counted (B, right). n = 3, **P < 0.01. (C and D) HMECs were transfected with various concentrations of Flag-tagged expression vector for NICD, pME-FNIC (2, 4, and 8 μg). Thirty-six hours after transfection, the cells were either lysed for western blot analysis or fixed for immunocytochemistry. (C) Western blot analysis of NICD and FGF-2 protein levels in control and pME-FNIC-transfected cells; representative western blot (C, left), cumulative densitometric data (C, right). n = 3, **P < 0.01 vs. control. (D) Immunofluorescent staining using anti-Flag and anti-FGF-2 antibodies in the NICD-transfected cells. Nuclei were counterstained with DAPI (blue). The arrows indicate the HMECs overexpressing Flag-NICD (red), in which strong FGF-2 signals (green) can be detected, whereas untransfected HMECs indicated by arrowheads show only very weak FGF-2-positive signals or no signal (original magnification, ×200). Scale bar: 50 μm. (E) HUVECs transfected with control vector or with pME-FNIC (3 μg) were pre-treated with or without DAPT for 2 h and then plated onto the surface of the GFR-Matrigel. Capillary-like tube formation was observed and photographs were taken after 5 h (E, left). The branch point number was counted (E, right). n = 3, **P < 0.01 vs. control. Scale bar: 50 μm.
IL-6 and VEGF have been recently reported by us and other investigators.\textsuperscript{7,8} The present study showed that DAPT treatment suppressed visfatin-induced up-regulation of IL-6 and VEGF (see Supplementary material online, Figure S5), suggesting that Notch signalling can be considered as a new mediator that links visfatin to potent angiogenic factors, including IL-6, VEGF, and FGF-2, in endothelial cells.

In this study, we checked the expression pattern of Notch receptors and its ligands in endothelial cells in the basal condition and after stimulation with visfatin (see Supplementary material online, Figure S6). VCAM-1 was used as a positive control for visfatin-induced gene.\textsuperscript{5} The level of Notch1 mRNA was dramatically elevated at 8 h after visfatin treatment and then gradually declined by 24 h. However, Notch4 showed opposite expression pattern compared with Notch1. In the case of Notch ligands, although Jagged-1 and Jagged-2 did not show any change in mRNA levels after visfatin treatment, DLL1 and DLL4 expression was increased by visfatin in a time-dependent manner. Thus, these results suggest the involvement of DLL receptors in visfatin signalling in human endothelial cells.

We are currently investigating the molecular mechanism underlying the up-regulation of Notch1, DLL1, and DLL4 genes by visfatin in human endothelial cells.

Recent study has evaluated the regulation of Jagged/Notch signalling by Erk1/2 pathways and has shown that Jagged-1 is an Erk1/2-dependent target of TLR signalling in macrophage.\textsuperscript{40} Considering Figure 5 FGF-2 promoter containing a functional CSL-binding site is transactivated by Notch1. (A) A schematic representation of the human FGF-2 promoter (1979 bp promoter region from \(-1818\) to \(+161\) bp with respect to the transcription start site) depicting a putative CSL-binding site spanning from \(-1456\) to \(-1450\) bp. (B) The HMECs were transiently transfected with the FGF-2 reporter vector containing a 1979 bp fragment [F2Pwt(1979)-Luc] and then pre-treated with or without DAPT for 1 h before stimulation with visfatin (500 ng/mL). After 48 h, the cell extracts were prepared and analysed for luciferase activity. \(n = 3\), \(**P < 0.01\). (C) Schematic representations showing the deletion and mutation constructs of the human FGF-2 promoter. (D) The FGF-2 promoter reporter assays were performed using F2Pwt(1979)-Luc, F2Pmut(1979)-Luc, and F2P(1042)-Luc. HMECs were transiently co-transfected with each of the indicated FGF-2 reporter vectors and pCMV-β-galactosidase, and then stimulated with or without visfatin (500 ng/mL) for 48 h, the cell extracts were prepared and analysed for luciferase activity. \(n = 3\), \(**P < 0.01\). (E) The HMECs were treated with or without visfatin (500 ng/mL) for 24 h. ChIP analysis was performed with anti-CSL or anti-c-Notch1 antibodies, as described in Section 2.
our recent report that Erk1/2 pathway activated by visfatin is involved, at least in part, in FGF-2 up-regulation, it is likely that FGF-2 up-regulation is attributable to visfatin-induced Notch1 signalling activation that is dependent on Erk1/2 activity. We therefore investigated the effect of Erk1/2 inhibitor U0126 on Notch1 activation by visfatin. U0126 treatment reduced the levels of c-Notch1 protein in visfatin-treated HMECs, suggesting that Erk1/2 activity may have an impact on Notch1 activation pathway in endothelial cells (see Supplementary material online, Figure S7). Additional works are needed to precisely delineate the molecular mechanisms responsible for the nature of crosstalk between Erk1/2 and Notch signalling pathways in visfatin-stimulated endothelial cells.

Dysregulated Notch signalling plays an important role in pathological conditions such as ischaemic disease, cancer, and inflammatory diseases including atherosclerosis. Furthermore, aberrant FGF-2 signalling contributes to many vascular diseases such as cancer and atherosclerosis as well as tumour angiogenesis. High levels of visfatin expression have been observed in various human diseases including atherosclerosis and cancer. Therefore, further understanding of the visfatin/Notch1/FGF-2 signalling axis will provide an attractive strategy to develop therapeutic regulators for understanding of the visfatin/Notch1/FGF-2 signalling contributions to many vascular diseases such as inflammatory diseases including atherosclerosis. Furthermore, aberrant FGF-2 signalling worsens brain damage and functional outcome in ischemic conditions such as ischaemic disease, cancer, and inflammatory diseases such as ischaemia or wound (see Supplementary material online, Figure S8).

In summary, our findings provide the first evidence of regulation of γ-secretase and Notch1 activation by a newly characterized adipokine, visfatin. Furthermore, we have demonstrated that FGF-2 acts as a downstream target gene of Notch1 signalling, contributing at least in part, to visfatin-induced endothelial angiogenesis. These results provide important clues towards the understanding of the mechanisms of vascular pathologies and new opportunities for therapeutic intervention by targeting the signalling axis of visfatin/Notch1/angiogenic factors, including IL-6, VEGF, and FGF-2.

Supplementary material

Supplementary material is available at Cardiovascular Research online.

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