Visfatin induces human endothelial VEGF and MMP-2/9 production via MAPK and PI3K/Akt signalling pathways: novel insights into visfatin-induced angiogenesis

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Aims Visfatin is a novel adipokine whose plasma concentrations are altered in obesity and obesity-related disorders; these states are associated with an increased incidence of cardiovascular disease. We therefore investigated the effect of visfatin on vascular endothelial growth factor (VEGF) and matrix metalloproteinases (MMP-2, MMP-9) production and the potential signalling cascades.

Methods and results In human umbilical vein endothelial cells (HUVECs), visfatin significantly and dose-dependently up-regulated gene expression and protein production of VEGF and MMPs and down-regulated expression of tissue inhibitors of MMPs (TIMP-1 and TIMP-2). The gelatinolytic activity of MMPs (analysed by zymography) correlated with mRNA and western blot findings. Interestingly, visfatin significantly up-regulated VEGF receptor 2 expression. Inhibition of VEGFR2 and VEGF [by soluble FMS-like tyrosine kinase-1 (sFlt1)] down-regulated visfatin-induced MMP induction. Visfatin induced dose- and time-dependent proliferation and capillary-like tube formation. Importantly, visfatin was noted to have anti-apoptotic effects. In HUVECs, visfatin dose-dependently activated PI3K/Akt (phosphatidylinositol 3-kinase/Akt) and ERK1/2 (extracellular signal-regulated kinase) pathways. The functional effects and MMP/VEGF induction were shown to be dependent on the MAPK/PI3K-Akt/VEGF signalling pathways. Inhibition of PI3K/Akt and ERK1/2 pathways led to significant decrease of visfatin-induced MMP and VEGF production and activation, along with significant reduction in endothelial proliferation and capillary tube formation.

Conclusion Our data provide the first evidence of visfatin-induced endothelial VEGF and MMP production and activity. Further, we show for the first time the involvement of the MAPK and PI3K/Akt signalling pathways in mediating these actions, as well as endothelial cell proliferation. Collectively, our findings provide novel insights into visfatin-induced endothelial angiogenesis.

KEYWORDS Matrix metalloproteinases; VEGF; MAPK; PI3K/Akt; Endothelial factors; Visfatin

1. Introduction

The increasing incidence of atherosclerotic cardiovascular disease (CVD) associated with obesity and the metabolic syndrome is one of the leading causes of mortality and morbidity.1 Recently, there has been significant interest in bioactive molecules secreted from adipose tissue, termed adipocytokines, and their interactions with vascular endothelium.2,3

A recently identified adipocytokine, visfatin (initially described as pre-B-cell colony-enhancing factor, PBEF), has been shown to be elevated in obesity, insulin resistance, type II diabetes mellitus, and pro-inflammatory states4; however, others have reported the contrary.5,6 We have previously shown elevated circulating visfatin levels in women with polycystic ovary syndrome, a pro-inflammatory condition known to predispose to cardiovascular risk and premature atherosclerosis.7 This is of interest, as it is increasingly evident from the literature that adipocytokines play a significant role in the induction of atherogenesis and dysregulated angiogenesis.8,9

Matrix metalloproteinases (MMPs) are proteolytic enzymes that remodel the extra-cellular matrix as part of an inflammatory response. Increased activity of MMPs have been implicated in atherosclerosis and CVD.10-13 One of the major MMP species in the vasculature are the gelatinases (MMP-2 and -9), regulating vascular matrix remodelling,14 with raised peripheral concentration of these MMPs reported in patients with acute coronary syndromes,15,16 and in cerebral ischaemia.12 MMPs are up-regulated by a variety of hormones, cytokines, and growth factors, including vascular endothelial growth factor (VEGF),17 VEGF, a homodimeric glycoprotein, plays an important role in vasculogenesis,
atherogenesis, and vascular remodelling in response to 'injury'.

With the aforementioned, we sought to study the possible interplay between visfatin and the pro-angiogenic molecules, VEGF and MMPs. In the present study, we found and report for the first time that visfatin dose-dependently increased MMP-2 and -9 and VEGF production. Visfatin’s proliferative and anti-apoptotic actions, along with up-regulation of VEGF and MMPs, were dependent on the MAPK and phosphatidylinositol 3-kinase (PI3K)/Akt and VEGF/VEGFR2 type-II receptor (VEGFR2) signalling cascades.

2. Materials and methods

2.1 Endothelial cell culture

Ethical approval for the procurement of human umbilical veins from healthy-term pregnancies during elective caesarean sections was obtained from the Local Research Ethics Committee, and all patients involved gave their informed consent, in accordance with the guidelines in The Declaration of Helsinki 2000. Human umbilical vein endothelial cells (HUVECs) were isolated and cultured as described previously18 (for details, see Supplementary material online). Similar experiments were performed in human microvascular endothelial cells (HMECs, Supplementary material online).

2.2 RNA isolation and real-time quantitative reverse transcription PCR

Quantitative PCR was performed on a Roche Light Cycler system (Roche Molecular Biochemicals, Manheim, Germany). For analysis, quantitative amounts of MMP-2, MMP-9, TIMP-1, TIMP-2, VEGF, and VEGFR2 were standardized against the housekeeping gene GAPDH (see Table 1 for primers for genes of interest). The mRNA levels were expressed as a ratio, using delta-delta method for comparing relative expression results between treatments in real-time PCR19 (for protocol conditions, see Supplementary material online).

2.3 Cell apoptosis

2.3.1 Annexin V staining: flow cytometry protocol

Annexin V-fluorescein isothiocyanate (FITC) staining was performed using annexin V-FITC apoptosis detection kit (Immunotech, Beckman Coulter, Marseille, France) according to the manufacturer’s instructions (for details of protocol conditions, see Supplementary material online).

2.4 Cell proliferation

2.4.1 Alamar blue cytotoxicity/proliferation assay

To determine the effect of visfatin on cell proliferation, the vital dye alamar blue was used. HUVECs were incubated at various time points (4–72 h) with varying concentrations of visfatin (0–100 nM) with or without VEGF at the optimized dose of 10 ng/mL (for details, see Supplementary material online).

2.5 MTS proliferation assay

Cell proliferation was also determined with CellTitier 96 AQueous One Solution Cell Proliferation Assay (MTS) kit (Promega, UK). Following visfatin treatment and addition of MTS reagent, absorbance was recorded. The percentage of the absorbance was calculated against untreated cells according to the manufacturer’s instructions (for details, see Supplementary material online).

2.6 In vitro angiogenesis assay

Angiogenesis was assessed by studying the formation of capillary-like structures by HUVEC on a Matrigel (BD Biosciences, San Jose, CA, USA) according to the manufacturer’s protocol. Following visfatin treatment for 24 h, capillary tube formation assay was performed (for details, see Supplementary material online).

2.7 Migration assay

Endothelial cell migration was performed using a protocol obtained from BD BioCoat Angiogenesis System and using a modified Boyden chamber. Briefly serum starved, Calcein-AM-labelled HUVECs were treated with or without visfatin. The migration induced was quantified by a fluorescence plate reader (for details, see Supplementary material online).

2.8 Gelatin zymography

Following optimization experiments (data not shown), the gelatinolytic activity of secreted MMP-2 and MMP-9 in the culture supernatants was measured by gelatin zymography, following 24 h visfatin treatment (for details, see Supplementary material online).

2.9 Western blot analysis

For MMPs, tissue inhibitors of MMP (TIMPs), and VEGF protein analyses, and their regulation by visfatin, HUVECs were serum starved overnight and then pre-treated with or without an MEK inhibitor, U0126 (Calbiochem), a PI3K inhibitor, LY 294002 (Calbiochem), a VEGF antagonist, soluble FMS-like tyrosine kinase-1 (sFlt1) (Calbiochem), and a VEGF receptor blocker, SU1498 (Calbiochem), followed by treatment with human recombinant visfatin [0–100 nM]; Axxorra Ltd, Nottingham, UK (ALX-201-336)] (for detailed protocol and antibody concentrations, see Supplementary material online).

2.10 Immunoprecipitation and PI3K activity assay

Serum-starved HUVECs were treated with or without visfatin (0–100 nM) or insulin (1 μM) (positive control) for various time points (0, 2, 5, 15, 20, 30 min). Cells were washed twice with ice-cold Tris-buffered saline, followed by Buffer A (for details, see Supplementary material online) and lysis buffer (Buffer A plus 1% NP-40 and 1 mM PMSF). The cells were scrapped and centrifuged for 10 min.

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Primers used for RT-PCR analysis</th>
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<tr>
<td>Gene/product size (bp)</td>
<td>Sense primer</td>
</tr>
<tr>
<td>MMP2 (179)</td>
<td>5'-tgcaagatgcttcgcttcgttc-3'</td>
</tr>
<tr>
<td>MMP9 (200)</td>
<td>5'-tgggattcagccttgcttc-3'</td>
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<tr>
<td>TIMP1 (173)</td>
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<tr>
<td>TIMP2 (175)</td>
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<tr>
<td>VEGF (196)</td>
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<td>VEGFR2 (218)</td>
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</tr>
<tr>
<td>GAPDH (185)</td>
<td>5'-gagaagttcggcttgct-3'</td>
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The supernatants were incubated with anti-PI 3 kinase antibody (Upstate Biotechnology, catalogue 06–195). Sixty millilitres of 50% slurry of Protein A-agarose beads in PBS were added to each of the tubes and incubated by constant mixing. Immunoprecipitated enzyme was collected by centrifugation and washed three times by each these buffers (A, B, and C) in sequence.

PI3K activity in these prepared cell lysates was evaluated using PI3 kinase ELISA kit (K1000, Echelon Biosciences, UT, USA) according to the manufacturer’s protocol (for details, refer to Supplementary material online).

2.11 Statistical analysis
All of the data in the present study are expressed as mean ± SEM. Differences between two groups were assessed using the Mann-Whitney U test. Comparisons among groups were made by ANOVA (non-parametric). When significance \( P < 0.05 \) was detected, a post hoc Dunns multiple-comparison test was performed. All statistical analyses were performed using Graph Pad software (version 4.0).

3. Results
3.1 Visfatin enhances MMP-2 and MMP-9 and decreases TIMP-1 and TIMP-2 mRNA levels in HUVECs
The angiogenic potential of HUVECs is greatly enhanced by the degradation of the extra-cellular matrix, where gellatinases MMP-2 and -9, and VEGF play a vital role. Enhanced MMP-2 and MMP-9 and a decrease in TIMP-2 and TIMP-1 mRNA were observed as early as 1 h but peaked at 4 h and then declined thereafter (data not shown). At 4 h, visfatin induced a dose-dependent increase in mRNA expressions of MMP-2 and MMP-9, which was significant at the maximum dose (Figure 1A and B: MMP-2 and -9, \( P < 0.01 \)). At 4 h, visfatin induced a significant dose-dependent increase, maximal response at 100 nM, in mRNA expressions of MMP-2 and MMP-9 (Figure 1A and B: MMP-2 and -9, \( P < 0.01 \)). However, mRNA levels of TIMPs, TIMP-1 and TIMP-2, regulators of MMP-9 and MMP-2 activity, respectively, concurrently decreased dose-dependently following visfatin treatment, with maximal effect at 100 nM (Figure 1C: TIMP-2, \( P < 0.05 \); Figure 1D: TIMP-1, \( P < 0.001 \)). VEGF, used as a positive control, revealed significant up-regulation of MMPs and down-regulation of TIMPs (Figure 1A–D: \( P < 0.001 \)).

3.2 Effect of visfatin on MMP-2 and MMP-9 protein expressions
3.2.1 Western blotting
Further, we measured protein expression of MMP-2 and MMP-9 following 24 h visfatin treatments (0–100 nM), by western blot analyses, and observed similar results as our mRNA findings (Figure 1E: MMP-2, six-fold increase, \( P < 0.001 \); Figure 1F: MMP-9, 5.2-fold increase, \( P < 0.001 \)).
Likewise, there was a significant decrease in TIMP-1 and TIMP-2 protein expressions (TIMP-1: *P* < 0.001; TIMP-2: *P* < 0.01; data not shown).

### 3.2.2 Zymography
Zymographic assessment of gelatinolytic activity of MMPs was performed and showed that following visfatin treatment, dose and time dependent; MMP-2 and MMP-9 activities in the conditioned media was significantly up-regulated, with maximal activity at 24 h and at 100 nM of visfatin (Figure 1G: MMP-2, 4.5-fold, *P* < 0.001 and Figure 1H: MMP-9, seven-fold, *P* < 0.001, when compared with basal).

### 3.3 Visfatin induces VEGF and VEGFR2 levels in HUVECs: role of VEGF/VEGFR2 in visfatin-induced MMP-2 and -9 production
Serum-starved HUVECs were treated with visfatin (0–100 nM) for 4 and 24 h. Visfatin induced a dose-dependent increase in VEGF mRNA (Figure 2A: *P* < 0.001). In a similar set of experiments, visfatin induced a dose-dependent (0–100 nM) increase of VEGF protein levels in cell lysates (Figure 2B: *P* < 0.001) and VEGF protein levels in conditioned media (Figure 2C: *P* < 0.001). Given that VEGF, acting through its receptor VEGFR2, is a known regulator of MMPs, we sought to determine whether visfatin-induced MMP production was VEGF dependent. VEGF significantly up-regulated VEGFR2 mRNA expression in HUVECs at 4 h (Figure 2D: *P* < 0.001), and interestingly the same was true for visfatin (Figure 2D: *P* < 0.001). In the light of visfatin’s stimulatory effects on VEGF, we employed sFlt1 and found a significant reduction in visfatin-induced VEGFR2 mRNA levels by 50% (Figure 2D: *P* < 0.01), suggesting both VEGF-dependent and VEGF-independent effects of visfatin on VEGFR2. Interestingly, prior to visfatin treatment of HUVECs, pre-incubation with sFlt1 resulted in significant reduction in both MMP-2 and -9 mRNA levels (Figure 2E and F: *P* < 0.05) and, more importantly, the gelatinolytic activity (Figure 2G and H: *P* < 0.01). Furthermore, HUVECs pre-incubated with VEGFR2 blocker, SU1498, for 1 h, followed by visfatin treatment, resulting in a significant down-regulation of mRNA levels (Figure 2E and F: MMP2, *P* < 0.01; MMP9, *P* < 0.05), and gelatinolytic activity (Figure 2G and H: *P* < 0.001). SU1498 (10 μM) was more efficacious at lowering MMP-9 than sFlt1 (*P* < 0.05), whereas in relation to MMP-2, this just failed to reach significance (*P* = 0.07).

![Figure 2](image_url)
These findings highlight the involvement of VEGF and its receptor in visfatin-induced MMP up-regulation.

3.4 Visfatin-induced endothelial cell proliferation
HUVEC proliferation was studied in a time-dependent manner (4–48 h), maximal response being noted at 24 h (data not shown). Treatment with visfatin (0–100 nM) or 10 ng/mL VEGF (positive control), at 24 h, led to a dose-dependent proliferative effect in visfatin/VEGF-treated samples [Figure 3A: 1.85-fold for VEGF (P < 0.001), and 1.65-fold at the maximal dose of visfatin (100 nM) compared with control; P < 0.01; n = 6 experiments]. These proliferative effects were confirmed by colorimetric Alamar blue cytotoxicity/proliferation assay (Supplementary material online, Figure S1C: 1.75-fold for VEGF (P < 0.001) and 1.55-fold at the maximal dose of visfatin, 100 nM, compared with control; P < 0.01; n = 6 experiments). Given that endothelial migration, similar to proliferation, is a critical step in angiogenesis, we assessed the migratory potential of visfatin.

3.5 Visfatin-induced endothelial cell migration
Serum-starved, Calcein-labelled HUVECs were subjected to migration assay, which were treated with dose-dependent visfatin (0–100 nM) and VEGF (10 ng/mL). The time points were 4, 8, 12, and 24 h. Visfatin increased migration in a dose-dependent and time-dependent manner, with a maximal effect at 100 nM and 24 h (Figure 3B: **P < 0.01, ***P < 0.001; visfatin treated vs. basal); VEGF, used as a positive control, also significantly increased HUVEC migration. Our data confirm the effect of visfatin on migration in HUVECs.

3.6 Anti-apoptotic effect of visfatin on endothelial cells
To evaluate whether the visfatin-induced proliferative effect was due to its anti-apoptotic activity, early- and late-stage apoptotic cells were analysed by Annexin V staining. Using the maximal proliferative dose of visfatin (100 nM), endothelial cells treated with both visfatin and H2O2 were subjected to flow cytometric analysis. Both, early- and late-stage apoptotic cells were sparse after combined treatment of visfatin and H2O2 (4%), compared with H2O2-treated

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Figure 3  Proliferative, migratory, and anti-apoptotic actions of visfatin. (A) Proliferation assay—MTS assay. Serum-starved HUVECs were treated with visfatin (0–100 nM)/VEGF (10 ng/mL) (positive control) for 24 h, and cell growth was assessed by MTS assay. Results were expressed as percentage of cells in relation to basal (untreated) and represent the mean of triplicates. ***P < 0.001, **P < 0.01, *P < 0.05 vs. control, n = 6. (B) Endothelial cell migration assay. Serum-starved Calcein-AM-labelled HUVECs were treated with visfatin (0–100 nM) for 4, 8, 12, and 24 h. VEGF (10 ng/mL) was used as a positive control. Migrated cells were quantified using a fluorescence plate reader. The migrated cells were expressed as the ratio of the fluorescence compared with the control. Results are means ± SE of six independent experiments. **P < 0.01, ***P < 0.001, vs. control. (C) Apoptosis assay. Annexin V staining for the assessment of apoptosis. (C1) and (C2) Flow cytometric analysis of HUVECs treated with H2O2 (200 μM) and visfatin (100 nM) + H2O2 (200 μM) for 8 h, respectively, and analysed by a four-colour flow cytometer. (C3) Percentage of treated cells that are live, early, and late apoptotic. (D)–(E) Visfatin-induced activation of ERK1/2. Serum-starved HUVECs were treated with (D) time (0–30 min) and (E) dose (0–100 nM) dependent visfatin, and phosphorylation of ERK1/2 was measured by western blot analysis. The results are represented as a ratio of phosphorylated to total protein and expressed as fold changes over basal. Results are means ± SEM of six independent experiments. ***P < 0.001, **P < 0.01, *P < 0.05 vs. basal, n = 6 experiments per group.
cells (65%; Figure 3C). These novel anti-apoptotic effects were further confirmed using DNA fragmentation assay (data not shown).

3.6 Visfatin-induced activation of ERK1/2

MAPK signalling pathways are involved in HUVEC proliferation. Interestingly, visfatin significantly phosphorylated both p42 and p44 (ERK1/2—extracellular signal-regulated kinase), maximally at 20 min, decreasing thereafter (Figure 3D: \( P < 0.001 \)). More importantly, visfatin dose-dependently (0–100 nM) phosphorylated ERK1/2 (Figure 3E: 23-fold, compared with controls; \( P < 0.001 \)). Visfatin-activated ERK1/2 was completely inhibited by U0126 (Supplementary material online, Figure S1A). Interestingly, visfatin had no effect on JNK activity (data not shown).

3.8 Visfatin-induced activation of PI3K/Akt signalling pathway

The PI3K/Akt pathway is known to regulate MMPs and VEGF, and play a role in angiogenesis. In order to address whether visfatin signals via this pathway in HUVECs, we treated HUVECs with visfatin and insulin (positive control for PI3K induction) compared with basal (untreated). Visfatin (0–100 nM) treated cell lysates showed a dose-dependent significant up-regulation in PI3K activity following 5 min of incubation [Figure 4A: visfatin (100 nM) showed a 2.1-fold increase compared with untreated cell lysates; \( P < 0.01 \)]. Treatment with insulin 1 \( \mu \)M (positive control) also showed a significant rise in PI3K activity (Figure 4A: 2.8-fold increase compared with untreated cell lysates; \( P < 0.001 \)).

Moreover, to investigate the downstream PI3K signalling pathway, we probed visfatin-treated cell lysates for Akt phosphorylation. On western blot analysis, we observed that visfatin time- and dose-dependently increased phosphorylation of Akt, with maximal response at 15 min (Figure 4B: \( P < 0.001 \)) of incubation with 100 nM visfatin (Figure 4C: 11.1-fold increase compared with basal; \( P < 0.001 \)). Both ERK1/2 phosphorylation and visfatin-activated Akt were significantly inhibited by the MEK inhibitor (U0126) and by the PI3K inhibitor, LY294002 (Supplementary material online, Figure S1A and B).

3.9 Involvement of ERK1/2 and PI3K/Akt signalling pathways in visfatin-induced MMP up-regulation and VEGF production

Given the involvement of MAPK and PI3K signalling in angiogenesis and visfatin’s pro-inflammatory effects, we sought...
to elucidate the involvement of MAPK and PI3K signalling in visfatin-induced MMP up-regulation and VEGF production.

HUVECs pre-incubated with an MEK inhibitor (U0126) and a PI3K inhibitor (LY294002), prior to visfatin treatment (100 nM), showed significant down-regulation of both MMP-2 and MMP-9 mRNA (Figure 5A and B: \( P < 0.001 \)) vs. visfatin-only treated). Moreover, the MMP-2 and MMP-9 gelatinolytic activities were significantly attenuated (Figure 5C and D: \( P < 0.001 \)) vs. visfatin-only treated). Similar findings were noted for VEGF mRNA expression, where both MEK and PI3K inhibitors significantly attenuated visfatin-induced VEGF induction (Figure 5E: \( P < 0.001 \)). VEGF protein production in cell lysates and conditioned media was significantly reduced by both MEK and PI3K inhibitors (Figure 5F and G: \( P < 0.001 \)). Similar down-regulatory effect was observed with VEGFR2 mRNA expression (Figure 5H: \( P < 0.001 \)).

3.10 Involvement of VEGF, PI3K, and MAPK signalling pathways in visfatin-induced gelatinolytic activity, endothelial cell proliferation, and capillary tube formation

Further experiments were conducted to elucidate the involvement of VEGF, MAPK, and PI3K signalling pathways on visfatin-induced gelatinolytic activity, endothelial proliferation, and capillary tube formation by using specific inhibitors. Visfatin-induced endothelial cell proliferation was significantly inhibited when HUVECs were pre-incubated with inhibitors: 10 \( \mu \text{M} \) of MAPK, PI3K, VEGF, and a global MMP inhibitor-GM6001 (Figure 6A: \( P < 0.001 \) vs. visfatin-only treated), respectively. Using the same inhibitors, visfatin-induced capillary tube formation was significantly attenuated (Figure 6B1: \( P < 0.001 \) vs. visfatin treated). When the condition media of the aforementioned proliferation assay was subjected to gelatin zymography, similar observations were noted (Figure 6C and D: \( P < 0.001 \) vs. visfatin only treated), implicating MMPs and VEGF in visfatin-induced HUVEC proliferation.

4. Discussion

Dysregulated angiogenesis is involved in conditions such as ischaemic heart disease, diabetes, or chronic inflammation, including atherosclerosis, and involves the VEGF–MMP system.\(^{20}\) Despite significant advances in therapeutic angiogenesis, the treatment of both macro- and micro-vascular ischaemic diseases remains a major concern. Extending diametrically opposed therapeutic advancements is necessary to deal with complex diseases such as diabetes and atherosclerosis.\(^{21}\) Although inhibition of endogenous growth...
factors seems beneficial in certain ophthalmic conditions, atherosclerotic plaques, and so on, the reverse is required in inducing angiogenesis for improving macro-vascular insufficiency, for example in peripheral limb ischaemia. Therefore, the study of VEGF–MMP system assumes crucial importance.

In the present study, we describe visfatin-induced production of VEGF and MMP-2 and -9 in both human micro- and macro-vascular endothelial cells. More importantly, we report that visfatin, whose plasma concentrations are altered in obesity and obesity-related disorders, induces migration, tube formation, and angiogenesis possibly through activation of VEGF–MMP pathways. In addition, we demonstrate for the first time the role of VEGF–MMP up-regulation and endothelial angiogenesis. During the preparation of our manuscript, a terse report by Kim et al. has demonstrated visfatin’s in vivo angiogenic potential in HUVECs via the ERK1/2 pathway. However, maintenance of capillary tube-like structures, a prerequisite for angiogenesis, requires anti-apoptotic mechanisms.

In our study, we extensively elucidate that ERK1/2 plays a role in visfatin-induced angiogenesis. In relation to its pro-angiogenic actions where visfatin activates PI3K/Akt signalling pathways, known to play a crucial role in angiogenesis, we also illustrate this function in tandem with MMP-2/9 and VEGF production. When HUVECs were pre-incubated with inhibitors of MEK and PI3K, visfatin-induced MMP expression and gelatinolytic activity were significantly reduced, supporting the involvement of these pathways in MMP induction. Likewise, these inhibitors decreased VEGF production and secretion. In addition, these inhibitors completely blocked visfatin-induced HUVEC proliferation and capillary-like tube formation. However, it is important to note that the observed reduction of visfatin-induced MMPs and VEGF by some of the kinase inhibitors resulted in below basal levels, possibly suggesting that the observed effects are not specific for visfatin. In relation to this, it is interesting to note that activation of Akt alone is sufficient for angiogenesis. Collectively, our observations highlight the importance of MAPK, and PI3K/Akt pathways in visfatin-induced VEGF production, MMP up-regulation, and endothelial angiogenesis.
Bearing our observations in mind, it is likely that the angiogenic property of visfatin may play a pathological role in the recruitment of blood inflammatory cells and the infiltration of macrophages and T-lymphocytes. Interestingly, Dahl et al. have recently suggested that visfatin may play a role in plaque destabilization, given that macrophages are laden with visfatin. Moreover, they have shown that visfatin induced an increase in MMP-9 activity in THP-1 monocytes. Our findings are therefore very timely and have revealed for the first time that visfatin directly induces VEGF production and secretion, as well as MMP-2 and MMP-9 production and gelatinolytic activity in HUVECs and HMECs. Both VEGF and MMP-2/-9 play critical roles in the initiation and progression of vascular pathology.29,30

A feature of early vascular remodelling is enhanced production of MMPs, in particular, MMP-2 and MMP-9, which are regulated by TIMP-2 and TIMP-1, respectively. Furthermore, activated MMPs contribute to a decrease in endothelial barrier function.35 Therefore, our observations of visfatin up-regulation of MMP-2/-9, with concurrent and dose-dependent decrease in TIMP-2 and TIMP-1, is of importance, as this would tip the MMP/TIMP balance in favour of matrix degradation. Furthermore, when the MMP inhibitor, GM6001, was pre-incubated with visfatin, there was a significant decrease in HUVEC proliferation and capillary tube formation, suggesting a potential causal relationship between visfatin-induced MMP activity and angiogenesis. In addition to MMPs, we have shown for the first time the effect of visfatin on the VEGF/VEGF2 system. Collectively, in tandem with Kim et al. and Dahl et al., our findings would tentatively support a role of visfatin in vascular pathologies.

MMPs are regulated by a number of cytokines and growth factors. VEGF is a specific endothelial mitogen, known to initiate and accelerate atherosclerosis, that acts via the VEGF type-II receptor (VEGFR2).40 We provide novel evidence that visfatin not only increases VEGF production, but significantly up-regulates VEGFR2. It is known that VEGF up-regulates VEGFR2 by a positive feedback mechanism; however, when pre-incubated with sFlt1, visfatin induction of VEGFR2 was attenuated by 50%, suggesting both VEGF-dependent and -independent mechanisms. In addition, visfatin-induced MMP expression and gelatinolytic activity were significantly reduced when pre-incubated with sFlt1 and SU1498; however, the VEGFR2 blocker compared with sFlt1 was significantly more potent at decreasing MMP-9 gelatinolytic activity. These observations were further extended to visfatin-induced endothelial proliferation and capillary tube formation. Our findings illustrate the crucial involvement of the VEGF system in visfatin-induced MMP up-regulation and angiogenesis. It should be emphasized that the inhibition of gelatinase activity by either the SU compound or sFlt1 is not selective, as sFlt1 scavenges VEGF and thereby affects activation of both R1 and R2; the SU compound is known to have limited selectivity for growth factor receptor tyrosine kinases and also blocks MAPK in a manner that is dependent on Raf-8 or MEK activation, but is not necessarily growth factor associated.41

At least two functionally distinct endothelial cell types, macro-vascular and micro-vascular, exist within most vascularized tissues. However, there are distinct morphogenetic, antigenic, and functional characteristics between micro-vascular and macro-vascular (HUVEC) endothelial cells, with differences in the secretion of vasoactive substances and the functional responses to external stimuli between these different cell types, which are likely to reflect differences in the activation of transcription factors that mediate signal transduction mechanisms in the two cell types. These differences notwithstanding, our functional effects of visfatin in both endothelial cells were similar.

In conclusion, we demonstrate for the first time the effects of visfatin on MMP up-regulation and VEGF production in human micro- and macro-vascular endothelial cells. Our findings suggest a functional interplay between visfatin and these pro-angiogenic molecules, via multiple signalling pathways including MAPK, PI3K/Akt, and VEGFR2. Finally, our data add to the diverse effects of visfatin, but more importantly reveal novel insights into the potential role(s) of visfatin in human CVD.

Supplementary material
Supplementary material is available at Cardiovascular Research online.

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