Visfatin activates eNOS via Akt and MAP kinases and improves endothelial cell function and angiogenesis in vitro and in vivo: translational implications for atherosclerosis

Lovren F, Pan Y, Shukla PC, Quan A, Teoh H, Szmitko PE, Peterson MD, Gupta M, Al-Omran M, Verma S. Visfatin activates eNOS via Akt and MAP kinases and improves endothelial cell function and angiogenesis in vitro and in vivo: translational implications for atherosclerosis. Am J Physiol Endocrinol Metab 296: E1440–E1449, 2009. First published April 7, 2009; doi:10.1152/ajpendo.90780.2008.—Improving endothelial nitric oxide synthase (eNOS) bioactivity and endothelial function is important to limit native, vein graft, and transplant atherosclerosis. Visfatin, a NAD biosynthetic enzyme, regulates the activity of the cellular survival factor, Sirt1. We hypothesized that visfatin may improve eNOS expression, endothelial function, and postnatal angiogenesis. In human umbilical vein (HUVEC) and coronary artery endothelial cells, we evaluated the effects of recombinant human visfatin on eNOS protein and transcript expression and mRNA stability, in the presence and absence of visfatin RNA silencing. We also assessed visfatin-induced protein kinase B (Akt) activation and its association with src-tyrosine kinases, phosphorylation of Ser1177 within eNOS in the presence and absence of phosphatidylinositol 3-kinase (PI 3-kinase) inhibition with LY-294002, and evaluated the contributory role of extracellular signal-regulated kinase (ERK1/2). Finally, we determined the impact of visfatin on HUVEC migration, proliferation, inflammation-induced permeability, and in vivo angiogenesis. Visfatin (100 ng/ml) upregulated and stabilized eNOS mRNA and increased the production of nitric oxide and cGMP. Visfatin-treated HUVEC demonstrated greater proliferation, migration, and capillary-like tube formation but less tumor necrosis factor-α-induced permeability; these effects were decreased in visfatin gene-silenced cells. Visfatin increased total Akt and Ser1177-phosphorylated Akt expression with concomitant rises in eNOS phosphorylation at Ser1177; these effects were blocked by LY-294002. Studies with PP2 showed that the nonreceptor tyrosine kinase, src, is an upstream stimulator of the PI 3-kinase-Akt pathway. Visfatin also activated mitogen-activated protein (MAP) kinase through PI 3-kinase, and mitogen/extracellular signal-regulated kinase inhibition attenuated visfatin-elicited Akt and eNOS phosphorylation. Visfatin-filled Matrigel implants showed an elevated number of infiltrating vessels, and visfatin treatment produced significant recovery of limb perfusion following hindlimb ischemia. These results indicate a novel effect of visfatin to stimulate eNOS expression and function in endothelial cells, via a common upstream, src-mediated signaling cascade, which leads to activation of Akt and MAP kinases. Visfatin represents a translational target to limit endothelial dysfunction, native, vein graft and transplant atherosclerosis, and improve postnatal angiogenesis.

nitric oxide; mice; endothelium; atherosclerosis; visfatin; protein kinase B; phosphatidylinositol 3-kinase; mitogen-activated protein kinase; endothelial nitric oxide synthase

THE ENDOTHELIUM PLAYS A CENTRAL ROLE in the maintenance of vascular homeostasis, and impaired endothelial function contributes to the development and clinical course of both native and vein graft atherosclerosis (3, 14, 22, 23, 27–29). Endothelial cell homeostasis is maintained largely through the synthesis of nitric oxide (NO), from the precursor L-arginine, under the influence of the enzyme nitric oxide synthase (eNOS) (17). NO serves several critical anti-inflammatory (9), anti-thrombotic (26), and anti-atherosclerotic (7) roles within blood vessels, in addition to promoting postnatal angiogenesis and reparative vasculogenesis (2).

Visfatin (nicotinamide phosphoribosyltransferase/pre-B cell colony-enhancing factor), has been described as a critical NAD biosynthetic enzyme that regulates the activity of Sirt1 in mammalian cells (19). Additional studies suggest that visfatin may function as a cytokine and enhance the maturation of B cell precursors in the presence of interleukin (IL)-7 and stem cell factor (21). Recent data have suggested that visfatin may also function analogous to a visceral fat adipocytokine and may alter insulin and glucose signaling, although this remains controversial (5). Evidence is accumulating to suggest that visfatin may serve as a cellular survival factor and may exert beneficial effects on both endothelial (8) and vascular smooth muscle cells (25). Indeed, in vascular smooth muscle cells, visfatin functions as a longevity protein, in part via optimizing Sirt1-mediated p53 degradation, whereas, in endothelial cells, visfatin may stimulate angiogenesis and augment endothelial survival (24).

Given the critical role of NO toward endothelial repair and regeneration, we hypothesized that visfatin may exert a direct effect to regulate eNOS expression. In this report, we define for
the first time a novel effect of visfatin to stimulate eNOS expression, in part through Akt and mitogen-activated protein (MAP) kinases, and improve endothelial cell function and angiogenic activity in vitro and in vivo.

METHODS

Materials. Recombinant human soluble visfatin (rh-visfatin) was purchased from Alexis Biochemicals. Predesigned annealed small-interfering RNA (siRNA) directed against human visfatin was obtained from Ambion. The dominant-negative mutant of protein kinase B (Akt) 1 (dnAkt) was obtained from VectorBiolabs. The following antibodies were purchased from Cell Signaling: rabbit polyclonal phospho-eNOS (Ser^1177), rabbit polyclonal phospho-Akt (Ser^473), rabbit polyclonal Akt, rabbit polyclonal phospho-extracellular signal-regulated kinase (ERK) 1/2, and rabbit polyclonal ERK1/2. Mouse monoclonal visfatin, mouse monoclonal eNOS, and mouse monoclonal actin antibodies were respectively obtained from Alpco Diagnostic, BD Bioscience, and Chemicon. All other chemicals, unless otherwise noted, were purchased from Sigma-Aldrich.

Cell culture. Human umbilical vein endothelial cells (HUVECs) and human coronary artery endothelial cells (HCAECs) were purchased from Cambrex. HUVECs, up to the seventh passage, were cultured in MCDB-131 complete medium (VEC Technologies) supplemented with 10% FBS. HCAECs were cultured in endothelial growth medium-2 (Cambrex) and used at passages two or three.

Visfatin measurement. Visfatin concentrations in the cell culture medium were determined with a human visfatin enzyme-linked immunosorbent assay kit (AdipoGen).

Endothelial cell transfection. Visfatin gene expression was silenced via siRNA transfection. Briefly, the siRNA (30 nM), 100 μl Opti-MEM (Invitrogen), and the transfection reagent siPORT NeoFX (Ambion) were incubated according to the manufacturer’s recommendations (Ambion). The cocktail was dispensed in empty six-well plates that were then plated with HUVECs (1 × 10^5/well). The transfection media were removed 24 h later, and the cells were maintained in MCDB-131 complete medium. Visfatin downregulation was confirmed by RT-PCR and Western blot techniques. In some experiments, HUVECs were transfected with dnAkt (multiplicity of infection 50) for 18 h.

RNA analysis. Total HUVEC and HCAEC RNA, extracted with the RNAeasy Plus Mini Kit (Qiagen), were reverse transcribed with Omniscript Reverse Transcriptase (Qiagen) before undergoing real-time PCR reactions on the ABI PRISM 7900HT system (Applied Biosystems). Oligonucleotide primers for human visfatin were designed from the GeneBank databases (NM_005746) using the ProbeFinder software (Roche Applied Science): (forward) 5'-AGGCTTGTGCATTCTTCCAGA and (reverse) 5'-TGCCCACTGTATTGATGATCC. Primer sequences for eNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) have been reported previously (10, 13). For each PCR assay, standard curves were prepared by serial dilutions of cDNA. The efficiencies of the amplifications with each primer set were calculated from the slope of the standard curve [efficiency = (10^(-1/slope)) − 1]. The mRNA expression of each target gene was normalized against the corresponding GAPDH expression [ratio = 2^[(Ct(sample) − Ct(control))]/(efficiencyGAPDH(sample) × efficiencyGAPDH(control − sample))] (18).

Western blotting. Western blot analysis was performed according to standard procedures. Proteins from whole cell lysates of HUVECs and HCAECs were separated on 4–12% Tris-glycine gels (Invitrogen) and transferred to nitrocellulose membranes (Invitrogen). Complete and uniform transfer was confirmed by Ponceau S staining. Membranes were probed with antibodies against visfatin, eNOS, phospho-eNOS, Akt, phospho-Akt, ERK1/2, phospho-ERK1/2, and actin followed by incubation with appropriate horseradish peroxidase-associated secondary antibodies before signals were visualized with the enhanced chemiluminescence detection system (Amersham Bioscience). Phospho-eNOS, phospho-Akt, and phospho-ERK1/2 measurements were performed in HUVECs that had been treated with the phosphatidylinositol 3-kinase (PI 3-kinase) inhibitor LY-294002 (50 μM) for 2 h, the tyrosine kinase inhibitor PP2 (10 μM) for 30 min, the mitogen/ extracellular signal-regulated kinase inhibitor PD-98059 (50 μM) for...
30 min, or transfected with dnAkt. All cells, regardless of pretreatment, were stimulated with visfatin (100 ng/ml) for 5–30 min.

**Immunostaining.** HUVECs and HCAECs were seeded on chamber slides (Lab-Tek) and grown to 70–80% confluence. Paraformaldehyde (4%)-fixed cells were incubated with anti-visfatin antibody (1:70) followed by an anti-mouse fluorescein isothiocyanate (FITC)-linked secondary antibody (Molecular Probes) (1:20). Nuclei were counterstained with 4′,6′-diamidino-2-phenylindole.

**Endothelial cell function.** Cell proliferation, tube formation, migration, and permeability were assessed in HUVECs treated with rh-visfatin (100 ng/ml) for 24 h and siRNA-transfected 24-h-starved HUVECs.

**Proliferation.** HUVECs, seeded at a density of $1 \times 10^4$ cells/well in 96-well plates, were treated with 100 ng/ml visfatin or transfected with visfatin siRNA before incubation with bromodeoxyuridine (BrdU) for 18 h (Roche Diagnostic) at a final concentration of 10 mM. BrdU incorporation was determined by a commercially available enzyme-linked immunosorbent assay (Roche Diagnostic).

**Tube formation assay.** HUVECs were seeded at $4 \times 10^4$/well in 96-well plates coated with 50 μl/well of ECMatrix (BD Bioscience).

![Figure 2](image_url) **Fig. 2.** Visfatin regulation of endothelial nitric oxide synthase (eNOS) mRNA and protein expression is concentration- and time-dependent. A: HUVECs were starved for 24 h before treatment with 0–100 ng/ml visfatin or 1 μM bradykinin (BK) for 24 h. B and C: starved HUVECs were incubated with 100 ng/ml visfatin for 0–72 h before mRNA and protein analyses. D: starved HUVECs were incubated with 100 ng/ml visfatin for up to 24 h before treatment with 5 μM actinomycin D. eNOS expression was evaluated 0, 8, and 24 h thereafter. GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Data are presented as means ± SE; *P < 0.05 vs. vehicle control.

![Figure 3](image_url) **Fig. 3.** Visfatin-induced eNOS stimulation in HUVECs leads to an increase in nitric oxide (NO) production. NO levels in the cell culture medium were measured after HUVECs were incubated with 100 ng/ml visfatin. Data are presented as means ± SE; *P < 0.05 vs. control (n = 5 experiments).

![Figure 4](image_url) **Fig. 4.** Visfatin modulates cGMP accumulation in mouse aortas. cGMP was measured in aortas that had been incubated with visfatin (100–500 ng/ml) or acetylcholine (10 μM). Values are means ± SE; *P < 0.05 vs. control (n = 3–4).
The extent of tube formation, 4 h after treatment with 100 ng/ml visfatin or transfection with visfatin siRNA, was determined in five random microscopic fields and quantified with the NIH ImageJ analysis software.

**Migration assay.** Visfatin-treated (100 ng/ml) or visfatin siRNA-transfected HUVECs (5 × 10⁵ cells in 500 μl of 0.5% BSA-supplemented medium) were placed in the upper chambers of modified fibronectin (50 μg/ml)-coated Boyden chambers (pore size 8 μm; BD Bioscience). After 4 h, migrating cells on the lower side of the chamber were fixed, stained with a Diff-Quik Stain (Dade Behring), and quantified in three random fields.

**Permeability.** Visfatin-treated (100 ng/ml) or visfatin siRNA-transfected HUVECs, seeded at a density of 2.7 × 10⁵ cells/well in collagen-coated Transwell inserts (12-well clusters, 0.4-μm pore size; Corning), were allowed to establish a monolayer that occluded the membrane pores. At initiation, the medium in the upper compartment of each insert was replaced with 0.5 ml of a FITC-BSA tracer solution (250 mg/ml in complete medium; Invitrogen), and in the lower compartment was replaced with 1.5 ml of fresh medium. Permeability was detected 12 h later by FITC-BSA transmigration from the upper chamber of the Transwell insert system to the lower chamber across the cell monolayer.

**Fig. 5.** Visfatin stimulates angiogenesis in HUVECs. A: endothelial cell proliferation, as determined by bromodeoxyuridine (BrdU) incorporation, was measured in HUVECs treated with visfatin (100 ng/ml) alone or transfected with visfatin small-interfering RNA (siRNA). Data are presented as means ± SE; *P < 0.05 vs. control (n = 5). B: tube-forming activity was determined in HUVECs 4 h after initiating treatment with visfatin (100 ng/ml) alone or coincubated with N⁵-nitro-L-arginine methyl ester (L-NAME, 0.1 mM) or transfection with visfatin siRNA. Data are presented as means ± SE; *P < 0.05 vs. control (n = 5). C: representative micrographs of tube-forming activity in HUVECs. D: endothelial cell migration was measured in HUVECs that had been treated with visfatin (100 ng/ml) alone or together with L-NAME (0.1 mM) for 24 h or transfected with visfatin siRNA. *P < 0.05 vs. control. E: cell permeability was determined in HUVECs treated with visfatin (100 ng/ml) for 24 h or transfected with visfatin siRNA before incubation with tumor necrosis factor (TNF)-α (10 ng/ml) for 6 h. Data are presented as means ± SE; *P < 0.05 vs. control (n = 6). F: visfatin mRNA and protein expression in endothelial cells transfected with visfatin siRNA (30 nM). PCR values are presented as means ± SE; *P < 0.05 vs. control (n = 3).
Visfatin is constitutively expressed in endothelial cells. In the first series of experiments, we evaluated whether visfatin is

Measurement of NO production. Nitrite and nitrate levels were used as surrogates to determine NO production. Total nitrite concentrations were spectrophotometrically measured at 540 nm with the Griess reagent. Specifically, nitrates were reduced with nitrate reductase (Roche) before nitrite levels were quantified in a total volume of 0.5 ml with 480 μl of culture supernatant, 10 μl of nitrate reductase (10 U/ml), and 10 μl of NADPH (5 mM in Tris-Cl) for 3 h at room temperature. Sample nitrite levels were calculated against a standard curve prepared with 0.1–10 μM NaNO₂.

Mice. All animal procedures were performed in accordance with the guidelines of the Canadian Council on Animal Care and approved by the Institutional Animal Care Committee.

Measurement of cGMP production. cGMP production was evaluated ex vivo by an enzyme immunoassay (Amersham). Isolated thoracic aortas from 10- to 12-wk-old male C57Bl/6 mice underwent a 2-min incubation with visfatin (100–500 ng/ml) or acetylcholine (10 μM) before being snap-frozen in liquid nitrogen and homogenized in ice-cold 6% trichloroacetic acid for retrieval of the supernatant fraction.

In vivo Matrigel plug assay. Matrigel HC (0.5 ml; BD Bioscience) was injected subcutaneously into the dorsal neck area of 8-wk-old male Balb/c mice. Briefly, the skin overlying the abdomen and left limb was incised to allow ligations of the proximal end of the femoral artery and the distal portion of the saphenous artery before complete excision of the femoral artery and its attached side branches. Immediately following the surgical procedure, the visfatin plasmid (500 μg; Origene) was injected intramuscularly at three locations of the adductor muscle and at two locations of the gastrocnemius muscle of one leg with the other leg serving as control. Visfatin gene expression in these muscles, 1 and 2 wk postprocedure, was confirmed by real-time PCR. The effect that visfatin administration had on perfusion recovery after induction of ischemia was determined by laser Doppler flow imaging at days 0 (postsurgery), 2, 7, and 14.

Statistics. All values are presented as means ± SE. The one-way ANOVA test, accompanied where appropriate by post hoc Tukey’s test, was used for comparing multiple treatment groups. Comparisons between two treatment groups were analyzed by the Student’s t-test. Differences were considered significant at P < 0.05.

RESULTS

Visfatin is constitutively expressed in endothelial cells. In the first series of experiments, we evaluated whether visfatin is
expressed in human endothelial cells in culture. Protein and transcript (mRNA) analyses revealed visfatin expression in cultured venous (HUVECs) and cultured arterial (HCAECs) endothelial cells (Fig. 1, A and B). Immunostaining localized visfatin to within the endothelial cytoplasm and nuclei in both HUVECs and HCAECs (Fig. 1C). Visfatin concentrations in the cell culture medium were below the lowest measurable level of 0.25 ng/ml.

Visfatin activates eNOS mRNA expression in a concentration- and time-dependent fashion and promotes NO release and cGMP production. We next determined whether visfatin activates eNOS expression in endothelial cells. As shown in Fig. 2A, rh-visfatin upregulated endothelial eNOS mRNA expression in a concentration-dependent manner with the highest concentration of visfatin used (100 ng/ml) evoking a response that was 214 ± 19% of the control group (P < 0.05). Importantly, elevations in HUVEC eNOS expression following incubation with visfatin (50 and 100 ng/ml) were similar to that detected after stimulation with 1 μM bradykinin (Fig. 2A), a known activator of eNOS. Silencing of the visfatin gene with visfatin siRNA diminished eNOS expression (53 ± 7% vs. siRNA control group, P < 0.05). Visfatin also elevated eNOS mRNA expression in a time-dependent manner (Fig. 2B), with the maximum response seen at 48 h postinitiation of incubation. Similarly, visfatin time-dependently upregulated eNOS protein expression in endothelial cells (Fig. 2C). The half-lives of eNOS mRNA under basal tissue culture conditions and following incubation with 100 ng/ml visfatin were 17 ± 3 and 30 ± 2 h, respectively (Fig. 2D), suggesting that visfatin may augment eNOS expression, in part through improving transcript stability.

The effects of visfatin to augment eNOS expression were associated with a net increase in NO release from endothelial cells. The increase in NO production was time-dependent, reaching a three-fold rise following incubation with visfatin for 72 h (P < 0.05) (Fig. 3). NO production following 24–72 h incubation with visfatin was abolished in endothelial cells coincubated with NG-nitro-L-arginine methyl ester (L-NAME, 1 mM) (data not shown).

Fig. 8. Quantitative temporal analyses of visfatin-induced eNOS, Akt, and ERK1/2 phosphorylation following LY-294002, PP2, and PD-98059 treatments. Data are presented as means ± SE; *P < 0.05 vs. control (n = 6).
To further corroborate the observed effects of visfatin on eNOS activation, we evaluated the production of cGMP, the key second messenger of NO activation, in mouse aortas following incubation with rh-visfatin. Visfatin exposure evoked a concentration-dependent increase in cGMP production, with 100 ng/ml visfatin increasing cGMP content by 350% over the control value (P < 0.05) (Fig. 4).

Visfatin promotes key NO-mediated functions in endothelial cells. We next evaluated whether visfatin-mediated upregulation of eNOS is associated with an improvement in NO-mediated endothelial cell functional responses. Endothelial cells treated with rh-visfatin (100 ng/ml) demonstrated greater proliferation, migration, and capillary-like tube formation consistent with a role to favorably modulate eNOS expression. Furthermore, visfatin treatment of endothelial cells attenuated tumor necrosis factor (TNF)-α-induced permeability compared with those in the vehicle-treated group, suggestive of a role of visfatin in improving endothelial barrier function (Fig. 5, A–E). Silencing of endogenous visfatin gene expression, as confirmed by real-time PCR and Western blotting (Fig. 5F), diminished endothelial cell proliferation, migration, and tube formation while increasing TNF-α-induced permeability (Fig. 5, A–E). To gain additional information on the mechanism of visfatin-stimulated NO production in HUVECs, we pretreated cells with l-NAME (0.1 mM). Tube formation and migration responses to visfatin were suppressed in endothelial cells previously incubated with l-NAME suggesting that eNOS activation is integral to visfatin-stimulated NO production (Fig. 5, B–D).

Visfatin stimulates eNOS phosphorylation at Ser1177 in part via PI 3-kinase/Akt activation. To determine if visfatin stimulation of eNOS requires eNOS phosphorylation at Ser1177, endothelial cells were incubated with visfatin (100 ng/ml) for up to 30 min. As shown in Fig. 6, eNOS phosphorylation at Ser1177 in HUVECs was observed as early as 5 min after visfatin stimulation, reaching a maximum response at the 30-min time point.

Several stimuli are known to activate eNOS through Akt-mediated phosphorylation of the enzyme. Hence we evaluated the involvement of Akt in the phosphorylation and stimulation of eNOS by visfatin. Akt is activated by PI 3-kinase through recruitment to the plasma membrane where Akt becomes phosphorylated at Ser473 and Ser308. Akt phosphorylation at Ser473 and ERK1/2 phosphorylation were respectively detected 5 and 10 min poststimulation with visfatin (Fig. 6).

LY-294002, a PI 3-kinase inhibitor, inhibited visfatin-evoked eNOS, Akt, and ERK1/2 phosphorylation (Fig. 6), indicating that PI 3-kinase is the upstream effector of ERK1/2 activation by visfatin.

Tyrosine kinases frequently serve as upstream stimulators of PI 3-kinase, and the involvement of nonreceptor tyrosine kinase (src family) has been demonstrated for eNOS stimulation by a number of factors. To determine the effects of the src kinase family as an activator of PI 3-kinase by visfatin, we evaluated the effect of PP2, a specific src kinase inhibitor, on visfatin-induced eNOS phosphorylation. As shown in Fig. 6, PP2 suppressed both eNOS and Akt phosphorylation and attenuated ERK1/2 activation. These data suggest that visfatin stimulates tyrosine kinases that are most likely src family kinases, which in turn activate the PI 3-kinase-Akt-MAP kinase pathway to ultimately lead to eNOS phosphorylation.

eNOS activation is ERK1/2- and Akt/PKB-dependent. To determine if ERK1/2 phosphorylation and Akt phosphorylation are involved in visfatin-mediated eNOS activation, endothelial cells were pretreated with PD-98059 and dnAkt. PD-98059 (50 μM) abrogated eNOS, Akt, and ERK1/2 activation by visfatin (Fig. 7). Visfatin-elicited eNOS and ERK1/2 phosphorylations were also attenuated by treatment with dnAkt (Fig. 7), suggesting the Akt activation is fundamental to ERK1/2 phosphorylation by visfatin and in turn phosphorylates eNOS.

Fig. 9. Visfatin stimulates neovascularization in vivo. Representative micrographs (A) and quantitative summary (B) of blood vessel infiltration in Matrigel sections stained with α-smooth muscle actin. Quantitative results are presented as means ± SE; *P < 0.05 (n = 5).
Visfatin modulates neovascularization in vivo. Having demonstrated that visfatin has a key role in modulating eNOS bioactivity and endothelial function in vivo, we evaluated the potential translational in vivo implications of these observations. To this aim, we monitored the effects of visfatin in a Matrigel plug assay. Compared with control samples, rh-visfatin-infused Matrigel implants showed a significantly greater number of infiltrating vessels (Fig. 9).

We next used the murine model of hindlimb ischemia to further characterize the in vivo relevance of the above-mentioned ex vivo observations. As assessed by laser Doppler perfusion, mice that received the visfatin plasmid showed improved limb perfusion at day 7 postsurgery, with significantly increased perfusion compared with untreated mice on day 14 postsurgery (Fig. 10).

DISCUSSION

Visfatin was cloned by Samal et al. (21) in 1994 from activated peripheral blood lymphocytes, as a factor that promotes differentiation of B cell precursors. Although the exact role of visfatin continues to be elucidated, a critical role in pathobiology is suggested by its high degree of conservation throughout evolution (4, 11, 12, 16). Accumulating evidence suggests that visfatin exerts important dual effects as an intracellular cell cycle regulatory protein (iNampt), in addition to secreted cytokine (eNampt), capable of modulating innate immunity and inflammatory responses across various cell types. From an intracellular standpoint, visfatin is a dimeric type II phosphoribosyltransferase that serves a critical role as the rate-limiting step in the biosynthesis of NAD from nicotinamide and regulates the activity of NAD-dependent deacetylase Sirt1 in mammalian cells (30). On the other hand, visfatin appears to play an important role as a cytokine, secreted extracellularly in response to inflammatory stimuli such as lipopolysaccharide, TNF-α, IL-1β, and IL-6, capable of modulating the innate immune response in states of systemic inflammation and sepsis (6, 15). Indeed, elegant experiments by Jia and colleagues (6), clearly demonstrate that visfatin is upregulated in neutrophils and macrophages, by proinflammatory stimuli of both host and microbial origin, and that it plays an essential role in inhibiting neutrophil apoptosis. Conversely, in vitro and ex vivo studies demonstrate that rh-visfatin activates CD14+ monocytes, inducing inflammatory cytokine elaboration while increasing the surface expression of costimulatory molecules CD54, CD40, and CD80 (15). Recent data have suggested that, in addition to the afore-mentioned roles, visfatin may function as an adipocytokine, released from visceral adipose tissue, capable of modulating insulin sensitivity (5). Although this effect remains controversial, elegant studies now suggest that, whereas extracellular visfatin does not have a direct insulin-mimetic effect, it may regulate insulin secretion within pancreatic β-cells through modulating NAD biosynthetic activity (20).

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Recent studies implicate an important role of visfatin as both an intracellular and extracellular regulator of vascular function. Indeed, visfatin has been described to extend the life span of smooth muscle cells, via augmenting SIRT1-mediated p53 degradation (24, 25). Furthermore, visfatin, in a NAD-dependent fashion, promotes the acquisition of a mature smooth

Fig. 10. Visfatin stimulates neovascularization in mice following hindlimb ischemia. A: Balb/c mice were subjected to unilateral (right) hindlimb ischemia before administration of the visfatin plasmid. Perfusion was assessed 0 and 14 days postprocedure using laser Doppler imaging. The highest perfusion level is shown in red. B: quantitative perfusion results 14 days after hindlimb ischemia was induced are presented as means ± SE; *P < 0.05 (n = 5).
muscle cell phenotype, an important step in terminating smooth muscle cell-mediated remodeling of diseased arteries. These data suggest that visfatin may function as a survival factor in smooth muscle cells and serve to limit aberrant vascular remodeling.

A few previous studies have evaluated the role of visfatin in endothelial cells and have suggested that visfatin may function to improve endothelial cell function and support postnatal angiogenesis (1, 8). Although the mechanism(s) of these effects remain unclear, they may involve activation of MAP kinase ERK-dependent pathways, upregulation of vascular endothelial growth factor, and/or stimulation of PI 3-kinase/Akt pathways.

eNOS plays a critical role in maintaining vascular homeostasis by exerting a plethora of anti-inflammatory and anti-thrombotic effects while actively promoting endothelial repair, regeneration, and postnatal neovascularization. In the present study, we hypothesized that visfatin may directly upregulate eNOS expression and, via this mechanism, may serve to regulate endothelial cell function. We make the novel observation that visfatin markedly upregulates protein and mRNA expression of eNOS in both human venous and coronary artery endothelial cells. eNOS bioavailability is regulated by at least three different mechanisms, including transcriptional upregulation of eNOS, posttranscriptional activation of eNOS, and reduction of reactive oxygen species-mediated breakdown of NO. We demonstrate that visfatin affects endothelial NO synthesis by increasing eNOS mRNA stability and posttranscriptional stimulation of eNOS mRNA. Because phosphorylation of Ser1177 within eNOS by Akt is critical for activation of eNOS, we investigated the effects of visfatin in this regard. These studies clearly indicate that visfatin activates protein kinase Akt, which leads to posttranscriptional activation of eNOS via phosphorylation of the amino acid Ser1177. We next probed the role of the PI 3-kinase pathway, which is known to play an important role in the regulation of eNOS synthesis by Akt. Inhibition of PI 3-kinase by LY-294002 inhibited visfatin-induced phosphorylation of Akt, demonstrating the specific effect of visfatin on the activation of the PI 3-kinase/Akt pathway. We have also demonstrated additional proximal signaling events. We demonstrate that a src family tyrosine kinase is a further upstream stimulator of the PI 3-kinase/Akt pathway by visfatin. MAP kinases are also known to play a role in eNOS regulation, in addition to PI 3-kinase/Akt activation. We have demonstrated that visfatin stimulates ERK1/2 phosphorylation and that src kinases are upstream activators of MAP kinase activation by visfatin. Furthermore, MAP kinase activation plays a role in visfatin-induced Akt phosphorylation and eNOS phosphorylation (Fig. 6). Both Akt and MAP kinases play an important role in endothelial survival, repair, and regeneration. Our results indicate that visfatin may modulate eNOS bioavailability through dual pathways and improve angiogenesis ex vivo and in vivo.

It is important to point out that, in addition to mediating eNOS activation, Akt may engage several NO-independent pathways, including glycojen synthesis kinase 3, p21/p27, EDG-1, and FOXO, which serve to regulate endothelial survival and function. Furthermore, Akt plays a direct role in the mobilization of bone marrow-derived endothelial progenitor cells, which play an important role in postnatal neovascularization. Therefore, the ability of visfatin to augment Akt phosphorylation may uncover many NO-dependent and -independent signaling pathways to improve vascular function.

Translational Implications

Taken together, the present study demonstrates, for the first time, a novel effect of visfatin to modulate eNOS expression and function in endothelial cells. This effect may occur via an upstream, src-mediated signaling cascade that leads to parallel activation of Akt and MAP kinases (Fig. 10). We suggest that approaches aimed at augmenting visfatin bioactivity may limit endothelial dysfunction and native and vein graft atherosclerosis and improve postnatal angiogenesis. Translational studies evaluating the role of visfatin to attenuate atherothrombosis are warranted.

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