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Visfatin exerts angiogenic effects on human umbilical vein endothelial cells through the mTOR signaling pathway

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ABSTRACT

The biologically active factors known as adipocytokines are secreted primarily by adipose tissues and can act as modulators of angiogenesis. Visfatin, an adipocytokine that has recently been reported to have angiogenic properties, is upregulated in diabetes, cancer, and inflammatory diseases. Because maintenance of an angiogenic balance is critically important in the management of these diseases, understanding the molecular mechanism by which visfatin promotes angiogenesis is very important. In this report, we describe our findings demonstrating that visfatin stimulates the mammalian target of the rapamycin (mTOR) pathway, which plays important roles in angiogenesis. Visfatin induced the expression of hypoxia-inducible factor 1α (HIF1α) and vascular endothelial growth factor (VEGF) in human endothelial cells. Inhibition of the mTOR pathway by rapamycin eliminated the angiogenic and proliferative effects of visfatin. The visfatin-induced increase in VEGF expression was also eliminated by RNA interference-mediated knockdown of the 70-kDa ribosomal protein S6 kinase (p70S6K), a downstream target of mTOR. Visfatin inactivated glycogen synthase kinase 3β (GSK3β) by phosphorylating it at Ser-9, leading to the nuclear translocation of β-catenin. Both rapamycin co-treatment and p70S6K knockdown inhibited visfatin-induced GSK3β phosphorylation at Ser-9 and nuclear translocation of β-catenin. Taken together, these results indicate that mTOR signaling is involved in visfatin-induced angiogenesis, and that this signaling leads to visfatin-induced VEGF expression and nuclear translocation of β-catenin.

1. Introduction

Obesity is strongly linked to diabetes mellitus and several other metabolic and cardiovascular disorders [1]. Recently, adipose tissue has emerged as an endocrine organ that is the primary producer of adipocytokines, a group of secreted, biologically active growth factors and cytokines that include adiponectin, interleukin-6, leptin, and visfatin (pre-B-cell colony-enhancing factor; PBEF) [2]. These adipocytokines have various biological functions and can have cardiovascular effects [3].

Visfatin was originally cloned from human peripheral blood lymphocytes and characterized as nicotinamide phosphoribosyltransferase (Nampt), an enzyme that synthesizes nicotinamide mononucleotide from nicotinamide [4,5]. Subsequently, it was recognized as an adipocytokine preferentially produced by visceral fat and renamed visfatin. Interestingly, visfatin has been reported to have insulin-mimetic effects in various cultured cell lines and in insulin-sensitive tissues, although these effects remain controversial [6,7].

Several recent studies have suggested that visfatin has angiogenic properties [8–11]. Angiogenesis, the process of new blood vessel formation from existent vasculature, is important not only in tissue homeostasis, embryonic development, and wound healing but also in some pathologic conditions, including tumor growth, diabetes, and inflammation [12]. Angiogenesis is also important as a therapeutic target for myocardial infarction, ischemic stroke, and other ischemic conditions.

Several studies have shown that visfatin levels are elevated in diabetes, cancer, and inflammatory disease [13–16]. Visfatin activates Akt (the serine/threonine protein kinase B) and induces the expression of vascular endothelial growth factor (VEGF) in human endothelial cells [9]. However, the precise downstream signaling mechanism underlying these effects and its ability to induce angiogenesis are not yet fully understood. Since maintaining angiogenic homeostasis is very important in preventing the progression of these diseases, understanding the molecular mechanisms by which visfatin promotes angiogenesis is an important research goal.

The serine/threonine kinase known as mTOR (mammalian target of rapamycin) is a critical regulator of cellular proliferation and metabolism. The mTOR pathway integrates the signals of various factors, including Ras, Akt, and NF-κB, that respond to nutrients,

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energy status, and growth factors [15,16]. Dysregulation of mTOR signaling is frequently associated with tumor growth, metastasis, and angiogenesis [17]. Rapamycin, an inhibitor of the mTOR pathway, is used clinically for anticancer therapy and to prevent stent restenosis [18].

In the present study, we investigated the possible involvement of the mTOR signaling pathway in visfatin-induced angiogenesis and characterized the downstream molecular effectors of visfatin. Our data show for the first time that visfatin induces angiogenesis in human endothelial cells *via* activation of the mTOR pathway. They also demonstrate that activation of the mTOR pathway increases VEGF protein expression and β-catenin nuclear translocation by inactivating glycogen synthase kinase 3β (GSK3β).

2. Materials and methods

2.1. Materials

Recombinant visfatin and a phosphoinositide 3 (PI3)-kinase inhibitor, LY294002 were purchased from Peprotech (Rocky Hill, NJ) and Calbiochem (San Diego, CA), respectively. Visfatin protein used in this study was >98% pure (SDS-PAGE analysis) and contained 0.01 ng/μg LPS as determined by the *Limulus* amebocyte lysate similar to the previous study [19]. Rapamycin, differentiation inducing factor-3 (DIF-3), lithium chloride (LiCl), and a specific competitive inhibitor of visfatin, FK866 were purchased from Sigma (St Louis, MO). Recombinant human VEGF was from R&D Systems (Minneapolis, MN). Growth factor-reduced Matrigel was purchased from BD Biosciences (San Jose, CA). The primary antibodies used in this study were specific for Ser-473-phosphorylated Akt, Ser-2448-phosphorylated mTOR, Ser-9-phosphorylated GSK3β, Thr-389-phosphorylated 70-kDa ribosomal protein S6 kinase (p70S6K), Ser-241-phosphorylated phosphoinositide-dependent kinase-1 (PDK1), HIF1α (Cell Signaling Technology, Beverly, MA), VEGF (R&D Systems), β-catenin (BD Biosciences), cyclin D1 (BD Biosciences), visfatin (Phoenix Pharmaceuticals, Belmont, CA), and β-actin (Sigma).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs; Clonetics, Walkersville, MD) at passages 3–6 were grown in SingleQuots endothelial cell growth medium-2 (EGM-2; Clonetics) at 37 °C in a humidified 5%-CO_2 atmosphere. The medium was replaced with endothelial cell basal medium-2 (EBM-2; Clonetics) supplemented with 0.1% fetal bovine serum (FBS) 5 h before all experimental treatments, with the exception of tube formation and aortic ring-sprouting assays.

2.3. Western blotting

Cells were lysed in a lysis buffer (Cell Signaling Technology) containing 50 mM NaF, 2 mM Na_3VO_4, 100 μg/ml phenylmethylsulfonyl fluoride, and protease inhibitor cocktail (Sigma). Protein concentrations in the lysate samples were determined using Bradford Assay Reagent (Bio-Rad Laboratories, Hercules, CA) with bovine serum albumin (BSA) as a standard. Equal amounts of cell lysates were subjected to SDS-PAGE, and the separated proteins were transferred to a polyvinylidene fluoride membrane (Millipore, Bedford, MA). The membrane was incubated with primary antibody overnight at 4 °C and then with the appropriate secondary antibody for 1 h at room temperature. Bands were detected using an ECL system (Santa Cruz Biotechnology, Santa Cruz, CA). All experiments were performed at least three times.

2.4. Tube formation assay

Tube formation assays were carried out using Matrigel according to the manufacturer's protocol (BD Biosciences). Briefly, HUVECs were cultured on the surface of the Matrigel, treated with visfatin (1 μg/ml) in the presence or absence of rapamycin (25 nM), and the extent of capillary tube formation 15 h after treatment was observed in three random microscopic fields. As a positive control, the cells were treated with VEGF (20 ng/ml) rather than visfatin.

2.5. Aortic ring-sprouting assay

Aortic ring-sprouting assays were performed using a protocol modified from a previous study [20]. Each well of a 24-well plate was coated with Matrigel, and the Matrigel was allowed to polymerize for 30 min at 37 °C. Aortas were extracted from 6-week-old male C57BL/6 mice, and the adipose tissue was carefully removed under a microscope. The aortas were cut into 1-mm-thick slices, rinsed four times with EGM-2 medium, placed on the Matrigel-coated plates, and covered with additional Matrigel. The Matrigel was allowed to solidify at 37 °C, and the EGM-2 medium was added. After 24 h, the medium was replaced with fresh EBM-2 media supplemented with 2% FBS, ascorbic acid, hydrocortisone, heparin, and amphotericin, and visfatin, rapamycin, and/or VEGF were added. After 4 days of incubation, vascular sprouts from the aortic tissues were photographed. Mice were housed and treated in accordance with the Animal Care Guidelines of the Korean National Institutes of Health Animal Facility.

2.6. 5-bromodeoxyuridine (BrdU) incorporation assay

BrdU incorporation was assayed using a Cell Proliferation ELISA BrdU (colorimetric) kit (Roche, Mannheim, Germany) according to the manufacturer's instructions. Briefly, HUVECs treated with visfatin in the presence or absence of rapamycin were labeled with BrdU for 8 h, fixed, and washed. Anti-BrdU-POD working solution and substrate solution were added, and BrdU incorporation was quantified by measuring the absorbance at 370 nM on an automated ELISA microplate reader (Molecular Devices, Sunnyvale, CA).

2.7. In vitro wound-healing migration assay

HUVECs were seeded into 12-well plates and grown to 90% confluence. Wounds were made by careful scraping of the cell layer with sterile cell scrapers. The medium was replaced with EBM-2 media supplemented with 0.1% FBS and visfatin with or without rapamycin. After 24 h at 37 °C, the cells were photographed, and the cells that had migrated into the wound area were counted manually. At least three independent experiments were performed.

2.8. Transfection of siRNA

HUVECs grown to 30% confluence were transfected with p70S6K or β-catenin siRNA (Invitrogen, Carlsbad, CA) using Lipofectamine RNAiMAX (Invitrogen) according to the manufacturer's protocol. After 48 h, the cells were treated with visfatin. The siRNA sequences were as follows: human p70S6K, 5′-UUC UCC UCC ACU GAG AUA CUC AAG G-3′ (sense) and 5′-CCU UGA GUA UCU CAG UGG AGG AGA A-3′ (anti-sense); human β-catenin, 5′-UUA CCA CUC AGA GAA GGA GCU GUG G-3′ (sense) and 5′-CCA CAG CUC CUU CUC UGA GUG GUA A-3′ (anti-sense). At a final siRNA concentration of 50 nM, the transfection efficiency was about 80% efficiency.

2.9. Immunocytochemical analysis

After HUVECs were cultured on four-well Lab-Tek chamber slides (Nalge Nunc, Rochester, NY), the medium was replaced with the EBM-