Visfatin: a new player in mesangial cell physiology and diabetic nephropathy

Hye Kyoung Song,1 Mi Hwa Lee,1 Bo Kyung Kim,2 Yun Gyu Park,2 Gang Jee Ko,1 Young Sun Kang,1 Jee Young Han,3 Sang Youn Han,4 Kum Hyun Han,4 Hyoung Kyu Kim,5 and Dae Ryong Cha1

1Department of Internal Medicine, Korea University, Ansan City, Kyungki-Do; 2Department of Pathology, Inha University, Incheon; 3Department of Internal Medicine, Inje University, Goyang City, Kyungki-Do; and 4Department of Internal Medicine, Korea University, Seoul, Korea

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Visfatin: a new player in mesangial cell physiology and diabetic nephropathy. Am J Physiol Renal Physiol 295: F1485–F1494, 2008. First published September 3, 2008; doi:10.1152/ajprenal.90231.2008.—Visfatin is an adipocytokine that improves insulin resistance and has an antidiabetic effect. However, the role of visfatin in the kidney has not yet been reported. In this experiment, the synthesis and physiological action of visfatin in cultured mesangial cells (MCs) were studied to investigate the role of visfatin in diabetic nephropathy. Visfatin was found synthesized in MCs as well as adipocytes. Visfatin synthesis was markedly increased, not by angiotensin II, but by high glucose stimuli. In addition, visfatin treatment induced a rapid uptake of glucose, peaking at 20 min after visfatin treatment in a dose-dependent manner. A small inhibiting RNA against insulin receptor significantly blocked visfatin-mediated glucose uptake. Visfatin stimuli also enhanced intracellular NAD levels, and treatment with FK866, which is a specific inhibitor of nicotinamide phosphoribosyltransferase (Nampt), significantly inhibited visfatin-induced NAD synthesis and glucose uptake. Visfatin treatment increased glucose transporter-1 (GLUT-1) protein expression in isolated cellular membranes, and pretreatment with cytochalasin B completely inhibited visfatin-induced glucose uptake. Moreover, immunofluorescent microscopy showed the migration of cytotoxic GLUT-1 into cellular membranes after visfatin treatment. In accordance with these results, the activation of protein kinase B was detected after visfatin treatment. Furthermore, visfatin treatment dramatically increased the synthesis of profibrotic molecules including transforming growth factor-β1, plasminogen activator inhibitor-1, and type I collagen, and pretreatment with cytochalasin B completely inhibited visfatin-induced upregulation of profibrotic molecules. These results suggest that visfatin is produced in MCs, which are a novel target for visfatin, and play an important role in the pathogenesis of diabetic nephropathy.

ADIPOCYTES ARE INSULIN-RESPONSIVE CELLS that take up glucose and store energy in the form of triglycerides, subsequently releasing the triglycerides as free fatty acids to circulation. However, adipocytes have recently attracted attention as dynamic endocrine cells that produce and secrete various bioactive molecules that are collectively named adipocytokines, some of which affect glucose homeostasis and insulin resistance of other tissues (19, 37). Among many adipocytokines, tumor necrosis factor-α, leptin, plasminogen activator inhibitor-1 (PAI-1), interleukin-6, resistin, visfatin, monocyte chemoattractant peptide-1, and adiponectin have been implicated as active molecules in the development of insulin resistance (1, 19, 27, 37).

Visfatin is a recently discovered adipocytokine produced and secreted mainly by visceral adipose tissue (12). Visfatin binds to and activates the insulin receptor and induces an insulin-like effect both in vitro and in vivo (12). However, visfatin is identical to pre-B cell colony-enhancing factor (29). The visfatin gene is expressed in adipocytes, where it is regulated, and the protein is expressed predominantly in visceral adipose tissue (24).

Visfatin expression and plasma levels of visfatin are associated with obesity in animals and humans (6, 12). Furthermore, mice on a high-fat diet showed higher plasma visfatin levels compared with mice fed normal chow (12). Visfatin treatment protects insulin resistance, exhibited as an insulin-mimetic effect. Visfatin treatment in diabetic mice improved insulin sensitivity, with the insulin-mimetic action of visfatin mediated through binding to the insulin receptor (12). A recent study demonstrated elevated plasma visfatin levels in patients with type 2 diabetes mellitus (8). Furthermore, Haider et al. (14) reported that increased plasma visfatin levels in morbidly obese Caucasians were reduced after a gastric banding operation.

A recent study, however, contradicts several previously reported results about visfatin and fails to confirm the original results in human diabetic patients (35). Plasma visfatin levels are not related to insulin sensitivity or insulin itself, and there is no difference in visfatin gene expression between visceral and subcutaneous adipose tissues (8). In addition, the origin of plasma visfatin is not yet defined. Whether visfatin is an actual adipocytokine has yet to be determined. Several reports provide evidence that visfatin is not an actual adipocytokine and is a new marker of inflammation. Visfatin expression is increased in foam cell macrophages within unstable atherosclerotic regions that play a role in plaque destabilization (10). Plasma visfatin concentration is related to endothelial dysfunction in type 2 diabetic patients and patients with chronic kidney disease (5, 34). In addition, visfatin expression is detected in synovial fibroblasts in patients with rheumatoid arthritis, and visfatin itself activates the NF-κB and related various proinflammatory cytokines in cultured synovial fibroblasts (7). Furthermore, visfatin is also reported as nicotinamide phosphoribosyltransferase (Nampt), which is an essential enzyme in the nicotinamide adenine dinucleotide (NAD) biosynthetic pathway (28). Collectively, these results suggest a new physiological role of visfatin in organ injury.

Address for reprint requests and other correspondence: D. R. Cha, Dept. of Internal Medicine, Korea Univ. Ansan-Hospital, 516 Kojan-Dong, Ansan City, Kyungki-Do, 425-020 Korea (e-mail: cdragn@unitel.co.kr).

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In this study, we investigated whether visfatin synthesis occurs in mesangial cells (MCs) and what regulates visfatin synthesis. In addition, we also examined the effect of visfatin on intracellular NAD levels and investigated whether visfatin activates glucose transport processes in MCs and whether this activation is insulin receptor mediated. In addition, to define the physiological action of visfatin, we evaluated the effect of visfatin in cultured MCs.

**MATERIALS AND METHODS**

**MC culture.** A portion of the renal cortex from Sprague-Dawley rats was obtained immediately after surgical nephrectomy and grown in Dulbecco’s modified Eagle’s medium supplemented with 17% fetal calf serum, 100 μg/mL penicillin/streptomycin, 1% HEPES, 2 g of sodium bicarbonate, and 2 mM l-glutamine. For evaluation of the effect of high glucose and angiotensin II on visfatin synthesis, subconfluent MCs were serum-starved for 24 h, and the medium was then exchanged with medium containing 30 mM glucose or angiotensin II and administered at a final concentration of 100 nM to the culture medium. All experimental groups were cultured in triplicate and harvested at 48 h for extraction of total RNA and protein.

**Cell viability assay.** A modified colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to quantify cell viability using the CellTitre 96 cell proliferation assay kit (Promega, Madison, WI) per the manufacturer’s instructions. Briefly, confluent cells were cultivated on 96-well tissue culture plates and made quiescent in serum-deprived conditions for 24 h. Cell were then incubated for 48 h in normal glucose medium with or without angiotensin II at a final concentration of 100 nM to the culture medium. In some wells, medium containing 30 mM l-glucose was used to observe the effect of high glucose stimulation on cell survival. The solubilized formazan product was then measured in an ELISA at 570 nm.

**Quantification of NAD biosynthesis.** Since visfatin has been reported as a Namp, which is the rate-limiting enzyme in the salvage pathway of NAD biosynthesis, we next investigated the effect of visfatin on intracellular NAD concentration. Cells were cultivated on six-well culture plates, serum-starved for 24 h, and then treated with 10 ng/mL visfatin with or without 1 nM FK866, a specific inhibitor of Namp (16), for 24 h. FK866 was supplied by Research Triangle Institute International (Research Triangle Park, NC). Intracellular NAD levels were determined using the EnzyChrom NAD assay kit (Bioassay Systems, Hayward, CA) per the manufacturer’s instructions. NAD levels were normalized by protein concentrations in cell lysates.

**Measurement of glucose uptake.** Cells were starved in serum-free medium for 16 h and then washed three times with PBS buffer. Cells were incubated in 1 ml of Krebs-Ringer phosphate-HEPES buffer [136 mM NaCl, 4.7 mM KCl, 1.2 mM CaCl2, 2.5 mM Na2HPO4, 10 mM HEPES, and 0.1% (wt/vol) bovine serum albumin, pH 7.4] for 30 min at 37°C. The cells were then treated with or without 100 ng/mL visfatin or 100 nM insulin and incubated for 15 min at 37°C. Cells were next incubated with 1 μCi of 2-deoxy-[1-14C]-D-glucose (2-DG) and incubated for the indicated times from 10 to 30 min. To exclude non-carrier-mediated glucose uptake, we pretreated cells with 10 μM cytochalasin B, an inhibitor of glucose transporters, for 30 min in control wells. In some wells, cells were pretreated with 10 μM cytochalasin B for 30 min before visfatin treatment. At the indicated time, the medium was collected, and cells were washed three times with PBS buffer and lysed in 0.1 N NaOH for 1 h at room temperature (RT). Total radioactivity from the medium (Rm) and total cell-associated radioactivity from lysates (Rt) were counted using a liquid scintillation counter. True glucose uptake was calculated as follows: radioactivity of glucose uptake = ([Rt/(Rm + Rt)]sample − [Rt/(Rm + Rt)])cytochalasin B. The radioactivity was normalized for total protein quantified using the Bradford method. Next, the dose dependency of visfatin in glucose uptake was determined. Because peak glucose uptake occurred at 20 min, different concentrations of visfatin at final concentrations of 1, 10, and 100 ng/mL were treated for 20 min, and glucose uptake was measured as described previously. Since visfatin is an important rate-limiting enzyme in NAD biosynthesis, we further investigated the effect of inhibition of Namp and nicotinamide mononucleotide (NMN), a product of the Namp reaction on glucose transport. Cells were treated with 10 ng/mL visfatin with or without 1 nM FK866, a specific inhibitor of Namp. In some wells, NMN was administered at a final concentration of 2 μM under visfatin and FK866 treatment, and glucose uptake was measured after 20 min.

**Stealth RNA interference for insulin receptor.** Since visfatin has been reported to have insulin-mimetic action through binding to the insulin receptor (12), we performed an experiment with a small interfering (si)RNA for the insulin receptor to investigate whether visfatin-induced rapid uptake of glucose is mediated by the insulin receptor or is independent of the insulin receptor. Rat insulin receptor mRNA was specifically knocked down using commercially available siRNA oligonucleotides. The sequences of Stealth siRNA were designed using BLOCK-IT RNAi Designer (Invitrogen Life Technologies, Gaithersburg, MD) to target bp 1692–1716 of the rat insulin receptor (sense strand, 5'-AUC AUG GAC CAU GCA GUA CCG A-3'; antisense strand, 5'-UCG CAA CUG CAU GUG UGC CCA UGA U-3'). These Stealth RNA oligonucleotides were synthesized by Invitrogen Life Technologies. Before transfection, rat MCs were maintained in DMEM with 10% fetal calf serum and then transfected with 100 pmol of Stealth siRNA using Lipofectamine 2000 (Invitrogen Life Technologies) per the manufacturer’s instructions. Since transfection for 48 h induced an 85% reduction in insulin receptor mRNA expression, MCs were transfected for 48 h with siRNA and then cultivated using medium containing serum-free and antibiotic-free DMEM. Cells were made quiescent for 16 h, and then cells were treated with different concentrations of visfatin at final concentrations of 1, 10, and 100 ng/mL for 20 min, and glucose uptake was measured as described previously. Stealth RNAi negative control duplexes were used as controls.

**Analysis of gene expression by real-time quantitative PCR.** Total RNA was extracted from cells with Trizol reagent and further purified using an RNeasy mini kit (Qiagen, Valencia, CA). Quantitative gene expression was performed on a LightCycler 1.5 system (Roche Diagnostics, Indianapolis, IN) using SYBR green technology. Sequence-specific primers for Visfatin, glucose transporter-1 (GLUT-1), transforming growth factor-β1 (TGF-β1), PAI-1, and procollagen Iα of type I collagen [procollagen Iα(I)] were used for the PCR reaction. The nucleotide sequences of each primer are as follows: visfatin, sense 5'-TGG CGT GAT AAG AAG AAC GA-3' and antisense 5'-ACT TCT TGG GCC TCC TGG AT-3'; GLUT-1, sense 5'-TGG ATG TGC GCT ATA ACA CC-3' and antisense 5'-ACA CCT CCC CCA CAT ACA TG-3'; TGF-β1, sense 5'-ATG GAC GAG TTT CGA TCC AGG-3' and antisense 5'-GTC CAG CAG CCT CCA AAT ATA GG-3'; PAI-1, sense 5'-ATG AGA TCA GTC ATG CCC CGG CAC CCA TCT TGG-3' and antisense 5'-GCA CGG AGA TGG TGC TAC CAT CAG ACT GTG-3'; procollagen Iα(I), sense 5'-TGG TTC TCC TCG AAA TGC TGG ACC-3' and antisense 5'-CAG GAG AAC CAG GAG AAC CAG G-3'; insulin receptor, sense 5'-GTT CAA GAC CAG ACC CCA AGG-3' and antisense 5'-GCG CAT ACA GAC GAA-3'; and β-actin sense 5'-TCA TGA TGT AGT CCT TCA CGA-3' and antisense 5'-TCT AGG CAC AAA GGT GTG-3'. Total mRNA was reverse-transcribed into cDNA using an iScript cDNA synthesis kit (Bio-Rad, Hercules, CA). Real-time RT-PCR was performed for 10 min at 50°C and for 5 min at 95°C. Subsequently, 45 cycles were applied, consisting of denaturation for 10 s at 95°C and annealing with extension for 30 s at 60°C. The ratio of each gene and β-actin level (relative gene expression number) was calculated by subtracting the threshold cycle number (Ct) of the target gene from that of β-actin and raising 2 to the power of this difference.
Protein extraction, fractionation, and Western blot analysis. To study total cellular lysates, cells were lysed in lysis buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, and 1 mM phenylmethylsulfonyl fluoride) and the lysate was boiled for 3 min. For microsomal preparations of cells, whole cell extracts were prepared by incubating the cells in lysis buffer (20 mM Tris-HCl, 100 mM NaCl, 5 mM MgCl2, 1% Nonidet P-40, 0.5% sodium deoxycholate, 10 mM β-glycerophosphate, 0.1 mM orthovanadate, and protease inhibitors) for 30 min. Cytosol and membrane fractions were prepared using the Qproteom cell compartment kit (Qiagen) per the manufacturer’s instructions. The protein concentration was measured by bicinchoninic acid protein assay (BCA; Pierce, Rockford, IL). Fifty micrograms of each protein sample were loaded for electrophoresis on a 10% SDS-polyacrylamide gel (Tefco, Tokyo, Japan) under denaturing conditions. The proteins were transferred onto a polyvinylidene difluoride membrane (Immobilon-P; Millipore, Bedford, MA) for 120 min at 250 mA. After the filter was blocked by incubating the membrane with blocking solution (TBST: Tris-buffered saline containing 150 mM NaCl, 50 mM Tris, 0.05% Tween 20, and 5% nonfat milk, pH 7.5) for 1 h at RT, the membrane was hybridized with rabbit polyclonal anti-visfatin antibody (1:500; Phoenix Pharmaceuticals, Burlingame, CA), rabbit polyclonal anti-PAI-1 antibody (1:200; Santa Cruz Biotechnology, Santa Cruz, CA), rabbit polyclonal anti-GLUT-1 antibody (1:500; Abcam, Cambridge, MA), rabbit monoclonal Akt and phospho-specific Akt antibody (1:5,000; Cell Signaling Technology, Danvers, MA), or mouse monoclonal anti-β-actin antibody (1:5,000; Sigma-Aldrich, St. Louis, MO) in blocking buffer overnight at 4°C. To confirm the equal loading and micromolar fractions of protein, we applied goat polyclonal anti-apoptosis-inducing factor antibody (1:200; US Biological, Swampscott, MA) to the membrane in blocking buffer overnight at 4°C. The filter was then washed four times with TBST and incubated with horseradish peroxidase-conjugated secondary antibody diluted 1:1,000 for 60 min at RT. Specific signals were detected using the enhanced chemiluminescence method (ECL; Amersham, Little Chalfont, UK).

Measurement of secreted visfatin and collagen in cultured supernatant. Secreted visfatin and total soluble collagen were measured in culture supernatants by using the visfatin enzyme immunoassay kit (Phoenix Pharmaceuticals) and the Sircol soluble collagen assay kit (Biocolor, Belfast, Northern Ireland) per the manufacturer’s instructions. For visfatin measurement, 50 μl of sample were incubated with 25 μl of primary antiserum and 25 μl of biotinylated peptide in a secondary antibody-coated plate at RT for 2 h. Plates were then washed five times with 300 μl of assay buffer, 100 μl of streptavidin-horseradish peroxidase solution was then added, and the plates were incubated at RT for 1 h. After being washed six times, 100 μl of substrate solution were incubated at RT for 1 h, and the reaction was terminated by adding 2 N HCl. The absorbance was then measured at 450 nm using an ELISA reader. To measure collagen, we added 1 ml of Sirius red reagent to 100 μl of test samples and mixed for 60 min at RT in a mechanical shaker. The collagen-dye complex was precipitated by centrifugation at 14,000 g for 10 min. To release the blue dye, we added 1 ml of alkali reagent (0.5 M NaOH) to the precipitate, and the absorbance was measured at 540 nm using an ELISA reader. Supernatant visfatin and collagen levels were expressed relative to the total protein concentration in each condition.

Immunofluorescence microscopy. To confirm the GLUT-1 activity and translocation of GLUT-1 after stimulation with visfatin in experimental cells, we performed immunofluorescent staining for GLUT-1. Cells were incubated in a four-chamber glass slide, and 50% confluent MCs were fixed at RT for 10 min using 75% ethanol. The slides were then blocked with 3% goat serum for 1 h at 37°C. The slides were then incubated at 4°C overnight with rabbit polyclonal anti-GLUT-1 antibody (1:200; Abcam) containing 0.2% Triton X-100. After being rinsed in ice-cold PBS, slides were incubated in a fluorescent tagging secondary antibody, Alexa Fluor 488 donkey anti-rabbit IgG antibody (1:200; Invitrogen, Carlsbad, CA) to visualize GLUT-1 for 1 h at 37°C and were examined under an immunofluorescence microscope (LSM 5 PASCAL; Carl Zeiss Microimaging, Berlin, Germany).

Statistical analysis. A nonparametric analysis was used because of the relatively few samples present. Results are means ± SE. A Kruskal-Wallis test was used for comparison of more than two groups, followed by a Mann-Whitney U-test for comparison using a microcomputer-assisted program with SPSS for Windows 10.0 (SPSS, Chicago, IL). A P < 0.05 was considered statistically significant.

RESULTS

MCs produce visfatin in response to glucose stimuli. To confirm the synthesis of visfatin in MCs, we performed RT-PCR. As a positive control, epididymal fat tissue and 3T3-L1 adipocytes were used because of their high expression of visfatin. As shown in Fig. 1A, visfatin was expressed in MCs as well as adipose tissue. Next, the effects of high glucose and angiotensin II on visfatin gene expression and protein synthesis

Fig. 1. Synthesis of visfatin and the effect of high glucose and angiotensin II on visfatin gene expression in cultured mesangial cells (MCs). A: PCR results for visfatin in cultured MCs. Representative RT-PCR results show 143-bp products. As a positive control, epididymal fat and 3T3-L1 adipocytes were used. MW, molecular weight. B: effects of high glucose and angiotensin II stimuli on visfatin gene expression. Expression of visfatin mRNA was measured using real-time PCR. Data are means ± SE. NG, normal glucose; HG, high glucose; ANG II, angiotensin II. ***P < 0.001 vs. NG.
were examined. Visfatin gene expression showed a fourfold increase compared with the controls after high glucose stimulation. However, angiotensin II stimulation did not induce any change in visfatin expression (Fig. 1B). To test the possibility that necrosis or apoptosis of cells under high glucose and angiotensin II stimulation leads to release of visfatin to culture medium, we performed an MTT assay. As shown in Fig. 2A, high glucose and angiotensin II stimuli did not induce any cytotoxic or proliferative effects in MCs. In line with the gene expression, visfatin protein synthesis was also dramatically increased in response to high glucose stimuli, and most of the synthesized visfatin was secreted into the culture supernatant (Fig. 2B and C).

Effect of Nampt inhibition on intracellular NAD concentration in MCs. To further characterize the physiological role of visfatin, we observed the effect of visfatin and FK866, a potent specific inhibitor of Nampt, on intracellular NAD levels. As shown in Fig. 3, 10 ng/ml visfatin enhanced NAD biosynthesis by 80% compared with controls. In addition, 1 nM FK866 significantly inhibited visfatin-induced NAD biosynthesis, suggesting that visfatin mediated NAD biosynthesis in cultured MCs.

Effect of visfatin on 2-DOG uptake in MCs. Because previous studies suggested visfatin exerts an insulin-like effect in adipocytes, glucose uptake occurring in MCs was determined using a 2-DOG uptake assay. First, the time course of 2-DOG uptake in MCs treated with 100 ng/ml visfatin was examined. As shown in Fig. 4A, visfatin treatment induced a rapid, approximately eightfold uptake of 2-DOG in MCs, with a maximal stimulation at 20 min and then a rapid decrease to control levels at 30 min. As expected, insulin treatment also induced a rapid uptake of 2-DOG, peaking at 20 min in MCs. Cytochalasin B, a generic glucose transporter inhibitor, completely abolished visfatin-stimulated glucose uptake to basal levels, showing that glucose uptake was mediated by glucose transporters (Fig. 4A). Furthermore, the stimulation of 2-DOG uptake by visfatin increased in a dose-dependent fashion from 1 ng/ml and reached a maximal stimulation at 100 ng/ml (Fig. 4B).

Effect of Nampt inhibition and NMN on 2-DOG uptake in MCs. To further elucidate the physiological action of Nampt and NAD on glucose transport induced by visfatin, we next examined the effect of FK866 and NMN under visfatin treatment on glucose uptake. As shown in Fig. 4C, FK866 treatment abolished visfatin-induced glucose uptake to near control levels. However, NMN treatment did not induce a significant change in glucose uptake.
Effect of Stealth siRNA of insulin receptor on visfatin-induced glucose uptake in MCs. We next performed an siRNA experiment to further confirm whether visfatin-induced rapid uptake of glucose is mediated by insulin receptor or independent of insulin receptor. As shown in Fig. 5A, silencing the rat insulin receptor by transfecting with 100 pmol of Stealth insulin receptor siRNA for 48 h induced 85% reduction in insulin receptor mRNA levels. Interestingly, knockdown of insulin receptor significantly reduced visfatin-induced glucose uptake (Fig. 5B). Even in the normal controls, knockdown of insulin receptor decreased basal glucose uptake 40%.

GLUT-1 is responsible for visfatin-induced glucose uptake. The ability of a glucose transporter to mediate visfatin-induced glucosetransport was investigated. Among the facilitative glu-
glucose transporters, GLUT-1 has been known as the major glucose transporter in MCs. Thus the possibility of visfatin stimulation increasing the expression of GLUT-1 was examined. As shown in Fig. 6, A and B, visfatin treatment induced the mRNA expression of GLUT-1 in a time- and dose-dependent manner. To further confirm the role of GLUT-1 in visfatin-induced glucose uptake, we performed immunofluorescence microscope examination using the GLUT-1 antibody. As shown in Fig. 6C, the cellular location of GLUT-1 was mainly distributed in cytoplasm in the resting state, but after stimulation with visfatin, GLUT-1 was translocated to the cell surface after 10 min of visfatin stimulation. Prior treatment with cytochalasin B inhibited visfatin-induced translocation of GLUT-1 into the cell surface membrane (Fig. 6C). To further confirm the translocation of GLUT-1 into the cellular membrane, we performed a Western blot analysis using the membrane fraction of the cells. As shown in Fig. 6D, GLUT-1 expression was dramatically increased in the plasma membrane of cells after visfatin stimulation, and cytochalasin B pretreatment abolished visfatin-induced GLUT-1 expression in the cell membrane. As a positive control, GLUT-1 translocation into the cell membrane induced by insulin stimulation was also observed (Fig. 6D).

Effect of visfatin on Akt activation and profibrotic molecule synthesis. Since visfatin increased glucose transport, the effect of visfatin on Akt activation, an important downstream component of the insulin signaling pathway, was examined next. As shown in Fig. 7A, the activation of Akt, assessed by measuring the levels of phospho-specific Akt, was found to rapidly increase in response to visfatin after 5 min. The maximal activity was observed at 30 min, at which point the level of Akt phosphorylation gradually decreased. Next, the effect of augmented glucose influx induced by visfatin on changes in the synthesis of profibrotic molecules such as TGF-β1, PAI-1, and collagen was evaluated. As shown in Fig. 7B, visfatin stimulation significantly increased the gene expression of TGF-β1, PAI-1, and type I collagen. Prior treatment with cytochalasin B

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**Fig. 6.** Effect of visfatin on glucose transporter-1 (GLUT-1) synthesis and location in cultured MCs. A: effect of visfatin on GLUT-1 gene expression. Different concentrations of visfatin at final concentrations of 1, 10, and 100 ng/ml were treated for 48 h and harvested for extraction of total RNA. B: visfatin was treated at a final concentration of 100 ng/ml for the indicated time intervals and harvested for extraction of RNA. Expression of GLUT-1 mRNA was measured by real-time PCR. C: immunofluorescent staining for GLUT-1 after visfatin stimulation. In the sixth well (f), cells were pretreated with 10 μM cytochalasin B for 30 min. a, Control (basal); b, 5 min after visfatin stimuli; c, 10 min after visfatin stimuli; d, 15 min after visfatin stimuli; e, 20 min after visfatin stimuli; f, 20 min after visfatin stimuli under prior treatment with 10 μM cytochalasin B. Membrane translocations of GLUT-1 are indicated by arrows. D: representative Western blot showing the effect of visfatin on the expression of GLUT-1 protein in cellular membranes. Cytosol and membrane fractions were prepared using a Qproteom cell compartment kit, and 50 μg of each protein sample were loaded for electrophoresis on a 10% SDS-polyacrylamide gel. GLUT-1 protein was detected as a single band of ~48 kDa. To confirm the equal loading and microsomal fractionation, we performed a Western blot for apoptosis-inducing factor (AIF). Data are means ± SE. *P < 0.05; **P < 0.01 vs. control.
completely abolished visfatin-induced TGF-β1, PAI-1, and type I collagen gene expression, which implies that overexpression induced by visfatin was through augmented glucose influx into cells. To further confirm the effect of visfatin on collagen protein synthesis and PAI-1 protein expression, we performed ELISA and Western blot analysis to evaluate the total collagen content in the supernatant and the cellular PAI-1 level. As shown in Fig. 7, C and D, collagen protein secretion and PAI-1 protein expression were markedly increased by visfatin treatment, and cytochalasin B treatment completely abolished visfatin-induced protein synthesis.

**DISCUSSION**

In the present study, visfatin was shown to be synthesized in MCs and to stimulate glucose uptake in glomerular MCs mediated by GLUT-1. In addition, high glucose stimulation was found to markedly upregulate visfatin synthesis but was not increased in response to angiotensin II stimulation. Furthermore, exogenous visfatin administration induced increased synthesis of profibrotic molecules, including TGF-β1, PAI-1, and type I collagen. These results suggest that endogenous visfatin produced by MCs can stimulate glucose uptake and is followed by accelerated glucose-mediated metabolic abnormalities in MCs.

Hyperglycemia in diabetes is associated with increased glucose uptake into cells and leads to metabolic alterations in cells, which have been considered an important pathophysiological mechanism for diabetic microvascular complications (2, 4, 9, 11). There are many results suggesting that increased glucose uptake by MCs induces mesangial extracellular matrix protein accumulation, which is a pathological feature of diabetic nephropathy (3, 15, 17, 25, 33). Heilig et al. (17) have reported that overexpression of GLUT-1 in MCs results in increased glucose uptake and extracellular matrix synthesis even in the normal glucose culture medium, which further supports a link between glucose uptake and matrix synthesis in MCs.

MCs, similarly to many other cells, take up glucose through glucose transporters that enhance glucose transport across the plasma membrane. The glucose uptake system in MCs has reported the presence of two types of glucose transporters, facilitative and sodium-coupled transporters (17, 18, 20, 21,
36). Previous reports suggest GLUT-1 is the predominant glucose transporter in MCs (21). In the present study, the expression of other reported GLUTs, GLUT4 and SGLT1, was examined, but other GLUTs were not detected in this study (data not shown).

In the present study, high glucose stimulation was found to significantly increase the visfatin synthesis, and most of the synthesized visfatin was secreted into the culture medium. Angiotensin II, another important mediator in diabetic nephropathy, did not induce upregulation of visfatin synthesis. This result is in agreement with a recent report that exogenous glucose infusion increased plasma visfatin levels in a healthy population (13). These results suggest the possibility that a high glucose condition in a diabetic environment could continuously stimulate visfatin synthesis from MCs in vivo.

Visfatin is a newly identified adipocytokine that is secreted from adipocytes and mimics insulin action in animals (12). Recent clinical studies show plasma levels of visfatin are increased in obese and type 2 diabetic patients and are related to insulin resistance (6, 8, 30). In addition, visfatin treatment in diabetic mice improved insulin sensitivity and protected insulin resistance in a high-fat diet induced in insulin-resistant animal models (12). However, recent studies also provide evidence that visfatin has proinflammatory properties and that visfatin promotes angiogenesis by activation of the MAPK pathway, suggesting a new physiological role of visfatin (5, 7, 10, 23, 26, 34).

In this study, we have provided evidence demonstrating that visfatin functions as Nampt and has an important role in NAD biosynthesis in mesangial cells. We observed that visfatin treatment markedly increased intracellular NAD concentrations and that FK866, which is a specific inhibitor of Nampt, significantly inhibited visfatin-induced NAD biosynthesis, suggesting that visfatin mediated NAD biosynthesis in cultured MCs. In addition, we have shown that visfatin-induced glucose uptake was significantly decreased by FK866, suggesting that NAD may be involved in the visfatin-mediated glucose uptake in MCs. However, NMN treatment, which is a product of Nampt reaction, did not restore the reduction of glucose uptake induced by FK866. These results are in line with a recent report that inhibition of Nampt caused defects in NAD biosynthesis and glucose-stimulated insulin secretion in pancreatic islets (28). However, in contrast with our result, the insulin-mimetic effect of visfatin was not observed in that study (28). This discrepancy may be caused by differences in culture condition and type of cells used in the study.

Although the molecular mechanism of underlying visfatin-mediated glucose uptake in MCs is not yet known, visfatin may interact with the insulin receptor tyrosine kinase and activate it like small-molecule insulin receptor activator (32). In support of this hypothesis, our interesting finding in this study is that knockdown of insulin receptor with siRNA blocked visfatin-induced glucose uptake, indicating that the visfatin effect is insulin receptor mediated. This is the first evidence that visfatin-mediated glucose uptake in MCs is through insulin receptor.

In the present study, the ability of visfatin to stimulate 2-DOG uptake in MCs in a dose-dependent manner was observed. In addition, the visfatin-induced increase in the 2-DOG uptake was abolished by the addition of cytochalasin B in visfatin-treated cells, suggesting that 2-DOG was taken up by MCs mainly through facilitative glucose transporters. The expression of GLUT-1, GLUT-4, and SGLT-1 was examined using real-time PCR in MCs. However, GLUT-4 and SGLT-1 could not be detected in this study. The results of PCR analysis indicate that exogenous visfatin treatment stimulates the expression of mRNA for GLUT-1 in a time- and dose-dependent manner in MCs.

In this current study, the effect of visfatin on translocation of GLUT-1 in MCs was further investigated. GLUT-1 was mainly detected within the cytoplasm in the resting state in MCs under an immunofluorescence microscope. However, after stimulation with visfatin, GLUT-1 was observed translocated into the plasma membrane of cells. This translocation of GLUT-1 was observed after 10 min of visfatin stimulation and peaked at 20 min of stimulation. These results are in agreement with the
2-DOG uptake results, showing maximal uptake at 20 min of visfatin stimulation. A Western blot analysis using the membrane fraction of cells was also performed to further confirm the translocation of GLUT-1 after visfatin stimulation. Similar to the result of the immunofluorescent microscopy, GLUT-1 protein expression was markedly increased after 20 min of visfatin stimulation. As expected, prior treatment with cytochalasin B completely inhibited visfatin-induced GLUT-1 translocation.

Because visfatin treatment increases the glucose uptake in MCs, the effects of visfatin treatment on the activation of downstream signaling molecules induced by glucose uptake in cells were examined. Protein kinase B activation was observed. Protein kinase B is an important downstream target for phosphatidylinositol 3-kinase with an important role in glucose metabolism, including the enhancement of glucose uptake in cells. The activity of Akt, assessed by measuring the levels of phospho-specific Akt, was found to rapidly increase in response to visfatin after 5 min and was maintained at a higher level for up to 1 h. This result suggested that visfatin treatment induced glucose uptake, followed by activation of Akt, the downstream signaling molecule of glucose influx into the cells.

A significant result from this study was that exogenous visfatin treatment in MCs induced increased synthesis of profibrotic molecules such as TGF-β1, PAI-1, and type I collagen. Various metabolic abnormalities under hyperglycemic conditions have been proposed to play an important role in the development of diabetic nephropathy (22, 31, 38, 39). Among these metabolic abnormalities, excessive glucose uptake into MCs is the first step of high glucose-induced metabolic alterations in cells. Visfatin produced from MCs under high glucose conditions was hypothesized to induce an increase in endogenous visfatin synthesis from MCs, and consequently, visfatin could stimulate glucose uptake and thus accelerate metabolic alterations, including the overproduction of profibrotic molecules. The interaction between visfatin and GLUT-1 shown in this study could provide a new physiological role of visfatin in the pathogenic mechanism of diabetic nephropathy. Although previous animal studies have suggested an anti-diabetic effect of visfatin in diabetes mellitus through an improvement in insulin resistance by increased glucose disposal in insulin target tissues, the present study shows that visfatin treatment in MCs induced rapid glucose uptake and increased profibrotic cytokine synthesis. These results are in agreement with very recent reports that circulating levels of visfatin are associated with endothelial dysfunction in patients with chronic kidney disease and that visfatin itself activates various proinflammatory cytokines in cultured synovial fibroblasts (5, 7, 34).

In conclusion, this study suggests a new physiological role of visfatin in the pathogenesis of diabetic nephropathy. Increased visfatin synthesis from MCs under high glucose conditions was hypothesized to possibly contribute to increased glucose influx into MCs, thereby accelerating diabetic nephropathy through the aggravation of metabolic alterations (Fig. 8). More in vivo studies are necessary to define the role of visfatin in the pathogenesis of diabetic nephropathy.

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