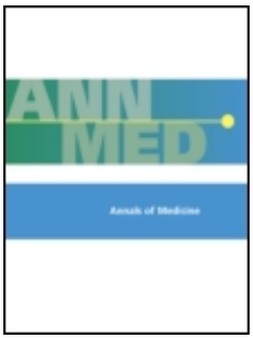

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ORIGINAL ARTICLE

Overexpression of visfatin/PBEF/Nampt alters whole-body insulin sensitivity and lipid profile in rats

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Abstract

Background. Visfatin/PBEF/Nampt is an adipose-derived hormone proposed to exert insulin-mimicking effects and play a positive role in attenuating insulin resistance. However, the precise mechanisms underlying the beneficial effects of visfatin/PBEF/Nampt on insulin sensitivity remain unknown.

Method. Euglycemic-hyperinsulinemic clamps were used in the same groups of rats to study the *in vivo* effect of visfatin/PBEF/Nampt on insulin sensitivity and glucose/lipid metabolism before and after the overexpression of visfatin/PBEF/Nampt protein, which was carried out by injection of pcDNA3.1-visfatin plasmid.

Results. On day 4 after plasmid injection, plasma visfatin/PBEF/Nampt protein levels were significantly increased and displayed a hypocholesterolemic effect in both normal-chow (NC) and high-fat diet (HT) animals with pcDNA3.1-visfatin treatment. A second glucose clamp also demonstrated increased insulin sensitivity in pcDNA3.1-visfatin animals. Consistent with the clamp data, the extent of insulin receptor substrate (IRS)-1 tyrosine phosphorylation in response to insulin was significantly enhanced in the liver and adipose tissues. In addition, the mRNA expression of peroxisome proliferator-activated receptor-γ (PPARγ) and sterol regulatory element-binding proteins 2 (SREBP-2) in the liver and adipose tissues was also significantly upregulated in these animals.

Conclusion. These results demonstrate that visfatin/PBEF/Nampt improves insulin sensitivity and exerts its hypocholesterolemic effects at least partially through upregulation of the tyrosine phosphorylation of IRS-1 protein and the mRNA levels of PPARγ and SREBP-2.

Key words: *Insulin sensitivity, lipid metabolism, PPARγ, SREBP-2, visfatin*

Introduction

Type 2 diabetes mellitus (T2DM) is a genetically heterogeneous disorder characterized by peripheral insulin resistance and reduced insulin secretion from pancreatic beta cells in the late stage of the disorder (1–3). Despite extensive studies, the underlying mechanism of these defects remains elusive. Several polypeptide hormones have been discovered that could represent functional links between energy balance and insulin resistance. These polypeptides include leptin, resistin, adiponectin, visfatin, apelin, and others (4–9). Among these polypeptide hormones, visfatin was recently identified as a novel adipocytokine implicated in the development of obesity-associated insulin resistance and diabetes mellitus (10). Visfatin is a nicotinamide phosphoribosyltransferase (Nampt). It was originally reported to be a cytokine that promotes

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Key messages

- Visfatin/PBEF/Nampt is an adipose-derived hormone proposed to exert insulin-mimicking effects and play a positive role in attenuating insulin resistance.
- The precise mechanisms underlying the beneficial effects of visfatin/PBEF/Nampt on insulin sensitivity remain largely unknown.
- Using twice EU clamps in animals serving as their own control, we investigated the *in vivo* effect of visfatin/PBEF/Nampt on insulin sensitivity and glucose-lipid metabolism by overexpressing visfatin/PBEF/Nampt protein in rats.

β cell maturation and therefore named 'pre-β cell colony-enhancing factor' (PBEF) (11). Thus, visfatin, PBEF, and Nampt refer to an identical protein with multiple biological functions.

Visfatin/PBEF/Nampt is preferentially expressed in visceral fat of both humans and animals as compared with subcutaneous fat. In humans, plasma visfatin/PBEF/Nampt concentrations correlate strongly with the amount of visceral fat. Most interestingly, visfatin mimics insulin effects in various rodent models of insulin resistance and obesity *in vivo*. A study by Fukuhara et al. showed that visfatin/PBEF/Nampt directly bound to and stimulated the insulin receptor through a different binding site of insulin (10). However, the action of visfatin/PBEF/Nampt on the insulin receptor needs to be further confirmed by other groups because the original study by Fukuhara et al. was retracted later on by the authors (12). Another study by Beltowski et al. showed that acute intravenous administration of visfatin reduced plasma glucose levels without affecting plasma insulin concentrations, which suggests that the hypoglycemic effect of visfatin/PBEF/Nampt is not through an insulin-stimulating mechanism (13). In addition, visfatin/PBEF/Nampt stimulates the differentiation of preadipocytes to mature fat cells, induces triglyceride accumulation, accelerates triglyceride synthesis from glucose, and induces the expression of genes encoding the adipose tissue-specific markers peroxisome proliferator-activated receptor-γ (PPARγ), fatty acid synthase, diacylglycerol acyltransferase, and adiponectin (14). In humans, plasma visfatin/PBEF/Nampt levels correlate significantly with percent body fat, body mass index, and visfatin/PBEF/Nampt mRNA level in visceral adipose tissue, but not with visceral fat mass or waist-to-hip ratio (WHR) (14). In two recent studies, plasma visfatin/PBEF/Nampt was shown higher in patients with T2DM than in normoglycemic controls (15,16). These data suggest that endogenous visfatin/PBEF/Nampt is involved in the regulation of glucose and lipid homeostasis.

The role of visfatin/PBEF/Nampt in the regulation of glucose and lipid homeostasis is arguable because several studies reported that plasma visfatin/PBEF/Nampt was reduced in obese patients and was not related to insulin resistance (17). The circulating visfatin level was also shown to correlate with inflammation, but not with insulin resistance (18). Thus, the relationship between visfatin/PBEF/Nampt and insulin resistance remains inconclusive. To further characterize the physiological role of visfatin/PBEF/Nampt, we examined the effects of overexpression of visfatin/PBEF/Nampt on glucose/lipid metabolism and insulin sensitivity in rats. We also examined the effects of visfatin/PBEF/Nampt on insulin receptor substrate (IRS-1), fibroplast growth factor-21 (FGF-21), and gene expression of selected metabolic enzymes.

Materials and methods

Construction of recombinant plasmids

The full-length visfatin cDNA (1476 bp) was amplified from rat adipose tissue using the 5′-GCGAATTCG CCACCATGAA T G CTGCGGC AGAAGC-3′ sense and 5′-GCC TCGAGCTAGT-GAG GCGCCACATCCT-3′ antisense primers by Reverse Transcription Polymerase Chain Reaction (RT-PCR). PCR products and pcDNA3.1(+) (Invitrogen, Carlsbad, CA) were digested with EcoR I and Xho I. The 1476 bp (visfatin cDNA) and 5428 bp (pcDNA3.1+) fragments were collected in low melting point agarose, then ligated using T_4 ligase at 16°C overnight. Escherichia coli (DH-5α) was transformed using the ligation products. Recombinant plasmids (pcDNA3.1-visfatin) were purified from transformed *E. coli*, screened on ampicillin plates, and verified by restriction endonuclease digestion and DNA sequencing.

Animals

The animal studies presented here were conducted in accordance with National Institutes of Health (NIH) Guidelines for Animal Care and approved by Chongqing Medical University. Four-week-old male sprague-drawley rat (SD) rats (EA Center, Chongqing, China) with average body-weight of 120 g were used in this study. Rats were housed individually in controlled light/dark (12 h/12 h) and temperature conditions with free access to water and

standard rat chow for 3 days. Rats were then randomly divided into three groups. Two groups ($n=10$ for each) were fed a normal-chow diet containing 60% carbohydrate, 21% protein, and 19% fat. The third group ($n=10$) was fed a high-fat diet containing 54% fat, 33% carbohydrate, and 13% protein. The content of fiber, vitamins, and minerals was the same in both diets.

Animal studies

The euglycemic-hyperinsulinemic clamps (EU clamp) were performed 16 weeks after the initiation of the special diet. Four days prior to the clamp study, animals were catheterized through the right jugular vein and the left carotid artery under pentobarbital sedation (50 mg/kg; Nembutal Abbott Laboratories, Abbott Park, IL). Catheters were 'tunneled' subcutaneously, exteriorized at the back of neck, and filled with heparinized saline. The jugular and carotid catheters were used for blood sampling and infusion, respectively.

On postoperative day 4, EU clamps were performed in awake and unrestrained rats as previously described (19). Briefly, insulin ($4.8\,\mathrm{mU}\cdot\mathrm{kg}^{-1}\cdot\mathrm{min}^{-1}$) was infused through the carotid artery catheter from 0 to 120 min during the EU clamps. Glucose concentrations were clamped at euglycemic levels by a variable rate infusion of 25% glucose. Blood glucose levels were monitored with a portable glucometer, and glucose infusion rates were adjusted every 5–10 min as needed. Blood samples (400 μL) were obtained from the jugular vein at 0 and 180 min for determination of levels of insulin, visfatin/PBEF/Nampt, free fatty acids (FFA), triglyceride (TG), and total cholesterol (TC). Whole-body insulin sensitivity was determined by the rate of glucose infusion that maintains euglycemia (glucose infusion rate, GIR).

At the end of the clamp, one group of the normal-chow fed rats (NT group; $n=10$) was given pcDNA3.1-visfatin plasmid at a dose of 300 μg in 300 μL phosphate buffer system (PBS) Another group of the normal-chow fed rats (NC group; $n=10$) was given pcDNA3.1(+) at the same dose. High-fat-fed rats (HT group; $n=10$) were given pcDNA3.1-visfatin plasmid at a dose of 300 μg in 300 μL PBS. For all three groups, the DNA was injected into the rectus femoris muscle using a disposable insulin syringe with a sterile 29-gauge needle. On postinjection day 4, EU clamps were performed again in the animals as described above to access the insulin sensitivity. At the end of the studies, rats were euthanized with overdosing of pentobarbital (180 mg/kg) after terminal blood sampling at 120 min. Tissues were harvested, immediately frozen in liquid nitrogen and stored at -80°C until analysis.

Western blot analysis

Plasma visfatin/PBEF/Nampt levels were quantified by Western blot analysis. One microliter of plasma was subjected to 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), transferred, blotted with primary goat anti-rat antibody for visfatin/PBEF/Nampt and FGF-21 (Santa Cruz Biotechnology, Inc., CA, USA) (1:5000 dilution). Secondary antibodies consisted of goat anti-rabbit Horseradish Peroxidase (HRP)-conjugated antibodies (Santa Cruz Biotechnology, Inc., CA, USA) used with enhanced chemiluminescent (ECL) reagents (Perkin Elmer, MA, USA). Binding was imaged on Kodak Maximum Sensitivity (MS) films and quantified with a Bio Imaging System Densitometer (Bio-Rad, CA, USA). The plasma of normal rat was used as the positive control and water as the negative control (data not shown).

RT-PCR analysis of gene expression

Total RNA was isolated from adipose tissue, liver, or muscle using Trizol reagent (Invitrogen, Carlsbad, CA, USA). RNA was quantified by A260, and its integrity verified by agarose gel electrophoresis using ethidium bromide for visualization. Reverse transcription was performed on 1 μg total RNA using RTase Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV) (RNase H, TaKaRa Bio Co. Ltd, Dalian) and Oligo deoxy-thymine (dT) 18 Primer (TaKaRa Bio Bio Co. Ltd, Dalian). The PCR reaction mixture contained in a final volume of 2 μL of the first strand cDNA, 2 μL of 10 pmol specific primers, 4 μL of 2.5 mM deoxy-ribonucleoside triphosphates (dNTP), 5 μL 10 × buffer, 2.5 U Taq DNA polymerase, 3 μL of 2.5 mM $MgCl_2$, dH_2O up to 50 μL. The PCR reaction mixture contained in a final volume of 2 μL of the first strand cDNA, 2 μL of 10 pmol specific primes, 4 μL of 2.5 mM dNTP, 5 μL 10 × buffer, 2.5 U Taq DNA polymerase, 3 μL of 2.5 mM $MgCl_2$, dH_2O upto 50 μL. Samples were incubated for an initial denaturation at 94°C for 3 min followed by 1 cycle consisting of 94°C for 30 s for degeneration, 55°C for 30 s for annealing, and 72°C for 40 s for extension. The final cycle included a prolonged extension phase performed at 72°C for 5 min for more intense extension. The intensities of PCR bands were quantified with a Bio Imaging System Densitometer (Bio-Rad, CA, USA). The sequences, product lengths, and

annealing temperatures of the primers are shown in Table I.

IRS-1 tyrosine phosphorylation assays

Dot-blot assay was used to study the effects of visfatin/PBEF/Nampt overexpression on insulin-stimulated tyrosine phosphorylation. To maximally stimulate protein tyrosine phosphorylation in adipose and liver tissues, insulin was injected via cardiac puncture at the dose of 0.5 U/kg bodyweight 4 days after plasmid treatment. Epididymal adipose tissue and liver were harvested after 5 min and snap-frozen in liquid N_2.

A total of 100 mg of liver or adipose tissue was homogenized in liquid nitrogen and treated with lysis buffer (150 mmol/L NaCl, 1% Nonidet P40 Lysis Buffer (NP-40), 0.5% deoxycholic acid sodium, and 50 mmol/L Tris, pH 8.0). The lysates were clarified by centrifugation (20,000 *g* at 4°C for 3 min). A total of 100 μL of supernatant was blotted onto a polyvinylidene fluoride (PVDF) membrane (0.22 μm) by vacuum using a Bio-Dot blotting apparatus (Bio-Rad, CA, USA). The membrane was blocked with buffer A (Tris Buffered Saline with Tween 20 (TBST) containing 1% blot-qualified bovine serum albumin (BSA)) at 30°C for 120 min. The membrane was incubated for 2 h at 30°C in buffer A containing a rabbit anti-IRS-1 phosphotyrosine antibody (IRS-1 tyrosine-632; Santa Cruz Biotechnology, Inc., CA). The membrane was then washed three times with TBST for 10 min before being incubated for 30 min at 30°C with horseradish peroxidase-labeled sheep anti-rabbit Immunoglobulin G (IgG) antibody (Santa Cruz Biotechnology, Inc., CA) diluted 1:5000 in TBST. Washing in TBST was repeated as described above, and the reactive dots were identified after a 5-min incubation in ECL, prepared according to the manufacturer's instructions. The intensity of the dots was quantified with a Bio Imaging System Densitometer (Bio-Rad, CA, USA).

Analytical procedures

Plasma insulin was measured in deproteinized serum by radioimmunoassay using rat insulin as standard (Linco, St. Charles, Missouri). Free-fatty acid (FFA) was measured in plasma with a kit from Wako Pure Chemicals, Richmond, VA. Plasma glucose was measured using the glucose oxidase method. Plasma triglyceride, total cholesterol, low-density lipoprotein cholesterol (LDL-C), and high-density lipoprotein cholesterol (HDL-C) concentrations were determined enzymatically.

Statistical analysis

Data are presented as mean ± SE. A repeated measures analysis of variance (repeated-measures ANOVA) was used to assess the results measured at consecutive multiple time points. A two-way design was used to incorporate additional effects of different

Table I. Characteristics of the specific primers used for Reverse Transcription Polymerase Chain Reaction (RT-PCR) analysis.

Gene	Forward and reverse primers	bp	Annealing temperature
PPARγ	5′-GATGACCACTCCCATTCCTTTG-3′ 5′-GATGCTTTATCCCCACAGACTC-3′	281	55°C
SREBP-2	5′-GGCTCTGGCCGCAATGTA- 3′ 5′-TGACCGAGG AGCG TGA GT- 3′	172	55°C
HMG CoA	5′-CCTGCGTGTCCCTGGTCCTA- 3′ 5′-CTTTGGGTTACTGGGTTTG G-3′	124	55°C
SCD-1	5′-CTACAAGCCTGGCCTCCTGC -3′ 5′-GGACCCCAGGGAA ACCAGGA -3′	226	55°C
PEPCK	5′-GCCTCCTCAGCTGCATAATGGTCT-3′ 5′-GAATGCTTTCTCGAAGTCCTCTTCTG-3′	376	55°C
ATGL	5′-GACCTGATGACCACCCTTTC-3′ 5′-CAGATACTGGCAGATGCTACC-3′	183	55°C
HSL	5′-CG CCTTACGGAGTCTATGC-3′ 5′-GCTGTCTGATGGCTCTGAGTT-3′	138	55°C
FGF-21	5′-GCCTCTAGGTTTCTTTGCC-3′ 5′-GACTCCTGGTTGCTCTTGG-3′	243	55°C
β-actin1	5′-CCACTGCCGCATCCTCTTCCTC-3′ 5′-TCCTGCT TGCTGATCCACATCT-3′	400	55°C
β-actin2	5′-GCTGTCCCTGTATGCCTC T-3′ 5′-GATGTCACGCACGATTTCC-3′	220	55°C

PPARγ = peroxisome proliferator-activated receptor-γ; SREBP-2 = sterol regulatory element-binding proteins 2; HMG CoA = 3-hydroxy-3-methylglutaryl coenzyme A reductase; SCD-1 = stearoylcoenzyme A desaturase-1; PEPCK = phosphoenolypyruvate carboxykinases; ATGL = adipose triglyceride lipase; HSL = hormone-sensitive lipase; FGF-21 = fibroplast growth factor-21.

experimental groups followed by a *post hoc* least significant difference (PLSD) test to compare two individual groups. Within-group comparisons were made using the paired Student's *t* test. Differences were considered statistically significant at $P<0.05$. All analyses were performed using SPSS (SPSS graduate pack; SPSS, Chicago, IL).

Results

Metabolic parameters

Compared to rats fed a regular diet (NC and NT), rats fed a high fat diet (HT) had significantly increased body-weight and plasma concentrations of FFA, triglycerides (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C) (Table II). On day 3 following plasmid injection, however, plasma levels of TC, HDL-C, LDL-C, and insulin were significantly decreased in the HT group ($P<0.05$). A decrease in TC and HDL-C levels was also observed in the NT group ($P<0.05$).

Plasma levels of visfatin/PBEF/Nampt

To study the effects of visfatin/PBEF/Nampt *in vivo*, we overexpressed the visfatin protein by injecting male Sprague-Dawley rats with pcDNA3.1-visfatin or control pcDNA3.1. The overexpression was carried out by a recombinant plasmid expressing the rat visfatin/PBEF/Nampt cDNA from the cytomegalovirus (CMV) promoter as described under the heading 'Materials and methods'. To confirm the high level of visfatin/PBEF/Nampt protein expression *in vivo*, we performed Western blotting on the plasma samples obtained 4 days after the injection. As shown in Figure 1, injection of pcDNA3.1-visfatin resulted in an increase in plasma levels of visfatin/PBEF/Nampt in the NT group (from 0.99 ± 0.08 to 1.54 ± 0.06 arbitrary units, $P<0.01$) and in the HT group (from 0.99 ± 0.16 to 1.92 ± 0.18 arbitrary units, $P<0.01$). This effect was not observed in the NC group injected with pcDNA3.1(+) (0.99 ± 0.05 versus 0.99 ± 0.04 arbitrary units, $P>0.05$).

The expression of FGF-21 mRNA and its plasma protein levels

Both plasma FGF-21 levels and hepatic expression of FGF-21 mRNA were significantly decreased in the NT group compared with the NC group (0.50 ± 0.02 versus 0.66 ± 0.04, $P<0.05$; and 1.88 ± 0.11 versus 3.04 ± 0.11, $P<0.01$, respectively; all data are expressed in arbitrary units). These differences were not detected between the HT and the NC groups (Figure 2).

Euglycemic-hyperinsulinemic clamp

To directly examine the quantitative effect of visfatin overexpression on insulin sensitivity, we performed euglycemic-hyperinsulinemic clamps on the same group of rats before and after the plasmid injection. Before the plasmid injection, there was no statistic difference in glucose infusion rate (GIR) required to maintain euglycemia among the three groups of rats. Injection of pcDNA3.1-visfatin resulted in a significant increase in GIR in the NT rats (by 28%) and in the HT rats (by 36%). The GIR did not change in the NC rats injected with pcDNA3.1(+) (Table II and Figure 3).

Table II. Laboratory data in rats injected with pcDNA3.1-visfatin versus control pcDNA3.1.

	NC ($n=10$)		NT ($n=10$)		HT ($n=10$)	
	Basal	After	Basal	After	Basal	After
Body-weight (g)	292.2 ± 4.8		289.4 ± 4.4		463.1 ± 10.1	
FBG (mmol/L)	6.0 ± 0.3	6.1 ± 0.4	5.6 ± 0.3	5.9 ± 0.4	6.7 ± 0.2	6.5 ± 0.2
FIns (mU/L)	23.2 ± 7.5	20.0 ± 4.7	19.0 ± 4.9	16.5 ± 6.8	24.4 ± 6.2	$14.5v\pm3.7^{a}$
TG (mmol/L)	$0.49v\pm0.13$	0.39 ± 0.15	0.47 ± 0.10	0.42 ± 0.07	$0.90\pm0.21^{c,d}$	0.86 ± 0.12
TC (mmol/L)	1.55 ± 0.11	1.55 ± 0.12	1.76 ± 0.22	1.31 ± 0.10^{a}	$2.36\pm0.22^{c,d}$	1.60 ± 0.21^{a}
HDL-C (mmol/L)	0.86 ± 0.02	0.89 ± 0.07	0.95 ± 0.15	0.59 ± 0.04^{a}	$1.41\pm0.24^{c,d}$	0.88 ± 0.11^{a}
LDL-C (mmol/L)	0.48 ± 0.10	0.47 ± 0.09	0.59 ± 0.09	0.52 ± 0.06	$0.76\pm0.17^{c,d}$	0.50 ± 0.09^{a}
FFA (μmol/L)	0.86 ± 0.29	1.03 ± 0.30	0.97 ± 0.18	0.93 ± 0.12	$1.16\pm0.18^{c,d}$	1.25 ± 0.15
GIR ($mg\cdot kg^{-1}\cdot min^{-1}$)	30.8 ± 2.0	30.1 ± 1.9	27.6 ± 1.7	35.3 ± 1.4^{b}	$24.0\pm1.2^{c,d}$	32.6 ± 1.2^{b}

Data are mean$\pm$SE. $^{a}P<0.05$. $^{b}P<0.01$, versus basal. $^{c}P<0.05$, versus NT group.
$^{d}P<0.05$, versus NC group.
NC = normal rats injected with pcDNA3.1(+); NT = normal rats injected with pcDNA3.1-visfatin; HT = high-fat rats injected with pcDNA3.1-visfatin; GIR = glucose infusion rates; FBG = fasting blood glucose; FIns = fasting plasma insulin; TG = triglyceride; TC = total cholesterol; HDL-C = high-density lipoprotein cholesterol; LDL-C = low-density lipoprotein cholesterol; FFA = free fatty acids; After = after plasmid injection.

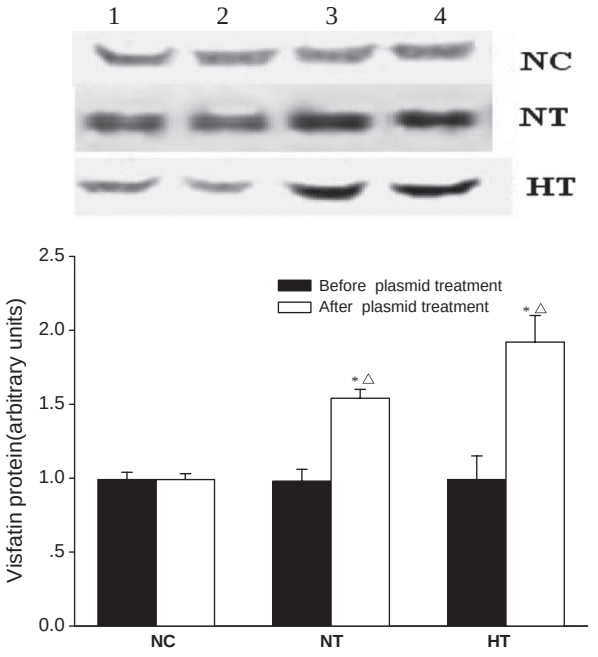

Figure 1. Plasma visfatin/PBEF/Nampt protein levels in normal controls treated with pcDNA3.1(+) (NC; $n=5$), normal-chow rats treated with pcDNA3.1-visfatin (NT; $n=5$), and high-fat-fed rats treated with pcDNA3.1-visfatin (HT; $n=5$) for 4 days. Each experiment was performed in triplicate. Plasma samples were taken under following experimental conditions: Lane 1, basal values; Lane 2, at the end of first clamp; Lane 3, day 4 after plasmid injection; and Lane 4, at the end of second clamp.

RT-PCR analysis of gene expression

Compared to NC (0.68 ± 0.09 arbitrary units), hepatic expression of PPARγ mRNA was significantly upregulated in NT (0.98 ± 0.08 arbitrary units, $P<0.05$) and HT (1.59 ± 0.15 arbitrary units, $P<0.01$). PPARγ mRNA expression in adipose tissues was significantly upregulated in the NT compared to NC (0.56 ± 0.04 versus 0.27 ± 0.07 arbitrary units, $P<0.01$). No difference was detected between the HT and the NC groups. Hepatic expression of SREBP-2 mRNA was significantly increased in both NT and HT groups compared to the NC (0.37 ± 0.06 and 0.36 ± 0.07 versus 0.21 ± 0.04 arbitrary units, both $P<0.05$). Expression of hepatic 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoA) mRNA was higher in the NT than in the NC (0.89 ± 0.12 versus 0.37 ± 0.07 arbitrary units, $P<0.01$). There was no difference in the hepatic mRNA levels of SREBP-2 and HMG CoA between the HT and the NC groups (Figure 4). The mRNA expression of PEPCK (phosphoenolpyruvate carboxykinase), SCD-1 (stearoylcoenzyme A desaturase-1), ATGL (adipose triglyceride lipase), and HSL (hormone-sensitive lipase) in the liver and adipose tissue was not different among the three groups (data not shown).

Insulin signaling studies

To explore the cellular mechanisms involved in the visfatin-induced insulin sensitivity, we obtained muscle and liver samples at the end of the euglycemic-hyperinsulinemic clamping study. These samples represented the fully 'insulinized' state at the termination of the glucose clamp study. Hypervisfatinemia increased IRS-1 tyrosine phosphorylation in response to insulin in the liver of the NT (1.41 ± 0.11, $P<0.01$) and the HT (1.20 ± 0.10, $P<0.05$) rats compared to the NC group (0.92 ± 0.08). A similar increase was seen in muscles of NT (2.18 ± 0.16, $P<0.01$) and HT

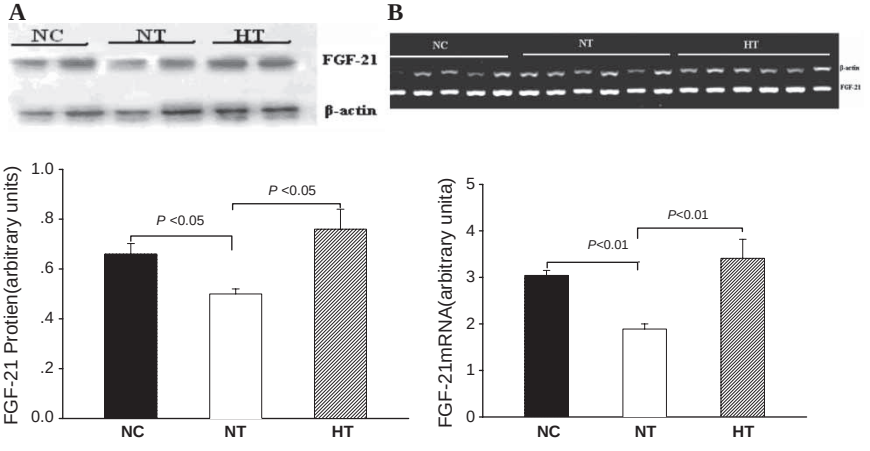

Figure 2. Effects of visfatin/PBEF/Nampt overexpression on fibroplast growth factor-21 (FGF-21) mRNA expression and its plasma protein levels. A: Plasma FGF-21 protein levels ($n=5$ in each group, and each experiment was performed in triplicate); B: FGF-21 mRNA expression in liver tissue ($n=5$ in normal-chow diet with pcDNA3.1(+) plasmid infusion (NC) group; $n=6$ in normal-chow diet with pcDNA3.1-visfatin plasmid infusion (NT) and High-fat diet with pcDNA3.1-visfatin plasmid infusion (HT) group; each experiment was performed in triplicate).

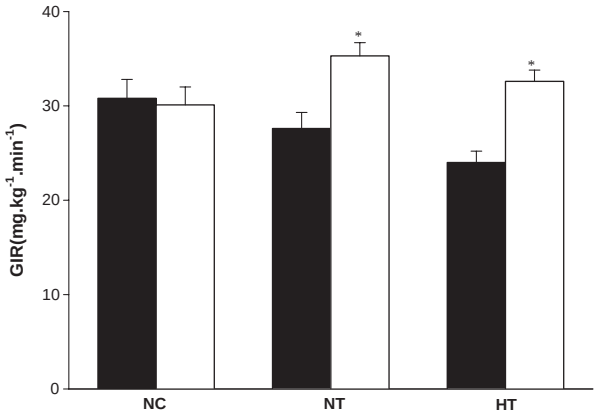

Figure 3. Glucose infusion rates (GIR) during EU clamping in normal controls treated with pcDNA3.1(+) (NC; $n=10$), normal-chow rats treated with pcDNA3.1-visfatin (NT; $n=10$), and high-fat-fed rats treated with pcDNA3.1-visfatin (HT; $n=10$) for 3 days. ■ Before plasmid treatment; □ After plasmid treatment; *indicates $P<0.01$ before and after injection of plasmid.

(1.66 ± 0.06, $P<0.05$) groups compared to NC (1.34 ± 0.04) (Figure 5).

Discussion

Recent work in obesity research has revealed that adipose tissue functions as an endocrine organ, producing a variety of adipocytokines that play important roles in metabolic homeostasis (20). The newly identified adipocytokine visfatin/PBEF/Nampt has piqued interest among researchers. Assessment of the biological function of visfatin/PBEF/Nampt revealed that it has a glucose-lowering effect and enhances glucose uptake *in vivo* and *in vitro*, similar to that induced by insulin. It is also reported that visfatin/PBEF/Nampt can directly activate insulin receptor (IR) in a manner distinct from insulin. In the adipocyte, visfatin/PBEF/Nampt exerts its functions through autocrine and paracrine pathways (10). These data suggest that visfatin/PBEF/Nampt is an important molecule linking adipocyte and blood glucose metabolism. To date, it has not been elucidated how visfatin/PBEF/Nampt modulates glucose and lipid metabolism. It is not known whether visfatin/PBEF/Nampt improves glucose and lipid metabolism by the activation of upstream insulin signaling components and/or by the regulation of expression of related genes. We present in this study that overexpression of visfatin/PBEF/Nampt in rats increases whole-body insulin sensitivity, probably through the activation of the insulin signaling pathway in the liver and adipose tissue. Overexpression of visfatin/PBEF/Nampt also results in the upregulation of PPARγ and SREBP-2 mRNA levels.

Visfatin/PBEF/Nampt-overexpressing rats were generated by intramuscular injection of a plasmid vector (pcDNA3.1-visfatin) containing a mouse visfatin/PBEF/Nampt cDNA. A significant increase in the plasma levels of visfatin/PBEF/Nampt protein was detected on postinjection day 4 in rats injected with pcDNA3.1-visfatin, but not observed in rats injected with a control plasmid vector pc DNA3.1(+). The expression of visfatin via plasmid pcDNA3.1-visfatin injection provided a good *in vivo* model for generating hypervisfatinemia and for the study of physiologic functions of visfatin/PBEF/Nampt.

We found that transgenic expression of visfatin/PBEF/Nampt led to decreased plasma levels of TC, HDL-C, and LDL-C in high-fat-fed rats. These findings suggest that visfatin/PBEF/Nampt plays an important role in cholesterol metabolism *in vivo*. In parallel, overexpression of visfatin/PBEF/Nampt also resulted in a significant improvement of insulin sensitivity as accessed by euglycemic-hyperinsulinemic clamps before and after the plasmid injection. As shown in Figure 3, before the plasmid injection the GIR required to maintain euglycemia was significantly lower in rats fed a high-fat diet (the HT group) than in rats fed a normal diet, indicating a high-fat-induced decrease in insulin sensitivity. Four days after the plasmid injection, a second euglycemic-hyperinsulinemic clamp was done in the same groups of rats. Injection of pcDNA3.1-visfatin increased significantly the GIR not only in the HT group but also in the NT group, indicating the improvement of insulin sensitivity. Injection of control vector pcDNA3.1(+) did not alter the insulin sensitivity in the NC group. These results demonstrate the hypoglycemic effect of visfatin/PBEF/Nampt, which is not associated with diet conditions. Further studies need to be done to evaluate the effect of visfatin on lipid metabolism under physiologic conditions.

Previous studies of the relationship between visfatin/PBEF/Nampt and insulin signaling yield controversial results. To explore the potential mechanisms underlying our observed effects of visfatin/PBEF/Nampt, we determined the activity of the upstream insulin-signaling pathway by measuring IRS-1 phosphorylation in skeletal muscle and in the liver. The levels of IRS-1 phosphorylation in the liver and muscle were increased in both NT and HT rats injected pcDNA3.1-visfatin, suggesting that the improvement of insulin sensitivity by visfatin/PBEF/Nampt is probably through the activation of insulin signaling at posttranscriptional stage. However, injection of pcDNA3.1-visfatin in the present study failed to alter the plasma triglyceride

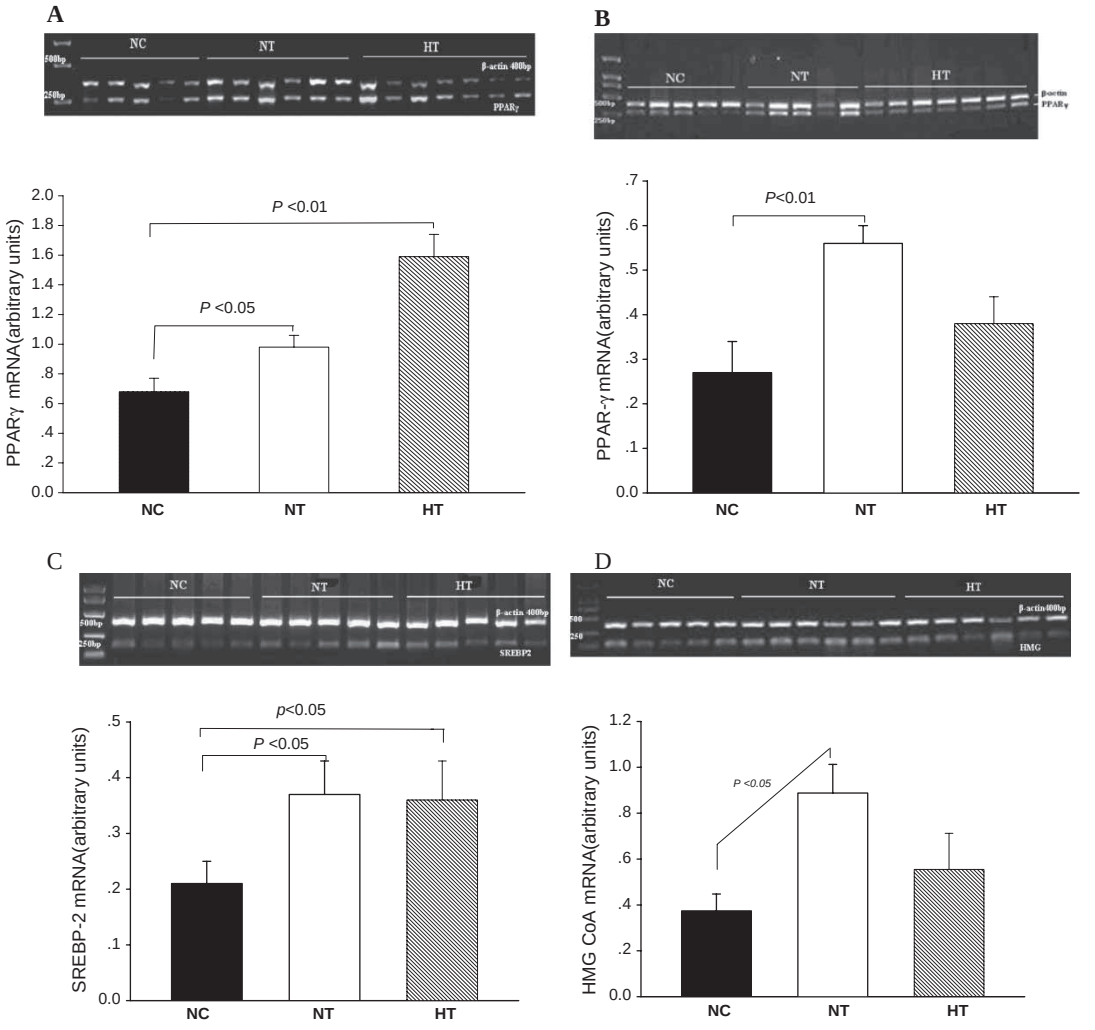

Figure 4. Effects of visfatin/PBEF/Nampt overexpression on peroxisome proliferator-activated receptor-gamma (PPARγ) and sterol regulatory element binding proteins 2 (SREBP-2) mRNA expressions. A: PPARγ mRNA expression in the liver; B: PPARγ mRNA expression in adipose tissues; C: SREBP-2 mRNA expression in the liver; D: 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMG CoA) mRNA expression in the liver. NC: normal-chow diet with pcDNA3.1(+) plasmid infusion; NT: normal-chow diet with pcDNA3.1-visfatin plasmid infusion; HT: High-fat diet with pcDNA3.1-visfatin plasmid infusion.

levels and the mRNA expression of downstream targets of insulin signaling, including PEPCK, ATGL, HSL, and SCD-1 in the liver and muscle (data not shown). These are key enzymes involved in hepatic gluconeogenesis, lipogenesis, and lipolysis (21–23). An explanation for these observations is that visfatin improves insulin sensitivity through its anti-inflammatory effect in addition to activation of the IRS-1, since insulin resistance is known to associate with the inflammatory process.

Despite its cholesterol-lowering effect, visfatin/PBEF/Nampt appears to have no effect on lipogenesis. Administration of pcDNA3.1-visfatin failed to alter the plasma levels of triglycerides and the mRNA expression of enzymes responsible for triglyceride synthesis and hydrolysis. On day 3 following pcDNA3.1-visfatin plasmid injection the plasma levels of TC, LDL-C, and HDL-C were significantly decreased, while SREBP-2 and HMG CoA mRNA expression was significantly increased in normal-chow-fed rats. In HT rats injected with the pc DNA3.1-visfatin plasmid, only SREBP-2 mRNA expression was increased. SREBP-2 is best known to regulate HMG CoA reductase, LDL receptor, and genes directly involved in cholesterol homeostasis (24,25). These results suggest that the increase in mRNA expressions of SREBP-2 and HMG CoA may be a secondary effect of hypervisfatinemia in normal-chow rats, probably due to diminished plasma cholesterol levels. However, pcDNA3.1-visfatin treatment in HT rats did not significantly increase HMG CoA mRNA expression, suggesting a suppressive effect of excess cholesterol uptake in high-fat diet rats. Taken together, visfatin/

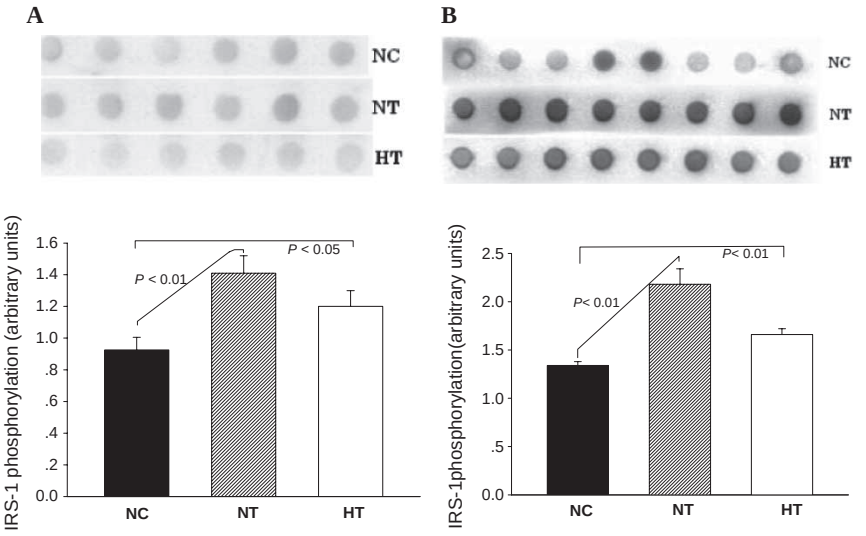

Figure 5. Effects of visfatin/PBEF/Nampt overexpression on the extent of insulin receptor susbstrate-1 (IRS-1) tyrosine phosphorylation. A: IRS-1 tyrosine phosphorylation in the liver ($n=6$ in each group and each experiment was performed in triplicate); B: IRS-1 tyrosine phosphorylation in muscle tissues ($n=8$ in each group and each experiment was performed in triplicate). NC: normal-chow diet with pcDNA3.1(+) plasmid infusion; NT: normal-chow diet with pcDNA3.1-visfatin plasmid infusion; HT: High-fat diet with pcDNA3.1-visfatin plasmid infusion.

PBEF/Nampt may exert a negative feedback through HMG CoA action in regulation of cholesterol homeostasis.

PPARγ is expressed primarily in adipose tissue and is an adipogenic factor that regulates the expression of genes associated with lipid metabolism (26,27). It is well known that PPARγ activation in adipose tissue is associated with an increase in the number of differentiated adipocytes with enhanced capacity to take up and store lipids leading to improved insulin sensitivity in other tissues (28,29). In our study, it was noteworthy that in normal-chow-fed rats with pcDNA3.1-visfatin injection, hypervisfatinemia increased PPARγ mRNA expression in the liver and adipose tissues, increased whole-body insulin sensitivity, and decreased circulating cholesterol levels. Therefore, it is possible that visfatin/PBEF/Nampt reduces insulin sensitivity and lipid profile by modifying PPARγ activities. However, PPARγ mRNA expression in adipose tissues was not significantly increased in the HT-fed pcDNA3.1-visfatin animals compared with normal controls. We speculate that the increasing insulin resistance in adipose tissues secondary to excess fat accumulation in the HT rats may lead to a downregulation of PPARγ mRNA.

To further explore the effects of elevated circulating visfatin/PBEF/Nampt levels on other adipokines, we measured the FGF-21 mRNA expression in the liver and its plasma protein levels. Both hepatic mRNA expression and plasma protein levels of FGF-21 were significantly reduced in pcDNA3.1-visfatin-injected NT but not HT rats. FGF-21 is a recently identified adipokine associated with energy homeostasis and insulin sensitivity (30,31). Our findings suggest a negative effect of visfatin/PBEF/Nampt on FGF-21 mRNA, and protein expressions may attribute to the visfatin-induced insulin sensitivity. In contrast, in HT rats, increasing insulin resistance may lead to a secondary downregulating of FGF-21 mRNA and protein expression.

In conclusion, we studied the effect of visfatin on insulin sensitivity using euglycemic-hyperinsulinemic clamps in the same group of hypervisfatinemic rats we created. We show for the first time *in vivo* that hypervisfatinemia leads to enhanced whole-body insulin sensitivity. Hypervisfatinemia also leads to reduced plasma concentrations of cholesterol and increased PPARγ mRNA expression in the liver and adipose tissue. Our study presents strong evidence suggesting that visfatin/PBEF/Nampt improves insulin sensitivity and has hypocholesterolemic effects, at least partially through upregulation of PPARγ activity. We speculate that visfatin/PBEF/Nampt may be a potential therapeutic target for the treatment of insulin resistance and atherosclerosis.

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