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ORIGINAL PAPER

Circulating and local visfatin/Nampt/PBEF levels in spontaneously hypertensive rats, stroke-prone spontaneously hypertensive rats and Wistar-Kyoto rats

Pei Wang · Hui Du · Ruo-Yu Zhang · Yun-Feng Guan · Tian-Ying Xu · Quan-Yi Xu · Ding-Feng Su · Chao-Yu Miao

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Abstract Visfatin (also known as nicotinamide phosphoribosyltransferase and pre-B cell colony-enhancing factor) is a multifunctional protein. Visfatin has been reported to be involved in several biological processes in the cardiovascular system, . However, the role of visfatin in hypertension is still unclear. In this study, we examined the circulating and local adipose visfatin levels in spontaneously hypertensive rats (SHR), stroke-prone spontaneously hypertensive rats (SHR-SP), and in their normotensive control Wistar-Kyoto (WKY). SHR and SHR-SP rats exhibited lower body weight, lower fat tissue and hypolipidemia. No differences of serum visfatin levels were observed in SHR/SHR-SP and WKY. Serum visfatin levels did not correlate to serum glucose, lipids, insulin, and fat pad weights, but significantly correlated to weights of skeletal muscle. Visfatin expression in visceral fat tissue was slightly lower in SHR-SP compared with that in WKY. Moreover, there were no significant differences of visfatin expression in skeletal muscles among WKY, SHR and SHR-SP. Finally, visfatin protein was detected in L6 rat skeletal muscle cell culture medium, indicating that visfatin was secreted from skeletal muscle cells. Thus, our results may provide useful information for understanding the characteristic of visfatin in hypertensive models, and support the view that visfatin may be a myokine.

Keywords Visfatin · Hypertension · Skeletal muscle · Spontaneously hypertensive rats · Stroke-prone spontaneously hypertensive rats · Myokine

P. Wang and H. Du contributed equally to this work.

P. Wang · H. Du · R.-Y. Zhang · Y.-F. Guan · T.-Y. Xu · Q.-Y. Xu · D.-F. Su · C.-Y. Miao (✉)
Department of Pharmacology, Second Military Medical University, 325 Guo He Road, Shanghai 200433, China
e-mail: chaoyumiao@yahoo.com.cn

C.-Y. Miao
Shanghai Key Laboratory of Vascular Biology, Ruijin Hospital and Shanghai Institute of Hypertension, Shanghai Jiaotong University School of Medicine, Shanghai, China

Introduction

Visfatin [also known as nicotinamide phosphoribosyltransferase (Nampt) and pre-B cell colony enhancing factor (PBEF)], is a new attractive protein. Visfatin was originally isolated from the peripheral blood lymphocyte [1] and characterized as a rate-limiting enzyme with Nampt activity, which is crucial for nicotinamide adenine dinucleotide (NAD^+) biosynthesis [2, 3]. Recently, visfatin was reported to be a new adipokine which produced and secreted mainly by visceral adipose tissue, and binding to and activating the insulin receptor, inducing an insulin-mimic effect both in vitro and in vivo [4], although this effect is still putative [5, 6]. Numerous studies have been published to address the possible associations between plasma visfatin levels and various metabolic disorders such as obesity, type 2 diabetes, and metabolic syndrome [7–9].

In the cardiovascular system, it has been reported that visfatin produces cardioprotection in ischemic myocardial injury [10], induces vascular endothelial angiogenesis [11] and inflammation [12]. In a previous study, we have demonstrated that perivascular adipose-derived visfatin regulates vascular smooth muscle cell growth and apoptosis via both endocrine and paracrine pathways though ERK1/2 and p38 signaling pathways [13]. However, little is known about the role of visfatin in hypertension. In a small sample in clinical research, Dogru et al. [14] have

shown that there was no change of blood visfatin levels in a specifically selected group of young hypertensive patients without obesity or lipid and glucose metabolic abnormalities. Additionally, in a very recent study, we have reported that serum visfatin was increased and associated with lipid metabolic abnormality in Lyon hypertensive (LH) rats [15].

Due to the tight associations between adipokines and cardiovascular diseases, we hypothesized that cardiovascular functions were related to visfatin expressions in hypertensive animal models. Therefore, in this study, we used two hypertensive animal models, spontaneously hypertensive rats (SHR) and stroke-prone spontaneously hypertensive rats (SHR-SP), to explore the potential associations between visfatin expressions and hypertension or other cardiovascular functions. The SHR strain is a well-known and most widely used genetic hypertensive animal model derived from Wistar-Kyoto (WKY) normotensive rats [16]. SHR-SP, a substrain of SHR which was established in 1974 by Okamoto et al. [17], develop more severe hypertension than SHR and mostly finally succumb to cerebral hemorrhage or infarction.

Materials and methods

Animals

Wistar-Kyoto, SHR, and SHR-SP rats were provided by the Animal Center of our university. Male rats aged 18–20 weeks were used for the experiments. All procedures were in accordance with institutional guidelines for animal care and the Guide for Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH publication no. 85–23, revised 1996).

Blood pressure measuring

Blood pressure and heart rate were continuously recorded in conscious unrestrained WKY, SHR and SHR-SP as described in our previous reports [18, 19]. The rats were anaesthetized with an intraperitoneal injection of ketamine (50 mg/kg) and diazepam (5 mg/kg). A floating polyethylene catheter was inserted into the lower abdominal aorta via the left femoral artery. After 2 days of recovery, animals were placed in individual cylindrical cages containing food and water. After approximately 14 h habituation, the blood pressure signals were digitized and processed by a blood pressure instrument (ALCBIO, Shanghai, China) for 3 h while moving freely in the cage. The mean values of these parameters over the last 1-h period for each rat were calculated.

Tissue samples and serum biochemical assays

Tissue samples and serum biochemical assays were performed as described previously [15]. Importantly, to avoid the potential influence of the intervention of measuring blood pressure on the blood visfatin level, another three subsets of these strains were used. After being fasted overnight and anesthetized with sodium pentobarbital (40 mg/kg, i.p.), blood, subcutaneous and visceral fat pads were carefully dissected from each animal according to anatomical landmarks as described previously [20]. Visceral fat tissue included omental and mesenteric fat tissue (all fat tissue along the mesentery, starting at lesser curvature of the stomach and ending at the sigmoid colon), retroperitoneal and perirenal fat tissue, and periepididymal fat tissue. The fat pads were weighed, and standardized to body weight according to previous reports [21]. The weight of gastrocnemius muscle was deemed as representative of skeletal muscle weight as described previously [22, 23]. In another three subsets of the rats, gastrocnemius, soleus and extensor digitorum longus (EDL) were isolated. Tissues were frozen at −80°C for western blot analysis and PCR analysis. Serum total cholesterol, high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, triglyceride, glucose, and insulin were measured with an autoanalyzer (Beckman Autoanalyzer; Beckman Instruments, Fullerton, CA, USA). The homeostasis model assessment-insulin resistance (HOMA-IR) index were calculated as previously [24]. HOMA-IR = fasting serum insulin (mIU/L) $\times$ fasting serum glucose (mmol/L)/22.5.

Enzyme immunoassay and western blotting analysis

Serum visfatin levels and local visfatin expression were determined by visfatin (C-terminal) enzyme immunoassay (EIA) kit (Phoenix, Belmont, CA, USA) and western blotting analysis, respectively, as described previously [15]. The EIA kit has an intraassay CV% of <5%, an interassay CV% of <12% and a sensitivity of 0.1 ng/ml. For western blotting analysis, tissues were homogenized by protein extraction reagent (Pierce, USA) supplemented with a protease inhibitor cocktail (Pierce), separated by 10% SDS-PAGE, transferred to nitrocellulose membranes, probed by rabbit polyclonal antibody against visfatin (1:1,000; Phoenix), mouse monoclonal antibody against glyceraldehyde 3-phosphate dehydrogenase (GAPDH, 1:1,000; Santa-Cruz, USA) overnight at 4°C. After incubating with corresponding secondary antibodies for 2 h, the signals were visualized with enhanced chemiluminescence reagent (Santa Cruz).

Reverse transcription polymerase chain reaction analysis

RNA extraction and reverse transcription polymerase chain reaction (RT-PCR) were performed as described previously [13]. Total RNA was extracted from several rat tissues using Trizol, and 1 µg RNA was reverse transcribed to cDNA. The following primers were used: rat visfatin (accession no. NM_177928), 5′ GAT TCT GGA AAT CCG CTC GA 3′ (sense) and 5′ TGA CTC TAA GGT AAG GTG GCA GC 3′ (antisense); rat β-actin, 5′ AGA CCT CTA TGC CAA CAC AGT GC 3′ (sense) and 5′ GAG CCA CCA ATC CAC ACA GAG T 3′ (antisense). The PCR product was subjected to electrophoresis on 1.2% agarose.

Cell culture

L6 rat skeletal muscle cells were cultured as described previously [25]. L6 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) in a humidified atmosphere of 95% air and 5% CO_2 at 37°C. Myoblasts at 50–60% confluence were differentiated into myotubes in DMEM containing 2% FBS for 3 days. Three days after the myotubes were formed, the medium was collected and concentrated around three times using Microcon YM-10 (Millipore) with 10-kDa molecular weight cutoff membranes.

Statistical analysis

Data are expressed as mean ± SEM. Data was analyzed by ANOVA followed by Tukey post hoc using SPSS software. The relationships between serum visfatin level and fat tissue weight and between serum visfatin level and serum biochemical parameters were assessed by linear regression analysis. Statistical significance was set at $P < 0.05$.

Results

Blood pressure levels of SHR and SHR-SP

Systolic and diastolic blood pressure were significantly higher in SHR and SHR-SP, compared with WKY rats (Fig. 1a, b). Heart rate was unchanged in these hypertensive rats (Fig. 1c). Furthermore, blood pressure levels in SHR-SP were markedly higher than those in SHR.

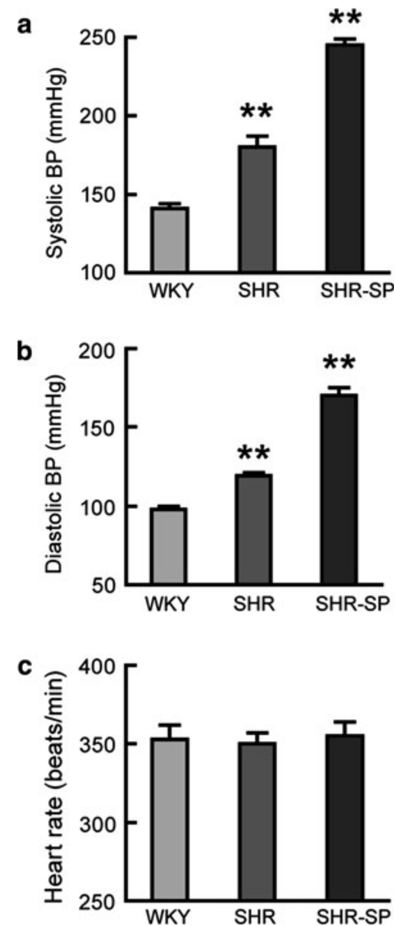

Fig. 1 **a, b** Blood pressure (*BP*) and **c** heart rate of Wistar-Kyoto rats (*WKY*, $n = 8$), spontaneously hypertensive rats (*SHR*, $n = 8$), and stroke-prone spontaneously hypertensive rats (*SHR-SP*, $n = 8$). $^{**}P < 0.01$ versus WKY

Differences of serum parameters and fat weights among SHR, SHR-SP and WKY

SHR and SHR-SP had lipid metabolic abnormality (Fig. 2a). They were characterized by lower serum levels of total cholesterol, HDL cholesterol and triglyceride. There were no significant differences in serum glucose levels, serum insulin levels, and HOMA-IR insulin resistance index in SHR and SHR-SP. The ratio of insulin to glucose was not different in WKY (3.71 ± 0.67), SHR (5.00 ± 0.31, $P > 0.05$ vs WKY) and SHR-SP (3.69 ± 0.34, $P > 0.05$ vs WKY).

Compared with WKY, body weight was lower in SHR and SHR-SP by 32 and 34%, respectively (Fig. 2b). The weight of subcutaneous fat tissue was insignificantly higher in SHR by 35%, whereas it was significantly lower in SHR-SP by 56%. The weight of visceral fat tissue was lower in SHR by about 50% and in SHR-SP by 55%. For different

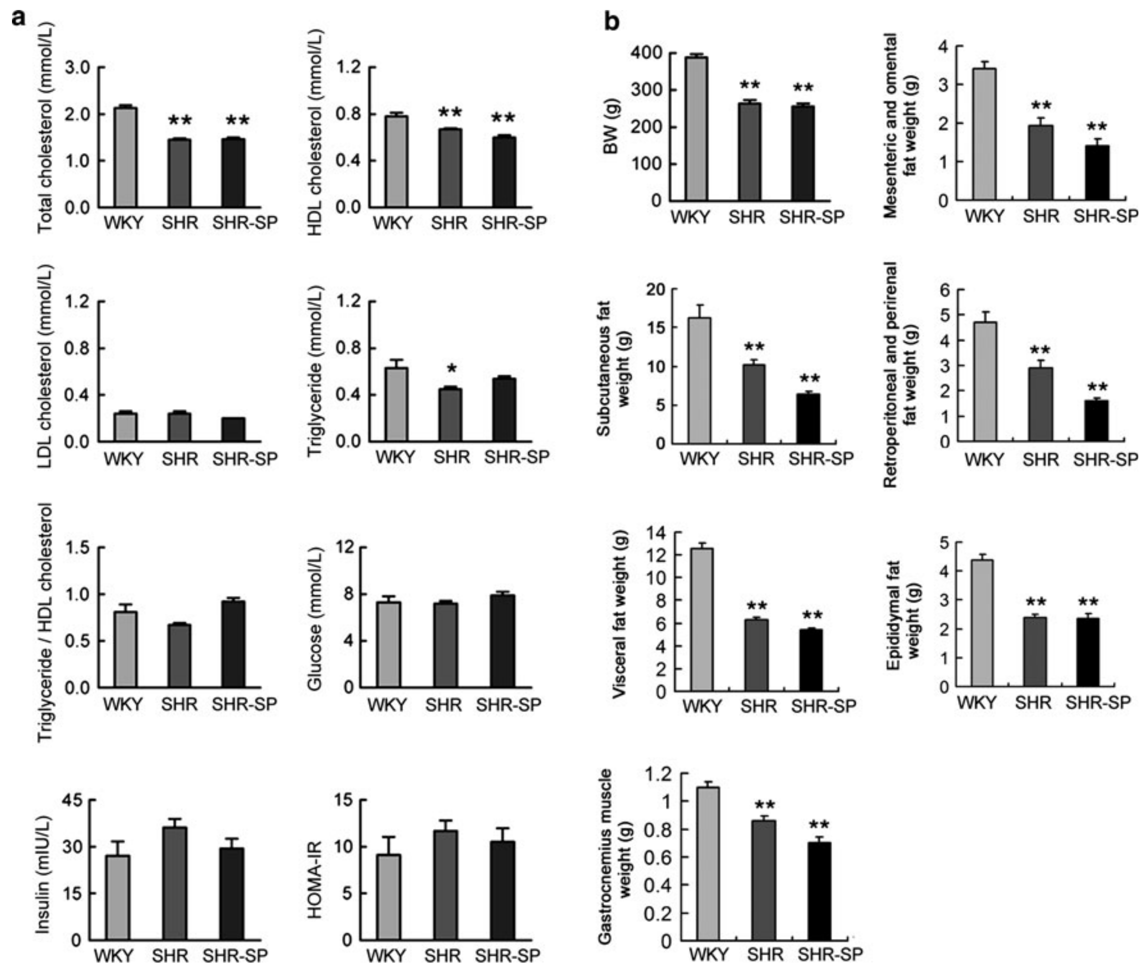

Fig. 2 Serum biochemical parameters (**a**), body weight (*BW*) and fat tissue weights (**b**) of Wistar-Kyoto rats (*WKY*, $n = 8$), spontaneously hypertensive rats (*SHR*, $n = 8$), and stroke-prone spontaneously hypertensive rats (*SHR-SP*, $n = 8$). $^{*}P < 0.05$, $^{**}P < 0.01$ versus WKY. *HDL* High-density lipoprotein, *LDL* low-density lipoprotein, *HOMA-IR* homeostasis model assessment insulin resistance

depots of visceral fat tissue, mesenteric and omental fat tissue was lower in SHR by 35% and in SHR-SP by 58%, retroperitoneal and perirenal fat tissue was lower in SHR by 39% and in SHR-SP by 67%, and epididymal fat tissue was lower in SHR by 46% and in SHR-SP by 43%.

Circulating visfatin levels and linear regression analysis

Serum visfatin levels were not statistically different in WKY, SHR and SHR-SP (Fig. 3), although there were declining trends in SHR and SHR-SP. Circulating visfatin levels did not correlated with blood pressure levels in these stains (data not shown). As shown in Fig. 4a, there were no significant correlations between circulating visfatin and glucose, lipids and insulin levels in whole SHR and SHR-SP as well as in separate SHR and SHR-SP (data not shown). Similarly, there were no significant correlations between circulating visfatin levels and body weight, subcutaneous fat tissue weight and visceral fat tissue weight in whole SHR and SHR-SP (Fig. 4b). Similar results were obtained in separate SHR and SHR-SP (data not shown). However, a significant correlation between circulating visfatin levels and skeletal muscle weight was observed ($P < 0.05$, Fig. 4b).

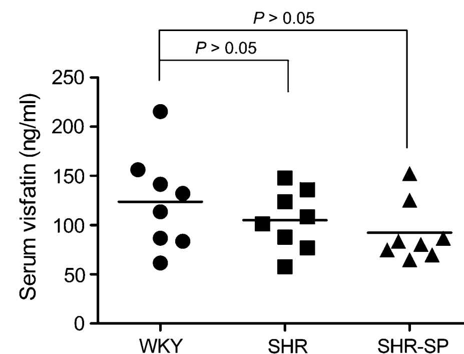

Fig. 3 Serum visfatin levels of Wistar-Kyoto rats (*WKY*, $n = 8$), spontaneously hypertensive rats (*SHR*, $n = 8$), and stroke-prone spontaneously hypertensive rats (*SHR-SP*, $n = 8$)

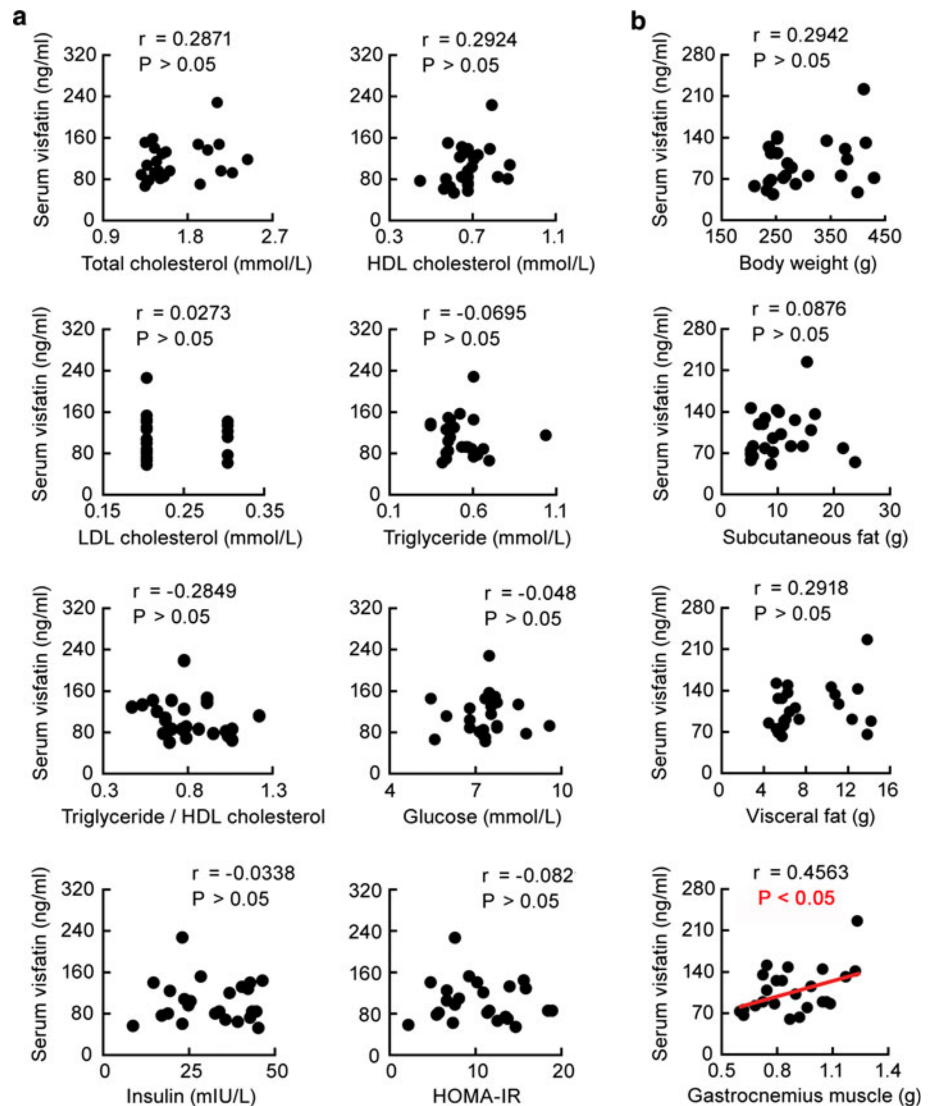

Fig. 4 Scatter plots showing the relationships between serum visfatin levels and serum lipids levels (**a**), and between serum visfatin levels and body weights, fat pad weights and skeletal muscle weights (**b**) in of Wistar-Kyoto rats (*WKY*, $n = 8$), spontaneously hypertensive rats (*SHR*, $n = 8$), and stroke-prone spontaneously hypertensive rats (*SHR-SP*, $n = 8$). Data were analyzed by linear regression analysis. Abbreviations as in Fig. 2

Local adipose visfatin levels in SHR and SHR-SP

In SHR, visfatin expression remained unchanged in both examined fat tissues, compared with WKY (Fig. 5a, b). In SHR-SP, visfatin expression was lower (~0.7-fold) in mesenteric visceral fat tissue, without significant change in inguinal subcutaneous fat tissue, compared with WKY (Fig. 5a, b).

Local skeletal muscle visfatin levels in SHR and SHR-SP

Because the linear analysis indicating visfatin levels were positively associated with skeletal muscle weights, we considered that the visfatin might be secreted by skeletal muscle as a myokine. To test this idea, we investigated the local skeletal muscle visfatin levels. There were no significant differences of visfatin protein (Fig. 6a) and mRNA (Fig. 6b) levels among the WKY, SHR and SHR-SP. These results excluded the possibility of hypertension altered visfatin expression in skeletal muscle, and suggested that the amount of skeletal muscle, but not the local visfatin expression levels in skeletal muscle, affected the circulating visfatin levels in rats.

To directly provide further evidence, we tested whether visfatin can be detected in the culture medium of skeletal muscle cells. Indeed, in the culture medium of L6 myotubes, visfatin was detected by Western blotting (Fig. 6c), further supporting the notion that visfatin may be a myokine.

Discussion

In addition to providing new information on body fat distribution of the SHR, SHR-SP and WKY rats, the present

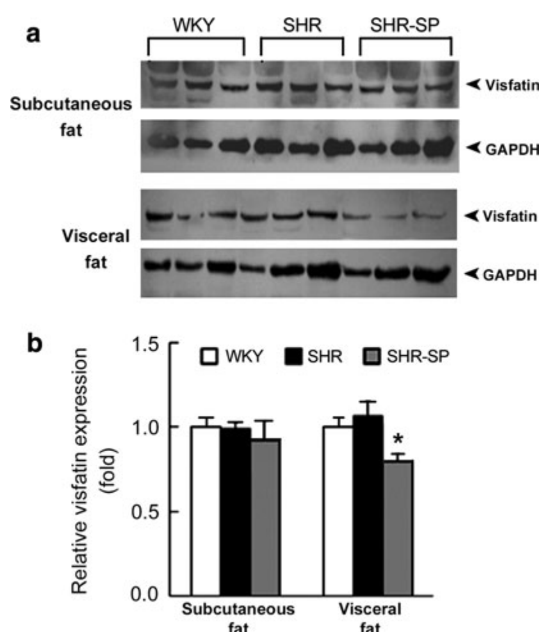

Fig. 5 Western blotting showing visfatin expression in inguinal subcutaneous fat tissue and mesenteric visceral fat tissue in Wistar-Kyoto rats (*WKY*), spontaneously hypertensive rats (*SHR*), and stroke-prone spontaneously hypertensive rats (*SHR-SP*). $*P < 0.05$ versus WKY. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control

study demonstrates that there were no differences in circulating visfatin levels in SHR and SHR-SP, compared with that in WKY. Interestingly, circulating visfatin levels positively correlated with skeletal muscle weights but not fat pads weights, lipids, glucose and insulin levels in SHR and SHR-SP, suggesting visfatin may be a myokine. The amount of skeletal muscle, but not the local visfatin expression levels in skeletal muscle, may affect the circulating visfatin levels in rats.

In our previous study, we showed that serum visfatin levels are enhanced and correlated to body weights, fat tissue weights and serum lipid levels in LH rats. Moreover, both excessive fat tissue and high visfatin protein expression in visceral fat tissue contribute to the elevation of serum visfatin concentrations in LH rats. LH, SHR and SHR-SP rats are different hypertensive models. Although they all have high blood pressure levels, LH exhibited high body weight, higher fat tissue and hyperlipidemia, whereas SHR and its sub-strain SHR-SP exhibited low body weight, reduced fat tissue and hypolipidemia (Figs. 1 and 2). Different from previous results was that serum visfatin levels were elevated and associated with lipids abnormalities in LH rats [15], but we did not observe similar results in SHR and SHR-SP in this study. Furthermore, a downward trend of visfatin serum levels was observed in lean hypertensive rats (Fig. 2). These indicate that a high circulating visfatin level appears only in obesity-related hypertension of LH. Our results support a clinical study, showing neither alteration of plasma visfatin levels nor correlation between plasma visfatin levels and blood pressure levels in a small group of young hypertensive patients without obesity and dyslipidemia [14].

At the examined age (18–20 weeks) in the present study, SHR-SP was very similar to SHR in all determined parameters except for higher blood pressure levels in SHR-SP than SHR. Also, similar results were obtained from the linear regression analysis using SHR and SHR-SP in the whole population and in two separate populations. Thus, SHR-SP was considered as a hypertensive model in the present study. It should be emphasized that at a later stage, SHR-SP mostly die from stroke, ischemic stroke in a majority and hemorrhagic stroke in a minority, with a lifespan of 45 weeks, whereas SHR is resistant to stroke, with a longer lifespan of 75 weeks [26, 27]. Obviously, in the present study, serum visfatin levels demonstrated a downward trend without statistical significance in SHR-SP. This seems in contradiction to a clinical study, showing an elevation of plasma visfatin levels in ischemic stroke patients [28]. The contradiction might be explained by a big difference in examined time; in the present study, serum visfatin was determined long before stroke occurrence, while in the clinical study, serum visfatin was determined after stroke, which perhaps reflects a compensatory reaction in the acute stage of illness. In any case, the role of visfatin in stroke remains to be further studied.

The most intersecting and notable point in this study may be that we found a positive correlation between serum visfatin levels and skeletal muscle weights in SHR and SHR-SP. The source of visfatin in vivo is still under investigation. In 2008, Krzysik-Walker et al. [29] showed that skeletal muscle, not the visceral adipose tissue, might be the primary source of visfatin in chickens, thereby raising the possibility that visfatin might be acting as a myokine affecting skeletal muscle growth and metabolism. Then, Fulco et al. [30] demonstrated that visfatin played an important role in skeletal myoblast differentiation. Moreover, intracellular visfatin (Nampt) was induced by exercise in skeletal muscles in humans [31] and rats [32]. The positive correlation between serum visfatin levels and skeletal muscle weights shown in our study supports the notion that visfatin may be a myokine. In fact, in L6 rat skeletal muscle cells culture medium, we detected the visfatin. This result further indicates visfatin can be secreted by skeletal muscle. However, the precise role of visfatin in skeletal muscle needs further deep investigation.

Collectively, in this study, we compared the circulating and local adipose visfatin levels among SHR, SHR-SP and WKY rats. No significant changes of circulating visfatin and no association between circulating visfatin levels and blood pressure, serum glucose, serum lipids, serum insulin,

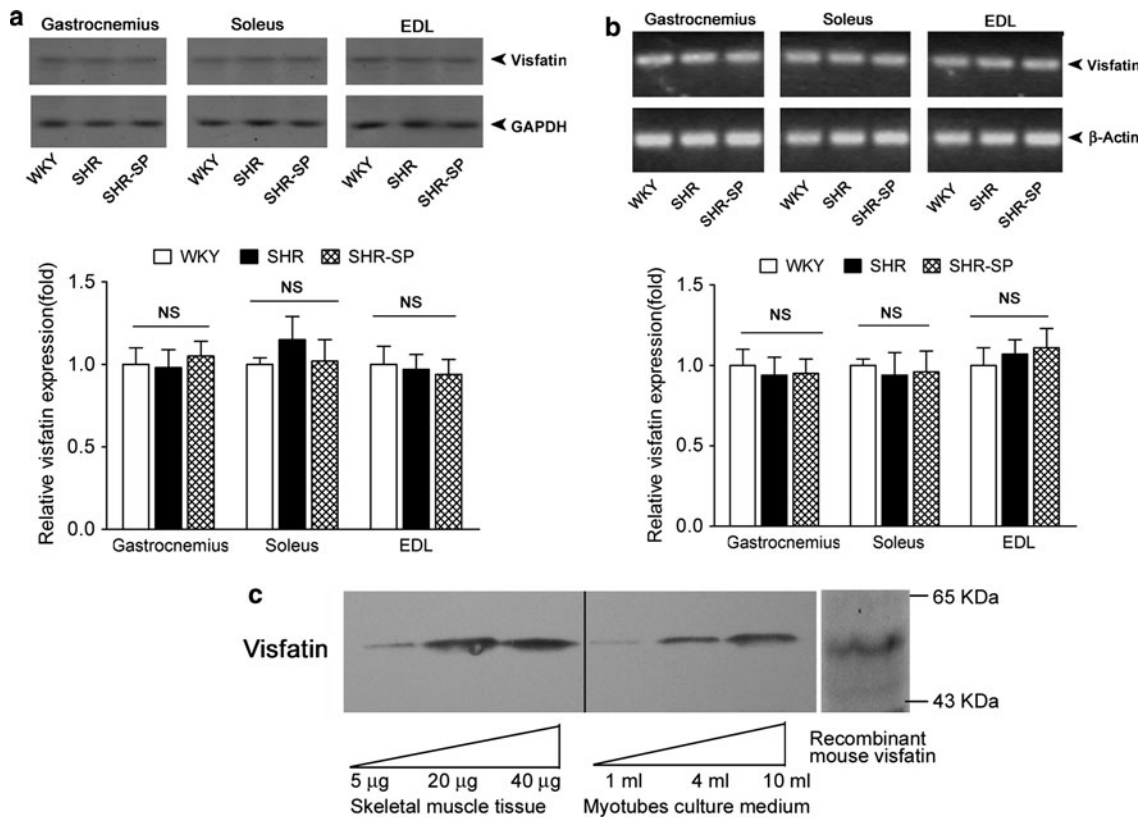

Fig. 6 Western blotting showing visfatin expression in skeletal muscle tissues and skeletal muscle cell culture medium. Visfatin protein (**a**) and mRNA (**b**) expression in *gastrocnemius*, *soleus* and extensor digitorum longus (*EDL*) in Wistar-Kyoto rats (*WKY*), spontaneously hypertensive rats (*SHR*), and stroke-prone spontaneously hypertensive rats (*SHR-SP*). *NS* Not significant versus WKY. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was used as a loading control. **c** Visfatin was detected in skeletal muscle tissue and L6 rat skeletal muscle cell culture medium. Recombinant mouse visfatin was used as a positive control

body weights and fat pad weights were observed. However, we found that circulating visfatin levels were positively correlated with skeletal muscle weights in these rats, and that visfatin can be secreted by skeletal muscle cells in vitro, suggesting the potential involvement of visfatin in the biological functions of skeletal muscles.

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Conflict of interest statement The authors declared no conflict of interest.

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