Am J Transl Res 2017;9(12):5299-5307
www.ajtr.org /ISSN:1943-8141/AJTR0065140

Original Article

FGF21 inhibitor suppresses the proliferation and migration of human umbilical vein endothelial cells through the eNOS/PI3K/AKT pathway

Yumei Li[1*], Jiangnan Huang[2*], Zhiyuan Jiang[2], Yang Jiao[3], Hui Wang[3]

[1]Centre for Cellular & Structural Biology, Sun Yat-sen University, Guangzhou City, Guangdong Province, P. R. China; [2]Department of Hypertension, First Affiliated Hospital of Guangxi Medical University, Nanning City, Guangxi Province, P. R. China; [3]College of Pharmacy, Guangxi Medical University, Nanning City, Guangxi Province, P. R. China. [*]Equal contributors.

Received July 8, 2017; Accepted October 18, 2017; Epub December 15, 2017; Published December 30, 2017

Abstract: In this study, we investigated molecular mechanism underlying the regulation of endothelial nitric oxide synthase (eNOS) expression by fibroblast growth factor 21 (FGF21). We analyzed FGF21 and eNOS expression in hypertensive and healthy (control) subjects (n=30/group). To evaluate the effects of FGF21 on endothelial cells, we transfected FGF21 mimics or FGF21 inhibitor into human umbilical vein endothelial cells (HUVECs). Cell proliferation was analyzed using the methyl thiazolyl tetrazolium assay, and cell migration and invasion were assessed using Transwell assays. In addition, eNOS, PI3K, and AKT mRNA in the HUVECs were evaluated by quantitative reverse transcription PCR, and p-eNOS, PI3K, and p-AKT were evaluated by Western blotting. Our results showed increased levels of FGF21 mRNA and eNOS mRNA/protein in the blood of hypertensive patients compared with healthy controls. The FGF21 inhibitor inhibited HUVEC growth, migration, and invasion and significantly decreased eNOS, PI3K, and AKT mRNA levels and p-eNOS, PI3K, and p-AKT protein levels in HUVECs. Treatment with VEGF and/or overexpression of eNOS partially restored cell proliferation and p-AKT levels. Taken together, our results indicate that FGF21 regulates eNOS through the PI3K/AKT pathway.

Keywords: Fibroblast growth factor 21, eNOS, HUVECs, PI3K/AKT, VEGF

Introduction

Fibroblast growth factor 21 (FGF21) is a member of the FGF family of proteins, which carries out diverse biological functions through various modes of action [1]. FGFs exert mitogenic and cell survival activities and are involved in embryonic development, cell growth, morphogenesis, tissue repair, tumor growth, and invasion [2, 3]. FGF21 is a secreted factor that is controlled by metabolic pathways such as the peroxisome proliferator-activated receptor (PPAR) γ [4] and PPARα [5] pathways. The role of FGF21 in cardiovascular disease, diabetes, metabolic disorders, and cancer has been extensively studied [6-8]. For example, FGF21 has been found to inhibit key processes in the pathogenesis of atherosclerosis, directly and indirectly protects against cardiovascular disease [9], and appears to act on endothelial cells to protect against atherosclerosis, improve lipid profiles, and decrease systemic inflammation [10].

There is increasing evidence that FGF21 regulates the expression of genes involved in cell differentiation, such as those in the Ets-like protein-1 pathway, thereby promoting the differentiation of cells into a contractile phenotype [11]. Tissue-specific metabolic actions of FGF21 have been described, including effects on insulin sensitivity and lipid metabolism [12]. In addition, FGF21 protects the liver against D-galactose-induced oxidative stress and apoptosis by activating the Nrf2 and phosphoinositide-3-kinase (PI3K)/AKT pathways [13]. PI3K-AKT signaling, which plays a crucial role in cell growth [14], is activated by many types of stimuli and promotes cell survival through the Bcl-2 family of proteins [15]. However, most studies of FGF21 have been conducted in rodent mod-

Table 1. Clinicopathologic characteristics of the study population

	Healthy subjects (n=30)	Hypertensive subjects (n=30)	P
Age, years	64.50±6.48	65.23±6.42	>0.05
Male, n (%)	17 (56.7%)	18 (60.0%)	>0.05
Smoker, n (%)	8 (26.7%)	10 (33.3%)	>0.05
FPG, mmol/L	5.35±1.92	5.18±2.21	>0.05
BMI, kg/m^2	24.38±2.15	25.15±3.21	>0.05

Abbreviations: BMI, body mass index; FPG, fasting plasma glucose. Results are expressed as n (%) or mean ± SD.

els; therefore, it is important to investigate its potential role in human diseases.

Although much is known about the effects of FGF21, the intracellular signaling pathways underlying these effects are unclear. A better understanding of biological pathways that lead to the development of cardiovascular disease may help identify new therapeutic targets. Our previous work showed that the gene encoding endothelial nitric oxide synthase (eNOS), which consists of 26 exons and 25 introns (7q35-36), is expressed primarily in endothelial cells [16], and previous studies have described the function of eNOS in human umbilical vein endothelial cells (HUVECs) [17, 18].

In this study we evaluated the expression of FGF21 and eNOS in patients with hypertension. In addition, we assessed the effect of FGF21 on HUVEC proliferation, migration, and invasion and eNOS expression and phosphorylation. We hypothesized that the effects of FGF21 were mediated through the PI3K/AKT signaling pathway.

Materials and methods

Patients and blood samples

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and national research committees and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all study participants. We recruited 30 hypertensive patients from October 2015 and August 2016 at the First Affiliated Hospital of Guangxi Medical University (Nanning, China). In addition, we recruited 30 healthy (control) participants. We collected two blood samples for the analysis of FGF21 and eNOS mRNA levels. Finally, we predicted the target gene.

ELISA

We evaluated eNOS protein levels in the blood samples by enzyme-linked immunosorbent assay (ELISA). ELISA plates were coated with the antigen and washed three times with 200 μl wash buffer. After adding 200 μl blocking buffer to the wells, the plates were incubated at 37°C for 1 h to block nonspecific binding sites. The plates were then washed three times with 200 μl wash buffer. Primary antibodies were added to each well, and the plates were incubated at 37°C for 1 h. After washing the plates five times with wash buffer, horseradish peroxidase-conjugated secondary antibodies were added, and the plates were incubated at 37°C for 35 minutes. The plates were then washed five times with wash buffer, and absorbance at 450 nm was detected.

Cell culture and transfection

HUVEC line was purchased from the Cell Bank of Shanghai (Shanghai, China) and cultured in Dulbecco's Modified Eagle's Medium (DMEM) with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (all from Thermo Fisher Scientific, Waltham, MA, USA). The cells were maintained at 37°C in an atmosphere of 5% CO_2. When the cells reached 80% confluence, they were transfected with FGF21 mimics (100 nM, Sigma-Aldrich, Milan, Italy), FGF21 inhibitor (100 nM, Sigma-Aldrich, Milan, Italy), or control plasmid (100 nM, Sigma-Aldrich, Milan, Italy) using Lipofectamine 3000 (Thermo Fisher Scientific) according to the manufacturer's instructions. The cells were incubated in Opti-MEM (Thermo Fisher Scientific) for 6 h after transfection and then maintained in DMEM for 48 to 72 h. Fluorescent images were used to monitor transfection efficiency.

Cell proliferation assay

Cell proliferation was evaluated as previously described [19]. Briefly, cells stably expressing FGF21 mimics, FGF21 inhibitor, or control plasmid were seeded in 96-well plates (2×10^4 cells/well) in DMEM containing FBS. After a 24-h

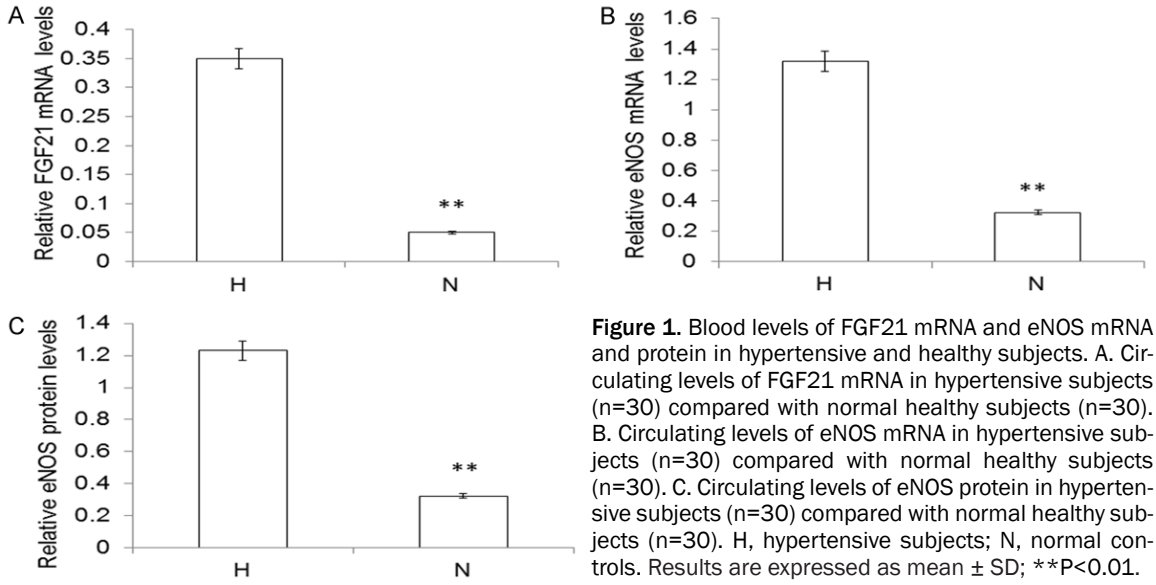

Figure 1. Blood levels of FGF21 mRNA and eNOS mRNA and protein in hypertensive and healthy subjects. A. Circulating levels of FGF21 mRNA in hypertensive subjects (n=30) compared with normal healthy subjects (n=30). B. Circulating levels of eNOS mRNA in hypertensive subjects (n=30) compared with normal healthy subjects (n=30). C. Circulating levels of eNOS protein in hypertensive subjects (n=30) compared with normal healthy subjects (n=30). H, hypertensive subjects; N, normal controls. Results are expressed as mean ± SD; **P<0.01.

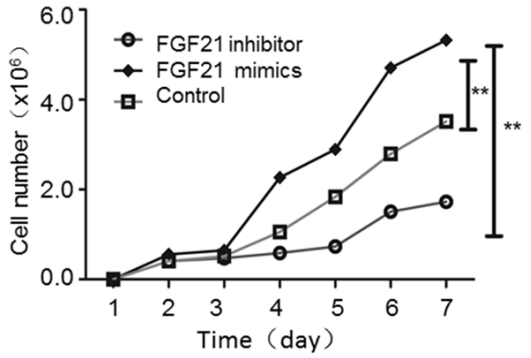

Figure 2. Effect of FGF21 on HUVEC proliferation. Proliferation of cells stably transfected with FGF21 mimics, FGF21 inhibitor, or control vector. Results are expressed as mean ± SD; **P<0.01 compared with control.

incubation, the culture medium was replaced with serum-free DMEM, and cell proliferation was assessed every 24 h for 7 days by adding 100 μl 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (5 mg/ml, Sigma-Aldrich, USA) to each well and incubating at 37°C for 4 h. The medium was carefully removed to avoid disrupting the formazan crystals, and 150 μl DMSO was added to solubilize the crystals. Absorbance was measured at 570 nm using a microplate reader (Cell Application, San Diego, CA, USA).

Colony formation assay

HUVECs stably expressing FGF21 mimics or FGF21 inhibitor were seeded in 6-cm cell culture plates (1×10^3 cells/plate). After incubation for 15 days, the cells were fixed with methanol and stained with 0.1% crystal violet. Visible colonies were counted.

Transwell migration and invasion assays

HUVECs were transfected with 50 nM FGF21 mimics, FGF21 inhibitor, or control plasmid. To evaluate cells migration 24 h after transfection, 2×10^5 cells were seeded in the top chamber of the Transwell insert containing the non-coated membrane. To evaluate cells invasion 24 h after transfection, 5×10^4 cells were seeded in the top chamber of the Transwell insert containing a membrane coated with Matrigel. In both assays, the top chamber contained serum-free DMEM, and the bottom chamber contained DMEM supplemented with 10% FBS as a chemoattractant. The plates were incubated for 24 to 36 h, and cells remaining on the top of the membrane were removed by a cotton swab. Migration and invasion were assessed using the QCM™ 24-Well Colorimetric Cell Migration Assay Kit according to the manufacturer's instructions [20]. Cells on the underside of the membrane were counted at ×100 magnification in at least three randomly selected fields.

Quantitative reverse transcription-polymerase chain reaction

Total RNA was extracted from cells using TRIzol reagent (Invitrogen, USA). Reverse transcription (RT) was performed using the Hairpin-it™

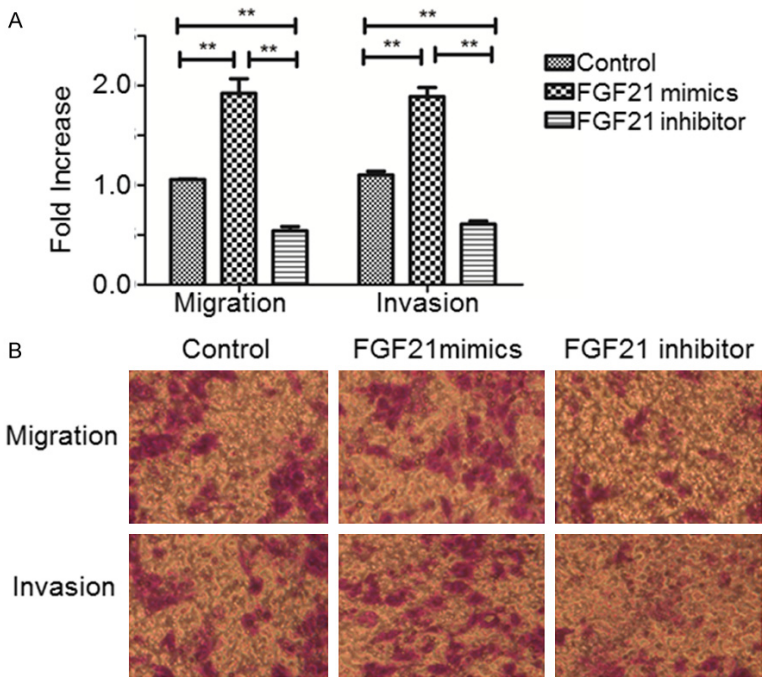

Figure 3. Effect of FGF21 on HUVEC migration and invasion. A, B. Migration and invasion of HUVECs transfected with FGF21 mimics, FGF21 inhibitor, or control vector, as assessed by Transwell assays. Results are expressed as mean ± SD; **P<0.01 compared with control.

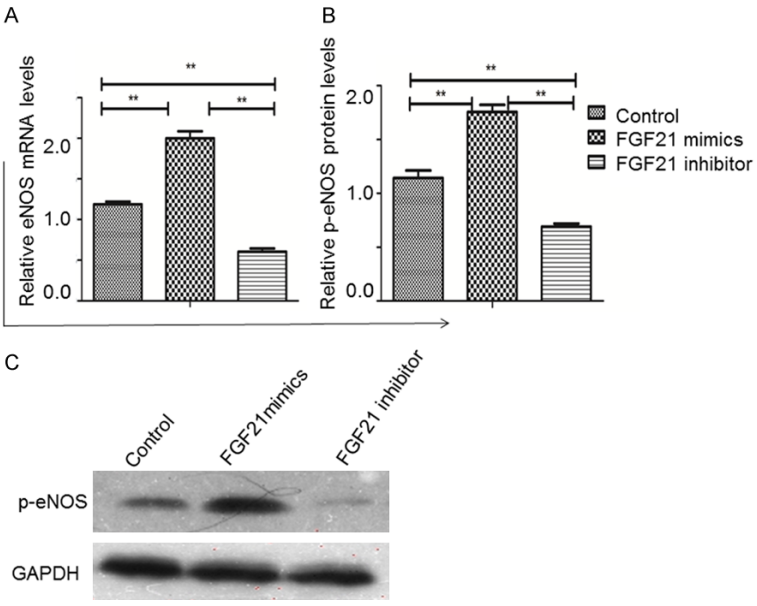

Figure 4. Effect of FGF21 on eNOS mRNA level and protein phosphorylation in HUVECs. A. Levels of eNOS mRNA in HUVECs transfected with FGF21 mimics, FGF21 inhibitor, or control vector were determined by qRT-PCR. B, C. Levels of p-eNOS were determined by Western blotting. Results are expressed as mean ± SD; **P<0.01 compared with control.

miRNA qPCR quantification kit (GenePharma, China). Quantitative PCR (qPCR) was performed in triplicate using SYBR Premix Ex Taq (TaKaRa, Japan) according to the manufacturer's instructions. The relative mRNA levels of FGF21, eNOS, PI3K, and AKT were determined using the 2-ΔΔCT analysis method [21], using U6 snRNA as the internal control.

Western blotting analysis

Western blotting analysis was performed as previously described [22]. Total protein was extracted from cells using lysis buffer and quantified using the Bradford method (Bio-Rad, USA). Protein was quantified using a bicinchoninic acid kit, separated by 12% sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to polyvinylidene difluoride membranes (Invitrogen, USA). The membranes were incubated with primary antibodies against p-eNOS (1:2000), PI3K (1:3000), and anti-p-AKT (1:3000) (Cell Signaling Technology) in 5% nonfat milk dissolved in Tris buffered saline with Tween 20 at room temperature for 1 h. The membranes were washed three times and incubated with secondary antibodies at room temperature for 1 h. The membranes were developed using enhanced chemiluminescence plus reagent and visualized using an electrochemiluminescence detection system (Invitrogen, USA). Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used as a loading control.

Statistical analysis

All experiments were performed in triplicate, and results are expressed as mean ± standard

deviation (SD). Statistical analyses were performed using SPSS 17.0 software (Chicago, USA). Groups were compared by two-tailed Student's t-test, followed by the Bonferroni correction for multiple comparisons; P<0.05 was considered significant.

Results

Blood FGF21 mRNA and eNOS mRNA/protein levels are upregulated in hypertensive patients

The demographic characteristics of the 30 hypertensive patients and 30 healthy subjects in this study did not differ significantly (**Table 1**). We evaluated the expression of FGF21 and eNOS in the study participants by analyzing blood levels of mRNA and protein. Compared to healthy subjects, hypertensive patients showed significantly higher levels of FGF21 mRNA (0.35±0.02 vs. 0.05±0.04; P<0.01) and eNOS mRNA (1.32±0.067 vs. 0.324±0.095; P<0.01) (**Figure 1A** and **1B**). Similarly, eNOS protein levels were significantly higher in hypertensive patients than in healthy subjects (1.23±0.13 vs. 0.365±0.032; P<0.01) (**Figure 1C**).

FGF21 decreases cell proliferation in vitro

To determine the effects of FGF21 on cell proliferation, HUVECs stably expressing FGF21 mimics or inhibitor were cultured in serum-free medium and counted daily. From day 1 to day 3, cell numbers did not differ significantly between treatment groups; however, from day 4 to day 7, cell proliferation was significantly higher in the FGF21 mimics group than in the control and FGF21 inhibitor groups (P<0.01; **Figure 2**). In addition, cell proliferation was significantly lower in the FGF21 inhibitor group than in the control group during that period (P<0.01). These results suggest that FGF21 stimulates the proliferation of HUVECs.

FGF21 inhibitor decreases HUVEC migration and invasion in vitro

We then assessed the effects of FGF21 on cell migration and invasion using cells stably expressing FGF21 mimics or inhibitor. The results of Transwell assay showed that migration and invasion were significant decreased in cells expressing FGF21 inhibitor compared with controls (**Figure 3**) and significantly higher in cells expressing FGF21 mimics compared with controls. These results suggest that FGF21 plays a role in cell migration and invasion.

FGF21 inhibitor decreases eNOS expression and phosphorylation in HUVECs

The effects of FGF21 on eNOS expression and phosphorylation were evaluated by qRT-PCR and Western blotting in cells stably expressing FGF21 mimics or inhibitor. Compared with the controls, eNOS mRNA levels were significantly decreased in cells expressing FGF21 inhibitor and significantly increased in cells expressing FGF21 mimics (P<0.01; **Figure 4A**). Similar results were observed for p-eNOS levels (P< 0.01; **Figure 4B** and **4C**). These results suggest that FGF21 increases eNOS mRNA levels and phosphorylated protein levels in HUVECs.

FGF21 inhibitor decreases PI3K/AKT mRNA levels and PI3K/p-AKT protein levels in HUVECs

Recent evidence suggests the involvement of the PI3K/AKT pathway in various malignancies [23-25]. To investigate whether PI3K/AKT signaling is involved in the effects of FGF21 in endothelial cells, we evaluated PI3K and AKT in HUVECs stably expressing FGF21 mimics or inhibitor. Our results showed that PI3K mRNA and protein levels were significantly decreased in cells expressing FGF21 inhibitor and increased in cells expressing FGF21 mimics compared with controls (P<0.01; **Figure 5**). Similarly, AKT mRNA levels were significantly lower in cells expressing FGF21 inhibitor than in controls and increased in cells expressing FGF21 mimics (P<0.01; **Figure 6A**). In addition, levels of p-AKT (Ser473) were significantly decreased in cells expressing FGF21 inhibitor and increased in cells expressing FGF21 mimics compared with controls (P<0.01; **Figure 6B** and **6C**). These results suggest that FGF21 inhibitor attenuates PI3K/AKT signaling in HUVECs.

VEGF and eNOS partially rescue the effects of FGF21 inhibition on HUVEC proliferation

We previously reported that vascular endothelial growth factor (VEGF) plays an important role in regulating eNOS expression and HUVEC proliferation [17, 18]. To investigate whether eNOS is involved in the effects of FGF21 on HUVEC proliferation, we transfected HUVECs with FGF21 inhibitor with or without eNOS and

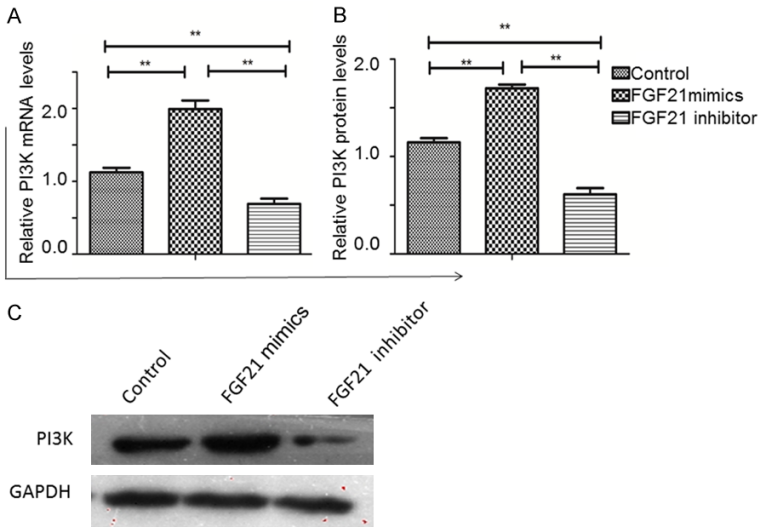

Figure 5. Effect of FGF21 on PI3K expression in HUVECs. A. PI3K mRNA levels in HUVECs transfected with FGF21 mimics, FGF21 inhibitor, or control vector were determined by qRT-PCR. B, C. PI3K protein levels were determined by Western blotting. Results are expressed as mean ± SD; *P<0.01 compared with control.

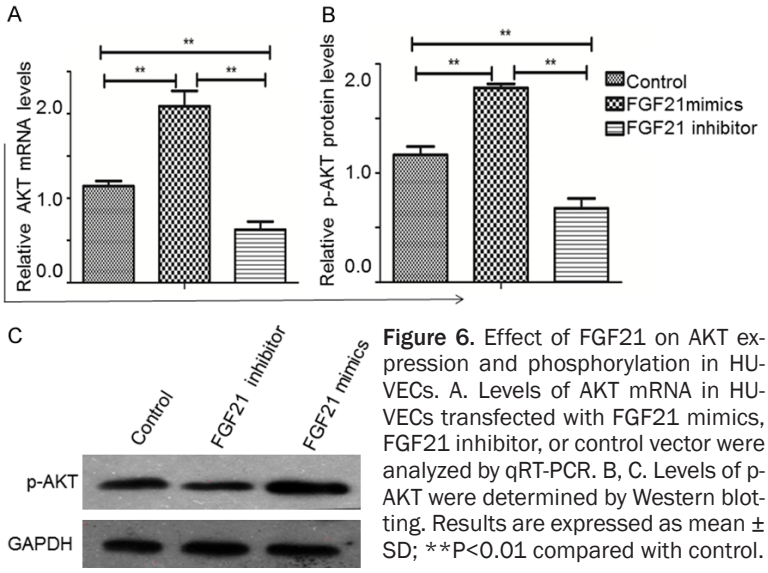

Figure 6. Effect of FGF21 on AKT expression and phosphorylation in HUVECs. A. Levels of AKT mRNA in HUVECs transfected with FGF21 mimics, FGF21 inhibitor, or control vector were analyzed by qRT-PCR. B, C. Levels of p-AKT were determined by Western blotting. Results are expressed as mean ± SD; **P<0.01 compared with control.

then treated the cells with VEGF. Results of the MTT assay showed that VEGF significantly increased proliferation in cells stably expressing FGF21 inhibitor (P<0.01; **Figure 7**). Proliferation of cells transfected with both FGF21 inhibitor and eNOS was higher than that of cells transfected with FGF21 inhibitor alone (P<0.01), and VEGF treatment further increased proliferation in cells cotransfected with FGF21 inhibitor and eNOS (P<0.01). These results support the involvement of eNOS in the effects of FGF21 on HUVEC proliferation.

VEGF and eNOS partially rescue the effects of FGF21 inhibition on AKT expression and phosphorylation in HUVECs

To evaluate whether eNOS is involved in the effects of FGF21 via PI3K/AKT signaling, we transfected HUVECs with FGF21 inhibitor with or without eNOS and then treated the cells with VEGF. Our results showed that VEGF increased AKT mRNA levels in cells stably expressing the FGF21 inhibitor (P<0.05; **Figure 8**), and cotransfection with eNOS also increased AKT mRNA levels compared with FGF21 inhibitor alone. The combination of VEGF and eNOS further increased AKT mRNA levels in cells expressing FGF21 inhibitor (P<0.01). Similar results were observed for p-AKT levels. These results suggest that inhibition of inhibition of FGF21 decreases PI3K/AKT signaling, which involves eNOS.

Discussion

Previous studies have described the role of FGF21 in metabolic diseases. For example, FGF21 is induced by fasting and consumption of a ketogenic diet; it also plays a key role in fatty acid oxidation in the liver [4, 26]. A recent study reported that FGF21 is associated with type 2 diabetes and cardiovascular disease [27, 28]. However, no published studies have evaluated circulating levels of FGF21 in patients with hypertension. In this study, we showed that FGF21 mRNA level was significantly elevated in the blood of hypertensive patients. Previous studies have reported eNOS overexpression in cardiovascular disease, which may be associated with disease progression and prognosis [29, 30]. In

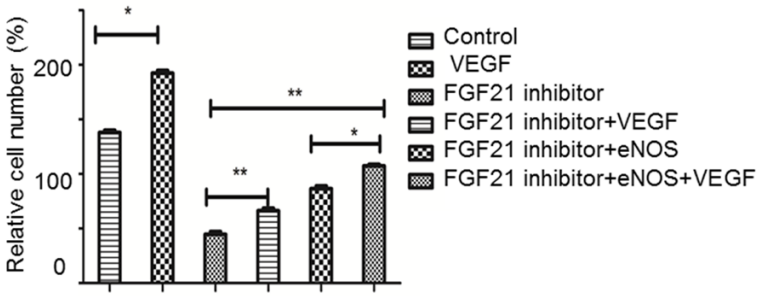

Figure 7. Effect of FGF21 and eNOS on HUVEC proliferation. Proliferation was evaluated in HUVECs stably transfected FGF21 inhibitor alone or in the presence of VEGF and/or eNOS. Results are expressed mean ± SD; *P<0.05, **P<0.01 compared with control.

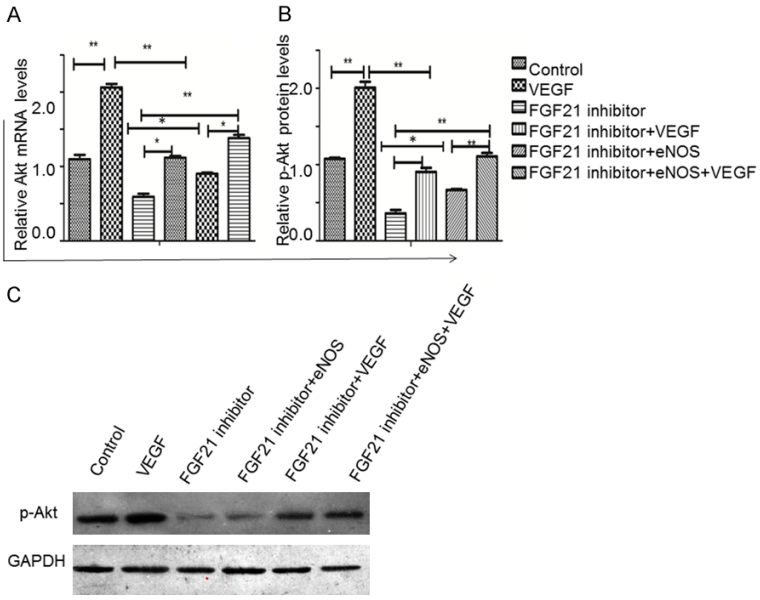

Figure 8. Effect of FGF21 and eNOS on AKT expression and phosphorylation. HUVECs were transfected with FGF21 inhibitor alone or in the presence of VEGF and/or eNOS. A. Levels of AKT mRNA were determined by qRT-PCR. B, C. Levels of p-AKT were determined by Western blotting. Results are expressed as mean ± SD; *P<0.05, *P<0.01 compared with control.

this study, we showed that eNOS mRNA and protein levels were elevated in the blood of hypertensive patients. We previously showed that eNOS mRNA levels in endothelial cells were decreased by overexpression of a 27-nt microRNA, and this effect was reversed by VEGF [17, 26]. The main findings of this study this study were that (1) inhibition of FGF21 decreases HUVEC proliferation, migration, and invasion; (2) inhibition of FGF21 significantly decreases eNOS, PI3K, and AKT mRNA levels and p-eNOS, PI3K, and p-AKT protein levels; and (3) eNOS overexpression partially restores cell proliferation and AKT expression/phosphorylation in HUVECs stably expressing an FGF21 inhibitor. Taken together, our results suggest that the effects of FGF21 on eNOS expression are mediated by the PI3K/AKT pathway.

This mechanism of gene regulation by FGF21 has been supported by several recent studies [31, 32]. In the present study, we hypothesized that FGF21 regulates eNOS through the PI3K/AKT pathway. Our results showed that FGF21 inhibitor substantially suppressed eNOS expression, which was associated with decreased levels of PI3K and p-AKT. Overexpression of eNOS partially restored p-AKT level and cell proliferation in HUVECs stably expressing an FGF21 inhibitor. These findings support the idea that eNOS expression is regulated by FGF21 via PI3K/AKT signaling.

The role of eNOS dysfunction in hypertension is well understood [33]. Downregulation of eNOS expression decreases the synthesis and the release of nitric oxide, which promotes vasorelaxation. Here we showed that FGF21 plays an important role in the regulation of eNOS at both the transcriptional and post-translational levels and acts, at least in part, through the PI3K/AKT pathway.

Acknowledgements

This study was funded by the National Natural Science Foundation of China (grant number 81360056) and the Youth Foundation of Guangxi Medical University, China (grant number GXMUYSF201531) and the excellent doctoral thesis cultivation Project of Guangxi Province, China (grant number YCBZ2014025).

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Hui Wang, College of Pharmacy, Guangxi Medical University, Nanning City, Guangxi Province, P. R. China. Tel: 86-0771-5358272; Fax: 86-0771-5358272; E-mail: wwhh-980@126.com; Dr. Jiangnan Huang, Department of Hypertension, First Affiliated Hospital of Guangxi Medical University, No. 22 Shuangyong Road, Nanning City 530021, Guangxi Province, P. R. China. Tel: 86-0771-5358272; Fax: 86-0771-5358272; E-mail: huangjn1998@163.com

References

[1] Ornitz DM and Itoh N. The fibroblast growth factor signaling pathway. Wiley Interdiscip Rev Dev Biol 2015; 4: 215-266.

[2] Li D, Wei X, Xie K, Chen K, Li J and Fang J. A novel decoy receptor fusion protein for FGF-2 potently inhibits tumour growth. Br J Cancer 2014; 111: 68-77.

[3] Ni B, Farrar JS, Vaitkus JA and Celi FS. Metabolic effects of FGF-21: thermoregulation and beyond. Front Endocrinol (Lausanne) 2015; 6: 148.

[4] Inagaki T, Dutchak P, Zhao G, Ding X, Gautron L, Parameswara V, Li Y, Goetz R, Mohammadi M, Esser V, Elmquist JK, Gerard RD, Burgess SC, Hammer RE, Mangelsdorf DJ and Kliewer SA. Endocrine regulation of the fasting response by PPARalpha-mediated induction of fibroblast growth factor 21. Cell Metab 2007; 5: 415-425.

[5] Contreras AV, Torres N and Tovar AR. PPAR-alpha as a key nutritional and environmental sensor for metabolic adaptation. Adv Nutr 2013; 4: 439-452.

[6] Stemmer K, Zani F, Habegger KM, Neff C, Kotzbeck P, Bauer M, Yalamanchilli S, Azad A, Lehti M, Martins PJ, Muller TD, Pfluger PT and Seeley RJ. FGF21 is not required for glucose homeostasis, ketosis or tumour suppression associated with ketogenic diets in mice. Diabetologia 2015; 58: 2414-2423.

[7] Lu J, Yu H, Mo Y, Ma X, Hao Y, Lu W, Li H, Bao Y, Zhou J and Jia W. Patterns of circulating fibroblast growth factor 21 in subjects with and without type 2 diabetes. PLoS One 2015; 10: e0142207.

[8] Cornu M, Oppliger W, Albert V, Robitaille AM, Trapani F, Quagliata L, Fuhrer T, Sauer U, Terracciano L and Hall MN. Hepatic mTORC1 controls locomotor activity, body temperature, and lipid metabolism through FGF21. Proc Natl Acad Sci U S A 2014; 111: 11592-11599.

[9] Lin Z, Pan X, Wu F, Ye D, Zhang Y, Wang Y, Jin L, Lian Q, Huang Y, Ding H, Triggle C, Wang K, Li X and Xu A. Fibroblast growth factor 21 prevents atherosclerosis by suppression of hepatic sterol regulatory element-binding protein-2 and induction of adiponectin in mice. Circulation 2015; 131: 1861-1871.

[10] Das JR, Gutkind JS and Ray PE. Circulating fibroblast growth factor-2, HIV-tat, and vascular endothelial cell growth factor-A in HIV-infected children with renal disease activate Rho-A and Src in cultured renal endothelial cells. PLoS One 2016; 11: e0153837.

[11] Ge X, Chen C, Hui X, Wang Y, Lam KS and Xu A. Fibroblast growth factor 21 induces glucose transporter-1 expression through activation of the serum response factor/Ets-like protein-1 in adipocytes. J Biol Chem 2011; 286: 34533-34541.

[12] Owen BM, Mangelsdorf DJ and Kliewer SA. Tissue-specific actions of the metabolic hormones FGF15/19 and FGF21. Trends Endocrinol Metab 2015; 26: 22-29.

[13] Wheler JJ, Atkins JT, Janku F, Moulder SL, Stephens PJ, Yelensky R, Valero V, Miller V, Kurzrock R and Meric-Bernstam F. Presence of both alterations in FGFR/FGF and PI3K/AKT/mTOR confer improved outcomes for patients with metastatic breast cancer treated with PI3K/AKT/mTOR inhibitors. Oncoscience 2016; 3: 164-172.

[14] Porta C and Figlin RA. Phosphatidylinositol-3-kinase/Akt signaling pathway and kidney cancer, and the therapeutic potential of phosphatidylinositol-3-kinase/Akt inhibitors. J Urol 2009; 182: 2569-2577.

[15] Liu CM, Ma JQ and Sun YZ. Puerarin protects rat kidney from lead-induced apoptosis by modulating the PI3K/Akt/eNOS pathway. Toxicol Appl Pharmacol 2012; 258: 330-342.

[16] Marsden PA, Heng HH, Scherer SW, Stewart RJ, Hall AV, Shi XM, Tsui LC and Schappert KT. Structure and chromosomal localization of the human constitutive endothelial nitric oxide synthase gene. J Biol Chem 1993; 268: 17478-17488.

[17] Yan L, Kang M, Qin Z, Zhang W, Li Y and Ou H. An intronic miRNA regulates expression of the human endothelial nitric oxide synthase gene and proliferation of endothelial cells by a mechanism related to the transcription factor SP-1. PLoS One 2013; 8: e70658.

[18] Li Y, Yan L, Zhang W, Hu N, Chen W, Wang H, Kang M and Ou H. Suppression of endothelial nitric oxide synthase expression and endothelial cell proliferation by an intronic 27-nt miRNA and it's a novel link to AP-1. Am J Transl Res 2015; 7: 285-297.

[19] Jiang J, Feng X, Zhou W, Wu Y and Yang Y. MiR-128 reverses the gefitinib resistance of the lung cancer stem cells by inhibiting the c-met/PI3K/AKT pathway. Oncotarget 2016; 7: 73188-73199.

[20] Zhang JG, Wang JJ, Zhao F, Liu Q, Jiang K and Yang GH. MicroRNA-21 (miR-21) represses tumor suppressor PTEN and promotes growth and invasion in non-small cell lung cancer (NSCLC). Clin Chim Acta 2010; 411: 846-852.

[21] Livak KJ and Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. Methods 2001; 25: 402-408.

[22] Li Y, Huang J, Jiang Z, Zhong Y, Xia M, Wang H and Jiao Y. MicroRNA-145 regulates platelet-derived growth factor-induced human aortic vascular smooth muscle cell proliferation and migration by targeting CD40. Am J Transl Res 2016; 8: 1813-1825.

[23] Fang WL, Huang KH, Lan YT, Lin CH, Chang SC, Chen MH, Chao Y, Lin WC, Lo SS, Li AF, Wu CW, Chiou SH and Shyr YM. Mutations in PI3K/AKT pathway genes and amplifications of PIK3CA are associated with patterns of recurrence in gastric cancers. Oncotarget 2016; 7: 6201-6220.

[24] Robbins HL and Hague A. The PI3K/Akt pathway in tumors of endocrine tissues. Front Endocrinol (Lausanne) 2015; 6: 188.

[25] Zhang C, Lan T, Hou J, Li J, Fang R, Yang Z, Zhang M, Liu J and Liu B. NOX4 promotes non-small cell lung cancer cell proliferation and metastasis through positive feedback regulation of PI3K/Akt signaling. Oncotarget 2014; 5: 4392-4405.

[26] Badman MK, Pissios P, Kennedy AR, Koukos G, Flier JS and Maratos-Flier E. Hepatic fibroblast growth factor 21 is regulated by PPARalpha and is a key mediator of hepatic lipid metabolism in ketotic states. Cell Metab 2007; 5: 426-437.

[27] Hecht R, Li YS, Sun J, Belouski E, Hall M, Hager T, Yie J, Wang W, Winters D, Smith S, Spahr C, Tam LT, Shen Z, Stanislaus S, Chinookoswong N, Lau Y, Sickmier A, Michaels ML, Boone T, Veniant MM and Xu J. Rationale-based engineering of a potent long-acting FGF21 analog for the treatment of type 2 diabetes. PLoS One 2012; 7: e49345.

[28] Brahma MK, Adam RC, Pollak NM, Jaeger D, Zierler KA, Pocher N, Schreiber R, Romauch M, Moustafa T, Eder S, Ruelicke T, Preiss-Landl K, Lass A, Zechner R and Haemmerle G. Fibroblast growth factor 21 is induced upon cardiac stress and alters cardiac lipid homeostasis. J Lipid Res 2014; 55: 2229-2241.

[29] Suvorava T, Nagy N, Pick S, Lieven O, Ruther U, Dao VT, Fischer JW, Weber M and Kojda G. Impact of eNOS-dependent oxidative stress on endothelial function and neointima formation. Antioxid Redox Signal 2015; 23: 711-723.

[30] Liu S, Premont RT and Rockey DC. G-protein-coupled receptor kinase interactor-1 (GIT1) is a new endothelial nitric-oxide synthase (eNOS) interactor with functional effects on vascular homeostasis. J Biol Chem 2012; 287: 12309-12320.

[31] Cyphert HA, Alonge KM, Ippagunta SM and Hillgartner FB. Glucagon stimulates hepatic FGF21 secretion through a PKA- and EPAC-dependent posttranscriptional mechanism. PLoS One 2014; 9: e94996.

[32] Li H, Gao Z, Zhang J, Ye X, Xu A, Ye J and Jia W. Sodium butyrate stimulates expression of fibroblast growth factor 21 in liver by inhibition of histone deacetylase 3. Diabetes 2012; 61: 797-806.

[33] Li Q, Youn JY and Cai H. Mechanisms and consequences of endothelial nitric oxide synthase dysfunction in hypertension. J Hypertens 2015; 33: 1128-1136.