Original Article
Effect of fosinopril on chemerin and VEGF expression in diabetic nephropathy rats

Haifeng Huang[1], Liping Hu[2], Jiancong Lin[1], Xiaoxiao Zhu[1], Weiling Cui[1], Wenming Xu[1]

Departments of [1]Internal Medicine, [2]Pharmacy, The Eastern Hospital of First Affiliated Hospital, Sun Yat-Sen University, Guangzhou 510700, China

Abstract: As the most common and severe complication of diabetes, diabetic nephropathy (DN) has been known to be related with angiotensin converting enzyme inhibitor (ACEI), which can reduce proteinuria and protect renal function. This study analyzed the effect of ACEI analog-fosinopril-on the expression of chemerin and vascular epithelial growth factor (VEGF), in an attempt to reveal the mechanism of ACEI analog on renal protection. A total of 45 SD rats were induced by sreptozotocin for diabetes and were given fosinopril via intragastric cannulation for 12 weeks. After sacrifice, serum and renal chemerin and VEGF contents were quantified by enzyme linked immunosorbent assay (ELISA) and Western blot method, in addition to biochemical laboratory examinations. In diabetic model rats, blood glucose, creatinine, urea nitrogen, 24-hour urinary protein, chemerin and VEGF protein contents were all signifi-cantly elevated when compared to those in control group ($P<0.05$). After fosinopril treatment, blood creatinine, urea nitrogen, 24-hour urinary protein, Chemerin and VEGF protein concentrations were significantly depressed ($P<0.05$ compared to model group). Positive relationships existed between renal chemerin, VEGF and urea protein levels. Fosinopril may protect renal tissues in diabetes by suppressing chemerin and VEGF protein expression.

Keywords: Fosinopril, diabetic nephropathy, chemerin, VEGF

Introduction

As one of the most common chronic diseases, more than 92 million diabetic patients now lived in China, along with about 148 million pro-phase patients [1]. Due to the transition of life styles in China, the incidence of diabetes is still increasing by years, with more cases of compli-cations. Diabetic nephropathy (DN) is the most common micro-vascular related complication of diabetes [2]. It is mainly manifested as thick-ening of basal membrane, proliferation of mesangial cells, increase of proteinuria and extracellular matrix (ECM), and has been sug-gested to be the leading cause for chronic kid-ney failure [3]. In US, more than 30% of termi-nal kidney failure cases were caused by DN [4], making it a major public health issues. Therefore, the further illustration of DN related mechanism is of critical importance for preven-tion and treatment against chronic renal failure.

DN occurrence requires the action of multiple factors, among which abnormal angiogenesis plays a crucial role [5]. As a novel adipocyte cytokine, chemerin is the ligand of G-protein coupled receptor-adipocyte cytokine receptor 1 (CMKLR1 or ChemR23), and has been shown to facilitate the angiogenesis of endothelial cells in both dose- and time-dependent manners [6, 7]. Studies have revealed the correlation between chemerin and insulin-resistance and diabetes [8, 9], and the potency of chemerin as biological marker of diabetes [10]. Chemerin has also been suggested to be related with dia-betic vascular diseases [11]. Further studies found the involvement of high serum chemerin level in the occurrence of DN [12]. The applica-tion of rosiglitazone can decrease expression of chemerin and ChemR23 expression in diabetic rat kidneys, thus protecting renal function [13]. Therefore, chemerin plays a critical role in both occurrence and treatment of DN.

Vascular endothelial growth factor (VEGF) can stimulate the proliferation of vascular endothe-lial cells and participate in angiogenesis. Previous studies have shown elevated serum

Table 1. General condition and biochemical indexes

Index	Control group	Model group	Fosinopril group	P value
Body weight (g)	400.16±25.26	296.15±28.74[a]	324.18±27.15[a,b]	<0.001
Kidney index (mg/g)	3.72±0.15	4.91±0.21[a]	4.14±0.18[a,b]	<0.001
Blood glucose (mM)	5.61±0.56	20.16±3.65[a]	19.23±2.98[a,b]	<0.001
Creatinine (μM)	48.29±4.15	100.76±5.12[a]	64.23±4.56[a,b]	<0.001
Urea nitrogen (mM)	6.23±1.15	17.58±2.36[a]	12.28±2.43[a,b]	<0.001
24 h urea protein (mg)	8.18±0.56	108.56±4.25[a]	79.43±4.13[a,b]	<0.001

Note: [a], $P<0.05$ compared to control group; [b], $P<0.05$ compared to model group.

Table 2. Serum levels of VEGF and chemerin

Group	N	VEGF (pg/ml)	Chemerin (pg/ml)
Control	15	102.34±13.45	458.58±40.69
Model	15	135.68±12.68[a]	568.26±42.36[a]
Fosinopril	15	116.35±11.98[a,b]	489.38±43.24[a,b]
F value		25.99	27.07
P value		<0.001	<0.001

Note: [a], $P<0.05$ compared to control group; [b], $P<0.05$ compared to model group.

VEGF level in early stage DN, and the correlation between VEGF genetic polymorphism and DN susceptibility [14-16]. Fasudil can protect kidney functions of DN rats via depressing VEGF level [17]. All those studies pointed the crucial role of VEGF on the occurrence and treatment of DN. Using appropriate measures to lower chemerin and VEGF expressions, therefore, may have clinical implications for DN. Current opinions believe the renal protective function of angiotensin-converting enzyme inhibitor (ACEI) via decreasing proteinuria and slowing disease progression [18], but leaving its detailed mechanism unclear. This study thus constructed a diabetic rat model, on which fosinopril, an ACEI analog, was applied for quantification of chemerin and VEGF expression, in an attempt to elucidate the protective mechanism of ACEI.

Materials and methods

Animal model and sample collection

A total of 45 healthy male Wistar rats (8 weeks old, body weight: 180~200 g) were provided by Guangdong Animal Experimental Center, China. After one-week acclimation and blood glucose test, rats were divided into control, model and drug treatment groups (N=15 each). In the latter two groups, animal were intraperitoneally injected with streptozotocin (STZ, 65 mg/kg, Sigma, US; freshly prepared in citric acid buffer) after 12-hour fasting. In the control group, equal volume of citric acid buffer was applied. 72 hours after injection, blood glucose level was quantified. Those with fasting blood glucose higher than 16.7 mM were defined as having diabetes. One week later, rats in the drug group received fosinopril via intragastric cannulation (5 mg/kg in distilled water, once daily for 12 weeks, purchased from Sino-American Shanghai Squibb Pharmaceuticals Ltd, China), while model and control group received equal volumes of distilled water. 12 weeks later, rats were sacrificed and collected for 24-hour urea, blood and renal tissue samples. Blood glucose level, along with creatinine and urea nitrogen was quantified by automatic biochemical analyzer. The 24-hour total urea protein was determined by Biuret method.

Enzyme-linked immunosorbent assay (ELISA)

Serum Chemerin and VEGF contents were measured by ELISA using test kits (Senxiong Biotech, China) following manual instruction. Collected blood samples were centrifuged at 1 500 g for 10 min. Supernatants were transferred into 96-well plate, along with blank control and standard samples. After 37°C incubation for 30 min, supernatants were discarded, followed by washing buffer for 5 changes. Enzyme-linked reagents were added into each well except the blank control for further incubation. Reagents A and B were then added, mixed and incubated in dark at 37°C for 15 min. The color development was stopped by adding quenching buffer. A microplate reader was used to measure optical density (OD) values of each well at 450 nm. A standard curve was firstly plotted using serially diluted standard samples. Sample concentration was then deduced from the regression function.

Western blot

Renal tissues were homogenized and extracted for total proteins using total protein extraction kit (BestBio, China). Proteins in supernatants
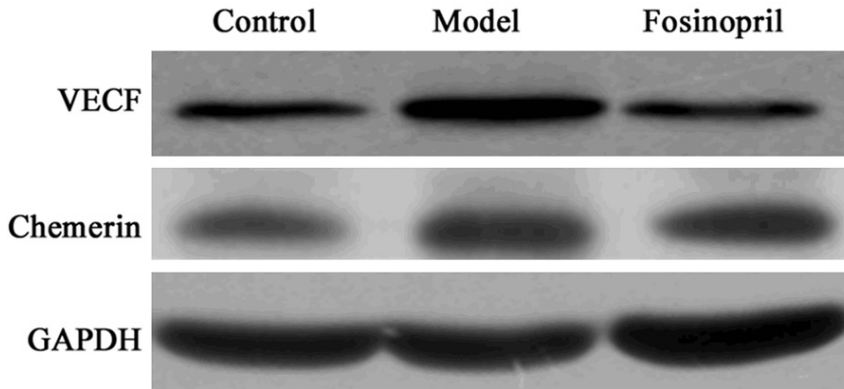
Figure 1. Representative protein bands showing Chemerin and VEGF proteins in rat renal tissues.

Table 3. Chemerin and VEGF protein level in renal tissues

Group	Control	Model	Fosinopril	F value	P value
Chemerin	0.51±0.11	0.89±0.14[a]	0.62±0.13[a,b]	35.40	<0.001
VEGF	0.48±0.12	0.82±0.16[a]	0.58±0.15[a,b]	21.98	<0.001

Note: [a], $P<0.05$ compared to control group; [b], $P<0.05$ compared to model group.
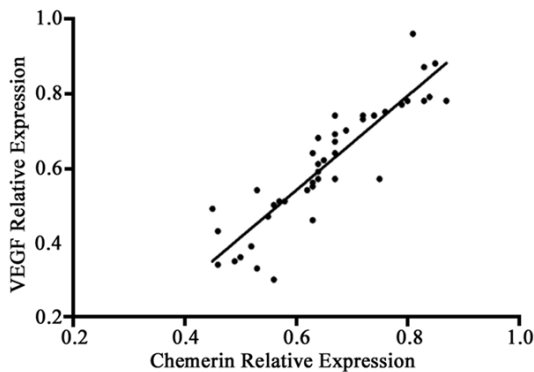

Figure 2. Correlation between Chemerin and VEGF protein levels in rat renal tissues.

were quantified by Coomssie Brilliant Blue protein assay kit (Meiji Biotech, China) and were loaded into SDS-PAGE for separation. After transferring to PVDF membrane, non-specific bindings were blocked by 5% defatted milk powders for 2 hours at room temperature. The membrane was rinsed in PBST and was incubated with primary antibody against chemerin, VEGF or GAPDH (Abcam, US) at 4°C overnight. On the next day, primary antibodies were washed by PBST, followed by secondary antibody incubation (1 hour at room temperature). ECL chromogenic substrates were applied on the membrane for development and exposure

in dark room. Images were captured and analyzed by GIS-202D system (Sigma, US). OD values of both chemerin and VEGF bands were normalized to GAPDH bands for calculating relative protein concentration.

Statistical analysis

SPSS13.0 software package was used to perform all statistical analysis. Multiple group comparison was done by analysis of variance (ANOVA). Pearson analysis was used to elucidate the correlation between two groups. The significant level was defined as 0.05.

Results

General condition and biochemical indexes

Controlled rats had normal neurological reflexes and mood, smooth furs and normal feeding and urea patterns. Other two groups, however, manifested with polydipsia, polyuria and polyphagia, along with retard response and hypomania. General conditions and laboratory results of all rats were shown in **Table 1**. Controlled rats had significantly elevated body weight than the other two groups (*P*<0.05). Model group had increased blood glucose, urea nitrogen, creatinine and 24-hour urea protein levels compared to control ones (P<0.05). The application of fosinopril significantly suppressed all those abovementioned indexes, suggesting the improvement of diabetes and DN.

Serum chemerin and VEGF level

We employed ELSIA to detect serum levels of chemerin and VEGF in all animals. As shown in **Table 2**, significant difference existed across three groups as shown by ANOVA (F=25.99 and 27.07, *P*<0.001 in both cases). Post-hoc com-

parison showed elevated chemerin and VEGF levels in both fosinopril and model groups when compared to control group (*P*<0.05) and lowered indexes in fosinopril group than model group (*P*<0.05). In a word, fosinopril can ameliorate the condition of DN by lowering chemerin and VEGF expression.

Protein levels of chemerin and VEGF in rat renal tissues

We further utilized Western blotting method to detect the protein level of chemerin and VEGF in rat renal tissues. As shown in **Figure 1** and **Table 3**, consistent results have been obtained compared to those blood indexes. Diabetic model rats had elevated chemerin and VEGF proteins in renal tissues compare to control group (*P*<0.05). The application of fosinopril effectively decreased expression of those two proteins (*P*<0.05).

Correlation between chemerin/VEGF protein and urea protein level

We used Pearson analysis to detect the relationship between chemerin or VEGF protein level with urea protein level. Results showed the positive correlation between chemerin or VEGF protein and urea protein level (r=0.563 and 0.625, *P*<0.001 in both cases). Further analysis revealed the positive correlation between chemerin and VEGF protein level (**Figure 2**, r=0.894, *P*<0.001).

Discussion

ACEI has been proved to have protective effects on renal functions in DN patients, but leaving its mechanism unclear. This study thus generated a diabetic rat model via STZ induction and injected ACEI analog-fosinopril-to observe the expression of chemerin and VEGF, both of which hallmarks are reflecting DN disease condition. In model rats, we found significantly elevated blood urea nitrogen, creatinine and 24-hour urea protein, all of which suggested the occurrence of renal damage. After the intervention by fosinopril, all those indexes were significantly depressed, as agreed with previous study [19]. Although having such renal protection function, fosinopril can only marginally depress blood glucose level, suggesting the minor role in correcting hyperglycemia. Such effects may be contributed by elevated insulin sensitivity of ACEI [20].

By detecting chemerin and VEGF expressions in both serum and renal tissues, we found significantly elevated expression of those two proteins, as agreed with previous reports [12, 14]. These results indicated the crucial role of chemerin and VEGF in pathogenesis of DN. After fosinopril intervention, expressions of those two proteins were significantly inhibited in both serum and renal tissues. Moreover, positive correlations exist between chemerin or VEGF with urea protein level, suggesting the potency of fosinopril in renal protection via depressing chemerin and VEGF. The expression of those two proteins was also positively correlated with each other, as suggested by previous findings showing that elevated chemerin lead to VEGF up-regulation [21]. These results suggest the regulation of VEGF expression by chemerin. The modulation of such signal transduction pathway, therefore, would be helpful to alleviate DN condition.

In summary, chemerin may participate in DN pathogenesis via up-regulating VEGF expression. Fosinopril may protect renal function via depressing chemerin and VEGF expression, providing further clues for the mechanism of ACEI analogs in renal protection. Further studies will be conducted in clinics to reflect the dynamic changes in human body.

Disclosure of conflict of interest

None.

Address correspondence to: Dr. Haifeng Huang, Department of Internal Medicine, The Eastern Hospital of First Affiliated Hospital, Sun Yat-Sen University, 183, Guangzhou East Road of Huangpu, Gangzhou 510700, China. Tel: +86-20-82377155; Fax: +86-20-82377155; E-mail: hhaifeng198@126.com

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