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Scutellarin Isolated from *Erigeron multiradiatus* Inhibits High Glucose-mediated Vascular Inflammation

Pei LUO,[a] Zheng-Huai TAN,[b] Zhi-Feng ZHANG,[a] Hao ZHANG,[a] Xian-Fu LIU,[c] and Zheng-Ji MO*,[a]

[a]*West China School of Pharmacy, Sichuan University, No. 17, Section 3, Ren-Min-Nan-Lu Road, Chengdu, Sichuan 610041, China,* [b]*Sichuan Institute of Chinese Materia Medica, Chengdu, Sichuan, China, and* [c]*Ganzi Institute of Drugs Control, Sichuan 626000, China*

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Erigeron multiradiatus (Lindl.) Benth is a traditional Tibetan medicine herb long used to treat various diseases related to inflammation. Our previous phytochemical studies on *E. multiradiatus* resulted in the isolation of scutellarin, which is a known flavone glucuronide with comprehensive pharmacological actions. In present study, we investigated the inhibition action of scutellarin on high glucose-induced vascular inflammation in human endothelial cells (ECV304 cells). Consistent with previous reports, exposure of ECV304 cells to high glucose for 24 h caused an increase of intercellular adhesion molecule-1 (ICAM-1) and monocyte chemoattractant protein 1 (MCP-1), and promoted cell adhesion between monocyte and ECV304 cells. However, pretreatment with scutellarin (0.1 and 1 μM) reversed these effects in a concentration-dependent manner. Scutellarin was able to inhibit the activation of NF-κB induced by high glucose in ECV304 cells. Furthermore, although oral administration of scutellarin (10 and 50 mg/kg) did not produce significant antihyperglycemic action, it lowered the serum MCP-1 levels significantly in alloxan-induced diabetic mice. Therefore, our results suggest that scutellarin has anti-inflammation effect that may afford some protection against hyperglycemia-induced vascular inflammatory both *in vitro* and *in vivo*.

Key words――scutellarin; adhesion molecule; nuclear factor-kappa B; endothelial cell

INTRODUCTION

Diabetes vascular complication, due to its high prevalence, is becoming probably the most important metabolic disease and is widely recognized as one of the leading causes of morbidity and mortality worldwide.[1,2] Diabetic chronic hyperglycemia, as a common fate for patients with diabetes mellitus, induces endothelial cell dysfunction, which can be defined as a partial or complete loss of vascular homeostasis including hemostasis, antigen presentation, permeability, inflammatory function, and angiogenesis.[3–8] Over the past decade, accumulating evidence has shown that endothelial dysfunction closely related with vascular inflammatory process might represent an early stage of vasculopathy that can lead to diabetic vascular disorders. Recently, reports have shown that the diabetic condition elevation of intracellular glucose causes overproduction of adhesion molecules leading to leukocyte-endothelium adhesion, which is considered one of the earliest events in vascular inflammation process. Therefore, up-regulation of endothelial cell adhesion molecules including ICAM-1 and MCP-1 is mediated by interactions between monocytes and endothelial cells *via* activation of nuclear factor-kappa B (NF-κB).[9,10] Therefore suppression of vascular inflammation might be beneficial for the treatment of diabetic vascular complications.

Erigeron multiradiatus (Lindl.) Benth is naturally distributed in the northern and southwestern mountain regions of China as well as Sikkim, Nepal, and Afghanistan.[11] This folk medicine has been widely used under ''meiduoluomi'' for the treatment of hypopepsia, enteronitis, diarrhea, hepatitis, adenolymphitis, rheumatism, and hemiparalysis.[12] Our previous phytochemistry studies reported that *E. multiradiatus* comprised a mixture of different compounds such as flavonoids, phenolic acids, monoterpenes, sterols, *etc*.[13,14] We successfully isolated scutellarin (Fig. 1) from the extract of *E. multiradiatus*.[14,15] Scutellarin is a known flavone glucuronide with comprehensive pharmacological actions, primarily derived from *Erigeron breviscapus* (Vant.) Hand-mazz. Since the 1970s, several studies showed the effectiveness of scutellarin on dilating blood ves-

*e-mail: mzj-121@scu.edu.cn

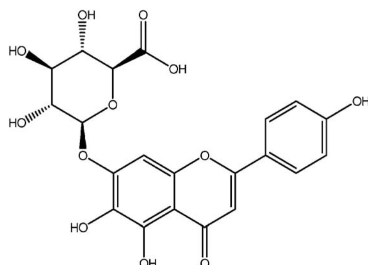

Fig. 1. Chemical Structure of Scutellarin

sels, improving microcirculation, increasing cerebral blood flow, and inhibiting platelet aggregation.[16–18] Scutellarin preparations have been suggested for clinical use to treat diabetes with vascular complications. However, as the main constituent in those preparations, scutellarin has not been investigated for its effect on hyperglycemia-induced endothelial dysfunction. Therefore the purpose of the present study was to verify scutellarin's inhibitory effect against vascular inflammation both in high glucose-cultured human endothelial cells (ECV304 cells) and alloxan-induced diabetic mice.

MATERIALS AND METHODS

Plant Material *E. multiradiatus* samples were collected in natural growth sites in Luhuo, Sichuan, China. These samples were authenticated by Prof. Hao Zhang (West China School of Pharmacy, Sichuan University, Chengdu, P. R. China.) using geographical origin identification, macroscopic identification, and microscopic identification. A voucher specimen was deposited at Herbarium Center of West China, School of Pharmacy, Sichuan University (NO. E12025).

Extraction and Isolation *E. multiradiatus* (the content of scutellarin is 1.1% w/w) was extracted with methanol. The yield (%, w/w dry plant material) of methanol extract is 20.3%. The extracts were subjected to column chromatographic separation on macroporous resins and Sephadex LH-20; pure compound was obtained and yielded (%, w/w) 32.3%. The chemical structure of the isolated compound was identified by comparison of spectral properties (MS, 1H-, 13C-NMR) with those reported in the literature.[14]

Reagents Cell culture materials were purchased from Gibco-BRL (MD, USA). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) and dimethylsulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The other chemicals used were of the highest analytical grade commercially available.

Cell Culture and Treatment ECV304 Cells, a spontaneously transformed line derived from the human umbilical vein endothelial cell,[19] were purchased from CCTCC (China). The cells were maintained at 37°C in a humidified atmosphere of 5% CO_2 on culture plates with 10% fetal bovine serum-supplemented DMEM medium. All the subsequent procedures were carried out under these conditions. ECV304 cells were pretreated with a series of concentrations of scutellarin (0.1 and 1 μM) for 24 h then stimulated with 30 mM glucose for 24 h. Cell treatment with scutellarin was conducted as follows: control (5.5 mM glucose), HG (30 mM glucose), low-concentration scutellarin (30 mM glucose plus 0.1 μM scutellarin), high-concentration scutellarin (30 mM glucose plus 1 μM scutellarin). All the glucose used in the present study was D-glucose.

Cell Viability and Morphology The MTT assay for cell survival assessment was performed as described by Mosmann.[20] ECV304 cells were seeded at 2×10^4 cell/well onto 96-well plates and preincubated for 24 h. Following incubation with various treatments, a volume of 200 μl of 0.5 mg/ml MTT in DMEM medium was added to each well. After incubation at 37°C for 4 h, the MTT solution was removed from the medium. The resultant formazan crystals were dissolved in 150 μl of dimethylulfoxide (DMSO) and the absorbance in each well was then read at 570 nm using a microplate reader (BioRad 3550, Bio-Rad Laboratories). Survival of control groups not exposed to high glucose was defined as 100 %. The numbers of surviving cells in the treated groups were expressed as a percent of control groups. Values are given as averages for triplicate determinations. For the morphology study, cells were grown on 6-well plates, treated with glucose and scutellarin, and observed under light microscopy (Nikon, Japan) then photographed.

Monocyte Adhesion Assay The monocyte-endothelial cell adhesion assay was modified from that of Cybulsky and Gimbrone.[21] 5×10^6 ECV304 cells/well were seeded onto 24-well plate and incubated with different treatments. After endothelial monolayers were washed three times with PBS, 1×10^5 monocytes/well were added and microplates incubated at 37°C for 60 min. The monocytes used for monocyte-

endothelial cell adhesion assay were isolated from peripheral human blood as described previously.[22] Theseafter the plates were sealed, inverted, and centrifuged for 5 min to separate nonadherent monocytes. Adherent cells were quantified by protein-quantitation methods. Monocyte adherence was calculated as the ratio of the protein quantitation of adhered monocytes to that of the total monocytes (1×10^5 cells) added.

Cell ELISA Enzyme immunoassay was used to determine the level of adhesion molecule expression on ECV304 cells as previously described.[23] Confluent cells were grown on 96-well plate and stimulated with 30 mM glucose in the presence or absence of different concentrations of scutellarin (0.1 and 1 μM). According to the manufacturer's protocol, after incubating endothelial cells under various conditions, the cells were washed 3 times with PBS and then fixed by 1% paraformaldehyde and exposed to the antiadhesion molecule antibody directed against ICAM-1, MCP-1 (purified mouse anti-human ICAM-1, MCP-1 antibody) at room temperature for 60 min, washed three times with PBS, and incubated for 1 h with a horseradish-peroxidase-conjugated goat antimouse IgG. The content of ICAM-1, MCP-1 was quantified by adding a peroxidase substrate solution and incubated at 37°C for 30 min. The reaction was stopped by addition of 5 NH_2SO4, and the optical density of each well was read using a microplate reader. The expressions were calculated as the ratio of the absorbance associated with various medium-treated versus control.

Preparation of Nuclear Extract and Western Blot Analysis Cells were pretreated with scutellarin for 24 h and stimulated with high glucose for 24 h. After treatment, the cells were harvested and washed twice with ice-cold PBS, and then resuspended in 1 ml of the same buffer. Nuclear extracts were prepared on ice by the method previously described by Kim *et al.*[24] After centrifugation at 13000 rpm, the cell pellet was suspended in ice-cold buffer A (10 mmol/l HEPES pH, 1.5 mmol/l $MgCl_2$, 0.2 mmol/l KCl 0.2 mmol/l phenylmethylsulphonylfluoride, 0.5 mmol/l dithothreitol), vortexed for 10 s, then centrifuged at 13000 rpm for 5 min. The nuclear pellet was then washed in 1 ml buffer (20 mM HEPES, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA) at 4200 g for 3 min, resuspended in 30 μl buffer, rotated at 4°C for 30 min, then centrifuged for 20 min. The supernatant was used as nucleus extract. The nucleus extracts were then analyzed for protein content using Bradford assay.

The above extract (70 μg) was separated on a 12% sodium dodecyl sulfate (SDS) polyacrylamide gel and transferred to PVDF membrane. Blots were then washed with H_2O, blocked with 5% skimmed milk powder in TBST [10 mM Tris-HCl (pH 7.6), 150 mM NaCl, 0.05% Tween-20] for 1 h, and immunoblotted using against NF-κB p65 and β-actin proteins (1 : 1000 at 4°C overnight) antibody (Santa Cruz Biotechnology, U.S.A). Then, the membrane was washed and primary antibodies were detected with goat anti-rabbit-IgG conjugated to horseradish peroxidase detected by an enhanced chemiluminescence procedure (Amersham, Buckinghamshire, England).

Experimental Animals Kunming mice weighing 20–25 g of either sex obtained from Animal House, Pharmacy Discipline, Sichuan University, Chengdu, were used. The animals were fed with a rodent standard diet with free access water ad libitum and were housed in rooms maintained at 25 ± 1°C with a 12 h light/dark cycle following international recommendations. The Animal Ethics Committees of the Faculty of Medicine, Sichuan University, approved all experimental protocols, in accordance with "Principles of Laboratory Animal Care and Use in Research" (Ministry of Health, Beijing, China).

Injecting alloxan at a dose of 60 mg/kg intravenously induced hyperglycemia in mice. Then, the mice were kept under observation and after 48 h were tested for serum glucose. Animals with a blood glucose level >16.7 mmol/l were considered diabetic and equally divided into 4 groups of 8 animals each. Another 8 normal mice served as control. The 5 groups were used in the antidiabetic study. Normal control group and model control group animals received orally distilled water. Group 3 was administered pioglitazone (5 mg/kg); group 4 scutellarin (10 mg/kg), and group 5 scutellarin (50 mg/kg). The animals were treated for 3 weeks. On the day 22, the animals were killed by decapitation and blood was collected from the arterial jugular and serum separated for estimation of blood glucose and MCP-1 levels (mouse MCP-1 ELISA kit from ShangHai Lengton Bioscience Co. LTD).

Statistical Analysis Statistical analysis of each set of data was carried out using one–way ANOVA

and Duncan's multiple range test with SPSS11.5, and p values <0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Effect of Scutellarin on ECV304 Cells Viability and Morphological Changes In this study, we carried out experiments to evaluate the cytotoxicity of scutellarin on ECV304 cells. The results suggest that scutellarin does not significantly alter viability of ECV304 cells until reaching a concentration of 100 μM (data not shown). As Fig. 2 shows, treatment with 30 mM glucose significantly caused cytotoxicity against ECV304 cells as detected by MTT assay. However, pre-treating the cells with different concentrations of scutellarin (0.1 and 1 μM) for 24 h markedly suppressed the damage of high glucose in a dose-dependent manner and maximum effects (91.7%) were observed at the highest concentration (1 μM). Morphological observation by phase microscopy showed that the cells were reduced by the treatment with 30 mM glucose. However, the morphology of scutellarin (0.1 and 1 μM) treated-cells was not different from that of 5.5 mM glucose-treated cells.

Effects of Scutellarin on Monocyte-Endothelial Cell Adhesion Mediated by High Glucose There is strong evidence for an involvement of monocyte activation in the pathogenesis of diabetes-associated vascular complications.[25] It has been shown previously that hyperglycemia induces increased adhesion of leukocytes (especially monocytes) to endothelial cells as an early event in atherosclerosis and certain inflammatory disorders in diabetes-induced endothelial dysfunction.[26] Inhibition of adhesion of monocytes is a pharmacological approach to attenuate hyperglycemia-induced vascular inflammatory disorders. To determine the effect of scutellarin on endothelial cell-monocyte interaction, we examined the adhesion of monocytes to ECV304 cells induced by high glucose. ECV304 cell monolayers were treated with 5.5 and 30 mM of glucose for 24 h and adhesion assays performed with monocytes. As shown in Fig. 3, the control group (5.5 mM glucose) showed minimal binding to monocytes, but when were treated with high glucose (30 mM) for 24 h, the adhesion of monocytes to ECV304 cells was found significantly to increase (69% *vs.* 25%, $n=3$, $p<0.01$). On the other hand, the pretreatment with scutellarin (0.1 and 1 μM) significantly attenuated the adherence of monocytes in a concentration-dependent manner (55%, 38% *vs.* 69%, $n=3$, $p<0.05$ or $p<0.01$).

Effects of Scutellarin on Level of High Glucose-induced ICAM-1 and MCP-1 in ECV304 Cells ICAM-1 and MCP-1 have been implicated as having an important role in the mechanism of inflammation in vascular disease.[27] According to Takami *et*

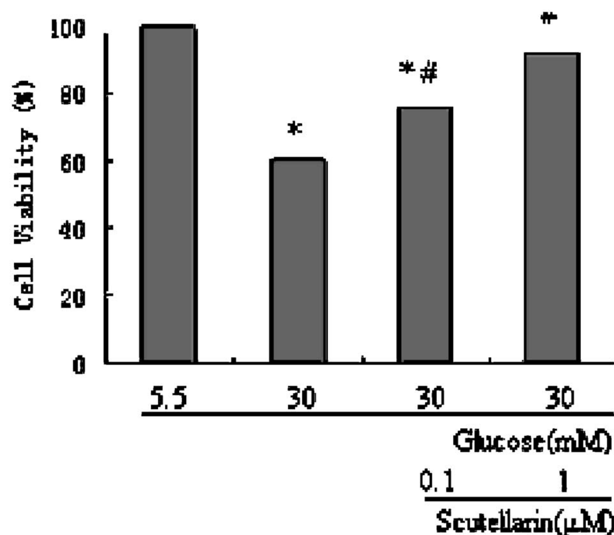

Fig. 2. Cell Viability

Cells were pre-cultured in serum-free medium in the presence or absence of scutellarin (0.1 and 1 μM) for 24 h, and then stimulated with 30 mM glucose for 24 h. $^{*}p<0.05$ compared with 5.5 mM glucose-treated values; $^{\#}p<0.05$ compared with 30 mM glucose-treated values.

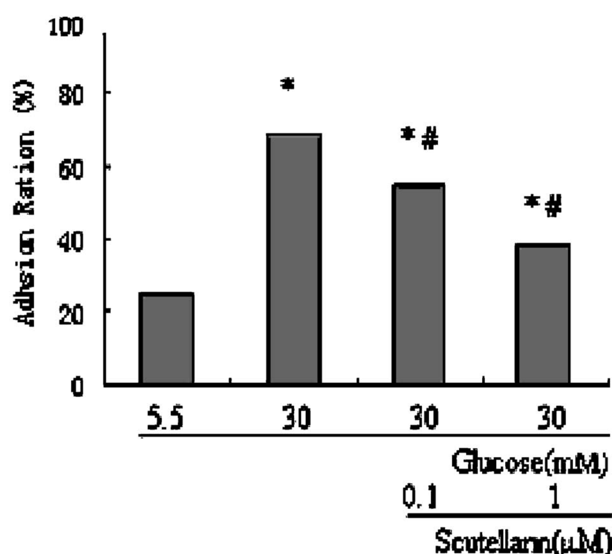

Fig. 3. Effects of Scutellarin on High Glucose-induced Monocyte Adhesion to ECV304 cells

$^{*}p<0.05$ compared with 5.5 mM glucose-treated values; $^{\#}p<0.05$ compared with 30 mM glucose-treated values.

al.,[28] expression of ICAM-1 was increased on the basis of diabetic vascular diseases. Baumgartner-Parzer *et al.*[29] also reported that incubation of human umbilical vein endothelial cells in 30 mM glucose for 24 h increased ICAM-1 expression. Many epidemiological studies have shown that the concentration of soluble ICAM-1 in patients with type 2 diabetes is significantly higher than that in healthy subjects.[30] In addition, MCP-1, a major chemotactic molecule of monocyte trafficking, generated within the vessel wall, has been observed in a variety of cell types including monocytes, vascular smooth muscle cells, and vascular endothelial cells in response to several different stimuli including hyperglycemia.[31] Recent findings seem to suggest that hyperglycemia could accelerate MCP-1 production in vascular cells. Thus glucose induction of MCP-1 might underlie the higher risk of cardiovascular diseases in hyperglycemic states. To investigate whether scutellarin inhibits vascular inflammation induced by hyperglycemia, we evaluated its effect on high glucose-caused expression of ICAM-1 and MCP-1 in ECV304 cells using ELISA. As shown in Fig. 4, cell ELISA results showed that high glucose (30 mM) increased ICAM-1 and MCP-1 contents in comparison with the 5.5 mM condition ($p<0.01$). Pretreatment with both concentrations of scutellarin blocked high glucose-induced increase of adhesion molecules expression as regards the same glucose conditions where no scutellarin was added.

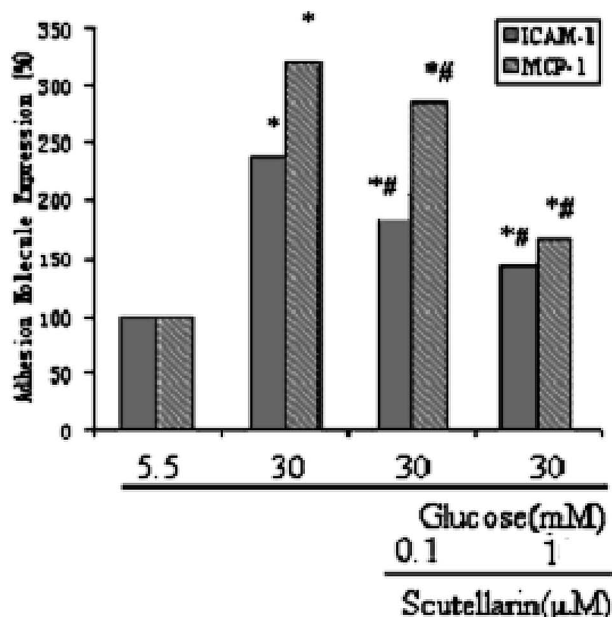

Fig. 4. Effect of Scutellarin on High Glucose-induced Increase of ICAM-1 and MCP-1 Expression Induced in ECV304 Cells

$^{*}p<0.05$ compared with 5.5 mM glucose-treated values; $^{\#}p<0.05$ compared with 30 mM glucose-treated values.

Effect of Scutellarin on High Glucose-induced NF-κB Activation We evaluated the effect of scutellarin on high glucose-induced NF-κB activation by western blotting analysis for NF-κB p65 protein level. NF-κB exists in the cytoplasm as a latent inactive transcription factor form bound to an inhibitory protein, I-κB. Upon stimulation of cells, NF-κB is released rapidly from I-κB, translocates the active dimer p50 and p65 to the nucleus, and activates target gene expression of several cytokines and chemotactic and matrix proteins involved in inflammation responses.[32,33] Inflammation at the cellular level can be described as an increase in the proinflammatory transcription factor nuclear factor NF-κB in the nucleus, with concomitant decreases in its inhibitors, I-κB. Previous studies have shown that high glucose-induced cell adhesion molecules expression may depend on activation of NF-κB. Morigi *et al.*[34] also reported that leukocyte adhesion to endothelial cells increases in conditions of high glucose concentration in a NF-κB-dependent fashion.[34] Accumulating evidence suggests that NF-κB, known to be the primary transcription factor responsible for inflammation during hyperglycemia, may be a key step in disease progression in microvascular complications in diabetes.[35] As shown in Fig. 5, translocation of NF-κB in the nuclear fractions of ECV304 cells was significantly increased by treatment with high glucose. However, scutallarin at a concentration $>0.1\,\mu$M decreased high glucose-induced p65 NF-κB expression levels.

Effect of Scutellarin on Blood Glucose and MCP-1 Level in Alloxan-induced Diabetic Mice At the third week, in the model control there was severe hyperglycemia as compared with the normal control

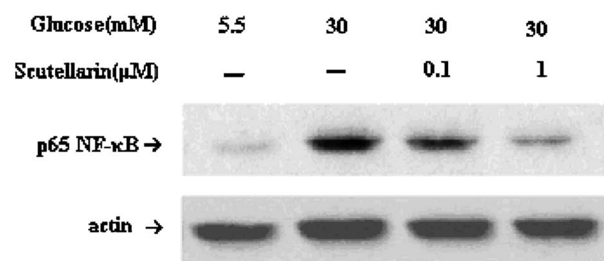

Fig. 5. Effect of Scutellarin on High Glucose-induced NF-κB Activation in ECV304 Cells

Table 1. Effect of Scutellarin on Blood Glucose and MCP-1 Level in Alloxan-induced Diabetic Mice

Samples	Normal	Model	Pioglitazone (5 mg/kg)	Scutellarin (10 mg/kg)	Scutellarin (50 mg/kg)
Blood glucose (mmol/l)	6.8±1.4	25.3±4.5**	14.6±4.2#	24.6±3.6	23.0±5.1
MCP-1 (pg/ml)	3.24±0.57	5.11±0.46**	4.36±0.48#	5.20±0.84	4.25±0.26#

Values are mean±S.E.M., ** $p<0.01$ when compared with Normal, # $p<0.05$ when compared to Model.

($p<0.001$). Comparing with the model control, pioglitazone significantly lowered the elevated blood glucose levels, but there was no significant decline in the two scutellarin-treated groups. It was also observed that due to diabetes there was an increase in serum MCP-1 levels in model groups ($p<0.01$). The pioglitazone treated animals showed a significant reversal in the levels as compared with the model animals. Scutellarin (50 mg/kg) treatment showed a similar reducing capacity. On the contrary, scutellarin (10 mg/kg) did not show any effect in lowering the MCP-1 level, as shown in Table 1.

CONCLUSION

In conclusion, the present data suggest that scutellarin isolated from *E. multiradiatus* might inhibit high glucose-induced monocyte-endothelial cells adhesion by decreasing ICAM-1 and MCP-1 levels in ECV304 cells as well as lower the elevated MCP-1 level in alloxan-induced diabetic mice. Here, we could conclude that the antidiabetic activity of scutellarin may not be attributed to its antihyperglycemic action, but might be associated with decreased expression of NF-κB, which is believed an important factor in the progression of vascular disease in diabetes. Therefore our study suggests that the inhibitory effect of scutellarin on high glucose-induced vascular inflammation is mediated via decreasing inflammatory factor levels and reducing NF-κB nuclear translocation, and this may provide therapeutical benefits in diabetic vascular inflammation-related endothelial dysfunction.

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