Scutellarein alleviates the dysfunction of inner blood-retinal-barrier initiated by hyperglycemia-stimulated microglia cells

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Abstract

● AIM: To investigate the alleviation of scutellarein (SN) against inner blood-retinal-barrier (iBRB) dysfunction in microglia cells stimulated by hyperglycemia and to elucidate the engaged mechanism.

● METHODS: Microglia BV2 cells were stimulated by using 25 mmol/L D-glucose. The same concentration of mannitol (25 mmol/L) was applied as an isotonic contrast. Real-time PCR, Western-blot assay and immunofluorescence staining assay was performed. The dysfunction of iBRB in vitro was detected by using transendothelial electrical resistance (TEER) assay. Additionally, the leakage of fluorescein isothiocyanate (FITC)-conjugated dextran (70 kDa) was detected.

● RESULTS: SN abrogated microglia BV2 cells activation and reduced the phosphorylated activation of extracellular signal-regulated protein kinase (ERK)1/2. SN also decreased the transcriptional activation of nuclear factor κB (NFκB) and the elevated expression of tumor necrosis factor α (TNFα), interleukin (IL)-6 and IL-1β in BV2 cells treated with D-glucose (25 mmol/L). SN attenuated iBRB dysfunction in human retinal endothelial cells (HRECs) or choroid-retinal endothelial RF/6A cells when those cells were treated with TNFα, IL-1β or IL-6, or co-cultured with microglia cells stimulated by D-glucose. Moreover, SN restored the decreased protein expression of tight junctions (TJs) in TNFα-treated HRECs and RF/6A cells.

● CONCLUSION: SN not only alleviate iBRB dysfunction via directly inhibiting retinal endothelial injury caused by TNFα, IL-1β or IL-6, but also reduce the release of TNFα, IL-1β and IL-6 from microglia cells by abrogating hyperglycemia-mediated the activation of microglia cells.

● KEYWORDS: scutellarein; blood-retinal-barrier; tight junctions; inflammation; tumor necrosis factor α

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INTRODUCTION

Blood-retinal barrier (BRB) is composed of an inner BRB (iBRB) and an outer BRB (oBRB). Previous studies have shown that the BRB breakdown occurs in many blinding retinal diseases including diabetic retinopathy (DR), which is a common and serious microvascular complication of diabetes mellitus (DM)[1]. With the development of social economy civilization, science and economy, the living standard of the people has been continuously improved. And the improvement of people’s living standard, at the same time, the prevalence rate of diabetes is increasing day by day. DR has become the main cause that leads to the blindness in adults in industrialized countries[1].

Retinal endothelial cells lining micro vessels are critical components that form iBRB[2-3]. In iBRB, tight junction (TJ) between neighboring cells forms an exceedingly tight seal and thus prevents potentially noxious materials (toxins, infections, endobiotics, endobiotics etc.) from entering and damaging retina, and it is also critical for the supplement of nutrients from blood[1-4]. Additionally, other cells including microglia cells and pericytes also play important roles in maintaining the homeostasis of iBRB[3].
Scutellarein (5,6,7,4’-tetrahydroxy flavone, SN) is the aglycone of scutellarin found in some traditional Chinese medicines like *scutellaria barbata* (Ban-Zhi-Lian), *scutellaria baicalensis* (Huang-Qin), *oroxylum indicum* (Mu-Hu-Die) and *erigerontis berba* (Deng-Zhan-Xi-Xin). Previous studies have demonstrated that SN has anti-cancer, neuroprotective and anti-inflammatory activities.[6-10] SN has already been reported to inhibit human retinal endothelial cells (HRECs) growth and reduce the hypoxia-induced vascular endothelial growth factor (VEGF) expression in HRECs, and all those contributed to its alleviation on DR development[11]. A previous study in our group showed that SN reduced the expression of TNFα in microglia cells through abrogating the activation of ERK1/2-NFκB signaling cascade, and it also directly weakened TNFα-induced BRB breakdown (including iBRB and oBRB) by our group showed that SN reduced the expression of TNFα to its alleviation on DR development.

### MATERIALS AND METHODS

**Reagents** Chemical structure of SN (purity 98%, Shanghai Yihong Bio-Chem Co., Ltd., China) is presented in Figure 1A. Antibodies: ionized calcium-binding adapter molecule1 (Iba1) and claudin-5 [GeneTax (Alton Parkway Irvine, CA, US)]; phospho-inhibitor of NFκB (IKK), NFκBp65, phospho-ERK1/2 (Thr202 and Tyr204), total-ERK1/2, Lamin-B1 and β-actin (Cell Signaling Technology, Danvers, MA, USA); claudin-1, occludin and claudin-19 (Santa Cruz, CA, USA). Peroxidase-conjugated goat anti-rabbit immunoglobulin G (IgG) (H+L) and anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA). Enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN, USA). BCA Protein Assay Kits and NE-PER cytoplasmic and nuclear extraction kits (ThermoFisher Scientific, Waltham, MA, USA). BCA Protein Assay Kits and NE-PER cytoplasmic and nuclear extraction kits (ThermoFisher Scientific, Waltham, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits (R&D, Minneapolis, MN, USA). Cell culture mediums, Trizol reagent and 4’,6-diamidino-2-phenylindole (DAPI; Life Technology Carlsbad, CA, USA). Recombinant TNFα, IL-1β and IL-6 (PeproTech, Cranbury, NJ, USA). Alexa 568 labeled goat anti-rabbit immunoglobulin G (IgG) (H+L) and anti-mouse IgG (H+L) (Jackson ImmunoResearch, West Grove, PA, USA). Primescript RT Master Mix and SYBR Premix DNA polymerase (TaKaRa, Otsu, Japan). FITC-dextran as described[12]. Permeability of endothelial cells was measured by calculating the leakage of FITC-dextran as described[12].

### Statistical Analysis

Data were expressed as mean±standard error of the mean (SEM). The significance of differences within groups was analyzed by using one-way ANOVA with LSD post hoc test. *P*<0.05 was considered as statistically significant differences.

### RESULTS

**Scutellarein Attenuated Microglia Cells Activation in vitro** Iba1 is commonly used to identify activated microglia cells[13]. Figure 1B shows that the quantity of activated microglia cells was increased when D-glucose (25 mmol/L) was added into BV2 cells, whereas SN (50 mmol/L) obviously decreased this increase. Meanwhile, SN (50 mmol/L) also decreased the elevated Iba1 expression induced by 25 mmol/L D-glucose in BV2 cells, whereas SN (50 mmol/L) obviously decreased this increase. Meanwhile, SN (50 mmol/L) also decreased the elevated Iba1 expression induced by 25 mmol/L D-glucose in BV2 cells (Figure 1C).

**Scutellarein Abrogated the D-glucose-initiated NFκB and ERK1/2 Activation in vitro** Figure 2A-2C shows that D-glucose (25 mmol/L) caused the nuclear translocation of NFκBp65 in BV2 cells, and SN (50 mmol/L) decreased this glucose and 10% FBS (Life Technology, Carlsbad, CA). D-glucose (25 mmol/L) was used to incubate with BV2 cells for the indicated times. At the same time, an isotonic contrast mannitol (25 mmol/L) was used. Cellular RNA was extracted and real-time PCR assay was performed as described in our previous published paper[12]. The results were analyzed by the 2-ΔΔ*CT* method and given as ratio compared with the control. The primer sequences are shown in Table 1. Protein expression was detected and calculated as described in our previous study[12]. Supernatants from cells were used for the ELISA assay. Cells were pre-incubated with SN (20, 50 mmol/L) for 6h, and then further incubated with D-glucose (25 mmol/L) for 24h. Immunofluorescence staining of Iba1 was conducted as described[12]. TEER was measured as described[12]. Permeability of endothelial cells was measured by calculating the leakage of FITC-dextran as described[12].

### Table 1 The list of primers for real-time PCR

<table>
<thead>
<tr>
<th>Target, primer</th>
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<tr>
<td>TNF-α</td>
<td>5’-AGGCCATCCCCCCTAAAAAGAT-3’</td>
</tr>
<tr>
<td>IL-1β</td>
<td>5’-AGTTGACGAGCCCCAAAG-3’</td>
</tr>
<tr>
<td>IL-6</td>
<td>5’-CGGAGAGGGAGCTCAGAG-3’</td>
</tr>
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<td>Actin</td>
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accumulation. Besides, SN (20, 50 mmol/L) reduced the increased IKK phosphorylation in BV2 cells incubated with D-glucose (25 mmol/L) (Figure 2A-2B). SN (50 μmol/L) and U0126 (20 μmol/L), the inhibitor of MEK1/2, also weakened the increased ERK1/2 phosphorylation stimulated by 25 mmol/L D-glucose in microglia BV2 cells (Figure 2D-2E). Moreover, SN (50 mmol/L) also reduced the up-regulated mRNA expression of IL-1β (Figure 3A) and IL-6 (Figure 3B). TNFα is another important pro-inflammatory cytokine. SN (20, 50 mmol/L) obviously reduced the up-regulated TNFα mRNA expression in D-glucose-treated BV2 cells (Figure 3C). Meanwhile, SN (20, 50 mmol/L) reduced the up-regulated TNFα amount in supernatants isolated from BV2 cells stimulated by D-glucose (Figure 3D).
Scutellarein Rescued the D-glucose-stimulated BV2 cells or TNFα-initiated iBRB Injury

As shown in Figure 4A, when incubating with D-glucose-treated BV2 cells, TEER value was obviously decreased in RF6A cells, and SN (20, 50 mmol/L) rescued this decrease (Figure 4A). When RF/6A cells were incubated with D-glucose-treated BV2 cells for 24h, the FITC-dextran leakage in RF/6A cells was obviously up-regulated. SN (20, 50 mmol/L) reduced this increased leakage (Figure 4B). SN (20, 50 mmol/L) also reversed the TNFα-induced reduction of TEER value and increase of FITC-dextran leakage in RF/6A cells (Figure 4C-4D). Meanwhile, SN (20, 50 mmol/L) restored the TNFα-induced decreased protein expression of claudin-1 and -19 in RF/6A cells (Figure 4E). SN (50 mmol/L) increased the TNFα-induced decreased expression of occludin in RF/6A cells (Figure 4E). SN (20, 50 mmol/L) and TNFα both had no obvious effects on cellular claudin-5 expression (Figure 4E).

When incubating with D-glucose-treated BV2 cells, TEER value was obviously decreased in HRECs, and SN (20, 50 mmol/L) enhanced this decrease (Figure 5A). When HRECs cells were incubated with D-glucose-treated BV2 cells for 24h, the FITC-dextran leakage in HRECs cells was obviously up-regulated. SN (20, 50 mmol/L) reduced this increased leakage (Figure 5B). Next, TEER value decreased when HRECs were stimulated with TNFα for 6 or 18h, while SN (20, 50 mmol/L) rescued this decrease (Figure 5C). And TNFα caused the increase of the FITC-dextran leakage in HRECs. However, SN (20, 50 mmol/L) reduced this increase (Figure 5D). Besides, induced by TNFα (20 ng/mL), the protein level of claudin-1, -19 and occludin decreased in HRECs, but SN (50 mmol/L) restored this decrease (Figure 5E). SN (20 mmol/L) also restored the TNFα-induced decreased expression of claudin-19 in HRECs (Figure 5E). SN (20, 50 mmol/L) and TNFα also had no obvious effects on cellular claudin-5 expression (Figure 5E).

Scutellarein Rescued the IL-1β or IL-6-mediated iBRB Injury

Both IL-1β and IL-6 down-regulated the TEER value of HRECs. However, SN (20, 50 mmol/L) rescued this decrease (Figure 6A-6B). Similarly, both IL-1β and IL-6 accelerated the leakage of FITC-dextran in HRECs, while SN (20, 50 mmol/L) also reversed this (Figure 6C-6D).

DISCUSSION

The persistent hyperglycemia will destroy the homeostasis of circulatory system, so there are many serious microvascular diabetic complications including DR. Microglia cells are the long-living resident cells regulating immune response in central nervous system (CNS). Thus it can be seen that the activation of microglia cells is considered to be closely linked to the progression of DR. NFκB is critically involved in regulating inflammatory responses by inducing the expression of various pro-inflammatory mediators, cytokines, etc. Next, we detected the inhibitory effects provided by SN against hyperglycemia-induced NFκB activation in vitro, which may be a useful help for its amelioration of DR. NFκB is critically involved in regulating inflammatory responses by inducing the expression of various pro-inflammatory mediators, cytokines, etc. Next, we detected the inhibitory effects provided by SN against hyperglycemia-induced NFκB activation in vitro, which may be a useful help for its amelioration of DR.
its phosphorylation will lead to the activation of microglia cells in both acute and chronic CNS diseases including Alzheimer’s disease and stroke\cite{12,21}, and also in DR development\cite{12,22-23}. Additionally, SN also abrogated ERK1/2 activation in BV2 cells induced by high concentration of D-glucose. The above results suggest that SN abrogated microglia cells activation and the subsequent TNFα release from hyperglycemia-stimulated microglia cells, which may contribute to the SN-provided improvement of DR.

Our previous study has shown that hyperglycemia-stimulated microglia BV2 cells caused obvious damage on both iBRB and oBRB\cite{12,22}. In this study, the hyperglycemia-stimulated microglia BV2 cells caused obvious damage on iBRB in vitro when retinal endothelial cells (including RF/6A cells and HRECs) were co-cultured with BV2 cells stimulated with D-glucose. However, SN rescued this iBRB dysfunction in vitro. TNFα, IL-1β and IL-6 are main pro-inflammatory cytokines released from the activated microglia cells, and previous studies showed that TNFα induced iBRB dysfunction\cite{24}. In this study, TNFα was found to induce obvious injury of iBRB in vitro. Additionally, IL-1β and IL-6 also induced iBRB injury in vitro. However, SN restored the iBRB dysfunction induced by TNFα, IL-1β or IL-6 in vitro. All these results clearly demonstrated that SN rescued the iBRB injury induced by hyperglycemia-stimulated microglia cells or pro-inflammatory cytokines such as TNFα, IL-1β and IL-6.

TJs are mainly composed of junctional adhesion molecules, occludin, claudins, zonula occludens-1 and -2, and TJs integrity is critical for maintaining the normal physiological function of iBRB\cite{3-4}. Previous studies have already shown that TNFα and IL-1β caused the reduced expression of TJs and thus led to the breakdown and unbalance of BRB\cite{25-26}. Claudin-1,
Figure 5 SN rescued the D-glucose-stimulated BV2 cells or TNFα-initiated iBRB injury in HRECs A, B: Upper-chamber seeded with HREC cells and lower-chamber seeded with BV2 cells in 24-well plates composed a co-cultured environment, and 6h before the D-glucose (25 mmol/L) stimulation on BV2 cells, SN (20, 50 μmol/L) was added into the bottom of transwells for pre-treatment. TEER (A) \((n=3)\) and FITC-dextran leakage (B) \((n=4)\) were determined. C, D: TNFα (20 ng/mL) was added into the plates under the chambers, and HRECs were cultured in the upper-chamber with the pretreatment with or without SN (20, 50 mmol/L) for 6h. TEER \((n=3; C)\) and FITC-dextran leakage \((n=4; D)\) were detected. E: The same treatment as C&D was performed and the cell samples were collected for detecting the content of claudin-1 
\((n=3)\), claudin-5 \((n=4)\), claudin-19 \((n=3)\) and occludin \((n=3)\) \(^aP<0.05, ^bP<0.01, ^cP<0.001\) versus control; \(^dP<0.05, ^eP<0.01, ^fP<0.001\) versus D-glucose-treated BV2 cells or TNFα.

Figure 6 SN rescued the IL-1β or IL-6-mediated iBRB injury in HRECs A, B: IL-1β (20 ng/mL) was added into the plates under the chambers, and HRECs were cultured in the upper-chamber with the pretreatment with or without SN (20, 50 mmol/L) for 6h. A: TEER was detected \((n=4)\). B: FITC-dextran leakage was detected \((n=3)\). C, D: IL-6 (20 ng/mL) was added into the plates under the chambers, and HRECs were cultured in the upper-chamber with the pretreatment with or without SN (20, 50 mmol/L) for 6h. C: TEER was detected \((n=4)\). D: FITC-dextran leakage was detected \((n=3)\) \(^aP<0.05, ^bP<0.001\) versus control; \(^cP<0.05, ^dP<0.01, ^eP<0.001\) versus IL-1β or IL-6.
-5 and -19 are classical claudin proteins generally expressed in BRB, and their decreased expression was associated with the development of DR\textsuperscript{[28-29]}. We found that SN restored the down-regulated expression of claudin-1 and -19 in TNFα-stimulated retinal endothelial cells. However, the expression of claudin-5 was not changed in TNFα-stimulated retinal endothelial cells. The reduced expression of occludin has already been found in BRB damage during DR development\textsuperscript{[28-29]}. TNFα reduced occludin expression in retinal endothelial cells, but SN restored the reduced occludin expression induced by TNFα in retinal endothelial cells. These above results imply that SN maintains BRB integrity by rescuing the TNFα-induced decreased expression of occludin, claudin-1 and -19.

In summary, our results demonstrate that natural product SN alleviated iBRB dysfunction mediated by hyperglycemia-stimulated microglia cells. The proposed engaged mechanism may be that SN abrogates the activation of ERK1/2-NFκB-initiated signaling cascade in hyperglycemia-stimulated microglia cells and decreased the subsequent TNFα, IL-1β or IL-6 expression in microglia cells. SN also directly rescued iBRB damage induced by pro-inflammatory cytokines by maintaining the integrity of TJs. Our study suggests that SN has a good prospect of further research and development for DR treatment.

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