The Effect and Mechanism of Emodin on the NO Secretion of Human Umbilical Vein Endothelial Cells (HUVECs) Induced by High Glucose

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Objective. To investigate the effects of emodin on nitric oxide (NO) secretion induced by high glucose in human umbilical vein endothelial cells (HUVECs) through the p-Akt signaling pathway.

Methods. Sensitivity of cells to emodin was determined by MTT assay to establish the experimental concentrations; then, HUVECs were treated with high-dose (33.3 mmol/L) glucose (HG), HG + emodin (HG+E), HG + the Akt phosphorylation inhibitor LY294002 (HG+LY), or HG+E+LY. The p-Akt (Ser 473) expression in 48 h was analyzed using Western blot. NO effect on the secretion of HUVECw was analyzed using nitrate reductase assay.

Results. The sensitive emodin concentration for HUVECs growth was 10 mol/L (P < 0.05). Compared with the HG group, NO secretion was significantly higher in the HG+E group (P < 0.05), whereas it was lowest in the HG+LY group (P < 0.05). Compared with the HG+LY group, NO secretion was increased in the HG+E+LY group (P < 0.05). The p-Akt protein expression was decreased in the HG+LY group when compared to the HG group (P < 0.05), while it significantly increased in the HG+E group (P < 0.05). Compared with HG+LY group, p-Akt protein expression was significantly higher in the HG+E+LY group (P < 0.05).

Conclusion. Emodin could improve the NO secretion of HUVECs by high glucose through the p-Akt signaling pathway.

1. Introduction

Insulin resistance (IR) is the characteristic of type 2 diabetes (T2DM). The inflammatory reaction and the functional disorder of vascular cells usually accelerated the growth of the IR [1]. The endothelial cells produce nitric oxide (NO), which is an important regulator of vessel relaxation. The reduction of endothelial cell NO is an important reason for its dysfunction, and the NO secretion is closely associated with the phosphorylation of nitric oxide synthase (eNOS) [2, 3]. It was discovered in the cellular experiments that the phosphorylation of the eNOS was related to the phosphatidylinositol 3-kinase/Akt (PI3K/Akt) transduction [4]. Current studies showed that the endothelial functional disorder of the T2DM patients was associated with the inhibition of PI3K/Akt/eNOS transduction [5]. Previous studies have shown that emodin has a potential role in improving the insulin resistance. In the differentiation studies of osteoblast (OB) cells, Lee et al. found that the emodin could regulate the Akt via PI3K activity to promote the differentiation of OB cells [6]. Therefore, this paper discussed whether the emodin would improve the NO secretion induced by high glucose through the p-Akt protein signal transduction to provide the experimental basis for
further exploration of the effect of emodin on the improvement function of diabetes insulin resistance and the vascular complications of diabetes.

2. Materials and Methods

2.1. Reagents and Equipment. Emodin (manufactured by US Sigma Corporation): weigh 1 mg of emodin and dissolve it in the DMSO, add 7.4 mL of high-pressure sterilized triple-distilled water, and then add 50 μL of 5 mol/L NaOH solution with pipettes to dissolve it into orange-red transparent solution, namely, emodin. The high-pressure sterilized filter is re-filtered via the clean bench. Take the filtered solution as the mother solution, with a concentration of 500 μmol/L. Use the high-pressure sterilized triple-distilled water to dilute the mother solution into the concentrations of 0.1 μmol/L, 5 μmol/L, 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L, and 30 μmol/L, respectively. Human umbilical vein endothelial cells (HUVECs) were purchased from the Pharmacology Laboratory Cell Center of Central South University; the NO test kit was from Nanjing Jiancheng Bioengineering Institute; p-Akt (Ser473) antibody, UK Abcam Corporation; rat anti-β-actin antibody, Beijing Zhongshan Gold Bridge Biotechnology Co., Ltd.; prestained protein gradient marker, Hangzhou Sijiqing Bioengineering Co., Ltd.; and LY294002, Shanghai Beyotime Biotechnology Co., Ltd. Preparation of LY294002: add 1083 μL DMSO into 1 mg of it to obtain 3 × 10⁻³ mol/L of stock solution, dilute it for two times to obtain 1 × 10⁻³ mol/L of application liquid, and then add 10 μmol/L of application liquid into 1 mL culture medium. The final concentration of the solution is 10 μmol/L. Low-sugar DMEM culture solution (US Gibco Corporation), fetal bovine serum, FBS (APP Corporation, Australia), D-glucose and trypsin powder (US Sigma Corporation), MTT (US Fluka Corporation), gel imaging analysis system GOS7500 (US UAP Corporation), inverted fluorescence microscope (Japan Olympus Company), and high-speed low-temperature centrifuge 5804R (German EPPendorf Company) were used.

2.2. High Glucose Modeling and Cell Culture. When the cells reached coalescence of 80% after HUVECs were cultured and abraded, discard the serum-contained culture medium and replace the culture medium without serum to culture for 24 h and then adopt the culture solution containing the glucose of different concentrations, 5 mmol/L, 11.1 mmol/L, 22.2 mmol/L, and 33.3 mmol/L, respectively. After observing it for 0 h, 24 h, 48 h, and 72 h, respectively, it was found that the optimal glucose concentration of the HUVECs in the high glucose model was 33.3 mmol/L after 48 h incubation. Experimental grouping was as follows: high glucose 33.3 mmol/L + emodin group (HG + emodin), high glucose + LY294002 group (HG + LY group), and high glucose + emodin group + LY294002 group (HG + emodin + LY group). After 48 h joint incubation, the NO in the cell-cultured supernatant of various groups was determined and the p-Akt protein level was detected with the Western blot method.

2.3. MTT Assay of Drug Sensitivity of Cells. Through the observation in the inverted microscope, cells were in good condition and the culture bottle was covered 90%. Discard the old culture medium, wash the bottle with PBS twice, digest it with trypsin, and add culture medium and count the plate counts. Add 5.0 × 10³ cells in each hole of 96-pore plate, and observe it with the inverted microscope after 12 hours of the inoculation. Discard the digested culture medium and use the PBS buffer solution to wash the cells twice after watching the cells adhered to the walls. Add 150 μL of culture medium of the emodin with separate concentrations of 0.1 μmol/L, 5 μmol/L, 10 μmol/L, 15 μmol/L, 20 μmol/L, 25 μmol/L, and 30 μmol/L into the corresponding 96-well pore plates, and after the culture of 24 hours, add 20 μL MTT with a concentration of 5 mg/mL into each hole. Discard the supernatant 4 hours later, add 150 μL DMSO into each hole and slightly shake it for 10 minutes (min) for crystal and dissolution. Measure the absorbency OD value under the 570 nm wavelength of the microplate reader. According to the formula, calculate HUVEC IR (survival rate) (%): (1 – OD mean value of test hole/mean value of controlled hole) × 100% and calculate IC50 values of various groups of cells.

2.4. Detection of NO with Nitrate Reductase. NO was quickly transformed into NO⁻² and NO⁻³ in human body, and NO⁻² was further transformed into NO⁻³. NO⁻³ was reduced to NO⁻² using the idiosyncrasy of nitrate reductase. Its concentration was determined via the degree of coloration. The collected culture solution was dissolved at the room temperature and then diluted. The specific operating steps were performed in strict accordance with the manual of the kit. By determining the absorbance value of various samples with the spectrophotometer at the optical path of 550 nm and 0.5 cm, the content of NO (μmol/L × 10⁵ cells/ml) in the culture solution was then calculated in strict accordance with the steps indicated in the manual of the reagent kit. The coefficient of variation within the assay was less than 10%. (1) Take 0.1 mL sample, add it into the mixture and mix them evenly, and then put it into the water bath for 60 min at 37°C; (2) add 0.21 mL of reagent 3 and reagent 4, respectively, fully swirl and mix them for 30 seconds, stew it for 40 min at room temperature, 3500 rpm/min, and a centrifuge operation of 10 min, and then take the supernatant for coloration; (3) take 0.5 mL supernatant and add 0.6 mL color reagents, mix them evenly, and stew it for 10 min at room temperature; set it to zero with the distilled water, and determine the absorbance with 721-type spectrophotometer with optical path of 550 nm and 0.5 cm; (4) substitute the absorbance value into the equation to calculate the content of NO.

2.5. Western Blot Analysis of p-Akt Protein Expression. Detection steps of the Western blot method: the laminated flat glass was assembled and fixed in the stand, the prepared separating gel and stacking gel were poured into the stand in an appropriate proportion, the comb was inserted into the proper position in the stacking gel, and then the stacking gel was polymerized for 30 min at room temperature. Then, the
protein solution volume determined by the Manual of Protein Quantitative Kit was taken as the loading volume (sample volume). After the loaded samples were energized and subjected to the gel electrophoresis by SDS-PAGE, they were subjected to the electric blotting for about 50 min. 5% skimmed milk was sealed by blocking solution and shaken slowly for one hour at room temperature, and then, the blocking solution was discarded. 1% skimmed milk was diluted and subjected to the primary antibody incubation overnight at 4°C (1:500) or for one hour at 37°C, with primary antibody for recovery, and the film was washed three times with TBST, about 10 min for each time. It was diluted and subjected to the secondary antibody incubation for one hour at 37°C (1:1000) and it was then developed.

2.6. Statistical Analysis. The relevant data obtained in the experiments were presented by \( \bar{x} \pm s \), and the statistical analysis of these data was performed with the software SPSS 16.0. The experiments were repeated three times. They were statistically significant at \( P < 0.05 \) through the one-way ANOVA analysis of differences between groups.

3. Results

3.1. Screening of Emodin Concentrations in Experiments. It was determined through the MTT experiments that the emodin could inhibit the growth of the HUVECs and promote their apoptosis in a dosage-dependent manner. The cell concentration with the survival rate of 90% was selected as the experimental concentration. The results in Table 1 showed that the survival rate of the HUVECs was higher than 91.3% when the emodin concentration was 0.1 \( \mu \text{mol/L} \), 5 \( \mu \text{mol/L} \), and 10 \( \mu \text{mol/L} \), respectively. There was no significant difference for the survival rate of the HUVECs between 0.1 \( \mu \text{mol/L} \) group and 10 \( \mu \text{mol/L} \) group (0.1 \( \mu \text{mol/L} \) group vs. 10 \( \mu \text{mol/L} \) group) \( (P > 0.05) \). There was a statistical difference for the survival rate of the HUVECs \( (P < 0.05) \) as the group of 15 \( \mu \text{mol/L} \), 20 \( \mu \text{mol/L} \), 25 \( \mu \text{mol/L} \), and 30 \( \mu \text{mol/L} \) compared with 10 \( \mu \text{mol/L} \) group. Therefore, the concentration of the emodin in the experiment adopted 10 \( \mu \text{mol/L} \).

3.2. Effect of Emodin on Supernatant NO Content of Cultured HUVECs Induced by High Glucose. In Table 2, under the emodin treatment, the NO secretion of HUVECs was increased in the HG + emodin group compared with the high glucose group (HG group) \( (* P < 0.05) \). Additionally, the NO secretion of HUVECs was reduced in the HG + LY294002 group compared with the HG group \( (** P < 0.01) \), while it was enhanced in the HG + emodin + LY294002 group compared with the HG + LY294002 group \( (** P < 0.05) \).

3.3. Effect of Emodin on p-Akt Protein Expression in HUVECs Induced by High Glucose. In Table 3 and Figure 1, the p-Akt protein expression was decreased in the HG (33.3 mmol/L) + LY294002 group compared with the 33.3 mmol/L of the high glucose group \( (** P < 0.01) \), whereas it was increased in the HG (33.3 mmol/L) + emodin group \( (** P < 0.05) \).

### Table 1: Effect of emodin with different concentrations with MTT method on the survival rate of HUVECs \((n=3, \bar{x} \pm S)\).

<table>
<thead>
<tr>
<th>Group (concentration, ( \mu\text{mol/L} ))</th>
<th>Cell survival rate</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>0.927 ± 0.043</td>
</tr>
<tr>
<td>5</td>
<td>0.920 ± 0.031</td>
</tr>
<tr>
<td>10</td>
<td>0.913 ± 0.032</td>
</tr>
<tr>
<td>15</td>
<td>0.872 ± 0.024**</td>
</tr>
<tr>
<td>20</td>
<td>0.815 ± 0.042**</td>
</tr>
<tr>
<td>25</td>
<td>0.652 ± 0.030**</td>
</tr>
<tr>
<td>30</td>
<td>0.490 ± 0.023**</td>
</tr>
</tbody>
</table>

Note: **\( P < 0.05 \) vs. 0.1 \( \mu \text{mol/L} \) group (**\( P < 0.05 \)).

### Table 2: Effect of emodin on culture supernatant NO content of HUVECs induced by 33.3 mmol/L of high glucose \((n=3, \bar{x} \pm S)\).

<table>
<thead>
<tr>
<th>Group</th>
<th>NO (( \mu\text{mol/L} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG</td>
<td>40.512 ± 5.83</td>
</tr>
<tr>
<td>HG + emodin</td>
<td>47.015 ± 5.02*</td>
</tr>
<tr>
<td>HG + LY294002</td>
<td>30.902 ± 4.35**</td>
</tr>
<tr>
<td>HG + emodin + LY294002</td>
<td>39.153 ± 4.04***</td>
</tr>
</tbody>
</table>

Note: compared with the high glucose group, \( ^* P < 0.05 \) and \( ** P < 0.01; \) compared with the HG + LY294002 group, \( *** P < 0.05 \).

### Table 3: Effect of emodin on p-Akt protein expression level in HUVECs induced by 33.3 mmol/L of high glucose \((n=3, \bar{x} \pm S)\).

<table>
<thead>
<tr>
<th>Group</th>
<th>p-Akt (grey level)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HG</td>
<td>0.61 ± 0.13</td>
</tr>
<tr>
<td>HG + emodin</td>
<td>0.87 ± 0.19*</td>
</tr>
<tr>
<td>HG + LY294002</td>
<td>0.22 ± 0.05**</td>
</tr>
<tr>
<td>HG + emodin + LY294002</td>
<td>0.59 ± 0.11***</td>
</tr>
</tbody>
</table>

Note: compared with the HG group, \( ^* P < 0.05 \) and \( ** P < 0.01; \) compared with HG + LY294002 group, \( *** P < 0.05 \).

4. Discussion

Under the pathological conditions, the vascular endothelial dysfunction was mainly characterized by the inflammatory response, endothelial denudation, increased endothelial vascular permeability, and decreased endothelial-dependent vasodilatation (EDD) [7]. These pathological changes are an important inducement of the development of insulin resistance. NO is one of the important biologically active substances secreted by endothelial cells. The vascular dysfunction is closely associated with the regulation of NO concentration and the important indicator of measuring the endothelial functions. Excessively secreted NO can induce or initiate the apoptosis procedures to induce the apoptosis of endothelial cells [8]. On the contrary, the decrease of secreted NO will cause the dysfunction of EDD, the intensified smooth muscle cell proliferation, and enhanced platelet aggregation and lead to the thrombosis to promote the development of atherosclerosis. The high glucose in patients with diabetes is an important factor of causing endothelial dysfunction, which not only affected the barrier of
endothelial functions but also led to the increase of constriction factors of angiotensin II secreted by endothelial cells and the decrease of relaxing factors of NO. Some studies also showed that the contracting function of vascular endothelial cells was increased and the vasodilatation was decreased and the blood flow velocity slowed down after the glucose load was given to the normal human. It could be inferred that the glucose concentration regulated upward the vasodilatation [9, 10], and the vascular endothelial dysfunction could be directly induced by the high glucose (hyperglycemia), thus affecting the endothelial secretion function.

Emodin is a derivative of hydroxyl anthraquinone. It can reduce the generation of ATP and raise the AMP/ATP proportion in the cells by inhibiting the oxygen consumption of skeletal muscle cell mitochondria, thus activating the energy switch AMPK in the cells and promoting the transformation of the glucose/dextrose into the cells and achieving the purpose of lowering the blood glucose so as to improve the insulin resistance [11, 12]. Some studies showed that the emodin extracted from certain herbs, e.g., rhubarb, Polygonum cuspidatum, was expected to bring new hope for treating type 2 diabetes (T2DM). After being fed to some rats induced by dietary obesity, emodin was found to reduce the insulin level in their blood glucose and serum and increase the insulin resistance level and the lipid level [13]. Our study indicates that the emodin is of the potential role of improving the insulin resistance, and it has attracted the attention of more and more researchers, but its specific functional mechanism is still not clear.

In this study, we found that 10 mol/L emodin was the best concentration for the logarithmic growth phase and the growth status of the cultured HUVECs. Previous studies have shown that one of insulin signal transductions like the phosphatidylinositol 3-kinase (PI3K) was activated by the insulin receptor substrate (IRS) to cause the dextrose/glucose to be absorbed by the skeletal muscle cells and promote the release of NO from the endothelial cells [14]. Hence, the damage of PI3K signal transduction is the prominent feature of the IR. In the circumstance of high concentration insulin, the damage of PI3K signal transduction of the insulin affects the activity of eNOS and reduces the generation of eNOS [15]. LY294002 is the specific inhibitor of the PI3K and acts on the active target of PI3K/Akt to inhibit the phosphorylation of the Akt and promote the apoptosis of the cells [16, 17].

Emodin can improve the NO secretion in the high glucose environment. By adding 10 μmol/L emodin for intervention and through the contrasts of HG 33.3 mmol/L group, HG 33.3 mmol/L + emodin group, HG 33.3 mmol/L + LY294002 group, and HG 33.3 mmol/L + emodin + LY294002 group, we found that the NO secretion in the HG + LY294002 group was the lowest, but this decrease could be reversed when intervened by the 10 μmol/L emodin. The results showed that the emodin could improve the damage of the HUVECs by high glucose.

This experiment for detecting the p-Akt protein expression also confirmed the positive effect of emodin on p-Akt protein expression level in HUVECs induced by 33.3 mmol/L of high glucose. Meanwhile, the result of p-Akt protein expression confirmed that LY294002, as a specific inhibitor of the PI3K, inhibited the Akt phosphorylation in the high glucose environment. Emodin could promote the expression of p-Akt protein in high glucose environment and also reverse the decrease of the p-Akt protein expression in the HG + LY294002 group, which was in accordance with the regulation effect of emodin on NO secretion as shown in MTT results.

To conclude, the studies showed that the emodin played a regulatory role in a variety of molecules in PI3K/Akt transduction and was closely related to the Akt transduction. Emodin might increase the NO secretion of endothelial cells by regulating the Akt transduction, thus playing the role of protecting the endothelial cells of diabetes and decreasing the insulin resistance.

Data Availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflicts of Interest

The authors declare that they have no conflicts of interest.

Authors’ Contributions

Yajia Li and Chunyan Xie contributed equally.
Acknowledgments

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