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# Protection of vascular endothelial cells from high glucose-induced cytotoxicity by emodin

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ABSTRACT

Induction of endothelial cytotoxicity by hyperglycemia in diabetes has been widely accepted. Emodin is a natural anthraquinone in rhubarb used for treatment of diabetes, but its mechanism of action is not fully understood. This study aimed to examine the potential beneficial effects of emodin on endothelial cytotoxicity caused by high glucose milieu. Culture of human umbilical vein endothelial cells (HUVECs) with high concentrations of glucose resulted in damage to the cells, leading to decreased formazan products by 14–27%, reduced DNA contents by 12–19%, and increased hypodiploid apoptosis by 40–109%. These adverse effects of high glucose could be prevented to a large extent by co-culture with 3 μM of emodin which *per se* did not affect HUVECs viability. In addition, CCL5 expression of HUVECs cultured in high glucose medium was significantly elevated at both mRNA and protein levels, an effect abolished after treatment with emodin. Moreover, the enhanced adhesion of monocytes to HUVECs (2.1–2.2 fold over control) and elevated chemotaxis activities (2.3–2.4 fold over control) in HUVECs cultured in high glucose medium were completely reversed by emodin. Emodin also suppressed activation of p38 MAPK and ERK1/2 due to high glucose. Our data demonstrated that endothelial cytotoxicity occurred clearly when HUVECs were exposed to high glucose milieu and emodin was able to alleviate the impairments. The protective effects of emodin might be related to the inhibition of CCL5 expression and subsequent cell stress/inflammatory events possibly mediated by activation of MAPK signaling pathways.



## 1. Introduction

Diabetes is a severe metabolic syndrome resulting in a host of cardiovascular and other complications. Hyperglycemia is an obvious disorder in diabetes, and the endothelial cytotoxicity due to hyperglycemia is widely recognized. Chinese herbal medicines have been applied in diabetes treatment for a long period of time. Emodin (3-methyl-1, 6, 8-trihydroxyanthraquinone) is an active anthraquinone constituent of rhubarb extract. It has been consumed as a traditional Chinese medicine in both single and combination prescriptions for treating fever, constipation and ringworm. Presently, emodin is used commonly for treatment of diabetic nephropathy and protection of kidney function in China [1,2]. The reported actions of emodin include anti-mutagenic, anti-cancer, anti-diuretic, antioxidant, anti-macrophage activation, anti-inflammation, anti-apoptosis and immunosuppressive activities [3–10]. Some studies showed that emodin was effective in ameliorating renal dysfunction in diabetic rats and the mechanism might involve its inhibition of activation of p38 MAPK pathway and down-regulation of the expression of fibronectin [11–13]. Other reports suggested that the activation of PPARγ and the modulation of metabolism-related genes were likely involved in the anti-diabetic effects of emodin [14]. However, the exact mechanisms of improving the diabetic anomalies by emodin remain to be fully understood. Therefore, the present study was performed to

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investigate the potential role of emodin in protecting vascular endothelial cytotoxicity induced by high glucose.

## 2. Materials and methods

### 2.1. HUVEC culture

Human umbilical vein endothelial cells (HUVECs) were cultured in MCDB-131 medium supplemented with 15% heat-inactivated fetal bovine serum (FBS), 17.7 U/ml heparin, 2.5 μg/ml ampicillin, 100 μg/ml streptomycin, and 20 ng/ml aFGF as described previously [15]. The pooled HUVECs were obtained from different donors for this study. All experiments were performed in cells at passages 5–8.

### 2.2. U937 cell culture

The U937 cells, a human monomyelocytic cell line, were cultured in suspension in RPMI 1640 medium supplemented with 10% FBS, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 50 μM 2-mercaptoethanol and 1 mM pyruvate at 37 °C in humidified air with 5% $CO_2$ (all reagents from Sigma–Aldrich). Medium was changed three times a week and cell concentrations maintained at 0.5–1.0 × $10^6$ cells/ml.

### 2.3. MTS assay

The cytotoxicity of high glucose and protective effects of emodin were assessed by 3-(4,5-dimethylthiazol-2-yl) -5-(3-carboxymethoxyphenyl) -2-(4-sulfophenyl)-2H-tetrazolium (MTS) assays [16]. Emodin (purchased from Nanjing Zhelang Medical Technology Co., Nanjing, China) was dissolved in dimethyl sulfoxide (DMSO), stored in aliquots at −20 °C and diluted further in cell culture medium as needed. DMSO (0.5%) was used as vehicle control for all experiments. HUVECs were cultured for 5 days with medium containing control (5.5 mM, i.e. 1 g/l) or elevated concentrations of glucose (22.2 or 44.4 mM; i.e. 4 or 8 g/l) in the absence or presence of emodin at various concentrations (1, 3, 10, 30 μM). After washing and pre-incubation in 100 μl medium for 30 min, cells were added with 20 μl mixture of MTS and phenazine methosulfate (Promega) at 37 °C for 1 h. The absorbance of colored formazan product at 490 nm was measured in an absorbance microplate reader (Sunrise, TECAN, Mannedorf, Switzerland).

### 2.4. Determination of DNA content

HUVECs were cultured for 5 days with medium containing control (5.5 mM) or elevated concentrations of glucose (22.2 or 44.4 mM) in the absence or presence of emodin. Cellular DNA was extracted and its contents were detected using the DNA-binding dye Hoechst 33258. A standard curve of DNA was set up by a serial dilution of standard DNA (100, 80, 60, 40, and 20 μg/ml in order). Both standard DNA and HUVEC samples were topped up to 400 μl in 48-well microplate with 1 μg/ml Hoechst 33258. After 30 min incubation in dark at 37 °C, the plate was subjected for measurement by using a fluorescence microplate reader (Tecan Infinite M200) with excitation wavelength at 346 nm and emission wavelength at 460 nm. All readings were corrected by the blank, and DNA content of each sample was calculated by comparison with the standard curve.

### 2.5. PI staining and flow cytometry

Apoptotic cell death was detected by propidium iodide (PI) staining and flow cytometry as described previously [17]. HUVECs were harvested and suspended in 0.5 ml PBS followed by fixation in 4.5 ml 70% ethanol at 4 °C for 2 h. Fixed cells were kept in −20 °C until performing PI staining. Cell pellets were washed in 5 ml PBS followed by re-suspension in 1 ml PI/Triton X-100 staining solution (20 μg/ml PI, 0.1% Triton X-100, and 0.2 mg/ml ribonuclease in PBS) for 30 min at room temperature. Flow cytometric analysis was carried out using a fluorescence-activated cell sorter (EPICA Elite ESP, Beckman Coulter, Hialeah, FL). Fluorescence (excitation at 488 nm and emission at 610 nm) data were collected on a linear scale and 1 × $10^4$ cells were evaluated for each sample. The flow rate was set to around 200 cells/s. Cells at sub-G1 phase reflecting hypodiploid apoptosis and other cell cycle stages were analyzed and calculated by using WinMDI software (Scripps Institute, La Jolla, CA).

### 2.6. Real-time RT-PCR analysis

Total RNA extraction using the RNeasy Mini Kit (Qiagen, Hilden, Germany) was performed according to the manufacturer's instructions. cDNA was synthesized through reverse transcription (Promega corporation, Madison, WI, USA). For SYBR green based real-time PCR, SYBR green master mix (Applied Biosystems, Foster city, CA, USA), forward and reverse primers, and cDNA template were mixed thoroughly. Quantitative results were generated by the ABI 7500 Fast System using SDS software. The relative gene expression levels were calculated compared to that of β-actin, which was used as the internal control. Forward and reverse primers were 5′-CCTGGCACCCAGCACAAT-3′ and 5′-GCCGATCCACACGGAGTACT-3′ for β-actin; 5′-CGCTGTCATCCTCATTGCTA-3′ and 5′-GCACTTGCCACTGGTGTAGA-3′ for CCL5.

### 2.7. Cell adhesion assay

U937 cells (monocytes) were loaded with 2,7-bis(2-carboxyethyl)-5(6)-carboxyfluorescenin acetoxymethylester (BCECF-AM, Molecular Probes) at 5 μM final concentration in RPMI 1640 medium (Invitrogen) containing 10% FBS at 37 °C for 1 h. The labeled cells were harvested by centrifugation and washed three times with PBS. Afterwards, the BCECF-labeled U937 monocytes (4 × $10^5$ cells/ml) were added into culture plates grown with HUVECs and co-cultured at 37 °C for 1 h. The unbound U937 cells were aspirated and the plates were washed three times with PBS gently. The quantitative results were obtained using a fluorescence plate reader (Tecan Infinite M200) at 485 nm excitation and 535 mM emission wavelength. The cells were also observed under fluorescence microscopy (AMG EVOS/fl).

### 2.8. Chemotaxis assay

Monocyte chemotactic activity was performed in 12-well Transwell chambers fitted with 5 μm pore polycarbonate membranes (Corning, USA). HUVECs were cultured in the lower chamber in the designated conditions. U937 cells loaded with BCECF-AM were added in the upper chamber at 4 × $10^6$ cells/ml and co-cultured with HUVECs in the lower chamber at 37 °C for 2 h. Cells remaining on top chamber of the filter were aspirated and the surface was washed twice with RPMI 1640 medium. The plate was then centrifuged (312 × *g*, 5 min) and the filter was removed. The cells were observed by fluorescence microscopy (AMG EVOS/fl). The quantitation of fluorescent cells was carried out using a fluorescence reader (Tecan infinite M200).

### 2.9. Assessment of CCL5 protein and activation of p38 MAPK and ERK1/2 by Western blotting

Following treatment with high glucose and emodin, HUVECs were harvested in lysis buffer (RIPA:PMSF:All-in-One = 100:1:1,