Emodin-8-*O*-glucuronic acid, from the traditional Chinese medicine qinghuobaiduyin, affects the secretion of inflammatory cytokines in LPS-stimulated raw 264.7 cells via HSP70

PING WANG[1], QUANYONG HE[2] and JIE ZHU[2]

[1]PET/CT Center, Hunan Provincial Tumor Hospital; [2]Department of Burns and Plastic Surgery, Third Xiangya Hospital, Central South University, Changsha, Hunan 410013, P.R. China

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Abstract. Qinghuobaiduyin (QHBDY) is a traditional Chinese medicine, which has an opsonization effect on the immune system. However, which chemical compound in QHBDY underlies the therapeutic effect remains to be elucidated. The present study was designed to investigate the effect of emodin-8-*O*-glucuronic acid, a chemical compound isolated from QHBDY, on the secretion of inflammatory cytokines in lipopolysaccharide (LPS)-stimulated Raw 264.7 cells. The compound was isolated from QHBDY and identified as emodin-8-*O*-glucuronic acid using liquid chromatography-mass spectrometry^n. The results obtained from an ELISA assay showed that emodin-8-*O*-glucuronic acid inhibited the elevated expression levels of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-10 in the LPS-stimulated Raw 264.7 cells, which occurred in a dose-dependent manner. In addition, emodin-8-*O*-glucuronic acid induced the expression of heat shock protein 70 (HSP70) in the LPS-stimulated Raw 264.7 cells, as demonstrated using western blot analysis. The effect of emodin-8-*O*-glucuronic acid on the secretion of TNF-α, IL-1β and IL-10 was attenuated by the knockdown of HSP70. In conclusion, the present study demonstrated that emodin-8-*O*-glucuronic acid effectively suppressed LPS-induced inflammatory cytokine secretion, and this effect was attained by the increased expression of HSP70.

Introduction

Qinghuobaiduyin (QHBDY) is a traditional Chinese medicine, which has been used in a clinical setting since 1995 to treat burns (1,2). It inhibits the expression of inflammatory mediators in the inflammatory response, and has an opsonization effect on the immune system (3,4). However, which chemical compound in QHBDY underlies the therapeutic effect remains to be elucidated.

In the present study, emodin-8-*O*-glucuronic acid was identified as a major chemical constituent of QHBDY. The effect and mechanisms of action of emodin-8-*O*-glucuronic acid, which is the analogue of emodin-8-*O*-β-D-glucoside, on inflammatory cytokine secretion were investigated in lipopolysaccharide (LPS)-stimulated Raw 264.7 cells. The current study aimed to increase current knowledge about emodin-8-*O*-β-D-glucoside, and aid in the modulation of inflammatory responses.

Materials and methods

Sample extraction. QHBDY was provided by the Chinese Medicine Laboratory of The Third Xiangya Hospital of Central South University (Changsha, China). The QHBDY water decoction was concentrated to an extract and dried for 2 h. The extract was then added to 0.5 ml methanol and ultrasound was performed to promote mixing of the solvent and the extract. Following centrifugation at 10,000 x g for 5 min at 4˚C, the supernatant was collected for analysis.

Liquid chromatography (LC)-mass spectrometry(MS)^n analysis. High performance liquid chromatography (HPLC) was performed on a Hypersil GOLD column (2.1x150 mm; 3 μm; Thermo Fisher Scientific, Inc., San Jose, CA, USA), using methanol and water as the mobile phase. MS was performed on an Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Inc.) using electrospray ionization. The auxiliary gas flow was set to 30 units and the sheath gas flow was set to 5 units. The temperature was set to 350˚C and the spray pressure was set at 45 psi. MS scans between 100 and 1,000 m/z were acquired. The major chemical constituents of QHBDY were analyzed using the PubMed database (http://pubchem.ncbi.nlm.nih.gov/search/).

Cell culture and cell transfection. The RAW 264.7 cells were purchased from the American Type Culture Collection

Correspondence to: Professor Jie Zhu, Department of Burns and Plastic Surgery, Third Xiangya Hospital, Central South University, 138 Tongzipo Road, Changsha, Hunan 410013, P.R. China
E-mail: zhu_jie4145@163.com

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(Manassas, VA, USA), and cultured at 37˚C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM; Invitrogen; Thermo Fisher Scientific, Inc.) containing 10% fetal bovine serum (Gibco; Thermo Fisher Scientific, Inc.). LPS was purchased from Sigma-Aldrich (St. Louis, MO, USA) and diluted with phosphate-buffered saline (PBS). The emodin-8-*O*-glucuronic acid was diluted with DMEM. The cells ($1x10^6$ cells/well) were treated with or without LPS (1 μg/ml) for 12 h at 37˚C in a 5% CO_2 atmosphere in the presence of various concentrations of emodin-8-*O*-glucuronic acid (0.1, 1 or 10 ng/ml; 24 h prior to LPS treatment). Transfection of the cells ($2x10^6$ cells/well) with HSP70 small interfering (si) RNA was performed using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc.), following the manufacturer's protocol.

Measurements of tumor necrosis factor-α (TNF-α), interleukin (IL)-1β and IL-10. The concentrations of TNF-α, IL-1β and IL-10 in the culture supernatant were determined using commercial TNF-α, IL-1β and IL-10 ELISA kits (BD Biosciences, San Diego, CA, USA), according to the manufacturer's protocols.

Western blot analysis. The cells were harvested and lysed in ice-cold lysis buffer [150 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Nonidet P-40, 2 mM EDTA, 50 mM sodium fluoride, 0.2% SDS, 100 mM sodium vanadate and 1 mM phenylmethyl-sulfonyl fluoride], and were then centrifuged at 150 x g for 5 min at 4˚C, following which the culture supernatant was collected. Protein quantification was performed using a Bradford Protein Assay kit (Beyotime Institute of Biotechnology, Shanghai, China). The proteins (20 μg) were separated on a 10% SDS polyacrylamide gel, following which they were electrotransferred onto a polyvinylidene fluoride membrane (EMD Millipore, Billerica, MA, USA). Following blocking with 5% non-fat milk, the membranes were washed with Tris-buffered saline with Tween-20 3 times followed by incubation with rabbit polyclonal antibody against HSP70 (dilution 1:800; cat. no. sc-33575; Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and rabbit polyclonal antibody against GAPDH (dilution 1:1,000; cat. no. sc-25778; Santa Cruz Biotechnology, Inc.) overnight at 4˚C, followed by incubation with goat anti-rabbit IgG (dilution 1:1,000; cat. no. sc-2004; Santa Cruz Biotechnology, Inc.) for 1 h at 37˚C. To correct for differences in protein loading, the blots were normalized against GAPDH. The signals were visualized using an ECL western blotting kit (Pierce Biotechnology, Inc., Rockford, IL, USA).

Statistical analysis. The results are expressed as the mean ± standard deviation and differences between two groups were assessed using Student's *t*-test. Statistical analysis was performed using SPSS software, version 19.0 (IBM SPSS, Armonk, NY, USA). $P<0.05$ was considered to indicate a statistically significant difference.

Results

Identification of QHBDY compounds. LC-MS^n detection was developed for the analysis of the major chemical constituents of QHBDY. By comparing with the data in the PubMed database, the major compound in the peak area at 447 m/z was identified as emodin-8-*O*-glucuronic acid (Fig. 1).

Effect of emodin-8-O-glucuronic acid on the expression of inflammatory cytokines in Raw 264.7 cells. The Raw 264.7 cells were treated with LPS for 12 h, and the expression levels of inflammatory cytokines, including TNF-α, IL-1β and IL-10, were examined using ELISA assays. As shown in Fig. 2, the expression levels of TNF-α, IL-1β and IL-10 were significantly increased in the supernatant of cells following treatment with LPS, compared with the levels following treatment with PBS.

The LPS-stimulated Raw 264.7 cells were then treated with various concentrations of emodin-8-*O*-glucuronic acid for 24 h, following which the expression levels of TNF-α, IL-1β and IL-10 were measured. The results from the ELISA assays revealed that the elevated expression levels of TNF-α, IL-1β and IL-10 in the LPS-stimulated Raw 264.7 cells were suppressed by emodin-8-*O*-glucuronic acid, and this occurred in a dose-dependent manner. Treatment with 10 ng/ml emodin-8-*O*-glucuronic acid resulted in a marked inhibitory effect on the expression levels of TNF-α, IL-1β and IL-10.

Effect of emodin-8-O-glucuronic acid on the expression of HSP70 in Raw 264.7 cells. The expression of HSP70 in Raw 264.7 cells treated with LPS were examined using western blot analysis. The results revealed that, compared with the cells in the control group, no significant alterations in the expression of HSP70 were identified in the Raw 264.7 cells treated with LPS.

The present study also examined the expression levels of HSP70 in LPS-stimulated Raw 264.7 cells following treatment with various concentrations of emodin-8-*O*-glucuronic acid for 24 h. It was shown that emodin-8-*O*-glucuronic acid significantly increased the expression of HSP70, in a dose-dependent manner, with the maximum effect at 10 ng/ml (Fig. 3).

HSP70 knockdown attenuates the effect of emodin-8-O-glucuronic acid on the expression of inflammatory cytokines. To investigate whether HSP70 mediates the effect of emodin-8-*O*-glucuronic acid on the expression levels of TNF-α, IL-1β and IL-10, HSP70 siRNA was transfected into the LPS-stimulated Raw 264.7 cells to knock down endogenous HSP70. As shown in Fig. 4, the increased expression of HSP70 observed following treatment with 10 ng/ml emodin-8-*O*-glucuronic acid was significantly decreased in the cells transfected with HSP70 siRNA.

Furthermore, the results revealed that HSP70 knockdown eliminated the decreased expression levels of TNF-α, IL-1β and IL-10 induced by emodin-8-*O*-glucuronic acid treatment in the LPS-stimulated Raw 264.7 cells (Fig. 5).

Discussion

Emodin-8-*O*-β-D-glucoside was first found as a component of the traditional Chinese medicinal herb, *Polygonum cuspidatum* Sieb. et Zucc (5). Xiang *et al* (6) reported that the chemical compound, emodin-8-*O*-β-D-glucoside can also be isolated from the herb *Polygonum amplexicaule* D.

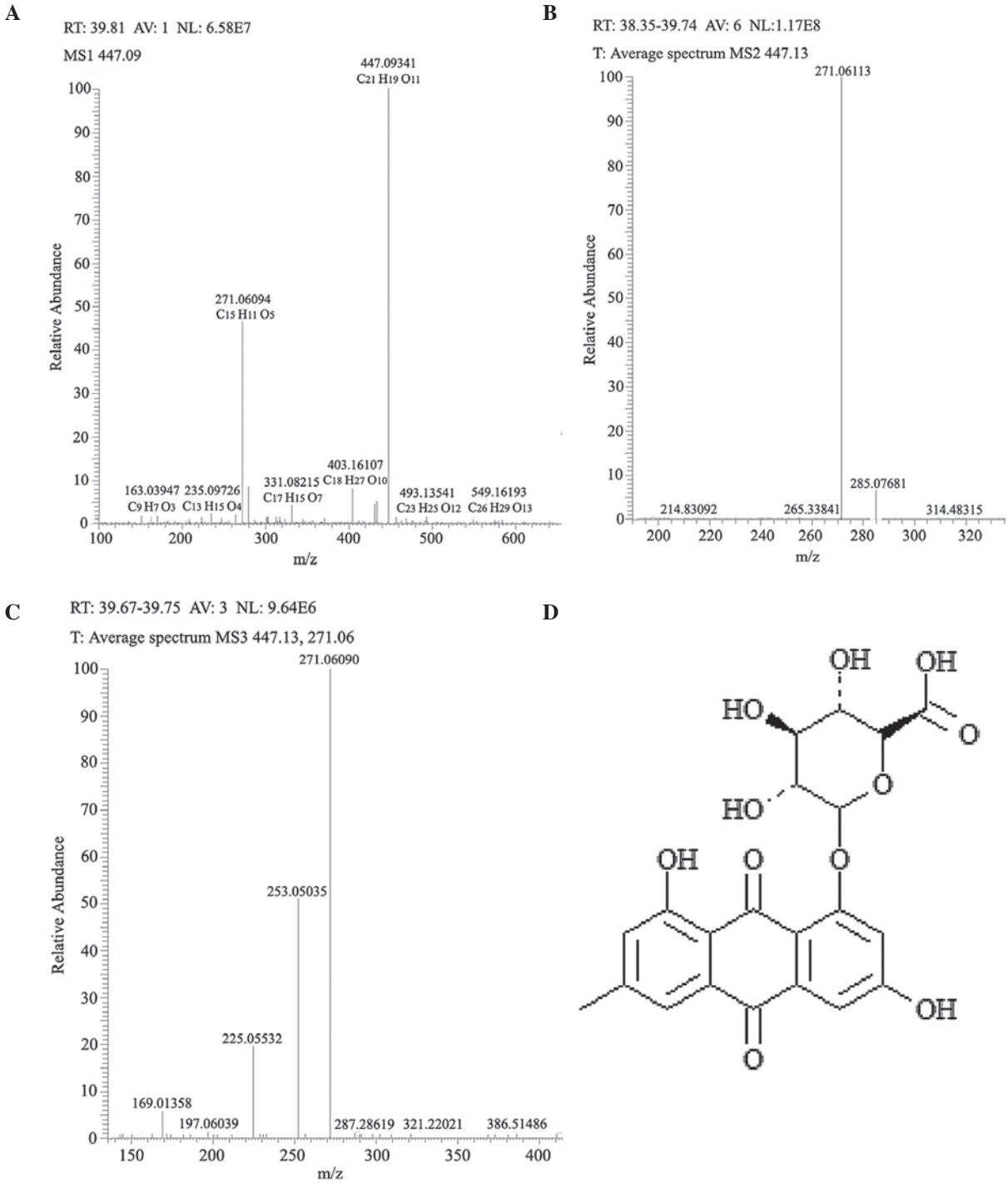

Figure 1. LC-MS^n analysis and the structure of emodin-8-*O*-glucuronic acid. (A-C) Results of the LC-MS^n analysis of emodin-8-*O*-glucuronic acid. (A) MS1, (B) MS2 and (C) MS3 spectra of emodin-8-*O*-glucuronic acid. (D) Chemical structure of emodin-8-*O*-glucuronic acid. LC-MS, liquid chromatography-mass spectrometry.

Don var. sinense Forb. (Polygonaceae). In the present study, it was revealed that emodin-8-*O*-glucuronic acid is a major component of QHBDY, and is the analogue of emodin-8-*O*-β-D-glucoside.

Emodin-8-*O*-β-D-glucoside exerts antioxidative effects and is widely used to treat acute hepatitis (7). A study by Wang *et al* (7) demonstrated that emodin-8-*O*-β-D-glucoside is able to provide neuroprotection against the focal cerebral injury induced by ischemia and reperfusion, and inhibit glutamate neurotoxicity through antioxidative mechanisms. Previously, it was found that emodin-8-*O*-β-D-glucoside directly stimulates the proliferation and differentiation of osteoblasts, thus promoting the healing of bone injury (6). Until now, there have been no reports regarding the potential effect of emodin-8-*O*-β-D-glucoside on immune reactions. Therefore, the present study performed *in vitro* experiments to investigate the functions of emodin-8-*O*-glucuronic acid.

TNF-α and IL-1β are key pro-inflammatory cytokines, and IL-10 is an anti-inflammatory cytokine, all of which are produced by inflammatory cells and are vital in the

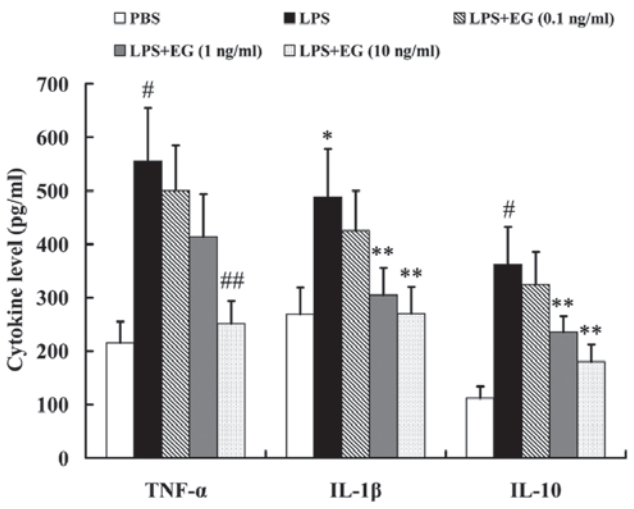

Figure 2. Effect of emodin-8-*O*-glucuronic acid on the expression of inflammatory cytokines in Raw 264.7 cells. *P<0.05 and $^{\#}$P<0.01, compared with the PBS group; **P<0.05 and $^{\#\#}$P<0.01, compared with the LPS group. PBS, phosphate-buffered saline; LPS, lipopolysaccharide; EG, emodin-8-*O*-glucuronic acid; TNF, tumor necrosis factor; IL, interleukin.

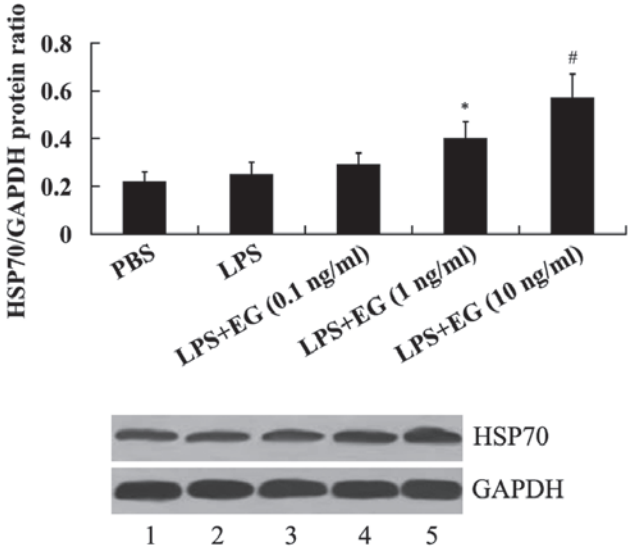

Figure 3. Effect of emodin-8-*O*-glucuronic acid on the expression of HSP70 in Raw 264.7 cells. Lane 1, PBS; lane 2, LPS; lane 3, LPS+EG (0.1 ng/ml); lane 4, LPS+EG (1 ng/ml); lane 5, LPS+EG (10 ng/ml). *P<0.05 and $^{\#}$P<0.01, compared with the LPS group. PBS, phosphate-buffered saline; LBS, lipopolysaccharides; EG, emodin-8-*O*-glucuronic acid; HSP70, heat shock protein 70.

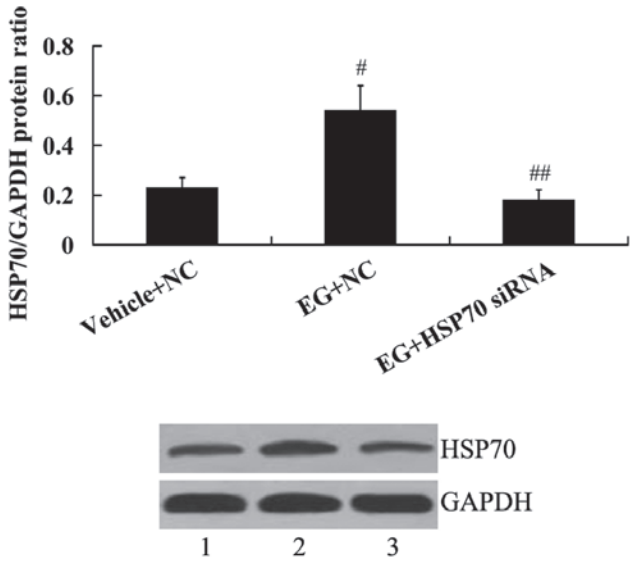

Figure 4. Expression of HSP70 in LPS-stimulated Raw 264.7 cells following transfection with HSP70 siRNA. Lane 1, Vehicle+NC; lane 2, EG+NC; lane 3, EG+HSP70 siRNA. $^{\#}$P<0.01 compared with the Vehicle+NC; $^{\#\#}$P<0.01 compared with the EG+NC. LPS, lipopolysaccharide; EG, emodin-8-*O*-glucuronic acid; HSP70, heat shock protein 70; NC, negative control; siRNA, small interfering RNA.

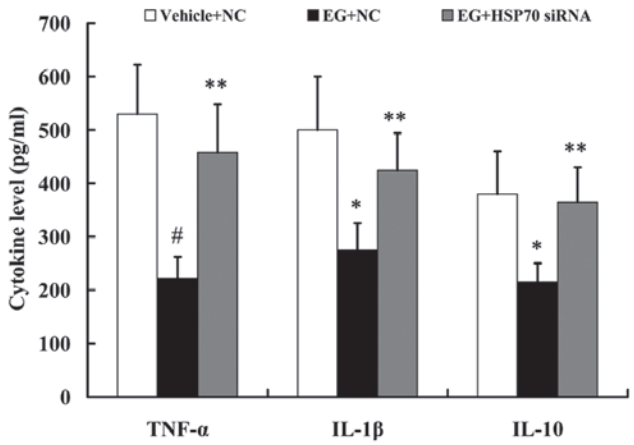

Figure 5. HSP70 knockdown attenuates the effect of emodin-8-*O*-glucuronic acid on the expression of inflammatory cytokines. *P<0.05 and $^{\#}$P<0.01, compared with the Vehicle+NC group; **P<0.05, compared with the EG+NC group. EG, emodin-8-*O*-glucuronic acid; NC, negative control; HSP70, heat shock protein70; TNF, tumor necrosis factor; IL, interleukin; siRNA, small interfering RNA.

inflammatory response (8-12). The abnormal production of these cytokines has been implicated in the pathogenesis of inflammation (13-16). LPSs are potent stimulators of inflammatory reactions (17,18). Investigating the changes in the expression of TNF-α, IL-1β and IL-10 may improve understanding of the effect of emodin-8-*O*-β-D-glucoside on inflammatory reactions. The present study demonstrated that emodin-8-*O*-glucuronic acid exerted inhibitory effects on the secretion of TNF-α, IL-1β and IL-10 in LPS-stimulated Raw 264.7 cells in a dose-dependent manner.

HSP70 is an ATPase, which protects against a variety of stresses, thus regulating multicellular physiological functions (18-21). It has been demonstrated that HSP70 is important in modulating the febrile response to LPS at the cell level and the whole organism level (22,23). The upregulation of HSP70 represses LPS-induced cytokine expression in animals and cultured macrophages (24-26). In our previous study (27), it was demonstrated that QHBDY induced the expression of HSP70 in a severe burn model. In the present study, the effect of emodin-8-*O*-glucuronic acid on the expression of HSP70 was investigated, and the findings suggested

that emodin-8-*O*-glucuronic acid induced the expression of HSP70 in the LPS-stimulated Raw 264.7 cells. Furthermore, it was found that the effect of emodin-8-*O*-glucuronic acid on the secretion of inflammatory cytokines was attenuated by the knockdown of HSP70, indicating that the effect of emodin-8-*O*-glucuronic acid was established through HSP70.

In conclusion, the present study provided the first evidence, to the best of our knowledge, that emodin-8-*O*-glucuronic acid effectively suppressed the LPS-induced secretion of TNF-α, IL-1β and IL-10 in Raw 264.7 cells, and that the mechanism underlying this effect was mediated by HSP70. These findings expand on current knowledge regarding QHBDY and emodin-8-*O*-glucuronic acid, and reveal a molecular mechanism underlying the opsonization effect of the traditional Chinese medicine.

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