

Dioscin attenuates oxLDL uptake and the inflammatory reaction of dendritic cells under high glucose conditions by blocking p38 MAPK

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Abstract. Dioscin has been shown to affect the regulation of metabolic diseases, including diabetes; however, the mechanism of action is still unclear. Under high glucose (HG) conditions, the expression of scavenger receptors and the uptake of oxidized low-density lipoprotein (oxLDL) are upregulated in dendritic cells (DCs), which are critical steps in atherogenesis and inflammation. In this study, the focus was on the impact of dioscin on the function of DCs. Immature DCs were cultured with: 5.5 mM glucose medium (control group); 30 mM glucose medium (HG group); HG + 10 mM dioscin; HG + 20 mM dioscin; HG + 30 mM dioscin; and HG + 40 mM dioscin. For subsequent experiments, 30 mM dioscin was used as the experimental concentration. Dichlorodihydrofluorescein fluorescence was used to measure the intracellular production of reactive oxygen species (ROS) in DCs. The expression levels of the scavenger receptors, including class A scavenger receptors (SR-A), CD36 and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) were determined via quantitative PCR. The protein expression of p38 mitogen-activated protein kinase (MAPK) was determined by western blotting. Furthermore, ELISA was used to detect the levels of interleukin (IL)-6, IL-10 and IL-12. Finally, DCs were incubated with diOlistic (Dil)-labeled oxLDL, and flow cytometry analysis was used to investigate the Dil-oxLDL-incorporated fraction. The incubation of DCs with dioscin inhibited the induction of ROS production, in a dose-dependent manner, under HG conditions. The upregulation of SR-A, CD36 and LOX-1 genes was partially abolished by dioscin, which also partially reversed p38 MAPK protein upregulation. Furthermore, increased secretion of IL-6 and IL-12, and decreased secretion of IL-10 in DCs, induced by HG, was also reversed by dioscin.

To conclude, dioscin could attenuate the production of ROS, inflammatory cytokine secretion and oxLDL uptake by DCs in HG conditions by preventing the expression of scavenger receptors and p38 MAPK, thus playing a positive role in preventing atherogenesis.

Introduction

The prevalence of diabetes mellitus has increased substantially in the past decade, with the number of diabetes cases reaching 387 million (8.3%) in 2014 globally (1,2). In type 2 diabetes, the rates of microvascular and macrovascular complications are 18.8 and 12.7%, respectively (3). Arteriosclerosis is the basis of vascular complications, and high glucose (HG) is the main factor in the development of arteriosclerosis (4,5). During the course of arteriosclerosis development, dendritic cells (DCs) play a key role; DCs move towards the vessel wall, and recognize autoantigens and oxidized low-density lipoproteins (oxLDLs) (6). oxLDL uptake by DCs may enhance the presentation of lipid and peptide antigens to natural killer T cells and T cells to further stimulate vascular inflammation and monocyte adhesion in the atherosclerotic plaque (7,8).

In DCs and intraperitoneal macrophages derived from diabetic mice, hyperglycemia increased reactive oxygen species (ROS) production (5,9). Mitogen-activated protein kinase (MAPK) is an important protein kinase activator and a promising target in the treatment of diabetes, obesity and cardiovascular diseases, due to its important roles in cell growth, cell differentiation and energy metabolism (10). HG can increase the amount of ROS, and this can induce hyperactivation of p38 MAPK in DCs, leading to oxLDL uptake by DCs and atherosclerosis (8,11,12).

Dioscin is a natural steroidal saponin that is isolated from certain medicinal plants or vegetables. Dioscin has numerous pharmacological actions, including anti-inflammatory, immunoregulatory, hypolipidemic, antiviral, antifungal and antitumor effects (13,14). Dioscin has been shown to have protective effects in liver, kidney and lung damage/cancers through various mechanisms. For example, dioscin can activate the Notch1/Jagged1 pathway to promote hepatoproliferation, thus facilitating liver regeneration after partial hepatectomy (15). Dioscin showed a protective effect against thioacetamide-induced acute liver injuries in rats and mice, possibly via the inhibition of oxidative stress and inflammation

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due to its action on the farnesoid X receptor/AMP-activated protein kinase (AMPK) signaling pathway (16). Dioscin has been found to also prevent nonalcoholic fatty liver disease by adjusting lipid metabolism via the sirtuin 1 (Sirt1)/AMPK signal pathway (17). Additionally, dioscin exhibited a protective effect against lipopolysaccharide-induced liver injury and acute lung injury via the alteration of the Toll-like receptor 4/myeloid differentiation primary response 88 pathway (18,19). *In vitro* dioscin attenuated cell damage and decreased renal injury in rats and mice treated with cisplatin through the microRNA-(miR)-34a/Sirt1 signaling pathway (20). During intestinal ischemia/reperfusion (II/R) injury, dioscin upregulated MAPK13 expression by decreasing miR-351-5p levels to inhibit inflammation and apoptosis, thus showing a protective effect (21). Dioscin exerted efficient anti-prostate cancer activity via activation of the estrogen receptor- β (22). Dioscin showed anti-pancreatic cancer effects via miR-149-3P-mediated inhibition of the Akt1 signaling pathway (23). In addition, previous studies have suggested that dioscin also has beneficial effects in the regulation of metabolic diseases, such as diabetes, osteoporosis, obesity and hyperuricemia (24-27). However, the mechanism by which dioscin regulates diabetes is still unclear. Since DCs have critical roles in arteriosclerosis development, this study focused on the impact of dioscin on the function of DCs.

Materials and methods

Generation of monocyte-derived DCs. Peripheral blood mononuclear cells were obtained from 9 healthy volunteers in the Chongqing Traditional Chinese Medicine Hospital between 1 March, 2018 and 30 September, 2018 (6 males and 3 females aged 26-35 years). Mononuclear cells were layered in lymphocyte separation solution and centrifuged for 20 min at 800 x g using discontinuity density gradient centrifugation at room temperature. The interface was recovered and washed three times with Hank's solution and centrifuged three times at 600 x g for 8 min at room temperature. The cells were magnetically sorted for CD14 after gradient centrifugation, adjusted to $5 \times 10^8/l$ using RPMI 1640 medium (Hyclone; GE Healthcare Life Sciences) and incubated at 37°C in an atmosphere of 5% CO₂ for 2 h. The non-adherent cells were removed, and the remaining adherent cells were incubated in 0.9 ml RPMI 1640 medium supplemented with 0.1 ml calf serum (Hyclone; GE Healthcare Life Sciences), 10 μ l 50 mg/l recombinant human granulocyte-macrophage colony-stimulating factor (PeproTech, Inc.) and 44 μ l recombinant human interleukin (IL)-4 (20 mg/l) (PeproTech, Inc.) at 37°C and an atmosphere of 5% CO₂. Immature DCs were harvested on day 6. Then, cells were exposed to glucose (5.5 and 30 mM) or dioscin at various concentrations (10, 20, 30 and 40 mM) with 30 mM HG for an additional 24 h (dioscin was purchased from Selleck Chemicals, with a purity of >97% and a molecular weight of 869.04 g/mol). The cells were further incubated for 2 days, and the immature DCs developed into mature DCs (8). Then, cells were incubated with FITC-CD1a (1:40; Bioscience, 11-0019-42), FITC-CD83 (1:40; Bioscience, 11-0839-42) and FITC-CD86 (1:40; Bioscience, MHCD8601) in 4°C for 30 min. Flow cytometry analysis (FlowJo v7.6.1, FlowJo LLC) was used to quantify the biomarkers CD1a, CD83 and CD86,

while a population of DCs matured. Not all cells in the study can be classed as mature DCs.

Measurement of intracellular ROS. Intracellular ROS production was detected using a ROS assay kit (Beyotime Institute of Biotechnology). Cells were treated as described above. In addition, after cells were treated with HG + 30 mM dioscin for 24 h, the culture medium was changed to one containing only 30 mM HG and then cultured for another 24 h (HG + Dioscin + HG group). The DCs were incubated at 37°C with 10 μ mol/l 2,7'-dichlorodihydrofluorescein diacetate (DCFH-DA) for 20 min and mixed once every 3-5 min. Then, the cells were washed 3 times using cell culture medium without serum to remove the DCFH-DA that had not entered the cells. Fluorescence intensity was measured at an excitation wavelength of 488 nm and an emission wavelength of 525 nm by a TECAN GENios Pro multifunction microplate reader (Tecan Group, Ltd.). The amount of oxygen radicals in each group was expressed as the fluorescence intensity with respect to the control group.

Quantitative (q)PCR. Total RNA was isolated and treated with DNase using TRIzol reagent (Thermo Fisher Scientific, Inc.). The primer sequences of different scavenger receptors, including class A scavenger receptors (SR-A), CD36 and lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in DCs were the same as those used in a previous study (8). SR-A was amplified using the sense primer 5'-TCC TCGTGTTCAGTTCTC-3' and antisense primer 5'-GCA ATTCTTCGTTTCCCCT-3'. CD36 was amplified using the sense primer 5'-CGCTGAGGACAACACAGTCT-3' and antisense primer 5'-GTTGTCAGCCTCTGTTCCAA-3'. LOX-1 was amplified using the sense primer 5'-GGGCTCATTTAA CTGGGAAA-3' and antisense primer 5'-GAAATTGCTTGC TGGATGAA-3'. β -actin was amplified using the sense primer 5'-CTCCATCCTGGCCTCGCTGT-3' and antisense primer 5'-GCTGTCACCTTACCGTTCC-3'. Total RNA was reverse transcribed according to the manufacturer's protocols using a ReverTra Ace- α -First Strand cDNA Synthesis kit (Toyobo Life Science). Fluorescence qPCR was performed with SYBR Green Real-Time PCR master mix (SinoBio Biotech) using a CFX96 real time system (Bio-Rad Laboratories, Inc.). The reaction program included 94°C for 5 min and 40 cycles of 94°C for 30 sec, 57°C for 30 sec, and 72°C for 30 sec. Relative gene expression was analyzed using the delta-delta threshold cycle (CT) method (28). The system software was used to analyze the gene expression, and the standard curve method was used to determine the copies of each mRNA molecule. The internal reference housekeeping gene β -actin was used to normalize the expression.

Western blot analysis. DCs were treated with HG or HG + 30 mM dioscin. Then, the cells were washed with ice-cold PBS and extracted in cold cell lysis buffer (Beyotime Institute of Biotechnology). The bicinchoninic acid protein assay reagent kit (Beyotime Institute of Biotechnology) was used to measure the protein concentration. SDS-PAGE (8%) was used to fractionate the proteins (20 μ g) in each experimental group, and they were then transferred to nitrocellulose membranes (EMD Millipore) and blocked with 5% nonfat

milk for 1 h at room temperature. The primary antibodies rabbit polyclonal anti-p38 MAPK (p38; 1:1,000; Cell Signaling Technology, Inc., 9212), rabbit polyclonal anti-p-p38 MAPK (Thr180/Tyr182; pp38; 1:500; Cell Signaling Technology, Inc., 4631), and monoclonal mouse anti- α -tubulin (1:1,000; Sigma Aldrich, T6199) were added and incubated for 12 h at 4°C. The goat polyclonal anti-mouse IgG-HRP (1:1,000; Santa Cruz Biotechnology, Inc., sc-2005) and anti-rabbit IgG-HRP (1:1,000; Santa Cruz Biotechnology, Inc., sc-2004) were used as secondary antibodies, and incubated for 2 h at room temperature. Steptavidin-HRP (Beyotime Institute of Biotechnology) was added and incubated for 2 h at 37°C and then an ECL detection system (Thermo Fisher Scientific, Inc.) was used to detect antigen-antibody complexes according to the manufacturer's protocol. The resulting bands were analyzed using ImageQuant LAS 4000 (GE Healthcare Life Sciences). All values were normalized to the tubulin loading control.

ELISA analysis. DCs were incubated with HG or HG + dioscin for 24 h, and the total proteins were extracted with extraction solution [200 μ l 5 M guanidine HCl, 50 mM Tris-HCl (pH 8.0) and protease inhibitor] and then centrifuged at 4°C at 12,000 x g for 30 min to remove insoluble materials. Levels of IL-6, IL-10, and IL-12 were measured using IL-6, IL-10 and IL-12 ELISA kits (R&D Systems, Inc, D6050, D1000B and D1200), respectively.

oxLDL uptake. DCs were incubated with 10 μ g/ml Dil-oxLDL (Beijing Union-Biology Co. Ltd.) for 60 min at 37°C after treatment with 5.5 or 30 mmol/l glucose for 24 h. Then, cells were harvested and washed with PBS three times at 4°C. Flow cytometry analysis (FlowJo v7.6.1, FlowJo LLC) was used to quantify the Dil-oxLDL-incorporated fraction.

Statistical analysis. All of the experiments on cells were repeated three times. Data were expressed as the mean \pm SD. One-way ANOVA followed by the Tukey-Kramer post hoc test (version 17.0; SPSS Inc.) was used to determine significant differences. $P < 0.05$ was considered statistically significant.

Results

Mature DCs. The mature DCs had a typical treelike shape (Fig. 1A). Costimulatory molecules, antigen-presenting molecules and adherence factors, including human leukocyte antigen, CD1a, CD80, CD83 and CD86, have been shown to be upregulated in mature DCs (29-32). In the current study, the expression of CD1a, CD83 and CD86 was observed in mature DCs. However, a number of, but not all, DCs showed signs of maturation (Fig. 1B-D).

Measurement of ROS production. The effect of dioscin on the production of ROS in DCs is shown in Fig. 2. Compared with the levels in the control group, HG induced the formation of ROS in human monocyte-derived DCs ($P < 0.01$). Compared with the levels in the HG group, 20-40 mM dioscin inhibited ROS production in HG conditions ($P < 0.05$). Moreover, the inhibitory effect of dioscin at 30 mM and 40 mM was similar; thus, 30 mM dioscin was selected as the reference concentration for subsequent experiments. In addition, the ROS

production in the HG + Dioscin + HG group was similar to those of cells treated only with 30 mM HG in the HG group ($P > 0.05$; Fig. S1).

Effect of dioscin on scavenger receptor expression. The effects of dioscin on the expression of SR-A, CD36 and LOX-1 are shown in Fig. 3A-C. Compared with the expression in the control group, the expression of SR-A, CD36 and LOX-1 mRNA was significantly increased in the HG group and HG + dioscin group ($P < 0.05$). Nevertheless, dioscin treatment led to a significant reduction in SR-A, CD36 and LOX-1 mRNA expression compared with that of the HG group ($P < 0.01$). The expression levels of the scavenger receptors SR-A, CD36 and LOX-1 in the HG + Dioscin + HG group were similar to those of cells treated only with 30 mM HG in HG group ($P > 0.05$; Fig. S2).

Effect of dioscin on p38 MAPK protein levels. The effect of dioscin on the protein level of p38 MAPK is shown in Fig. 3D and E. Compared with the level in the control group, the protein level of p38 MAPK was increased significantly in the HG group ($P = 0.006$). Nevertheless, dioscin treatment led to a significant reduction in the p38 MAPK protein level compared with that of the HG group ($P = 0.015$).

Effect of dioscin on DC cytokine secretion. The effects of dioscin on the cytokine secretion of IL-6, IL-10 and IL-12 are shown in Fig. 3F-H. Compared with the levels of the control group, the levels of IL-6 and IL-12 were markedly increased in the HG group, whereas HG significantly reduced the IL-10 level ($P < 0.05$). Furthermore, when compared with the levels of the HG group, the levels of IL-6 and IL-12 were significantly reduced, and the IL-10 level was significantly elevated in the HG + dioscin group ($P < 0.05$).

Effect of dioscin on the oxLDL uptake by DCs. Compared with the uptake in the control group, the oxLDL uptake by DCs was markedly increased in the HG group ($P < 0.05$). However, when compared with the uptake in the HG group, the oxLDL uptake by DCs was significantly reduced in the HG + dioscin group ($P < 0.05$), as shown in Fig. 4.

Discussion

Type 2 diabetes has become a major public health problem and may lead to a number of severe complications, including: Diabetic nephropathy; diabetic eye complications; diabetic foot; diabetic cardiovascular complications; diabetic cerebrovascular disease; and diabetic neuropathy (1,2). The major cause of type 2 diabetes is the disturbance of carbohydrate metabolism and insulin resistance (3). Current drug therapies for diabetes include sulfonylurea, biguanide, euglycemic agent, α -glucosidase inhibitors, food glucose regulators and dipeptidyl peptidase-4 inhibitors (33). However, these treatments have various side effects, including weight gain, hypoglycemia and drug resistance, with high rates of secondary failure (33). Therefore, it is necessary to discover new antidiabetic drugs with higher curative effects, but fewer side effects. Recently, a variety of natural compounds have been shown to have effects on type 2 diabetes through various mechanisms, such

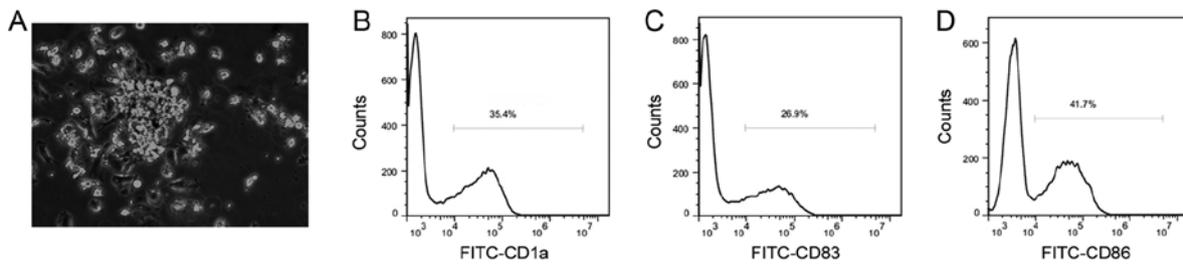


Figure 1. Characteristics of mature DCs. (A) Images of mature DCs with typical treelike shape (magnification, x200). Expression of (B) CD1a, (C) CD83 and (D) CD86. DCs, dendritic cells.

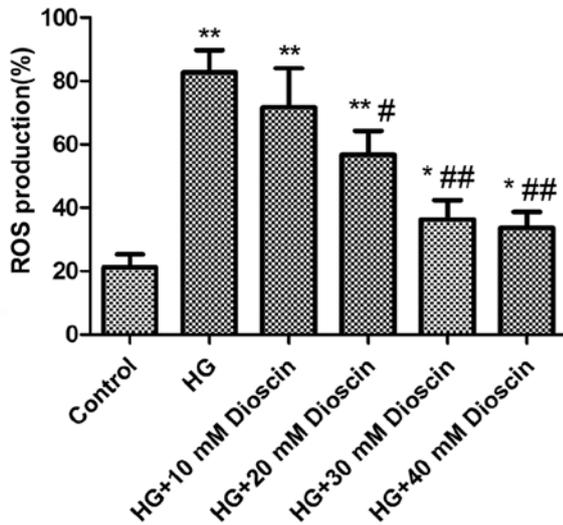


Figure 2. Effect of dioscin on ROS production by DCs. HG induced the formation of ROS in DCs, while 20-40 mM dioscin inhibited ROS production in HG conditions. * $P < 0.05$, ** $P < 0.01$ vs. control group; # $P < 0.05$, ## $P < 0.01$ vs. HG group. ROS, reactive oxygen species; DCs, dendritic cells; HG, high glucose.

as flavonoids, polyphenols, terpenoids, alkaloids, saponins and quinones (34).

Dioscin is a natural product that has been shown to have beneficial effects on the inhibition of ischemic stroke-induced inflammation and protective effects against doxorubicin-induced cardiotoxicity. It has also been shown to inhibit colon cancer cell growth through the inhibition of inflammation, oxidative stress and apoptosis in previous studies (24,35-37). A previous study showed that the total saponins from *Dioscorea nipponica* Makino, including dioscin, could significantly reduce blood glucose levels, improve the levels of oral glucose and insulin tolerance, and increase body weight and serum insulin levels via the regulation of MAPKs, NF- κ B, peroxisome proliferator-activated receptor γ and glycogen synthase kinase-3 β pathways (38). Wei *et al* (39) reported that dioscin may prevent myocardial injury in diabetic rats by upregulating the nitric oxide-soluble guanylyl cyclase-cGMP-protein kinase G pathway. Dioscin also attenuated high-fat diet-induced insulin resistance of adipose tissue by regulating the insulin receptor substrate-1/PI3K/Akt signaling pathway in an animal model (40). DCs are antigen-presenting cells that can activate T cells and stimulate vascular inflammation in the process of atherosclerosis, thus playing an important

role in its development (41-43). In the present study it was hypothesized that dioscin may have some effects on DCs.

Only mature DCs can play a role in the immune reaction. oxLDL can induce the maturation of DCs (8,11-12). In the current study, it was found that under HG, the expression of scavenger receptors, including SR-A, CD36 and LOX-1, was upregulated, as well as oxLDL uptake. Scavenger receptor upregulation and oxLDL uptake are considered critical steps in the initiation and progression of atherosclerosis in diabetes patients (8,11). Dioscin could decrease the expression of the three scavenger receptors and oxLDL uptake, thereby preventing DC maturation and inhibiting atherosclerosis in patients with diabetes in the early stage of its development.

Dioscin showed anti-liver fibrosis effects via Sirt1/nuclear related factor 2-mediated inhibition of the p38 MAPK pathway (44). Under HG conditions, the production of intracellular ROS was increased and the p38 MAPK pathway was activated, upregulating the expression of scavenger receptors and subsequently activating the NF- κ B pathway (45). Glucose-induced oxidative stress led to the activation of p38 MAPK in macrophages and DCs (8,46), which can lead to oxLDL uptake, and oxLDL-presenting DCs can activate naive T-cells to induce the inflammatory cascade and promote the formation of atherosclerotic plaques (8,11). In the current study, it was observed that dioscin partially reversed p38 MAPK protein upregulation in HG conditions. However, only one protein was observed and it was not at mRNA level, which is a limitation of this work.

Hu *et al* (15) reported that dioscin reduced ROS levels caused by hypoxia-reoxygenation injury in IEC-6 cells and showed a protective effect against II/R injury by modulating the miR-351-5/Sirt6 signal to reduce oxidative stress. Qiao *et al* (47) reported that dioscin reduced ROS levels in renal tissue and showed protective effects against fructose-induced renal damage by modulating Sirt3-mediated oxidative stress, renal fibrosis, lipid metabolism and inflammation. Dioscin significantly attenuated NRK-52E cell injury and reduced ROS levels *in vitro* (48). In the present study, it was found that dioscin could reduce ROS generation in DCs under HG conditions. Moreover, there was a significant dose-effect relationship in the inhibition of ROS generation. However, in the study by Lv *et al* (49), dioscin showed noteworthy anticancer activity in glioblastoma cells by promoting ROS accumulation, inducing DNA damage and activating mitochondrial signaling pathways, which seems contrary to the results of the present study. In human gastric carcinoma MGC-803 cells, dioscin caused DNA damage and mitochondrial changes, and

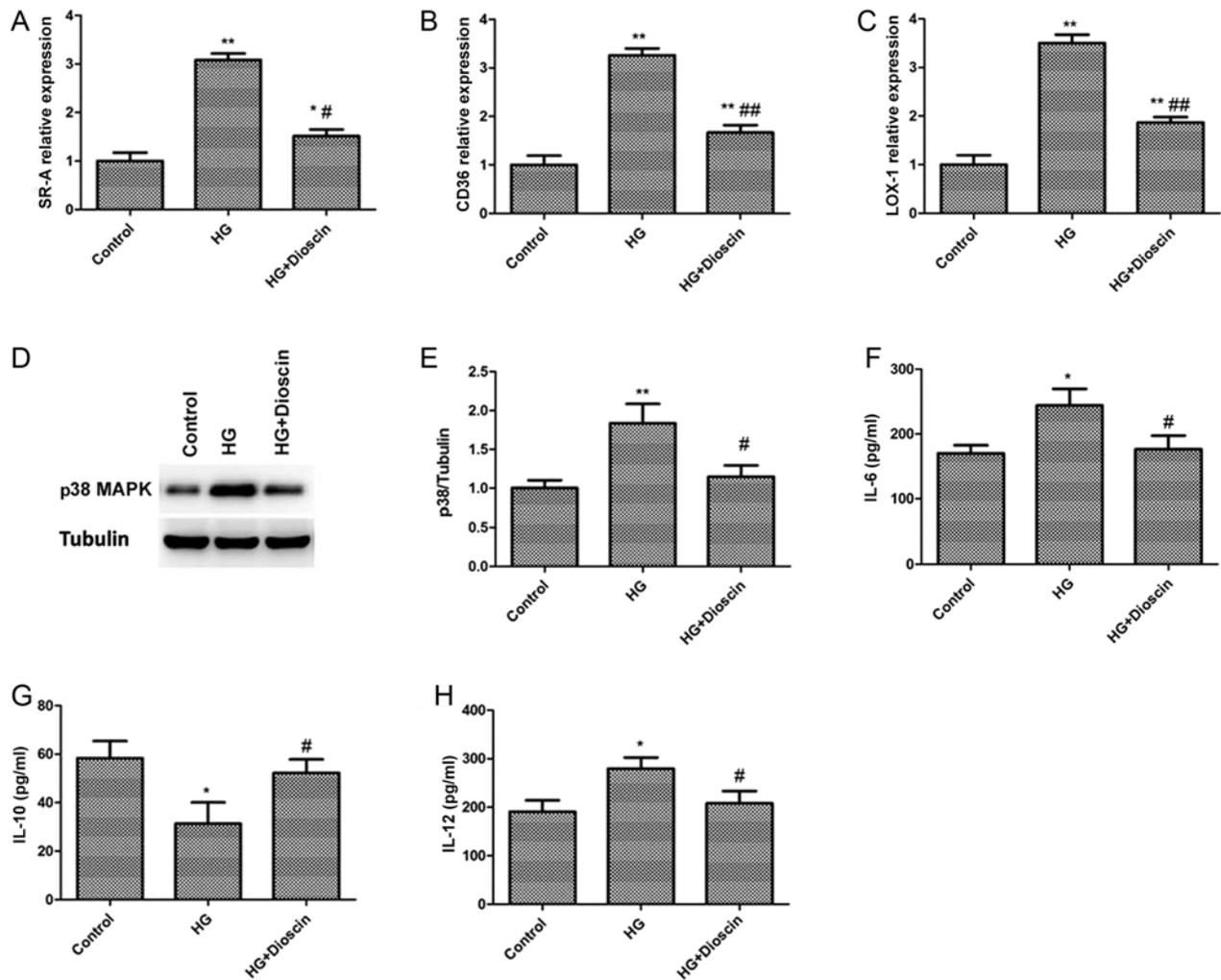


Figure 3. Effect of dioscin on expression of molecules. Bar graphs showing that the upregulation of (A) class A scavenger receptors, (B) CD36 and (C) lectin-like oxidized low-density lipoprotein receptor-1 genes was partially abolished by dioscin. (D) Western blot and (E) bar graph showing that dioscin partially reversed p38 MAPK protein upregulation. Bar graph showing that HG-induced (F) increased secretion of IL-6, (G) decreased secretion of IL-10 and (H) increased secretion of IL-12 in DCs was reversed by dioscin. *P<0.05, **P<0.01 vs. control group; #P<0.05, ##P<0.01 vs. HG group. HG, high glucose; MAPK, mitogen-activated protein kinase; DCs, dendritic cells; IL, interleukin.

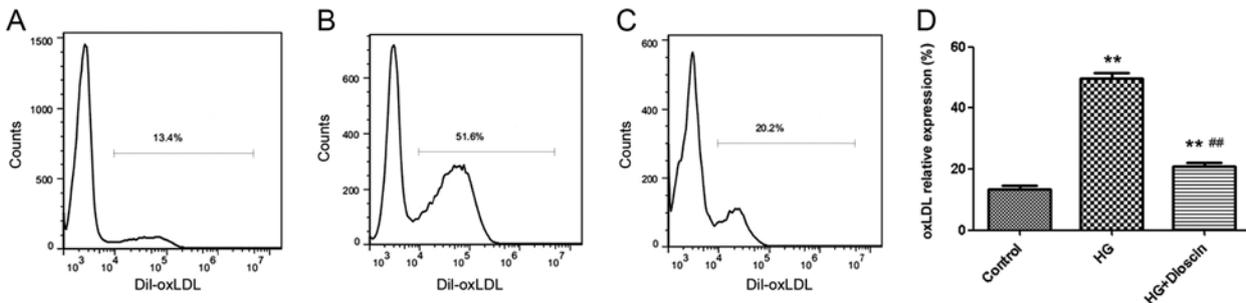


Figure 4. Effect of dioscin on expression of oxLDL. (A) Expression of oxLDL in control group. (B) oxLDL uptake by DCs was markedly increased in the HG group. (C) oxLDL uptake by DCs was significantly reduced in the HG + dioscin group. (D) Statistical analysis of oxLDL uptake by DCs in the three groups. **P<0.01 vs. control group; ##P<0.01 vs. HG group. oxLDL, oxidized low-density lipoprotein; DCs, dendritic cells; HG, high glucose; DiI, diIolistic.

induced ROS generation (50). The reason for the divergence in the effect of dioscin on ROS is not known. In the present study it was hypothesized that the effect of dioscin on ROS generation depends on the cell types. For tumor cells, dioscin promotes ROS accumulation to show anticancer effects; however, for other cell types, dioscin inhibits ROS generation

to show protective effects, and the mechanism needs further investigation.

In the presence of HG, the production of proinflammatory cytokines, such as IL-6 and IL-12, was increased in human DCs and then induced DC maturation. In the early stage of type 2 diabetes mellitus, the IL-6 level was evaluated, and it

was shown to have a critical role in the development of atherosclerosis (51). In T helper cell differentiation within the plaque and T cell recruitment into the plaque, IL-12 appears to play important roles (52). In the current study, it was also discovered that dioscin could reverse the effects of HG-induced increases in the production of IL-6 and IL-12 by DCs. Considering the role of IL-6 in the formation of atherosclerotic lesions and IL-12 in plaque destabilization, these findings suggested that dioscin potentially inhibits atherosclerosis by weakening DC maturation and immunity. IL-10 is an anti-inflammatory cytokine that can downregulate the inflammatory response and antagonize inflammatory mediators in various inflammatory diseases, including atherosclerosis (8,53), which suggests that dioscin potentially inhibits atherosclerosis by promoting the production of IL-10 in DCs. In addition, the ROS production and expression levels of the scavenger receptors SR-A, CD36 and LOX-1 in HG + Dioscin + HG group were similar to those of cells only treated with 30 mM HG in HG group. This suggested that the reported effects are reversed when dioscin is removed from cell culture.

In conclusion, this study demonstrated that dioscin could attenuate the production of ROS, inflammatory cytokine secretion and oxLDL uptake by DCs. This is by preventing the expression of scavenger receptors and p38 MAPK, thus having a positive role in preventing atherogenesis.

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Availability of data and materials

The datasets used and/or analyzed during the present study are available from the corresponding author upon reasonable request.

Authors' contributions

YiL, YoL and TY performed the experiments; MW designed the study and reviewed the manuscript; YiL analyzed and interpreted the data; MW acquired the data and drafted and edited the manuscript.

Ethics approval and consent to participate

Healthy volunteers provided informed consent, and the present study was approved by the Ethics Committee of the Chongqing Traditional Chinese Medicine Hospital.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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