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Puerarin inhibits hyperglycemia-induced inter-endothelial junction through suppressing endothelial Nlrp3 inflammasome activation via ROS-dependent oxidative pathway

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Declarations of interest
None

Abstract

\textit{Background:} Recent studies indicate that vascular complications are closely related to diabetes mellitus; in particular, inflammatory-mediated endothelial dysfunction plays a crucial role in diabetes-induced cardiovascular diseases. Therefore, exploring effective methods to suppress endothelial dysfunction via inhibition of inflammatory responses is imperative. Puerarin (Pu), a flavonoid common in \textit{Pueraria}, has been widely and successfully used to treat cardiovascular diseases in China for many years. However, information on its protective properties in hyperglycemia-induced vascular complications is insufficient.

\textit{Hypothesis/Purpose:} In this study, we investigate the protective effects of puerarin against high glucose-induced endothelial dysfunction and the underlying mechanism of the flavonoid.
Methods: we investigated the protective effects of Pu against hyperglycemia-induced inter-endothelial junction by permeability and transendothelial electrical resistance (TEER) assay. In addition, changes in the Nlrp3 inflammasome activation via reactive oxygen species (ROS)-dependent oxidative pathway were investigated using western blot, immunofluorescence microscopy analyses and flow cytometry. ROS scavenger and Nlrp3 gene silencing were used to determine the roles of the ROS-Nlrp3 pathway involved in the molecular mechanism of Pu.

Results: Our findings demonstrate that puerarin inhibits high glucose-induced Nlrp3 inflammasome formation and activation, as shown by fluorescence confocal microscopy and Western blot. Puerarin decreases Nlrp3 protein, which is a critical factor necessary to form an inflammasome complex. We demonstrate that puerarin exerts anti-oxidation and ROS scavenged effects, similar to apocynin (APO). Interestingly, thioredoxin-interacting protein (TXNIP) protein and TXNIP binding to Nlrp3 markedly decreased with puerarin treatment. Together with these changes, puerarin could decrease high mobility group box 1 (HMGB1) release from mouse vascular endothelial cell (mMVECs). We also demonstrate the decreased expression of the tight junction proteins ZO-1/ZO-2, which are related to endothelial permeability after stimulation by high glucose in endothelial cells. Puerarin could recover the gap junction protein and decrease monolayer cell permeability in endothelial cells. In conclusion, we reveal a new protection mechanism of puerarin that inhibits Nlrp3 inflammasome activation and decreases subsequent caspase-1 activation, triggering the release of HMGB1 by reducing ROS generation.

Conclusions: Our findings indicate that puerarin exhibits immense potential and specific therapeutic value in hyperglycemia-related cardiovascular disease and the development of innovative drugs.

Key word: Nlrp3 inflammasome, Hyperglycemia, Endothelium, Tight junction proteins, ROS

Abbreviations: Pu, puerarin; TEER, transendothelial electrical resistance; ROS, reactive oxygen species; APO, apocynin; TXNIP, thioredoxin-interacting protein; HMGB1, high mobility group box 1; mMVECs, mouse vascular endothelial cell

Introduction

Vascular complications are the primary cause of death and disability in patients with diabetes mellitus (Mozaffarian, 2016; Teliti et al., 2018), one of the most common diseases and major public health problems in the world. Endothelial dysfunction is defined as the early onset of various hyperglycemia-associated vascular diseases, such as atherosclerosis, which triggers vascular inflammation and the consequent formation of atherosclerotic lesions. Diabetes has been postulated to have a close relationship with cardiovascular disease (Tabit et al., 2010), and low levels of blood sugar are critical to prevent cardiovascular disorders (Luan, 2009). In particular, the
progress of hyperglycemia-induced vascular endothelium inflammation results in endothelial barrier dysfunction, which eventually leads to diabetes-associated vasculopathy (van den Oever et al., 2010). Despite this knowledge, however, the detailed molecular mechanism accounting for the effect of hyperglycemia-induced endothelial dysfunction has not been completely understood. Previous in vitro and in vivo studies have confirmed that hyperglycemia can abnormally activate Nlrp3 inflammasomes and induce endothelial injury (Chen et al., 2016). Therefore, suppressing Nlrp3 inflammasomes could be a new approach in reducing hyperglycemic toxicity and preventing the onset of vascular complications.

Nlrp3 inflammasomes belong to the family of nucleotide-binding and oligomerization domain-like receptors and have been extensively studied and shown to play a critical role in hyperglycemia-associated vascular disease (Chen et al., 2015a; Chen et al., 2015b; Chen et al., 2016; Esser et al., 2014; Zhang et al., 2015). Nlrp3 inflammasome monomers form a high-molecular-weight inflammasome complex containing Nlrp3, Apoptosis-associated speck-like protein containing a CARD (ASC), and cysteine-aspartic proteases 1 (caspase 1) (Zhou et al., 2011). As a pattern recognition receptor, Nlrp3 is upregulated when it is stimulated by excessive production of the reactive oxygen species (ROS)-induced TXNIP and recruited by ASC (Liu et al., 2014). Pro-caspase-1 is then converted to its active form (cle-caspase-1) and subsequently activates its substrates, such as high mobility group box-1 protein (HMGB1) (Lu et al., 2012), leading to tight junction disruption and consequent endothelial permeability (Huang et al., 2012). Oxidative and ER stresses as responses to the stimulus of ROS are crucial to Nlrp3 inflammasome activation (Martinon, 2010), causing premature senescence and cellular dysfunction of endothelial cells (Hayashi et al., 2014). In the present study, we investigate whether ROS-dependent oxidative stress plays is a key promoter in the process of hyperglycemia-induced Nlrp3 inflammasome activation and endothelial barrier dysfunction in ECs.

_Pueraria lobata_ (Willd.) Ohwi, one of the major traditional Chinese herbal medicines, has been widely used in China for hundreds of years to treat hyperglycemia and its vascular complications (Wu et al., 2013), such as diabetes, angina pectoris, and arteriosclerosis (Woo et al., 2013). Previous research indicates that puerarin (daidzein-8-C-glucoside-7,4′-dihydroxy-8-C glucosylisoflavone), the major component of _P. lobata_ (Willd.) Ohwi, has a specific hypoglycemic effect and a wide range of anti-oxidant and anti-inflammatory activities (Hsu et al., 2003; Wang et al., 2016). The flavonoid exerts its effects mainly by protecting neuronal cells from ROS-induced retinal damage (Zhang et al., 2008). However, the protective effect of puerarin against endothelial dysfunction in
the pathogenesis of hyperglycemia-induced vascular complications and the underlying mechanism remains largely unknown. This knowledge gap will be addressed in the present research.

**Methods**

1. **Cell culture and treatment**

The mMVEC line was purchased from ATCC (CRL-2586). Cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (Gibco, USA) with 1% penicillin-streptomycin (Gibco, USA) and 10% fetal bovine serum (Gibco, USA) under a humid atmosphere of 5% CO₂ at 37 °C. Cells were passaged by trypsinization (Trypsin/EDTA; Sigma, USA) when they had spread to more than 80% of the bottom of the culture dish. Cells were then divided into six groups: control (0.1% DMSO), model (0.1% DMSO + HG), and Pu 1, 10, 25 and 50 (5, 10 and 20 μM Pu + HG, respectively). In the Pu groups, mMVEC cells were co-cultured with high glucose (HG, 30 mM) in a cell incubator and treated with different concentrations of Pu for 24 h. Where inhibitors were used, the cells were treated with APO (100 μM).

2. **Immunofluorescence microscopy analysis**

The mMVECs were grown on glass coverslips fixed with 4% buffered paraformaldehyde. The cells were then stained using goat anti-ZO-1 (1:200, Invitrogen, CA, USA), goat anti-ZO-2 (1:200, Invitrogen), rabbit anti-Nlrp3 (1:200; Abcam, Cambridge, UK), mouse anti-caspase-1 (1:200; Santa, MA, USA), rabbit anti-ASC (1:100, Santa), and rabbit anti-TXNIP (1:200; Santa). After incubation with primary antibodies, the samples were washed and labeled with the corresponding Alexa Fluor-488 and Alexa Fluor-555 conjugated secondary antibodies (Invitrogen,). Fluorescence was visualized with a Zeiss LSM800 microscope. Co-localization was analyzed by Image Pro Plus software, and the co-localization coefficient was calculated using Pearson’s correlation coefficient as previously described.

3. **Western blot analysis**

Total protein was extracted using RIPA buffer (Thermo, USA). The supernatant was centrifuged at after 10,000 g for 15 min at 4 °C and protein concentration was measured with a BCA Protein Assay Kit (Beyotime, China). Cell homogenates were denatured with reducing Laemmli SDS-sample buffer and boiled in a metal bath for 5 min at 95 °C. Equal amounts of the protein samples were separated by 12% SDS-PAGE and transferred onto a PVDF membrane. The membrane was incubated with primary antibodies at 4°C overnight and then treated with anti-rabbit IgG (1:2,000; CST, MA, USA) or anti-mouse IgG (1:2,000; CST) for 2 h at room temperature. The primary antibodies were anti-NLRP3 (1:1,000; CST), anti-Caspase-1 (8:5,000; Santa), anti-ASC (8:5,000; Santa),
anti-TXNIP (1:1,000; Santa), and anti-HMGB1 (1:500; Santa). Anti-β-actin (1:1,000; BOSTER, Beijing, China) was used as an internal control. The target bands were detected and analyzed using Image J software (NIH, USA).

4. Reactive oxygen species assay

The mMVECs were treated as described above. Intracellular ROS levels were measured by DCFH-DA, and mitochondrial superoxide generation was measured by MitoSOX Red (Invitrogen). Briefly, the cells were washed three times with PBS, incubated with DCFH-DA (1:1,000) for 30 min, and then washed three times with ice-cold DMEM solution to remove the remaining fluorescence dye. The fluorescence intensity of different parts of the cell was measured by fluorescence microscopy with excitation and emission wavelengths of 488 and 525 nm, respectively. Cells were also incubated with MitoSOX red (5.0 μM) for 15 min as described, digested using 0.25% trypsin, and then washed three times with PBS supplemented with 1% BSA to remove the remaining fluorescence dye. Measurements were performed using flow cytometry (BD Biosciences, USA) with excitation and emission wavelengths of 488 and 585 nm, respectively (Mukhopadhyay et al., 2007; Rajesh et al., 2007). Levels of catalase (CAT), glutathione (GSH), superoxide dismutase (SOD), and malondialdehyde (MDA) in other parts of the cells were measured using kits (Jiancheng Company, China).

5. Plasmid construction and nucleofection

The gRNA sequences of the CRISPR/Cas9 gene of Nlrp3 were designed by the CRISPR Design tool (http://crispr.mit.edu/). Then, gRNA sequences (Nlrp3 gRNA sequence: 5’-GACGAGTGTCCGTTGCAAGC-3’) were synthesized and inserted into the BbsI-digested px459 plasmid. Gene editing in cells was performed by Lipofectamine 3000 transfection according to the manufacturer’s instructions (Invitrogen). Briefly, a master mix of plasmids was prepared by dilution in Opti-MEM™ Medium (Gibco, USA) and then addition of P3000™ and Lipofectamine™ 3000 reagent. Incubation for 15 min at room temperature was subsequently performed. The transfected cells were incubated in the medium with 2.5μg/ml puromycin to screen out gRNA plasmid-containing cells, and transfection efficiency was analyzed by Western blot.

6. Permeability assay

The mMVECs were cultured and subjected to transendothelial electrical resistance (TEER) assay as described below. Next, 600 μl of fresh media was added to the lower well and 100 μl of culture medium containing 40 kDa fluorescein isothiocyanate (FITC)-dextran (Sigma, 1 mg/ml) was added to the upper well of each insert. The plate was incubated for 30 min at 37 °C, and the samples were collected from the upper and lower chambers for
measurement by a fluorescent microplate reader (Molecular Devices, USA). Permeability was calculated as follows: 
Permeability = FL/FU, where FL is the fluorescence of the lower chamber and FU is the fluorescence of the upper chamber.

7. Transendothelial electrical resistance (TEER) assay

The mMVECs were plated on the upper portion of 24-well transwell chambers (0.4 μm pore size, 0.3 cm² effective growth area, Corning) at a density of 1.0 × 10⁴ cells/well. After attaching with confluence to the bottom of the insert (48 h), the cells were treated without or with Pu and HG as indicated. The TEER of the cell layers was measured using a Millicell electrical resistance apparatus (Millipore, USA), and readings were recorded immediately. TEER values are calculated as: TEER = resistance × filter area (Ω cm²).

8. Statistical analysis

Results are expressed as mean ± SEM and analyzed using SPSS 21.0. The LSD or Dunnett’s test was used for comparison of multiple groups based on homogeneity of variance. P < 0.05 or P < 0.01 was considered to indicate statistical significance.

Result

1. Puerarin inhibits HG-induced Nlrp3 inflammasome formation and activation

Nlrp3 inflammasomes have been demonstrated to be the principal initiating mechanism triggering endothelial dysfunction (Esser et al., 2014). Therefore, this study determined the effects of puerarin on hyperglycemia-induced Nlrp3 inflammasome formation in mMVECs. First, we analyzed inflammasome formation in mMVECs by confocal microscopy. Co-localization of Nlrp3 (green) and ASC (red), as shown by yellow staining, decreased in puerarin treatment groups compared with that in the model group (P < 0.01). Similar results were observed in the co-localization coefficient of HG-induced Nlrp3 (green) and Caspase-1 (red) (P < 0.01), which indicates that puerarin could inhibit the aggregation and formation of the inflammasomes. Next, we detected whether puerarin could block activation of Nlrp3 inflammasomes in response to HG. Increased expression of active caspase-1 stimulated by HG indicated increased cleavage of pro-caspase-1 into bioactive caspase-1 (P < 0.01; Fig. 1). Thus, puerarin could inhibit the formation and activation of Nlrp3 inflammasomes in HG-stimulated mMVECs.

2. Puerarin down-regulates the protein expression of Nlrp3 inflammasome components

To confirm that HG-induced the expression levels of Nlrp3 and ASC after puerarin treatment, which indicates that puerarin could reduce the expression of Nlrp3 inflammasome molecules (P < 0.01; Fig.2). This finding reveals
that puerarin down-regulates Nlrp3 and ASC expression. Our data thus far suggest that puerarin could abrogate the activation of Nlrp3 inflammasomes in response to HG stimuli in mMVECs.

3. **Puerarin inhibits HG-induced Nlrp3 inflammasomes by a suppressing oxidative stress**

   The effects of puerarin on HG-induced ROS production are shown in Fig. 3. The results of microscopic and FACS analyses illustrate that intracellular ROS production and mitochondrial superoxide were obviously elevated in mMVECs stimulated by HG (P < 0.01). After treatment, intracellular ROS production and mitochondrial superoxide were significantly decreased in the puerarin (10, 25, and 50 μM) groups compared with those in the model group (P < 0.01), which indicates that puerarin could attenuate intracellular ROS and mitochondrial superoxide accumulation. Prior treatment with puerarin or APO also significantly decreased intracellular ROS and mitochondrial superoxide accumulation compared with those in the HG group (P < 0.01). Prior treatment with puerarin or APO also significantly attenuated the expression of Nlrp3 protein (P < 0.01; Fig. 3). These results reveal that puerarin down-regulates Nlrp3 expression through antioxidant pathways. Assays on several oxidative indices showed significant alterations, including increases in MDA and decreases in GSH and CAT in the HG group (Supplementary Material, Fig. S1). MDA content decreased and GSH and CAT levels increased in the puerarin (10, 25, and 50 μM) group compared with those in the model group (P < 0.01, P < 0.05). SOD activities did not obviously change.

4. **Puerarin attenuates HG-induced TXNIP-NLRP3 binding**

   ROS is an agonist of Nlrp3 inflammasome and promotes the assembly of the Nlrp3 complex via the ROS-sensitive TXNIP protein (Zhou et al., 2010). HG stimulation significantly increased TXNIP expression and co-localization of Nlrp3 (green) and TXNIP (red). Treatment with Pu or APO significantly attenuated HG-induced TXNIP expression; co-localization of Nlrp3 and TXNIP also decreased in the Pu treatment groups (P < 0.01; Fig. 4). Our data reveal that Pu could block the recruitment of TXNIP to the Nlrp3 inflammasome fractions, and the related mechanism appears to involve reduction of intracellular ROS production.

5. **Puerarin inhibits Nlrp3 inflammasome mediated HMGB1 release by HG**

   HMGB1 release is a downstream product of Nlrp3 inflammasome activation and destroys the permeability of the endothelium. HG-induced HMGB1 release increased and treatment with puerarin (10, 25, and 50 μM) significantly decreased HMGB1 in the cell medium (P < 0.01). Prior treatment with puerarin or APO significantly suppressed HMGB1 release into the cell medium compared with that in the HG group (P < 0.01; Fig. 5).

6. **Puerarin recovers HG-induced disruption of tight junction proteins**
Recent studies demonstrate that the function, including the tight junction, of mMVECs is injured by HG stimulation (Chen et al., 2016). We examined whether puerarin or APO could prevent the disassembly of junction proteins in mMVECs by inhibiting Nlrp3 inflammasome activation. HG stimulation markedly decreased the fluorescence intensity across cell–cell contacts of ZO-1 and ZO-2 at cell junctions. Treatment with puerarin (10, 25, and 50 μM) significantly recovered cellular tight junction. Prior treatment with puerarin or APO could also prevent HG-induced disruption of cell–cell contacts. Finally, to directly monitor the protective effect of puerarin in HG-induced cell junction dysfunction related to Nlrp3 inflammasome activation, Nlrp3 shRNA plasmid transfection was applied. mMVECs transfected with Nlrp3 shRNA plasmids showed inhibited HG-induced disruption of cell junctions. After treatment with puerarin, faint alterations of cell–cell contact in HG-induced disrupted cells, as shown in Fig. 6. Thus, these results indicate that puerarin could maintain cellular tight junction protein and relate to suppressing ROS level and deactivating NLRP3 inflammasome activation.

7. Puerarin recovers HG-induced permeability increase of the endothelial cell monolayers

Tight junction proteins control the passage of macromolecules in the endothelium (Brown et al., 2007). We demonstrated that HG induces the dysfunction of endothelial cells, which showed obviously increased permeability compared with that of control group (P < 0.01). After treatment, the penetration of cells was significantly recovered in the puerarin (10, 25, and 50 μM) groups compared with that in the model group (P < 0.01). Prior treatment with puerarin or APO also prevented increases in permeability in mMVECs compared with that in the HG group (P < 0.01). mMVECs transfected with Nlrp3 shRNA plasmids showed inhibited increases in permeability, but no obvious differences between groups treated with or without puerarin were found. Hence, we assume that puerarin exerts protective effects against the HG-induced dysfunction of mMVECs and could be crucial targets for suppressing ROS levels and NLRP3 inflammasome activation.

Discussion

Our studies have demonstrated that hyperglycemia induces NLRP3 inflammasome activation and endothelium dysfunction (Chen et al., 2016). Pu exerts protective effects against inflammation in vascular diseases(Woo et al., 2013). However, the cellular mechanism of puerarin in modulating endotheliocyte functions remains largely unknown. This study aimed to determine whether puerarin exhibits protection against hyperglycemia-induced endothelium dysfunction through its antioxidant effects, which are related to the ROS-TXNIP-Nlrp3 pathway. Our data reveal that puerarin inhibits hyperglycemia-induced Nlrp3 inflammasome activation, which is associated with
ROS production and results in up-regulation of tight and adherent junction proteins through HMGB1 inhibition the endothelial cells.

Hyperglycemia could cause a non-classic inflammation response in the vascular endothelium (Lontchi-Yimagou et al., 2013) and contributes to the inflammation response through the aggregation of intracellular ROS (Newsholme et al., 2007), which plays a critical role in Nlrp3 inflammasome activation (Martinon, 2010). Hyperglycemia could lead to oxidative stress in mitochondria (Rajesh et al., 2007), and four known pathways could be activated in turn, including the polyol, hexosamine, protein kinase C, and advanced glycation end-products pathways (Evans et al., 2002). This findings suggests a cause–effect relationship between oxidative stress and Nlrp3 inflammasome formation and activation. As a traditional Chinese medicine, *P. lobata* (Willd.) Ohwi is a popular herb that has been widely and successfully used for many years. Puerarin (daidzein-8-C-glucoside) is a major isoflavonoid derived from *P. lobata*. It is reported to possess many biological activities, including cardio-protection, neuroprotection, antioxidant, and anti-inflammation (Woo et al., 2013; Wu et al., 2013). Nevertheless, evidence to support its protective properties on hyperglycemia-induced vascular complications is lacking. Therefore, the present study aimed to explore whether puerarin is involved in inhibiting hyperglycemia-induced endothelial Nlrp3 inflammasome activation and endothelial dysfunction.

As a large intracellular signaling platform, Nlrp3 inflammasomes function in the innate immune response, including activation of caspase-1 and subsequent maturation and secretion of biologically active HMGB1 (Willingham et al., 2009). Our previous study confirmed that HG could significantly induce Nlrp3 inflammasome activation in endothelial cells, thus indicating that Nlrp3 inflammasome is an important mediator in the process of hyperglycemic toxicity in cardiovascular diseases (Chen et al., 2016). The results of this study show that pre-treatment with puerarin could dose-dependently attenuate hyperglycemia-induced up-regulation of Nlrp3 inflammasome formation and expression Nlrp3 protein, which, in turn, suggests that puerarin may exert its action against endothelial dysfunction by inhibiting the hyperglycemia-induced Nlrp3 inflammasome pathway (Figs. 1 and 2). Our findings reveal that the endothelial protective effect of puerarin is closely related to Nlrp3 inflammasomes.

To further explore the mechanism of the flavonoid, we focused on how puerarin attenuates Nlrp3 inflammasome activation. At least three mutually non-exclusive mechanisms are known to be involved in the activation of Nlrp3 inflammasomes, including the K⁺ efflux, generation of ROS, and lysosome membrane permeabilization pathways (Jin and Flavell, 2010). Puerarin has also been shown to exert a critical antioxidative
effect (Guerra et al., 2000). In the present study, we confirmed that puerarin confers anti-oxidation effects (Figs. 3A–3D). ROS are chemically reactive species containing oxygen, including peroxides, superoxides, and hydroxyl radical, among others. ROS production plays a significant role in the pathogenesis of diabetes-associated endothelial dysfunction, and mitochondrial generation of superoxide appears to play the most crucial role in diabetic complications (Eletr and Keith, 1972; Rajesh et al., 2007). Decomposition of ROS by several antioxidant enzymes, including SOD, CAT, and GSH, which catalyze the dismutation of superoxide into water and oxygen, is necessary to maintain the balance of oxidative stress. We found that puerarin could recover CAT and GSH activity and reduce MDA production; however, its influence in SOD activity is limited (Figs. S1A-D). Further experimentation showed that puerarin’s ability to inhibit ROS generation is closely related to its effects on mitochondrial superoxide generation (Figs. 3E–3F). Other native compounds, such as cannabidiol (Rajesh et al., 2007), also provide this pharmacological effect, so we speculate that puerarin restores CAT and GSH function, which converts hydrogen peroxide (H$_2$O$_2$) into water and oxygen. Previous studies have suggested that H$_2$O$_2$ activates the NF-κB pathway in endothelial cells (Csiszar et al., 2006a; Csiszar et al., 2006b), and our data show that hyperglycemia-induced ROS generation may increase Nlrp3 expression through activation of NF-κB, which is decreased by puerarin or APO (Figs. 3G–3H). The assembly of Nlrp3 inflammasome complex is associated with the release of TXNIP from oxidized TRX and the binding of TXNIP to NLRP3 thought intracellular ROS accumulation, resulting in activation of caspase-1 and subsequent HMGB1 release (Liu et al., 2014). We demonstrated in this work that puerarin also regulates hyperglycemia-induced assembly of Nlrp3 inflammasome by regulating TXNIP expression and binding (Fig. 4). Our findings suggest that ROS-TXNIP is critical for Nlrp3 inflammasome activation in endothelial cells during HG stimulation and may trigger and promote endothelial cell dysfunction. Thus, the effects of puerarin may be associated with suppression of Nlrp3 inflammasome assembly and activation. Pharmacologic intervention with puerarin confirmed that diminished NLRP3 inflammasome activation and post-transcriptional modification of Nlrp3 are attributable to reductions in ROS and inhibition of TXNIP expression.

Our previous study has shown that activation of Nlrp3 inflammasomes by injurious factors results in endothelial tight junction disruption and consequent endothelial permeability, which is dependent on the increased release of HMGB1 in cultured mMVECs (Chen et al., 2016). In the present work, our data confirmed that puerarin decreases HMGB1 release from endothelial cells (Fig. 5). Changes in HMGB1 were consistent with recent findings that Nlrp3 inflammasome activation could lead to translocation and secretion of HMGB1 by immune cells (Lotze
Other studies also demonstrate that extracellular HMGB1 enhances the cellular permeability of the endothelial monolayer via its receptor for the advanced glycation end-product-mediated pathway (Huang et al., 2012). Thus, the expression of tight junction proteins ZO-1/2 could be considered a marker of the cellular permeability of the endothelium. In this research, we demonstrated the decreased expression of ZO-1/ZO-2, which are related to endothelial permeability, after stimulation by HG in endothelial cells; by contrast, puerarin could recover the gap junction protein in endothelial cells (Figs. 6 and 7). We demonstrated that the inhibition of Nlrp3 inflammasome activation and protection of endothelial cell function afforded by puerarin are related to decreased ROS. To verify that puerarin exerts protective effects through inhibition of the ROS-TXNIP-Nlrp3 pathway, we treated endothelial cells with APO, which acts as a scavenger of the reaction products of H₂O₂ (Heumuller et al., 2008; Jaquet et al., 2009), as a positive control drug (Touyz, 2008). We demonstrated that puerarin inhibits hyperglycemia-induced Nlrp3 inflammasome formation and HMGB1 release, similar to the effects of APO; the function of tight junction cells in the endothelium was also protected. Our data reveal that Nlrp3 gene silencing prevents the protective effect of puerarin in hyperglycemia-induced tight junction dysfunction. The present study demonstrates that ROS scavenging and Nlrp3 gene silencing attenuate hyperglycemia-induced tight junction disruption and enhances the permeability of endothelial monolayers in mMVECs. Thus, puerarin may recovers inter-endothelial junction disruption by inhibiting ROS, which is triggered by endothelial Nlrp3 inflammasome activation and release of HMGB1.

In summary, we confirm, for the first time, that puerarin exerts protective effects on hyperglycemia-associated inter-endothelial junction disruption. The present results reveal a new protection mechanism of puerarin that inhibits Nlrp3 inflammasome activation and decreases subsequent caspase-1 activation, triggering the release of HMGB1 by reducing intracellular ROS generation. Overall, our findings indicate that puerarin exhibits immense potential and specific therapeutic value in hyperglycemia-related cardiovascular disease and the development of innovative drugs.

Conflicts of Interest
None.

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Reference


**Figure Legend**

Fig. 1. Puerarin inhibits HG-induced Nlrp3 inflammasome formation and activation. mMVECs were stimulated with HG (30mM) and treated with Pu (1, 10, 25, and 50 μM). After 24 h of incubation, the cells were fixed in paraformaldehyde for immunofluorescence assay or extracted by using RIPA buffer for Western blot assay. (A) Fluorescence images indicating the effect of Pu on the co-localization of Nlrp3 (green) with ASC (first line, red) or caspase-1 (second line, red) in HG-induced mMVECs. (B, C) Quantitative data of the co-localization efficiency (Pearson’s correlation coefficient) of Nlrp3 with ASC or caspase-1 (n= 4). (D) Western blot analysis showing the effect of Pu on HG-induced pro-caspase-1 (Pro-casp1) and cleaved caspase-1 (Cle-casp1). (E) Summary of Western blot results (n = 4). *P < 0.01 compared with the control group. **P < 0.01 compared with the model group.
Fig. 2. Puerarin down-regulates the protein expression of Nlrp3 inflammasome components. The mMVECs were stimulated with HG (30mM) and treated with Pu (1, 10, 25 and 50 μM). HG (30 mM)-induced mMVECs were also
treated with or without Pu (50 μM) in the presence of PBS (control) or a ROS inhibitor (APO, 100 μM). After 24 h of incubation, the mMVECs were extracted using RIPA buffer for Western blot assay. (A, B) Western blot analysis and summarized data showing the effect of Pu on the protein expression levels of Nlrp3 (n = 4). (C, D) Western blot analysis and summarized data showing the effect of Pu on the protein expression levels of ASC (n = 4). *P < 0.05, **P < 0.01 compared with the model group.

Fig. 3. Puerarin inhibits HG-induced Nlrp3 inflammasome activation by suppressing oxidative stress. The mMVECs were stimulated with HG (30mM) and treated with Pu (1, 10, 25 and 50 μM). HG (30 mM)-induced mMVECs were also treated with or without Pu (50 μM) in the presence of PBS (control) or a ROS inhibitor (APO, 100 μM). After 24 h of incubation, the mMVECs were stained with DCFH-DA (1:1,000) for 30 min and MitoSOX
Red (5.0 μM) for 15 min, and the fluorescence intensity of the cells was measured through microscopy and FACS. (A) Fluorescence images showing HG-induced mMVECs treated with Pu or APO and stained with DCFH-DA (green). (B, C) Quantitative data showing the effect of APO and Pu on HG-induced intracellular ROS accumulation (n = 4). (D) Flow cytometry data showing HG-induced mMVECs treated with Pu or APO and stained with MitoSOX Red. (E, F) Quantitative data showing the effect of APO and Pu on HG-induced mitochondrial superoxide formation (n = 4). (G, H) Western blot analysis and summarized data showing the effect of APO and Pu on the HG-induced protein expression levels of Nlrp3 (n = 3). *P < 0.01 compared with the control group. **P < 0.01 compared with the model group.
Fig. 4. Puerarin attenuates HG-induced TXNIP-NLRP3 binding. The mMVECs were stimulated with HG (30mM) and treated with Pu (1, 10, 25 and 50 μM). HG (30 mM)-induced mMVECs were also treated with or without Pu (50 μM) in the presence of PBS (control) or a ROS inhibitor (APO, 100 μM). After 24 h of incubation, the mMVECs were fixed in paraformaldehyde for immunofluorescence assay or protein-extracted using RIPA buffer for Western blot assay. (A) Fluorescence images indicating the effect of Pu on co-localization of Nlrp3 (green) with TXNIP (red) in HG-induced mMVECs. (B) Quantitative data of the co-localization efficiency (Pearson’s correlation coefficient) of Nlrp3 with TXNIP (n = 4). (C, D) Western blot analysis and summarized data showing the effect of Pu on the protein expression levels of TXNIP (n = 4). (E, F) Western blot analysis and summarized data showing the effect of APO and Pu on HG-induced protein expression levels of TXNIP (n = 3). *P < 0.01 compared with the control group. **P < 0.01 compared with the model group.
A

Nrp3/TXNIP

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TXNIP

β-actin

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TXNIP/β-actin

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TXNIP

β-actin

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F

TXNIP/β-actin

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Ctrl HG Pu

Con HG Pu

Con HG Pu

Ctrl HG Pu

Ctrl HG Pu

APO
Fig. 5. Puerarin inhibits Nlrp3 inflammasome mediated HMGB1 release by HG. The mMVECs were stimulated with HG (30mM) and treated with Pu (1, 10, 25 and 50 μM). HG (30 mM)-induced mMVECs were also treated with or without Pu (50 μM) in the presence of PBS (control) or a ROS inhibitor (APO, 100 μM). After 24 h of incubation, the cells and cell medium were extracted using RIPA buffer for Western blot assay. (A, C) Western blot analysis and summarized data showing the effect of Pu on HG-induced HMGB1 release (n = 4). (B, D) Western blot analysis and summarized data showing the effect of APO and Pu on HG-induced HMGB1 release (n = 3). *P < 0.01 compared with the control group. **P < 0.01 compared with the model group.

Fig. 6. Puerarin recovers the HG-induced disruption of tight junction proteins. The mMVECs or Nlrp3 shRNA plasmid-transfected mMVECs were stimulated with HG (30mM) and treated with Pu (1, 10, 25 and 50 μM) or a ROS inhibitor (APO, 100 μM). After 24 h of incubation, the mMVECs were fixed in paraformaldehyde for
immunofluorescence. (A) Representative fluorescence images showing the cell membrane fluorescence of ZO-1 (first line) and ZO-2 (second line) (n = 3). (B) Tight junctions are represented by histograms of ZO-1 (first line) and ZO-2 (second line) fluorescence intensity (RFI) as indicated by dotted lines across two cell–cell contacts. (C) Representative fluorescence images showing the effect of APO and Pu on the HG-induced cell membrane fluorescence of ZO-1 (first line) and ZO-2 (second line) (n = 3). (D) Tight junctions are represented by histograms of ZO-1 (first line) and ZO-2 (second line) fluorescence intensity (RFI) as indicated by dotted lines across two cell–cell contacts. (E) Representative fluorescence images showing the effect of treatment with or without puerarin on the HG-induced cell membrane fluorescence of ZO-1 (first line) and ZO-2 (second line) in Nlrp3 shRNA plasmid-transfected cells (n = 3). (F) Tight junctions are represented by histograms of ZO-1 (first line) and ZO-2 (second line) fluorescence intensity (RFI) as indicated by dotted lines across two cell–cell contacts.
Fig. 7. Puerarin recovers the HG-induced permeability of the endothelial cell monolayer. The mMVECs or Nlrp3 shRNA plasmid-transfected mMVECs were stimulated with HG (30mM) and treated with Pu (1, 10, 25 and 50 μM) or a ROS inhibitor (APO, 100 μM). After 24 h of incubation, the mMVECs were measured by permeability assay. (A) TEER analysis and summarized data showing the effect of Pu (n = 4). (B) Permeability analysis and summarized data showing the effect of Pu (n = 4). (C) TEER analysis and summarized data showing the effect of APO and Pu (n = 4). (D) Permeability analysis and summarized data showing the effect of APO and Pu (n = 4). (E) TEER analysis and summarized data showing the effect of treatment with or without puerarin on transfected cells (n = 4). (F) Permeability analysis and summarized data showing the effect of treatment with or without puerarin on Nlrp3 shRNA plasmid-transfected cells (n = 4).  *P < 0.01 compared with the control group.  **P < 0.01 compared with the model group.
A

B

C

D

E

F

TEER values (Ohm x cm²)

Permeability (% of Ctrl)

TEER values (Ohm x cm²)

Permeability (% of Ctrl)

TEER values (Ohm x cm²)

Permeability (% of Ctrl)

TEER values (Ohm x cm²)

Permeability (% of Ctrl)

HG
Pu (μM)

-  

+  

+  

+  

+  

+  

-  

-  

1  

10  

25  

50  

Vehl HG Pu

Vehl HG-Pu

Vehl HG Pu

Vehl HG Pu

Scramble

shNLRP3

**

#
Graphical abstract