The effect of puerarin against IL-1β-mediated leukostasis and apoptosis in retinal capillary endothelial cells (TR-iBRB2)

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Purpose: Blood–retinal barrier (BRB) breakdown, the early hallmark of diabetic retinopathy (DR), is thought to depend on retinal inflammation and cell damage. The proinflammatory factor interleukin-1β (IL-1β) was demonstrated to cause inflammation as well as cell apoptosis during the process of BRB breakdown. This study extensively evaluated the protective effect of puerarin, a major active component extracted from the traditional herb Radix puerariae, against IL-1β-induced cell dysfunction in TR-iBRB2 cells, a retinal capillary endothelial cell line.

Methods: TR-iBRB2 cells were pretreated with IL-1β (10 ng/ml) for 24 h and then exposed to puerarin (0, 10, 25, and 50 μM) for another 24 h. Leukocyte endothelial adhesion was assessed through a cell-based assay using lymphoblastoid cells. Cell apoptosis was evaluated with flow cytometry, and the expression of adhesion molecules and apoptosis-related molecules was assessed with western blot analysis.

Results: Our data showed that puerarin attenuated IL-1β-mediated leukostasis and cell apoptosis in TR-iBRB2 cells. Furthermore, puerarin strikingly prevented IL-1β-induced molecular events of the upstream and downstream signaling pathways involved in this cellular process.

Conclusions: These findings may significantly contribute to better understanding of the protective effect of puerarin, in particular for DR, as well as provide novel insights into the potential application of this compound in DR therapy.
exhibits other endothelial cell properties [1], and further investigated the protective role of puerarin in this cellular process, in case it could be a potential therapeutic agent.

METHODS

Materials: Puerarin was obtained from the National Institute for the Control of Pharmaceutical and Biologic Products (Beijing, China). Human recombinant IL-1β was purchased from Peprotech (Rocky Hill, NJ). Dulbecco’s modified eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco (Grand Island, NY). Endothelial cell growth factor (ECGF) and calcein AM (C-AM) was purchased from Sigma (St. Louis, MO). The annexin V-fluorescein isothiocyanate (FITC) and propidium iodide (PI) double staining kit was obtained from BD Biosciences (San Jose, CA). Caspase-3 substrate DEVD-AFC was purchased from R&D Systems (Minneapolis, MN). Dimethyl sulfoxide (DMSO), sodium bicarbonate, penicillin-streptomycin, trypsin, RNase A, polyvinylidene difluoride (PVDF) membrane, and enhanced chemiluminescence (ECL) assay kit were from Beyotime (Nantong, China). The following antibodies were used: vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1; Abcam, Cambridge, MA), Bax (Biomol, Hamburg, Germany), and Bcl-2 and caspase-3 (Oncogene, Cambridge, MA). Horseradish peroxidase (HRP) conjugated goat anti-mouse and anti-rabbit secondary antibodies were purchased from Santa Cruz (Santa Cruz, CA).

Cell culture and treatment: All animal experiments were done in accordance with the Guideline for the Care and Use of Laboratory Animals on the University of Sydney, and with the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. Rat retinal capillary endothelial cells (TR-iBRB2) were isolated from the retina of male transgenic rats (line no. 1507–5) as previously described [1] and cultured in low-glucose DMEM containing 10% heat inactivated FBS, 20 mM sodium bicarbonate, 15 ng/ml embryonic carcinoma-derived growth factor (ECGF), 0.1 mg/ml streptomycin, and 100 IU/ml penicillin. Briefly, eyes were enucleated from a Tg rat and retinas were gently separated from the retinal pigmented epithelial cell layer, dissected into small pieces and homogenized using four strokes of a 1 ml teflon-glass homogenizer (Wheaton, Millville, NJ). The tissue debris was then centrifuged for 5 min at 4 °C and 600 g. The homogenate was resuspended in 1 ml 0.1% collagenase/dispose (Boehringer Mannheim, Mannheim, Germany) and incubated in a water bath at 37 °C with frequent agitation. After centrifugation, the pellet obtained consisted of the retinal capillary-rich fraction. The retinal capillary-rich fraction was seeded onto rat tail collagen type I-coated tissue culture dishes (Becton Dickinson, San Jose, CA). The cells were cultured in DMEM containing 20 mM sodium bicarbonate, 15 ng/ml ECGF, 10 U/ml heparin, 100 U/ml penicillin, 100 mg/ml streptomycin, 2.5 mg/ml amphotericin B, and 20% FBS at 37 °C in a humidified atmosphere of 5% CO₂/air. After cell attachment, the temperature was reduced from 37-33 °C to activate SV40 large T-antigen and the culture medium was changed to DMEM containing 20 mM sodium bicarbonate, 15 ng/ml ECGF, 100 U/ml penicillin, 100 mg/ml streptomycin, and 10% FBS. Cells were maintained at 33 °C in a humidified atmosphere containing 5% CO₂. For the experiments, the cells were detached, reseeded in plates, and then incubated with or without drugs for the indicated time.

Leukocyte-endothelial cell adhesion assay: TR-iBRB2 cells pretreated with the indicated drugs were plated at a density of 1.2×10⁴ cells/well in flat clear-bottom black 96-well plates,

![Figure 1. The effect of puerarin against IL-1β-induced leukocyte adhesion to TR-iBRB2. All data were expressed as mean ± standard deviation (SD) of three experiments (triplicate repeats in each experiment). **p<0.01 versus control; #p<0.05; ###p<0.01 versus IL-1β alone.](image-url)
and cultured to confluence overnight. Lymphoblastoid cells were harvested and suspended in RPMI 1640 with 10% FBS and then incubated with 2 mM C-AM for 30 min at 37 °C. After incubation, the cells were washed with PBS (1X; 137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4) three times to remove the free C-AM. The suspension of the C-AM labeled cells (5×10⁵ cells/well) was added to the monolayer of TR-iBRB2 cells for 90 min at 37 °C. After incubation, non-adherent cells were removed by washing with cell culture.

C-AM fluorescence in cells was measured in a fluorescence plate reader (Molecular Devices, Sunnyvale, CA) at an excitation wavelength of 490 nm and an emission wavelength of 530 nm [15].

**Cell apoptosis assay:** Apoptosis of cells was examined with double staining with annexin V-FITC and PI. After treatment with the indicated drugs, the cells were washed twice with ice-cold PBS and resuspended in 300 μl binding buffer (Annexin V-FITC kit, Becton-Dickinson) containing 10 μl of Annexin V-FITC stock and 10 μl of PI. After incubation for 15 min at room temperature in the dark, the samples were then analyzed with flow cytometry. The annexin V⁺/PI⁻ cells were considered apoptotic cells, the percentage of which was calculated with CellQuest software (Becton-Dickinson).

**Caspase activity assays:** Caspase activity was assessed according to the manufacturer’s instructions. Cells were collected and lysed in caspase assay buffer containing 50 mM Tris-HCl (pH 7.4), 1 mM EDTA, 10 mM ethylene glycol tetraacetic acid (EGTA), 10 mM digitonin, and 2 mM dithiothreitol (DTT). Then the ice-cold cell mixtures were centrifuged, and the suspensions were transferred into new tubes. After quantification, 5 μg of protein was incubated with the caspase-3 specific substrate for 1 h at 37 °C. Caspase activity was determined by measuring the OD₄₅₀ of the released p-nitroaniline (pNA) [16].

**Measurement of cytochrome c release:** For measuring the cytochrome c release, the cytosol and mitochondrial fractions were prepared as described previously [17]. Briefly, cells were harvested by centrifugation at 600 ×g for 10 min at 4 °C. The cell pellets were washed once with ice-cold PBS and resuspended with five volumes of buffer A (20 mM Hepes-KOH, pH 7.4, 10 mM KCl, 1.5 mM MgCl₂, 1 mM sodium EDTA, 1 mM sodium EGTA, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 250 mM sucrose. The cells were homogenized with 10 strokes of a Teflon homogenizer, and the homogenates were centrifuged twice at 750 ×g for 10 min at 4 °C. The supernatants were centrifuged at 10,000 ×g for 15 min at 4 °C, and the resulting mitochondria pellets were resuspended in buffer A containing 250 mM sucrose and frozen at –80 °C. The supernatants were further centrifuged at 16,000 ×g for 1 h at 4 °C, and the resulting supernatants were frozen at –80 °C for further experiments. After treatment with the indicated drugs, mitochondrial and cytosolic fractions were extracted from the cells using the ApoAlert Cell Fractionation Kit (Clontech, Mountain View, CA) according to the manufacturer’s instructions. Cytochrome c expression was determined using a monoclonal antibody through western blotting.

**Western blot analysis:** For western blotting analysis, approximately 1×10⁶ cells were collected and lysed in ice-cold RIPA buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EGTA, 1 mM EDTA, 20 mM NaF, 100 mM Na₃VO₄, 1% Nonidet P40 (NP-40), 1% Triton X-100, 1 mM phenylmethylsulfonyl fluoride (PMSF), 10 mg/ml aprotinin and 10 mg/ml leupeptin) for 30 min. The protein concentration was
determined with the Bradford method [18], and 50 μg protein from each sample was used for western blot analysis. Proteins were separated by electrophoresis on 10% sodium dodecyl sulfate-PAGE (SDS–PAGE) and transferred electrophoretically to PVDF membranes. After blocking with 5% bovine serum albumin (BSA) in the mixture of Tris-buffered saline and Tween-20 (TBST) for 1 h, the membranes were incubated with primary antibody overnight and followed by incubation with secondary antibody for 1 h at room temperature. Protein bands were visualized using the ECL assay kit. The density of each band was normalized to the expression of β-actin.

Statistical analysis: Biostatistical analyses were performed using the SPSS 16.0 software package. All experiments were repeated three times. Results of multiple experiments were expressed as mean ± standard deviation (SD). A p value less than 0.05 was accepted as statistically significant.

RESULTS

Puerarin attenuates IL-1β-induced leukocyte adhesion to TR-iBRB2 cells: Leukocyte adhesion to endothelial cells can trigger the disorganization of endothelial cell adherence and tight junctions as well as vascular leakage. The breakdown of the blood–retinal barrier followed the onset of diabetes-associated retinal leukostasis [19]. We first investigated whether IL-1β can induce leukocyte adhesion to TR-iBRB2 cells in vitro. The results indicated that after treatment of IL-1β (10 ng/mL) for 24 h, leukocyte adhesion to TR-iBRB2 cells was obviously increased (68.24% ± 5.35% of the control), whereas this effect was prevented by puerarin in a dose-dependent manner (Figure 1).

Puerarin inhibits IL-1β-induced expressions of VCAM-1 and ICAM-1: To investigate whether cell adhesion molecules...
Figure 4. The effect of puerarin against IL-1β-induced alternation of Bcl-2 family expression in TR-iBRB2 cells. 

A: The expression levels of Bax and Bcl-2 were detected using an immunoblotting assay. 

B: Densitometry analysis of Bax and Bcl-2 protein expression. All data are expressed as mean ± standard deviation (SD) of three experiments, and each experiment included triplicate repeats. 

Figure 5. The effect of puerarin against IL-1β-induced cytochrome c release in TR-iBRB2 cells. The levels of cyto- and mito-cytochrome c were detected with immunoblotting assay after treatment with interleukin (IL)-1β (10 ng/ml) in the absence or presence of the indicated concentrations of puerarin. All data are representative of three independent experiments.

Figure 6. The effect of puerarin against IL-1β-induced activation and protein cleavage of caspase-3 in TR-iBRB2 cells. 

A: The activity of caspase-3 was determined as described in the Methods section. All data are expressed as mean ± standard deviation (SD) of three experiments, and each experiment included triplicate repeats. 

B: Caspase-3 cleavage was assessed with western blot analysis. All data are representative of three independent experiments.
including VCAM-1 and ICAM-1 were involved in IL-1β-induced leukostasis, VCAM-1 and ICAM-1 expression was analyzed. The data demonstrated that IL-1β (10 ng/ml) stimulation significantly induced higher expression of VCAM-1 and ICAM-1 in TR-iBRB2 cells compared with control (7.74 and 16.12 folds of control, respectively). However, cotreatment with puerarin (10, 25, and 50 μM) dose-dependently attenuated the effect induced by IL-1β (Figure 2). These results suggest that puerarin inhibits IL-1β-induced leukostasis mainly by regulating the expression of VCAM-1 and ICAM-1.

**Puerarin attenuates IL-1β-induced cell apoptosis in TR-iBRB2 cells**: Apoptosis of TR-iBRB2 cells treated with the indicated drugs was assessed with annexin V-PI dual-staining assay. The results showed the percentage of apoptotic cells induced by IL-1β (10 ng/ml) increased from 0.09% ± 0.04% to 56.82% ± 6.37% compared to the control. However, with the cotreatment of puerarin (10, 25, and 50 μM), cell apoptosis decreased to 34.13% ± 4.39%, 18.53% ± 1.97%, and 7.94% ± 2.13%, respectively (Figure 3). These results indicate that puerarin can suppress IL-1β-induced apoptosis in TR-iBRB2 cells.

**Puerarin inhibits IL-1β-induced mitochondrial dysfunction in TR-iBRB2 cells**: Apoptosis of retinal endothelial cells may be one mechanism of BRB breakdown, which is reported to be regulated by the mitochondrial pathway [20]. We investigated the expression of apoptosis-related proteins, Bax and Bcl-2, in TR-iBRB2 cells, under the treatment of IL-1β (10 ng/mL) in the presence and absence of puerarin for 24 h. Our results showed that IL-1β treatment upregulated the expression of Bax but downregulated that of Bcl-2; however, cotreatment with puerarin (10, 25, and 50 μM) significantly attenuated this effect in a dose-dependent manner (Figure 4). Release of cytochrome c from mitochondria to cytosol is a major feature of cell apoptosis [21]. In our present study, IL-1β (10 ng/ml) treatment significantly increased cytochrome c levels in cytosol and decreased the levels in mitochondria in TR-iBRB2 cells (Figure 5). However, this effect was dramatically reversed by cotreatment with puerarin. Furthermore, the activation status of caspase-3 was also investigated. Caspase-3 activity in TR-iBRB2 cells was markedly increased after treatment with IL-1β for 24 h. The results were then reversed when the cells were cotreated with puerarin (Figure 6A). In addition, as shown in Figure 6B, puerarin cotreatment also resulted in the pronounced attenuation of caspase-3 cleavage elevation induced by IL-1β in TR-iBRB2 cells. These results demonstrate that puerarin inhibits IL-1β-induced cell apoptosis by regulating the mitochondrial pathway.

**DISCUSSION**

BRB breakdown is the leading cause of vision loss in DR [2]. Diabetic endothelial dysfunction, such as leukostasis and cell apoptosis, plays a central role in the pathogenesis of DR, explaining the initial change in the blood–retinal barrier [22]. A previous study demonstrated that IL-1β is upregulated in the retina of experimental diabetes, which may be important for promoting BRB breakdown, in particular, for regulating endothelial dysfunction [8-10,23]; however, the mechanism has not been investigated until now. In this study, using TR-iBRB2, a retinal capillary endothelial cell line that characterizes the endothelial cell markers and transport activity [1], we found that IL-1β markedly induced leukostasis and cell apoptosis in vitro. Thus, IL-1β may be a promising target for suppressing retinal complications of diabetes.

Puerarin, an active ingredient extracted from the root of *Radix puerariae* with anti-inflammatory activities [24], has been studied as an antihyperglycemic agent in the ability of increasing insulin sensitivity and protecting the pancreatic islets [25,26]. Additionally, puerarin effectively inhibits the retinal pigment epithelial cells and pericyte apoptosis [27,28]. In this study, the mechanism of puerarin against IL-1β-mediated cell dysfunction was investigated using TR-iBRB2 cells. Our results indicated that puerarin...
not only attenuated the leukostasis caused by IL-1β treatment but also inhibited IL-1β-induced cell apoptosis in TR-iBRB2 cells. Furthermore, we explored the molecular mechanisms underlying the effect of puerarin against the stimulation of IL-1β in TR-iBRB2 cells.

Previous studies demonstrated that diabetes increased leukocyte adhesion to retinal vessels and consequently induced BRB breakdown [23,29]. The occurrence of retinal leukostasis is dependent on adhesion molecules such as ICAM-1, and the integrity of the BRB is maintained by tight junctions between vascular endothelial cells [30]. Adhesion molecules on the cell surface are upregulated in response to cytokines, and are the basis of leukocyte–endothelium interactions and an important characteristic of inflammatory reactions [31,32]. The upregulation of expression of each adhesion molecule was detected in the retinas of diabetic rats [33]. Increased expression of VCAM-1 in the retinal endothelium resulting in leukocyte adhesion played a functional role in the development of retinal angiogenesis and a proliferative diabetic retinopathy [34]. In our present study, the direct treatment of IL-1β led to an increase in leukostasis and upregulation of VCAM-1 and ICAM-1 expression in TR-iBRB2 cells. However, puerarin significantly attenuated IL-1β-mediated leukocyte adhesion to TR-iBRB2 cells by inhibiting the expression of cell adhesion molecules.

Apoptosis of human retinal microvascular endothelial cells and retinal pigment epithelium cells, which is mainly regulated by the mitochondrial pathway, may be one mechanism of the BRB breakdown [20]. IL-1β, known as a “multi-functional” proinflammatory cytokine, can induce apoptosis by promoting the expression of Bax, inhibiting the expression of Bel-2, and increasing caspase-3 activity [10,35,36]. Our results demonstrated that the upregulation of Bax and the downregulation of Bel-2 expression were induced by IL-1β in TR-iBRB2 cells. Cotreatment with puerarin significantly attenuated or restored this effect in a dose-dependent manner. Furthermore, puerarin inhibited the caspase-3 activity and cleavage in TR-iBRB2 cells induced by IL-1β. These results suggest that the mitochondria-initiated intrinsic pathway might be involved in IL-1β-mediated apoptosis in TR-iBRB2 cells. Puerarin cotreatment significantly inhibited apoptosis and prevented damage by interfering with the mitochondrial pathway in TR-iBRB2 cells.

Of note, although the protective effects of puerarin against IL-1β-induced endothelial cell damage were observed in our in vitro model, it is not clear whether this compound can protect the BRB from IL-1β-induced inflammatory response and breakdown in vivo. Therefore, further efforts are needed to figure out the effect of puerarin against BRB breakdown in vivo as well as the precise molecular mechanisms underlying this effect.

In our present study, we extensively evaluated the protective effect of puerarin against IL-1β-induced leukostasis and cell apoptosis in TR-iBRB2 cells (Figure 7). Together, our findings may significantly contribute to better understanding of the therapeutic mechanism of puerarin as well as form the basis of puerarin as a therapeutic agent for diabetic retinopathy.

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