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Puerarin suppresses AGEs-induced inflammation in mouse mesangial cells: A possible pathway through the induction of heme oxygenase-1 expression

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ABSTRACT

Puerarin is a natural product isolated from *Puerarin lobata* and has various pharmacological effects, including anti-hyperglycemic and anti-allergic properties. In the present study, we investigated the effect of puerarin against advanced glycation end products (AGEs)-induced inflammation in mouse mesangial cells. Puerarin acts by inducing the expression of heme oxygenase-1 (HO-1) in a dose- and time-dependent manner. Puerarin was able to enhance phosphorylation of protein kinase C (PKC) δ, but not PKC α/β II, in a time-dependent manner. Induction of HO-1 expression by puerarin was suppressed by GF109203X, a general inhibitor of PKC, and by rottlerin, a specific inhibitor of PKC δ. However, induction was not suppressed by Gö6976, a selective inhibitor for PKC α/β II. Additionally, the knockdown of endogenous PKC δ by small interfering RNA (siRNA) resulted in the inhibition of HO-1 expression and Akt phosphorylation. Puerarin increased antioxidant response element (ARE)-Luciferase activity in a dose- and time-dependent manner in transfected mouse mesangial cells. Mutation of the ARE sequence abolished puerarin-induced HO-1 expression. Furthermore, puerarin treatments resulted in a marked increase in NF-E2 related factor-2 (Nrf-2) translocation, leading to up-regulation of HO-1 expression. However, transfection of Nrf-2 specific siRNA abolished HO-1 expression. Pretreatment with puerarin inhibited the expressions of COX-2, MMP-2 and MMP-9. But, these effects were reversed by ZnPP, an inhibitor of HO-1. Taken together, our results demonstrate that puerarin-induced expression of HO-1 is mediated by the PKC δ-Nrf-2-HO-1 pathway and inhibits *N*-carboxymethyllysine (CML)-induced inflammation in mouse mesangial cells.

Introduction

Puerarin (daidzein-8-C-glucoside) is an isoflavone glycoside isolated from the root of *Pueraria lobata* which has been used in traditional Korean medicine for the treatment of various diseases for thousands years. Puerarin has been studied for its use as an anti-hyperglycemic agent, which increases insulin sensitivity and protects pancreatic islets (Hsu et al., 2003; Xiong et al., 2006; Xu, 2003). Additionally, puerarin has been reported to effectively inhibit advanced glycation end products (AGEs) formation, one of the typical risk factors of diabetic complications (Kim et al., 2006). AGEs are a heterogeneous family of compounds derived from the reactions of reducing sugars with amino groups in proteins through a series of oxidative and non-oxidative reaction. AGEs have been reported to form in several pathophysiological situations associated with inflammation, such as diabetes and aging (Anderson et al., 1999; Shleicher et al., 1997). AGEs activate NADPH oxidase and this may lead to the production of reactive oxygen species, which can stimulate vascular cell adhesion molecule-1 expression (Wautier et al., 2001) and inflammatory gene expression through nuclear factor-κB (NF-κB) activation (Yan et al., 1994). Also, several lines of evidence suggest that AGEs are involved in the development of glomerular renal disease of diabetes (Beisswenger et al., 1993; Horie et al., 1997). Especially, glucose-modified proteins and *N*-carboxymethyllysine (CML)-protein adducts (the predominant AGEs) are increased in glomerular mesangial cells (Skolnik et al., 1991; Doi et al., 1992; Shleicher et al., 1997).

Heme oxygenase (HO-1) is the rate-limiting enzyme in the conversion of heme into biliverdin, releasing free iron and carbon monoxide. Biliverdin is rapidly metabolized to bilirubin, a potent antioxidant. Induction of the HO-1 gene is primarily regulated at the transcriptional level, and its inducibility by various inducers is linked to the transcription factor nuclear factor erythroid2-related 2 (Nrf-2) (Alam and Cook, 2003). Under normal conditions, Nrf-2 is sequestered in the cytoplasm by binding to Keap1, an actin-binding protein (Itoh et al., 2004). Several electrophilic antioxidants disrupt this complex, freeing Nrf-2 for translocation to the nucleus, where it then binds to the

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antioxidant response element (ARE) sequences in the HO-1 promoter (Kang et al., 2001; Owuor and Kang, 2002). The mechanism by which Nrf-2 is liberated from the Keap1–Nrf-2 complex remains to be established. Several studies have suggested that the mechanisms of HO-1 induction involve pathways of MAPK, JNK, PI3K/Akt and Nrf-2 (Alvarez-Maqueda et al., 2004; Buckley et al., 2003; Anwar et al., 2005; Martin et al., 2004). Nrf-2 has been known to induce expression of HO-1. Although it has been reported the mechanism of HO-1, the inhibitory effect of HO-1 on AGEs products mediated inflammation has not been established.

Thus, we hypothesized that puerarin induces HO-1 expression via Nrf-2 pathways in mouse mesangial cells and shows the protective effect of HO-1 on AGEs-induced inflammation. In the present study, we investigated whether puerarin could induce HO-1 expression in a PKC δ-Nrf-2-dependent pathway in mouse mesangial cells. We further examined the inhibitory effect of puerarin-mediated HO-1 on AGEs-induced inflammation.

Materials and methods

Chemicals. Puerarin was isolated as described previously (Kim et al., 2006). *N*-carboxymethyllysine (CML) was purchased from CircuLex (Nagano, Japan). Actinomycin-D, cycloheximide, rottlerin and zinc protoporphyrin (ZnPP) IX were obtained from Sigma Chemical (St. Louis, MO, USA). Bisindolylmaleimide (GF109203X) and Gö6976 were purchased from Calbiochem (EMD Biosciences Inc, San Diego, CA, USA). An MMP-2 ELISA kit was obtained from R&D systems (Minneapolis, MN, USA). Luciferase reporter gene fusion constructs for wild type and GC mutant AREs were made as described previously (Moehlenkamp and Johnson, 1999). These ARE-Luciferase and GC mutant-ARE constructs were provided by Dr. Young-Joon Surh (Seoul National University). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell culture. SV40-transformed mouse mesangial cells (MES-13 cells) were purchased from the American Type Culture Collection and were cultured at 37 °C in a 5% CO_2 atmosphere in DMEM plus F-12 media (3:1) supplemented with 5% fetal bovine serum, penicillin (100 U/ml), streptomycin (100 μg/ml) and HEPES buffer (14 mM). Cells were incubated with or without puerarin, an inducer, for the indicated time periods.

Western blotting and RT-PCR. Western blotting was performed as previously described (Kim et al., 2007). The primary antibody against HO-1 (SPA-894) was obtained from Stressgene (Ann Arbor, MI, USA). Histone H1 (SC-8030) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Nrf-2 (ab31163) was purchased from Abcam (Cambridge, UK). p-p38 (#9211), p-AKT (#9271), p-PKC δ (#9374), p-PKC α/β II (#9375), p-PKC ζ/λ (#9378), MMP-9 (#2270), p38 and AKT were obtained from Cell Signaling Technology (Danvers, MA, USA). COX-2 (160126) was purchased from Cayman Company (Ann Arbor, MI, USA). β-actin (A-1978) was obtained from Sigma (St. Louis, MO, USA). Total RNA was isolated using the TRIzol reagent kit. A total of 1 μg of RNA was used for the reverse transcription (RT) reaction, which also contained the AccuPower RT premix and random hexamers. Total cDNA corresponding to 1 μg of RNA was used in PCR reactions. The following PCR primers were used in this study: HO-1, 5′-GCA TAA ATT CCC ACT GCC AC-3′ (sense) and 5′-TTA CCTTCC CGA ACA TCG AC -3′ (antisense) and β-actin, 5′-CCA GAT CAT GTT TGA GAC CT-3′ (sense) and 5′-AAT GTA GTT TCA TGG ATG CC-3′ (antisense). Amplification products were resolved by 1.2% agarose gel electrophoresis. The gel was stained with ethidium bromide and photographed under ultraviolet light. All primers were purchased from Bioneer (Daejeon, Korea).

RNA interference by siRNA of PKC δ and Nrf-2. Predesigned siRNAs against mouse PKC δ (sc-36246), Nrf-2 (sc-37049) and control scrambled siRNA (sc-37007) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Mouse mesangial cells were transfected with double-stranded siRNAs (40 nmol/ml) for 6 h using the LipofectAMINE™ 2000 reagent according to the manufacturer's protocol (Invitrogen Company Carlsbad, CA, USA) and recovered in fresh media containing 5% FBS overnight. Knockdown of PKC δ and Nrf-2 protein expression was confirmed by western blotting using a protein-specific antibody.

Immunohistochemical staining. Cells grown on chamber slides were treated with puerarin for 3 h, washed with PBS and fixed with 4% paraformaldehyde for 30 min. After permeabilization, cells were blocked with 8% BSA in PBS for 1 h and were then incubated with rabbit anti-Nrf-2 antibody (1:500 dilution) for 2 h. Cells were then incubated with the secondary antibody, Texas-red-conjugated goat anti-rabbit antibody (1:500 dilution, Vector Laboratories Burlingame, CA, USA) for 30 min. After washing with PBS, the cells were counterstained with 1 μg/ml of 4′, 6-diamidino-2-phenylindole (DAPI) for 3 min. The cells were finally mounted with mounting medium (Dako, Hamburg, Germany) and were analyzed on a fluorescence microscope (BX-51 Olympus, Tokyo, Japan).

Preparation of cytosolic and nuclear extract. Mouse mesangial cells (1×10^7 cells in 100 mm dish) were either treated with puerarin or not, then harvested, washed with PBS, centrifuged and resuspended in ice-cold buffer A (10 mM HEPES (pH 7.0) 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.2 mM PMSF). After a 10 min incubation on ice, the cells were centrifuged again and the supernatants (cytosolic extracts) were collected and stored at −70 °C. The pellets were resuspended in buffer C (20 mM HEPES (pH 7.9), 20% glycerol, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM DTT and 0.2 mM PMSF) and incubated for 20 min at 4 °C. After vortexing, the resulting suspension was centrifuged, and the supernatant (nuclear extract) was stored at −70 °C for the Nrf-2 translocation experiments. The protein concentration of the nuclear extract was determined using a BCA assay kit (Pierce Company, Rockford, IL, USA).

Transient transfection and reporter gene assay. Cells were seeded at a density of 1×10^5 cells in 6-well plates and grown to 60–70% confluence. A total of 1 μg of the luciferase reporter plasmid construct harboring the ARE promoter or the equivalent amount of the control plasmid (pTi) was transfected using LipofectAMINE™ 2000 reagent according to the manufacturer's protocol (Invitrogen Company Carlsbad, CA, USA). CMV-β-galactosidase was used to correct for the transfection efficiency (Lee et al., 2001). Eight hours after transfection, the cells were treated with puerarin and cell lysis was carried out using the reporter lysis buffer. After mixing the cell extract with a luciferase substrate (Promega, Madison, WI, USA), luciferase activity was determined using a luminometer (Synergy HT, Winooski, VT, USA). Luciferase activity was expressed as the relative intensity of luminescence ± standard deviation (S.D.) and was normalized to β-galactosidase activity.

Measurement of prostaglandin E_2 (PEG_2) and MMP-2 levels. The level of PGE_2 and the accumulation of MMP-2 in the cell culture medium were measured using ELISA Kits (Cayman Chemical, Ann Arbor, MI, USA and R&D Systems, Minneapolis, MN, USA).

Statistical analysis. All experiments were repeated at least three times and all values are represented as means ± S.D. The results were subjected to an analysis of the variance (ANOVA) using the Tukey test to analyze differences. Values of $^*P<0.05$, $^{**}P<0.01$ were considered statistically significant.

Results

Effect of puerarin-induced HO-1 expression in mouse mesangial cells

To examine whether puerarin can induce HO-1 expression in mouse mesangial cells, changes of HO-1 expression were analyzed