Puerarin Suppresses Macrophage Activation via Antioxidant Mechanisms in a CaPO4-Induced Mouse Model of Aneurysm

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Summary
Aneurysm is characterized by balloon-like expansion of the arterial wall and eventual rupture of the aorta. The pathogenesis of aneurysm is associated with the degradation of matrix proteins by matrix metalloproteinases (MMPs) produced by activated macrophages. Although aneurysm is associated with significant mortality and morbidity, surgical intervention is the only proven treatment strategy. Therefore, development of therapeutic agents for aneurysm is greatly anticipated. Here, we demonstrated the protective effects of the major isoflavone puerarin, which is found in kudzu roots and vines. Aneurysms were surgically induced in ten-wk-old male mice using CaPO4. Subsequently, animals were intra-peritoneally injected daily with puerarin at 2.5 mg/kg body weight or with vehicle alone for 2 wk. CaPO4-induced aneurysm was significantly suppressed by puerarin administration. In subsequent macrophage activation assays using Tumor necrosis factor (TNFα) and CaPO4 crystals in vitro, puerarin decreased Mmp9 mRNA expression and secreted protein levels. Moreover, induction of IκB, ERK, and p38 phosphorylation by TNFα and CaPO4 in macrophages was suppressed by puerarin treatments. Finally, puerarin attenuated reactive oxygen species production, following induction by TNFα and CaPO4. Taken together, the present data demonstrate that puerarin suppresses macrophage activation by inhibiting IκB, ERK, and p38 activity and reactive oxygen species production in a CaPO4-induced mouse model of aneurysm.

Key Words kudzu, puerarin, aneurysm, macrophage activation

Aneurysms are caused by balloon-like expansions of aortic walls and occur predominantly in men over 65 y of age (1). Progressive weakening of the artery wall can lead to rupture if left untreated and carries a high risk of mortality (2). The pathogenesis of aneurysm is closely associated with chronic inflammation of the arterial wall, local activation of proteases, and degradation of matrix proteins by matrix metalloproteinases (MMPs) produced by activated macrophages (3, 4). Currently, surgical interventions include open repair or endovascular stent graft placement. Although these procedures are the only proven treatments for aneurysm, they are associated with significant morbidity and mortality risks, warranting the urgent development of alternative therapeutic strategies.

Puerarin (daidzein-8-C-glycoside) is an isoflavone found in roots and vines of the kudzu plant (Pueraria lobata) and has been used widely as a traditional Chinese herbal medicine for various diseases (5–7). The structural formula of puerarin is shown in Fig. 1. Several studies report antioxidant and anti-inflammatory effects of puerarin in mice. Among these, puerarin reportedly protects against CCl4-induced oxidative stress and inflammation in the kidneys via the ERK/Nrf2/ARE pathway (8) and against nickel-induced TLR4/p38/CREB associated oxidative stress and inflammation in the liver (9). However, the effects of puerarin on the formation of aneurysms have not been extensively studied.

In this study, we investigated the protective effects of puerarin against CaPO4-induced aneurysms in mice and showed that puerarin suppresses macrophage activation via antioxidant mechanisms.

MATERIALS AND METHODS

Materials. Tumor necrosis factor (TNFα) was purchased from Peprotech (Rocky Hill, NJ). Puerarin was purchased from Sigma-Aldrich (St. Louis, MO). All reagents and chemicals were of laboratory grade.

Cell culture. Murine RAW264.7 monocyte cells were purchased from American Type Culture Collection (Manassas, VA) and maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 10% fetal bovine serum (FBS), 100 IU/mL penicillin, and 100 μg/mL streptomycin. Prior to macrophage activation, cells

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were plated at $5 \times 10^5$ cells per well in 6-well plates and maintained in minimal essential medium alpha (MEMα) supplemented with 10% charcoal-stripped FBS and antibiotics.

**Cell viability assays.** RAW264.7 cells (4,000 cells per well) were cultivated in 96-well plates 1 d before treatments and were then cultured with or without TNFα and CaPO4 in the presence or absence of puerarin. Subsequently, cells were combined with MTT solution [3-(4,5-dimethyl-thiazol-2-yl)-2,5-diphenyltetrazolium bromide (50 ng/well)] and incubated for 4 h. Acid–isopropanol (0.04 N HCl in isopropanol) and 3% sodium lauryl sulfate were then added and mixed to dissolve reduced MTT crystals (formazan) in the cells, and absorbance was determined at 595 nm with a reference wavelength of 655 nm using a microplate reader.

**Generation and treatment of the mouse model of Aneurysm.** Ten-week-old male C57BL/6 mice were obtained from the Jackson Laboratory (Bar Harbor, ME). The procedures for creating our CaPO4-induced mouse model of aneurysm were previously described (10). We applied the procedure for the infrarenal aorta to the carotid artery in this study. Briefly, 0.5-μl CaCl2-soaked gauze was applied to the carotid artery in a perivascular manner for 10 min. The gauze was then replaced with phosphate buffered saline (PBS)-soaked gauze for 5 min, and the incised area was sutured. The diameter of the artery at the time of the initial surgery and sacrifice was measured with an electronic digital caliper (VWR International, West Chester, PA). All animal procedures were conducted in accordance with experimental protocols that were approved by the Institutional Animal Care and Use Committee at the University of Wisconsin, Madison (Protocol M02394).

**Histological and immunohistochemical analyses.** Murine carotid artery tissues were fixed in 4% paraformaldehyde (PFA) overnight. Samples were then embedded with optical cutting temperature compound (Sakura, Alphen aan den Rijn, the Netherlands) and were frozen, and were then sectioned at a thickness of 6 μm. Histological analyses were performed with hematoxylin and eosin (HE) stained sections using a light microscope (Olympus BH-2; Olympus, Tokyo, Japan). Immunofluorescent staining was performed using rat anti-MOMA-2 (ab33451, Abcam Cambridge, MA) and donkey anti-rat Alexa 488 (Molecular Probes). Nuclei were stained using TO-PRO 3 (Molecular Probes). Sections were visualized under a fluorescence microscope, and digital images were generated using a Nikon A1R Laser Scanning Confocal imaging system with appropriate argon beam lasers.

**Western blotting.** Proteins from cultured cells were extracted using a radioimmunoprecipitation assay buffer containing a protease inhibitor cocktail (Cell Signaling Technology, Danvers, MA) at 0–4°C. Subsequently, 20-μg lysates were separated on 8–12% polyacrylamide gels in Laemmli sample buffer and were then electrophoretically transferred onto polyvinylidene difluoride membranes. Primary antibodies for rabbit MMP-9 antibody (sc-10737), rabbit p-IkB antibody (sc-101713), rabbit IkB antibody (sc-371), rabbit ERK antibody (sc-94), rabbit p38 antibody (sc-535), and mouse α-Tubulin (sc-23948) antibody were purchased from Santa Cruz Biotechnology (Dallas, TX). Rabbit p-ERK antibody (9101S) and rabbit p-ERK antibody (9101S) were purchased from Cell Signaling Technology. Primary antibodies were detected using a horseradish peroxidase-conjugated secondary antibody and were visualized using an Enhanced Chemiluminescence kit (Thermo Scientific, Rockford, IL) or SuperSignal West Femto Maximum Sensitivity Substrate (Thermo Scientific).

**Quantitative real-time PCR.** Total RNA was extracted from RAW264.7 cells using an RNeasy Plus Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. Quantitative real-time PCR was performed using SYBR Green dye with a real-time PCR instrument (ABI, Foster City, CA). Primer sequences were as follows: Mmp9: F, 5′-CATTCGGTGATAAGAGT-3′ and R, 5′-GGTACCTGAGTCCACT-3′; Gapdh: F, 5′-TGTTGAAGGTCTGGAAAC-3′ and R, 5′-AAATGAAGGCTGTGATGG-3′. Mmp9 expression levels were normalized to those of Gapdh.

**Intracellular reactive oxygen species (ROS) detection.** The intracellular ROS level was measured using an OxiSelect Intracellular ROS Assay Kit with green fluorescence (Cell Bios, San Diego, CA). The fluorescent probe 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA) incorporated into cells was deacetylated by cellular esterases to non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH) and then rapidly oxidized to highly fluorescent 2′,7′-dichlorodihydrofluorescein (DCF) by different kinds of ROS. The fluorescence intensities of DCF were determined using a fluorometric plate reader.

**Statistical Analysis.** Data are reported as means ± standard deviations (SD). Statistical analyses were performed using the GraphPad Prism program version 4.00 (GraphPad Software, Inc., San Diego, CA). Comparisons between groups at single time points were performed using Student’s t-tests. Multiple comparisons among treatments were performed using one-way analysis of variance followed by Tukey’s range test. For statistical analysis in Fig. 3B, two-way analysis of variance followed by Tukey’s test was performed. Differences were considered significant when $p<0.05$. 

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Fig. 1. Chemical structure of puerarin.
Puerarin Suppresses Macrophage Activation

RESULTS

Protective effects of puerarin on the formation of CaPO₄-induced aneurysms in mice

Puerarin (2.5 mg/kg/d) was administered intraperitoneally for 2 wk. As shown in Fig. 2A and B, puerarin injections significantly suppressed the formation of aneurysms according to fold increases in maximum diameters of the arteries between initial surgery and sacrifice (Fig. 2B; 1.9±0.3 vs. 1.5±0.2; p<0.05). Subsequently, we determined whether puerarin suppressed macrophage accumulation. In these experiments, activated monocytes and macrophages were identified according to MOMA-2 expression in immunohistochemical analyses, and were mostly localized in the adventitia of mice with CaPO₄-induced aneurysms (Fig. 2C). Numbers of monocytes and macrophages were markedly decreased in puerarin-injected mice compared with untreated mice controls. These results suggest that puerarin suppresses a CaPO₄-induced aneurysm by inhibiting macrophage activation.

Inhibitory effects of puerarin on TNFα+CaPO₄-induced macrophage activation

Previously, we demonstrated macrophage activation following treatment with TNFα and CaPO₄ (11) using MMP9 as a marker of classical macrophage activation in RAW264.7 cells (12, 13). As shown in Figs. 3A and B, TNFα+CaPO₄ treatment significantly increased MMP9 mRNA and protein expression compared with that in non-treated controls. Puerarin treatment dose-dependently suppressed TNFα+CaPO₄-induced Mmp9 mRNA expression at 50–200 μM but did not affect Mmp9 expression in untreated cells (Fig. 3A). Relative expression of Mmp9 in cells treated with 100- and 200-μM puerarin was significantly lower than that in controls (41.9±5.7 vs. 31.9±5.9, p<0.05 and 41.9±5.7 vs. 9.4±2.0, respectively, p<0.01). MMP9 is synthesized as an inactive pro-enzyme (pro-MMP9) and is activated by proteolytic removal of an amino-terminal following secretion into the extracellular space (14). Thus we next measured the levels of active Mmp9 by Western blotting. The levels of active Mmp9 were decreased after puerarin treatment, following induction by TNFα+CaPO₄ (Fig. 3B, 10.7±1.0 vs. 7.6±2.1, p<0.05). However, MTT assays revealed no significant differences in cell proliferation among the six study groups (Fig. 3C). Taken together, these data suggest that puerarin suppresses TNFα+CaPO₄-induced macrophage activation without affecting cell viability.

Inhibitory effect of puerarin on the phosphorylation of IκB, ERK, and p38 in TNFα+CaPO₄-activated macrophages

The transcription factor NF-κB plays important roles in macrophage activation (15). Specifically, translocation of NF-κB to the nucleus is followed by phosphorylation, ubiquitination, and proteasomal degradation of IκBα (16). In addition, MAPK signaling pathways, including ERK and p38, are activated during oxidative stress and inflammation in macrophages (17, 18).
Thus, in the present study, the involvement of NFκB/H9260B and MAPK signaling pathways in puerarin suppression of macrophage activation was investigated using Western blotting. In these experiments, puerarin treatments decreased p-IκB, p-ERK, and p38 protein expression in TNFα/CaPO4-activated macrophages (Fig. 4). These data suggest that suppression of NFκB and MAPK signaling is involved in the protective effects of puerarin against macrophage activation.

Inhibitory effects of puerarin on TNFα/CaPO4-induced reactive ROS production

Induction of NFκB and MAPK signaling pathways by oxidative stress was diminished in the presence of puerarin. Thus, because Han et al. previously reported radical scavenging activities of puerarin in vitro (19, 20), we determined intracellular ROS levels in TNFα/CaPO4-activated macrophages (Fig. 4). These data suggest that suppression of NFκB and MAPK signaling is involved in the protective effects of puerarin against macrophage activation.

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the present model of the aneurysm, CaPO4 induces
pressed the formation of aneurysms in male mice (31).

In this study, we tried to elucidate the mechanism by
which puerarin decreases MMP9 expression, although
there exist multiple mechanisms underlying this effect.
Phosphorylation levels of IκB, ERK, and p38 were deter-
mained following activation by TNFα+CaPO4. In these
experiments, puerarin suppressed phosphorylation of
IκB, p38, ERK, and JNK, and attenuated reactive oxygen
species production. Tobari et al. reported that ROS-NFκB
acts as the crucial signal in transforming growth fac-
tor-β1-induced MMP-9 expression (41). There are also
some reports that the suppression of NF-κB and MAPKs
activation contribute to decrease MMP9 expression in
macrophages (42, 43). Therefore, we suggest that the
effect of puerarin is, at least in part, due to inhibiting
IκB, ERK, and p38 activity via antioxidant mechanisms.

A single oral administration of puerarin to rats shows
rapid clearance from the blood (t1/2 = 1.7 ± 0.6 h) and
low bioavailability (44). Thus, in this study, puerarin

While arterial aneurysms predominantly occur in the
abdominal aorta, we applied CaPO4 to the adventitia of
carotid arteries to induce the formation of aneurysms.
As well as abdominal aneurysm, by application of CaCl2
to the adventitia of carotid arteries, arterial dilatation
was reportedly accompanied by elastin calcification, loss
of VSMCs, and marked infiltration of inflammatory cells
such as neutrophils, lymphocytes, monocytes, and mul-
tinucleated giant cells (33). Although another group
has utilized the carotid artery as a model for aortic aneu-
rysm (34), possible physiological differences between
these anatomical structures make the carotid artery an
imperfect model and is a limitation of this study.

Plasma TNFα concentrations were reportedly higher
in patients with intracranial aneurysms than in age-
matched healthy controls (35), and have been shown to
play important roles in the pathogenesis of aneurysms
(36, 37). Moreover, vascular calcification deposits com-
prise hydroxyapatite and other forms of CaPO4 crystals,
which have proinflammatory effects and induce apop-
tosis (10). Accordingly, we previously reported activa-
tion of RAW264.7 macrophages using TNFα+CaPO4 as
a model of calcification in aneurysmal aortas. In the
present TNFα+CaPO4-activated macrophages, MMP9
mRNA expression and secreted protein levels were ele-
vated but were not increased in the presence of puer-
arin. When we examined the accumulation of puerarin
in RAW264.7 cells by high performance liquid chromato-
graphy, the time-dependent accumulation of puerarin
in RAW264.7 was observed (data not shown). These
results suggest that puerarin is incorporated in macro-
phages and suppress macrophage activation.

Because isoflavones reportedly act as estrogen mimetics
(38, 39), the protective effects of puerarin may
reflect estrogen-like activities. However, our previous
experiments showed that uterine atrophy due to estrogen
deficiency was unaltered by a puerarin-rich diet in
OVX mice (7, 40). Moreover, although the puerarin agly-
cone daidzein bound to estrogen receptors, puerarin did
not bind estrogen receptors α or β (40). Therefore, the
mechanism by which puerarin suppresses the formation of
aneurysms may not involve estrogen-like activities.

In this study, we tried to elucidate the mechanism by
which puerarin decreases aneurysms in male mice (31)
using an elastase-induced model of the aneurysm. In
the present model of the aneurysm, CaPO4 induces
apoptosis of vascular smooth muscle cells with subse-
quent macrophage infiltration, leading to the formation
of aneurysms. This process resembles the pathophysiol-
ogy of the aneurysm, which proceeds with the destruc-
tion of elastin and collagen by MMPs, vascular smooth
muscle cell apoptosis/necrosis, and macrophage infiltra-
tion through vessel walls (32).

**DISCUSSION**

Isoflavones have several positive effects (21–24) and
are principally found as glycosides bound to sugars in
plants (25). Following oral administration, O-glycoside
isoflavones are hydrolyzed by β-glucosidase to aglycones
(26). Puerarin has a C-glycoside bond that is resistant
to degradation by mouse microflora (27). Prasain et al.
(28) showed that non-metabolized puerarin and a small
amount of its glucuronide were predominantly absorbed
and detected at the early stage of feeding in the serum of
puerarin-fed rats. Moreover, orally administered puer-
arin was widely distributed in tissues (kidney, liver, lung,
pancreas, heart, eye, and brain) of rats (28–30), sug-

**Fig. 5.** Inhibitory effects of puerarin on TNFα+CaPO4-induced ROS production. Inhibitory effects of puerarin on ROS levels during TNFα+CaPO4-induced macrophage activation; RAW 264.7 cells were seeded in 96-well plates (4,000 cells/well) containing DMEM and were incubated for 24 h. Subsequently, the culture medium was replaced with MEM-α containing 10% FBS and puerarin. After 48 h, cells were gently washed twice with PBS, and dichloro-dihydro-fluores-
cencin diacetate (DCFH-DA) was added and incubated for 60 min at 37°C in the dark. After washing cells twice with PBS, the fluorescence intensities of 2',7'-dichloro-
fluorescein (DCF) were determined using a fluorometric plate reader. Relative fluorescence intensities are means ± SD; * p<0.05.
was intraperitoneally administrated to mice to enhance its bioavailability. However, 100 μM or larger concentrations of puerarin required for in vitro assay were shown to be higher than the approximately 20 μM concentrations of puerarin in blood when puerarin was intraperitoneally administered to mice (45). The macrophage activation assay using RAW264.7 might not reflect the in vivo response of macrophages. Therefore, we propose further studies using bone marrow-derived primary cell culture to resolve the differences between in vivo and in vitro.

In summary, the present data show inhibitory effects of puerarin on the formation of aneurysms in mice and suppression of macrophage activation in arterial walls. Moreover, in vitro assays showed inhibitory effects of puerarin on macrophage activation, likely reflecting antioxidant activities. Although further research is required, the present study warrants consideration of puerarin as a novel therapeutic agent for an aneurysm.

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