Puerarin Ameliorates Experimental Alcoholic Liver Injury by Inhibition of Endotoxin Gut Leakage, Kupffer Cell Activation, and Endotoxin Receptors Expression

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ABSTRACT

Puerarin, an isoflavone component extracted from Kudzu (Pueraria lobata), has been demonstrated to alleviate alcohol-related disorders. Our study examined whether puerarin ameliorates chronic alcoholic liver injury through inhibition of endotoxin gut leakage, the subsequent Kupffer cell activation, and endotoxin receptors expression. Rats were provided with the Liber-DeCarli liquid diet for 8 weeks. Puerarin (90 mg/kg or 180 mg/kg daily) was orally administered from the beginning of the third week until the end of the experiment. Chronic alcohol intake caused increased serum alanine aminotransferase, aspartate aminotransferase, hepatic gamma-glutamyl transpeptidase, and triglyceride levels as well as fatty liver and neutrophil infiltration in hepatic lobules as determined by biochemical and histologic assays. A significant increase of liver tumor necrosis factor α was detected by enzyme-linked immunosorbent assay. These pathologic effects correlated with increased endotoxin level in portal vein and upregulated protein expression of hepatic CD68, lipopolysaccharide-binding protein, CD14, Toll-like receptor 2, and Toll-like receptor 4. Meanwhile, the intestinal microvilli were observed to be sparse, shortened, and irregularity in distribution under the transmission electron microscope in conjunction with the downregulated intestinal zonula occludens-1 protein expression. These hepatic pathologic changes were significantly inhibited in puerarin-treated animals as were the endotoxin levels and hepatic CD68 and endotoxin receptors. Moreover, the pathologic changes in intestinal microvillus and the decreased intestinal zonula occludens-1 were also ameliorated with puerarin treatment. These results thus demonstrate that puerarin inhibition of endotoxin gut leakage, Kupffer cell activation, and endotoxin receptors expression is involved in the alleviation of chronic alcoholic liver injury in rats.

Introduction

Puerarin [7-hydroxy-3-(4-hydroxyphenyl)-1-benzopyran-4-one 8-β-D-glucopyranoside] (Supplemental Fig. 1) is an isoflavone component extracted from Kudzu (Pueraria lobata), a medicinal herb that has been used to treat alcohol abuse in traditional Chinese medicine for more than a millennium. Puerarin has been demonstrated to alleviate alcohol-related disorders, including suppressing alcohol consumption and preference in rodents (Lin et al., 1996; Overstreet et al., 1996) and humans (Penetar et al., 2012) and reducing the anxiety symptoms associated with alcohol withdrawal (Overstreet et al., 2003). Recently, puerarin protection against liver injury has been reported (Zhang et al., 2006; Zhao et al., 2010; Liu et al., 2012). However, the effects of puerarin on alcoholic liver injury induced by long-term ethanol (EtOH) intake and potential effective mechanisms have yet to be examined.

Endotoxin, a toxic lipopolysaccharide (LPS) component of Gram-negative gut bacteria, has been revealed to play an essential role in the pathologic progression of alcoholic liver injury, in addition to alcohol metabolism and oxidant stress (Rao et al., 2004; Seth et al., 2011). LPS levels in the portal vein are elevated in patients with alcoholic liver cirrhosis (Szabo and Bala, 2010), and this has been confirmed in the alcoholic liver disease (ALD) rodent model (Tsukamoto et al., 2008). Increased plasma LPS levels in continuous intragastric alcohol-fed rodents are correlated with the grade of liver injury histology (Tsukamoto et al., 2008).

Abbreviations: ALD, alcoholic liver disease; ALT, alanine aminotransferase; AST, aspartate aminotransferase; EtOH, ethanol; GGT, gamma-glutamyl transpeptidase; KCs, Kupffer cells; LBP, lipopolysaccharide-binding protein; LPS, lipopolysaccharide; mCD14, membrane-bound CD14; MD-2, myeloid differentiated protein-2; TEM, transmission electron microscope; TG, triglyceride; TJ, tight junctions; TLR, Toll-like receptor; TNF-α, tumor necrosis factor α; ZO-1, zonula occludens-1.
The mechanisms of EtOH-induced endotoxemia include diminished phagocytosis of Kupffer cells (KCs), bacterial overgrowth in the small intestine, alteration of gastrointestinal epithelial barrier function, and increased intestinal permeability (Rao et al., 2004). Potent host response to endotoxin is involved in the ordered interactions of endotoxin with receptors, lipopolysaccharide-binding protein (LBPs), membrane-bound CD14 (mCD14) or soluble CD14, and extracellular soluble myeloid differentiated protein-2 (MD-2) or MD-2 bound to Toll-like receptor 4 (MD-2-TLR4). When gut-derived endotoxin is released into the circulation, it binds to LBP with high affinity. LBP increases the exposure of the normally concealed hydrophobic lipid A, catalyzing the extraction of individual endotoxin molecules by soluble CD14 or mCD14 (Lee et al., 1993; Gioannini and Weiss, 2007). The endotoxin monomer is then rapidly delivered to MD-2 or MD-2-TLR4, which results in activation of target cells such as KCs (Nagai et al., 2002; Park et al., 2009) to promote nuclear factor-κB translocation into nuclear and transcription of proinflammatory cytokines, especially tumor necrosis factor α (TNF-α) (Su et al., 2000). TNF-α can further increase gut permeability and induce apoptosis and the production of other cytokines, which perpetuates and progresses liver injury (Bradham et al., 1998). A significant body of evidence indicates that endotoxin plays a crucial role in hepatocellular damage by activating KCs to secrete proinflammatory cytokines, especially TNF-α. The mechanism of KC activation releasing TNF-α to promote liver injury induced by gut-derived endotoxin in ALD is illustrated in Supplemental Fig. 2.

Our study investigated the effects of puerarin on endotoxin gut leakage, KC activation, and endotoxin receptors expression in chronic alcoholic liver injury in rats.

Materials and Methods

Animals and Treatments. Male Sprague-Dawley rats (160 ± 10 g), obtained from the Shanghai Laboratory Animal Center of the Chinese Academy of Sciences, were acclimatized for 7 days after delivery. All rats were maintained on a reverse 12-hour light/dark cycle. Animal handling and procedures were performed according to international guidelines for the use and care of laboratory animals. The experimental protocol was approved by the local ethics committee.

The rats were divided into four groups: control (n = 10), EtOH (n = 10), EtOH plus high-dosage puerarin (180 mg/kg daily, n = 10), and EtOH plus low-dosage puerarin (90 mg/kg daily, n = 10) group. The Lieber-DeCarli control and EtOH liquid diets were prepared according to the Lieber-DeCarli diet prescriptions (Lieber and DeCarli, 1982), as previously described elsewhere (Fang et al., 2006). Rats in the control group ingested the Lieber-DeCarli liquid control diet, and the other rats ingested the Lieber-DeCarli liquid alcohol diet. One liter of the liquid diet contains 1000 Kcal energy: EtOH provided 36% of the energy in the liquid alcohol diet, which was substituted with dextrin-maltose in the liquid control diet. The reagents in the Lieber-DeCarli formula were purchased from Sinopharm Chemical Reagents (Shanghai, People’s Republic of China).

Rats were single-cage reared, and their liquid diet was ingested via free feeding without additional water or chow for 8 weeks. At the beginning of the third week, the rats in the high- and low-dosage puerarin groups were administrated puerarin (Shanghai Winherb Medical &T Development, Shanghai, People’s Republic of China) by gavage at 180 mg/kg or 90 mg/kg daily, respectively; the other rats received an equal volume of sterile water. At the end of the eighth week, the rats were anesthetized with pentobarbital sodium (Sigma-Aldrich, St. Louis, MO) (45 mg/kg i.p.). Liver tissue, intestinal samples, and serum were collected and stored in −80°C for histologic, biochemical, immunohistochemical, and Western blot analysis. For endotoxin detection, 2 ml of blood from the portal vein was collected in a pyrogen-free, heparin-pretreated tube.

Histologic Examination. Liver and ileum tissues were formalin-fixed and embedded in paraffin. Sections (4 μm thick) were stained with hematoxylin and eosin and examined under a light microscope (Olympus Medical Systems, Tokyo, Japan). Frozen hepatic tissue (7 μm thick) was embedded in optimum cutting temperature compound (Sakura Finetek USA, Torrance, CA) and stained with oil red O reagent (Sinopharm Chemical Reagents) for hepatic lipid observation under a light microscope (Olympus Medical Systems).

Histologic liver damage was evaluated via an index that was calculated by adding the scores of macrovesicular steatosis, microvesicular steatosis, inflammation, and necrosis, as previously described elsewhere (Keshavarzian et al., 2001). The severity of macrovesicular steatosis was scored as 0 (no hepatocytes), 1 (<25% hepatocytes), 2 (26–50% hepatocytes), 3 (51–75% hepatocytes), and 4 (>75% hepatocytes) containing several droplets of fat. Macrovesicular steatosis was rated 0 to 4, according to increasing degree, similar to microvesicular steatosis. The severity of the inflammation was scored as 0 to 4: none (0), minimal (1), mild (2), moderate (3), and severe (4); it was based on the degree of portal and lobular inflammation and evidence of piecemeal and spotty necrosis. The degree of necrosis and necrotic hepatocytes was scored as 0 to 4: none, minimal, mild, moderate, and severe, respectively (Keshavarzian et al., 2001).

Intestinal injury was evaluated by the following parameters: epithelial cell injury/loss, mucin (goblet cell) loss, mucosal edema, and the degree of inflammatory cells within the lamina propria and in the epithelial layer (intraepithelial lymphocytes) (Keshavarzian et al., 2001). As previously described elsewhere (Keshavarzian et al., 2001), the degree of epithelial cell injury/loss was graded as follows: 0 (none), 1 (focal superficial epithelial cell injury), 2 (one to two glands lost with or without mild superficial ulceration), 3 (three glands lost with or without moderate areas of ulceration), or 4 (more or four glands lost with or without severe mucosal ulceration). The degree of cellular mucin depletion was graded as 0 (none), 1 (focal), 2 (one to two glands lost), 3 (three to four glands lost), or 4 (greater than five glands lost). The degree of lamina propria edema was assessed as 0 (none), 1 (focal), 2 (superficial), 3 (superficial to mild), and 4 (diffuse edema). The inflammatory infiltrate was scored qualitatively as 0 (no increased inflammation), 1 (mild), 2 (moderate), 3 (severe and focal), and 4 (severe and diffuse).

Ileum samples from two random rats in every group were prepared for transmission electron microscope (TEM) analysis. As previously described elsewhere (Gul et al., 2013), ileum tissues were immersed in 2% glutaraldehyde (pH 7.4) and chilled for 2 hours. After rinsing several times in cold sodium phosphate buffer, the tissues were then postfixed in 1% osmium tetroxide solution for 2 hours at 4°C. After being dehydrated in a series of graded EtOH, tissues were placed into propylene oxide and embedded in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate, and were examined by TEM (Philips Tecnai-12; Biotwin, Amsterdam, The Netherlands).

Serum Alanine Aminotransferase and Aspartate Aminotransferase Assays. The activity of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in serum was determined with the corresponding biochemical assay kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, People’s Republic of China).

Hepatic Gamma-Glutamyl Transpeptidase Assay. Liver tissue (100 mg) was homogenized in 1 ml of 0.9% NaCl and then centrifuged at 1000 g for 15 minutes at 4°C. The supernatant was removed into clean tubes and was centrifuged at 3,000g for 10 minutes, avoiding the upper adipose and removing the transparent
for gamma-glutamyl transpeptidase (GGT) assay with a commercial biochemical assay kit (Nanjing Jiancheng Bioengineering Institute).

**Hepatic Triglyceride Assay.** Liver tissue (200 mg) was homogenized in 3 ml of ethanol-acetone mixture (1:1 in volume). The total hepatic triglyceride (TG) was extracted in the medium at 4°C overnight then was centrifuged at 1000 g for 20 minutes at 4°C; the supernatant was removed for the TG assay with a commercial TG analysis kit (Donggu Bioengineering, Zhejiang, People's Republic of China).

**Endotoxin Assay in Plasma from Portal Vein.** Blood collected from portal vein was centrifuged at 500 g for 15 minutes at 4°C. The plasma was removed immediately for analysis with the Pyrochrome Limulus Amebocyte Lysate kit (Associates of Cape Cod, East Falmouth, MA), according to the manufacturer's instructions.

**Immunohistochemical Assessment of Hepatic C6D8.** As previously described elsewhere (Peng et al., 2008), 4-μm-thick paraffin sections were used for immunohistochemical assessment. To detect C6D8 expression, we used the commercially available antibodies monoclonal anti-rat C6D8 (AbD Serotec, Raleigh, NC) and horseradish peroxidase-linked goat anti-mouse IgG (Santa Cruz Biotechnology, Santa Cruz, CA). Diamino benzidine was applied as a chromogen, and hematoxylin was used for counterstaining. The negative control was performed on liver sections from EtOH-fed rats, where the C6D8 antibody was alternated with phosphate-buffered saline buffer.

**Measurement of TNF-α Content by Enzyme-Linked Immunosorbent Assay.** Liver TNF-α was isolated as previously described elsewhere (Lambert et al., 2003) and was determined using a commercially available enzyme-linked immunosorbent assay kit (Invitrogen, Camarillo, CA) according to the manufacturer's instructions. The results were corrected by protein quantification with a commercially available bicinchoninic acid protein concentration assay kit (Beyotime Institute of Biotechnology, Jiangsu, People's Republic of China) and were expressed as μg/mg of protein.

**Determination of C6D8 and Endotoxin Receptors in Liver Tissue and Intestinal Zonula Ocludens-1 Protein Expression by Western Blot Analysis.** As described previously (Peng et al., 2008; Peng et al., 2009), the total protein was extracted from liver and intestine tissues, and was analyzed with a bicinchoninic acid protein concentration assay kit (Beyotime Institute of Biotechnology). Western blot analysis was performed to evaluate the protein expression of hepatic C6D8, LBP, CD14, TLR2, TLR4 and intestinal zonula occludens-1 (ZO-1) using commercial antibodies: mouse anti-rat glyceraldehyde-3-phosphate dehydrogenase antibody (Kangchen Bio-Tech, Shanghai, People's Republic of China); mouse anti-rat C6D8 antibody (AbD Serotec); mouse anti-rat CD14 antibody, goat anti-rat LBP antibody, rabbit anti-TLR4 antibody, rabbit anti-rat ZO-1 antibody (Santa Cruz Biotechnology); rabbit anti-rat TLR2 antibody (Epitomics, Burlingame, CA); goat anti-mouse IgG, goat anti-rabbit-IgG peroxidase-linked antibody (Santa Cruz Biotechnology); and rabbit anti-goat IgG, peroxidase-linked antibody (Jackson ImmunoResearch Laboratories, West Grove, PA). Tissue lysates were separated by electrophoresis in 10% SDS-PAGE separating gel, with the Bio-Rad electrophoresis system (Bio-Rad Laboratories, Hercules, CA). The enhanced chemiluminescence kit (Pierce Biotechnology, Rockford, IL) and the Fuji FR-980 image analysis system (Shanghai Fuji, Shanghai, People's Republic of China) were employed for revealing and quantitative analysis of the blots. Results were expressed as the ratio of protein to glyceraldehyde-3-phosphate dehydrogenase.

**Statistical Analysis.** For parametric data, results were expressed as mean ± S.D. The data were analyzed using one-way analysis of variance followed by the least significant difference post hoc test. We employed a t-test for the comparison of two parameters, and P < 0.05 was considered statistically significant. The non-parametric data were analyzed by Kruskal-Wallis H-test for comparison of more than two groups; subsequently, the Nemenyi test was employed for pairwise comparisons of multiple independent groups.

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**Results**

**Liquid Diet Intake, Body and Liver Weight.** At the end of the eighth week, there were no statistically significant differences in the diet intake and body weight among the groups (P > 0.05, Table 1). The liver/body weight ratio of the rats fed with EtOH liquid diets was statistically significantly increased when compared with rats in the control group (P < 0.01, Table 1), but there was no statistically significant difference between the puerarin-treated and EtOH groups (P > 0.05, Table 1).

**Effects of Puerarin on Liver Injury and Steatosis Induced by Lieber-DeCarli Diet.** Liver injury was examined by biomarkers of liver damage and histologic changes in liver tissue. The serum ALT and AST activities statistically significantly increased in the EtOH group compared with the control rats: ALT, 124.02 ± 41.68 versus 21.81 ± 7.90 U/l (P < 0.01); AST, 81.25 ± 30.16 versus 26.70 ± 7.99 U/l (P < 0.01) (Fig. 1C). The serum ALT of the rats in the puerarin group was obviously decreased: 180 mg/kg daily of puerarin, 67.76 ± 19.60 versus 124.02 ± 41.68 U/l (P < 0.01); 90 mg/kg daily of puerarin, 89.45 ± 19.21 versus 124.02 ± 41.68 U/l (P < 0.05) (Fig. 1C). The serum AST activity in the puerarin group demonstrated similar trends: 180 mg/kg daily of puerarin, 49.70 ± 15.33 versus 81.25 ± 30.16 U/l (P < 0.01); 90 mg/kg daily of puerarin, 59.28 ± 12.25 versus 81.25 ± 30.16 U/l (P < 0.05) (Fig. 1C). On the other hand, the hepatic GGT activity obviously increased after EtOH liquid diet intake: 49.75 ± 3.97 versus 10.83 ± 2.11 U/μg protein. (P < 0.01) (Fig. 1C). After puerarin administration, hepatic GGT activity statistically significantly decreased: 180 mg/kg daily of puerarin, 15.78 ± 2.69 versus 49.75 ± 3.97 U/μg protein. (P < 0.01); 90 mg/kg daily of puerarin, 14.01 ± 1.45 versus 49.75 ± 3.97 U/μg protein. (P < 0.01), respectively (Fig. 1C).

After chronic alcohol intake for 8 weeks, microvesicular steatosis was observed in the most regions of the lobules, and macrovesicular steatosis was found predominantly in centrilobular regions. Lipid vacuoles occupied much of the hepatocyte cytoplasm, and the nucleus and other organelles were pushed to the periphery of the cell. Some hepatocytes appeared bloated, with a wispy, rarefied cytoplasm. The inflammatory cells such as neutrophil and lymphocytes were scattered located in the pericellular region (Fig. 1A). Analysis of the histologic liver damage index scores showed that the scores of alcohol intake rats (median 9, range: 6–10) increased significantly (P = 0) compared with control (median 0). Puerarin of

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**TABLE 1**

<table>
<thead>
<tr>
<th>Groups (n = 10)</th>
<th>Liquid Diet Intake</th>
<th>Body Weight</th>
<th>Liver Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>ml</strong></td>
<td><strong>g/kg body weight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2726 ± 75</td>
<td>262 ± 8</td>
<td>20.06 ± 2.99</td>
</tr>
<tr>
<td>Ethanol</td>
<td>2707 ± 218</td>
<td>257 ± 50</td>
<td>28.07 ± 5.70**</td>
</tr>
<tr>
<td>Puerarin, 180 mg/kg/d</td>
<td>2701 ± 214</td>
<td>266 ± 31</td>
<td>29.30 ± 2.73**</td>
</tr>
<tr>
<td>Puerarin, 90 mg/kg/d</td>
<td>2715 ± 123</td>
<td>264 ± 22</td>
<td>30.90 ± 3.61**</td>
</tr>
</tbody>
</table>

**P < 0.01 compared with control, d, daily.**
high-dosage treatment ameliorated the pathologic changes significantly (median 4.5, range: 3–6; \( P = 0.04 \)).

Oil red O staining was used to visualize hepatic lipid deposition. In the EtOH-fed group, severe macrovesicular steatosis was observed along with neutrophil and lymphocyte infiltration. Puerarin treatment (180 mg/kg daily [d]) alleviated these pathologic changes significantly. (B) Oil red O staining for liver tissue. Large droplets colored with oil red O were widespread in the hepatic lobules of EtOH-fed rats. In the puerarin-treated groups (180 mg/kg or 90 mg/kg daily), the droplets of oil red O deposit were smaller and limited, which indicated that hepatic steatosis was mitigated with puerarin administration (Fig. 1B).

The total TG extracted by liver-tissue homogenization was tested. As expected, long-term alcohol exposure elicited an almost 5-fold increase in hepatic TG levels as compared with control animals: 103.53 ± 13.59 versus 22.39 ± 9.19 mg/g tissue (\( P < 0.01 \)) (Fig. 1D). The levels of hepatic TG decreased remarkably in the animals treated with high-dosage puerarin: 180 mg/kg daily of puerarin 65.97 ± 27.54 versus 103.53 ± 13.59 mg/g tissue (\( P < 0.01 \)); 90 mg/kg daily of puerarin 85.53 ± 30.58 versus 103.53 ± 13.59 mg/g tissue (\( P > 0.05 \)) (Fig. 1D).
Effects of Puerarin on Endotoxin Level and Hepatic TNF-α Concentration. The link between endotoxin and liver injury in ALD has been well demonstrated (Rao et al., 2004). In our study, the endotoxin level in the portal vein was found to be remarkably increased after long-term EtOH intake: 0.54 ± 0.09 versus 0.37 ± 0.05 EU/ml ($P < 0.01$) (Fig. 2A); however, it was decreased in the puerarin-treated groups: 180 mg/kg daily of puerarin 0.40 ± 0.05 versus 0.54 ± 0.09 EU/l ($P < 0.01$); 90 mg/kg daily of puerarin 0.46 ± 0.12 versus 0.54 ± 0.09 EU/l ($P > 0.05$) (Fig. 2A).

Endotoxemia activates KCs to produce proinflammatory factors, which subsequently promote liver injury. As the primary proinflammatory factor induced by gut-derived endotoxin, TNF-α plays an important role in mediating parenchymal cell injury in ALD. In our study, hepatic TNF-α was observed to be statistically significantly increased after 8 weeks of EtOH intake compared with the control group: 88.18 ± 12.08 versus 33.76 ± 6.50 μg/mg protein. ($P < 0.05$) (Fig. 2B). However, with puerarin administration, hepatic TNF-α obviously decreased: 180 mg/kg daily of puerarin 61.19 ± 13.87

![Graph A](image1)

![Graph B](image2)

![Control](image3)
![EtOH](image4)
![Puerarin (180mg/kg.d)](image5)
![Puerarin (90mg/kg.d)](image6)

![Pathologic changes](image7)

![Western blot analysis](image8)

**Fig. 2.** Effects of puerarin on endotoxin levels in the portal vein, hepatic TNF-α concentration, intestine injury, and ZO-1 protein expression. (A) Endotoxin level in the portal vein determined by limulus amebocyte lysate kit. (B) Hepatic TNF-α content analyzed by enzyme-linked immunosorbent assay (ELISA). Values are mean ± S.D., $n = 10$ animals/group. (C) Pathologic intestinal changes were observed in the H&E stained sections. No obvious intestinal injury was found in the control or EtOH-fed rats. (D) Intestinal ultrastructure viewed by TEM. Microvilli were sparse, shortened, and irregular in distribution in the EtOH-fed rats. In the puerarin group, no obvious pathologic changes in the microvilli of the intestine were seen. (E) Western blot analysis for intestinal ZO-1 protein expression. These data were representative of three independent experiments. **$P<0.01$ compared with control; *$P<0.05$, **$P<0.01$ compared with EtOH.**
versus 88.18 ± 12.08 μg/mg protein. (P < 0.05); 90 mg/kg daily of puerarin 67.13 ± 10.35 versus 88.18 ± 12.08 μg/mg protein. (P < 0.05) (Fig. 2B).

Effects of Puerarin on Ingestion Intensity and Tight Junction, ZO-1, Protein Expression. Endotoxins normally penetrate the gut epithelium only in trace amounts because of the intestinal barrier (Mathurin et al., 2000; Parlesak et al., 2000; Lambert et al., 2003). The disruption of the epithelial barrier’s function by EtOH results in increased intestinal permeability to injurious factors such as LPS and contributes to endotoxemia in ALD (Rao et al., 2004).

In our study, when compared with the control rats, the hematoxylin and eosin stained sections of intestinal tissue from rats with chronic alcohol intake showed focal to superficial lamina propria edema and mild increased inflammatory but no obvious epithelial cell injury/loss or cellular mucin depletion (Fig. 2C). The histologic injury scores of the intestinal tissue showed no statistically significant differences among the control (median 0.5, range: 0–1), EtOH (median 1.5, range: 1–3), or daily puerarin (180 mg/kg, median 1, range: 0–3; 90 mg/kg, median 1, range: 0–1) groups. These results were consistent with those of other studies (Persson et al., 1990; Mathurin et al., 2000; Rao et al., 2004).

However, the ultrastructural findings were more pronounced (Fig. 2D). With TEM observation, the microvilli on the epithelial cells were found to be thin and scattered, shortened, and irregular in distribution in the EtOH-diet rats; these ultrastructural alterations of microvilli were not apparent in the puerarin-treated animals (Fig. 2D). The barrier function of the intestinal epithelium is provided by tight junctions (TJ), highly specialized junctional complexes located at the apical end of epithelial cells. ZO-1 is a well-studied TJ protein. In our study, ZO-1 protein expression was found to be significantly downregulated by EtOH intake compared with control rats (Fig. 2E). Meanwhile, after puerarin treatment, the protein expression of ZO-1 in intestine tissue was clearly upregulated (Fig. 2E).

Effects of Puerarin on KC Activation and Protein Expression of Endotoxin Receptors in Liver. CD68/macrosialin, a transmembrane protein expressed by activated tissue macrophages (Rabinowitz and Gordon, 1991), was detected as a marker of activated KCs. Immunohistologic assays found little CD68-positive staining in the hepatic sinusoidal of control rats (Fig. 3A); in the livers of EtOH-diet rats, the CD68-positive staining in hepatic tissue was strong and diffuse, especially accompanied by steatosis (Fig. 3A). The CD68-positive staining was thin and decreased in the liver sections of puerarin group (Fig. 3A) compared with the EtOH group. (The negative control of the CD68 immunostaining is shown in Supplemental Fig. 3.) The protein expression tested by Western blot assay revealed that the CD68 expression in the liver was upregulated significantly after chronic alcohol intake; after puerarin treatment, the protein expression of CD68 was remarkably downregulated compared with the EtOH group (Fig. 3B).

LB, the essential protein for LPS transfer in the circulation, is predominantly produced by the liver (Su et al., 1994). LB binds to LPS with high affinity to form the LPS-LBP complex, transferring LPS to the surface receptors on target cells (KCs) such as mCD14. TLRs are pattern-recognition receptors that recognize the signals of LPS delivered by LB and CD14, thus activating downstream cascades. As was shown by the protein expression assay, in the EtOH-diet group, the protein expression of endotoxin receptors (such as LBP, CD14, TLR4, and TLR2) in liver tissue was remarkably increased as compared with the control group. With puerarin administration, the protein expression of endotoxin receptors was significantly downregulated (Fig. 4).

Discussion

ALD encompasses a spectrum of injury, ranging from simple steatosis to hepatitis and cirrhosis. Even if fatty drops disappear after abstinence, steatosis still increases the susceptibility of hepatocytes to further injury (Teli et al., 1995; Day and James, 1998). Continued ingestion of alcohol results in subsequent steatohepatitis, characterized by neutrophil infiltration, hepatocyte degeneration, ballooning, and onotic necrosis (Bautista, 2002; French, 2002). At this latter stage, normal hepatic histology is rarely recovered, even after EtOH withdrawal (French, 2002). Alcoholic steatohepatitis triggers the pathologic progress to fibrosis followed by cirrhosis; this stage appears to represent a rate-limiting step in the progression of ALD (Galambos, 1972; Diehl, 2002). Therefore, blocking or reversing the early phase histopathologic changes (steatosis and steatohepatitis) is a key strategy in ALD treatment.
By use of the Lieber-DeCarli model, which mimics the early stage of human ALD, our study has confirmed the effects of puerarin on alcoholic liver injury and steatosis. Hepatic histologic alteration and the biomarkers of liver damage in serum or liver tissue induced by the EtOH diet were ameliorated with puerarin treatment. The lipid deposition and increased hepatic TG concentration that accompanied EtOH intake were remarkably inhibited in the puerarin-treated animals.

Endotoxin-induced liver injury plays an important role in ALD. Plasma LPS levels in patients with ALD are higher compared with levels in healthy subjects (Parlesak et al., 2000) and patients with nonalcoholic liver disease (Fukui et al., 1991). It also has been observed the alcoholic liver injury in rats is associated with increased levels of plasma endotoxin (Mathurin et al., 2000; Tsukamoto et al., 2008). Furthermore, administration of antibiotics (Adachi et al., 1995) and probiotic bacteria (Wang et al., 2012) to rodents reduces the growth of Gram-negative bacteria in the intestinal lumen and prevents EtOH-induced endotoxemia and liver injury. It has been suggested that in ALD the endotoxin levels at specific locations such as in portal blood might be more important than the levels in systemic plasma (Lambert et al., 2003). In our study, endotoxemia in the portal vein and obvious heightened TNF-α levels in the liver tissue of the EtOH-diet group reproduced the important role of gut-derived endotoxin in the pathogenesis of ALD. Meanwhile, puerarin’s inhibition of endotoxemia and hepatic TNF-α release supports the hypothesis that, in addition to inhibiting alcohol intake (Lin et al., 1996; Overstreet et al., 1996) and reducing oxidation and modulation activity of cytochrome P450 (Zhao et al., 2010), the potential mechanism of puerarin’s amelioration of alcoholic liver injury is probably related to the pathway of gut-derived endotoxin-activating KCs.

One mechanism by which alcohol induces endotoxemia is through alteration of the gastrointestinal epithelial barrier function, which subsequently increases intestinal permeability to endotoxins (Rao et al., 2004). The barrier function of the intestinal epithelium is provided by epithelial cells and the paracellular apical junction complex, including the tight and...
adherence junctions (Farhadi et al., 2003). TJ are composed of several transmembrane proteins such as occludin and claudins and intracellular molecules such as ZO-1. We observed gross morphologic changes in ileum, the ultrastructure of epithelial cells, and ZO-1 protein expression in intestinal tissue. As previously demonstrated by others (Persson et al., 1990; Mathurin et al., 2000; Rao et al., 2004), no obvious morphologic changes were found under light microscope after long-term EtOH exposure or oral puerarin administration. But the ultrastructural finding of pathologic changes in the microvilli of epithelial cells was accompanied by decreased protein expression in intestinal TJ, the ZO-1 expression in the EtOH-diet rats indicated intestine barrier function injury. With puerarin administration, the pathologic changes in the microvilli were mitigated, and ZO-1 protein expression was upregulated, which was probably associated with the mechanisms by which puerarin inhibits gut leakage induced by alcohol. Moreover, oxidative stress has been suggested to critically mediate alcohol-induced intestinal barrier dysfunction (Kaur et al., 1998; Keshavarzian et al., 2009). Inflammatory responses and cytokines, especially TNF-α, have also been found to increase TJ permeability (Suenaaert et al., 2002; Ma et al., 2004). Puerarin’s inhibition of the oxidative stress (Hwang et al., 2011; Liu et al., 2012) and TNF-α production (Liu et al., 2010; Huang et al., 2012) found in various diseases provides an indirect explanation of its protection of gut epithelial barrier function after alcohol intake. However, the precise mechanism by which puerarin inhibits the gut leakage induced by alcohol needs more investigation.

KCs, the resident macrophages in the liver, are one of the major sources of TNF-α induced by LPS. They ensure maximal liver function by removing bacteria and phagocytosing foreign materials (Ajakaiye et al., 2011). KCs are quiescent in the absence of stimulatory agents. In ALD, gut-derived endotoxins could active KCs to release several inflammatory cytokines, most significantly TNF-α, contributing to subsequent liver damage. We found that CD68, a marker of activated KCs (Rabinowitz and Gordon, 1991), was increased in the liver tissue of the EtOH-diet rats, indicating that alcohol had induced more KC activation; this was confirmed by the remarkable heightened hepatic TNF-α content we also found in the EtOH-diet group. Meanwhile, in the animals treated with puerarin, the expression of CD68 protein was downregulated, which obviously indicates inhibition of KC activation.

To activate KCs, endotoxin signal delivery depends on a series of endotoxin receptors. LBP, which is produced mostly by hepatocytes (Su et al., 1994), is secreted into the bloodstream where it binds with high affinity to the lipid A portion of LPS and catalyses the transfer of individual LPS molecules to cell-surface receptors such as mCD14, forming a monomeric LPS-CD14 complex (Lee et al., 1993; Gioannini and Weiss, 2007). With the stimulation of proinflammatory cytokines, LBP is constitutively synthesized in hepatocytes (Elsing et al., 2011); this is consistent with our results: in the rats on a long-term EtOH diet, protein expression of hepatic LBP was markedly upregulated, in contrast to its downregulation after puerarin administration.

Molecular CD14 anchors on the membranes of peripheral or liver-resident microphages (KCs) through glycosylphosphatidylinositol (GPI). The LPS-CD14 complex activates cells through TLR4 (Poltorak et al., 1998; Hoshino et al., 1999). It has been established that TLR4 is the specific receptor of LPS from Gram-negative bacteria (Uesugi et al., 2001; Su, 2002). Signaling through TLR4 requires MD-2, a secreted protein that is closely associated with the extracellular domain of TLR4 (Shimazu et al., 1999). We found that the protein expression of CD14 and TLR4 in liver tissue was up-regulated by chronic EtOH intake and was significantly inhibited by puerarin administration.

In addition to LPS, other bacterial components are likely translocated to the portal blood in alcoholics, since disruption of intestinal barrier by EtOH increases permeability for macromolecular substances in general (Rao, 2009). TLR2 appears to primarily respond to Gram-positive bacteria-derived lipoteichoic acid, peptidoglycan, and mycobacterial lipoarabinomannan (Akira et al., 2001). In addition to TLR4, increased expression of TLR2 has been observed in wild-type mice under the Lieber-DeCarli chronic-alcohol feeding model, where feeding with alcohol results in sensitization to liver inflammation and damage because the administration of TLR2 ligands increases the expression of TNF-α (Gustot et al., 2006). On the other hand, MD-2 also enables TLR2 to respond to endotoxin protein-free LPS and enhances TLR2-mediated responses to both Gram-negative bacteria and their LPS (Dziarski et al., 2001). Similarly, the cytokines induced by LPS, such as interleukin-1β (IL-1β) or TNF-α, upregulate TLR2 mRNA expression in rat hepatocytes both in vivo and in vitro (Liu et al., 2000). We found the protein expression of TLR2 to be remarkably upregulated in the liver tissue of the EtOH-diet rats and inhibited by puerarin administration.

Our study supports puerarin as a potential reagent for protecting against chronic alcoholic liver injury and steatosis. Puerarin’s inhibition of alcohol-induced endotoxin gut leakage, KC activation, and protein expression of endotoxin receptors may be associated with the potential mechanisms of its pharmacologic effects on alcoholic liver injury.

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Authorship Contributions

Participated in research design: Hu, Peng.
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