Ethanol extraction of *Picrorhiza scrophulariiflora* prevents renal injury in experimental diabetes via anti-inflammation action

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Abstract

There is evidence that inflammatory processes are involved in the development and/or progression of diabetic nephropathy. However, effective treatment for inflammation in the kidneys of diabetic is practically unknown. The rhizomes of *Picrorhiza scrophulariiflora* (PS) are a traditional medication long used to treat inflammatory diseases. The aim of the present study was to test the hypothesis that the ethanol extract of PS (EPS) may reduce inflammation in patients with diabetic kidneys. Streptozotocin-induced diabetic rats were randomly assigned to two groups treated with a gavage of either EPS or vehicle. A group of non-diabetic control rats was treated concurrently. Compared with vehicle-treated diabetic rats, EPS-treated animals displayed a significant decrease in renal macrophage infiltration and overexpression of chemokine (C-C motif) ligand 2 (CCL2) and TGFβ1. This was associated with attenuation of the structural and functional abnormalities of early diabetic nephropathy, such as glomerular hypertrophy, mesangial expansion, and albuminuria. Administration of EPS significantly reduced NADPH oxidase-dependent superoxide generation and decreased expression of malondialdehyde and advanced oxidation protein products in diabetic kidney. These data suggest that EPS might improve diabetic nephropathy, probably through inhibition of redox-sensitive inflammation.


Introduction

Diabetic nephropathy is a major complication of diabetes and a leading cause of end-stage renal failure in many countries. Although hyperglycemia is the underlying metabolic abnormality, many other mechanisms have also been proposed for the development of diabetic nephropathy (Schrijvers et al. 2004). Increasing evidence shows that aberrant inflammatory responses, such as macrophage infiltration and overexpression of proinflammatory cytokines, are present in the diabetic kidney (Ihm 1997, Sassy-Prigent et al. 2000, Chow et al. 2004) and linked to renal damage and fibrosis in both humans and animal models (Furuta et al. 1993, Young et al. 1995, Sassy-Prigent et al. 2000, Chow et al. 2004, Yozai et al. 2005). Although the involvement of inflammatory processes in the development and/or progression of diabetic nephropathy has been recognized, intervention for inflammation in the diabetic kidney is practically unknown.

*Picrorhiza* is a perennial herb belonging to the family of Scrophulariaceae. The dried rhizomes of *Picrorhiza* (a traditional medication) have long been used to treat inflammatory diseases such as arthritis and asthma in Southeast Asia (Yang et al. 2003). Two species of *Picrorhiza*, *Picrorhiza kurrooa* (PK) and *Picrorhiza scrophulariiflora* (PS), have been identified with similar characteristics in morphology, histology, bitterness, and the known active chemical components (Zhang et al. 1965). *P. kurrooa* grows on the western slopes of the Himalayas in India and *P. scrophulariiflora* is found in Tibet and China. *Picrorhiza* is almost insoluble in water but soluble in organic solvents (*Luper* 1998, Smit et al. 2000). Although many components of *Picrorhiza* have not been identified, evidence has shown that organic solvent extracts of *Picrorhiza*, such as picroside I, II, and kutkoside, have antioxidant activity similar to that of superoxide dismutase (*Chander* et al. 1992). Therefore, we hypothesized that the ethanol extract of PS (EPS) would decrease inflammation in the diabetic kidney and thus slow down the development of the early structural and functional abnormalities which lead to diabetic nephropathy.

Materials and Methods

Preparation of EPS

The dried rhizomes of *P. scrophulariiflora* (2 kg, Tongrentang Group Co., Beijing, China) were ground and extracted with hot ethanol (60 °C, 500 ml, 12 h) five times. Ethanol was removed in vacuo and the residues (478 g) were dried in 60 °C for 12 hours. HPLC analysis showed that the extract...
contained two known active components of *P. scrophulariiflora*, picroside I (4.5%) and picroside II (8.4%). We prepared three batches of the extract, which contained similar quantities of picroside I (5.5 ± 0.4%) and picroside II (9.2 ± 0.9%). The extracts were stored at −20 °C and a homogeneous suspension of EPS in water was prepared before gavage. The same batch of EPS was used for the experiments.

**Animal model**

Experimental diabetes was induced in male Sprague–Dawley rats (initial weight 230–250 g, obtained from the Southern Medical University Animal Experiment Center) by i.p. injection of STZ (50 mg/kg; Sigma) after overnight fasting (Shi et al. 2008). Animals with non-fasting serum glucose levels >20 mmol/l 1-week post-injection of STZ were included in the study as diabetic models. Diabetic rats were randomized into two groups (n = 12 in each group), matched for body weight and blood glucose, and treated as follows: group 1, gavage of EPS suspension (400 mg EPS/kg per day), and group 2, gavage of water. The amount of EPS administered was according to our preliminary data in accordance with guidelines set by the Animal Experiment Committee of Southern Medical University.

At the end of 5 or 10 weeks of treatment, the animals (n = 6 in each group at each time point) were anesthetized with sodium pentobarbital and exsanguinated. The kidneys were collected after perfusion with 50 ml ice-cold normal saline. The 24-h urine samples were collected in metabolic cages at end of the study period. All animal procedures were in accordance with guidelines set by the Animal Experiment Committee of Southern Medical University.

**Renal function and urine examination**

Serum and urine creatinine levels were determined using a commercial kit (sarcosine oxidase–peroxidase–antiperoxidase; Zixing, Shanghai, China), according to the manufacturer’s instructions. The creatinine clearance rate (Ccr) was calculated as described and factored for body weight (Li et al. 2007a). 24 hr UAE was measured using the Coomassie Blue method (Lott et al. 1983).

Urinary chemokine (C–C motif) ligand 2 (CCL2) and transforming growth factor, beta 1 (TGFB1) were quantified by ELISA using the commercial kits (BioSource, Camarillo, CA, USA), according to the manufacturer’s instructions.

**Renal morphometric analysis**

Renal morphometric analysis was performed as described previously (Sassy-Prigent et al. 1995). A transversal slice at the level of the hilus was fixed in periodate–lysine–paraformaldehyde solution and embedded in paraffin. Sections (2 μm thick) of the renal tissues were stained with periodic acid–Schiff for morphometric analysis. The measured glomerular parameters included 1) total glomerular surface area (Ag) and 2) mesangial surface area/glomerular tuft surface area. In each renal section, the observer blindly measured at least 30 consecutive unselected glomeruli randomly distributed over the depth of the cortex. Polar glomerular sections were not measured. Individual glomerular volume (Vg) was calculated as Vg = 1.25 × (Ag)3/2 (Li et al. 2007a).

**Renal immunohistochemical analysis**

The immunoperoxidase staining was performed as described (Taal et al. 2001). Macrophage infiltration was detected with monoclonal anti-ED-1 (Serotec, Oxford, UK), TGFB1 expression with polyclonal rabbit anti-rat TGFB1 (Santa Cruz Biotechnology, Santa Cruz, CA, USA), CCL2 with polyclonal rabbit anti-rat-CCL2 (Boster Biologic, Wuhan, China), and fibronectin expression with monoclonal anti-rat fibronectin (Lab Vision Corporation, Fremont, CA, USA). Control experiments included omission of the primary antibodies with substitution of non-immune rabbit or mouse IgG.

Macrophage infiltration was quantified by counting the number of ED-1-positive cells in 30 glomerular profiles and in 20 randomly chosen 0.3-mm areas of the tubulointerstitium for each kidney (Li et al. 2007a). The intensity of glomerular staining of TGFB1, CCL2, and fibronectin was evaluated at a 400× magnification on a 0–3 scale as follows: 0, no staining; 1, weak and spotty intraglomerular staining; 2, moderate and segmental intraglomerular staining; and 3, strong and diffuse (involving >50%) intraglomerular staining. Likewise, the intensity of tubulointerstitial staining in cortical areas was assessed at 250× magnification on a 0–3 scale as follows: 0, no staining; 1, <25% involvement; 2, 25–50% involvement; and 3, >50% involvement. All glomeruli (at least 20) in 20 randomly selected cortical tubulointerstitial areas of each sample were evaluated.

**Real-time RT-PCR**

Total RNA was prepared using the RNA extraction kit with DNase I treatment following the manufacturer’s instructions (Promega). First-strand cDNA was synthesized with random primers and the ReverTra Ace-α cDNA Synthesis Kit (Toyobo Co., Ltd, Osaka, Japan). Primers were designed using the Primer Express oligo design software (Applied Biosystems, Foster City, CA, USA) and synthesized by Invitrogen. All primer sets were subjected to rigorous database searches to identify potential conflicting transcript matches to pseudogenes or homologous domains within related genes. The sequences of the real-time PCR primers are as follows. TGFB1: sense 5′-GTGGGCTGAACCAAG-GAGACG-3′, antisense 5′-TGATCCCTATGGATTTCCA CGT-3′; CCL2: sense 5′-CAGATGCAGTTAATGCCC.
CCA-3′, antisense 5′-CCTGCTGCTGGATTCTTT-3′; and β-actin: sense 5′-CTGAAAGATGACCCAGATCA-3′, antisense 5′-GTGGTACGACCAGAGGCATACA-3′. The SYBR Green I assay and the MX3005P thermocycler (Stratagene, La Jolla, CA, USA) were used for detecting the products from the reverse-transcribed cDNA samples. β-actin was used as the normalizer. PCRs for each sample were performed in duplicate, and the relative gene expressions were analyzed as described previously (Livak & Schmittgen 2001).

Western blot
Renal cortex homogenates in ice-cold mild lysis buffer were centrifuged at 1000 g, 4 °C for 15 min, and supernatants were collected and resolved with SDS-PAGE as described previously (Guo et al. 2008). After blocking, the membranes were incubated with the rabbit polyclonal anti-TGFB1 or goat polyclonal anti-CCL2 antibody (Santa Cruz Biotechnology), reacted with the HRP-linked anti-rabbit IgG, and then detected by ECL (Pierce, Rockford, IL, USA).

Detection of lipid peroxides and advanced oxidation protein products in renal tissue
Lipid peroxides in the renal cortical homogenate were measured as thiobarbituric acid reactive substances (TBARS) by fluorometric assay (Yagi 1976). The level of advanced oxidation protein products (AOPP) in renal tissue was measured as described previously (Li et al. 2007a).

ROS production in renal tissue
NAD(P)H-dependent superoxide (O2−) production was assessed by lucigenin–enhanced chemiluminescence as described previously (Li et al. 2002). Tissue homogenate (100 μg/well) was placed in wells of a 96-well microplate. Immediately before recording, dark-adapted lucigenin (100 μm) with or without NAD(P)H (100 μmol/l) was added to each well. Light emission was recorded every minute for 30 min (VICTOR V Wallac 1420; PerkinElmer, Turku, Finland). Data are presented as counts/min/100 μg of protein. To confirm the intracellular source of O2−, the procedure was repeated in the presence of NAD(P)H oxidase inhibitor (i.e. diphenyleneiodonium (DPI) 10–100 μmol/l), a nitric oxide synthase inhibitor (N ω-nitro-l-arginine methyl ester (L-NAME) 100–1000 μmol/l), a xanthine oxidase inhibitor (oxyurinol, 10–100 μmol/l), a mitochondria inhibitor (rotenone, 2.5–250 μmol/l), and a cyclooxygenase inhibitor (indomethacin, 10 μmol/l).

Statistical analysis
Data are presented as mean ± s.e.m. One-way ANOVA was used for analyzing differences in variables between groups at the same time point. When P was ≤ 0.05, the least significant difference method was used for comparison. Independent sample t-test was used for analyzing the difference in the same variable between two time points. SPSS 11.5 software was used for statistical analysis. P ≤ 0.05 indicated a statistically significant difference.

Results

Characteristics of experimental animals
Compared with normal controls, diabetic rats had hyperglycemia, lower body weight, and nephromegaly (Table 1; P < 0.05). The renal index ratio at the 10th week was significantly lower in the EPS-treated group than the vehicletreated diabetes group (P < 0.05). However, EPS had no obvious effect on body weight and blood sugar in rats with diabetes. No toxicity of EPS was observed in the EPS-treated group during the study period.

EPS treatment relieved inflammatory response in diabetic kidney
At the 10th week, infiltration of ED-1-positive cells in the glomeruli and interstitium was significantly greater in diabetic rats than in normal controls (P < 0.05). EPS treatment significantly decreased the renal macrophages infiltration in diabetic animals (P < 0.05; Fig. 1).

Urinary CCL2 excretion and expression of Ccl2 mRNA and protein in renal tissue were significantly higher in diabetic rats than in normal rats (P < 0.05). Compared with the vehicle-treated group, the EPS-treated group showed a significantly lower level of urinary CCL2, decreased expression of CCL2 in glomeruli and interstitium at both the mRNA and protein levels (P < 0.05; Fig. 2).

Table 1. Biochemical and metabolic parameters for all animals at weeks 5 and 10a

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Week 5 (n=6)</th>
<th>Week 10 (n=6)</th>
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<tbody>
<tr>
<td>Body weight (g)a</td>
<td>226.17±35.76†</td>
<td>223.00±20.82†</td>
</tr>
<tr>
<td>Group 1 (DM+EPS)</td>
<td>223.00±20.82†</td>
<td>218.83±12.89†</td>
</tr>
<tr>
<td>Group 2 (DM+vehicle)</td>
<td>231.33±37.11†</td>
<td>230.00±13.87†</td>
</tr>
<tr>
<td>Group 3 (control)</td>
<td>326.50±43.98</td>
<td>370.00±36.48</td>
</tr>
<tr>
<td>Kidney hypertrophy index (g/kg)a</td>
<td>5.16±0.46‡</td>
<td>5.26±0.40‡</td>
</tr>
<tr>
<td>Group 1 (DM+EPS)</td>
<td>5.26±0.40‡</td>
<td>5.26±0.40‡</td>
</tr>
<tr>
<td>Group 2 (DM+vehicle)</td>
<td>5.21±0.42‡</td>
<td>5.27±0.38‡</td>
</tr>
<tr>
<td>Group 3 (control)</td>
<td>2.98±0.27</td>
<td>3.18±0.43</td>
</tr>
<tr>
<td>Blood glucose (mmol/l)x6</td>
<td>32.88±2.56‡</td>
<td>35.67±3.18‡</td>
</tr>
<tr>
<td>Group 1 (DM+EPS)</td>
<td>35.67±3.18‡</td>
<td>35.67±3.18‡</td>
</tr>
<tr>
<td>Group 2 (DM+vehicle)</td>
<td>32.00±2.34‖</td>
<td>35.10±3.88‡</td>
</tr>
<tr>
<td>Group 3 (control)</td>
<td>9.92±1.38</td>
<td>10.75±1.24</td>
</tr>
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DM, diabetes mellitus; EPS, ethanol extraction of Picrorhiza scrophulariiflora.
*ANOVA, P < 0.001; †P < 0.001 versus group 1; ‡P < 0.05 versus group 2.
Data are expressed as mean ± s.e.m.
Blood glucose concentration was measured by an automatic biochemistry analyzer (AU5000; Olympus, Tokyo, Japan).
Compared with normal rats, diabetic rats showed significantly increased urinary excretion of TGFB1 and renal expression of TGFB1 at both the mRNA and protein levels (P < 0.05). At the 5th and 10th treatment week, the urinary TGFB1 excretion, TGFB1 immunostaining score, and expression of Tgfb1 mRNA and protein were significantly lower in the EPS-treated group when compared with the vehicle-treated diabetic animals (Fig. 3).

EPS treatment improved diabetic kidney abnormalities

Compared with normal controls, diabetic rats had an increased glomerular volume and mesangial area at the 10th week (Fig. 4A). EPS treatment significantly reduced the glomerular volume and mesangial area in diabetic animals compared with vehicle-treated diabetic rats (P < 0.05; Fig. 4B and C).

EPS treatment reduced UAE and Ccr in diabetic rats

The 24-h UAE and Ccr were significantly increased in diabetic rats compared with normal controls. At the 5th and 10th week, UAE and Ccr were significantly decreased by EPS treatment in diabetic rats (P < 0.05; Table 2).

EPS treatment decreased oxidative stress in the diabetic kidney

Higher levels of TBARS and AOPP were found in the renal cortex lysates from diabetic rats compared with those from normal controls. The NAD(P)H-dependent generation of superoxide was increased in renal cortex in diabetic rats at the 10th week. The levels of TBARS, AOPP, and superoxide generation were significantly decreased in EPS– than vehicle–treated group (P < 0.05; Fig. 5A–C). In diabetic rats, superoxide generation in renal tissue could be significantly inhibited by the NAD(P)H oxidase inhibitor DPI, but not by other enzyme inhibitors (Fig. 5C).

Discussion

This study demonstrated that the rhizome of P. scrophularia-flora, a traditional medication with potential anti-inflammatory activity, improved early diabetic nephropathy in a STZ-induced diabetic model. Administration of EPS significantly attenuated kidney inflammation in this model, as evidenced by a decrease in macrophage infiltration and downregulated expression of CCL2 and TGFB1 at both the gene and protein levels. The renal benefit of EPS was further
supported by the finding that EPS administration improved
the early structural and functional abnormalities of diabetic
nephropathy, such as glomerular hypertrophy, mesangial
expansion, and albuminuria. To the best of our knowledge,
this is the first study demonstrating the in vivo renal protective
effects of P. scrophulariiflora in diabetes.

Diabetic nephropathy is generally considered a non-immune
disease. However, aberrant inflammatory responses, such as
macrophage infiltration and overexpression of CCL2, have been
demonstrated in the diabetic kidney (Ihm 1997, Sassy-Prigent
associated inflammatory processes have been found to occur
during early diabetic renal change as well as in established
diabetic nephropathy in both humans and animal models, and
have been linked to glomerular and tubular damage and renal
fibrosis (Furuta et al. 1993, Young et al. 1995, Sassy-Prigent et al.
2000, Chow et al. 2004, Yozai et al. 2005). Although these
reports support the notion that inflammatory processes are
involved in the development and/or progression of diabetic
nephropathy, little is known about the effect of anti-
inflammatory interventions in the diabetic kidney. Consistent
with previous studies, the present study showed increased
oxidative stress in diabetic kidneys, as demonstrated by increased
generation of superoxide and oxidative products such as TBARS
and AOPP. Reactive oxygen species generated in oxidative stress
have been found to signal the activation of TGFB1, the main
inflammatory transcription factor that triggers transcription of
several inflammatory mediators (Kusch & Medford 1999).
These inflammatory mediators can act in concert promoting tissue damage, particularly through leukocyte recruitment and cell proliferation (Stenvinkel et al. 2003). The present study demonstrated, in vivo, that administration of EPS significantly attenuated oxidative stress in the diabetic kidney, as evidenced by a reduction in superoxide production and decrease in TBARS and AOPP in renal tissue. This was accompanied by an improvement in renal inflammation, including decreased macrophage influx and downregulated expression of CCL2 and TGFB1. Further support for the anti-inflammatory effects of EPS comes from a study showing that organic extracts of P. scrophulariiflora inhibit non-specific and specific immune responses in vitro and improve carrageenan-induced paw edema in vivo (Smit et al. 2000).

Increasing evidence indicates that cellular inflammation may play an important role in the onset and progression of diabetic nephropathy (Navarro & Mora 2005), as macrophage infiltration was frequently found in the diabetic kidney at different stages of diabetic nephropathy and correlated with the progression of kidney disease (Coimbra et al. 2000, Sassy-prigent et al. 2000, Chow et al. 2004). It was further shown that intrarenal accumulation of macrophages was associated with prolonged hyperglycemia in type 2 diabetic nephropathy (Chow et al. 2004) and promoted by the increased expression of intercellular adhesion molecules (ICAM1; Sugimoto et al. 1997), E- and P-selectin (Shikata & Makino 2001), and CCL2 (Kato et al. 1999, Aoyama et al. 2002, Shi et al. 2008) in both diabetic patients and animal models. Previous reports and our preliminary study found that early insulin treatment significantly decreased renal macrophage infiltration in STZ-induced diabetic rats, supporting a link between hyperglycemia and cellular inflammation in this model (Wu et al. 2006). The present study showed that EPS administration, without altering glycemic levels, inhibited...
renal macrophage infiltration and improved early structural and functional abnormalities of diabetic nephropathy in STZ-induced diabetic rats. This might be important, as we provided new data supporting that an anti-inflammatory intervention is effective in diabetic nephropathy even in the presence of hyperglycemia. Consistent with our results, treatment with an angiotensin-converting enzyme inhibitor or an angiotensin II receptor blocker has been shown to suppress renal expression of CCL2 and to attenuate renal injury without altering glycemia control in diabetic rats (Kato et al. 1999, Li et al. 2003). The precise mechanisms by which EPS prevented cellular inflammation in the diabetic kidney remain to be studied. However, it might be related to inhibition of overexpression of chemokines such as CCL2, of adhesion molecules such as ICAM1 (Yang et al. 2006) and E- and P-selectin. Upregulation of TGFB1 may be the result of the initial inflammatory process, since it was turned on by oxidative stress and attenuated with the suppression of macrophage influx. The role of TGFB1 in growth and regeneration after initial damage has been linked to the fibrotic processes and progression of nephropathy (Liu 2006).

The chemical component responsible for the anti-inflammatory effects of EPS remains to be studied. Different components have been described in P. scrophulariiflora extracted by different organic solvents. The presence of apocynin was determined by HPLC in light petroleum, diethyl ether, and ethyl acetate extracts, while androsin, its O-β-D-glucoside, and picroside II were found in diethyl ether, ethyl acetate, and methanol extracts (Smit et al. 2000).

In the present study, compounds with small molecular mass picroside I (MW 492 kDa) and picroside II (MW 512 kDa) were identified in the ethanol extracts of P. scrophulariiflora. The significant antioxidant properties of picroside I and II have been demonstrated in both in vitro and in vivo studies (Chander et al. 1992, Gao & Zhou 2005, Li et al. 2007b).
Although we cannot exclude the possible effects of unidentified components in EPS, picroside I and II appear to play a major role in the in vivo anti-inflammation activity.

In this study, the dose of EPS for intervention is high (400 mg/kg per day), it may be related to the relatively lower levels of picrosides (12%) in the extract. For future clinical use, further effort will be required to improve the extraction and levels of picrosides (12%) in the extract. For future clinical use, (400 mg/kg per day), it may be related to the relatively lower

In conclusion, we demonstrate that administration of EPS reduces inflammation and attenuates the development of early structural and functional abnormalities of the diabetic kidney. The renal benefit of EPS might be associated with the inhibition of redox-sensitive inflammation.

Declaration of interest

No potential conflict of interest relevant to this article was reported.

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References


EPS inhibits inflammation in the diabetic kidney


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