Umbelliferone ameliorates renal function in diabetic nephropathy rats through regulating inflammation and TLR/NF-κB pathway

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[ABSTRACT] Diabetic nephropathy (DN) is a leading cause of renal failure, contributing to severe morbidity and mortality in diabetic patients. Umbelliferae (Umb) has been well characterized to exert protective effects in diabetes. However, the action and mechanism of Umb in DN remains unclear. In this work, we studied the effect of Umb in a streptozotocin (STZ)-induced DN rat model and explore its underlying mechanism. DN rats were treated with Umb (20, 40 mg·kg⁻¹) or irbesartan (15 mg·kg⁻¹) for 4 weeks. Levels of serum glucose, insulin, blood urea acid, creatinine, triglycerides (TG) and total cholesterol (TC) were measured by commercial assay kits, respectively. Histopathological changes and inflammatory cytokine levels including IL-6, IL-1β and TNF-α in the kidney were also evaluated. Alterations in the expression of podocin, CD2AP and TLR/NF-κB were assessed by western blotting. Our results showed that Umb reduced renal injury in DN rat model, as evidenced by the decrease in blood glucose, 24 h urinary protein, serum creatinine, and blood uric acid. Umb also significantly ameliorated the renal histopathological alteration, and down-regulated the expression of epithelial-to-mesenchymal transition-related molecular markers podocin and CD2AP. Moreover, Umb inhibited TLR2, TLR4, MyD88 expressions, NF-κB activation and considerably reduced levels of other downstream inflammatory molecules (TNF-α, IL-6, IL-1β). These findings indicated that Umb improved renal function through regulating inflammation and TLR/NF-κB pathway, suggesting the potential efficacy of Umb in DN treatment.

[KEY WORDS] Diabetic nephropathy; Umbelliferone; Streptozotocin; Inflammation; TLR/NF-κB pathway


Introduction

Diabetes mellitus is a serious chronic disease which affects millions of people worldwide. It is characterized by acquired deficiency of insulin and the ineffectiveness of the insulin generation [¹]. Chronic hyperglycemia contributes to glycation of body proteins which consequently results in a variety of complications affecting nerves, eyes, livers and kidneys [2]. Among these, diabetic nephropathy (DN) is one of the most serious microvascular complications of diabetes that seriously affects patients’ quality of life [³]. Pathologically, DN is characterized by a series of renal structure abnormalities such as increased urinary albumin excretion, increased basement membrane thickening and podocyte injury. Currently, DN is considered to be a progressive disease that involves several mechanisms, with changes in glomerular haemodynamics causing renal lesions, oxidative stress, inflammatory response, and fibrosis [⁴]. Accumulating evidence indicates that inflammatory mechanisms play a significant role in development and progression of DN [⁵]. In this context, tumor necrosis factor-alpha (TNF-α) and nuclear...
Materials and Methods

Main reagents and kits

Umb (purity 98%) was purchased from National Institutes for Food and Drug Control (Beijing, China). Streptozotocin (STZ) and IRB were obtained from Sigma (St Louis, MO, USA). Glucose, uric acid, creatinine, triglycerides (TG) and total cholesterol (TC) commercial kits were provided by Jiancheng Bioengineering Institute (Nanjing, China) and insulin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Nanjing KeyGen Biotech. Co., Ltd. (Nanjing, China). Primary antibodies against TLR, MyD88, p-NF-κBp65, NFKBp65, p-IκBα, IκBα were produced by Cell Signaling Technology (Danvers, USA).

Experimental animals

Male Sprague-Dawley rats weighing 200–220 g were obtained from Jiangning Qinglongshan Animal Cultivation Farm (Nanjing, China). Animals were housed at 23 ± 2 °C with 50% ± 5% humidity and with a 12 h light/dark cycle, and had free access to a standard rat pellet diet and tap water. The rats were allowed to acclimatize to their new location for 7 days prior to experiments. All the experimental protocols were approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine, and were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals (NIH Publications No. 8023, revised 1978).

Induction of experimental diabetes mellitus and experimental design

Diabetes was induced in the overnight fasted rats by single intraperitoneal injection (i.p.) of streptozotocin (135 mg/kg body weight) dissolved in 0.1 mol L⁻¹ citrate buffer (pH 4.5). Normal rats received only citrate buffer. After 3 days of STZ injection, blood glucose levels from the non-fasted rats were measured. Animals with 200 mg dL⁻¹ and above confirmed diabetic and were used for the study. After establishing the diabetic model, the rats were divided into five groups (n = 10 in each group) as follows: control, STZ, STZ + IRB (15 mg kg⁻¹), STZ + Umb (20 mg kg⁻¹) and STZ + Umb (40 mg kg⁻¹) groups. This doses of Umb and IRB have been proved to be effective and safe [19–20]. Umb and IRB were dissolved in 0.5% carboxymethyl cellulose-Na (CMC-Na, Solarbio, Beijing, China) and administered by gavage, once a day for 28 days. Rats in control and STZ groups were orally administrated with the same volume of 0.5% CMC-Na. On day 29, the animal study was terminated by sacrificing the animals to collect blood and kidney for further analysis. Serum and tissues collected were stored at −80 °C till further analysis.

Oral glucose tolerance test (OGGT)

On the last week of the experimental period, OGGT was performed. The rats were orally administered with 30% glucose (1.5 g kg⁻¹) after overnight fasting for 14 h after compounds treatment. Blood samples were collected from the orbit vein at 0, 30, 60, 90, 120 min after glucose
administration. The glucose levels were determined using serum glucose commercial kits according to the manufacturer’s instruction.

**Insulin assay**

The serum insulin levels were assayed by an enzyme-linked immunosorbent assay based on the manufacturer’s instruction.

**Detection of urinary albumin excretion**

Prior to sacrifice, rats were placed in metabolic cages to collect 24 h urine for measurement of albumin levels. The urine samples from each rat were combined and centrifuged. Aliquots of the supernatant were collected and frozen at –70 °C. The albumin levels were determined using an enzyme-linked immunobead assay with an anti-rat albumin antibody.

**Biochemical analysis**

Blood uric acid, creatinine, triglycerides (TG) and total cholesterol (TC) were determined using commercial kits provided by Jiancheng Bioengineering Institute (Nanjing, China) according to the experimental protocol.

**Histopathological procedures**

Paraffin-embedded kidney tissues were used for histopathological staining. The hematoxylin-eosin staining and Schiff periodic acid shift staining were applied to examine the tissue injury. The presence of tubular atrophy, connective tissue changes, fibrosis, inflammation, degeneration of tubular epithelial, congestion, and glomerular damage was evaluated to confirm the kidney damage. The histopathological evaluation was carried out by two pathologists in blinded manners.

**Determination of TNF-α, IL-1β, and IL-6 in kidney tissue**

Inflammatory cytokines including IL-6, IL-1β and TNF-α in the kidney were measured using ELISA kits according to the manufacturer’s instructions. The optical density (OD) of each well was read at 450 nm, and the concentrations of inflammatory cytokines were quantified by reference to the standard curves.

**Western blot analysis**

Kidney protein was extracted with lysis buffer for 30 min on ice and then centrifuged at 1 2000 r·min⁻¹ for 5 min at 4 °C to remove the debris. Total protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Nanjing, China). The samples were loaded on SDS-polyacrylamide gel electrophoresis and transferred onto the polyvinylidene difluoride membrane. The membrane was incubated with primary antibody in blocking buffer overnight at 4 °C, and then treated with secondary antibody for 1 h at room temperature after washing with TBST. The blotted protein bands were established and fixed by an ECL Advanced kit. Image analysis software was applied to quantify protein levels.

**Statistical analysis**

The data in the figures were expressed as means ± SDs. Assessment were analyzed by one-way analysis of variance (ANOVA) with Tukey multiple comparison test. All data were analyzed with GraphPad software, where \( P < 0.05 \) was considered significant.

**Result**

**Effects of Umb on body weight and kidney weight**

Diabetes is commonly characterized with body weight loss, which may be due to the protein wasting in a situation of unavailability of carbohydrate for utilization as an energy source. The treatment of diabetes is traditionally accompanied with body weight gain. In the present study, five diabetic groups were established at the beginning with the original body weight in each group of 265.6 ± 5.9, 261.9 ± 6.2, 263.6 ± 9.4, 267.7 ± 8.4, and 261.4 ± 10.4, respectively. After 4 weeks’ treatment, all the diabetic rats exhibited obviously gain in body weight with the increase rates of 77.1%, 15.1%, 33.6%, 33.6%, and 40.1%, separately (Table 1). The body weight of diabetic rats was still significantly lower than the normal rats (\( P < 0.01 \)). Also, significant increase of kidney weight was observed in diabetic rats (\( P < 0.01 \)). Administration of Umb (20 and 40 mg·kg⁻¹) could significantly increase the body weight and reduce the kidney weight of diabetic rats (\( P < 0.01 \)). The changes in body weights indicated that Umb could effectively prevent the loss of body weight in diabetic rats.

**Table 1** Effects of Umb on body weight and kidney weight in DN rats (means ± SD, \( n = 10 \))

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>STZ</th>
<th>IRB</th>
<th>Umb (mg·kg⁻¹)</th>
<th>20</th>
<th>40</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (0 week)</td>
<td>265.6 ± 5.9</td>
<td>261.9 ± 6.2</td>
<td>263.6 ± 9.4</td>
<td>267.7 ± 8.4</td>
<td>261.4 ± 10.4</td>
<td></td>
</tr>
<tr>
<td>Body weight (4 week)</td>
<td>470.3 ± 17.5</td>
<td>301.5 ± 22.9</td>
<td><strong>352.2 ± 17.9</strong></td>
<td><strong>357.6 ± 19.1</strong></td>
<td><strong>366.1 ± 20.6</strong></td>
<td></td>
</tr>
<tr>
<td>Kidney/mg</td>
<td>3036.3 ± 110.2</td>
<td>3845.1 ± 141.7</td>
<td><strong>3188.2 ± 119.2</strong></td>
<td><strong>3455.5 ± 131.1</strong></td>
<td><strong>3264.0 ± 128.7</strong></td>
<td></td>
</tr>
</tbody>
</table>

**Effects of Umb on oral glucose tolerance property and insulin resistance**

To investigate the blood glucose homeostasis, glucose tolerance ability of all the experimental rats was evaluated using OGTT test. As shown in Fig. 1, the STZ-induced diabetic rats showed significantly higher glucose level than the normal rats before glucose administration (\( P < 0.05 \)). After oral administration of glucose, the blood glucose level of all groups reached the peak at 60 min, and then the normal control reversed to the basal level after 120 min. However, the blood glucose level of diabetic rats was consistently maintained in a high level throughout the whole time (Fig.
Compared with diabetic control, administration of IRB and Umb resulted in a significant suppression on the blood glucose level \( (P < 0.05 \text{ or } P < 0.01) \). The results suggested that the impaired glucose tolerance in STZ-induced diabetic rats could be improved by Umb \[22\).

Insulin resistance usually attributes to the insulin deficiency, which leads to the failure of glucose homeostasis. To investigate the effects of Umb on the insulin resistance in STZ-induced diabetic rats, the serum insulin level was determined after 4 weeks’ treatment. As shown in Fig. 1B, the diabetic rats administrated with Umb \((40 \text{ mg} \cdot \text{kg}^{-1})\) exhibited a remarkable increase in insulin level compared with diabetic control. The results indicated that Umb was beneficial for the amelioration of insulin resistance in diabetic rats.

**Effects of Umb on serum profiles levels**

Liver is the primary organ for lipid metabolite and maintaining cholesterol homeostasis \[23\]. Chronical hyperlipidemia causes liver injury as well as insulin resistance substantially \[24\]. Thus, strategies aimed at preventing dyslipidemia are essential for the treatment of diabetes mellitus. Generally, dyslipidemia is characterized with elevated TC, TG, LDL-C levels and decreased HDL-C level in serum \[25\]. In the present study, a remarkable disorder in lipid profiles was observed in STZ-induced diabetic mice (Figs. 2A and 2B). After Umb treatment, significant decreases in serum TG, TC levels were observed with the reduction rates of 38.10%, and 15.0%, separately, compared to the diabetic control \( (P < 0.01) \), which is in corroboration with previous research work, which suggested that Umb was helpful for the normalization in lipid profiles in diabetic rats \[18\].

![](image1.png)

*Fig. 1 Effects of Umb on the blood glucose and serum insulin levels. (A): OGTT; (B): serum insulin levels. Rats were intraperitoneally injected with 135 mg·kg\(^{-1}\) of STZ. IRB (15 mg·kg\(^{-1}\)) and Umb (20, 40 mg·kg\(^{-1}\)) were intragastrically treated for consecutive 28 days. Values are expressed as means ± SD, \( n = 10 \). *\( P < 0.05 \), **\( P < 0.01 \) vs control; †\( P < 0.05 \), ‡\( P < 0.01 \) vs STZ*
Fig. 2  Effects of Umb on TG (A), TC (B), uric acid (C), creatinine (D), 24-h urinary protein levels (E). Values are expressed as means ± SD, n = 10. *P < 0.05, **P < 0.01 vs control; †P < 0.05, ‡P < 0.01 vs STZ

Effects of Umb on diabetic kidney injury

To assess the renal damage, biochemical markers such as blood uric acid (BUA), creatinine (Cre), and 24-h urinary protein (24-h U-PRO) were evaluated in all the groups. As shown in Figs. 2C–2E, a dramatic increase in levels of BUA, Cre and 24-h urinary protein were observed (all P < 0.01), indicating renal functional impairment. Treatment with Umb at the dosages of 20 and 40 mg·kg⁻¹, however, could significantly reduce the levels of BUA, Cre, and 24-h urinary protein in rats compared with the STZ-induced diabetic model group (For 40 mg·kg⁻¹, all P < 0.01). These results indicate that the therapeutic effect of Umb against diabetes-related kidney injury may be dose-dependent. In the present rat model, treatment with irbesartan elicited a similar biochemical response on rats as treatment with Umb, suggesting that Umb or irbesartan exerted an improved effect on renal functional impairment in diabetic rats.

Effects of Umb on renal tissue morphology

The impact of Umb treatment on pathological changes in the kidney tissues of rats was investigated with H&E and PAS. As shown in Fig. 3A, the animals in normal control group were observed with normal kidney architecture. STZ treatment produced obvious glomerular damage, whereas, treatment with Umb markedly attenuated the histopathological changes. Consistent with H&E staining, deleterious structural changes (e.g. brush border membrane loss, thickened basement membranes, PAS-positive materials deposition, and cast formation) were detected in the kidney sections of rats in STZ-induced diabetic model group. Administration of Umb abrogated these deleterious effects (Fig. 3B), suggesting that Umb has a potential to protect against diabetes-related kidney injury.

Fig. 3  Histological examination of the kidney sections. Kidney tissue sections from rats in the control, STZ, Irbesartan (15 mg·kg⁻¹), Umb (20mg·kg⁻¹) and Umb (40mg·kg⁻¹) were stained with HE (A) or PAS (B). Ultrathin kidney 50-nm-thick sections were examined under an electronic microscope.

Regulation of Umb on expression of epithelial-to-mesenchymal transition related molecular markers podocin and CD2AP in renal cortex tissues of rats

Researches have shown that high glucose could lead to epithelial-to-mesenchymal transition (EMT) in podocytes, which eventually caused the proteinuria occurrence and
development of diabetes mellitus. Inhibition of podocytes EMT could attenuate urinary protein and renal injury of diabetic nephropathy. After 4 weeks of treatment, the EMT related molecular markers podocin and CD2AP protein expression levels were measured to explore the mechanism of protective effect of Umb on renal impairment. The western blotting for podocin and CD2AP in renal tissues of the five groups was shown in Fig. 4. Podocin and CD2AP protein expression was decreased significantly in diabetic model group when compared to the control group. Treatment with Umb at dosages of 20 and 40 mg/kg significantly restored the decreased protein (Fig. 4).

**Effect of Umb on pro-inflammatory cytokines**

Pro-inflammatory cytokines like TNF-α, IL-1β, and IL-6 play an important role in the development of diabetes-related kidney injury. To explore the effects of Umb on inflammation, the levels of TNF-α, IL-6 and IL-1β were analyzed in the kidney. It was observed that STZ treatment resulted in significant ($P < 0.01$) increase in the levels of TNF-α, IL-1β, and IL-6 in model group, as compared to normal rats (Fig. 5). Treatment with Umb at both doses markedly ($P < 0.01$) restored the levels of all the pro-inflammatory cytokines studied to normal, compared to diabetic STZ rats (Fig. 5).

![Fig. 4](image-url)  
**Fig. 4** Effect of Umb on expression of epithelial-to-mesenchymal transition related molecular markers podocin and CD2AP in renal cortex tissues of rats. Values are expressed as mean ± SD, $n = 5$, *$P < 0.05$, **$P < 0.01$ vs control; †$P < 0.05$, ††$P < 0.01$ vs STZ.
Fig. 5  Effects of Umb on proinflammatory cytokines in the kidney. Values are expressed as mean ± SD, n = 10, *P < 0.05, **P < 0.01 vs control; *P < 0.05, **P < 0.01 vs STZ

Effect of Umb on TLR/NF-κB signaling pathway in renal tissues of rats

We further investigated whether Umb affected the activation of TLR/NF-κB pathway, the essential signaling transduction pathway involved in inflammation response. The western blot results showed that the expression levels of TLR2, TLR4 and MyD88 were increased 2.67-fold, 3.53-fold and 2.37-fold post STZ injection (Figs. 6A–6D). The nucleus accumulation of NF-κB (p65) was augmented by STZ (Figs. 6E–6F). Administration of Umb (20, 40 mg·kg⁻¹) suppressed diabetic-induced changes in TLR/NF-κB pathway related molecules (Figs. 6A–6F).
Fig. 6 Effect of Umb on TLR/NF-κB pathway-related pathway. Values are expressed as mean ± SD. Values are expressed as means ± SD, n = 5, *P < 0.05, **P < 0.01 vs control; #P < 0.05, ##P < 0.01 vs STZ.

Discussion

Previous studies have demonstrated that treatment with Umb presented attenuation of renal damage in type I diabetic rats [19]. However, the mechanisms underlying the action of Umb have not been clarified. The metabolic inflammation activated by TLRs is crucial for the pathogenesis of diabetic renal injuries, particularly in the early process of DN [26]. Therefore, the current study investigated the effect of Umb treatment on TLR/NF-κB pathway expression in the kidney tissues of rats in DN. The finding of present study suggested that treatment with Umb significantly reduce the levels of blood glucose, total TC, TG, uric acid and 24 h U-PRO, particularly when a higher dose of Umb (40 mg·kg⁻¹) was used in rats. Uric acid, creatinine are considered as the reliable renal function indices. The elevated content of creatinine and uric acid are the key features of DN. Administration of 40 mg·kg⁻¹ Umb provoked a comparative therapeutic effect compared with irbesartan, the positive control in the present experimental system. Treatment with Umb also significantly ameliorate the renal histopathological alteration, and down-regulate the expression of EMT-related molecular...
markers podocin and CD2AP in renal cortex tissues of rats. In addition, treatment with Umb dramatically reduced the levels of pro-inflammatory cytokines TNF-α, IL-1β, and IL-6, and inhibited the relative expression of TLR/NF-κB signaling which is crucial in the pathogenesis of DN.

Hyperglycemia is the main factor in the progression of diabetic nephropathy with increasing morbidity and mortality in patients. The pathogenesis of DN is related to aberrant action of cell signaling, abnormal immune and inflammatory responses, increased levels of the cytokines, TGF-β1 and NF-κB [27]. In the present study, HE stained kidney tissues of diabetic animals showed significant glomerular damage. Furthermore, PAS staining was identified in the kidney sections of rats in the model group, and stromal hyperplasia, glomerular basement membrane thickening along with increased mesangial matrix expansion were detected. Moreover, the values of EMT related molecular markers podocin and CD2AP were significantly increased in rats from the diabetic model group. Notably, upregulated pro-inflammatory cytokines (TNF-α, IL-1β, and IL-6) levels, and TLR2, TLR4, MyD88, p-NF-κB and p-IκB expression levels were detected in the kidney tissues of rats in the model group. These data indicated that during the early process of diabetes mellitus, aberrant activation of TLR/NF-κB signaling contributes to pro-inflammatory factor over-expression, as well as hyperglycemia-induced functional impairment and structural damage in the kidneys of rats. The present findings were in agreement with those from previous studies, which suggested that TLR2 and TLR4 are associated with acute kidney injury, chronic kidney diseases, and the occurrence of DN [28-31]. Therefore, the TLR/NF-κB signaling pathway may be a potential target for the development of therapies for DN.

Umb, namely 7-hydroxycoumarin, is a derivative of coumarin widely existing in plants from the Umbelliferae family like asafoetida (Ferula assafoetida), caraway (Carum carvi), coriander (Coriandrum sativum), and fruits like golden apple (Aegle marmelos), bitter orange (Citrus aurantium) as well as banana [32]. Umb displayed potent anti-inflammatory activity and provides protection from hyperglycemia-induced kidney injury. It is associated with the downregulation of TGF-β1 level [19]. The present study indicated that treatment with Umb significantly reduced uric acid, creatinine and 24h U-PRO. Treatment with Umb also found to reduce the glomerular damage, mesangial matrix expansion as well as the extent of fibrosis in HE and PAS stained kidney tissues. These findings indicated that Umb is able to protect hyperglycemia-induced kidney injury in animals and notably, that treatment with a higher dose of Umb (40 mg kg⁻¹) provided a comparative protective effect compared with irbesartan treatment. The present results are consistent with those from a previous study, which used a DN model [19]. Furthermore, the present study indicated that Umb treatment significantly attenuated hyperglycemia-upregulated TLR2, TLR4, MyD88, p-NF-κB and p-IκB expressions in the kidney tissues of rats. Given that TLR/NF-κB signaling positively regulates pro-inflammatory responses, it is possible that Umb may inhibit activation of the TLR/NF-κB pathway and in turn, mitigate hyperglycemia-induced inflammation, such as TNF-α, IL-1β, and IL-6, to protect the kidney from hyperglycemia-induced injury.

In conclusion, the present study demonstrated that treatment with Umb mitigated hyperglycemia-induced kidney impairment in a rat model of diabetes mellitus. Furthermore, treatment with Umb significantly mitigated hyperglycemia-induced upregulated TLR2, TLR4, MyD88, NF-κB and IκB expression in the kidney of diabetic rats, suggesting that the aberrant activation of TLR/NF-κB signaling may contribute to the DN development. The present findings may provide new insights into the molecular mechanisms underlying the action of Umb in treatment of DN. However, further studies are warranted before clinical application.

References


