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Original Article

Taxifolin attenuates diabetic nephropathy in streptozotocin-induced diabetic rats

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Abstract: The current study evaluates the effects of taxifolin (TA) on streptozotocin (STZ)-induced diabetic nephropathy in rats. We performed oral glucose tolerance tests (OGTTs) to determine blood glucose levels. We also measured the following biochemical parameters: uric acid, creatinine, and serum insulin. Kidney pathology was examined by staining sections with hematoxylin-eosin (H&E). The expression of Caveolin-1 and NF-κB was analyzed by qRT-PCR and western blotting. TA significantly reduced the concentrations of blood glucose, uric acid, creatinine, and serum insulin in STZ-induced diabetic rats. Pathological changes in kidneys of diabetes rats were alleviated by TA. Our data indicate that TA restored the levels of Caveolin-1/NF-κB signaling-related mRNA and proteins in diabetes rats. These combined results suggest that TA might mitigate the effects of STZ-induced diabetes.

Keywords: Taxifolin, streptozotocin (STZ), inflammation, Caveolin-1, NF-κB

Introduction

Diabetes mellitus (DM) is a serious chronic disease that affects millions of people worldwide. It is characterized by acquired insulin deficiency and ineffectiveness of the insulin that is generated [1]. Chronic hyperglycemia contributes to glycation of body proteins which consequently results in a variety of complications affecting nerves, eyes, livers and kidneys [2]. Diabetic nephropathy (DN) is one of the most serious microvascular complications of diabetes. DN is characterized by a renal structure abnormalities and inflammatory dysfunction. Inflammatory cytokines synthesized in several renal cells types involved in the progression of DN [3].

Streptozotocin (STZ) is an antibiotic produced by *Streptomyces achromogenes*. And it is a chemotherapy agent for treating pancreatic and other cancer types. It is commonly used to induce murine DM due to its toxic effects that cause oxidative damage and mimic cognitive decline, and hepatic and renal lesions [4]. Previous investigators have studied kidney injuries in STZ-induced diabetic mice [5].

Caveolae were first described in the 1950s; they were observed as small, 50-100 nm, cave-like invaginations in the plasma membrane. Caveolin formation requires certain structural components: Caveolin-1, Caveolin-2, and Caveolin-3. Each of these coat proteins have specific roles that vary in different cell types. As the key downstream effector, NF-κB is the key downstream effector of Caveolin-1; it is the regulator that controls inflammatory cytokine transcription, including IL-1β, IL-6, and TNF-α [6]. A previous study reported that Caveolin-1 could regulate the NF-κB pathway and participate in mediating inflammatory reaction [7].

Taxifolin (TA), also named dihydroquercetin, is a common flavonoid found in Pinaceae tree family, such as *Pseudotsuga taxifolia, Taxus chinensis, Cedrus deodara and Pinus roxburghii.* TA has a long medicinal history, and has been used clinically for the treatment of cardiovascular and cerebrovascular diseases [8]. Few studies have examined the effects of TA on diabetic. This study reports that TA exerts protective effects on renal injury in STZ-induced diabetic rats, and explores its potential mechanism.

Materials and methods

Experimental reagents

Taxifolin (TA, purity 98%) was purchased from National Institutes for Food and Drug Control (Beijing, China). Streptozotocin (STZ) was obtained from Sigma (St Louis, MO, USA). Glucose, uric acid, creatinine, and commercial kits were provided by Jiancheng Bioengineering Institute (Nanjing, China). The insulin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Nanjing KeyGEN Biotech. Co., Ltd. (Nanjing, China). Primary antibodies against Caveolin-1, p-NF-κBp65, NFκBp65, p-IκBα, and IκBα were produced by Cell Signaling Technology (Danvers, USA).

Animals

This study used 50 male rats (8 weeks old, weighing 200-220 g), which were obtained from the Animal Center of Wenzhou Medical University (Wenzhou, China). Animals were allowed to acclimatize to their new location for 7 days before experiments. All experimental procedures were performed in accordance with the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals.

Treatments

DM was induced by a single intraperitoneal administration of 135 mg/kg of streptozotocin (STZ, Sigma Chemical Co., St. Louis, MO, USA), which was dissolved in sodium citrate buffer at pH 4.2-4.5. Control animals were injected with the vehicle (sodium citrate buffer, pH 4.2-4.5). Rats were randomly assigned to five groups of 10 animals in each group as follows: control, STZ, STZ+ Allopurinol (ALL, 5 mg/kg), STZ + TA (10 mg/kg), and STZ + TA (20 mg/kg). After the seventh day post-STZ injection, TA (10 or 20 mg/kg/day) was intragastrically administrated by gavage for 28 consecutive days. Blood samples were collected from the orbit on day 29, and were centrifuged at 4,500 rpm for 15 min. Rats were sacrificed and kidneys were removed for further experiments.

Oral glucose tolerance test (OGTT)

Animals within each group (n = 10) were weighed, fasted for 14 h, and then treated with 30% glucose (1.5 g/kg) via oral administration. Blood samples were collected from the orbit vein at different times from 0 to 120 min, and the blood glucose levels were measured using commercial serum glucose kits.

Insulin assay

Serum insulin levels were measured using a commercial enzyme-linked immunosorbent assay kit according to the manufacturer's instruction.

Biochemical analyses

Kits for determining uric acid, and creatinine commercial kits were provided by Jiancheng Bioengineering Institute (Nanjing, China). The biochemical analyses for each component were conducted according to our study design and the recommended experimental protocols.

Histological evaluation

Rats were sacrificed, the tissue samples were collected immediately, fixed in 4% paraformaldehyde solution for 48 h, and then embedded in paraffin. Sections of 5 μm thickness were dewaxed according to the standard protocol. Then the histopathological evaluation was carried out by two pathologists in blinded manners.

RNA analysis

Total RNA was extracted using TRIzol reagent according to the manufacturer's instructions. Total RNA (2 μl) was reverse transcribed in a 20 μl reaction using the Quant Reverse Transcriptase kit (TianGen Biotech Co., Ltd.). Real-time quantitative PCR analyses were performed using a 7500 Real-Time PCR system (Thermo Fisher Scientific, Applied Biosystems). The following primers were used for qPCR amplification:

Caveolin-1 forward, 5'-GACCAAAGGAGCCAACGGAG-3', reverse, 5'-GACCACGTCGTCGTTGAGAT-3'; IκBα forward, 5'-CTGTTGAAGTGTGGGGCTGA-3', reverse, 5'-AGGGCAACTCATCTTCCGTG-3'; NF-κBp65 forward, 5'-CATGGATCCCTGCACACCTT-3', reverse, 5'-CTCAGCATGGAGAGTTGGCA-3'; GAPDH forward, 5'-AGTGCCAGCCTCGTCTCATA-3', reverse, 5'-GGTAACCAGGCGTCCGATAC-3'.

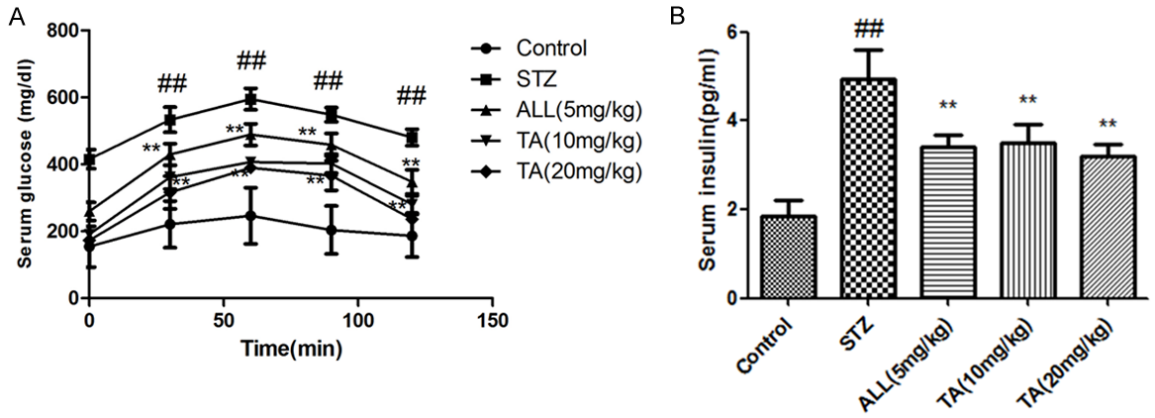

Figure 1. Effects of TA on blood glucose and serum insulin levels in STZ-induced diabetic rats. A: OGTT; B: Serum insulin levels. Rats were intraperitoneally injected with 135 mg/kg of STZ and then intragastrically treated with ALL (5 mg/kg) or TA (10 and 20 mg/kg) for consecutive 28 days. Values are expressed as means ± SDs. $^{\#\#}P<0.01$ compared with control; $^{**}P<0.01$ compared with the STZ-induced DM model.

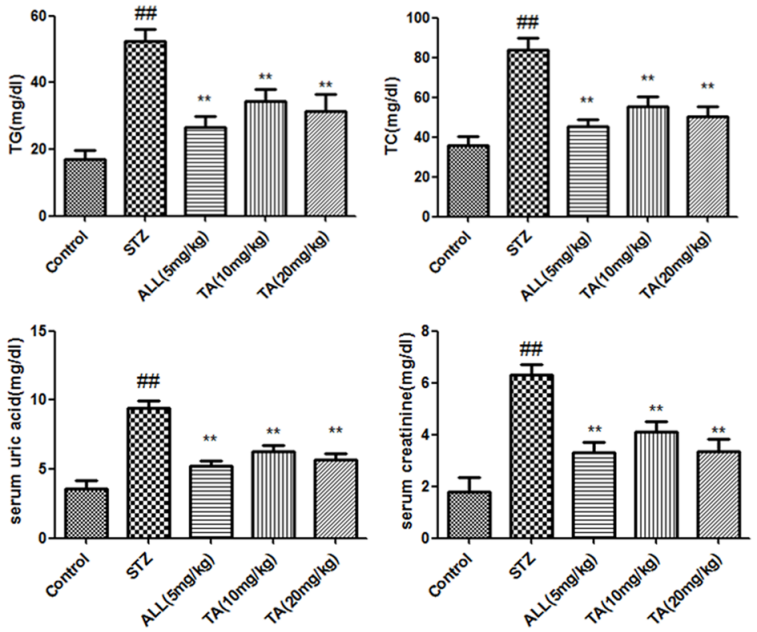

Figure 2. Effects of TA on uric acid and creatinine levels in STZ-induced diabetic rats. Rats were intraperitoneally injected with 135 mg/kg of STZ and then intragastrically treated with ALL (5 mg/kg) or TA (10 and 20 mg/kg) for consecutive 28 days. Values are expressed as means ± SDs. $^{\#\#}P<0.01$ compared with control, $^{**}P<0.01$ compared with the STZ-induced DM model.

Western blot analysis

Kidney protein was extracted in lysis buffer for 30 min on ice. The extract was centrifuged at 12,000 rpm for 5 min at 4°C to remove debris. Total protein concentration was determined using the bicinchoninic acid (BCA) protein assay kit (Beyotime, Nanjing, China). The samples were examined with SDS-polyacrylamide gel electrophoresis, and then transferred onto polyvinylidene difluoride membrane. The membrane was incubated with primary antibody overnight at 4°C blocked in skim milk. Then, the membrane was washed three times in TBST and treated with secondary antibody for 1 h at room temperature. The blotted protein bands were visualized and fixed by using the ECL Advanced kit. Image analysis software was applied to quantify protein levels.

Statistical analysis

Data are expressed as means ± SDs. Statistical significance was determined with one-way analysis of variance (ANOVA) and the Tukey multiple comparison test. All data were analyzed with GraphPad software, and $P<0.05$ was considered as significant.

Results

Effects of TA on OGTT and serum insulin resistance

As shown in **Figure 1**, STZ induced increases in blood glucose and serum insulin content as determined by OGTT. The results show that STZ-induced diabetic rats had greater increases in blood glucose levels than rats in the

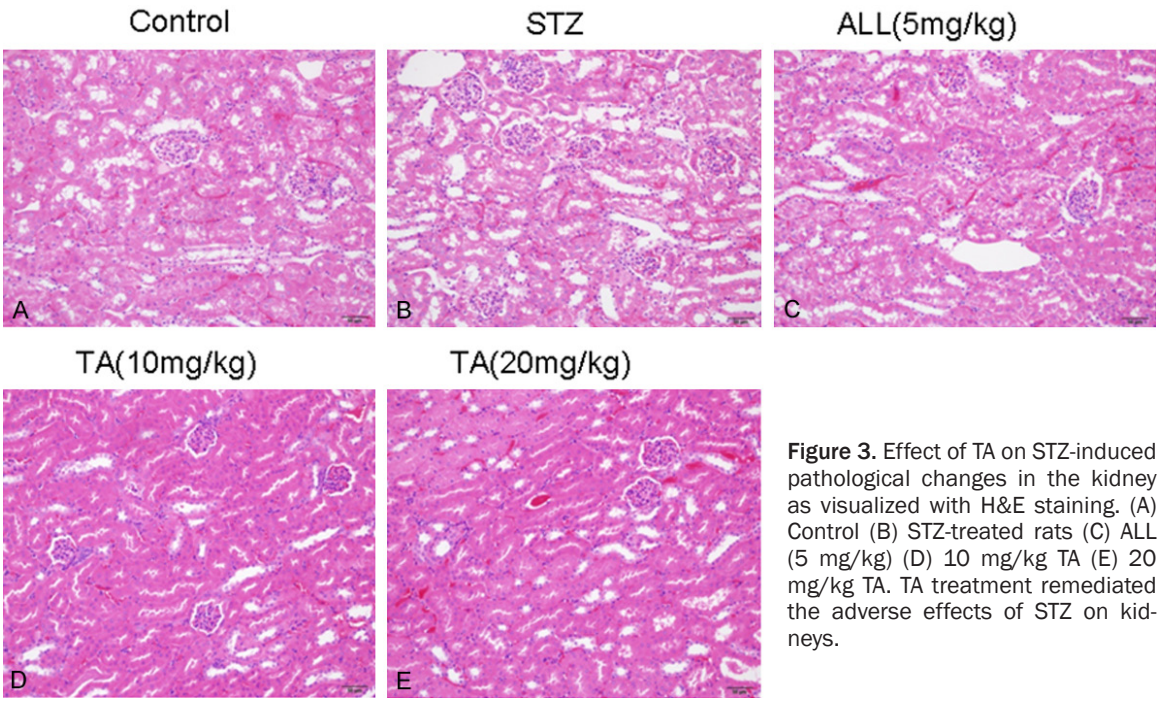

Figure 3. Effect of TA on STZ-induced pathological changes in the kidney as visualized with H&E staining. (A) Control (B) STZ-treated rats (C) ALL (5 mg/kg) (D) 10 mg/kg TA (E) 20 mg/kg TA. TA treatment remediated the adverse effects of STZ on kidneys.

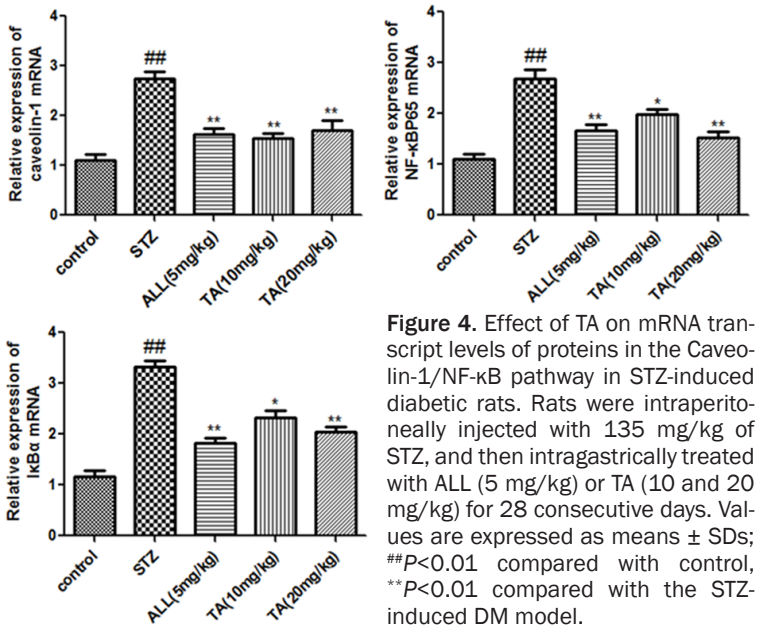

Figure 4. Effect of TA on mRNA transcript levels of proteins in the Caveolin-1/NF-κB pathway in STZ-induced diabetic rats. Rats were intraperitoneally injected with 135 mg/kg of STZ, and then intragastrically treated with ALL (5 mg/kg) or TA (10 and 20 mg/kg) for 28 consecutive days. Values are expressed as means ± SDs; $^{\#\#}P<0.01$ compared with control, $^{**}P<0.01$ compared with the STZ-induced DM model.

control group at different time points, suggesting that STZ also induced insulin resistance. However, TA (10 and 20 mg/kg) treatment significantly remediated blood glucose and serum insulin levels, which then maintain blood glucose homeostasis.

Effects of TA on renal function

The uric acid and creatinine levels were relatively higher in STZ-induced diabetic rats than in the control group. TA (10 and 20 mg/kg) treatment dramatically reduced the levels of uric acid and creatinine compared with those levels in the DM group, indicating that renal function improved after TA intervention (**Figure 2**).

Effects of TA on histological examination

Histological assessment of diabetic rats suggest necrosis symptoms as evidenced by thinned renal cortex, narrowed glomerular, increased matrix in mesangium, and vacuolar degeneration of glomerular epithelial cells. By contrast, STZ-induced diabetic rats treated with TA (10 and 20 mg/kg) had significantly improved histological results, suggesting that TA confers beneficial effect on renal tissues in STZ-treated rats (**Figure 3**).

Effects of TA on the Caveolin-1/NF-κB pathway

The expression of Caveolin-1/NF-κB pathway-related mRNA and protein was measured to evaluate the underlying mechanism of TA remediation of STZ-induced DM. The mRNA expression of Caveolin-1, IκBα, and NF-κB was signifi-

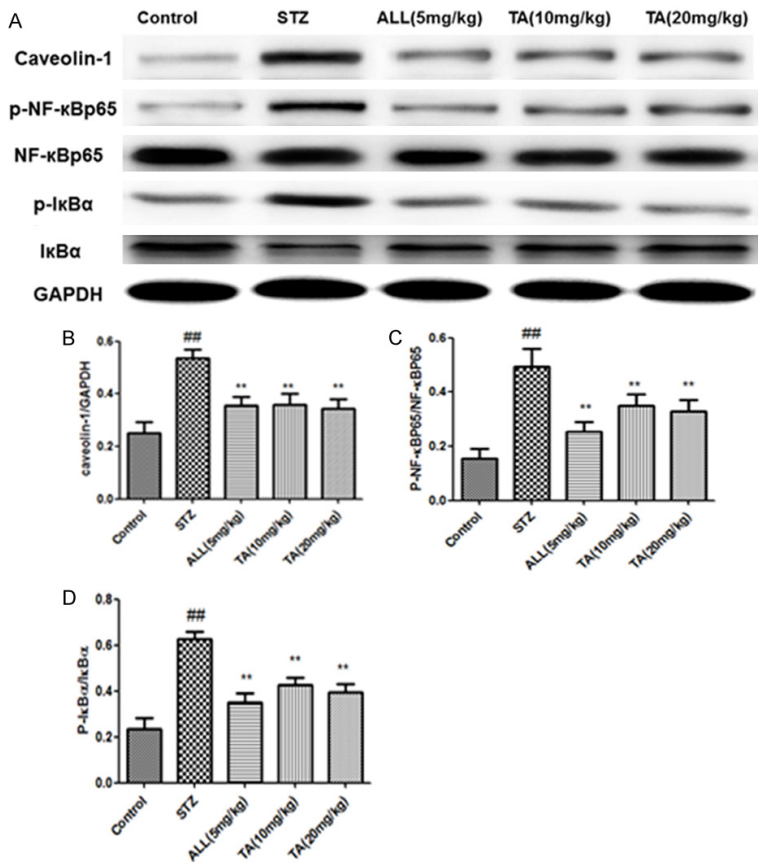

Figure 5. Effect of TA on Caveolin-1/NF-κB pathway-related protein levels in STZ-induced diabetic rats. A. Representative western blot. B-D. Protein quantification. Rats were intraperitoneally injected with 135 mg/kg of STZ, and then intragastrically treated with ALL (5 mg/kg) or TA (10 and 20 mg/kg) for 28 consecutive days. Values are expressed as means ± SDs; $^{\#\#}P<0.01$ compared with control, $^{**}P<0.01$ compared with the STZ-induced DM model.

cantly increased in the STZ-induced animals compared with the control group, as well as a markedly reduced in the TA treatment group (**Figure 4**). Western blot analyses revealed that the levels of Caveolin-1/NF-κB pathway-related proteins were lower in STZ treated animals, in contrast with the control group, while different degrees of reversion were observed with TA (10, 20 mg/kg) treatments (**Figure 5**).

Discussion

The results of this study indicate that TA treatment of STZ-induced diabetic rats reduces the levels of blood glucose, uric acid, and creatinine, and remediates STZ-induced the histopathological alterations. TA also reduces the expression of Caveolin-1/NF-κB related mRNA and proteins.

Hyperglycemia is the main factor that stimulates the progression of DN, which causes increasing morbidity and mortality in patients. DN pathogenesis is related to biochemical imbalances, metabolic and inflammatory processes. In our study, significant decreased in blood glucose and increase in plasma insulin levels were found in diabetes, whereas TA treatment mitigated the levels of glucose and insulin in the rat DM model. Uric acid, and creatinine are considered as reliable indices of renal function. Elevated creatinine content and low uric acid concentration are key features of DN. Our results indicated that TA treatment improved the renal function. The STZ-induced diabetic rats had thickened glomerular basement membrane and enlarged adipose tissues, whereas TA treatment reduced the size of adipocytes. H&E staining suggested that TA effectively remediated STZ-induced pathological alterations in kidney tissues.

Caveolin-1 is a 22-kDa membrane protein with pleiotropic cellular functions (e.g., cholesterol homeostasis, proliferation, and signal transduction). It has been postulated that Caveolin-1 modulates innate immunity and inflammation. Caveolin-1 regulates receptor signaling in membranes by directly binding to the receptor or downstream molecules, and has been implicated as a modulator of innate immunity and inflammation. One study showed that glucose was associated with Caveolin-1 expression [9]. We showed that Caveolin-1 expression was higher in STZ-induced diabetic rats, and TA reduced the expression of Caveolin-1 in STZ-induced DM.

NF-κB is a crucial therapeutic target for interventions in several inflammatory disorders including DN [10]. In diabetic animals, NF-κB is regulated by IκBα, which is activated by the upstream effector IKKs. The IKK complex contains two isoforms: IKKα and IKKβ. TLR4 triggers the phosphorylation of IKKα and IKKβ which induces IκBα activation. Then, IκBα is

phosphorylated and degraded. Subsequently, NF-κB translocates into the nucleus and triggers the expression of its target genes including TNF-α, IL-6 and IL-1β. These genes cause persistent and enhanced inflammation [11, 12]. Tumor necrosis factor (TNF-α) is implicated with the development of metabolic disorder, de novo fatty acid production, and the promotion of TG and TC synthesis [13]. IL-6 is considered as an inflammatory cytokine that can cause insulin resistance [14]. IL-1β is also involved in aberrant intraglomerular hemodynamics by inducing prostaglandin production. Our results indicate that TA reduced the expressions of NF-κB pathway.

In conclusion, we investigated the protective effects of TA on STZ-induced DN, and found that it conferred beneficial effects possibly via the Caveolin-1/NF-κB pathway. Further studies are warranted before clinical applications.

Disclosure of conflict of interest

None.

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