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Original article

Role of cyclooxygenase-2 in the development of interstitial fibrosis in kidneys following unilateral ureteral obstruction in mice

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ABSTRACT

Unilateral ureteral obstruction (UUO) induced tubulointerstitial fibrosis in kidneys mimics the pathogenesis of chronic kidney diseases and is considered a suitable model for studying the mechanisms leading to fibrosis. To study the role of cyclooxygenase-2 (COX-2) in kidney fibrosis, we investigated whether a selective COX-2 inhibitor, celecoxib, affected renal interstitial fibrosis during UUO in mice. To induce UUO, the left proximal ureter was ligated in male C57BL/6 mice. The mice were fed a diet with or without celecoxib from the day of UUO induction. Following UUO, the renal pelvis was observed to be dilated and the kidney cortex was significantly thinner than that of sham-operated mice. Immunofluorescent staining of type I, III, and IV collagen in UUO kidneys revealed that interstitial collagen deposition was significantly increased in the celecoxib-treated group. Expression of type I, III, and IV collagen in UUO kidneys was also significantly higher in the celecoxib-treated group than in the vehicle-treated group. In the celecoxib-treated group, mRNA levels of TGF-β/FGF-2 were also significantly higher than those in the vehicle-treated group. The present study demonstrates that COX-2 plays a protective role against fibrosis in UUO kidneys and suggests that supplementation of COX-2 products, such as PG analogues, will be a good option for preventing interstitial fibrosis.

1. Introduction

Renal fibrosis is characterized by glomerulosclerosis and tubulointerstitial fibrosis, and is the final common phenotype of a wide variety of chronic kidney diseases (CKD). Progressive CKD frequently causes widespread tissue fibrosis that leads to the complete destruction of kidney tissues and renal failure [1]. The pathogenesis of renal fibrosis is characterized by excessive accumulation and deposition of extracellular matrix (ECM) components, including collagen. However, it is not clear what factors regulate the development of fibrosis. A better understanding of this mechanism will provide novel insights into developing new therapeutic strategies. In recent years, significant progress has been made in understanding the cellular and molecular mechanisms that mediate the development of renal fibrosis [2,3]; however, there are limited data on the lipid factors that regulate fibrogenesis in the kidneys.

Unilateral ureteral obstruction (UUO) induces tubulointerstitial fibrosis in the kidneys similar to that of CKDs, and is considered a suitable model for studying the mechanisms leading to tubulointerstitial fibrosis [4]. It has been reported that the proinflammatory and vasoactive metabolites of arachidonic acid (prostanoids), such as prostaglandin (PG) E_2, are generated in the kidneys during the development of tubulointerstitial fibrosis induced by UUO [5,6]. Cyclooxygenases (prostaglandin endoperoxide synthase) are rate-limiting enzymes that catalyze the committed step in prostaglandin PG biosynthesis. Mammalian cells contain two related but unique isozymes: cyclooxygenase (COX)-1 and COX-2 [7]. COX-1 is expressed constitutively and is involved in the production of PGs that modulate normal physiological functions in several organ systems, including the kidneys, gastrointestinal (GI) tract, and platelets. By contrast, COX-2 expression is induced by bacterial endotoxins, cytokines, and growth factors, and is involved in the production of PGs that modulate pathophysiological events in development, cell growth, tumorigenesis, and inflammation. The uniquely different phenotypes of COX-1 and COX-2 knockout mice further confirm that the two isozymes are likely involved in the generation of PGs in different pathophysiological settings.

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In this study, we examined the roles of the PG-generating enzyme COX-2 in the development of interstitial fibrosis in UUO models using the selective COX-2 inhibitor, celecoxib [8]. The accumulation of type I, III, and IV collagens in UUO kidneys was significantly increased in mice treated with celecoxib. Conversely, COX-2 suppressed tubulointerstitial fibrosis in UUO kidneys, possibly via the downregulation of TGF-β and FGF-2. The present study demonstrates that COX-2 plays a protective role against fibrosis in UUO kidneys and suggests that supplementation with PG analogues may be a viable option for preventing the interstitial fibrosis observed in CKD.

2. Methods

2.1. Animals

Male 8-week-old C57BL/6 WT mice were obtained from CLEA Japan (Tokyo, Japan). All experiments were performed in accordance with the guidelines for animal experiments established by the Kitasato University School of Medicine.

2.2. Unilateral ureteral obstruction (UUO) model

UUO surgery was performed under isoflurane anesthesia. A median abdominal incision was made and the left proximal ureter was ligated at two points using 3-0 silk. The incision was closed with wound clips (AUTOCLIP, 9 mm, ALZET, USA). In sham-operated mice, the left ureter was exposed but not ligated.

2.3. Drugs

Mice in the treated group were fed a powdered diet containing celecoxib (150 mg per 100 g, equal to approximately 100 mg/kg/day/mouse, Pyser) starting after UUO surgery. Vehicle-treated mice were maintained on a powdered diet without celecoxib. Kidney samples were harvested at day 5.

2.4. Tissue harvesting

Kidney samples were collected at 0, 3, 5, 7, or 14 days after UUO. Day 0 kidney samples were collected prior to any surgical procedures. Under isoflurane anesthesia, mice were perfused with PBS from the left ventricle. The left kidney was harvested immediately and cut into transverse pieces for RT-PCR, frozen sections, paraffin sections, and Sircol collagen assay.

2.5. Histological examination

Kidney tissues were fixed in 4% paraformaldehyde at 4 °C overnight and embedded in paraffin for histological analysis. Paraffin-embedded tissues were cut into 4 μm sections and stained with Masson's trichrome stain. Kidney cortex thickness was calculated as the average of three distances in each section.

2.6. Immunofluorescent staining

For frozen sections, kidney tissues were frozen immediately with liquid nitrogen. The samples were cut into 4 μm sections from the cortical side. Cryosections were blocked with 1% BSA in PBST (0.1% TritonX-100 in PBS) for 1 h at room temperature and then incubated overnight at 4 °C with one of the following primary antibodies: (a) anti-type I collagen antibody (1:100 dilution, Abcam, ab21286), (b) anti-type III collagen antibody (1:300 dilution, Abcam, ab7778), and (c) anti-type IV collagen antibody (1:200 dilution, Cosmo Bio Co. Ltd., LSL-LB-1403). After washing in PBS, the sections were incubated for 1 h at room temperature with Alexa Fluor® 488 Donkey Anti-rabbit IgG (1:500 dilution, Molecular Probes). Five randomly selected cortical interstitial fields at × 400 magnification from each animal were photographed, excluding glomeruli and large vessels. The immunoreactive interstitial area was calculated by ImageJ image analysis software and expressed as the percent total area. All images were captured with a Biozero BZ-9000 series fluorescence microscope (Keyence, Japan).

2.7. Sircol collagen assay

Total soluble collagen was determined using Sircol collagen assay kit (Biocolor, UK). In brief, kidney samples were frozen immediately with liquid nitrogen and stored at −80 °C. Samples were taken from the cortex and weighed used less than 10 mg. Samples were diced and incubated in 1.2 mL of pepsin solution at a concentration of 0.5 mg/mL in 0.5 M acetic acid at 4 °C stationary overnight. Next day, the tubes were centrifuged at 15,000 r.p.m. at 4 °C for 15 min. A 1 mL aliquot of supernatant was transferred to a new tube. The supernatant was then neutralized and concentrated using accessory solution according to manufacturer's instruction. Collagen pellet and 100 μL of collagen standard were mixed with 1 mL of Sircol dye reagent and incubated for 30 min. The collagen-dye complex was dissolved in alkali reagent and the absorbance of the solution was read at 555 nm. Results were expressed as μg/mg kidney cortex weight.

2.8. Real-time RT-PCR

Total RNA was extracted from decapsulated kidney tissues using TRIzol reagent (Gibco-BRL, Life Technologies, Rockville, MD, USA) and single-stranded cDNA was generated from 1 μg of total RNA via reverse transcription using ReverTra Ace® qPCR RT Kit (TOYOBO CO., LTD., Osaka, Japan) according to the manufacturer's instructions. Real-time PCR was performed using SYBR® *Premix Ex Taq*™ II (Tli RNaseH Plus, Takara Bio, Inc. Shiga, Japan). The gene-specific primers used are listed below.

GAPDH	Forward:	5′-ACATCAAGAAGGTGGTGAAGC-3′
	Reverse:	5′-AGGTGGAAGAGTGGGAGTTG-3′
Col1a1	Forward:	5′-AGGCATAAAGGGTCATCGTG-3′
	Reverse:	5′-GACCGTTGAGTCCGTCTTTG-3′
Col3a1	Forward:	5′-AGGCAACAGTGGTTCTCCTG-3′
	Reverse:	5′-GACCTCGTGCTCCAGTTAGC-3′
Col4a1	Forward:	5′-CCATAGAGAGAAGCGAGATGTT-3′
	Reverse:	5′-CAGAGGCGAGCATCATAGTTA-3′
COX-2	Forward:	5′-TGGGTGTGAAGGGAAATAAGG-3′
	Reverse:	5′-CATCATATTTGAGCCTTGGGG-3’
TGF-β	Forward:	5′-AACAATTCCTGGCGTTACCTT-3′
	Reverse:	5′-TGTATTCCGTCTCCTTGGTTC-3′
FGF-2	Forward:	5′-GGCTGCTGGCTTCTAAGTGTG-3′
	Reverse:	5′-TTCCGTGACCGGTAAGTATTG-3′

PCR was performed in 20 μL reactions according to the manufacturer's protocol, and the reaction mixture was subjected to 40 cycles of amplification in a DNA thermal cycler. Data were normalized to the expression level of GAPDH in each sample.

2.9. Statistical analysis

All results are expressed as means ± SEM. Comparisons between the two groups were performed with Student's *t*-test. Values of $P < 0.05$ were considered statistically significant.