

## Effects of Celecoxib and Nordihydroguaiaretic Acid on Puromycin Aminonucleoside-Induced Nephrosis in the Rat

The selective cyclooxygenase-2 (COX-2) and 5-lipoxygenase (LOX) inhibitors might inhibit prostaglandin synthesis and reduce proteinuria. The present study was designed to investigate the anti-proteinuric effects of nordihydroguaiaretic acid (NDGA) as compared with celecoxib in puromycin aminonucleoside (PAN) nephrosis rats. Fifty five male Sprague-Dawley rats were divided into 4 groups; A, normal control; B, PAN group; C, PAN+COX-2 inhibitor (celecoxib) group; and D, PAN+5-LOX inhibitor (NDGA) group. After induction of PAN nephrosis through repeated injections of PAN (7.5 and 15 mg/100 g body weight), rats were treated with celecoxib, NDGA, or vehicle for 2 weeks. Twenty four hour urine protein excretions were significantly lower in PAN+celecoxib and PAN+NDGA groups than in PAN group. Serum creatinine (SCr) concentrations and 24 hr urine creatinine clearances (CCr) were not significantly different in the four groups. Electron microscopy showed that podocyte morphology was changed after the induction of PAN nephrosis and was recovered after celecoxib or NDGA administration. Celecoxib significantly recovered the expressions of nephrin, CD2AP, COX-2, and TGF- $\beta$ . NDGA also recovered TGF- $\beta$  expression, but did not alter the expressions of nephrin, CD2AP and COX-2. The present study suggested that celecoxib and NDGA might effectively reduce proteinuria in nephrotic syndrome without impairing renal function.

**Key Words :** Puromycin Aminonucleoside; Proteinuria; Celecoxib; Nordihydroguaiaretic Acid

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## INTRODUCTION

Diseases that affect podocytes typically present with proteinuria, with or without nephrotic syndrome. However, it should be noted that not all cases with nephritic range proteinuria are caused by podocyte diseases, because the glomerular filtration barrier also contains glomerular endothelial cells and glomerular basement membrane (GBM) (1). Moreover, fixed anionic charges on glomeruli are believed to form an important component of the glomerular filtration barrier. Thus several studies have been reported that the changes of anionic sites of the GBM might cause proteinuria (2-4). These studies suggest that proteinuria can be induced by a combination of various factors, i.e., both structural and functional factors, and that it can be reduced by addressing the factors concerned.

Nonsteroidal anti-inflammatory drugs (NSAIDs) actively inhibit both cyclooxygenases (COX-1 and COX-2), and thereby block prostaglandin production. Anti-proteinuric effect of NSAIDs in nephrotic syndrome is presumed to be due to reductions in renal plasma flow and glomerular filtration rates. Nevertheless, their use is limited by various side effects caused by COX-1 inhibition. Therefore the therapeutic applications of selective COX-2 inhibitor might result in more selective inhibition of inflammatory processes without influencing basal

COX-1-dependent prostaglandin synthesis (5, 6).

The 5-lipoxygenase (LOX) inhibitor, nordihydroguaiaretic acid (NDGA), is a typical lignin found in the creosote bush (*Larrea tridentata* or *Larrea divaricata*) which has a worldwide distribution. These plants are used as traditional medicines for more than 50 illnesses, including rheumatism, diabetes, gallbladder and kidney stones, and sterility (7). LOX inhibitors have also been compared with other NSAIDs, COX inhibitors, and leukotriene inhibitors in terms of their anti-inflammatory, anti-thrombotic, and anti-platelet effects (8, 9). Recently it was reported that NDGA could reverse the oxidative stress, and reduce proteinuria in diabetic nephropathy rats (10).

Therefore we hypothesized that proteinuria might be related to both the COX and LOX pathways. The present study was designed to investigate the therapeutic efficacy of NDGA as compared with celecoxib in puromycin aminonucleoside (PAN) nephrosis rats.

## MATERIALS AND METHODS

### Animals

Fifty five male Sprague-Dawley rats, which initially weighed

250-300 g, were kept in individual metabolic cages with free access to standard chow and water. The rats were divided into 4 groups; A (n=10), normal control; B (n=15), PAN group; C (n=15), PAN+celecoxib group; and D (n=15), PAN+NDGA group. They were subjected to standard laboratory tests for 4 weeks before being sacrificed for kidney removal. All experiments were performed in accordance with the NIH's *Guiding Principles in the Care and Use of Laboratory Animals*.

### Drugs and reagents

PAN (6-dimethylamino-9[3'-amino-3'-deoxyribose] purine, Sigma Chemical Co., St. Louis, MO, U.S.A.) was prepared for i.p. injection (7.5 and 15 mg/100 g body weight). Celecoxib (Celebrex®, Pfizer Inc., Seoul, Korea) was dissolved in drinking water for gavage (4 mg/100 g body weight/day). NDGA (Sigma Chemical Co.) was prepared in dimethyl sulfoxide (DMSO, Sigma Chemical Co.) solution for s.c. injection (1 mg/100 g body weight/day). All drugs administered i.p. or s.c. were prepared in a constant volume (0.1 mL/100 g body weight) at the beginning of each experiment.

### Treatment schedule

After 3 days of acclimation, rats were injected i.p. with PAN 7.5 mg/100 g body weight (day 0), and then with 15 mg/100 g body weight 7 days later (day 7). Control animals received an identical volume of vehicle. On day 14, serum and 24 hr urine samples were obtained to confirm the onset of proteinuria in the nephrotic range.

After the induction of PAN nephrosis, rats were treated with celecoxib, NDGA, or vehicle from day 14 to 27. Fifteen nephrotic rats were gavaged daily with celecoxib (4 mg/100 g body weight/day), 15 were injected s.c. daily with NDGA (1 mg/100 g body weight/day), and 15 were injected s.c. with vehicle. Ten control rats were also injected s.c. with vehicle.

On days 21 and 28, serum and 24 hr urine samples were obtained to determine blood and urine chemistries. Twenty four hour urine creatinine clearances (CCr) was used as surrogate of glomerular filtration rate (GFR) (Fig. 1).

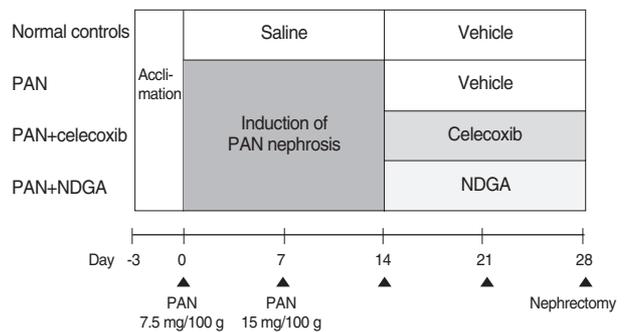


Fig. 1. Schematic of the treatment schedule. After three days of acclimation, test animals were injected intraperitoneally on day 0 with PAN 75 mg/kg, and then 7 days later with 150 mg/kg. Control animals were administered an identical volume of normal saline. After inducing PAN nephrosis, all animals were treated with celecoxib (40 mg/kg/day), NDGA (10 mg/kg/day), or vehicle up to Day 28. Serum and 24 hr urine samples were obtained on days 0, 7, 14, 21, and 28 (▲). All rats were sacrificed on day 28, and kidneys were removed immediately for histological examinations.

Table 1. Changes in serum and 24 hr urine parameters in the four experimental groups

	Group A (normal controls)	Group B (PAN)	Group C (PAN+Celecoxib)	Group D (PAN+NDGA)
Serum albumin (mg/dL)				
Day 7	1.6±0.1	1.3±0.4	1.0±0.2	1.1±0.1
Day 14	1.4±0.1	1.3±0.4	1.2±0.4	1.2±0.2
Day 21	1.5±0.2	1.8±0.2	1.6±0.4	1.7±0.1
Day 28	1.5±0.4	1.3±0.4	1.2±0.3	1.1±0.2
24 hr urine protein (mg/dL)				
Day 7	63.7±22.5	57.8±22.6	57.6±22.4	45.4±20.2
Day 14	53.4±22.6	253.2±95.8*	298.6±69.9*	183.7±80.7*
Day 21	44.6±20.5	619.1±132.3*	653.6±185.6*	672.9±61.8*
Day 28	55.1±26.5	704.4±225.7*	177.4±119.9 <sup>†</sup>	254.2±207.2 <sup>†</sup>
Serum creatinine (mg/dL)				
Day 7	0.57±0.16	0.89±0.18	0.94±0.22	0.97±0.19
Day 14	0.59±0.23	0.56±0.05	0.58±0.09	0.59±0.12
Day 21	0.63±0.10	0.47±0.07	0.57±0.13	0.47±0.05
Day 28	0.60±0.16	0.40±0.06	0.59±0.25	0.44±0.05
24 hr urine creatinine clearance (mL/min)				
Day 7	1.12±0.27	0.74±0.36	0.68±0.21	0.58±0.20
Day 14	1.43±0.74	1.60±0.35	1.46±0.44	1.14±0.26
Day 21	1.38±0.55	2.25±0.97	1.87±0.55	1.95±0.33
Day 28	1.36±0.48	2.61±0.92	2.28±1.04	2.31±0.33

All values are expressed in mean±SD. \* $p<0.05$  vs. group A, <sup>†</sup> $p<0.05$  vs. group B. PAN, puromycin aminonucleoside; NDGA, nordihydroguaiaretic acid.

**Kidney tissue preparation**

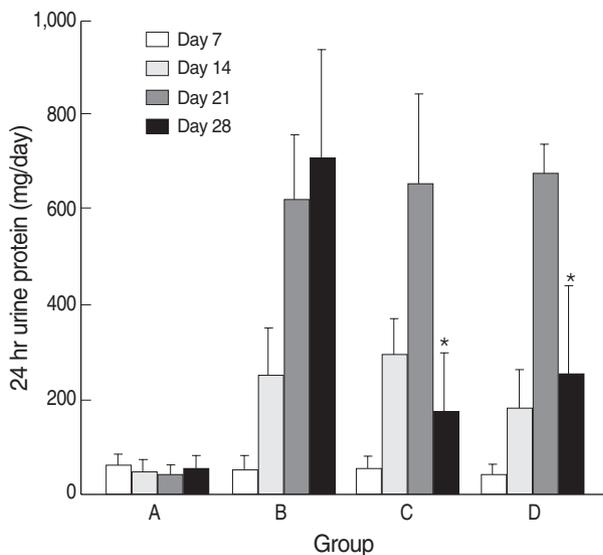
On day 28, all rats were sacrificed under general anesthesia using i.p. injection of ketamine. Kidneys were removed immediately for histological examinations and for isolation of glomeruli. Kidney sections were fixed in 10% neutral-buffered formalin, blocked in paraffin wax, and stained with hematoxylin-eosin (H&E) and periodic acid-Schiff (PAS) reagent. To evaluate glomerular sclerotic changes, we observed at least 100 glomeruli in each section. For electron microscopy, kidney sections were fixed in 2.5% glutaraldehyde, postfixed in 1% osmium, and embedded in epoxy resin. Ultrathin sections were stained with uranyl acetate.

**Isolation of glomeruli and reverse transcription-PCR**

Rats were perfused through left ventricles with saline. Kid-

**Table 2.** Sequences of PCR primers

Gene	Direction	Sequence	Size
GAPDH	Forward	GGCGTCTTCACCACCATGGAG	477
	Reverse	GCCTGCTTCACCCTTCTTG	
Nephrin	Forward	AGCCTCTTGACCATCGCTAA	293
	Reverse	GTCCTCGCCTTCAGCACCTG	
CD2AP	Forward	CCAGCTCCAAAACCTGACC	496
	Reverse	GGGTTGGAGAATGTCCACC	
COX-2	Forward	CCAGTATCAGAACCGCATTGC	464
	Reverse	CAAGTTCTACCATGGTCTCCC	
TGF- $\beta$ 1	Forward	CAACAATTCCTGGCGATAACC	468
	Reverse	GCTCCAAATGTAGGGGCAGGG	



**Fig. 2.** Effects of celecoxib and NDGA on 24 hr urine protein excretion in PAN-induced nephrosis rats. Twenty four hour urine protein excretion amounts were significantly lower in groups C and D on day 28 than in group B (\* $p < 0.05$  vs. group B). Group A, normal controls; group B, a PAN group; group C, a PAN+celecoxib group; group D, a PAN+NDGA group.

neys were removed and cortices were finely minced, sieved through sequential sieves, and digested in collagenase. Total glomerular RNA was extracted using TRIzol<sup>®</sup> (Invitrogen, Carlsbad, CA, U.S.A.). The levels of the mRNAs of nephrin, CD2AP, COX-2, and TGF- $\beta$  were determined by reverse transcriptase-polymerase chain reaction (RT-PCR) using PCR Pre-Mix<sup>®</sup> (Bioneer, Daejeon, Korea) using the primers shown in Table 2.

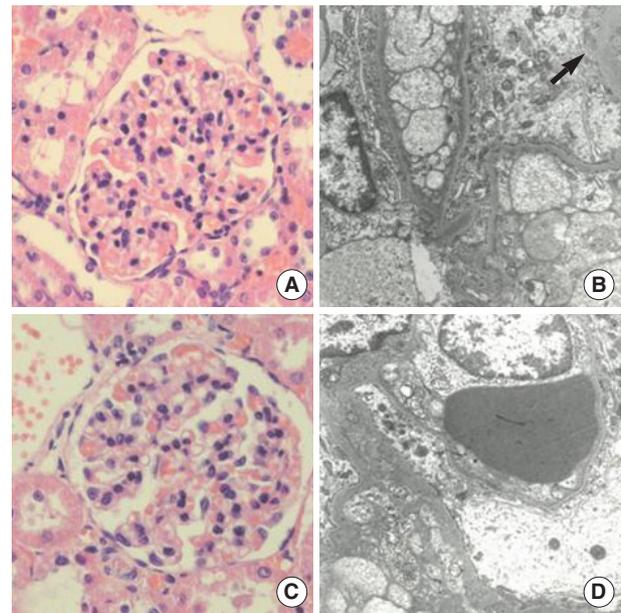
**Statistical analyses**

All values are presented as means  $\pm$  SD. Repeated measures ANOVA and post-hoc analysis (Tukey test) were used for the analysis, and differences were considered significant when  $p$  values were  $< 0.05$ .

**RESULTS**

**Effects of celecoxib and NDGA on proteinuria**

On day 7, seven days after the first PAN (7.5 mg/100 g body weight) injection, all rats in the treatment groups showed physiological proteinuria ( $63.7 \pm 22.5$ ,  $57.8 \pm 22.6$ ,  $57.6 \pm$



**Fig. 3.** Representative pathologic features of a PAN and a PAN+celecoxib groups. (A) Light microscopic feature of a glomerulus in PAN group. Small segmental proliferations with matrix expansion and adhesions between the glomerular tuft and Bowman's capsule were observed (PAS stain,  $\times 300$ ). (B) Electron microscopic feature of mild foot processes changes (arrow) such as effacement, fusion and microvilli in PAN group. (C) Light microscopic feature of a glomerulus in PAN+celecoxib group. Slight expansion of mesangial matrix was observed (PAS stain,  $\times 300$ ). (D) Electron microscopic feature of non-specific foot processes changes in PAN+celecoxib group.

22.4, and  $45.4 \pm 20.2$  mg/24 hr, respectively) and increased weights ( $353.6 \pm 23.4$ ,  $338.6 \pm 28.8$ ,  $353.6 \pm 10.7$ , and  $349.3 \pm 20.9$  g, respectively) versus their baseline weights ( $279.3 \pm 10.6$ ,  $274.3 \pm 10.6$ ,  $291.4 \pm 8.0$ , and  $290.7 \pm 12.4$  g, respectively).

On day 14, seven days after the second PAN (15 mg/100 g body weight) injection, rats in groups B, C, and D exhibited proteinuria in the nephrotic range of  $253.2 \pm 95.8$ ,  $298.6 \pm 69.9$ , and  $183.7 \pm 80.7$  mg/24 hr, whereas rats in group A showed physiological proteinuria of  $63.7 \pm 22.5$  mg/24 hr ( $p < 0.05$  vs. group A). Mean serum albumin concentrations in groups B,

C, and D tended to be lower  $1.3 \pm 0.4$ ,  $1.2 \pm 0.4$ , and  $1.2 \pm 0.2$  g/dL, respectively, than in group A  $1.4 \pm 0.1$  g/dL.

On day 21, seven days after initiating celecoxib and NDGA treatment, group C and D rats still showed elevated levels of proteinuria ( $653.6 \pm 185.6$  and  $672.9 \pm 61.8$  mg/24 hr,  $p < 0.05$  vs. group A).

On day 28, group C and D rats exhibited significant reductions in proteinuria ( $177.4 \pm 119.9$  and  $254.2 \pm 207.2$  mg/24 hr), whereas group B rats showed a further increase in proteinuria ( $704.4 \pm 225.7$  mg/24 hr,  $p < 0.05$  group C vs. group B and group D vs. group B, Table 1, Fig. 2).

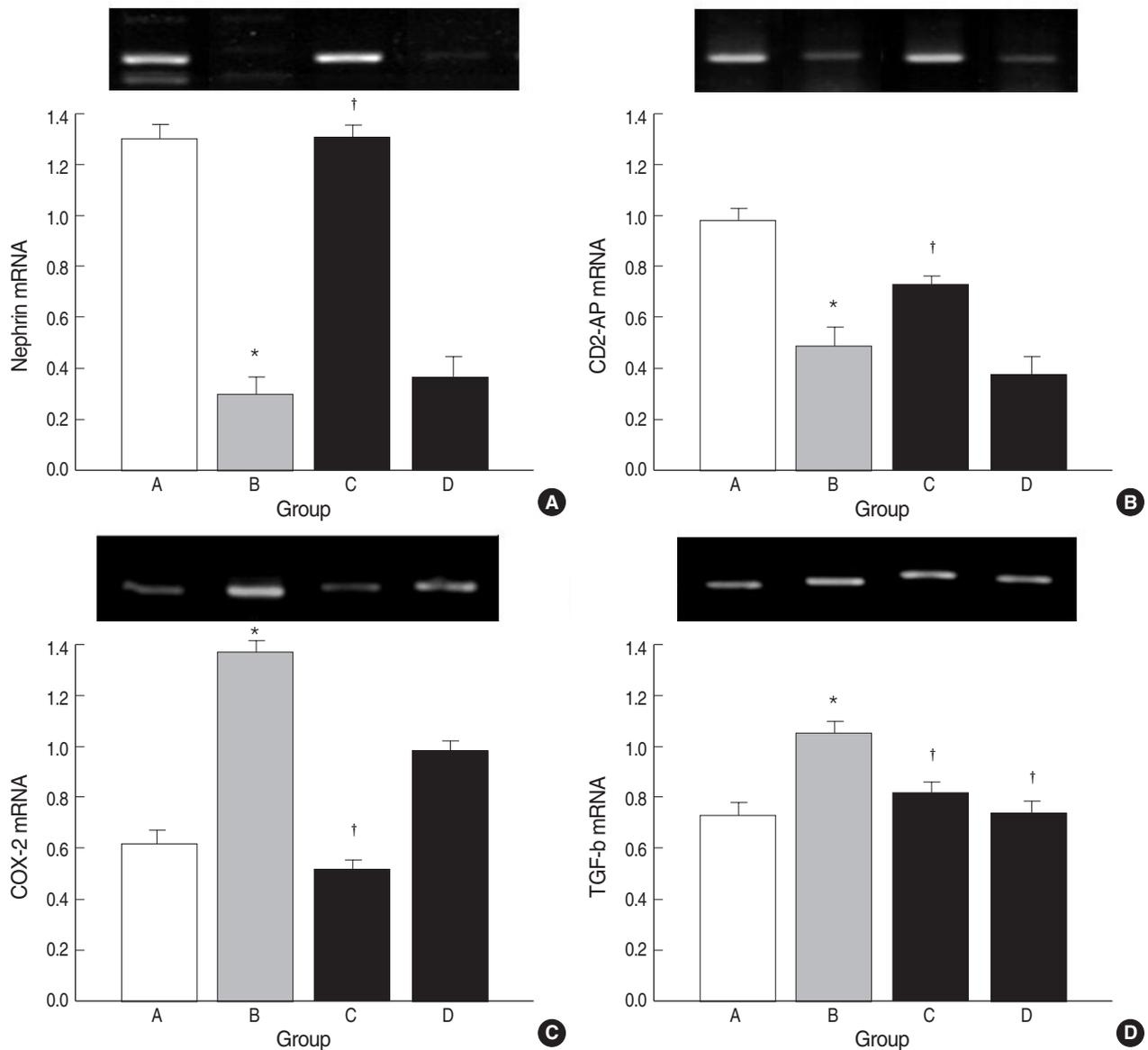


Fig. 4. Effects of celecoxib and NDGA on nephrin, CD2AP, COX-2, and TGF- $\beta$  mRNA expressions. (A) Nephryn mRNA expressions decreased after administering PAN in PAN group, and restored significantly in PAN+celecoxib group. (B) CD2AP mRNA expressions decreased in PAN group, and restored significantly in PAN+celecoxib group. (C) COX-2 mRNA expressions increased in PAN group, and restored significantly in PAN+celecoxib group. (D) TGF- $\beta$  mRNA expressions increased in PAN group, and decreased significantly in PAN+celecoxib and PAN+NDGA groups. \* $p < 0.05$  vs. normal controls; † $p < 0.05$  vs. PAN group.

### Effects of celecoxib and NDGA on renal functions

In groups C and D, SCr tended to decrease, and 24 hr urine CCr to increase. However there were no significant changes during the experiment ( $p > 0.05$ , Table 1).

### Effects of celecoxib and NDGA on renal morphology

Light microscopy revealed small segmental proliferations with matrix expansion and adhesions between the glomerular tuft and Bowman's capsule in group B, C, and D. However, focal segmental or global sclerotic lesions were not observed. Electron microscopy revealed variable degrees of foot processes fusion, effacement, and microvillous transformation in group B, but not in groups C and D on day 28 (Fig. 3).

### Effects of Celecoxib and NDGA on the expressions of nephrin, CD2AP, COX-2, and TGF- $\beta$

PAN significantly reduced the expressions of nephrin and CD2AP, and increased the expressions of COX-2 and TGF- $\beta$  ( $p < 0.05$  vs. group A, respectively). However, celecoxib significantly restored the expressions of nephrin, CD2AP, COX-2, and TGF- $\beta$  ( $p < 0.05$  vs. group B, respectively). NDGA also restored TGF- $\beta$  expression ( $p < 0.05$  vs. group B), but did not alter the expressions of nephrin, CD2AP and COX-2 (Fig. 4).

## DISCUSSION

The PAN nephrosis model used in the present study, is a well-described animal model of human idiopathic nephrotic syndrome, and has been used extensively to study the fundamental processes of proteinuria (11-13). PAN is a nephrotoxin and may injure podocytes and alter the selectivity of the GBM. However, the precise mechanisms that underlie PAN-induced proteinuria are not well understood.

Sprague-Dawley rats that were injected with a single dose of PAN developed significant proteinuria several days later. In PAN-induced nephrosis, the onset of proteinuria coincides with the development of focal defects in podocyte epithelium, which expose the outer surface of the GBM to the urinary space (14, 15). However these morphological and functional changes are recovered within 1-2 weeks after the induction of PAN nephrosis. Recent studies showed that the repeated injections of PAN induced specific focal glomerulosclerosis lesions, but with an incidence of only 10-30% (16-19). Therefore we attempted to repeat injections twice with an interval of seven days to maintain the proteinuria in the nephrotic range with the absence of self-reduction over the course of the experiments. In the present study, after the first PAN injection (7.5 mg/100 g body weight), no significant increase in proteinuria was observed, but the second injection (15 mg/100 g body weight) induced a significant and sustained increase in pro-

teinuria over a period of two weeks. However, no glomerulosclerotic lesions were found in the present study.

In models of progressive glomerular injury, COX-2 inhibitors decrease proteinuria and retard progressive glomerulosclerosis (20-22). LOX inhibitors have also been studied as potential anti-inflammatory agents with anti-thrombotic and anti-platelet effects (8, 9). In the present study, anti-proteinuric effects of celecoxib and NDGA suggest that hemodynamic improvements are associated with eicosanoids in the arachidonic acid pathway, and with anti-inflammatory and immune modulating effects. On the contrary to the previous reports, our study showed the sustained increase in 24 hr urine CCr in PAN-treated groups (B, C, and D). These findings are thought to be the results of hyperfiltration in the early stage of acute kidney injury caused by PAN injections. LOX inhibitors have been reported to have protective effects on ileal motor disturbances induced by endotoxins, to promote adaptation following massive bowel resection, and to promote correction of coronary spasms of immune origin (23-25). These findings suggest that LOX inhibitors can reduce proteinuria due to their anti-inflammatory and protective effects on vascular smooth muscle.

PAN causes podocyte injury and reduces the expressions of nephrin and CD2AP. In the present study, celecoxib restored the expressions of nephrin and CD2AP, but NDGA did not alter their expressions. Furthermore, whereas celecoxib restored COX-2 expression, NDGA increased it through complementary enhancing the COX pathway. It appears that overexpression of COX-2 by NDGA predisposed podocyte injury, which negated its beneficial anti-inflammatory effect (26). PAN caused focal mesangial expansion and fibrosis, and increased the expression of TGF- $\beta$ . TGF- $\beta$  can cause extracellular matrix (ECM) accumulation by enhancing the productions of collagen and fibronectin by glomerular mesangial cell, by suppressing the expression of ECM-degrading protease, and by increasing the synthesis of ECM protease inhibitors (27-29). In this study, we found that celecoxib and NDGA reduced TGF- $\beta$  expression, which suggests the possible involvements of the COX and LOX pathways in TGF- $\beta$ -induced ECM accumulation and proteinuria.

In conclusion, our results support the involvements of the COX and LOX pathways in PAN-induced proteinuria. Celecoxib and NDGA could reduce proteinuria without impairing renal function, by promoting hemodynamic and anti-inflammatory improvements. In particular, celecoxib might reduce proteinuria, restore the expressions of nephrin and CD2AP, and suppress the expressions of COX-2 and TGF- $\beta$ . Further investigations should be conducted to elucidate the roles of these pathways in the pathogenesis of proteinuria, as this would help identify the anti-proteinuric effects of celecoxib and NDGA in progressive glomerular disease.

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