**Effect of N-Acetyl Cysteine on Renal Interstitial Fibrosis in Mice**

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This study examined the effect of N-acetyl cysteine (NAC), a reactive oxygen species (ROS) inhibitor, on renal interstitial fibrosis induced by unilateral ureteral obstruction (UUO) in mice. UUO led to a significant increase in the fibrotic area of obstructed kidneys, which was attenuated by NAC (84.8 mg/kg/d) in the drinking water. Renal expression of type III collagen and tumor necrosis factor (TNF)-α mRNAs was elevated in UUO mice and inhibited by NAC. Extracellular signal-regulated kinase (ERK1/2) phosphorylation was significantly elevated by UUO, and NAC significantly attenuated the elevation. UUO inhibited the activity of glutathione peroxidase, while NAC restored its activity. Together, the results of this study suggest that renal interstitial fibrosis induced by UUO was ameliorated by NAC via several mechanisms including increased glutathione peroxidase activity, reduced phosphorylation of ERK1/2, and reduced expression of TNF-α and type III collagen mRNAs.

**Key words** N-acetyl cysteine; fibrosis; glutathione peroxidase; tumor necrosis factor (TNF)-α; type III collagen; extracellular signal-regulated kinase

### INTRODUCTION

Progressive interstitial renal fibrosis, which is characterized by the accumulation of excessive amounts of extracellular matrix components and proliferation of interstitial fibroblasts, is a common final pathological pathway of chronic kidney diseases. The unilateral ureteral obstruction (UUO) model generates renal fibrosis leading to tubulointerstitial fibrosis.\(^1\) UUO causes ischemia, mechanical stretch, oxidative stress, and hypoxia, resulting renal tubular cell injury.\(^2\)

Many reports indicate that UUO leads to enhance renal production of reactive oxygen species (ROS). ROS are produced as a result of normal cellular metabolism and affect a variety of physiological processes.\(^3\) ROS overproduction, however, can cause cell damage and trigger inflammation, which plays a role in the pathogenesis of many diseases. N-Acetyl cysteine (NAC) is an exogenous antioxidant that acts as a scavenger of a free radicals scavenger. It is a precursor of glutathione and one of the most a critical intracellular antioxidant, and it has been reported to ameliorate angiotensin II-mediated renal fibrosis in the UUO mouse model.\(^5\) Xia et al. reported that NAC alleviated contrast agent-induced kidney injury in UUO-induced rats.\(^5\) In this manner, NAC may have a protective effect against renal obstruction.

We previously showed that, amloidipine, a calcium channel blocker, ameliorates renal interstitial fibrosis induced by UUO by inhibiting the expression of heat shock protein (HSP) 47 and type IV collagen and reducing c-Jun-N-terminal kinase (JNK) phosphorylation.\(^6\) Administration of the cell permeable superoxide dismutase (SOD) mimetic manganese (III) tetrakis(1-methyl-4-pyridyl) porphyrin significantly attenuated the increases of HSP47 expression following ischemia and reperfusion in mice.\(^6\) Therefore, the present study examined the effect of NAC on renal interstitial fibrosis induced by UUO in mice.

### MATERIALS AND METHODS

**Materials** We purchased NAC from FUJIFILM Wako Pure Chemical Corporation (Osaka, Japan). Anti-extracellular signal-regulated kinase (ERK1/2), anti-phospho-ERK1/2, anti-JNK, anti-phospho-JNK, anti-phospho-p38 mitogen-activated protein kinase (MAPK), and anti-p38MAPK antibodies were obtained from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). All other drugs and chemicals were of reagent grade or the highest available quality.

**Animals** Male BALB/cCrSlc mice (age 5 weeks) were obtained from Japan SLC Inc. (Hamamatsu, Japan). The mice were group housed (3–5 per cage) with a 12-h light/dark schedule for 1 week or more prior to UUO surgery. The experiments had been approved by the Ethics Committee for Animal Experiments consistent with the Guidelines for Animal Experiments of Takasaki University of Health and Welfare and the Japanese Government Animal Protection and Management Law. Efforts were made to reduce the number of animals used in experiments and minimize suffering.

**Experimental Protocols** UUO was carried out as reported previously.\(^5\) Briefly, a midline abdominal incision was made under anesthesia (medetomidine hydrochloride: 0.3 mg/kg, midazolam: 4 mg/kg, butorphanol 5 mg/kg, intraperitoneally (i.p.)); the left ureter was exposed and ligatured with 4–0 silk. Mice that received sham surgery (anesthesia with ureter exposure but not ligation) were used as controls. At day 14 following UUO induction, samples of kidney tissue were obtained in anesthetized mice, and were stored immediately at −80°C until use. Mice received NAC daily in the drinking water following UUO surgery.

**Histopathological Analyses** Kidney sample were embedded in paraffin and sectioned 1 μm thick. Masson’s trichrome was used to stain the sections for examination of interstitial fibrosis. The area of kidney tissue with interstitial fibrosis in the outer medulla was measured with image analysis software.
Western Blotting Total protein was extracted from homogenized kidney using Pro-Prep Protein Extraction Solution (iNtRON Biotechnology, Sungnam, Korea). Western blotting was carried out as reported previously. Polyvinylidene difluoride (PVDF) membranes were incubated for 2 h with 0.5% skim milk in Tris-buffered saline with Tween 20, and incubated with a 1:1000 dilution of antibodies for ERK1/2, phospho-ERK1/2, JNK, phospho-JNK, p38MAPK, or phospho-p38MAPK for 2 h at 25°C. The membranes were then incubated with anti-rabbit horseradish peroxidase-conjugated immunoglobulin G (IgG) for 2 h at 25°C. An ECL assay kit (GE Healthcare UK Ltd., Little Chalfont, U.K.) was used to measure immunoreactivity and a luminescent image analyzer (LAS-3000, FUJIFILM, Tokyo, Japan) was used for signal detection. Band density was measured with Image Gauge (FUJIFILM). Data are expressed as a ratio relative to the total amount of ERK1/2, JNK or p38MAPK.

Real-Time RT-PCR A GenElute Mammalian Total RNA Miniprep Kit (Sigma-Aldrich, St. Louis, MO, U.S.A.) was used to extract total RNA, following the manufacturer’s instructions. Real-time PCR analysis was carried out with an Mx3000P analyzer (Stratagene, La Jolla, CA, U.S.A.). cDNA amplification was carried out using SYBR Premix Ex Taq (TaKaRa Bio, Otsu, Shiga, Japan). The primer sequences were as follows: transforming growth factor-β (TGF-β), 5'-CCG CAA CAA CGC CAT CTA TGA-3' (sense) and 5'-GGG GGT CAG CAG CCG GTT AC-3' (antisense); Type III collagen, 5'-TGG TCC TCA GGG GTG TAA AGG-3' (sense) and 5'-GTC CAG CAT CAC CTT TTG GT-3' (antisense); TNF-α, 5'-GCA TGA TCC GGC AGC GTG AA-3' (sense) and 5'-AGA TCC ATG CCG TTG GCC AG-3' (antisense); KIM-1, 5'-TTA AAC CAG AGA TTC CCA CA-3' (sense) and 5'-TTG GAG GAG TGG AGG TAG AGA-3' (antisense); SOD2, 5'-AAC TCA GGT CGT CTC TCG GC-3' (sense) and 5'-GAA CTT TGG ACT CCC ACA GA-3' (antisense); glycerol-3-phosphate dehydrogenase (G3PDH), 5'-ACC ACA GTT ACC CAT GCC-3' (sense) and 5'-TCC ACC ACC CTT CTG GTG TA-3' (antisense). The conditions for real-time PCR were 95°C for 2 min, followed by 30 cycles of 15 s at 95°C, 30 s at 56°C, and 30 s at 72°C. Gene expression was normalized to the levels of G3PDH.

Measurement of Glutathione Peroxidase Activity Glutathione peroxidase activity was measured using a Glutathione Peroxidase Assay Kit from Northwest Life Science Specialties (Vancouver, WA, U.S.A.) according to the manufacturer’s instructions.

Protein Assay Protein concentrations were measured using a bicinchoninic acid assay (Thermo Fisher Scientific, Rockford, IL, U.S.A.) with bovine serum albumin used as a standard.

Data Analysis All data are represented as the mean ± standard error of the mean (S.E.M.). Statistical differences between values were determined by one-way ANOVA and Dunnett’s test for multiple comparisons. Statistical significance was defined as p-value < 0.05.

RESULTS

UUO-Induced Histological Damage and Fibrosis Mice received NAC daily in the drinking water. The average daily NAC dose was at 84.8 ± 7.5 mg/kg/d. At day 14 of UUO induction, the area of renal fibrosis was assessed in tissue sec-
tions stained with Masson’s trichrome. Partial tubule dilation was evident UUO mice and the damage was reduced by NAC. The estimated fibrotic area was increased significantly following UUO, and the increase was diminished significantly by NAC (Fig. 1).

Although we measured serum creatinine and blood urea nitrogen, there is no significant difference between untreated and NAC-treated UUO mice (data not shown).

**mRNA Expression**  
TGF-β is known to mediate the production of collagen during fibrogenesis, and KIM-1 is a general marker of injury that regulates inflammation. Therefore, we used real-time RT-PCR to assess the TGF-β and KIM-1 induction in UUO mice. The levels of TGF-β and KIM-1 mRNA were elevated following UUO, and this effect was diminished...
Numerous reports have revealed that NAC has beneficial effect against ROS. Zukowski and associates reported that NAC prevents the development of oxidative stress, improves insulin sensitivity, and lowers concentrations of insulin in the serum of rats with insulin resistance induced by a high-sugar diet. In addition, Bmi-1 is involved maintaining mitochondrial function protecting against DNA damage. Bmi-1−/− mice showed fibrosis of the renal interstitium, tubular atrophy, severe impairment of kidney function and decreased proliferation of renal cells. NAC has been shown to ameliorate renal interstitial fibrosis in Bmi-1−/− mice. Although we investigated whether the production of hydrogen peroxide was affected by UUO with or without NAC, NAC did not affect the production of hydrogen peroxide (data not shown). Therefore, we thought that NAC may ameliorate the renal interstitial fibrosis without direct antioxidant effect. Although the precise protective effect of NAC on UUO-induced renal interstitial fibrosis should be needed for further investigations, our study indicates that NAC may have beneficial anti-fibrotic effects in addition to inhibiting ROS.

We showed in a previous study that meloxicam, a cyclooxygenase (COX)-2 inhibitor, ameliorates the renal interstitial fibrosis induced by UUO by inhibiting collagen expression and reducing ERK1/2 and JNK phosphorylation. Similarly, in the present study, NAC ameliorated UUO-induced renal interstitial fibrosis by inhibiting ERK1/2 phosphorylation and expression of TNF-α and type III collagen. Cheng and co-workers reported that UUO-induced renal interstitial fibrosis is responsible for phosphorylation of ERK1/2. Moreover, U0126, a specific inhibitor of ERK1/2 kinase, suppressed the terbinafine-induced increase of TNF-α levels in human monocytic THP-1 cells. Another specific inhibitor of ERK1/2 kinase, PD98059, has been reported to prevent the increase of type III collagen mRNA levels induced by aldosterone in rat renal fibroblast NRK-49F cells. Therefore, it is possible that inhibition of ERK1/2 activation by NAC ameliorated the renal interstitial fibrosis.

Glutathione peroxidase belongs to a family of selenoproteins whose function is to catalyze the reduction of hydrogen peroxide. NAC is a byproduct of glutathione, which is an efficient cofactor for glutathione peroxidase. Kinter et al. reported a significant decrease in glutathione peroxidase activity in UUO kidneys relative to sham-operated kidneys. Therefore, we reasoned that the activity of glutathione peroxidase would also be increased by NAC. In fact, our results showed that glutathione peroxidase activity was decreased following UUO, and NAC diminished this effect. Consequently, it seems that NAC restored this repressive process of ROS, then affects renal fibrosis.

In summary, this study showed that an ROS inhibitor, NAC, ameliorated UUO-induced renal interstitial fibrosis in mice by inhibiting ERK1/2 phosphorylation and the expression of TNF-α and type III collagen with an increase in glutathione peroxidase activity. Therefore, combination therapy with an ROS inhibitor and another renal protective medication may improve the outcomes of patients with renal interstitial fibrosis.

**Conflict of Interest** The authors declare no conflict of interest.

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Fig. 4. Effect of NAC Treatment on Glutathione Peroxidase (GPx) Activity in Mouse UUO Kidney

GPx activity were measured in kidneys 14 d post sham operation, UUO, or UUO and NAC treatment. Column represent mean ± S.E.M. *p < 0.05.

by NAC, although the effect was not statistically significant (Figs. 2A, B).

TNF-α has been reported to be involved in proliferation and apoptosis of renal tubular and interstitial cells in obstructive renal injury. TNF-α mRNA levels were higher subsequent to UUO, and NAC significantly inhibited the hyperexpression of TNF-α (Fig. 2C). The level of collagen mRNA expression was measured to determine the role of collagen expression in renal fibrosis induced by UUO. Administration of NAC led to a significant reduction in type III collagen mRNA (Fig. 2D). Intracellular SOD protects tissues against oxidative stress by catalyzing the conversion of superoxide radicals into less toxic ROS. SOD-1 is localized in the cytoplasmic compartments with copper and zinc in its catalytic center, while SOD-2 is located in the mitochondria and contains manganese in its catalytic center. The level of SOD-2 mRNA were reduced following UUO, and the reduction was partially attenuated by NAC, although the effect was not statistically significant (Fig. 2E).

**MAPK Family Phosphorylation** Since MAPKs play a role fibrogenesis, we investigated whether the activity of MAPK was affected by UUO with or without NAC. ERK1/2 phosphorylation was a significantly increased following UUO (Fig. 3A) and significantly attenuated by NAC administered after the induction of UUO. JNK and p38 MAPK, which are members of MAPK family, were not affected under the conditions examined (Figs. 3B, C).

**Glutathione Peroxidase Activity** Glutathione peroxidase catalyze the reduction of various peroxides. Since UUO is known to induce glutathione peroxidase activity, we examined the effect of NAC on the activity of glutathione peroxidase following UUO. Glutathione peroxidase activity was decreased after UUO, and this effect was diminished by NAC (Fig. 4).

**DISCUSSION**

This study demonstrated that NAC, an ROS inhibitor, ameliorated renal interstitial fibrosis induced by UUO in mice. Furthermore, this effect of NAC was associated with inhibition of ERK1/2 phosphorylation, increased expression of TNF-α and type III collagen mRNAs, and increased glutathione peroxidase activity.
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