Advanced oxidation protein products contribute to renal tubulopathy via perturbation of renal fatty acids

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Abstract

Background: Renal proximal tubulopathy plays a crucial role in kidney disease, but its molecular mechanism is incompletely understood. Since proximal tubular cells consume much energy during reabsorption, the relationship between fatty acids (FAs) and proximal tubulopathy has been attracted attention. The purpose of this study is to investigate the association between change in renal fatty acids (FAs) composition and tubulopathy.

Methods: Mice with cisplatin-induced nephrotoxicity was used as AKI model and 5/6-nephrectomized mice was used as CKD model. Renal FAs composition in mice was measured by GC-MS. Human tubular epithelial cells (HK-2 cells) was used for in vitro study.

Results: In kidneys of AKI mice, increased stearate (C18:0) and decreased palmitate (C16:0) were observed, accompanied by increasing expression of the long-chain FAs elongase Elovl6. Similar results were also obtained in CKD mice. We show that C18:0 has higher tubular toxicity than C16:0 via induction of ER stress. Using adenovirus-expressing Elovl6 or siRNA for Elovl6 in HK-2 cells, it is demonstrated that increased Elovl6 expression contributes to tubulopathy via increasing C18:0. Elovl6-knock out suppressed the increased serum creatinine level, renal ER stress and inflammation as observed after 5/6 nephrectomy. Advanced oxidation protein products (AOPPs), specifically an oxidized albumin, was identified as inducing Elovl6 via the mTORC1/SREBP1 pathway.

Conclusions: AOPPs may contribute to renal tubulopathy via perturbation of renal FAs through Elovl6 induction. The perturbation of renal fatty acids induced by the AOPPs-Elovl6 system could be a potential target for the treatment of tubulopathy.
**Introduction**

Recent studies have demonstrated that proximal tubulopathy plays an important role in the onset and development of chronic kidney disease (CKD). Thus, its contribution to renal pathogenesis has attracted attention. For example, proximal tubular necrosis and inadequate tubular repair induced by acute kidney injury (AKI) have been shown to lead to the progression of CKD\(^1\). In this regard, Takaori et al. revealed that proximal tubule injury can induce several features of CKD, such as glomerulosclerosis and fibrosis using mice with proximal tubule-specific adjustable injury\(^2\). In mice with diabetic nephropathy, metabolic abnormalities in the proximal tubule also contribute to subsequent damage in glomerular lesions\(^3,4\). Although these reports suggest an important role for proximal tubulopathy in renal pathophysiology, the molecular mechanisms and factors that cause proximal tubulopathy are still incompletely understood.

Several studies have demonstrated the close relationship between fatty acids (FAs) and proximal tubulopathy. To date, the mechanism for FA-induced tubulopathy proposed comprises FA bound-urinary albumin being taken up by proximal tubular cells *via* an albumin receptor such as megalin/cubilin or CD36, with subsequent FA-induced tubular damage\(^5,6\). In fact, Soumura et al. showed in mouse proximal tubular cells that long-chain saturate fatty acid (SFA) bound-albumin induced inflammatory pathways such as monocyte chemoattractant protein-1 (MCP-1) induction *via* activation of the PKC/NF-κB signaling pathway\(^7\). Yasuda et al. also reported that SFA bound-albumin exacerbated endoplasmic reticulum (ER) stress\(^8\) that affects kidney disease\(^9\). In contrast, it has also been reported that long-chain unsaturated fatty acids such as oleate (C18:1) suppress SFA-induced inflammation\(^7,8\). In addition, there have been several reports in which metabolic effects of FAs-bearing albumin on proximal tubule induces tubulopathy\(^10-15\).
These findings suggest the importance of renal FA species in tubulopathy. Traditionally, the fluctuations of renal FA species or composition have been mainly explained by the effect of FAs that flowed into the renal tubular lumen in albuminuria after passing through the glomerulus.

In addition to the FAs entering the tubule, recent studies demonstrated that intracellular FA composition is also affected by intracellular FA metabolism via their elongation and desaturation. To date, several reports have studied the perturbation of FA composition and its contribution to organ pathology\textsuperscript{16-18}. In fact, Matsuzaka et al. demonstrated that the perturbation of hepatic FA composition accelerated the progression of nonalcoholic hepatitis\textsuperscript{16}. Similar association has also been reported in alveolar type II epithelial cells\textsuperscript{17} and vascular smooth muscle cells\textsuperscript{18}. However, there is limited information about the relationship between renal FA composition and tubulopathy, although proximal tubular cells require high FA metabolism to produce ATP for maintaining their functions, such as reabsorption etc.

The purpose of this study is to clarify the relationship between the change in renal FA composition and proximal tubulopathy. Initially, using AKI model mice, renal FA composition was determined by GC-MS analysis to identify the FA species which could contribute to tubulopathy. Secondly, we explored the endogenous factors that could induce renal FA fluctuation. Finally, the molecular mechanism of tubulopathy induced by renal FA fluctuation was investigated using an immortalized human renal proximal tubule epithelial cell line.
Materials and Methods

Animal experiments

All animals were purchased from Japan SLC and maintained in a room with controlled temperature (21-23°C) and a 12 hr dark/light cycle (light 8 am-8 pm) and freely provided with food and water. All animal experiments were conducted using procedures approved by the experimental animal ethics committee at Kumamoto University. Male ICR mice (4-week-old) were randomized before intraperitoneal administration of 15 mg/kg cisplatin (RandaTM) (Nippon Kayaku Co., Ltd., Tokyo, Japan). Control mice were administrated with saline. Mice were sacrificed on day 4 after administration of saline or cisplatin. 5/6-nephrectomized (Nx) mice were produced using a two-step surgery using male ICR mice (5-week-old) according to previous reports19. In brief, 2/3 of the right kidney was removed, and 1 week later the left kidney was removed. For mice on a high stearate (C18:0) diet, high C18:0 diet (Kbt oriental Co., Ltd., Tosu, Japan) was fed to male ICR mice lacking the left kidney for 8 weeks. Dietary FA compositions are shown in Supplemental Table 1. For advanced oxidation protein products (AOPPs)-overloaded mice, male ICR mice (4-week-old) were intraperitoneally administered with AOPPs (150 mg protein/kg/day) for 7 weeks. As a control, defatted normal albumin (150 mg protein /kg/day: the same protein amount as AOPPs) was also administered to male ICR mice (4-week-old) for 7 weeks.

Measurement of FAs by GC-MS

Chloroform (1 mL) was added to freeze-dried kidney tissue pieces (100 mg) and incubated at 50°C for 30 min. After removing tissue remains, the extract was evaporated. FAs from total lipid
were methylated using a FA methylation kit (Nacalai Tesque, Kyoto, Japan) and measured by GC-MS (Agilent 5975C inert XL MSD) using a capillary column (Agilent VF-WAXms 0.25 µm x 30 m x 0.25 mm). Nonadecanoic acid (Sigma-Aldrich, St. Louis, MO.) was used as internal standard.

Real time RT-PCR analysis

Total RNA from kidney or cells was isolated and real-time PCR measurements were performed using methods previously described20. The primers used for mRNA detection are shown in Supplemental Table 2. The threshold cycle (Ct) values for each gene were normalized by subtracting the Ct value calculated for GAPDH.

Preparation of AOPPs

Commercial human serum albumin solution (Japan Blood Products Organization, Tokyo, Japan) was defatted using the charcoal procedure described by Chen21, deionized, freeze dried, and then stored at -20°C until use. AOPPs was prepared in vitro as described previously22. Briefly, 300 µM albumin was incubated with 100 mM chloramine-T (Nacalai Tesque, Kyoto, Japan) in 67 mM phosphate buffer (pH 8.0) for 1 hr at 37°C. The sample was dialyzed against phosphate-buffered saline (PBS) (pH 7.4) for 2 days to remove chloramine T and to stop the oxidation reaction. The protein solution was frozen at -80°C, then lyophilized. This AOPPs activity was 205.6 µmol/g protein.

Measurement of AOPPs activity
The AOPPs activity was determined as described previously\textsuperscript{23}. 200 \mu L of 10-fold diluted serum samples (dilution in PBS) were placed in a 96-well plate and mixed with 25 \mu L of 20\% acetic acid and 10 \mu L of 1.16 M potassium iodide. In standard wells, 200 \mu L of chloramine-T solution was added as the standard curve, followed by addition of 25 \mu L of 20\% acetic acid and 10 \mu L of 1.16 M potassium iodide. The absorbance of the reaction mixture at 340 nm was read immediately in a microplate reader. Potassium iodide and acetic acid were purchased from Nacalai Tesque (Kyoto, Japan).

**In situ hybridization**

Elovl6 mRNA localization in paraffin-embedded kidney tissue (4 \mu m thickness) was determined using the Manual RNAscope\textsuperscript{\textregistered} 2.5 HD-RED assay (Advanced Cell Diagnostics, Hayward, CA) according to the manufacturer’s protocols (QUICK GIDE: RNAscope\textsuperscript{\textregistered} 2.5 RED for FFPE). The following set of RNAscope probe was used in this study: 20ZZ probe named Mm-Elovl6 targeting 144-1211 of NM\_130450.2.

**Cell culture**

Human renal proximal tubule epithelial cell line (HK-2 cells) were cultured in DMEM/Ham’s F-12 medium (FUJIFILM Wako Pure Chemical, Osaka, Japan) with 10\% fetal bovine serum (Corning, NY) and 1\% antibiotic-antimycotic mixed stock solution (penicillin 100 U/mL, streptomycin100 \mu g/mL, amphotericin B 0.25 \mu g/mL) (Nacalai Tesque, Kyoto, Japan) at 37 \degree C in an atmosphere containing 5\% CO\textsubscript{2}. HK-2 cells were confirmed to express CD36 and Megalin protein\textsuperscript{22}. HK-2 cells were seeded at 1.5 \times 10\textsuperscript{5} cells/well in 6-well plates and exposed to fatostatin
(Cayman Chemical Company, Ann Arbor, MI.), 25-hydroxyvitamin D3 (LKT Laboratories, Inc., St. Paul, MN.) or rapamycin (LC Laboratories, Inc., Woburn, MA.) for 4 hr, then incubated with AOPPs for 24 hr. As control, albumin was used at the same concentration as the protein level of AOPPs.

**Cell experiments with free fatty acids (FFAs)**

FFAs were dissolved in 50% ethanol at 60°C. FFAs solution was mixed with 20% defatted albumin solution in PBS (pH 7.4) at a 1:5 molar ratio at 37°C. The solution was passed through a 0.2 µm filter. HK-2 cells were seeded at 1.0 × 10^4 cells/well in 96-well plates and incubated with FFAs for 24 hr to evaluate cell viability using cell counting kit-8 (Dojindo Molecular Technologies, Inc., Kumamoto, Japan). HK-2 cells were seeded at 2.0 × 10^5 cells/well in 6-well plates and incubated with 100 µM FFAs for 24 hr to determine CHOP mRNA levels. Sodium palmitate (C16:0) and sodium stearate (C18:0) were purchased from Sigma-Aldrich (St. Louis, MO.).

**Preparation of recombinant adenovirus and adenovirus treatment**

The recombinant adenovirus plasmid coding for mouse Elovl6 cDNA were a kind gift from Dr. Matsuzaka (Tsukuba University). Recombinant adenovirus was produced in HEK293 cells and purified by calcium chloride density gradient centrifugation. HK-2 cells were infected with adenovirus-expressing GFP (Ad-GFP: control) or Elovl6 (Ad-Elovl6) at a multiplicity of infection of 100 for 16 hr.
RNAi treatment to knock down Elovl6

HK-2 cells (5.0 × 10⁴ cells) were incubated with Stealth RNAi™ for human Elovl6 (siElovl6) or Stealth RNAi™ for Negative Control (Med GC) as control siRNA (siControl) with Lipofectamine™ RNAiMAX at an siRNA concentration of 50 nM in Opti-MEM I® for 4 hr (siElovl6 sense: 5’-GAACAUGUCAGUGUUGACUUUACAA, antisense: 5’- UUGUAAAGUCAACACUGACAGUUC). Stealth RNAi™ for human Elovl6, siRNA Negative Control (Med GC), Lipofectamine™ RNAiMAX and Opti-MEM I® were purchased from Thermo Fisher Scientific (Waltham, MA).

Cell experiments with serum samples from hemodialysis patients

Nine stable CKD patients (9 men) receiving dialysis aged from 69 to 86 (average 81.0 ± 1.6) years old who had been admitted to the Department of Nephrology of the Akebono Clinic of Japan, in October 2018 were enrolled in this study. As control, healthy volunteers (6 men and 4 women) aged 21 to 29 years old (average 23.6 ± 0.7) were used. HK-2 cells were seeded at 1.0 × 10⁵ cells/well in 6-well plates. The cells were incubated with medium containing 10% serum sample for 24 hr. The study complies with the Declaration of Helsinki, and was approved by the ethics committees of the Faculty of Life Sciences at Kumamoto University.

Generation of Elovl6-KO mice

Male C57BL/6J WT mice (8-week-old, Japan SLC) or Elovl6-KO mice (8~9-week-old, CARD, Kumamoto University, Japan) were randomized by body weight. Elovl6-KO mice were efficiently generated by reproductive technology using ultrasuperovulation technique at the
Institute of Resource Development and Analysis Center for Animal Resources and Development (CARD), Kumamoto University, Japan\textsuperscript{25}. Elovl6-KO embryo was donated from Dr. Takashi Matsuzaka, University of Tsukuba\textsuperscript{24}.

**Statistical analyses**

The correlation data was evaluated using Pearson’s correlation analysis. The means for two group data were compared by the unpaired t-test. The means for groups were compared by analysis of variance using Tukey’s multiple comparison method (using Graph Pad Prism 8, San Diego, CA). A probability value of $P<0.05$ was considered to be significant.
Results

Increased renal C18:0 and Elovl6 in tubulopathy in mice with cisplatin-induced nephrotoxicity

Initially, we determined renal FA composition in mice with cisplatin-induced nephrotoxicity as an AKI model. 4 days after cisplatin treatment, blood urea nitrogen (BUN) and serum creatinine were significantly enhanced (Table 1). At this point, the renal content of palmitic acid (C16:0) was decreased, while that of stearic acid (C18:0) was increased (Figure 1A). These fluctuations in renal FAs suggest a contribution of elongation of very long chain fatty acids protein 6 (Elovl6), which is a microsomal enzyme that regulates the elongation of C12-16 SFAs and monounsaturated FAs, particularly C16:0 to C18:0. In fact, Elovl6 mRNA level in renal tissue of cisplatin-treated mice was significantly increased compared with control mice (Figure 1B). In addition, renal Elovl6 mRNA levels were positively correlated with kidney injury molecule-1 (KIM-1), which is a tubulopathy marker ($R^2=0.591$, $p<0.001$) (Figure 1B and C).

Next, because SFA induces ER stress, we evaluated the change in mRNA levels of renal CHOP, an ER stress marker, in cisplatin-treated mice. Renal CHOP mRNA levels were significantly increased (Figure 1B) and also positively correlated with renal Elovl6 mRNA levels ($R^2=0.456$, $p<0.01$) (Figure 1D). To date, there is no information regarding the localization of Elovl6 in kidney. Thus, in situ hybridization was performed to determine renal Elovl6 localization. As shown in Figure 1E, we found that Elovl6 mRNA was mainly located in renal tubular cells (proximal and distal tubular cells) in addition to partially in the renal interstitial area, and the expression was increased in cisplatin-treated mice. These data suggest that in cisplatin-induced
AKI mice increased renal C18:0 via Elovl6 induction might contribute to tubulopathy through ER stress.

**Involvement of C18:0 and Elovl6 in tubulopathy in HK-2 cells**

The cytotoxic effects of C18:0 and C16:0 were compared using HK-2 cells. As shown in Figure 2A, both C18:0 and C16:0 induced cytotoxicity in a dose-dependent manner (100-500 µM), but C18:0 had higher cytotoxicity than C16:0. Similarly, 100 µM of C18:0 induced an increase in CHOP mRNA expression, but 100 µM of C16:0 did not (Figure 2B). These data also indicate that the increased Elovl6 expression and C18:0 in kidney (Figure 1B) might contribute to the cisplatin-induced tubulopathy through activation of ER stress. To confirm the role of Elovl6, HK-2 cells were infected with adenovirus-Elovl6 with C16:0 as an Elovl6 substrate. CHOP mRNA expression was significantly increased in cells treated with adenovirus-Elovl6 and C16:0 (Figure 2C). Furthermore, treatment with siRNA targeting Elovl6 (Figure 2D) suppressed ER stress induced by addition of 200 µM C16:0 (Figure 2E). In addition, feeding with a high C18:0 diet for 8 weeks significantly increased renal KIM-1 mRNA levels compared with a control diet in unilateral nephrectomy mice (Figure 2F), suggesting that loading with-C18:0 induced tubulopathy *in vivo*.

**Exploring endogenous substances that induce renal Elovl6 expression**

To explore the factors that induce renal Elovl6 expression in CKD, we performed *in vitro* experiments using HK-2 cells. As shown in Figure 3A, the expression of Elovl6 mRNA in HK-2 cells was significantly increased when the cells were treated with 10 % serum from hemodialysis
patients compared to cells treated with control serum. These results suggest that humoral factors increased in serum from CKD patients, including uremic toxins were involved in the induction of Elovl6. Here, we examined the effect of 3 uremic toxins (indoxyl sulfate, parathyroid hormone (PTH (1-34)) and advanced oxidation protein products (AOPPs)) as well as C16:0, angiotensin II (AII) and hydrogen peroxide on Elovl6 mRNA expression in HK-2 cells. AOPPs were prepared by treatment of defatted albumin with chloramine-T (oxidant) as previously reported\textsuperscript{22}. As shown in Figure 3B, AOPPs increased the Elovl6 mRNA level, while the other treatments did not. These data suggest that AOPPs act as an endogenous inducer of Elovl6 in kidney disease. The activity of serum AOPPs was increased in serum from hemodialysis patients (Figure 3C). Interestingly, AOPPs activity in patient’s serum showed a significant positive correlation with Elovl6 mRNA expression in HK-2 cells (Figure 3D) (R\textsuperscript{2}=0.460, p<0.01). These data suggest that AOPPs contribute to increased renal Elovl6 expression. In fact, in cisplatin-treated mice, increased serum and renal AOPPs activities were observed (Table 1) and renal AOPPs activity demonstrated a significant positive correlation with renal Elovl6 mRNA levels (R\textsuperscript{2}=0.772, p<0.001) (Figure 3E).

**Perturbation of renal FA composition induced by AOPPs**

To further confirm the ability of AOPPs to act as an Elovl6 inducer, we performed in vivo experiments using AOPPs-overloaded mice. Similar to the cisplatin-treated mice, increased C18:0 and decreased C16:0 were observed in the kidney of AOPPs-overloaded mice compared with those of albumin-overloaded mice (Figure 4A). Then, AOPPs-overloaded mice showed increased serum creatinine level as compared with albumin-treated group (Table 2). These data suggest that AOPPs-induced abnormal renal FA metabolism could contribute to renal dysfunction. In addition,
the mRNA levels of Elovl6, KIM-1 and CHOP were significantly increased in kidneys of these mice (Figure 4B) and Elovl6 mRNA was positively correlated with KIM-1 mRNA ($R^2=0.697$, $p<0.001$ (Figure 4C)) and CHOP mRNA ($R^2=0.300$, $p=0.05$ (Figure 4D)). These results indicate that AOPPs increase renal Elovl6 expression and C18:0, and subsequently cause tubulopathy.

**Mechanism of Elovl6 induction by AOPPs**

To investigate the mechanism of Elovl6 induction by AOPPs, first, we examined the time-dependence of AOPPs-induced Elovl6 in HK-2 cells. As shown in Figure 5A, Elovl6 mRNA expression was induced by AOPPs in a time-dependent manner. Next, the effects of AOPPs on the upstream regulators of Elovl6, such as sterol regulatory element-binding protein 1 (SREBP-1) and peroxisome proliferator-activated receptor-α (PPARα)$^{26}$ were examined 6 hr after adding AOPPs to HK-2 cells because Elovl6 induction was not observed at this time point (Figure 5A). The results demonstrate that AOPPs significantly increased SREBP1 mRNA, but did not affect PPARα mRNA (Figure 5B). A similar induction of renal SREBP1 mRNA was also observed in cisplatin-treated mice and AOPPs-overloaded mice (Figure 1B and 4B). At 24 hr after addition of AOPPs, the inhibitors of SREBP1 such as fatostatin and 25-hydroxyvitamin D3 (25(OH)D) significantly suppressed AOPPs-induced Elovl6 mRNA expression (Figure 5C). Furthermore, rapamycin, an inhibitor of mTORC1 which is an upstream signal molecule of SREBP1$^{27}$, also suppressed AOPPs-induced Elovl6 mRNA expression (Figure 5C). These data suggested that AOPPs induce Elovl6 mRNA expression via the mTORC1/SREBP1 pathway. In addition, Figure 5D also showed that AOPPs induced CHOP mRNA expression (ER stress), and rapamycin treatment suppressed AOPPs induced-CHOP expression. The data suggested that AOPPs have an
ER stress-inducing action. Therefore, the pathway AOPPs/mTORC1/SREBP1/Elovl6/ER stress could be one of the AOPPs-induced tubular injury pathways.

**Involvement of Elovl6 in tubulopathy of 5/6-nephrectomized CKD mice**

We further investigated whether AOPPs-induced tubulopathy also occurs in CKD conditions using 5/6-nephrectomized mice (5/6 Nx mice). At 4 weeks after 5/6-nephrectomy, serum AOPPs activity was significantly increased compared with control mice (Table 3). At the same time, a decreased C16:0 and increased C18:0 in kidneys of 5/6 Nx mice were observed (Figure 6A). In addition, renal Elovl6, KIM-1, CHOP and SREBP1 mRNA were significantly increased in 5/6 Nx mice (Figure 6B) and Elovl6 mRNA was significantly correlated with KIM-1 mRNA ($R^2=0.715$, $p=0.001$ (Figure 6C)) and CHOP mRNA ($R^2=0.443$, $p<0.05$ (Figure 6D)). Furthermore, increased proinflammatory cytokines IL-1β, TNF-α or fibrotic factor and TGF-β (Figure 6B) were positively correlated with Elovl6 mRNA levels (data not shown; IL-1β mRNA: $R^2=0.622$, $p<0.01$; TNF-α mRNA: $R^2=0.595$, $p<0.01$; TGF-β mRNA: $R^2=0.729$, $p<0.001$). These results suggest that increased renal C18:0 could also contribute to renal injury in CKD via Elovl6 induction.

**Contribution of Elovl6 on renal function of 5/6-nephrectomized CKD mice**

Finally, we evaluated the contribution of Elovl6 on renal function of 5/6-nephrectomized CKD mice by using Elovl6-KO mice. In Elovl6-KO mice, no renal Elovl6 mRNA expression was observed, while the renal Elovl6 mRNA expression in WT 5/6 Nx mice was significantly increased as compared with WT control mice (Figure 7A). Increased renal C18:0 content (Figure
7B), renal mRNA expression of KIM-1, CHOP, TNF-α, F4/80, TGF-β and Colla2 (Figure 7C) after 5/6 Nx treatment were suppressed in Elovl6-KO mice. Importantly, Elovl6-KO suppressed the increased serum creatinine level (Table 4), renal ER stress and inflammation as observed in WT mice after 5/6 nephrectomy. These data suggested that increased renal Elovl6 expression could contribute to the progression of renal disease.
Discussion

In the present study, we investigated the association between changes in renal FA composition and renal pathology using cisplatin-treated AKI mice and 5/6 Nx CKD mice. As a result, we found an increase in renal C18:0 accompanied by increasing renal Elovl6 expression in both sets of mice. In vitro studies using HK-2 cells showed that compared to C16:0, C18:0 had higher cytotoxic effect through induction of ER stress. In addition, we demonstrated that AOPPs was involved in an increase of Elovl6 expression via the mTORC1/SREBP1 pathway. Furthermore, Elovl6-KO mice attenuated renal dysfunction after the 5/6 Nx treatment. These data indicate that the perturbation of renal FAs composition via the AOPPs-Elovl6 system contributes to proximal tubulopathy.

Albuminuria, increased by the progression of CKD, is known to induce proximal tubular damage via tubulointerstitial injury and infiltration of inflammatory immune cells. In fact, albuminuria is a risk factor for renal prognosis and cardiovascular disease, and it has been demonstrated that a decrease in albuminuria leads to improvement in renal prognosis\(^2\). However, albuminuria in patients with minimal change nephropathy (MCN) is less involved in the decline of renal function\(^9\). Furthermore, ADVANCE study showed that the differences in renal prognosis were also observed in diabetic patients with a similar amount of albuminuria\(^10\). This clinical evidence indicates that not only changes in albumin amount but also in the quality of albumin in urine could be important for the progression of kidney damage.

Oxidative stress is elevated during renal impairment, with subsequent oxidative-modification of albumin\(^3\). In contrast to MCN patients, patients with focal segmental glomerulosclerosis (FSGS) with albuminuria showed poor renal prognosis. Higher levels of
oxidative stress and accumulation of oxidatively-modified albumin were observed in patients with FSGS compared to MCN patients\textsuperscript{32}. These reports suggest that oxidative stress/oxidatively-modified albumin have the potential to exacerbate the progression of kidney disease. Of the oxidized albumins, AOPPs is the most excessively oxidized form and is a result of oxidation by hypochlorous acid (HOCl) derived from neutrophil myeloperoxidase. Several studies have reported that plasma AOPPs levels increased with CKD progression\textsuperscript{23}. In the present study, plasma and renal AOPPs levels in two nephropathy mice models were about two-times higher than those in control mice (Table1, 3). These findings indicate the possibility that oxidized-albumin, especially AOPPs in serum and urine are associated with the exacerbation of proximal tubulopathy. Previously, Li et al. have demonstrated that AOPPs-overload induced renal fibrosis, the increase in BUN and urinary protein in 5/6-nephrectomized CKD mice\textsuperscript{33}. Here, our data suggested that AOPPs contribute to renal dysfunction by affecting renal Elovl6 expression.

AOPPs leak into the renal tubular lumen after passing through glomeruli or leaks from blood vessels into the renal tissue stroma. Thereafter, AOPPs are taken up by tubular cells via CD36, an AOPPs receptor. Zhang et al. reported that AOPPs stimulate mTOR signaling after being taken up via CD36 into HK-2 cells\textsuperscript{34}. The present study shows that rapamycin, a mTOR inhibitor, suppresses the AOPPs-induced Elovl6 expression (Figure 5C), suggesting that mTOR signaling contributes to Elovl6 induction by AOPPs. mTORC1, one of the catalytic subunits of mTOR, contributes to the transcriptional regulation of lipid metabolism by accelerating the transfer of SREBP1 to the Golgi and the nucleus\textsuperscript{27}. Recently, Asano et al. showed that 25-hydroxyvitamin D (25(OH)D), the precursor of active vitamin D, inhibited SREBP1 activation\textsuperscript{35}. Since the plasma 25(OH)D level was decreased in CKD patients\textsuperscript{36}, it is likely that SREBP1
activity could be increased in CKD patients. In fact, it was reported that SREBP1 activation contributed to the exacerbation of renal pathology. Mustafa et al. also reported that fatostatin, a SREBP1 inhibitor, suppressed interstitial renal fibrosis in a mouse model. In this study, we showed that 25(OH)D and fatostatin suppressed AOPPs-induced Elovl6 expression (Figure 5C). These data suggest that SREBP1 inhibitor could be an effective therapeutic agent against kidney disease caused by the perturbation of renal FAs.

In contrast to these reports, Oishi et al. demonstrated that SREBP1 plays an important role in the convergence of inflammation by macrophages. Xu et al. reported that a molecule downstream of SREBP1, stearoyl-CoA desaturase-1 (SCD1), contributed to attenuate lipotoxicity in diabetic nephropathy mice by producing monounsaturated fatty acids. In addition, Van Krieken et al. showed that inhibition of SREBP1 by fatostatin did not show a sufficient renoprotective effect in mice with diabetic nephropathy. Therefore, it is likely that Elovl6, a downstream molecule of SREBP1, rather than SREBP1 itself is a potent therapeutic target for kidney disease.

Baldwin et al. demonstrated that SFAs with longer chain length had higher cytotoxic activity. For example, in a rat pancreatic cell line the cytotoxic activity of SFAs increased in the following order, myristic acid (C14:0) < C16:0 < C18:0. Recently, Palu et al. reported that Elovl6 deficiency reduced ER stress, and subsequently inhibited cell injury, while dietary supplementation with C18:0 caused the activation of ER stress. Moreover, Karasawa et al. demonstrated that C18:0 underwent intracellular crystallization to enhance the NLRP3 inflammasome in macrophage cells. These reports support our conclusion that increased renal C18:0 caused by Elovl6 induction contributes to tubulopathy. However, the mechanism by which
C18:0 induces ER stress remains unknown. In proximal tubular cells, the accumulation of ceramide, which is biosynthesized from fatty acyl-CoA and serine in the ER, induced apoptosis\textsuperscript{45}. Interestingly, Ting et al. demonstrated that an inhibitor of acyl-CoA synthetase attenuated C18:0-induced cytotoxicity in vascular smooth muscle cells\textsuperscript{46}. Since Elovl6 is localized in the ER, it is possible that C18:0 synthesized by Elovl6 in the ER could be incorporated into ceramide\textsuperscript{47}, and hence cause tubulopathy. Further studies of C18:0 crystallization and incorporation into ceramide are required.

Our data showed that increased C14:0 and C18:0, but decreased C16:0 were observed in AKI or CKD mice model (Figure 1A, 6A). The data suggested that the relatively enhanced Elovl6 activity toward C16:0-acyl-CoA but not toward C12:0- or C14:0-acyl-CoA. Further investigation would be needed to clarify the possibility of enhanced Elovl6 activity toward C16:0-acyl-CoA in our mice model in the future.

The present study is focused on the fluctuation of C16:0 and C18:0, which are known to exist most abundantly as SFA in the body and proposes a mechanism for tubulopathy through Elovl6 induction. However, the results observed in figure 1A and figure 6A show that other FAs also changed in AKI and CKD mice. In particular, C14:0 and \( \omega 6 \) FAs arachidonic acid (AA, C20:4) were increased, while \( \omega 3 \) FAs eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6) were decreased in both mice models. Of these changes, the increased renal AA and decreased renal \( \omega 3 \) FAs are consistent with previous reports on the change of serum FAs composition in CKD patients\textsuperscript{48}. Moreover, these FAs are metabolized into active substances such as prostanoid and leukotriene. The AA metabolites have an inflammatory effect, but the \( \omega 3 \) FAs metabolites showed anti-inflammatory effects\textsuperscript{49}. Therefore, the changes in composition of FAs
such as AA and ω3 FAs in the kidney may also contribute to the progression of renal injury. Importantly, it was reported that exogenous ω3 FAs-treatment suppressed SFAs-induced cytotoxicity and also decreased the activity of SREBP1 and Elovl6 in hepatocytes. These findings suggest that administration of ω3 FAs could prevent the progression of renal pathology via suppression of Elovl6 activity. As such further studies are required in the future.

The previous reports have shown that exogenous albumin-bound FA is involved in tubular injury in non-defatted albumin or C16:0-loaded albumin loading experiments. On the other hand, the present study showed that the change of FA metabolism in kidney also occurred under renal disease, and that cytotoxic C18:0 was increased. Figure 3B shows that C16:0-bound albumin did not induce Elovl6 expressions. On the other hand, in the state where Elovl6 is induced, the supply of exogenous albumin-bound FA may be considered to further exacerbate Elovl6-mediated tubular injury. Therefore, it could be necessary to study the vicious cycle between oxidized albumin and FA-loaded albumin in the future. Kang et al. reported that the decreased β-oxidation contributed to kidney fibrosis development. The data indicated that a fundamental pathophysiologic mechanism of proximal tubule lipotoxicity could be due to the decreased β-oxidation, which also results in toxic accumulation of fatty acids. Therefore, the further studies regarding on the relative contributions of fatty acid synthesis versus degradation such as β-oxidation would be needed.

In this study, we found a novel mechanism for tubulopathy in which oxidized albumin (AOPPs) acts on tubular cells, and abnormal fatty acid metabolism (increased proportion of C18:0) induces tubular damage via the mTORC1/SREBP1/Elovl6 pathway.
Thus, the AOPPs-Elovl6 system could serve as a potential target for the treatment of renal injury.

Disclosures
The authors declare no competing financial interests.

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Supplementary material

Table S1. FA composition of control diet and high stearate (C18:0) diet.
Table S2. The primers used for mRNA detection.

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Table 1 Body weight, renal parameters and AOPPs activity of cisplatin-induced nephrotoxicity mice

|                          | Saline (n=7) | Cisplatin (n=8) |
|--------------------------|--------------|-----------------|
| Body weight (g)          | 29.27±0.56   | 17.83±0.69\(^a\) |
| BUN (mg/dL)              | 19.43±1.08   | 44.50±6.12\(^a\) |
| Serum creatinine (mg/dL) | 0.13±0.01    | 0.29±0.04\(^a\) |
| Serum AOPPs activity (µM)| 60.23±7.92   | 103.36±6.89\(^a\) |
| Renal AOPPs activity (nmol)/tissue weight (mg) | 0.12±0.02 | 0.27±0.03\(^a\) |

Data are expressed as the mean±SE. \(^a\)p<0.01 compared with saline.

BUN, blood urea nitrogen
Table 2 Renal parameters of AOPPs-overloaded mice

|                        | Control (n=6) | AOPPs (n=5)  |
|------------------------|--------------|--------------|
| BUN (mg/dL)            | 26.43 ± 1.41 | 25.93 ± 1.73 |
| Serum creatinine (mg/dL)| 0.13 ± 0.03 | 0.27 ± 0.03  |

Data are expressed as the mean±SE. *p<0.01 compared with control.

BUN, blood urea nitrogen
Table 3 Body weight, renal parameters and AOPPs activity of 5/6-nephrectomized mice

|                                | Control (n=6) | 5/6-nephrectomized (n=5) |
|--------------------------------|---------------|----------------------------|
| Body weight (g)                | 46.57 ± 0.82  | 41.62 ± 1.44<sup>a</sup>  |
| BUN (mg/dL)                    | 24.15 ± 1.02  | 44.50 ± 7.21<sup>a</sup>  |
| Serum creatinine (mg/dL)       | 0.24 ± 0.02   | 0.57 ± 0.04<sup>b</sup>   |
| Proteinuria (mg/day)           | 1.45 ± 0.44   | 6.42 ± 3.02                |
| Serum AOPPs activity (µM)      | 67.56 ± 8.98  | 122.62 ± 8.41<sup>b</sup>  |

Data are expressed as the mean±SE. <sup>a</sup>p<0.05, compared with control. <sup>b</sup>p<0.01, compared with control

BUN, blood urea nitrogen
Table 4 Renal parameters of 5/6-nephrectomized (5/6Nx) Elovl6 KO mice

|                | WT Control (n= 4) | WT 5/6Nx (n=5) | KO 5/6Nx (n=5) |
|----------------|------------------|----------------|----------------|
| BUN (mg/dL)    | 27.18 ± 0.95     | 73.20 ± 3.73\(^a\) | 64.95 ± 3.00\(^a\) |
| Serum creatinine (mg/dL) | 0.21 ± 0.01     | 0.56 ± 0.05\(^a\) | 0.35 ± 0.01\(^{a,b}\) |

Data are expressed as the mean±SE.

\(^a\)p<0.05 compared with WT control, \(^b\)p<0.05 compared with WT 5/6Nx.

BUN, blood urea nitrogen
Figure legends

Figure 1 Change in renal FA composition and Elovl6 expression in mice with cisplatin-induced nephrotoxicity

Male ICR mice (4-week-old) were administered with saline (control) or cisplatin (15 mg/kg). 4 days after administration, mice were sacrificed and blood or kidney tissue collected. (A) The renal FA composition was determined using GC-MS. (B) mRNA expression of target genes in kidney tissue was measured by quantitative RT-PCR. Data are expressed as means ±SE (n=7~8). *p<0.05, **p<0.01 compared with control. Correlation between renal Elovl6 mRNA expression and renal mRNA levels of (C) KIM-1 and (D) CHOP are shown (n=15). (E) Elovl6 mRNA localization in paraffin-embedded kidney tissue was evaluated using in situ hybridization methods. White arrows denote Elovl6 mRNA expression. Scale bar represents 50 µm.

Figure 2 Comparative cytotoxicity of stearate (C18:0) and palmitate (C16:0) and Elovl6/C18:0-induced tubulopathy

(A) The dose-dependent effects of C18:0 or C16:0 on cell viability of HK-2 cells were evaluated using a cell counting kit-8. (B) The effects of 100 µM C18:0 and 100 µM C16:0 on CHOP mRNA levels in HK-2 cells. (C) The effect of 100 µM C16:0 (as a substrate for Elovl6) on CHOP mRNA levels in Elovl6-overexpressing HK-2 cells. HK-2 cells were infected with adenovirus-expressing GFP (Ad-GFP) or mouse Elovl6 (Ad-Elovl6) (upper panel). *p<0.05 compared with control/Ad-GFP; †p<0.05 compared with C16:0/Ad-GFP; ††p<0.05 compared with control/Ad-Elovl6. (D) Suppression of Elovl6 mRNA expression using the Stealth RNAi™: negative control (siControl) or Elovl6 (siElovl6) in HK-2 cells. (E) The effect of 200 µM C16:0 on CHOP mRNA levels in
Elovl6 knock-down HK-2 cells. Data are expressed as means±SE (n=4~6). *p<0.05 compared with control/siControl; †p<0.05 compared with C16:0/siControl †p<0.05 compared with control/siElovl6. (F) Male ICR mice (5-week-old) were subject to unilateral nephrectomy treatment. A control diet or high stearate (C18:0) diet was fed to uninephrectomy mice for 8 weeks and renal KIM-1 mRNA levels evaluated. Data are expressed as means±SE (n=7~8). *p<0.05 compared with control diet.

**Figure 3 Exploring endogenous substances that induce Elovl6 expression**

(A) Elovl6 mRNA levels in HK-2 cells was measured after incubation with 10% serum from healthy subjects or hemodialysis patients. Data are expressed as means±SE (n=7~8). **p<0.01 compared with healthy subjects. (B) The effect of 3 uremic toxins (1 mM indoxyl sulfate, 10 nM PTH (1-34), 250 µM AOPPs), 200 µM C16:0, 1 µM angiotensin II (AII) and 50 µM hydrogen peroxide on Elovl6 mRNA expression in HK-2 cells. Data are expressed as means±SE (n=4~6). *p<0.05 compared with control. (C) Serum AOPPs activity from healthy subjects or hemodialysis patients was measured. Data are expressed as means±SE (n=7~8). **p<0.01 compared with healthy subjects. (D) Correlation between Elovl6 mRNA level and serum AOPPs activity in serum (n=15). (E) Renal AOPPs activity was correlated with Elovl6 mRNA level in kidney of cisplatin-treated mice (n=10).

**Figure 4 Changes in renal FA composition and Elovl6 expression in AOPPs-overloaded mice**

Male ICR mice (4-week-old) were administrated daily with AOPPs (150 mg protein/kg, ip) or albumin as a control (150 mg protein /kg, ip) solution. After 7 weeks of administration, mice were
sacrificed and blood or kidney tissue was collected. (A) Renal FA composition were determined by GC-MS methods. (B) mRNA levels of target genes in kidney tissue were measured by quantitative RT-PCR. Correlation between renal Elovl6 mRNA expression and renal mRNA levels of (C) KIM-1 and (D) CHOP (n=13). Data are expressed as means±SE (n=6–7). *p<0.05 and **p<0.01 compared with albumin.

Figure 5 Mechanism of Elovl6 induction by AOPPs

(A) The time-dependency of 250 µM AOPPs-induced Elovl6 mRNA expression in HK-2 cells were determined 24 hr after incubation. (B) SREBP1 and PPARα mRNA levels were determined 6 hr after incubation with 250 µM AOPPs. (C) Elovl6 and (D) CHOP mRNA expression in HK-2 cells in the presence of SREBP1 or mTOR inhibitor were determined by quantitative RT-PCR. Data are expressed as means±SE (n=4–6). *p<0.05 compared with control. #p<0.05 compared with AOPPs in the absence of inhibitor.

Figure 6 Study using 5/6-nephrectomized CKD mice

5/6-nephrectomized (Nx) mice were produced by two-step surgery using male ICR mice (5-week-old). 4 weeks after 5/6 Nx treatment, the mice were sacrificed and blood or kidney tissue was collected. (A) Renal FA composition were determined by GC-MS methods. (B) mRNA levels of target genes in kidney tissue were measured by quantitative RT-PCR. Data are expressed as means±SE (n=5–6). *p<0.05 and **p<0.01 compared with control. Correlation between renal Elovl6 mRNA expression and renal mRNA levels of (C) KIM-1 and (D) CHOP (n=11).
**Figure 7 Study using Elovl6 KO mice with 5/6-nephrectomy**

At 8 weeks after 5/6 Nx treatment, WT or Elovl6-KO mice were sacrificed and blood or kidney tissue was collected. (A) Elovl6 mRNA levels in kidney tissue were measured by quantitative RT-PCR. (B) Renal C18:0 amounts were determined by GC-MS methods. (C) mRNA levels of target genes in kidney tissue were measured by quantitative RT-PCR. Data are expressed as means±SE (n=4~5). *p<0.05 compared with WT control.

**Figure 8 Schematic summary of AOPPs-induced tubulopathy through mTOR/SREBP1/Elovl6 pathway**

Albumin was oxidized to produce AOPPs. AOPPs act on the tubular epithelium to activate the mTORC1/SREBP1/Elovl6 pathway. Increased Elovl6 expression leads to increased C18:0 which contributes to aggravate ER stress and tubulopathy.
Figure 1

(A) % of total fatty acids in kidney

(B) Relative mRNA level in kidney

(C) KIM-1 mRNA level in kidney

(D) CHOP mRNA level in kidney

(E) Saline vs Cisplatin
Figure 3

(A) 
Elovl6 mRNA level /GAPDH (% of healthy subjects)

(B) 
Elovl6 mRNA level /GAPDH (% of control)

(C) 
Serum AOPPs activity (μM)

(D) 
Serum AOPPs activity (μM)

(E) 
Elovl6 mRNA level in kidney /GAPDH (% of saline)

Renal AOPPs activity (nmol) /kidney tissue weight (mg)

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y = 0.63x + 7.89
R² = 0.460
P < 0.01

Healthy subjects
Hemodialysis patients

Healthy subjects
Hemodialysis patients
Figure 4

(A) Percentage of total fatty acids in kidney

(B) Relative mRNA level in kidney

(C) KIM-1 mRNA level in kidney

(D) CHOP mRNA level in kidney

\[ y = 2.37x - 100.21 \]
\[ R^2 = 0.697 \]
\[ P < 0.001 \]

\[ y = 0.20x + 87.81 \]
\[ R^2 = 0.300 \]
\[ P = 0.05 \]
Figure 5

(A) Elovl6 mRNA level /GAPDH (% of control)

(B) Relative mRNA level /GAPDH (% of control)

(C) Elovl6 mRNA level /GAPDH (% of control)

(D) CHOP mRNA level /GAPDH (% of control)
Figure 6

(A) Relative mRNA level in kidney /GAPDH (% of control)

(B) Elovl6 mRNA level in kidney /GAPDH (% of control)

(C) y = 9.63x – 834.06
R² = 0.715
P = 0.001

(D) y = 2.68x – 150.54
R² = 0.443
P < 0.05
Figure 7

(A) The amount of C18:0 (μg) / kidney tissue (mg)

(B) Relative mRNA level in kidney /GAPDH (% of WT control)

(C) Elovl6 mRNA level in kidney /GAPDH (% of WT control)

WT control
WT 5/6Nx
KO 5/6Nx

WT control
WT 5/6Nx
KO 5/6Nx

WT control
WT 5/6Nx
KO 5/6Nx

P < 0.05

P < 0.05

KIM-1
CHOP
TNF-α
F4/80
TGF-β
Col1a2

* P < 0.05
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Figure 8

Oxidative stress

Albumin → AOPPs

Lumen

AOPPs

Renal tubular epithelial cell

mTORC1 → SREBP1 → Elov16 → C18:0 → ER stress → Tubulopathy

Blood vessel

AOPPs

AOPPs receptor (CD36 etc.)

Rapamycin

Fatostatin

25(OH)D