Renoprotective effect of the xanthine oxidoreductase inhibitor topiroxostat on adenine-induced renal injury

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Kamijo-Ikemori A, Sugaya T, Hibi C, Nakamura T, Murase T, Oikawa T, Hoshino S, Hisamichi M, Hirata K, Kimura K, Shibagaki Y. Renoprotective effect of the xanthine oxidoreductase inhibitor topiroxostat on adenine-induced renal injury. Am J Physiol Renal Physiol 310: F1366–F1376, 2016. First published March 30, 2016; doi:10.1152/ajprenal.00517.2015.—The aim of the present study was to reveal the renoprotective action of a xanthine oxidoreductase (XOR) inhibitor, topiroxostat (Top), compared with another inhibitor, febuxostat (Feb), in an adenine-induced renal injury model. We used human liver-type fatty acid-binding protein (L-FABP) chromosomal transgenic mice, and urinary L-FABP, a biomarker of tubulointerstitial damage, was used to evaluate tubulointerstitial damage. Male transgenic mice (n = 24) were fed a 0.2% (wt/wt) adenine-containing diet. Two weeks after the start of this diet, renal dysfunction was confirmed, and the mice were divided into the following four groups: the adenine group was given only the diet containing adenine, and the Feb, high-dose Top (Top-H), and low-dose Top (Top-L) groups were given diets containing Feb (3 mg/kg), Top-H (3 mg/kg), and Top-L (1 mg/kg) in addition to adenine for another 2 wk. After withdrawal of the adenine diet, each medication was continued for 2 wk. Serum creatinine levels, the degree of macrophage infiltration, tubulointerstitial damage, renal fibrosis, urinary 15-F2t-isoprostane levels, and renal XOR activity were significantly attenuated in the kidneys of the Feb, Top-L, and Top-H groups compared with the adenine group. Serum creatinine levels in the Top-L and Top-H groups as well as renal XOR in the Top-H group were significantly lower than those in the Feb group. Urinary excretion of L-FABP in both the Top-H and Top-L groups was significantly lower than in the adenine and Feb groups. In conclusion, Top attenuated renal damage in an adenine-induced renal injury model.

topiroxostat; xanthine oxidoreductase; xanthine oxidoreductase inhibitor; liver-type fatty acid-binding protein; adenine; tubulointerstitial damage

XANTHINE OXIDOREDUCTASE (XOR) is known to be a pivotal enzyme in purine catabolism, e.g., the conversion of hypoxanthine to xanthine and xanthine to uric acid. Its inhibitor, (XOR-I), is used to treat hyperuricemia, which is widely known to be an aggravative factor of chronic kidney damage (CKD) (3, 4, 42) in addition to diabetes, hypertension, and hyperlipemia. Some clinical trials have suggested that XOR-I slows the progression of renal dysfunction in patients with hyperuricemia and CKD (6, 7, 28, 36). Furthermore, the protein structure of XOR indicates that it is converted to xanthine oxidase (XO) under tissue hypoxic conditions with subsequent tissue damage, and XO produces ROS (23, 35). Therefore, XOR-I may effectively inhibit the progression of renal damage by preventing ROS production (17, 32).

Topiroxostat (Top) is an orally administered nonpurine analog and is a selective XOR-I (29, 30). It forms a covalent linkage to molybdenum via an oxygen in the hydroxylation reaction intermediate and interacts with amino acid residues of the solvent channel (21). Because Top has high bioavailability and safety in animals, it was approved to manage hyperuricemia and gout in clinical practice (33). Recently, a multicenter, double-blind study (7) reported that Top potently decreased the level of albuminuria in patients with hyperuricemic and mild renal dysfunction. Therefore Top, as well as other XOR-Is, e.g., febuxostat (Feb), may also inhibit the progression of renal damage.

The present study aimed to reveal the renoprotective action of Top against adenine-induced renal damage, in which model activation of XOR is induced and metabolites produced by its activation lead to the renal injury. To precisely evaluate the degree of renal injury using the marker urinary liver-type fatty acid-binding protein (L-FABP) (11), human L-FABP chromosomal transgenic (Tg) mice (12) in which human L-FABP was expressed in proximal tubules of the cortex were used, because L-FABP is not expressed in mouse kidneys (37). Because urinary L-FABP accurately reflects the degree of oxidative stress, peritubular blood flow, and tubulointerstitial damage (14–16), it is a novel tubular marker to predict renal prognosis in CKD (11, 13, 19) or for early detection of diabetic nephropathy (1, 16, 27) and acute kidney disease (acute kidney injury) (20, 32, 39) in clinical practice.
Table 1. Body weight and consumption of the adenine diet

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Adenine Group</th>
<th>Feb Group</th>
<th>Top-L Group</th>
<th>Top-H Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight, g</td>
<td>20.6 ± 0.6</td>
<td>20.0 ± 0.3</td>
<td>20.3 ± 0.8</td>
<td>20.1 ± 0.6</td>
</tr>
<tr>
<td>0 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>18.9 ± 0.5</td>
<td>19.5 ± 0.2</td>
<td>19.9 ± 0.8</td>
<td>19.8 ± 0.5</td>
</tr>
<tr>
<td>2 wk</td>
<td>17.9 ± 0.5</td>
<td>20.7 ± 0.4</td>
<td>21.4 ± 0.7</td>
<td>21.7 ± 0.4</td>
</tr>
<tr>
<td>3 wk</td>
<td>18.8 ± 0.7</td>
<td>22.6 ± 0.2*†</td>
<td>23.6 ± 0.6*†</td>
<td>23.7 ± 0.5*†</td>
</tr>
<tr>
<td>4 wk</td>
<td>20.4 ± 0.6</td>
<td>24.1 ± 0.2*†</td>
<td>24.9 ± 0.6*†</td>
<td>24.8 ± 0.4*†</td>
</tr>
<tr>
<td>Adenine consumption, mg·kg⁻¹·day⁻¹</td>
<td>318.3 ± 29.5</td>
<td>294.0 ± 35.3</td>
<td>311.0 ± 46.1</td>
<td>392.5 ± 40.9</td>
</tr>
<tr>
<td>0 wk</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1 wk</td>
<td>383.8 ± 22.2</td>
<td>355.2 ± 51.3</td>
<td>404.0 ± 32.2</td>
<td>408.0 ± 44.1</td>
</tr>
<tr>
<td>2 wk</td>
<td>409.5 ± 72.9</td>
<td>357.2 ± 55.7</td>
<td>446.5 ± 51.3</td>
<td>487.0 ± 74.8</td>
</tr>
</tbody>
</table>

Values are means ± SE. Feb, febuxostat; Top-L, low-dose topiroxostat; Top-H, high-dose topiroxostat. *P < 0.05 vs. the same group at week 0; †P < 0.05 vs. the adenine group at the same time point.

MATERIALS AND METHODS

Animals. This study was approved by the Institutional Animal Care and Use Committee (no. 2014-88) and was performed in the CMIC Bioresearch Center (Hokuto, Yamanashi, Japan), which is certified by the Association for the Assessment and Accreditation of Laboratory Animal Care International (no. 001182) to obtain high-quality data. Human L-FABP Tg mice were generated as previously described (World Intellectual Property Organization patent WO0073791) (11). Seven- to ten-week-old male human L-FABP Tg mice (n = 24, body weight: 21.3–28.7 g) on a C57/BL6 background were evaluated.

Model of adenine-induced renal damage. Male Tg mice (n = 24) were fed a 0.2% (w/w) adenine-containing diet ad libitum (from week 0) (40). Two weeks after the initiation of this diet, renal dysfunction of these mice was confirmed (at week 0), and they were divided into the following four groups: the adenine group was given only the diet containing adenine (adenine group) and the Feb, high-dose Top (Top-H), and low-dose (Top-L) groups were given the diet containing an XOR-I, such as Feb (3 mg/kg), Top-H (3 mg/kg), or Top-L (1 mg/kg), in addition to adenine for another 2 wk. Top is a product of Sanwa Kagaku Kenkyusho, and Feb was synthesized in the laboratory of Sanwa Kagaku Kenkyusho.

To examine the effect of XOR-I in the phase of adenine-independent renal injury, the adenine diet was stopped at 2 wk. After withdrawal of this diet, each medication was continued for an additional 2 wk (week 4). All animals had free access to tap water. Body weight was measured every week from weeks 0 to 4, and consumption of the adenine diet was measured every week from weeks 0 to 2. For urine and serum collections at weeks −2, 0, 2, and 4, all mice were housed overnight individually in metabolic cages with free access to tap water. Mouse kidneys were collected at week 4 for various analyses.

Serum and urinary biochemistry. Serum blood urea nitrogen (BUN) was measured using the urease-LEDH method [Jatro LQ UN Rate (A) II, LSI Medience, Tokyo, Japan]. Serum and urinary creatinine were measured using the Jaffe method (LabAssay Creatinine, WAKO Pure Chemical Industries, Osaka, Japan). Urinary parameters are reported as ratios relative to urinary creatinine levels. Urinary albumin was determined using the Mouse Albumin ELISA kit (Bethyl Laboratories). Urinary human L-FABP was measured using a two-step sandwich ELISA procedure (human L-FABP ELISA kit, CMIC, Tokyo, Japan) (11).

Renal histological and morphometric analysis. For light microscopic analysis, kidneys were dehydrated and embedded in paraffin. Serial sections (2 μm thick) were obtained for conventional histological assessments such as periodic acid-Schiff staining and Masson trichrome staining and for immunohistochemistry. Tubulointerstitial injury in periodic acid-Schiff-stained sections was categorized as tubular dilation with epithelial atrophy and tubular atrophy. Interstitial fibrosis in Masson trichrome-stained sections was defined as the area of blue colors due to extracellular matrix accumulation. Under magnification (×100), 10 nonoverlapping fields from the entire cortical and outer medulla areas were selected. Areas of tubulointerstitial injury were measured manually by drawing around them, and areas of interstitial fibrosis were measured automatically by separating for the blue color using the image analyzer (version 6.4, Auto/Manual Measurement Software, WinRoof, Mitani, Tokyo, Japan). Degrees of both tubulointerstitial injury and interstitial fibrosis were evaluated as ratios relative to the entire cortical and outer medulla areas.

![Fig. 1. Time-related changes in serum blood urea nitrogen (A) and serum creatinine levels (B). Time course of *P < 0.05 vs. the same group at week 0; †P < 0.05 vs. the adenine group at the same week; §P < 0.05 vs. the febuxostat (Feb) group at the same week; ¶P < 0.05 vs. the low-dose topiroxostat (Top-L) group at the same week. Top-H, high-dose topiroxostat.](http://ajprenal.physiology.org/)
Immunohistological analysis. Tissues fixed in methyl Carnoy solution were embedded in paraffin. An indirect immunoperoxidase method was used to identify the antigens, as previously described (8, 9). Macrophages were identified using rat monoclonal antibody F4/80 (1:200, xBMA Biomedicals, Augst, Switzerland). Aquaporin-1 as a proximal tubular marker was identified using rabbit polyclonal antibodies (1:400, Santa Cruz Biotechnology). Collagen type I and collagen type III were identified using rabbit polyclonal antibodies (1:200, Cedarlane Laboratories).

Fig. 2. Time-related changes in urinary albumin (A) and urinary human liver-type fatty acid-binding protein (L-FABP) levels (B). Time course of *P < 0.05 vs. the same group at week 0; †P < 0.05 vs. the adenine group at the same week; §P < 0.05 vs. the Feb group at the same week.

Fig. 3. Evaluation of hypoxia and oxidative stress. A: gene expression of hypoxia-inducible factor (HIF)-1α mRNA transcripts in the kidney. B: protein expression of HIF-1α in the kidney. C: gene expression of heme oxygenase (HO)-1 mRNA transcripts in the kidney. D: immunohistological staining using an antibody against HO-1. Original magnification: ×100. E: areas in D were assessed quantitatively as described in MATERIALS AND METHODS. *P < 0.05 vs. the adenine group at the same week.
Tissues were fixed in 10% buffered formalin and embedded in paraffin. An indirect immunoperoxidase method was used to identify heme oxygenase (HO)-1 using rabbit polyclonal antibody to HO-1 (1:400, Enzo Life Science, Farmingdale, NY). Myofibroblasts were identified using mouse monoclonal antibody to α-smooth muscle actin (α-SMA; 1:800, Sigma-Aldrich, St. Louis, MO). Sections for staining of HO-1 were heated in 0.01 M sodium citrate buffer (pH 6.0) for antigen retrieval.

Visualization was performed by incubation with polymeric horseradish peroxidase-conjugated secondary antibodies (ready-to-use, ImmPRESS Polymer Detection kit, Vector Laboratories, Burlingame, CA). Peroxidase activity was revealed by the diaminobenzidine reaction (Liquid DAB+, DAKO Japan, Tokyo, Japan), and sections were counterstained with hematoxylin.

Ten nonoverlapping fields from the entire cortical and outer medulla areas were selected. Degrees of macrophage infiltration and interstitial myofibroblasts in the cortical and outer medulla interstitium were measured automatically by separating for positively stained areas of F4/80 and α-SMA and are expressed as ratios of F4/80- and α-SMA-positive areas relative to the entire cortical and outer medulla areas under ×100 magnification, as measured with the image analyzer (WinRoof). Similarly, positive areas for aquaporin-1, collagen type I, collagen type III, and HO-1 were measured automatically by separating for positively stained areas for aquaporin-1, collagen type I, collagen type III, and HO-1 and are expressed as ratios of the aquaporin-1-positive area, collagen type I-positive area, collagen type III-positive area, and HO-1-positive area relative to the entire cortical and outer medulla areas.

Localization of human L-FABP and XO. Tissues were fixed in 10% buffered formalin and embedded in paraffin. To evaluate the expression of human L-FABP and presence of XO relative to the production of ROS under pathological conditions, immunohistochemistry was performed using a mouse adenine phosphoribosyltransferase monoclonal antibody against human L-FABP (1:1,000, CMIC), a rabbit polyclonal antibody against XO (1:50, Santa Cruz Biotechnology), and a rabbit polyclonal antibody to aquaporin-1 (1:200) in the serial sections. Apart from the sections fixed in the methyl Carnoy solution, sections fixed in formalin. Therefore, regarding aquaporin-1 staining, the sections were washed in 0.01 M sodium citrate buffer (pH 6.0). Sections were incubated with polymeric horseradish peroxidase-conjugated secondary antibodies (ImmPRESS Polymer Detection kit, Vector Laboratories). Peroxidase activity was revealed by the diaminobenzidine reaction (Liquid DAB+, DAKO), and sections were counterstained with hematoxylin.

Measurement of monocyte chemoattractant protein-1 by ELISA. Proteins were extracted from frozen kidneys, and protein concentrations were measured as previously described (8, 9). The TaqMan real-time PCR was performed using an Applied Biosystems Step One Plus TM Real Time PCR System (Applied Biosystems). Real-time PCR was used to quantify mRNA levels of HIF-1α, HO-1, and GAPDH. mRNA levels of HIF-1α were measured to evaluate the degree of renal hypoxia, and mRNA levels of HO-1 were measured to evaluate the degree of oxidative stress. Expression levels of these mRNAs in each sample were normalized to GAPDH expression levels.

Measurement of urinary 15-F2-isoprostane. To evaluate the degree of oxidative stress at week 4 compared with that at week 0, urinary 15-F2-isoprostane at weeks 0 and 4 was measured using a Urinary Isoprostane Assay Kit (Detroit R&D) (2, 24), and its levels are reported as ratios relative to urinary creatinine levels.

Measurement of urinary kidney injury molecule-1. To evaluate the degree of loss of protein from the apical membrane, urinary kidney injury molecule (KIM)-1 at week 4 was measured using a mouse TIM-1/Kim-1 ELISA kit (R&D Systems), and its levels are reported as ratios relative to urinary creatinine levels.

Measurement of XOR by liquid chromatography with Fourier transform mass spectrometry. Kidney homogenates were centrifuged for 60 min at 105,000 g at 4°C (25). These cytosols were added to reaction mixtures containing [15N2]xanthine (0.8 mM), NAD+ (1 mM), and oxonate (0.013 mM). Tris·HCl buffer (20 mM, pH 8.5) was added to adjust the total volume to 150 μl for each reaction mixture. These mixtures were incubated at 37°C for 30 min, and 50 μl of 200 μmol/l [13C2,15N2]uric acid were then added as an internal standard. Subsequently, the mixture was heated for 5 min at 95°C to stop the reaction. The resulting suspensions were centrifuged for 10 min at 15,000 g and 4°C. Supernatants were filtered through ultrafiltration membranes (Amicon Ultra-0.5 centrifugal filter devices, 3K, Millipore), and production levels of [15N2]uric acid were measured by liquid chromatography-mass spectroscopy (LTQ-Orbitrap, Thermo Fisher Scientific). Each activity was expressed as [15N2]uric acid in nanomoles per minute per milligram of protein.

Real-time quantitative PCR analysis. Total RNA was extracted and reverse transcribed as previously described (8, 9). The TaqMan real-time PCR was performed using an Applied Biosystems Step One Plus TM Real Time PCR System (Applied Biosystems). Real-time PCR was used to quantify mRNA levels of HIF-1α, HO-1, and GAPDH. mRNA levels of HIF-1α were measured to evaluate the degree of renal hypoxia, and mRNA levels of HO-1 were measured to evaluate the degree of oxidative stress. Expression levels of these mRNAs in each sample were normalized to GAPDH expression levels.

Western blot analysis. Protein (30 μg) extracted from frozen kidneys were separated by 4–20% Tris·HCl SDS-PAGE (Bio-Rad, Hercules, CA). After proteins had been transferred from the gel to a polyvinylidene difluoride membrane using the iBlot Dry Blotting Device (Hercules, CA), membranes were blocked in 3% BSA (Sigma-Aldrich) diluted in PBS and Tween 20. A primary antibody of 1:200 diluted mouse monoclonal antibody against hypoxia-inducible factor (HIF)-1α (Abcam) was incubated overnight at 4°C. After a wash, the primary antibody was detected with 1:2,000 diluted horseradish peroxidase-conjugated rabbit anti-mouse antibody (Abcam) for 1 h at room temperature. Subsequently, the chemiluminescent signal labeled using ECL Prime Western Blotting Detection Reagent (Amersham Biosciences, Uppsala, Sweden) was detected using a charge-coupled device camera system (LAS-4000, Fuji Photo Film, Tokyo, Japan). The membrane was then incubated at 50°C for 30 min in stripping buffer (2% SDS, 100 mM 2-mercaptoethanol, and 12% of 0.5 M Tris·HCl, pH 6.8) to remove the probes. The reprobing procedure was performed further with the 1:500 diluted mouse monoclonal antibody to GAPDH (Millipore, Billerica, MA). HIF-1α protein was normalized to GAPDH.

Measurement of urinary kidney injury molecule-1. To evaluate the degree of loss of protein from the apical membrane, urinary kidney injury molecule (KIM-1) at week 4 was measured using a mouse TIM-1/Kim-1 ELISA kit (R&D Systems), and its levels are reported as ratios relative to urinary creatinine levels.

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Statistical analysis. All values are expressed as means ± SE. Statistical significance was set at $P < 0.05$. Differences from week 0 in each group were analyzed using the Steel test. Four groups were analyzed by one-way ANOVA followed by the Mann-Whitney $U$-test.

RESULTS

Body weight and consumption of feed. At week 0, body weights were similar in the four groups. In the adenine group,
body weight did not change from weeks 0 to 4. In the Feb, Top-L, and Top-H groups, body weight increased significantly at weeks 3 and 4 compared with week 0 within the same groups, and the levels were significantly higher than those in the adenine group at the same time points \( P < 0.05 \); Table 1.

The amount of adenine feed consumed relative to body weight was similar in the four groups (Table 1).

**Serum and urinary biochemistry.** Both serum BUN (Fig. 1A) and creatinine (Fig. 1B) levels increased significantly at week 0 compared with week \(-2\), and the levels at week 0 were similar in the four groups. In the adenine group, both serum BUN and creatinine levels further increased significantly at week 2 compared with week 0 but then decreased to week 0 levels by week 4. In the Feb, Top-L, and Top-H groups, serum BUN levels did not increase at week 2, and they had decreased significantly by week 4 compared with week 0. At weeks 2 and 4, serum BUN levels in the Feb, Top-L, and Top-H groups were similar and significantly lower than those in the adenine group. In the Feb group, serum creatinine levels did not increase at week 2 and were not decreased significantly at week 4 compared with week 0. In the Top-L and Top-H groups, serum creatinine levels did not increase at week 2, but they were decreased significantly at week 4 compared with week 0. At week 4, serum creatinine levels in the Feb, Top-L, and Top-H groups were similar and significantly lower than those in the adenine group. At week 4, serum creatinine levels in the Feb, Top-L, and Top-H groups were significantly lower than those in the adenine group, and the levels in the Top-L and Top-H groups were significantly lower than those in the Feb group.

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**Fig. 7.** Histological findings. A–D: representative images of immunohistological staining using an antibody against aquaporin-1 (A), \( \alpha \)-smooth muscle actin (\( \alpha \)-SMA; B), collagen type I (C), and collagen type III (D). Original magnification: \( \times 100 \). E–H: areas in A–D were assessed quantitatively as described in MATERIALS AND METHODS. \*\( P < 0.05 \) vs. the adenine group at the same week.
Fig. 8. Urinary kidney injury molecule (KIM)-1 levels at week 4. *P < 0.05 vs. the adenine group at the same week.

Furthermore, these levels in the Top-H group were significantly lower than those in the Top-L group.

Urinary albumin levels (Fig. 2A) were increased significantly at week 0 compared with week −2, and the levels at week 0 were similar in the four groups. In the adenine group, urinary albumin levels at week 2 were similar to those at week 0 and were decreased at week 4, but this level was not significantly lower than that measured at week 0. In the Feb, Top-L, and Top-H groups, urinary albumin levels at weeks 2 and 4 decreased significantly compared with week 0, and the levels at weeks 2 and 4 were similar. Urinary albumin levels were significantly lower in the Top-L group at week 2 (P < 0.05) and in the Feb, Top-L, and Top-H groups at week 4 (P < 0.01) compared with levels in the adenine group measured at the same time points.

Urinary L-FABP levels (Fig. 2B) were increased significantly at week 0 compared with week −2, and the levels at week 0 were similar in the four groups. In the adenine group, urinary L-FABP levels were further increased significantly at week 2 compared with week 0 and had decreased to week 0 levels by week 4. In the Feb, Top-L, and Top-H groups, urinary L-FABP levels did not increase at week 2, but they decreased significantly at week 4 in the Top-L and Top-H groups compared with week 0, but not in the Feb group. At week 2, urinary L-FABP levels in the Feb, Top-L, and Top-H groups were similar and significantly lower than those in the adenine group. At week 4, urinary L-FABP levels were significantly lower in the Top-L and Top-H groups compared with the adenine or Feb groups. These levels in the Feb group at week 4 tended to be lower than those in the adenine group, but this difference was not significant.

Evaluation of hypoxia and oxidative stress. HIF-1α mRNA levels were measured to evaluate the degree of renal hypoxia. Gene expression levels of HIF-1α (Fig. 3A) in kidneys of the Feb and Top-H groups were significantly lower than in the kidneys of the adenine group (P < 0.05). Regarding the protein expression of HIF-1α, Western blot analysis of HIF-1α revealed that HIF-1α protein levels in kidneys of the Feb, Top-L, and Top-H groups were significantly lower than in kidneys of the adenine group (P < 0.05; Fig. 3B).

HO-1 mRNA levels were measured to evaluate the degree of oxidative stress. HO-1 gene expression levels (Fig. 3C) in kidneys of the Feb, Top-L, and Top-H groups were significantly lower than in the kidneys of the adenine group (P < 0.05). HO-1 protein was detected using immunohistochemical analysis. Although HO-1 was expressed in proximal tubules of the four groups, its expression levels in kidneys of the Feb, Top-L and Top-H groups were significantly lower than in the adenine group (P < 0.05; Fig. 3, D and E).

Although urinary 15-F2t-isoprostane levels at week 0 were similar in the four groups, these levels were significantly higher at week 4 compared with week 0 in the adenine group (Fig. 4). In the other groups, urinary 15-F2t-isoprostane levels were similar between weeks 0 and 4. Urinary 15-F2t-isoprostane levels in the Feb, Top-L, and Top-H groups at week 4 were significantly lower than in the adenine group (P < 0.01).

Expression of MCP-1 in the kidney. MCP-1 is an inflammatory cytokine released to the interstitium of kidneys from the proximal tubules. Protein expression levels of MCP-1 in the Feb, Top-L, and Top-H groups were significantly lower than those measured in the adenine group (P < 0.01; Fig. 5A).

Evaluation of macrophage infiltration. To examine the degree of tubulointerstitial inflammation, the degree of macrophage infiltration was evaluated by immunohistochemical analysis using rat monoclonal antibody F4/80. Macrophage infiltration into the kidneys of the Feb and Top-H groups was significantly reduced compared with the adenine group (P < 0.05; Fig. 5, B and C).

Renal histological and morphometric analysis. In all of the groups, periodic acid-Schiff-stained sections revealed tubulo-
interstitial damage, including dilatation of tubules and degeneration of proximal tubular epithelial cells (Fig. 6A). In particular, severe tubulointerstitial damage was observed in the adenine group. The damaged areas in the kidneys from the Feb, Top-L, and Top-H groups were significantly smaller than those in the adenine group ($P < 0.01$; Fig. 6B).

Masson’s trichrome staining revealed the presence of tubulointerstitial fibrosis (blue staining; Fig. 6C) in the adenine group. The degree of tubulointerstitial fibrosis in the Feb, Top-L, and Top-H groups was significantly lower than in the adenine group ($P < 0.01$; Fig. 6D).

**Immunohistological analysis of aquaporin-1, α-SMA, collagen type I, and collagen type III.** To search for the presence of proximal tubules that either were resistant to adenine-induced nephropathy or had regenerated after such nephropathy, immunohistochemical analysis was carried out to detect aquaporin-1 expression (Fig. 7A). Aquaporin-1-positive areas in the Feb, Top-L, and Top-H groups were significantly larger than those in the adenine group ($P < 0.05$; Fig. 7E).

To examine the degree of tubulointerstitial fibrosis, α-SMA, collagen type I, and collagen type III expressions were detected using immunohistochemical analysis. α-SMA-positive cells (Fig. 7, B and F) and deposition levels of collagen type I (Fig. 7, C and G) and collagen type III (Fig. 7, D and H) on the kidneys in the Feb, Top-L, and Top-H groups were significantly lower than in the adenine group ($P < 0.05$).

**Evaluation of urinary KIM-1.** Urinary KIM-1 levels in the Feb, Top-L, and Top-H groups were significantly lower than in the adenine group at week 4 ($P < 0.05$; Fig. 8).

**Evaluation of renal XOR activity.** Activity of renal XOR in the Feb, Top-L, and Top-H groups was significantly lower than in the adenine group ($P < 0.05$; Fig. 9). XOR activity in the Top-H group was significantly lower than that in the Feb group ($P < 0.05$).

**Expression of human L-FABP and XO in the kidney.** Human L-FABP was expressed in aquaporin-1-positive proximal tubules in mouse kidneys of the adenine group (Fig. 10). XO was detected generally or partially in the proximal tubules, which were double positive (L-FABP+/aquaporin-1+).

**DISCUSSION**

This study, which used a model of adenine-induced nephropathy, showed that both Top and Feb suppressed renal dysfunction to a similar level in the same phase (week 2) of adenine-dependent renal injury. At week 4, both Top and Feb inhibited the activation of XOR, downregulated HIF-1α (hypoxia) and HO-1 (oxidative stress) expression in the kidneys, inhibited the production of inflammatory cytokines and renal 15-F2t-isoprostane, and attenuated tubulointerstitial damage and renal dysfunction. Furthermore, both serum creatinine and urinary L-FABP levels were significantly lower in mice that received Top-H and Top-L compared with mice that received Feb, indicating that Top may have a renoprotective effect in renal disease. This could be due to XOR activation that is more effective than in Feb mice.

Uric acid levels in rodents are lower than those in humans due to the presence of uricase, a hepatic enzyme that degrades uric acid to allantoin. Since rodents lack uricase, uric acid levels increase in rodents and lead to oxidative stress due to the formation of ROS. This leads to renal injury.

**Fig. 10.** Localization of xanthine oxidase (XO) activity, human L-FABP, and aquaporin-1 in serial sections of the kidney in the adenine group. In aquaporin-1- and human L-FABP-positive tubules (arrow), expression of XO was observed generally or partially. Original magnification: ×400.

**Fig. 11.** Mechanism by which XOR inhibitor (XOR-I) inhibited the progression of renal injury in adenine-induced nephropathy. Adenine in the chow is primarily metabolized to 2,8-dihydroxyadenine (DHA) by XOR in the liver after intestinal absorption, and an increase in DHA concentration is induced. Next, DHA is precipitated in renal tubules because of its low solubility. Deposition of DHA crystals or tubular occlusion by DHA crystals injures renal tubular epithelial cells and causes inflammatory injury with subsequent fibrotic changes. Tubular occlusion causes stress to not only tubular cells but also to the peritubular capillary, thereby leading to tubular hypoxia. Under tubular hypoxia, degradation of ATP rather than production of ATP is accelerated, inducing an increase in hypoxanthine, which causes activation of renal XOR with production of ROS. This leads to tubulointerstitial damage and renal dysfunction. Tubular hypoxia and increased ROS accelerate urinary excretion of human L-FABP. While mice were fed the adenine diet, XOR-I may have inhibited XOR in both the liver and kidneys. After withdrawal of the diet, XOR-I may have inhibited renal XOR with a subsequent progression of renal damage.
uric acid to allantoin, and, therefore, hyperuricemia rarely occurs (38). Renal toxicity in adenine-induced nephropathy, therefore, is induced by 2,8-dihydroxyadenine (DHA) (18), a metabolic product of adenine catalyzed by XOR (Fig. 11). DHA will precipitate in renal tubules because of its low solubility. Deposition of DHA crystals or tubular occlusion by DHA crystals injures renal tubular epithelial cells and causes inflammatory injury with subsequent fibrotic changes (26). Tubular occlusion causes stress to not only tubular cells but also to the peritubular capillary, leading to tubular hypoxia (10). The primary target of adenine-induced nephropathy is the tubulointerstitium. Especially in the proximal tubules, production and degradation of ATP lead to active reabsorption of massive substances under aerobic conditions. Under tubular hypoxia, degradation of ATP rather than production of ATP is accelerated, thereby increasing the induction of hypoxanthine (34). This, in turn, causes activation of renal XO converted from XOR with the subsequent production of ROS (23, 35) and leads to tubulointerstitial damage and renal dysfunction (31). Because urinary 15-F_{2\alpha}-isoprostane levels after withdrawal of the adenine diet (at week 4) were significantly higher than those at week 0 in the adenine group, this result indicated that the oxidative stress continued generating after the adenine diet was stopped. Tubular hypoxia and increased ROS accelerate urinary excretion of human L-FABP (16). While mice were fed the adenine diet, Top or Feb may have inhibited both XOR activity in the liver and kidneys. After withdrawal of the adenine diet, they may have inhibited renal XOR activity, leading to the subsequent progression of renal damage. XOR-I retards the progression of renal disease by direct or indirect actions of DHA, and this may be an important strategy for treating progressive renal disease.

The dosages of Top administered to the mice in the present study, 1 or 3 mg/kg, are equivalent to dosages of Top prescribed to humans in clinical practice, whereas the Feb dosage given to the mice, 3 mg/kg, is greater than the maximum dose of 60 mg once daily for humans. Nevertheless, renal XOR activity was significantly lower in Top-H group than in the Feb group. Because Top-H group showed stronger XOR inhibitory potency compared with the Feb group, both attenuation of renal function and decreases in urinary L-FABP levels were significantly facilitated in Top-H group compared with the Feb group. Although comparison between Top and Feb on renoprotective effects has not been performed in clinical study for patients with CKD, Top with stronger XOR inhibitory potency might prevent the progression of CKD more than Feb.

Urinary albumin, which is considered a glomerular marker, has been reported to be useful for the detection of tubular dysfunction (41) and in the prediction of acute kidney injury after cardiopulmonary bypass surgery (43). Although urinary L-FABP, a tubular marker, increased significantly in the adenine group from weeks 0 to 2, urinary albumin levels were similar between weeks 0 and 2. In adenine-induced nephropathy, the changes in serum BUN and creatinine were similar to changes in urinary L-FABP, and, therefore, renal function deteriorated along with the progression of tubulointerstitial damage. However, urinary albumin did not reflect the change in renal function. This suggested that the sensitivity of urinary albumin as a tubular marker was lower than that of urinary L-FABP.

The mice used in this study expressed human L-FABP in their proximal tubules (12). L-FABP is known to have antioxidant effects and to inhibit the progression of renal damage in various renal injury models (16). In this study, XO expression was observed in the proximal tubules along with expression of human L-FABP. However, others have reported that the degree of renal damage in L-FABP Tg mice that received adenine was not significantly attenuated compared with that in wild-type mice (40); thus, a renal protective function of L-FABP was not observed. Therefore, it was considered that the expression of human L-FABP in the proximal tubules did not influence the renal protection of Top, consistent with results of the present study.

As a limitation, because it was difficult to perform the in vitro study correspondent to the adenine model, the detail mechanism by which Top works on cell function and pathways of oxidative stress was not elucidated in this study. Further study is needed to clarify the point.

XOR activation is known to contribute to the progression of hypertension (22), unilateral ureteric obstruction (31), and ischemia-reperfusion injury (5) by not only adverse effects of hyperuricemia produced by XOR activation but also by oxidative stress induced by XO conversion from XOR. Therefore, XOR-I is an important agent to prevent the progression of tubulointerstitial damage in kidney injury. This study demonstrates that Top attenuates tubulointerstitial damage and renal dysfunction in adenine-induced nephropathy. We propose that Top could be translated for wide therapeutic use to prevent disease progression in patients with various renal diseases.

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DISCLOSURES

T. Sugaya is the Director and Senior Scientist and T. Oikawa is a Scientist of CMIC HOLDINGS Company, Limited, the company that produced the kits for L-FABP analysis. C. Hibi, T. Nakamura, and T. Murase are employees of Sanwa Kagaku Kenkyusho Company, Limited., the company that produced topiroxostat. None of the other authors have conflicts of interest or financial disclosures of any relevance to the present study.

AUTHOR CONTRIBUTIONS


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