Nephrotoxicity Induced by a Single Dose of Adenine: Effects of 4-Aminopyrazolo[3,4-*d*]pyrimidine and Allopurinol

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The effects of allopurinol and 4-aminopyrazolo[3,4-*d*]pyrimidine (4APP) on adenine-induced renal injury in mice were examined. Plasma urea nitrogen (UN) and creatinine levels increased after the oral administration of adenine to mice. However, plasma UN and creatinine levels decreased inversely with the dose of 4APP when a different dosage of 4APP was administered together with adenine. Yet 4APP did not have any effect on the UN or creatinine levels when 4APP was administered after adenine administration. Plasma UN and creatinine levels increased in the allopurinol-administered group as in the adenine-administered group. Moreover, from light microscopic observation by hematoxylin-eosin staining, microvacuolic changes in the proximal tubuli were detected in the mouse kidney in the adenine-administered group, and epithelial cell loss, degeneration and microvacuolic changes in the proximal tubuli were observed in the mouse kidney in the adenine-and-allopurinol-administered group. However, there were no changes in the proximal tubuli in the mouse kidney in the adenine-and-4APP-administered group.

These findings suggested that 4APP inhibits the action of adenine in the mouse kidney.

Keywords adenine; allopurinol; 4-aminopyrazolo[3,4-*d*]pyrimidine; urea nitrogen; creatinine; microvacuolic change

Adenine is one of the most important components of nucleotides, and large amounts of adenine are found and ingested in food. Adenine is converted into adenosine 5'-monophosphate (AMP) by adenine phosphoribosyl transferase, and then into ATP for use by the body.¹⁾ However, Yokozawa *et al.*²⁾ have reported that renal injury occurred when food containing adenine was given to rats for a month, which they speculated was caused by the accumulation of 2,8-dihydroxy adenine (2,8-DHA) in the renal tubuli^{3,4)} and by the formation of guanidino compounds.⁵⁾ Xanthine oxidase (XOD) acts upon hypoxanthine and xanthine and produces uric acid,¹⁾ and XOD acts directly upon adenine and produces 2,8-DHA.⁶⁾ Therefore, XOD plays a large role in the adenine metabolic system. In addition, this enzyme is said to produce active oxygen.⁷⁾ Guanidino compounds have also been reported to be produced by active oxygen⁸⁾ and free radicals, participating in the induction of renal toxicity from adenine administration.⁹⁾ In our previous report, renal injury occurred in mice after a single administration of adenine.¹⁰⁾ However, there has been some doubt about the action of XOD on adenine-induced renal injury. Therefore, we investigated the cause of adenine-induced renal injury by using two adenine analogs, allopurinol and 4-aminopyrazolo[3,4-*d*]pyrimidine (4APP).

MATERIALS AND METHODS

Animals Six week-old male ddY strain mice were purchased from Japan SLC Co. (Shizuoka, Japan) and housed for one week prior to the start of the experiments, during which time they were fed standard mouse food (MF, Oriental Yeast Co., Ltd., Tokyo) and given tap water *ad libitum*.

Chemicals Adenine, allopurinol, 4APP and 2,8-DHA, whose structures are shown in Fig. 1, were obtained from

Sigma Chemical Co. (MO, U.S.A.).

Experimental Design The dosage of adenine and its analogs was based on the LD₅₀ value of adenine (745 mg/kg)¹¹⁾ and our previous report.¹²⁾

Administration of Adenine, Allopurinol and 4APP After one night of fasting, the mice were divided into six groups of six mice each. They were orally administered a solution (10 ml/kg) containing saline (control group), 175 mg/kg of adenine (ade group), allopurinol (allo group), 4APP (4APP group), adenine (175 mg/kg) and allopurinol (175 mg/kg) administered simultaneously (ade+allo group), and adenine (175 mg/kg) and 4APP (175 mg/kg) administered simultaneously (ade+4APP group).

Effect of the Dosage of 4APP on Adenine-Induced Renal Injury 4APP was administered orally together with adenine (175 mg/kg) to each group of mice at concentrations of 0, 1, 5, 10, 20, 40, 80, and 175 mg/kg. In the control group, saline alone was administered.

Effect of the Time of 4APP Administration on Adenine-Induced Renal Injury 4APP (175 mg/kg) was administered to the adenine-administered mice at 3, 1.5 h before, simultaneously with, 3 or 6 h after adenine administration.

Observation and Analyses Twenty-four hours after adenine administration, all mice were anesthetized, heparinized blood specimens were collected, and the kidneys were removed. In the other group, urine was collected at 24 h.

Fig. 1. Structures of Adenine, Allopurinol and 4APP

Each left kidney was homogenized with 0.25 M sucrose and the lipid peroxide level was measured by the method of Ohkawa *et al.*¹³⁾ After centrifugation, the superoxide dismutase (SOD) activity of the cytosol fraction was measured by the method of Minami and Yoshikawa,¹⁴⁾ and XOD activity was measured by the method of Hashimoto.¹⁵⁾ Each value was normalized with respect to protein, which was measured by the method of Lowry *et al.*¹⁶⁾ Plasma urea nitrogen (UN), creatinine and uric acid levels were measured with an autoanalyzer (705, Hitachi Co., Tokyo, Japan) by the urease and indophenol method (Nescauto UN; Nippon Shoji Co., Osaka), picric acid method (Nescauto CRE; Nippon Shoji Co.), and uricase and TOOS method (Nescauto UA; Nippon Shoji Co.). Urinary *N*-acetyl- β -D-glucosaminidase (NAG) activity and uric acid levels were measured by the NAG rate test (Shionogi & Co., Ltd., Osaka) and Nescauto UA kits. Urinary NAG activity and uric acid levels were corrected with respect to creatinine, which was measured with a Nescauto CRE kit using an autoanalyzer. Furthermore, a 10% homogenate was prepared with saline from each right kidney, and an equivalent volume of 10% trichloroacetic acid (TCA) was added. After centrifugation, the content of 2,8-DHA was assayed by means of high-performance liquid chromatography using a Shimadzu LC-6A and a UV-VIS spectrophotometric detector (SPD-6AV) (Shimadzu Co., Kyoto, Japan). A 10 μ l specimen that was extracted by 10% TCA solution was injected and eluted by a mixture of 50 mM NaH_2PO_4 and acetonitrile (98:2) at a flow rate of 1.0 ml/min at room temperature on a Cosmosil 5C18-AR column (250 $\times$ 4.6 mm i.d., Nakalai Tesque Inc., Kyoto, Japan) and the eluent was monitored at 305 nm.

Pathological Examinations Twenty-four hours after each drug administration, mice were anesthetized and perfused with saline. For light microscopy, the kidneys from individual mice were fixed in 10% formalin, embedded in paraffin, sectioned, and stained with hematoxylin-eosin (HE) and periodic acid Schiff reaction (PAS).

Statistical Analysis All results are given as the mean values $\pm$ S.D. Differences among the groups were determined by a one-way analysis of variance, and the comparison of means among the groups was evaluated by using the method of Dunnett.

RESULTS

As shown in Fig. 2, the plasma UN and creatinine levels were increased in the ade group. However, these levels in the allopurinol- or 4APP-administered group did not differ from those in the control group. In the ade + allo group, these levels were increased. However, these levels in the ade + 4APP group were lower than those in the ade group and did not differ from the levels in the control group. The plasma uric acid level was not increased in mice administered adenine. However, the level in the 4APP-administered group was significantly decreased and the same phenomenon was observed when 4APP and adenine were simultaneously administered.

The urinary NAG activity was increased in the adenine-administered group. However, the NAG activity in the 4APP-administered group did not differ from the level in the control group. The urinary uric acid level did not differ between the control and adenine-administered groups, although the uric acid level decreased in the

Fig. 2. Effects of Allopurinol and 4APP on the Plasma UN, Creatinine and Uric Acid Levels of Adenine-Administered Mouse

Twenty-four hours after various reagents were administered to each mouse group, plasma specimens were collected and UN and creatinine level measured. Control (C) group, saline given orally; ade group, adenine (175 mg/kg) given orally; allo group, allopurinol (175 mg/kg) given orally; 4APP group, 4APP (175 mg/kg) given orally; ade + allo group, adenine (175 mg/kg) and allopurinol (175 mg/kg) given orally, simultaneously; ade + 4APP group, adenine (175 mg/kg) and 4APP (175 mg/kg) given orally, simultaneously. Each value is the mean $\pm$ S.D. of 6 mice. a) $p < 0.05$, b) $p < 0.01$ vs. control group.

Fig. 3. Effects of Allopurinol and 4APP on the Urinary NAG Activity and Uric Acid Levels of Adenine-Administered Mouse

Urine was collected 24 h after the administration of various reagents, and NAG activity, uric acid and creatinine levels were measured. NAG activity and uric acid values were normalized with respect to creatinine level. Control group, saline given orally; adenine group, adenine (175 mg/kg) given orally; allopurinol group, allopurinol (175 mg/kg) given orally; 4APP group, 4APP (175 mg/kg) given orally; adenine+allopurinol group, adenine (175 mg/kg) and allopurinol (175 mg/kg) given orally, simultaneously; adenine+4APP group, adenine (175 mg/kg) and 4APP (175 mg/kg) given orally, simultaneously. Each value shows the mean $\pm$ S.D. of 6 mice. *a* $p < 0.05$ vs. control group.

Fig. 4. HE Staining of Mouse Kidney

(A) control, (B) adenine administered, (C) adenine and allopurinol administered, and (D) adenine and 4APP administered. Twenty-four hours after the administration of various reagents, the kidneys perfused with saline were removed and fixed in 10% formalin, embedded in paraffin, sectioned and stained with HE.

allopurinol-administered group (Fig. 3).

After HE staining, microvacuolic changes of the proximal tubuli in the kidney were observed in the ade

group. In the ade+allo group, epithelial cell loss and degeneration and microvacuolic changes of the proximal tubuli in the kidney were also observed. However, these

changes were not detected in the ade+4APP group (Fig. 4). Glomeruli and tubular basement membranes in the mouse kidney after PAS staining did not differ with the group (data not presented).

SOD and XOD activities were reduced in the ade group. However, when either allopurinol or 4APP was administered together with adenine, SOD activity did not differ from that in the control group. XOD activity was reduced when either adenine or allopurinol was administered, compared to that of the control group. There was no difference in XOD activity between the control and the ade+4APP groups. The lipid peroxide level did not differ (Table I).

As shown in Fig. 5, 2,8-DHA was produced in the kidney in the ade group. However, the 2,8-DHA content in the kidney in the ade+allo group was lower than that in the ade group.

When 4APP was administered at different doses together with adenine, plasma UN and creatinine levels decreased

TABLE I. SOD and XOD Activities and Lipid Peroxide Level in Mouse Kidney

	SOD activity (U/mg protein)	XOD activity (U/mg protein)	Lipid peroxide (nmol MDA/mg protein)
Control	5.31±0.60	15.40±4.02	3.30±0.56
Adenine	3.53±0.33 ^{a)}	6.22±1.13 ^{b)}	3.85±0.64
Allopurinol	4.19±0.30	8.40±2.32 ^{a)}	3.32±0.28
4APP	4.26±0.95	16.25±2.97	3.17±0.66
ade+allo	4.40±0.37	8.58±4.49 ^{a)}	3.06±0.27
ade+4APP	4.78±0.91	9.96±2.74	3.04±0.45

MDA: malondialdehyde. Lipid peroxide in 10% homogenate was assayed, and SOD and XOD activities in the cytosol fraction were measured. Each value was normalized with respect to protein. Each value shows the mean±S.D. of 6 mice. ^{a)} $p < 0.05$, ^{b)} $p < 0.01$ vs. control group.

inversely with the dose of 4APP (Fig. 6).

Figure 7 shows the levels of plasma UN and creatinine when the administration time of 4APP changed and 4APP was administered together with adenine. Those levels did not differ from the levels in the control group when 4APP was administered before the administration of the adenine, or simultaneously with adenine. However, when 4APP was administered after adenine administration, those levels were increased.

Fig. 5. 2,8-DHA Content in the Kidney

The concentration of 2,8-DHA in the kidney was measured by the HPLC method. HPLC conditions were shown in the Materials and Method. Each value shows the mean±S.D. of 6 mice. ^{a)} $p < 0.01$ vs. control group.

Fig. 6. Effects of Various Doses of 4APP on the Plasma UN and Creatinine Levels of Adenine-Administered Mouse

Twenty-four hours after administration of 4APP at various doses, together with adenine (175 mg/kg), to each mouse group, plasma samples were collected and UN and creatinine levels were measured. UN; creatinine. Control group, saline given orally; adenine group, adenine (175 mg/kg) given orally; adenine+4APP group, adenine (175 mg/kg) and various doses of 4APP (1, 10, 20, 40, 80 or 175 mg/kg) given orally, simultaneously. Each value shows the mean±S.D. of 6 mice. ^{a)} $p < 0.05$, ^{b)} $p < 0.01$ vs. control group.

Fig. 7. Effect of the Administration Time of 4APP on Adenine-Induced Mouse Renal Injury

▨, UN; ▤, creatinine. 4APP (175 mg/kg) was administered to the adenine-administered mice at the following times: 3 h before (group 1), 1.5 h before (group 2), simultaneously (group 3), 3 h after (group 4), and 6 h after adenine administration (group 5). Control group, saline given orally; adenine group, adenine (175 mg/kg) given orally. Twenty-four hours after the administration of adenine, all mice were anesthetized, and heparinized blood specimens were collected. Results are given as the mean $\pm$ S.D. of 6 mice. *a)* $p < 0.01$ vs. control group.

DISCUSSION

Renal injury occurs after the oral administration of adenine to mice and rats and after the intravenous administration of adenine to dogs.^{2,10,17} There are two pathways of adenine metabolism: (1) Adenine is converted by adenine phosphoribosyl transferase into adenine nucleotide and is utilized in the body, and is finally converted to uric acid by purine 5'-nucleotidase, purine nucleotide phosphorylase, and XOD. (2) XOD acts directly upon adenine to produce 2,8-DHA.¹ 2,8-DHA is an insoluble substance and 2,8-DHA crystals were precipitated in the proximal tubuli when the rat was fed an adenine diet.^{3,4} Moreover, 2,8-DHA urolithiasis developed in a boy who lacked adenine phosphoribosyl transferase.¹⁸ Therefore, XOD plays an important role in the adenine metabolic systems.

As shown in Fig. 1, 4APP and allopurinol are analogs of adenine. Both 4APP and allopurinol have a pyrazolo-[3,4-*d*]pyrimidine base, while adenine itself has a purine base. Allopurinol inhibits the activity of XOD.¹⁹ In this experiment, the plasma UN and creatinine levels in the allopurinol-and-adenine-administered group were increased, although the 2,8-DHA content in the allopurinol-and-adenine-administered group was lower than that in the adenine-administered group. However, the injury of renal tubuli probably occurred because of the increase in urinary NAG activity (Fig. 3). NAG is a lysosomal enzyme located in the renal tubular epithelial cells.^{20,21} Moreover, changes in the proximal tubuli in the kidney were detected by light microscopic observation with HE staining in the ade and ade+allo groups (Fig. 4), although no changes were observed in the glomeruli and tubular basement membranes with PAS staining (data not presented). These findings suggest that adenine induced renal injury, especially to the proximal tubuli, and that allopurinol does

not affect the adenine-induced renal injury. On the other hand, when 4APP, whose structure has amino group instead of allopurinol, was administered together with adenine, the plasma UN and creatinine levels and the urinary NAG activity were not changed from those in the control group. Moreover, the changes of proximal tubuli in the kidney were not detected in the ade+4APP group (Fig. 4). Therefore, 4APP is considered to inhibit adenine-induced renal injury. 4APP has been found to affect the function of the liver and inhibit the hepatic release of lipoprotein, and it remarkably decreases the serum cholesterol level.²²⁻²⁵ Iwamoto and Martin Jr.²⁶ compared the effects of 4APP and allopurinol on *de novo* purine biosynthesis of rat hepatoma cells. It has also been reported that 4APP causes a repression of an early function in the *de novo* pathway, although allopurinol acts by end product-mediated inhibition and/or depletes the cell of substrates. In our experiment, the effects of allopurinol and 4APP on adenine-induced renal injury were different. 4APP inhibited the action of adenine in the mouse kidney dose-dependently (Fig. 6). Plasma UN and creatinine levels were also decreased when 4APP was administered with adenine simultaneously, or before the adenine administration (Fig. 7). Moreover, the decrease in renal XOD and SOD activity in the mouse kidney in the adenine-administered group was improved by the administration of 4APP. Therefore, 4APP acts to inhibit adenine-induced renal injury, whereas free radicals may induce renal injury. Several guanidino compounds, which are induced by active oxygen, are found to be increased in uremic biological fluids, and some guanidino compounds may act as uremic toxins.²⁷⁻³⁰ Yokozawa *et al.*³¹ reported that the production of methylguanidine from creatinine in adenine-induced renal failure rats was higher than in normal rats. Therefore, it is important to measure the levels of guanidino compounds. However, Orita *et al.*³² reported

that the serum methylguanidine level abruptly rose when the serum creatinine level was above 7 mg/dl. These findings suggest that guanidino compounds have little effect on our experimental mice. Although the reason the level of plasma uric acid, the final metabolic substance of adenine, in the adenine-administered group did not differ from the control group is not clear, it is thought that 4APP inhibits the adenine metabolic system, since the plasma uric acid level in the 4APP-administered group decreased.

In conclusion, the decrease in SOD and XOD activities may play a role in adenine-induced renal injury and 4APP inhibits this injury.

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