Possible Mechanism Responsible for Allopurinol-Nephrotoxicity: Lipid Peroxidation and Systems of Producing- and Scavenging Oxygen Radicals

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Abstract—In order to elucidate toxic and protective mechanisms responsible for allopurinol-induced nephrotoxicity in rats, we investigated changes in plasma creatinine concentration, renal lipid peroxidation, and renal activities of xanthine oxidase, superoxide dismutase and catalase, as enzymatic factors in producing and scavenging oxygen radicals. The rats received subcutaneous injections of allopurinol in a dose of 100 mg/kg body weight, once a day for 3 days. In comparison to the control rats, the following changes were observed in the allopurinol-administered rats: an increase in plasma creatinine concentration, increases in renal contents of malonaldehyde, hypoxanthine and xanthine, and an increase of renal activity of xanthine oxidase, and decreases in renal activities of superoxide dismutase and catalase. Peaks in these changes were observed coincidentally on the third day after the administration of the drug was started. Afterwards, these parameters all returned to the control levels. These results strongly suggested that the allopurinol nephrotoxicity was attributed to the increase of lipid peroxidation which had been caused both by an increase in the ability of producing the oxygen radicals and by a decrease in the ability of scavenging the radicals.

Allopurinol (4-hydroxyprazolo (3,4-d)pyrimidine), a structural analog of hypoxanthine, has been used widely and through long duration for relieving hyperuricacidemia (1, 2). In spite of the usefulness of this drug, Selye (3) and others (4, 5) have reported that allopurinol in large doses can induce a marked nephrotoxicity in experimental animals. In man, glomerulonephritis and acute interstitial nephritis have been described as part of the hypersensitivity reaction to allopurinol (6–8). Also, as one of the causes of nephrotoxicity, oxypurinol, the relatively insoluble end-product of allopurinol, was observed to be precipitated in renal collecting tubule (4). However, in spite of these vigorous studies, the precise mechanism(s) responsible for the nephrotoxicity has not been well established even in experimental animals.

Recently, as one of the major mechanisms of nephrotoxicity induced by drugs, free-radicals production has been suggested in relation to lipid peroxidation in cellular membranes: In this lipid peroxidation, free-radicals including superoxide radical, hydroxyl radical, hydrogen peroxide and singlet oxygen, have been considered to play biologically important roles (9). Allopurinol and its metabolite, oxypurinol, are inhibitors of xanthine oxidase (10, 11). This xanthine oxidase, which converts hypoxanthine to xanthine and xanthine to uric acid, is known to produce the superoxide radicals during oxidizing processes (12, 13). Thus, we hypothetically considered that through the production of superoxide radicals, changes in renal activity of xanthine oxidase participated in the etiology of the allopurinol-induced nephrotoxicity.

Based on this view, we investigated the changes in lipid peroxidation and the changes in renal activities of xanthine oxidase, superoxide dismutase and catalase as en-
zymatic factors in producing and scavenging oxygen radicals (12, 14) in the kidneys of the allopurinol-administered rats.

Materials and Methods

Chemicals: Allopurinol and oxypurinol were purchased from Sigma Chemicals (St. Louis, MO, U.S.A.), and malonaldehyde-bis (dimethyl)acetal purchased from Aldrich Chem. Co. (Milwaukee, WI, U.S.A.). Hypoxanthine and xanthine were obtained from Wako Pure Chemicals Co. (Osaka, Japan), and the enzymes used were all purchased from Boehringer Mannheim GmbH (Mannheim, F.R.G.). All other reagents used were of the highest grade available.

General procedures: Male Wistar rats weighing 200–220 g (Clea Japan Inc., Tokyo, Japan) were housed in ordinary cages and allowed free access to water and standard diet pellets (MF; Oriental Yeast Co., Chiba, Japan). The rats were maintained in a temperature (25±2°C), humidity (relative humidity, 55±10%), and light (12 hr light, 12 hr dark) controlled room. In order to avoid diurnal changes, dosage and other operations to the rats were done between 10 a.m. to 11 a.m. One hundred mg of allopurinol was suspended in 4 ml of saline, and this suspension was subcutaneously injected to the rats in a volume of 4 ml/kg body weight, once a day for 3 days: the dose was 100 mg/kg body weight/day. The control rats received the same volume of saline. The body weight in each rat was measured every day at 10 a.m.

In the first, second, third, fourth, fifth, sixth, eighth and tenth days after the starting of the first administration of allopurinol, operations to prepare kidney homogenate and plasma were carried out. Under ether anesthesia, the abdominal cavity was opened through a ventral incision. Blood was taken from the abdominal aorta, put into a heparinized tube, and centrifuged (1,700×g, 10 min, 4°C) for separation of plasma. Following taking the blood, both kidneys were removed. The right and left kidneys were singly homogenized by glass-teflon homogenizers, so that 10 ml of homogenate contained one kidney. The right kidney was homogenized with 0.25 M sucrose containing 3 mM ethylene diamine tetraacetic acid (EDTA) disodium salt, in order to avoid possible lipid peroxidation during the homogenization. To eliminate the possibility that EDTA might have an effect on the enzymatic determinations described below, the left kidney was separately homogenized only with 0.25 M sucrose. The homogenate from the right kidney was used for determination of malonaldehyde, and the homogenate from the left kidney was used for determinations of the enzymatic activities and contents of protein, hypoxanthine, xanthine, allopurinol and oxypurinol.

Analytical methods: Plasma creatinine concentration was determined by the method of Bonsnes and Taussky (15). Protein content was measured by the method of Lowry et al. (16). Renal malonaldehyde, as an index in lipid peroxidation, was determined by the method of Uchiyama and Mihara (17) using the thiobarbituric acid reaction (18). Malonaldehyde, as a standard, was prepared from malonaldehyde-bis(dimethyl)acetal by the method of Esterbauer and Slater (19). In terms of determinations of renal activities of xanthine oxidase, superoxide dismutase and catalase, the homogenate was treated by Triton X-100 (1% v/v, final concentration). The xanthine oxidase activity with xanthine as the substrate was determined by the method of Fried and Fried (20). For determination of renal superoxide dismutase activity, the homogenate treated by Triton X-100 was sonicated using a Branson Sonifier (Branson Sonic Power Co.; Danbury, CT, U.S.A.) according to the method of Stein et al. (21). Superoxide dismutase activity was determined by the method of Elstner and Heupel (22), and the activity was calculated using logit paper (23). Catalase activity was determined with hydrogen peroxide as the substrate (24), and the activity was represented in international units.

In the above spectrophotometric assays, a Hitachi-320 spectrophotometer with a constant temperature cuvette holder (Hitachi Ltd., Tokyo, Japan) was used. Contents of hypoxanthine, xanthine, allopurinol and oxypurinol in the homogenate were determined by the high-performance liquid chromatographic (HPLC) method according
to Putterman et al. (25). The HPLC system consisted of a Model 510 solvent delivery pump fitted with a Model 710B WISP autosampler and a Model 481 Lambda-Max LC spectrophotometer, all from Waters Assoc. (Milford, MA, U.S.A.). A Model 3390A Hewlett-Packard Recording Integrator (Hewlett-Packard Co.; Avondale, PA, U.S.A.) was used to determine peak areas. All separations were carried out on a Radial PAK C18 column (8 mm x 10 cm; particle size, 10 μm; Waters Assoc.).

Statistics: Results are given as the mean±standard error (S.E.). Statistical significance was assessed by Student's t-test; P values of less than 0.05 were considered significant.

Results

As a marker for the general condition of the animals, body weight gains were investigated. The body weight gains showed no significant differences between the control and the allopurinol-administered groups at each time point throughout the whole process.

As to changes in plasma creatinine concentration, Fig. 1 shows the results. As compared with the control group, the plasma creatinine concentration in the allopurinol-administered group significantly increased from the second day, reached a maximal level on the third day, and returned to the control level on the fourth day and thereafter.

Next, changes in wet weights, contents of protein, hypoxanthine, xanthine, allopurinol and oxypurinol, and activities of xanthine oxidase, superoxide dismutase and catalase were investigated in the left kidney. In autopsy, a swelling was macroscopically observed in the administered group, from the first to the fourth day. Figure 2 shows changes of wet weights and total protein contents in left kidneys. In the control group, the wet weight and total protein content were almost constant throughout the whole process. In contrast, the wet weight and protein content in the administered group showed their highest values on the third day, showing significant differences in comparison to the control group.

Figure 3 shows changes in total contents of hypoxanthine and xanthine in left kidneys. As compared with the control group, renal hypoxanthine content in the administered group increased significantly from the second to the fourth day, showing their higher values on the third and fourth day. Renal xanthine content in the administered group showed a significantly high value only on the third day.

Table 1 shows changes of allopurinol and

![Fig. 1. Changes of plasma creatinine concentrations. Allopurinol was subcutaneously administered to rats, once a day for 3 days, with the dose being 100 mg/kg/day. The day when the administration was started was designated as Day 0. Number of rats was 6 for each time point. Points and bars represent the mean±S.E. Asterisks denote significant differences between control- and administered groups at each time point: *, P<0.05; **, P<0.01. Abbreviation: Ad., administration of allopurinol.](image-url)
oxypurinol contents in left kidneys. Detection limits (signal to noise=3:1) of allopurinol and oxypurinol were 73.5 and 65.0 nmole/g wet weight of kidney tissue, respectively, in the present HPLC method. Allopurinol was not detected in the kidney even in the administered group. Oxypurinol was detected in the second, third and fourth day in a few cases of the administered group. The content and number of cases in its detection were highest on the third day.

Figure 4 shows changes in total activities of xanthine oxidase in left kidneys. The activity in the allopurinol-administered group clearly increased from the first day. In particular, the highest activity was observed on the third day, with the value being approximately 4 times that of the control one. In the sixth day, the activity was still higher than the control value, though showing a tendency to approach the control level.

Figure 5 shows changes in total activities of superoxide dismutase and catalase in left kidneys. In terms of superoxide dismutase, the activity in the administered group decreased from the second day, showing significant differences in comparison to the control group. The lowest activity was observed on the third day, with the value being approximately 65% of the control one. Later than the fifth day, the activities approached the control level.

In terms of catalase, the activity in the administered group decreased from the first day, showing significant differences in comparison to the control group. The lowest activity was observed on the third day, with the value being approximately 70% of the control one. The activity was still lower on the sixth day, as compared with the control.

Fig. 2. Changes of wet weights and total protein contents in left kidneys. Wet weights, upper figure; total protein contents, lower figure. Explanations are as in Fig. 1.

Fig. 3. Changes of total contents of hypoxanthine and xanthine in left kidneys. Hypoxanthine, upper figure; xanthine, lower figure. Explanations are as in Fig. 1.
Table 1. Changes of total contents of allopurinol and oxypurinol in left kidneys of allopurinol-administered rats.

<table>
<thead>
<tr>
<th>Day</th>
<th>Content (nmole/kidney)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Allopurinol</td>
</tr>
<tr>
<td>0</td>
<td>n.d.</td>
</tr>
<tr>
<td>1</td>
<td>n.d.</td>
</tr>
<tr>
<td>2</td>
<td>n.d.</td>
</tr>
<tr>
<td>3</td>
<td>n.d.</td>
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<tr>
<td>4</td>
<td>n.d.</td>
</tr>
<tr>
<td>5</td>
<td>n.d.</td>
</tr>
<tr>
<td>6</td>
<td>n.d.</td>
</tr>
<tr>
<td>8</td>
<td>n.d.</td>
</tr>
<tr>
<td>10</td>
<td>n.d.</td>
</tr>
</tbody>
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Number of rats in each group was 6, and values in parentheses represent the number of rats in which oxypurinol was detected. Based on their data, values were calculated and expressed as values of the mean±S.E. Detection limits of allopurinol and oxypurinol in the present HPLC method were 73.5 and 65.0 nmole/g wet weight of kidney tissue, respectively. Abbreviation: n.d., not detected.

Fig. 4. Changes of total activities of xanthine oxidase in left kidneys. Explanations are as in Fig. 1.

Discussion

As to experiments producing allopurinol-nephrotoxicity in rats, Selye (3) reported that a subcutaneous administration of allopurinol in a dose of 400 mg/kg body weight for 4 days caused pathologically characteristic
nephropathy in the rat. Also, Wexler and Greenberg (5) reported that in the subcutaneous administration in three doses of 50, 100 and 400 mg/kg for 10 days, the 50 mg/kg dosing caused no changes, the 100 mg/kg dosing caused a moderate nephropathy in 74% of the rats, and the 400 mg/kg dosing caused a severe nephropathy in 100% of the rats. Based on these reports, we tentatively considered that a dose level of 100 mg/kg for 3 days would bring a reversible nephropathy to rats: In fact, this dosing brought such reversible nephrotoxic changes as reported here, and we could investigate how much the systems that produce and scavenge oxygen radicals participated in damaged- and regenerated stages of the allopurinol-induced nephrotoxicity.

As to the representation of the contents and activities of several determinations in this study, all were expressed in values per one (left) kidney. It has been generally known that the protein content and the tissue wet weight sometimes vary corresponding to the degree of damage in the concerned organ. Practically, this study showed the maximal increases in the wet weight and the total protein content of the kidney on the third day after starting the allopurinol administration. (Fig. 2) Between these two, the total protein content in the kidney showed a relatively less increase in comparison to the wet weight. This fact indicated that the allopurinol-administration brought a swelling of the kidney. Based on

![Graph of superoxide dismutase and catalase activities](image)

**Fig. 5.** Changes of total activities of superoxide dismutase and catalase in left kidneys. Superoxide dismutase, upper figure; catalase, lower figure. Explanations are as in Fig. 1.

![Graph of malonaldehyde formation](image)

**Fig. 6.** Changes of malonaldehyde formation in right kidneys. Malonaldehyde formation was determined in the right kidney, while the content of formed malonaldehyde was expressed in nmole per one left kidney through calculation of the wet weights of both the kidneys. Other explanations are as in Fig. 1.
this fact, we considered that the protein content and the tissue wet weight were fluctuating factors in this kind of toxicological examination, and adopted neither values per protein amount nor values per tissue wet weight in cases of representations of substance contents and enzymatic activities.

The plasma creatinine was investigated as an index for evaluation of nephrotoxic effects induced by allopurinol. The allopurinol-administered group showed its maximal increase on the third day. (Fig. 1) Also, the items determined in this study showed their peaks on the third day with a coincidence in time. In particular, the lipid peroxidation evaluated by the formation of malonaldehyde, was also recognized to be increased most highly at that time point. These findings suggested that the renal damage was most severely induced on the third day.

As represented in the section Introduction, free-radicals production has been raised as one of the major mechanisms of nephrotoxicity induced by drugs, in relation to lipid peroxidation: concerning several drugs and chemicals such as cephaloridine, adriamycin, paraquat, carbon tetrachloride and nitrofurantoin, the lipid peroxidation has been considered as a main mechanism of the tissue injuries (26–29). Peroxidation of lipids usually involves the reaction of free radicals and polyunsaturated lipid to form free radical intermediates, and the free radicals in biological systems have been considered to be produced from interaction of oxygen or other compounds with a free electron during oxidation-reduction reactions, or with the activity of enzymes such as xanthine oxidase or the mixed function oxidases of the endoplasmic reticulum (30). Among these possibilities on production of cytotoxic free radicals, oxygen free radicals including superoxide radical, hydroxyl radical, hydrogen peroxide and singlet oxygen, might be considered to be biologically important in the etiology of this allopurinol-nephrotoxicity. Supporting this idea is the fact that allopurinol-administration caused the increments in the malonaldehyde formation and xanthine oxidase activity and the declines in the superoxide dismutase and catalase activities in the kidney: their peaks were all observed coincidentally on the third day (Figs. 4, 5 and 6). In addition, renal contents of hypoxanthine and xanthine, as the substrates of the xanthine oxidase, showed their highest values on the third day (Fig. 2). These findings of the increments in the xanthine oxidase activity as well as in the contents of its substrates in the kidney, strongly supported the participation of the oxygen radicals in the etiology of the allopurinol nephrotoxicity.

Xanthine oxidase converted allopurinol into oxypurinol (31). In the case of considering relations of xanthine oxidase with allopurinol and its metabolite of oxypurinol, both the substances were not only inhibitors but also substrates of the xanthine oxidase (31, 32). Concerning these two substances, it is known that allopurinol is rapidly metabolized to oxypurinol and that oxypurinol is reabsorbed in the renal tubules and then relatively gradually excreted into the urine (32). In this work, the allopurinol was not detected in the kidney throughout the whole process, while the oxypurinol was detected from the second to the fourth day, with the highest levels detected on the third day (Table 1). This fact was in accordance with the above report (32), and the residual oxypurinol seemed likely to have toxic effects on the kidney. Providing that 1 g of kidney tissue contained 1 ml of water (i.e., the tissue weight is essentially equal to the water content) and providing that the oxypurinol is distributed homogeneously in the kidney, its concentration (approximately 0.6 mM on the third day) might be high enough to inhibit the xanthine oxidase (10). Our finding that there was an increase in formation of malonaldehyde indicated that the system for producing oxygen radicals was promoted in spite of the presence of the inhibitors in the renal cells, and it suggested that the inhibitors were localized and isolated from the cellular component(s) containing xanthine oxidase. As to the increment in xanthine oxidase activity and the decrements in superoxide dismutase- and catalase activities, we speculated as follows: (A) The increment of xanthine oxidase activity was due to enzymatic induction, as one of the self-defensive systems, for exclusion of the
two toxic substances from the body. (B) In the process of metabolizing the two toxic substances, oxygen radicals produced injured the subcellular fractions containing superoxide dismutase and catalase or directly affected the two enzymes.

The primary defense against the above cytotoxic oxygen radicals is provided by enzymes that catalytically scavenge the intermediates of oxygen reduction: The superoxide radicals are eliminated by superoxide dismutase, which catalyzes its conversion to hydrogen peroxide plus oxygen, and hydrogen peroxide is removed by catalase, which converts hydrogen peroxide to water plus oxygen (12, 33, 34). Also, the metal catalyzed reaction of these superoxide radicals and hydrogen peroxide resulted in the formation of an extremely powerful oxidant, the hydroxyl radical is eliminated according to catalysis of hydrogen peroxide by catalase (14). Our results denoted that these two enzymes, which played an important role for protections of the cells, were diminished by the allopurinol-administration. (Fig. 5) Thus, through the lipid peroxidation, the nephrotoxic mechanism of the allopurinol might be explained enzymatically by both the increments in the ability of producing the cytotoxic oxygen radicals and the declines in the ability to scavenge the radicals. In addition, concerning the items investigated in this study, the coincidences among the time processes in the durations of renal injury and regeneration were also considered to support strongly our view of the etiology of the allopurinol-induced nephrotoxicity.

Histologically, renal lesions derived from allopurinol administration have been reported to appear in the glomeruli, the proximal and distal convoluted tubules of the cortex, the collecting tubules of the medulla, the papillary ducts of Bellini in the pelvis and the interstitium of the kidney (3, 5, 7, 8). The lesions consisted mainly of polymorphonuclear leucocytes and crystals (3, 5). Kann et al. (4) reported that the crystals in renal tubules were oxypurinol. Our biochemical work was concerned with the changes in the whole kidney. Thus, as to which portions of nephron segments and interstitium were damaged, it remained to be investigated. However, concerning the above histological reports, our finding that oxypurinol was highly observed in the allopurinol-administered group might suggest the presence of oxypurinol in the renal tubules (3–5). In order to combine the histological reports with our biochemical data, biochemical work should be done at the nephron level.

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