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Xanthine oxidase-derived reactive oxygen species contribute to the development of D-galactosamine-induced liver injury in rats

YOSHIJI OHTA[1], TATSUYA MATSURA[2], AKIRA KITAGAWA[3], KENJI TOKUNAGA[4], & KAZUO YAMADA[2]

[1]*Department of Chemistry, Fujita Health University, Toyoake, Aichi 470-1192, Japan,* [2]*Division of Medical Biochemistry, Department of Pathophysiology and Therapeutic Science, Faculty of Medicine, Tottori University, Yonagao, Tottori 683-8503, Japan,* [3]*Department of Nutrition, Faculty of Wellness, Chukyo Women's University, Ohbu, Aichi 474-8651, Japan, and* [4]*Department of Clinical Medical Technology, Kagawa Prefectural College of Health Science, Mure-cho, Kagawa 761-0123, Japan*

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Abstract

We examined whether xanthine oxidase (XO)-derived reactive oxygen species (ROS) contribute to the development of D-galactosamine (D-GaIN)-induced liver injury in rats. In rats treated with D-GaIN (500 mg/kg), liver injury appeared 6 h after treatment and developed until 24 h. Hepatic XO and myeloperoxidase activities increased 12 and 6 h, respectively, after D-GalN treatment and continued to increase until 24 h. D-GalN-treated rats had increased hepatic lipid peroxide (LPO) content and decreased hepatic reduced glutathione (GSH) and ascorbic acid contents and superoxide dismutase (SOD), catalase and Se-glutathione peroxidase (Se-GSHpx) activities at 24 h, but not 6 h, after treatment. Allopurinol (10, 25 or 50 mg/kg) administered at 6 h after D-GalN treatment attenuated not only the advanced liver injury and increased hepatic XO activity but also all other changes observed at 24 h after the treatment dose-dependently. These results suggest that XO-derived ROS contribute to the development of D-GaIN-induced liver injury in rats.

Keywords: *D-Galactosamine, liver injury (rat), xanthine oxidase, reactive oxygen species metabolism, lipid peroxidation, neutrophil infiltration*

Introduction

Recently, much attention has been paid to the role of reactive oxygen species (ROS) such as superoxide radical ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical in the development of various types of liver injury in humans and experimental animals [1]. Xanthine oxidase (XO) generates large quantities of $O_2^{\cdot-}$ and H_2O_2 in the presence of hyoxanthine or xanthine [2,3]. It has been shown that XO activity increases in the serum and liver of patients with various types of liver injury [4–6]. It has also been shown that XO plays a critical role in the development of liver injuries induced by hepatotoxicants such as carbon tetrachloride, chloroform and thioacetamide [7–9], endotoxin [10], bile duct ligation [11], ischemia-reperfusion [12] and hypoxia-reoxygenation [13] in rats, although XO plays no role in the development of liver injury induced by acetaminophen or bromobenzene [9].

D-Galactosamine (D-GaIN)-induced liver injury is one of the experimental liver injury models which have been used most frequently, because this liver injury is very similar to acute vital hepatitis in humans [14,15]. It has been shown in D-GaIN-treated rats that the hepatic level of lipid peroxide (LPO), which is produced via ROS, increases during liver injury development [16–22] or before and during liver

Correspondence: Y. Ohta, Department of Chemistry, School of Medicine, Fujita Health University, Toyoake, Aichi 470-1192, Japan. Tel: 81 562 93 2573. Fax: 81 562 93 4593. E-mail: yohta@fujita-hu.ac.jp

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injury development [23]. It has also been shown in D-GaIN-treated rats that the hepatic levels of antioxidants such as reduced glutathione (GSH) and ascorbic acid and the hepatic activities of antioxidant enzymes such as superoxide dismutase (SOD) (an enzyme to scavenge $O_2^{\cdot-}$), catalase (an enzyme to decompose H_2O_2) and Se-glutathione peroxidase (Se-GSHpx; an enzyme to metabolize both H_2O_2 and lipid hydroperoxides using GSH as a co-substrate) are reduced during liver injury development [18,19,21,22]. The severity of liver injury in D-GaIN-treated rats is known to decrease with attenuation of increased hepatic LPO level by the simultaneous administration of SOD [16]. In addition, it has been suggested that ROS derived from activated polymorphonuclear leukocytes, i.e. neutrophils, extends liver cell necrosis through an increase in hepatic lipid peroxidation in rats with D-GaIN-induced hepatitis [20]. There are reports showing that administration of allopurinol, an XO inhibitor, in addition to SOD or catalase, protects against D-GaIN/endotoxin-induced hepatitis in mice [24,25]. However, it has not been clarified yet whether or how XO-derived ROS contributes to the development of D-GaIN-induced liver injury.

In the present study, therefore, we attempted to clarify whether XO-derived ROS contributes to the development of D-GaIN-induced liver injury in rats. Namely, we first examined the changes in hepatic xanthine dehydrogenase (XD) + XO and XO activities and XO/XD + XO ratio and serum XO activity and uric acid concentration with liver injury development in D-GaIN-treated rats. Next, we examined the effect of allopurinol administration on liver injury development and the changes in hepatic XO activity, LPO, GSH and ascorbic acid contents and SOD, catalase and Se-GSHpc activities and in serum XO activity and uric acid concentration with liver injury development in D-GaIN-treated rats. There are reports showing that activated neutrophils convert XD to XO by secretion of elastase in close proximity to cultured endothelial cells [26,27] and that $O_2^{\cdot-}$ generated by the xanthine–XO system induces neutrophil adhesion to cultured endothelial cells [28,29]. Therefore, the activity of myeloperoxidase (MPO), an index of neutrophil infiltration [30–32], was determined in the liver of D-GaIN-treated rats with and without allopurinol administration in order to elucidate whether XO-derived ROS affects neutrophil infiltration into the liver of D-GaIN-treated rats.

Materials and methods

Chemicals

D-GalN (HCl salt), xanthine, leupeptin, 3,3′,5,5′-tetramethylbenzidine (TMB), bovine serum albumin and bovine erythrocyte SOD were obtained from Sigma Chemical Co. (St Louis, MO, USA); 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate), dithiothreitol (DTT), horse radish peroxidase, leupeptin, phenylmethylsulfonylfluoride and bovine milk XO from Roche-Diagnostic Co. (Tokyo, Japan); NAD^+, NADPH and yeast uricase from Oriental Yeast Co. (Tokyo, Japan); allopurinol, L-ascorbic acid, 2,2′-dipyridil, 5,5′-dithiobis(2-nitrobenzoic acid) (Ellman's reagent), ethylenediaminetetraacetic acid (EDTA), GSH, 2-thiobarbituric acid and other reagents from Wako Pure Chemicals Industry Ltd. (Osaka, Japan).

Animals

Male Wistar rats aged 6 weeks were purchased from Nippon SLC Co. (Hamamatsu, Japan) and fed commercial rat chow, Oriental MF (Oriental Yeast Co., Tokyo, Japan) with free access to water for 1 week before use for the experiments. All animals received humane care in compliance with the guidelines of the Management of Experimental Animals in Fujita Health University, Japan.

Induction of liver injury

D-GaIN, which was dissolved in 0.9% NaCl after neutralization with 1 M NaOH, at a dose of 500 mg/kg body weight (BW) was intraperitoneally injected to fed rats (7 weeks old) to produce liver injury. Rats without D-GaIN injection received an intraperitoneal injection of the same volume of 0.9% NaCl. These animals were starved with free access to water after D-GaIN injection.

Allopurinol administration

A solution of allopurinol (10 mg/ml) was prepared as follows: 2, 5 or 10 mg of allopurinol was dissolved in a limited volume of 1 M NaOH, pH-adjusted to 10.9 with 1 M HCl and then diluted with distilled water in a final volume of 1.0 ml. Each allopurinol solution at a dose of 2 ml/kg BW was orally administered to rats injected with and without D-GaIN using a stomach tube at 6 h after the injection. Namely, 10, 25 or 50 mg/kg BW of allopurinol was administered. These doses of allopurinol were determined according to the report of Klein et al. [33] which indicates that orally administered allopurinol inhibits XO activity in the liver of rats without exerting an antioxidant effect at doses of 2–50 mg/kg BW. Rats without allopurinol administration orally received an equal volume of a diluted NaOH solution (pH 10.9) prepared without allopurinol at the same time point.

Sample preparations

Rats were sacrificed after collecting blood from the vena cava caudalis under ether anesthesia at 0, 6, 12,

18 or 24 h after D-GaIN injection. Immediately after sacrifice, livers were perfused with ice-cold 0.15 M NaCl through the portal vein to remove residual blood in the tissue as much as possible and then removed from the body. The isolated liver was washed well in ice-cold 0.15 M KCl, clotted on a filter, weighed and frozen on dry ice as soon as possible. The collected blood was kept on ice for 30 min and then separated into serum by centrifugation at 4°C. The isolated liver and serum were kept at −80°C until use. For hepatic XD + XO and XO assays, a part of the right large lobe of each liver was homogenized in 9 volumes of an ice-cold buffered solution (pH 7.8) containing 0.05 M potassium phosphate, 0.1 M EDTA, 0.5 mM DTT, 0.5 mg/ml of leupeptin and 0.2 mM phenylmethylsulfonylfluoride using a glass homogenizer with a Telfon pestle as described in our previous report [7]. The homogenate was dialyzed against 100 volumes of the same buffered solution without DTT at 4°C for 60 min using a microdialysis device (MWCO = 3500 daltons; Bio-Tec International Inc., Belleuve, WA, USA) to remove endogenous substrates. Another part of the right large lobe of each liver was homogenized in 9 volumes of ice-cold 0.15 M KCl containing 1.0 mM EDTA using a glass homogenizer with a Telfon pestle. For the determinations of hepatic LPO, GSH and ascorbic acid, the prepared homogenate itself was used. For the assays of hepatic SOD, catalase, Se-GSHpx and MPO, the prepared homogenate was sonicated on ice for 30 s × 2 times using a Handy Sonic model UR-20P (Tomy Seiko Co., Tokyo, Japan). The sonicated homogenate was dialyzed against 100 volumes of 0.05 M Tris–HCl buffer (pH 7.4) at 4°C for 60 min using the above-described microdialysis device. For hepatic MPO assay, this dialyzed homogenate was incubated at 60°C for 60 min to inactivate catalase present in the homogenate as described by Schierwagen et al. [31].

Assays of serum transaminases, XO, and uric acid

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were assayed using a commercial kit, Iatorzyme TA-L_Q (Dai-Iatron Co., Tokyo, Japan). Both activities are expressed as an international unit (IU/l). Serum XO was assayed at 30°C by the method of Majkic-Singh et al. [34] using xanthine as a substrate. This assay method is based on the oxidation of 2,2′-azino-di-(3-ethylbenzthiazoline-6-sulfonate) mediated by horse radish peroxidase and H_2O_2 produced in the metabolism of uric acid, a product of the XO-catalyzed reaction, to allantoin by exogenously added uricase. One unit (U) of serum XO is defined as the amount of enzyme forming 1 μmol uric acid per min. Serum uric acid was determined using a commercial kit, URIC ACID C-Test Wako (Wako Pure Chemicals Industry Ltd., Osaka, Japan).

Assays of hepatic enzymes and components

Hepatic XD + XO and XO were assayed in the presence and absence of 1.0 mM NAD^+, respectively at 30°C by the method of Hashimoto [35] using xanthine as a substrate. The activity of XD + XO or XO was assessed by measuring the increase in absorbance at 292 nm following the formation of uric acid. One unit (U) of hepatic XD + XO or XO is defined as the amount of enzyme forming 1 μmol uric acid per min. Hepatic SOD, catalase and Se-GSHpx were assayed by the methods of Oyangui [35], Bergmeyer [37] and Hochstein and Utley [38], respectively. SOD activity was measured at 37°C by the XO–NH_2OH method using purified bovine erythrocyte SOD (5000 units/mg solid) as a standard. The activity is expressed as the amount of the erythrocyte SOD showing activity equivalent to the activity determined in the $O_2^{\cdot-}$-generating system composed of xanthine and bovine milk XO. Catalase activity was measured at 37°C by recording H_2O_2 decomposition at 240 nm. One unit (U) of this activity is defined as the amount of enzyme decomposing 1 μmol H_2O_2 as a substrate per min. Se-GSHpx activity was determined at 37°C by recording the decrease in absorbance at 340 nm following the oxidation of NADPH in the presence of H_2O_2, GSH, yeast glutathione reductase and NaN_3 as a catalase inhibitor. One unit (U) of this activity is defined as the amount of enzyme oxidizing 1 μmol NADPH/min. Hepatic MPO was assessed by the H_2O_2-dependent oxidation of TMB (dissolved in dimethylsulfoxide) at 37°C according to the method of Suzuki et al. [39]. This TMB oxidation was measured spectrophotometrically at 655 nm. One unit (U) of this enzyme activity is expressed as the amount of enzyme causing a change in absorbance of 1.0 per min at 655 nm. Hepatic LPO was assayed spectrophotometrically by the method of Ohkawa et al. [40] using the thiobarbituric acid reaction except that 1.0 mM EDTA was added to the reaction mixture. The content of LPO is expressed as the amount of malondialdehyde (MDA) equivalents. Hepatic GSH was assayed spectrophotometrically by the method of Sedlak and Lindsay [41] using Ellman's reagent. Hepatic ascorbic acid was assayed spectrophotometrically by the method of Zannoi et al. [42] using 2,2′-dipyridil and L-ascorbic acid as a standard. Hepatic protein was assayed by the method of Lowry et al. [43] using bovine serum albumin as a standard.

Statistical analysis

Results obtained are expressed as means ± SD. Results were statistically analyzed by computerized statistical package (ANOVA, StatView). Each mean value was compared by one-way analysis of variance and Fischer's protected significant difference for multicomparison at the *post hoc* test. The minimum level of significance was set at $P < 0.05$.

Results

Changes in serum AST, ALT, and XO activity and uric acid concentration in D-GaIN-treated rats

Serum ALT and AST activities, indices of liver cell damage, in rats treated with D-GaIN (500 mg/kg BW) were significantly higher than those in control rats at 6, 12, 18, and 24 h, but not 3 h, after treatment (Figure 1(A),(B)). The D-GalN-treated group had significant increases in serum XO activity and uric acid concentration at 12, 18 and 24 h, but not 3 and 6 h, after treatment when compared with the control group (Figure 1(C),(D)). The increases in serum ALT, AST and XO activities and uric acid concentration in the D-GalN-treated group occurred time-dependently (Figure 1).

Changes in hepatic XD + XO and XO activities and XO/XD + XO ratio in D-GaIN-treated rats

Hepatic XD + XO activity in D-GalN-treated rats was significantly, although slightly, less than that in control rats only at 24 h after the treatment (Figure 2(A)). Hepatic XO activity and XO/XD+ XO ratio were significantly higher in the D-GalN-treated group than in the control group at 12, 18 and 24 h, but not 3 and 6 h, after the treatment (Figure 2(B),(C)). The increases in hepatic XO activity and XO/XD+ XO ratio in the D-GalN-treated group occurred time-dependently (Figure 2(B),(C)).

Effect of post-allopurinol administration on serum ALT and AST activities in D-GaIN-treated rats

Oral administration of allopurinol (10, 25 or 50 mg/kg BW), an XO inhibitor, to D-GaIN-treated rats at 6 h after the treatment significantly attenuated the increased serum ALT and AST activities found at 24 h after the treatment in a dose-dependent manner (Figure 3). However, the serum ALT and AST activities in the D-GaIN-treated group post-administered with allopurinol (50 mg/kg BW) were still significantly higher than those in the D-GalN-treated group found at 6 h after the treatment ($P < 0.05$; Figure 3). There were no changes in serum ALT and AST activities in D-GalN-untreated rats administered with the same doses of allopurinol (Figure 3).

Effect of post-allopurinol administration on serum uric acid concentration and XO activity and hepatic XO activity in D-GalN-treated rats

Oral administration of allopurinol (10, 25 or 50 mg/kg BW) to D-GaIN-treated rats at 6 h after the treatment significantly attenuated the increased serum uric acid concentration and XO activity and hepatic XO activity found at 24 h after the treatment in a dose-dependent manner (Figure 4). The serum uric acid concentration and XO activity and hepatic XO activity in D-GaIN-treated rats post-administered with allopurinol (50 mg/kg BW) were almost equal to those in control rats (Figure 4). Allopurinol given to D-GalN-untreated rats at the same doses significantly reduced

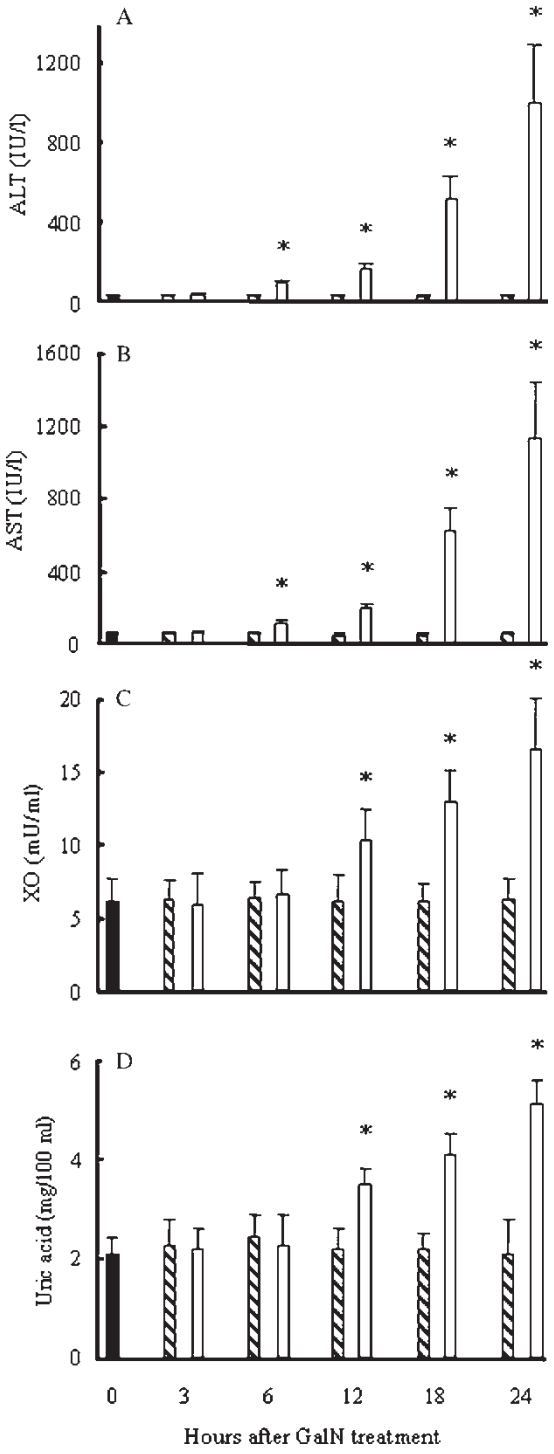

Figure 1. Changes in serum: (A) ALT; (B) AST; (C)XO; and (C) activities and (D) uric acid concentration following D-GalN treatment in rats. Rats were treated with (open bar) and without (hatched bar) D-GaIN (500 mg/kg BW, i.p.). Each value is a mean ± SD. ($n = 5–7$). *, Significantly different from rats without D-GalN treatment, $P < 0.05$.

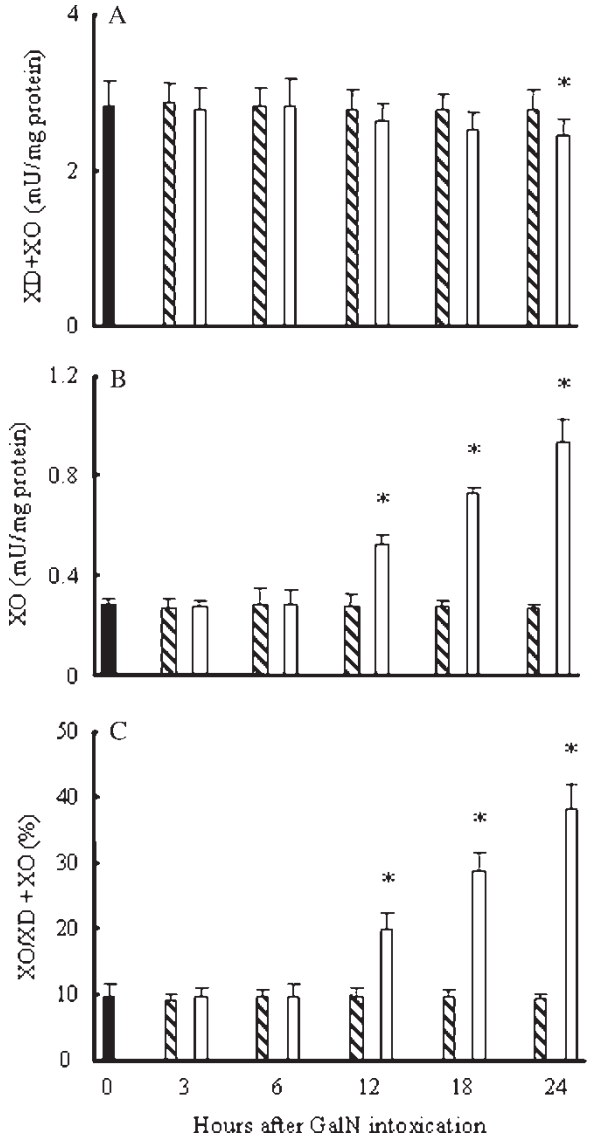

Figure 2. Changes in hepatic (A) XD + XO and (B) XO activities and (C) XO/XD + XO ratio following D-GalN treatment in rats. Rats were intoxicated with (open bar) and without (hatched bar) D-GaIN (500 mg/kg BW, i.p.). Each value is a mean ± SD. ($n = 5–7$). ⋆, Significantly different from rats without D-GalN treatment, $P < 0.05$.

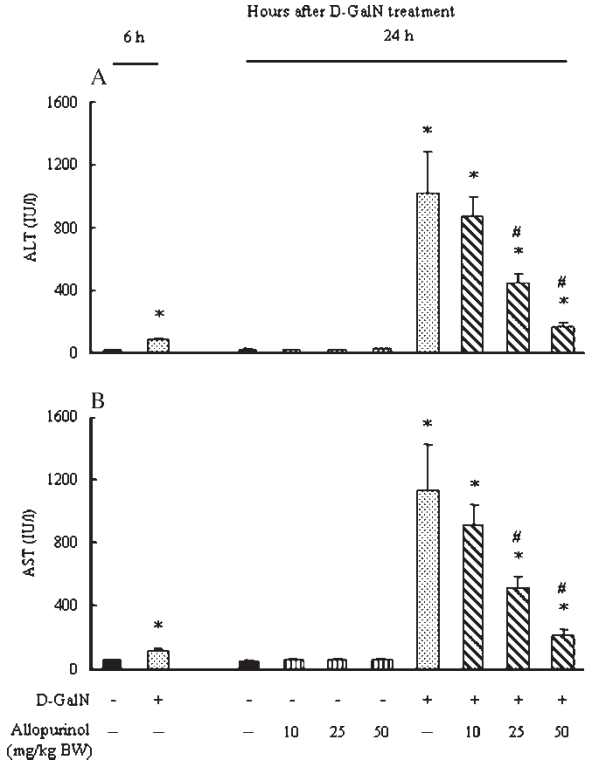

Figure 3. Effect of post-allopurinol administration on serum (A) ALT and (B) AST activities in rats intoxicated with D-GaIN. Rats treated with and without D-GaIN (500 mg/kg BW, i.p.) orally received allopurinol (10, 25 or 50 mg/kg BW) at 6 h after the treatment. The animals were sacrificed 6 and 24 h after D-GaIN treatment. Each value is a mean ± SD. ($n = 6–8$). ⋆, Significantly different from control rats, $P < 0.05$; #, significantly different from rats treated with D-GaIN alone, $P < 0.05$.

the serum XO activity and uric acid concentration dose-dependently and its dose of 50 mg/kg BW decreased the hepatic XO activity significantly, although slightly.

Effect of post-allopurinol administration on LPO, GSH, and ascorbic acid contents and SOD, catalase, and Se-GSHpx activities in D-GaIN-intoxicated rats

There were no differences in hepatic LPO, GSH and ascorbic acid contents and SOD, catalase and Se-GSHpx activities between D-GalN-treated and untreated control rats (Figures 5 and 6). Allopurinol (10, 25 or 50 mg/kg BW) administered to D-GaIN-treated rats at 6 h after the treatment significantly attenuated the increased hepatic LPO content and the decreased GSH and ascorbic acid contents found at 24 h after D-GalN treatment in a dose-dependent manner (Figure 5). The hepatic LPO content in D-GaIN-intoxicated rats post-administered with allopurinol (50 mg/kg BW) was still significantly higher than that in control rats, while the hepatic GSH and ascorbic acid contents in the former group were still significantly lower than those in the latter group (Figure 5). The post-administered allopurinol significantly attenuated the decreased hepatic SOD, catalase and Se-GSHpx activities found at 24 h after D-GaIN treatment dose-dependently but the hepatic SOD, catalase and Se-GSHpx activities in D-GalN-treated rats post-administered with allopurinol (50 mg/kg BW) were still significantly lower than those in control rats (Figure 6). D-GalN-untreated rats administered with the same doses of allopurinol showed no changes in hepatic LPO, GSH and ascorbic acid contents and SOD, catalase and Se-GSHpx activities (Figures 5 and 6).

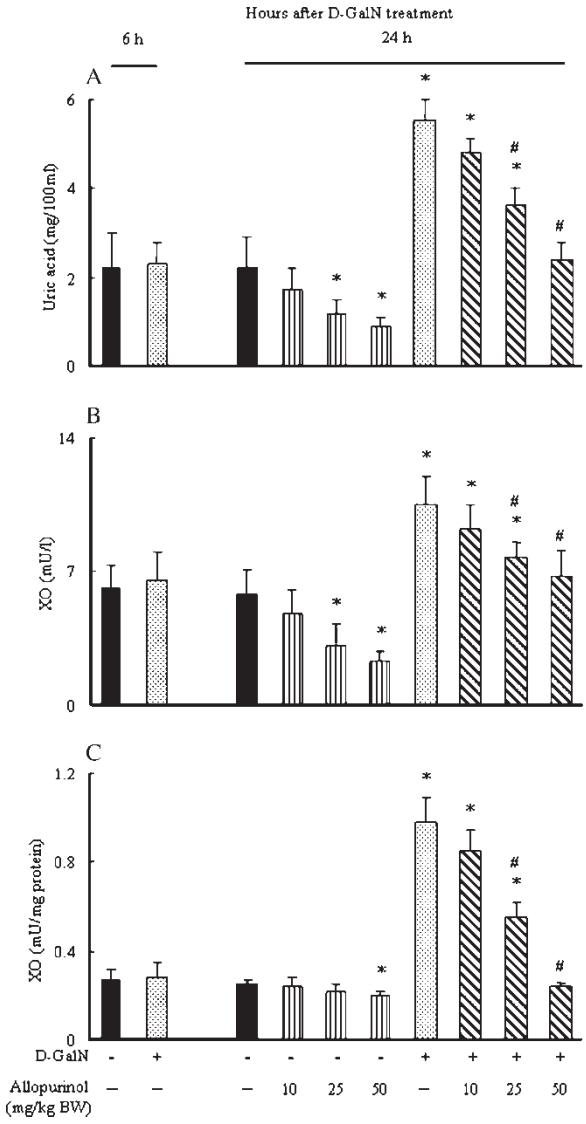

Figure 4. Effect of post-allopurinol administration on serum (A) uric acid concentration and (B) XO activity and (C) hepatic XO activity in rats treated with D-GaIN. Rats treated with and without D-GaIN (500 mg/kg BW, i.p.) orally received allopurinol (10, 25 or 50 mg/kg) at 6 h after the treatment. The animals were sacrificed 6 and 24 h after D-GaIN treatment. Each value is a mean ± SD. ($n = 6–8$). ⋆, Significantly different from control rats, $P < 0.05$; #significantly different from rats treated with D-GaIN alone, $P < 0.05$.

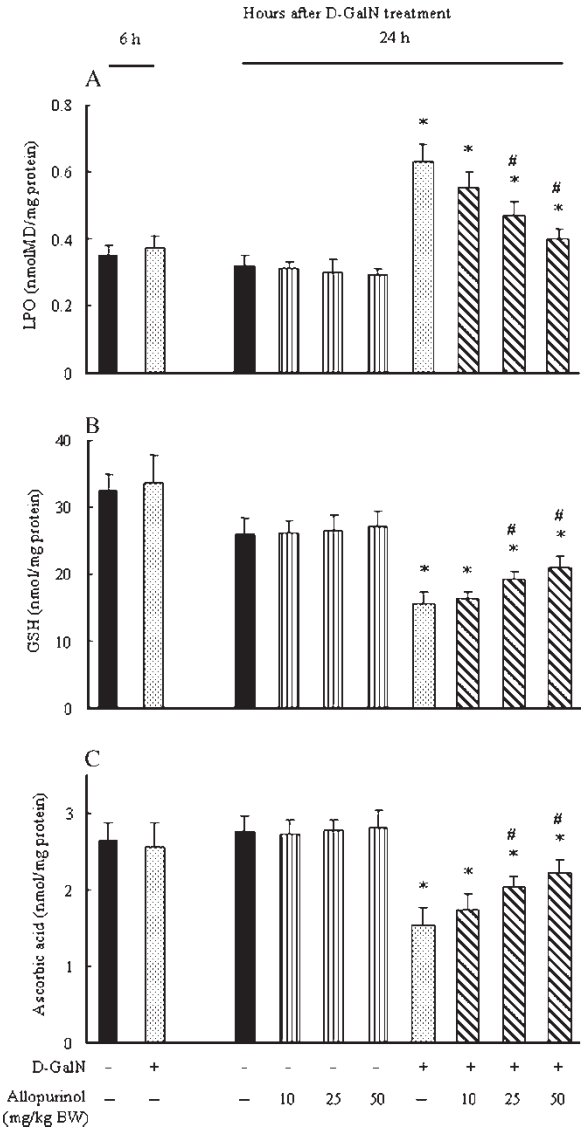

Figure 5. Effect of post-allopurinol administration on hepatic (A) LPO, (B) GSH, and (C) ascorbic acid contents in rats treated with D-GaIN. Rats treated with and without D-GaIN (500 mg/kg BW, i.p.) orally received allopurinol (10, 25 or 50 mg/kg) at 6 h after the treatment. The animals were sacrificed 6 and 24 h after D-GaIN treatment. Each value is a mean ± SD. ($n = 6–8$). ⋆, Significantly different from control rats, $P < 0.05$; #, significantly different from rats treated with D-GaIN alone, $P < 0.05$.

Change in hepatic MPO activity and effect of post-allopurinol administration on the change in hepatic MPO activity in D-GalN-treated rats

The D-GalN-treated group had significantly higher hepatic MPO activity than the control group at 6, 12, 18, and 24 h after treatment and the increase in hepatic MPO activity in the D-GalN-treated group occurred time-dependently (Figure 7). Allopurinol (10, 25 or 50 mg/kg BW) administered to D-GaIN-treated rats at 6 h after the treatment significantly reduced the increase in hepatic MPO activity found at 24 h after the treatment dose-dependently (Figure 8). The hepatic MPO activity in D-GalN-treateed rats pose-administered with allopurinol (50 mg/kg BW) was still significantly higher than control rats but was not significantly different from that in D-GalN-treated rats found at 6 h after the treatment (Figure 8). D-GalN-untreated rats given the same doses of allopurinol showed no change in hepatic MPO activity (Figure 8).

Discussion

In the present study, rats treated with D-GalN (500 mg/kg BW) showed apparent liver injury at 6 h

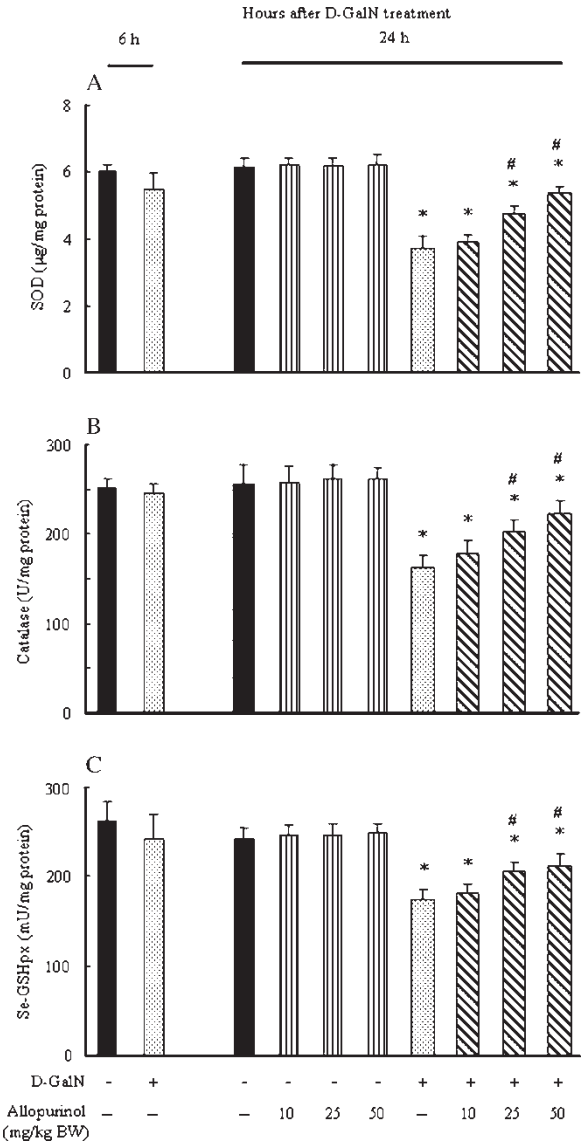

Figure 6. Effect of post-allopurinol administration on hepatic (A) SOD; (B) catalase; and (C) Se-GSHpx activities in rats treated with D-GaIN. Rats treated with and without D-GaIN (500 mg/kg BW, i.p.) orally received allopurinol (10, 25 or 50 mg/kg) at 6 h after the treatment. The animals were sacrificed 6 and 24 h after D-GaIN treatment. Each value is a mean ± SD. ($n = 6–8$). *, Significantly different from control rats, $P < 0.05$; #, significantly different from rats treated with D-GaIN alone, $P < 0.05$.

after the treatment and the liver injury developed time-dependently until 24 h, judging from the levels of serum ALT and AST, indices of liver cell damage. XO activity and XO/XD + XO ratio in the liver of rats treated with D-GalN began to increase after the appearance of liver injury, i.e. at 12 h after the treatment and continued to increase until the advanced stage of the injury, i.e. 24 h after the treatment, while XD + XO activity in the liver decreased slightly at the advanced stage of the injury. These results indicate that hepatic XO activity increases with the development of D-GalN-induced liver injury in rats and that the increase in hepatic XO

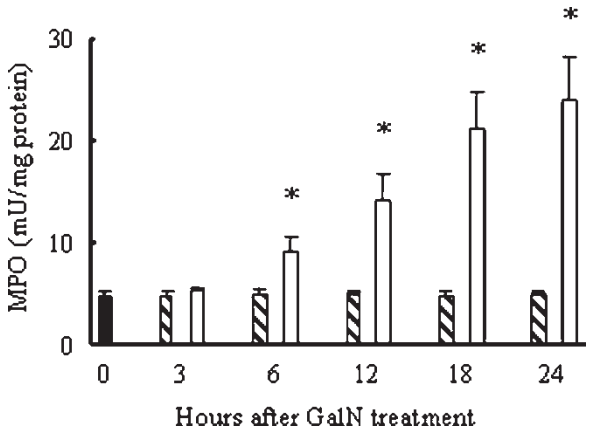

Figure 7. Change in hepatic MPO activity following D-GalN treatment in rats. Rats were treated with (open bar) and without (hatched bar) D-GaIN (500 mg/kg BW, i.p.). Each value is a mean ± SD. ($n = 5–7$). *, Significantly different from rats without D-GalN treatment, $P < 0.05$.

activity with liver injury development is due to the conversion of XD to XO in the tissue. Allopurinol, an XO inhibitor, (10, 25 or 50 mg/kg BW) administered orally to D-GalN-treated rats at the early stage of liver injury, i.e. at 6 h after the treatment, prevented the increase in hepatic XO activity found at the advanced stage of the injury in a dose-dependent manner and caused a complete prevention of the increase in hepatic XO activity at a dose of 50 mg/kg BW. The orally post-administered allopurinol attenuated the development of D-GalIN-induced liver injury in a dose-dependent manner but its dose of 50 mg/kg BW did not prevent the liver injury development

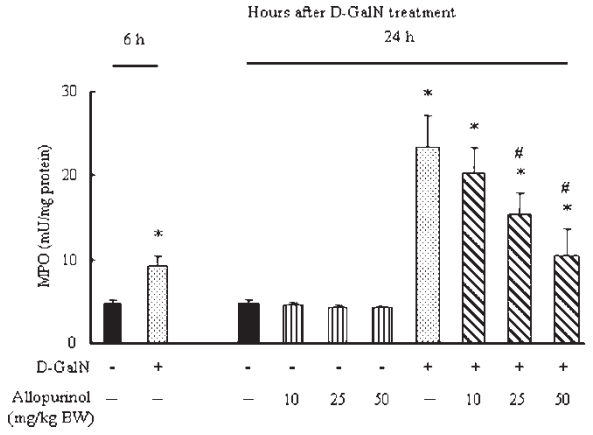

Figure 8. Effect of post-allopurinol administration on hepatic MPO activity in rats treated with D-GaIN. Rats treated with and without D-GaIN (500 mg/kg BW, i.p.) orally received allopurinol (10, 25 or 50 mg/kg) at 6 h after the treatment. The animals were sacrificed 6 and 24 h after D-GaIN treatment. Each value is a mean ± SD. ($n = 6–8$). *, Significantly different from control rats, $P < 0.05$; #, significantly different from rats treated with D-GaIN alone, $P < 0.05$.

completely. These results indicate that XO activity increases through XD to XO conversion in the damaged liver of D-GalN-treated rats, and suggest that XO increasing in the damaged liver of D-GalN-treated rats plays an important role in liver injury development.

In the present study, both serum XO activity and uric acid concentration began to increase after the appearance of D-GalN-induced liver injury, i.e. at 12 h after the treatment and they continued to increase until the advanced stage of the injury. Thus, the increases in serum XO activity and uric acid concentration coincided with the above-described increase in hepatic XO activity. However, it seems unlikely that the increase in XO activity in the serum of D-GalN-treated rats is due to the release of XO from the liver tissue, because the hepatic XD + XO activity showed little change, as described above.

There are reports showing that hepatic lipid peroxidation is enhanced during the development of D-GalN-induced liver injury in rats [16–22]. In the present study, an increase in hepatic LPO content in D-GalN-treated rats was observed at the advanced stage of liver injury, but not at the early stage of the injury. Allopurinol administered at the early stage of D-GalN-induced liver injury prevented the increased hepatic LPO content found at the advanced stage of the injury dose-dependently, although its dose of 50 mg/kg BW did not prevent the increase in hepatic LPO content completely. There was no change in hepatic LPO content in D-GalN-untreated rats given the same doses of allopurinol. Allopurinol and its main *in vivo* metabolite, oxypurinol, function as a scavenger of ROS *in vitro* [44,45]. However, Klein et al. [33] reported that while orally administered allopurinol at doses of 2–50 mg/kg BW effectively suppressed XO activity in the liver and intestine of rats, antioxidant activity was not seen even in its doses up to 100 mg/kg BW. Accordingly, it can be thought that ROS derived from XO increasing in the damaged liver of D-GalN-treated rats contribute, at least in part, to the increase in lipid peroxidation found in the tissue, which may be linked to the development of D-GalN-induced liver injury.

It has been reported that the hepatic levels of antioxidants such as GSH and ascorbic acid and the hepatic activities of antioxidant enzymes such as SOD, catalase and Se-GSHpx are reduced during liver injury development in D-GaIN-treated rats [18,19,21,22]. In the present study, GSH and ascorbic acid contents and SOD, catalase and Se-GSHpx activities in the liver of D-GaIN-treated rats unchanged at the early stage of liver injury but they all decreased markedly at the advanced stage of the injury. In addition, allopurinol administered to D-GalN-treated rats at the early stage of liver injury attenuated all these decreases found at the advanced stage of the injury dose-dependently, although its dose of 50 mg/kg BW did not prevent these decreases completely. These results suggest that ROS derived from XO increasing in the liver of D-GalN-treated rats causes disruption of the antioxidant defense system in the liver tissue, although the disruption mechanism is unknown at present. This disruption of the hepatic antioxidant defense system may be linked to the development of D-GalN-induced liver injury in rats.

It has been shown that the number of neutrophils infiltrating into liver tissues begins to increase at the early stage of D-GalN-induced liver injury in rats and further increases with the development of the liver injury [20,46]. It has been suggested that ROS derived from activated neutrophils extends liver cell necrosis through an increase in hepatic lipid peroxidation in rats with D-GaIN-induced hepatitis [20]. It is known that $O_2^{\cdot-}$ generated by the xanthine–XO system induces neutrophil adhesion to cultured endothelial cells [28,29]. In the present study, MPO activity, an index of tissue neutrophil infiltration [30–32], in the liver of D-GalN-treated rats began to increase at the early stage of liver injury and continued to increase until the advanced stage of the injury. The increase in hepatic MPO activity started before the onset of an increase in serum XO activity in D-GalN-treated rats. Allopurinol administered to D-GalN-treated rats at the early stage of liver injury attenuated the increases in hepatic MPO and serum XO activities found at the advanced stage of the injury dose-dependently. The hepatic MPO and serum XO activities in the D-GalN-treated rats administered with allopurinol (50 mg/kg BW) were almost equal to those in D-GalN-treated rats found just before allopurinol administration. These results suggest that ROS derived from XO increasing in the serum of D-GalN-treated rats mediates enhanced neutrophil infiltration into the liver tissue. This XO-mediated neutrophil infiltration into the liver of D-GalN-treated rats may contribute to liver injury development.

In conclusion, the results of the present study indicate that XO activity increases in the serum and liver tissue of D-GalN-treated rats and the increase in XO activity in the serum and liver tissue is closely associated with liver injury development. The results also suggest that ROS derived from XO increasing in the liver of D-GalN-treated rats causes an increase in lipid peroxidation and disruption of the antioxidant defense system in the liver tissue and that ROS derived from XO increasing in the serum of D-GalN-treated rats mediates enhanced neutrophil infiltration into the liver tissue. Therefore, these events caused by ROS derived from XO increasing in the serum and liver tissue of D-GalN-treated rats may contribute to liver injury development. However, further investigation is required to clarify the exact contribution of XO-derived ROS to the development of D-GalN-induced liver injury.

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