

**Establishment of a mouse model of enalapril-induced liver injury and investigation  
of the pathogenesis**

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**Running title:** Mechanism of enalapril-induced liver injury

**Abbreviations:** ACE, angiotensin-converting enzyme; ALT, alanine aminotransferase; AST, aspartate aminotransferase; BSO, L-buthionine-*S,R*-sulfoximine; CYP, cytochrome P450; DEX, dexamethasone; DILI, drug-induced liver injury; ELP, enalapril; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GATA3, GATA binding protein 3; GSH, glutathione; GSSG, glutathione disulfide; HO-1, heme oxygenase-1; IL, interleukin; MDA, malondialdehyde; MIP-2, macrophage inflammatory protein-2; MPO, myeloperoxidase; ROR $\gamma$ t, retinoid-related orphan receptor  $\gamma$ t; PCN, pregnenolone-16 $\alpha$ -carbonitrile; ROS, reactive oxygen species; RT, reverse transcription; T-bet, T-box expressed in T cells; TNF $\alpha$ , tumor necrosis factor  $\alpha$

**Keywords:** enalapril, drug-induced liver injury, oxidative stress, dexamethasone, tempol.

## **Abstract**

Drug-induced liver injury (DILI) is a major concern in drug development and clinical drug therapy. Since the underlying mechanisms of DILI have not been fully understood in most cases, elucidation of the hepatotoxic mechanisms of drugs is expected. Although enalapril (ELP), an angiotensin-converting enzyme inhibitor, has been reported to cause liver injuries with a low incidence in humans, the precise mechanisms by which ELP causes liver injury remains unknown. In this study, we established a mouse model of ELP-induced liver injury and analyzed the mechanisms of its hepatotoxicity. Mice that were administered ELP alone did not develop liver injury, and mice that were pretreated with a synthetic glucocorticoid dexamethasone (DEX) and a glutathione synthesis inhibitor L-buthionine-(*S,R*)-sulfoximine (BSO) exhibited liver steatosis without significant increase in plasma alanine aminotransferase (ALT). In mice pretreated with DEX and BSO, ALT levels were significantly increased after ELP administration, suggesting that hepatic steatosis sensitized the liver to ELP hepatotoxicity. An immunohistochemical analysis showed that the numbers of myeloperoxidase-positive cells that infiltrated the liver were significantly increased in the mice administered DEX/BSO/ELP. The levels of oxidative stress-related factors, including hepatic heme oxygenase-1, serum hydrogen peroxide and hepatic malondialdehyde, were elevated in the mice administered DEX/BSO/ELP. The involvement of oxidative stress in ELP-induced liver injury was further supported by the observation that tempol, an antioxidant agent, ameliorated ELP-induced liver injury. In conclusion, we successfully established a model of ELP-induced liver injury in DEX-treated steatotic mice and demonstrated that oxidative stress and neutrophil infiltration are involved in the pathogenesis of ELP-induced liver injury.

## Introduction

Drug-induced liver injury (DILI) is a serious health problem and a leading cause of death from acute liver failure.<sup>1</sup> DILI is also a major challenge for the pharmaceutical industry because the occurrence of DILI can lead to the termination of drug development, failure of drug approval, and post-market withdrawal of drugs.<sup>2</sup> A number of drugs have been withdrawn from the market worldwide due to their hepatotoxicity, including bromfenac, lumiracoxib, troglitazone, and trovafloxacin.<sup>3,4</sup> Although the potential toxicity of drug candidates is mostly detected in nonclinical safety studies in drug development, there are many cases in which toxicity is revealed only after marketing.<sup>5</sup> Thus, there is a need to better manage safety liabilities and understand mechanisms of toxicity before advancing drug candidates into clinical development.

Although pathogenesis of clinical DILI is not fully understood, two major pathways, direct hepatotoxicity and adverse immune reactions, have been proposed as mechanisms by a number of clinical and non-clinical studies.<sup>6</sup> Direct hepatotoxicity is caused by the direct action of a drug or its reactive metabolites, which can bind to cellular macromolecules such as proteins, lipids, and nucleic acids, leading to protein dysfunction, lipid peroxidation and oxidative stress. In acetaminophen<sup>7</sup>, azathioprine<sup>8</sup> and isoniazid<sup>9</sup>-induced liver injuries, oxidative stress (reactive oxygen species, ROS) and mitochondrial dysfunction play a critical role in hepatotoxicity. Additionally, there is growing evidence that immune and inflammatory responses are associated with the pathogenesis of DILI.<sup>10</sup> We previously reported that helper T (Th) cell-related factors are involved in the pathogenesis of carbamazepine<sup>11</sup>, halothane<sup>12</sup> and methimazole<sup>13</sup>-induced liver injuries. Because mechanisms of DILI vary among drugs, the elucidation of drug-specific mechanisms associated with DILI is expected. An animal model of DILI may provide the *in vivo* mechanisms for preclinical drug

development.

Enalapril (ELP) is an angiotensin-converting enzyme (ACE) inhibitor that is widely used in the treatment of cardiovascular diseases, including hypertension and heart failure. Although ACE inhibitors are generally well-tolerated, they have been reported to cause hepatotoxicity with a low incidence in humans.<sup>14-16</sup> The numbers of published case reports of liver injury caused by ACE inhibitors were 25 (enalapril), 16 (captopril), 14 (lisinopril), 4 (ramipril), 3 (fosinopril), and 2 (benazepril).<sup>17</sup> In another study using the WHO individual case safety report database (VigiBase), the numbers of “*over all liver injury*” reports caused by ACE inhibitors were 706 (enalapril), 643 (captopril), and 378 (lisinopril).<sup>18</sup> Considering the two studies, enalapril shows the relatively highest number of reports for liver injury in the ACE inhibitors, suggesting existence of drug-specific reasons but not pharmacological actions for the hepatotoxicity. In a case report, ELP-induced liver injury occurred in a patient who had liver steatosis.<sup>19</sup> In addition, patients who developed ELP hepatotoxicity were reported to be accompanied by fever, urticarial rash and eosinophilia, suggesting that immunologic reaction participated in ELP-induced liver injury.<sup>20</sup> In rats, it was reported that pretreatment with pregnenolone-16 $\alpha$ -carbonitrile (PCN) that induces cytochrome P450 (CYP) 3A enzyme followed by ELP administration produced a 2-fold increase in plasma alanine aminotransferase (ALT) levels compared with ELP administration alone.<sup>21</sup> In addition, pretreatment of L-buthionine-*S,R*-sulfoximine (BSO) and diethyl maleate that caused glutathione (GSH) depletion following ELP administration showed a 6-fold increase in plasma ALT levels compared with ELP administration alone in rats.<sup>21</sup> These previous findings may indicate that the steatotic phenotype, CYP3A induction, GSH depletion, and/or immunological reactions have suspected roles in the pathogenesis of ELP-induced liver injury. At present, however, the involvement of

oxidative stress and immune responses has not been reported in ELP-induced liver injury.

In this study, we sought to establish an ELP-induced liver injury model in mice for clarifying the mechanism of toxicity. A synthetic glucocorticoid dexamethasone (DEX) was used to cause hepatic steatosis in mice. By co-administration with or without DEX and BSO, we found that a steatotic phenotype and GSH depletion, rather than P450 induction, is essential to causing exacerbation of ELP-induced liver injury. Furthermore, we investigated the involvement of immune responses and oxidative stress in ELP-induced liver injury.

## **Materials and Methods**

### **Materials.**

ELP maleate, BSO, DEX, reduced GSH and oxidized GSH were purchased from Wako Pure Chemical Industries (Osaka, Japan). Imidapril was kindly provided by the Mitsubishi Tanabe Pharma Corporation (Osaka, Japan). Tempol (4-hydroxy-2,2,6,6-tetramethylpiperidine 1-oxyl) and PCN were purchased from Sigma-Aldrich (St. Louis, MO). Rat anti-mouse Gr-1 (RB6-8C5) monoclonal antibody (mAb) and rat IgG2b isotype control were purchased from TONBO Biosciences (San Diego, CA). RNAiso Plus and SYBR Premix Ex Taq (Tli RNaseH Plus) were purchased from Takara (Otsu, Japan). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). A ReverTra Ace qPCR RT kit was purchased from Toyobo (Osaka, Japan). Fuji DRI-CHEM slides of GPT/ALT-PIII, GOT/AST-PIII and TBIL-PIII used to measure ALT, aspartate aminotransferase (AST) and total bilirubin (TBIL), respectively, were purchased from FujiFilm (Tokyo, Japan). A rabbit polyclonal antibody against human myeloperoxidase (MPO) was purchased from Dako (Carpinteria, CA). All other chemicals used in this study were of analytical grade or the highest grade commercially available.

### **Animals.**

Male Balb/cCrSlc mice (8-week-old) were obtained from Japan SLC (Hamamatsu, Japan). The mice were housed under a 12-h light/dark cycle (lights on 9:00–21:00 h) in a controlled environment (temperature  $23 \pm 2^\circ\text{C}$  and humidity  $55 \pm 10\%$ ) in the institutional animal facility with *ad libitum* access to food and water. The animals were acclimatized before the experiments. All animal experiments were approved by the Animal Care Committee of Nagoya University Graduate School of

Medicine and were carried out in accordance with the guidelines established by the Institute for Laboratory Animal Research of the Medical School of Nagoya University, Japan.

### **DEX, BSO and ELP administration.**

DEX was suspended in corn oil (6 mg/ml) and intraperitoneally (i.p.) administered to mice at a dose of 60 mg/kg/day for 3 days. The dosing regimen of DEX was adopted based on our previous report to induce hepatic CYP3A expression and cause hepatic steatosis in mice.<sup>22</sup> BSO was dissolved in water (70 mg/ml) and intraperitoneally administered to the mice at a dose of 700 mg/kg 1 h prior to ELP administration. The 700 mg/kg dose of BSO was selected because a decrease in hepatic GSH level was confirmed at this dosage.<sup>23</sup> Twenty-four hours after the final DEX administration, ELP suspended in 0.5% carboxymethyl cellulose (100 mg/ml) was orally (p.o.) administered to the mice at a dose of 1000 mg/kg (approximately one-third of the p.o. LD<sub>50</sub>) after overnight fasting. Two hours after ELP administration, the mice were again allowed access to food *ad libitum*. Another ACE inhibitor drug, imidapril (1000 mg/kg, p.o.), was administered to mice under the same dosing regimen as that for ELP. At 0, 3, 6 and 24 h after ELP administration, blood was collected from the jugular veins and plasma ALT, AST and TBIL levels were measured using Fuji DRI-CHEM slides of GPT/ALT-PIII, GOT/AST-PIII and TBIL-PIII, respectively. At 3 and 6 h after ELP administration, the mice were sacrificed under isoflurane anesthesia, the blood was collected from the inferior vena cava, and the liver was excised from the left lateral lobe. A portion of each excised liver was fixed in 10% formalin neutral buffer solution, and each remaining liver sample was immediately frozen and stored at -80°C until further experiment.



### **Histopathological examination**

The formalin-fixed liver specimens were paraffin-embedded, sectioned, and stained with hematoxylin and eosin (H&E) for histopathological examination. To evaluate neutrophil infiltration, the sections were immunostained with a rabbit polyclonal antibody against human MPO as previously described.<sup>24</sup> The average number of MPO-positive cells was counted using 3 randomly selected fields per section from each animal at a 400-fold magnification (0.1 mm<sup>2</sup> each) and was expressed as cells/mm<sup>2</sup>.

### **Tempol treatment.**

Tempol was dissolved in phosphate buffered saline (pH 7.4, 20 mg/ml) and intraperitoneally administered to mice at a dose of 200 mg/kg simultaneously with ELP administration. The dosing regimen of tempol treatment was adopted according to the previous report.<sup>8</sup> The blood and liver samples were collected 6 h after ELP and tempol administration.

### **Anti-Gr-1 treatment.**

An anti-Gr-1 monoclonal antibody or rat IgG2b isotype control (150  $\mu$ g) was intraperitoneally administered to mice 36 h prior to ELP administration. The dose of anti-Gr-1 treatment was adopted according to the previous report.<sup>25</sup> The blood was collected at 6 h after ELP administration. To count the numbers of neutrophils in blood, the blood samples were analyzed using VetScan HM5 (Abaxis, Union City, CA) according to the manufacturer's instructions.

### **Real-time reverse transcription (RT)-PCR.**

Total RNA was isolated from the livers using RNAiso Plus, according to the manufacturer's instructions. RT was performed using a ReverTra Ace qRT-PCR kit, according to the manufacturer's instructions. In brief, 2 µg of total RNA was mixed with an appropriate volume of five-fold RT buffer, enzyme mix, primer mix, and nuclease-free water to adjust the total volume to 20 µl. The RT reaction was carried out at 37°C for 15 min and 98°C for 5 min. The expression of the macrophage inflammatory protein-2 (MIP-2), interleukin-1β (IL-1β), tumor necrosis factor α (TNFα), T-box expressed in T cells (T-bet), GATA binding protein 3 (GATA3), retinoid-related orphan receptor γt (RORγt), heme oxygenase-1 (HO-1) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was quantified by real-time PCR using a primer pair (Table.1). Real-time PCR was performed using an Mx3000P instrument (Agilent Technologies, Santa Clara, CA), with an initial denaturation at 95°C for 30 s, followed by 40 amplification cycles of 95°C for 5 s and 60°C for 30 s. The relative mRNA expression levels were calculated by the  $2^{-\Delta\Delta Ct}$  method, where  $\Delta\Delta Ct = [\Delta Ct \text{ of the target gene (sample from each time point)} - \Delta Ct \text{ of endogenous control GAPDH (sample from each time point)}] - [\Delta Ct \text{ of the target gene (vehicle control sample from each time point)} - \Delta Ct \text{ of GAPDH (vehicle control sample from each time point)}]$ .

### **Hepatic GSH measurement.**

The livers collected 6 h after ELP administration were homogenized in ice-cold 5% sulfosalicylic acid and centrifuged at 8000 g at 4°C for 10 min. The total GSH and disulfide GSH (GSSG) concentrations were measured in the supernatant as previously described.<sup>26,27</sup> The reduced GSH levels were calculated by subtracting GSSG from total

GSH levels.

### **Plasma hydrogen peroxide level.**

Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) levels in the plasma 3 and 6 h after ELP administration were measured using a Hydrogen Peroxide Assay kit (Bio Vision, Milpitas, CA), according to the manufacturer's instructions.

### **Hepatic MDA measurement.**

Hepatic malondialdehyde (MDA) levels were measured using a TBARS Microplate Colorimetric Assay kit (Oxford Biomedical Research, Oxford, MI) as previously described with minor modifications.<sup>28</sup> Briefly, a reaction mixture containing liver homogenate, 10 mM potassium phosphate buffer (pH 7.4), trichloroacetic acid and thiobarbituric acid (50 mg/ml) was incubated at 65°C for 1 h and then cooled. Following centrifugation at 13000 g at 4°C for 5 min, the supernatant was transferred to a 96-well plate, and the absorbance at 532 nm was measured.

### **Statistical analysis.**

The data are presented as the mean  $\pm$  SEM. Statistical analyses were performed using JMP Software (JMP Pro, Version 11.2.1; SAS Institute Inc., Cary, NC). The statistical significance between the two groups was determined by two-tailed Student's *t* test and between multiple groups by Dunnett's test. Values of *P* < 0.05 were considered statistically significant.

## Results

### Establishment of an ELP-induced liver injury model in mice.

To establish an ELP-induced liver injury model, male Balb/c mice were pretreated with DEX (60 mg/kg, i.p.) once daily for 3 days, then BSO (700 mg/kg, i.p.) 23 h after the final DEX treatment, and then these mice were administered ELP (1000 mg/kg, p.o.) 24 h after the final DEX treatment. The plasma ALT and AST levels were significantly increased in the DEX/BSO/ELP-treated group compared with the vehicle control group. The ALT and AST levels peaked at 6 h after ELP administration and decreased at 24 h in the DEX/BSO/ELP group (Figures 1a and 1b). In addition, TBIL levels were significantly higher in the DEX/BSO/ELP group 6 h after ELP administration compared to the vehicle control group (Figure 1c). The ALT levels were not significantly changed in the ELP only group (Figure 1a). To determine which of DEX and BSO were critical for ELP-induced liver injury, we examined the effects of withdrawal of DEX and/or BSO from the established DEX/BSO/ELP regimen on ALT levels. Plasma ALT levels at 6 h after ELP administration were not increased in any of the ELP alone-, BSO/ELP-, and DEX/ELP-treated groups (Figure 1d), demonstrating that the pretreatment of both DEX and BSO is essential for ELP-induced liver injury. Since DEX is a CYP3A inducer, we performed immunoblot analysis to examine CYP3A expression levels in ELP-induced liver injury model. The expression levels of CYP3A were increased in the DEX/BSO- and DEX/BSO/ELP-treated group (Supplemental figure 1). To examine the involvement of CYP3A induction in ELP-induced liver injury, we pretreated mice with PCN for 4 consecutive days and subsequently administered BSO and ELP. The plasma ALT levels 6 h after ELP administration showed slight elevation ( $153 \pm 17$  U/L,  $n = 5$ ) in the PCN/BSO/ELP group (Supplemental figure 2a). In addition, we confirmed that CYP3A expression levels were increased in the

PCN/BSO/ELP-treated group (Supplemental figure 2b). Because ELP is converted to enalaprilat, the active metabolite of ELP, in the intestine and liver, we administered enalaprilat to DEX/BSO-pretreated mice to examine the effects of enalaprilat on liver injury. As the result, six hours after the administration, the plasma ALT level was increased, which was comparable to that in the DEX/BSO/ELP-treated group (Supplemental figure 3). Another ACE inhibitor, imidapril, which has never been reported to cause hepatotoxicity in humans, did not increase the plasma ALT level under the same dosing conditions as ELP (Figure 1e). This result indicates that the pharmacological activity did not contribute to the ELP-induced hepatotoxicity. The liver histopathology 6 h after ELP or vehicle dosing revealed microvesicular steatosis in the DEX/BSO- and DEX/BSO/ELP-treated groups (Figure 2a). The number of MPO-positive cells in these two groups was significantly higher than in the vehicle control group (Figures. 2b and 2c), but it was even higher in the DEX/BSO/ELP-treated group than that in DEX/BSO-treated group (Figure 2c), suggesting that neutrophil infiltration was involved in the hepatotoxicity.

### **Expression levels of immune-, inflammatory- and oxidative stress-related genes in ELP-induced liver injury.**

To investigate the mechanism of ELP-induced liver injury in mice, we measured hepatic mRNA expression levels of inflammatory-related factors (MIP-2, TNF $\alpha$  and IL-1 $\beta$ ; Figure 3a), immune-related transcription factors (T-bet, GATA3, and ROR $\gamma$ t; Figure 3b) and an oxidative stress-related gene (HO-1; Figure 3c) at 3 and 6 h after ELP administration. The mRNA expression level of MIP-2, which is involved in neutrophil recruitment, was significantly increased in the DEX/BSO/ELP-treated group compared with the vehicle control group 6 h after ELP administration. In contrast, the mRNA

expression levels of TNF $\alpha$  and IL-1 $\beta$  were significantly decreased or not changed in the DEX/BSO/ELP-treated group. Similarly, the mRNA expression levels of T-bet, GATA3, and ROR $\gamma$ t, which are master regulators of Th1, Th2 and Th17 cells, respectively, were decreased in the DEX/BSO/ELP-treated group compared with vehicle control group. Importantly, the mRNA expression levels of HO-1, a marker of oxidative stress, were significantly increased only in the DEX/BSO/ELP-treated group. These data suggest that oxidative stress and neutrophil recruitment are involved in ELP-induced liver injury.

#### **Involvement of oxidative stress in ELP-induced liver injury.**

To further investigate the involvement of oxidative stress in ELP-induced liver injury, we measured hepatic GSH, plasma hydrogen peroxide, and hepatic MDA levels. GSH is an intracellular anti-oxidant that prevents oxidative stress. Total GSH, GSSG and GSH were significantly reduced in the DEX/BSO- and DEX/BSO/ELP-treated groups 6 h after ELP administration (Figures 4a to 4c). Total GSH and GSH were significantly reduced in the DEX/BSO/ELP-treated group compared with the DEX/BSO group. The hepatic GSH/GSSG ratio, a marker of oxidative stress, was significantly decreased in the DEX/BSO/ELP-treated group compared with the DEX/BSO group. The plasma levels of hydrogen peroxide, one of the reactive oxygen species (ROS), were significantly increased in the DEX/BSO/ELP-treated group 3 and 6 h after ELP administration (Figure 5a). Moreover, the levels of hepatic MDA, a lipid peroxidation marker, were significantly increased in the DEX/BSO/ELP group compared with the DEX/BSO group 6 h after ELP administration (Figure 5b). These data suggested that oxidative stress, including ROS production and lipid peroxidation, may contribute to ELP-induced liver injury.

### **Effects of anti-oxidant treatment on ELP-induced liver injury.**

To investigate whether oxidative stress contributes to the pathogenesis of ELP-induced liver injury, mice administered DEX/BSO/ELP were treated with an ROS scavenger tempol (200 mg/kg, i.p.) simultaneously with ELP administration. Plasma ALT levels were significantly decreased 6 h after ELP administration in the tempol-treated group compared with the vehicle control group (Figure 6a). In addition, tempol treatment tended to suppress hepatic HO-1 mRNA expression (Figure 6b). These data indicated that oxidative stress was critical in the pathogenesis of ELP-induced liver injury.

### **Effects of neutrophil depletion on ELP-induced liver injury.**

To determine whether neutrophil infiltration is involved in ELP-induced liver injury, we treated an anti-Gr-1 antibody, a neutrophil depleting agent, to DEX-administered mice before BSO and ELP administration. The number of blood neutrophils in the DEX/BSO/ELP-treated group were significantly higher than that in the DEX/BSO-treated group. Anti-Gr-1 treatment effectively reduced the number of blood neutrophils by 70% in ELP-induced liver injury (Figure 7a). Importantly, plasma ALT levels were significantly decreased in the anti-Gr-1 antibody-treated group compared with the isotype control-treated group (Figure 7b). These results indicated that neutrophil infiltration contributes to the pathogenesis of ELP-induced liver injury.

## Discussion

We successfully established a mouse model of ELP-induced liver injury by pretreatment with DEX and BSO and found that pretreatment with both agents is essential to causing the hepatotoxicity of ELP (Figure 1a-1d). DEX is known to inhibit acyl-CoA dehydrogenation and to impair mitochondrial fatty acid  $\beta$ -oxidation, leading to hepatic steatosis in humans as well as in rodents.<sup>29</sup> It has been shown that the development of steatosis increases the sensitivity of the liver to other hits such as oxidative stress and inflammation, leading to liver damage in nonalcoholic fatty liver disease.<sup>30-32</sup> In support of this notion, our study demonstrated that ELP treatment elicited ALT elevation in mice developing hepatic steatosis. Considering that ELP was reported to cause hepatotoxicity in a patient who had hepatic steatosis<sup>19</sup>, our animal model would adequately mimic the toxicity observed in humans. From these findings, liver steatosis caused by DEX might increase the sensitivity to ELP hepatotoxicity and further oxidative stress damage may accentuate the pathogenesis of ELP-induced liver injury.

In a previous study using rats, pretreatment with PCN for 4 consecutive days, followed by ELP (1500 mg/kg i.p.) administration, increased ALT levels (approximately 250 U/L, n = 6)<sup>21</sup>, suggesting CYP3A induction may play a role in ELP-induced liver injury. However, the ALT levels showed only a 2-fold elevation compared with ELP administration alone. Our study also showed a slight elevation in ALT levels (153 $\pm$ 17 U/L, n = 5) in the PCN/BSO/ELP-treated mice (Supplemental figure 2a). Considering the fact that oxidative reactive metabolites of ELP have not been reported previously, CYP3A induction plays a minor role in ELP-induced liver injury. In addition, we investigated the toxicological role of enalaprilat, active metabolite of ELP. The plasma ALT levels of the DEX/BSO/ Enalaprilat-treated group were comparable to those in the DEX/BSO/ELP-treated group (Supplemental figure 3). From this result, we suspected



that enalaprilat that is converted from enalapril in the intestine and liver would be mainly contribute to the liver injury.

Our data strongly suggest that oxidative stress was critical in the pathogenesis of ELP-induced liver injury because ALT elevation was accompanied by increases in hepatic mRNA expression of HO-1, serum hydrogen peroxide levels and hepatic MDA levels in the group administered DEX/BSO/ELP. Supporting our results, a previous study using a cDNA microarray showed that ELP administration (1800 mg/kg, p.o.) to rats altered the expression of oxidative stress-responsive genes (glutathione-S-transferase, alpha type 2 and NAD(P)H dehydrogenase quinone 1) in the liver.<sup>33</sup> However, administration of ELP alone was not sufficient to cause ALT elevation, probably due to the threshold of detoxification pathways against oxidative stress. GSH is a major antioxidant that eliminates ROS and drug reactive metabolites.<sup>34,35</sup> For example, hydrogen peroxide is detoxified to H<sub>2</sub>O and O<sub>2</sub> by glutathione peroxidase, which needs GSH as the electron donor.<sup>34</sup> ROS can attack polyunsaturated fatty acids and initiate lipid peroxidation to produce MDA and 4-hydroxy-2-nonenal.<sup>36</sup> Lipid peroxidation products are implicated to induce inflammation via activation of Kupffer cells.<sup>30</sup> In this study, under the hepatic GSH-depleted condition by pretreatment with BSO, ROS could not be detoxified sufficiently, and excess ROS in the steatotic liver would lead to liver injury. The involvement of oxidative stress in ELP-induced liver injury was further supported by the observation that tempol, which scavenges free radicals,<sup>37</sup> ameliorated ELP-induced liver injury. Acetaminophen-<sup>38</sup> and azathioprine-<sup>8</sup> induced liver injury in mice was attenuated by tempol treatment. Thus, our results support a role for oxidative stress in ELP-induced liver injury.

There is growing evidence that immune and inflammatory responses are associated with the pathogenesis of DILI.<sup>39</sup> In this study, however, mRNA expression

levels of most inflammation-related factors and immune-related transcription factors did not increase in the DEX/BSO/ELP group (Figures 3a and 3b). This result is probably due to the anti-inflammatory and immunosuppressive effects of DEX.<sup>40</sup> Although DEX suppressed inflammatory responses, we demonstrated for the first time that innate immune cells play an important role in the pathogenesis of ELP-induced liver injury as follows. The expression levels of MIP-2 and neutrophil infiltration were increased in the group administered DEX/BSO/ELP (Figures. 2b and 3a). It was reported that oxidative stress induces MIP-2 expression in liver cells including hepatocytes and Kupffer cells.<sup>41</sup> Therefore, increased oxidative stress in the steatotic liver is likely to trigger induction of hepatic MIP-2 levels to induce neutrophil infiltration into the liver. Moreover, neutrophil depletion by anti-Gr-1 alleviated ELP-induced liver injury (Figure 7b). The deleterious role (causing additional liver injury) of neutrophil infiltration in liver injury was demonstrated in models such as ischemia-reperfusion, obstructive cholestasis and DILI ( $\alpha$ -naphthylisothiocyanate and halothane).<sup>10</sup> The infiltrated neutrophils generate ROS in close to hepatocytes. The generated ROS including hydrogen peroxide and hypochlorite can diffuse cell membrane and increase an intracellular oxidant stress.<sup>42</sup> Thus, ROS generated from infiltrated neutrophil is one of the causal factors of ELP-induced oxidative stress. Taken together, neutrophil infiltration into the liver is another important factor in ELP-induced liver injury.

The dosage levels of ELP in this study (1000 mg/kg) are obviously high compared with clinical situation (maximum daily dose 40 mg for a human). However, dose is not a sole indicator when extrapolating animal safety profiles of drugs into human *in vivo* because toxicities of drugs are affected by the species differences in absorption, metabolism, and detoxification between experimental animals and humans. This is also true for ELP because the absorption of ELP in rats (34%)<sup>43</sup> is much lower

than that of human (60%)<sup>44</sup>. It was previously reported that detecting responses of oxidative stress and reactive metabolites in rodents given high dose of drugs showed the potential for identification of adverse responses seen in humans.<sup>33</sup> Therefore, the tests for risk assessment adopted frequently performed with high dose (high maximally tolerated dose) of drug. Generally, drugs were given at a single dose at about 2/3 of the LD<sub>50</sub>.<sup>33, 45</sup> The LD<sub>50</sub> of ELP in male mouse is 3696 mg/kg. From these reasons, in order to detect hepatotoxic signature worth for clinical practice, 1000 mg/kg dosage was adopted in the present study.

Our method showed the usefulness of diseased animals in detecting DILI. Generally, safety profiles of drug candidates are gathered using normal animals on current pre-clinical safety assessment. This is considered to be one of the reasons why the numbers of discontinuation of drug development and the withdrawal of drugs from the market due to hepatotoxicity are still high.<sup>46</sup> Actually, concordance of toxicity between preclinical safety assessment and human toxicity is low.<sup>5</sup> Considering the increasing prevalence of hypertension, obesity, and fatty liver in the world, safety assessment of drug candidates using diseased animals may answer specific question with respect to safety aspects of certain drugs. In this study, we used steatotic mice to assess ELP-induced liver injury because clinical evidence showed that hypertensive patients have a significantly higher prevalence of fatty liver (30.9%) than healthy control subjects (12.7%).<sup>47</sup> Although our mouse model mimicking steatosis does not always emulate the human situation in every aspect, the established diseased animal model would allow us to better predict possible drug toxicity to humans.

In conclusion, we first established a mouse model of ELP-induced liver injury by pretreatment with DEX and BSO. It was demonstrated that oxidative stress and neutrophil infiltration are mainly involved in the pathogenesis of ELP-induced

hepatotoxicity. Our animal model indicates hepatic steatosis and oxidative stress are risk factors for ELP-induced liver injury, and the usefulness of using diseased mice in understanding the factors that contribute to DILI.

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### **Conflicts of interest**

None of the authors have any conflicts of interest related to this manuscript.

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## Figure legends

**Figure 1** Plasma ALT, AST and total bilirubin levels in ELP-induced liver injury.

Male BALB/c mice (8-week-old) were pretreated with DEX (60 mg/kg, i.p.) once daily for 3 days and BSO (700 mg/kg, i.p.) 1 h prior to ELP administration. Twenty-four hours after the final DEX treatment, the mice were administered ELP (1000 mg/kg, p.o.). (a and b) Time-dependent changes in plasma ALT and AST levels at 0, 3, 6 and 24 h after ELP administration with or without DEX and BSO treatment. (c) Plasma total bilirubin levels 6 h after ELP administration. (d) Plasma ALT levels 6 h after ELP administration with or without DEX and/or BSO treatment. (e) Plasma ALT levels 6 h after ELP or imidapril administration. Mice were administered with imidapril (1000 mg/kg, p.o.) under the same dosing regimen as that for ELP. The data are presented as the means  $\pm$  SEM (n = 4 to 5 mice per each group). The differences compared with the vehicle control group (a, b and c), the ELP group (d), or the DEX/BSO group (e) at each time point were considered significant at  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ .

**Figure 2** Histopathological examination of the livers in the ELP-induced liver injury model.

Liver tissues were collected 6 h after ELP or vehicle administration and stained with hematoxylin and eosin (H&E) (a) or immunostained with an anti-MPO antibody (b).

The number of MPO-positive cells was counted (c). The data are presented as the means  $\pm$  SEM (n = 4 to 5 mice per each group). The differences compared with the vehicle control group were considered significant at  $**P < 0.01$ ,  $***P < 0.001$ , and the differences between the DEX/BSO-treated group and DEX/BSO/ELP group were considered significant at  $\#P < 0.05$ .

**Figure 3** Hepatic mRNA expression levels of inflammatory-related factors (a), Th-related transcription factors (b), and an oxidative stress-related gene (c) 3 and 6 h after ELP administration. The relative mRNA expression levels were measured by real-time RT-PCR and normalized to that of GAPDH mRNA. The fold induction of mRNA levels is shown compared with the vehicle control group at each time point. The data are presented as the means  $\pm$  SEM (n = 4 to 5 mice per each group). The differences compared with the vehicle control group were considered significant at  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ .

**Figure 4** Hepatic GSH contents in ELP-induced liver injury.

Hepatic total GSH (a), GSSG (b), GSH (c) contents and GSH/GSSG ratio (d) 6 h after ELP administration. The data are presented as the means  $\pm$  SEM (n = 4 to 5 mice per each group). The differences compared with the vehicle control group were considered significant at  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ , and the differences between the DEX/BSO group and the DEX/BSO/ELP group were considered significant at  $\#P < 0.05$ . NS, not significant.

**Figure 5** Plasma hydrogen peroxide levels and hepatic MDA levels in ELP-induced liver injury.

(a) Plasma hydrogen peroxide levels 3 and 6 h after ELP administration. (b) Hepatic MDA levels 6 h after ELP administration. The data are presented as the means  $\pm$  SEM (n = 4 to 5 mice per each group). The differences compared with the vehicle control group at each time point were considered significant at  $*P < 0.05$ ,  $**P < 0.01$ , and  $***P < 0.001$ , and the differences between the DEX/BSO group and the DEX/BSO/ELP group were considered significant at  $\#\#P < 0.01$ .

**Figure 6** Effects of tempol treatment in ELP-induced liver injury.

Mice pretreated with DEX and BSO were treated with tempol (200 mg/kg, i.p.) and ELP (1000 mg/kg, p.o.) simultaneously. Plasma ALT levels (a) and hepatic HO-1 mRNA expression levels (b) 6 h after tempol and ELP administration. The relative expression levels were normalized to that of GAPDH mRNA. The fold induction of mRNA levels is shown compared with the DEX/BSO/ELP group. The data are presented as the means  $\pm$  SEM (n = 5 mice per each group). The differences compared with the DEX/BSO/ELP group were considered significant at  $**P < 0.01$ .

**Figure 7** Effects of neutrophil depletion on ELP-induced liver injury.

An anti-Gr-1 monoclonal antibody was intraperitoneally administered to mice 36 h prior to ELP administration. The numbers of neutrophils in blood (a) and plasma ALT levels (b) at 6 h after ELP administration. The data are presented as the means  $\pm$  SEM (n = 4-6 mice per each group). The differences compared with the DEX/BSO-treated group were considered significant at  $**P < 0.01$  and  $***P < 0.001$  and the differences between the DEX/BSO/ELP/Isotype IgG2b control-treated group and DEX/BSO/ELP/Anti-Gr-1-treated group were considered significant at  $\#P < 0.05$  and  $##P < 0.01$ .

**Table 1.** Primer sequences for real-time RT-PCR.

Gene	Sequence (5' to 3')
GATA3	F GGAGGACTTCCCCAAGAGCA
	R CATGCTGGAAGGGTGGTGA
HO-1	F GACACCTGAGGTCAAGCACA
	R ATCACCTGCAGCTCCTCAAA
IL-1 $\beta$	F GTTGACGGACCCCAAAGAT
	R CACACACCAGCAGGTTATCA
MIP-2	F AAGTTTGCCTTGACCCTGAAG
	R ATCAGGTACGATCCAGGCTTC
ROR $\gamma$ t	F AGCGCACCAACCTCTTTTC
	R CCCACATCTCCACATTGA
T-bet	F TGCCCGAACTACAGTCACGAAC
	R AGTGACCTCGCCTGGTCAAATG
TNF $\alpha$	F TGTCTCAGCCTCTTCTCATTCC
	R TGAGGGTCTGGGCCATAGAAC
GAPDH	F AAATGGGGTGAGGCCGGT
	R ATTGCTGACAATCTTGAGTGA

F: forward, R: reverse, GATA3: GATA binding protein 3, HO-1: heme oxygenase-1, IL-1 $\beta$ : interleukin-1 $\beta$ , MIP-2: macrophage inflammatory protein-2, ROR $\gamma$ t: retinoid-related orphan receptor  $\gamma$ t, T-bet: T-box expressed in T cells, TNF $\alpha$ : tumor necrosis factor  $\alpha$ , GAPDH: glyceraldehyde-3-phosphate dehydrogenase

Figure. 1

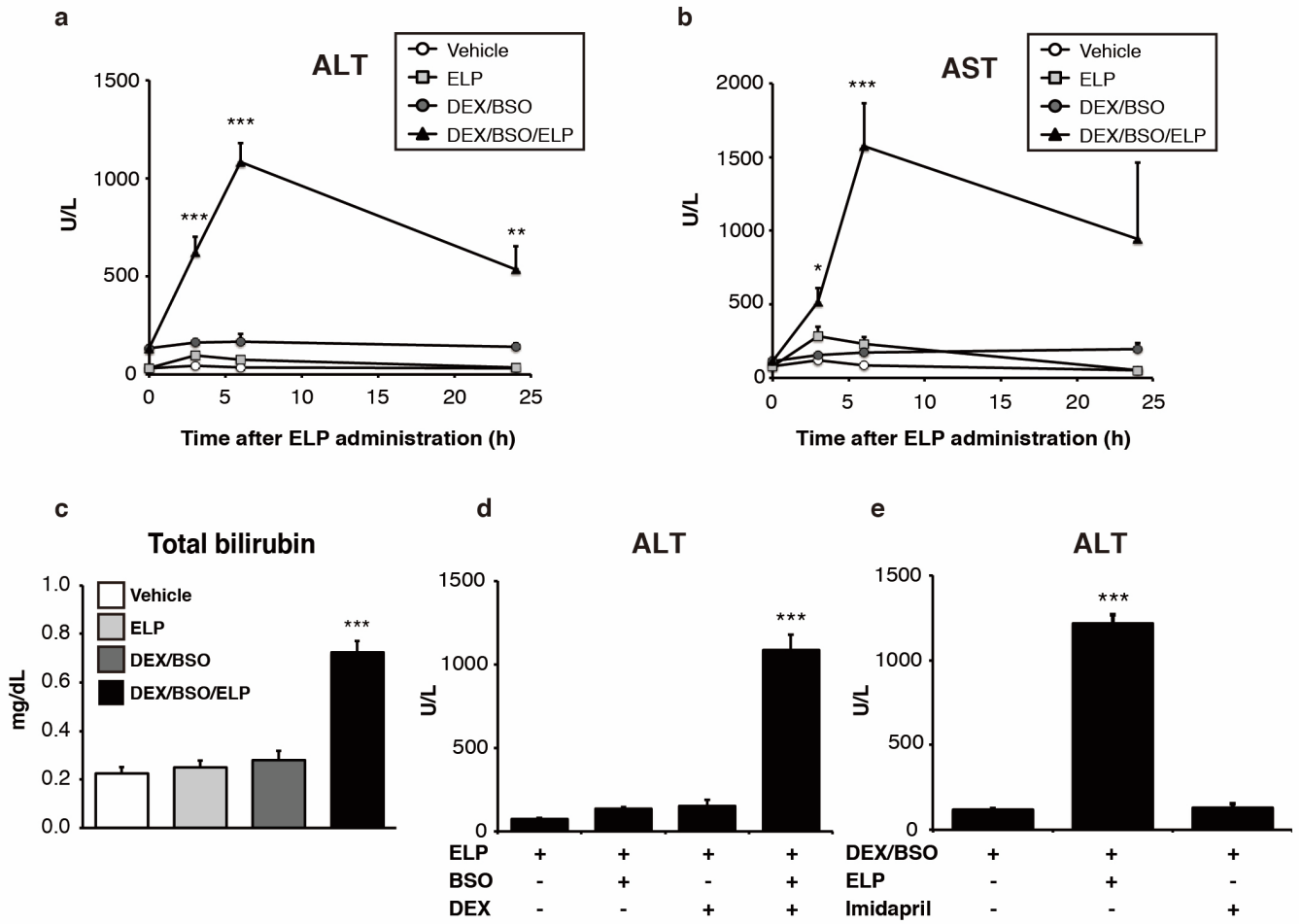


Figure. 2

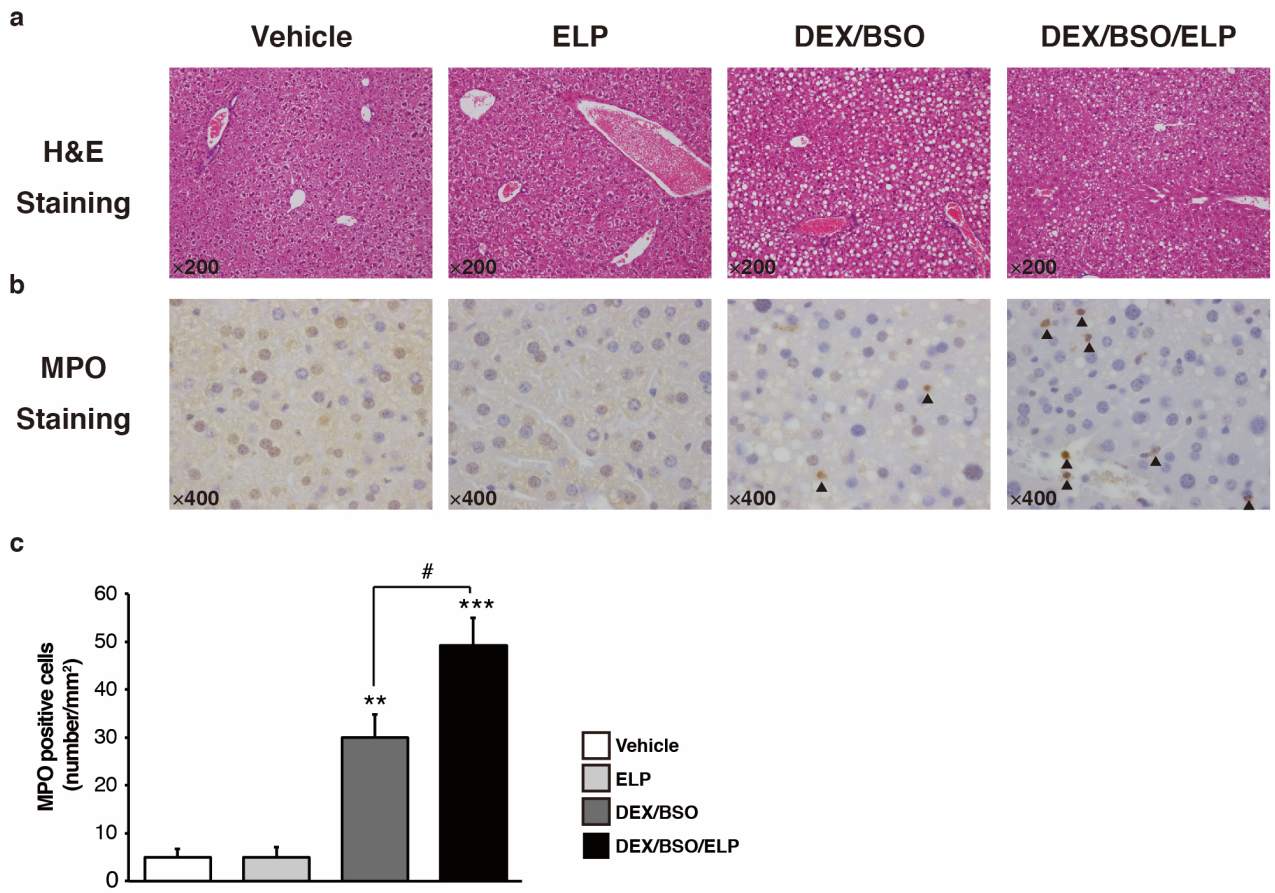


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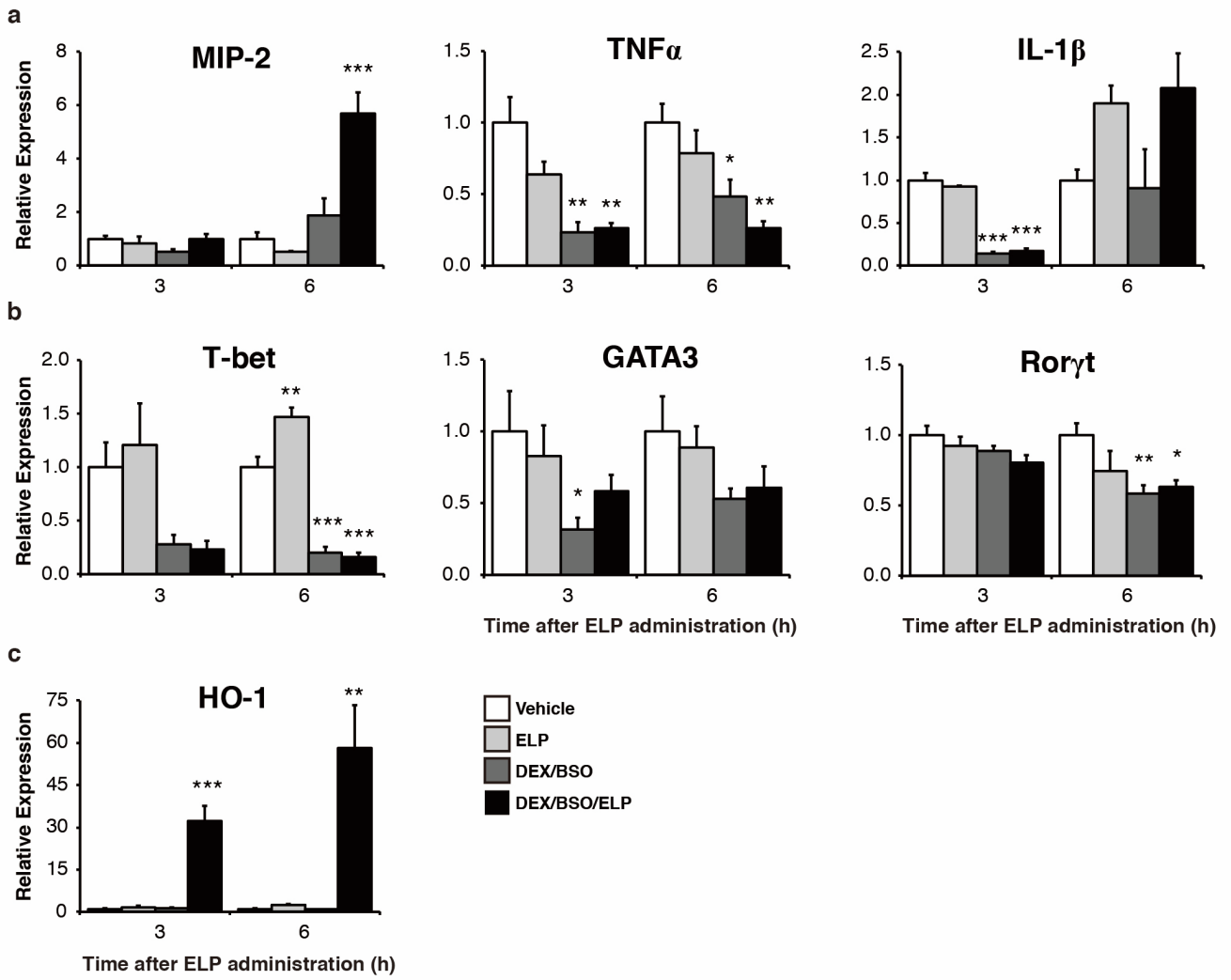




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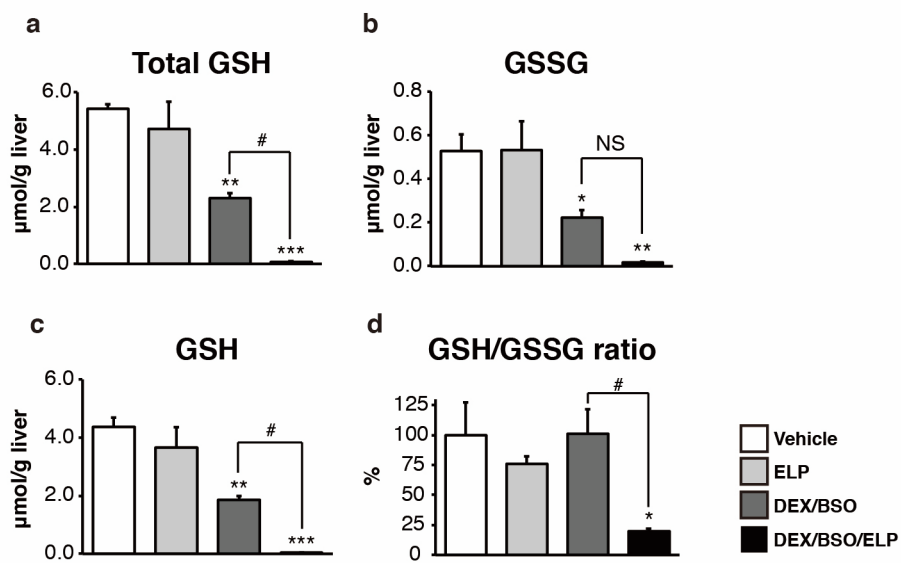


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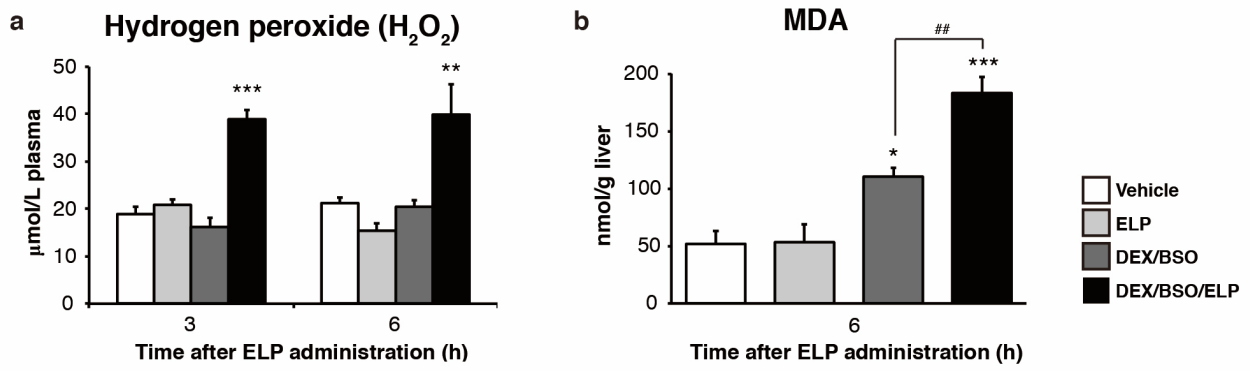


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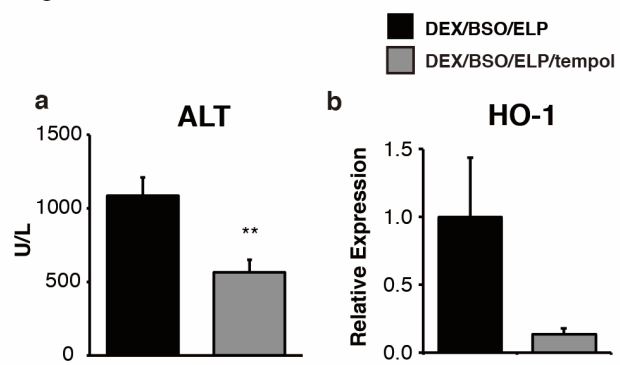
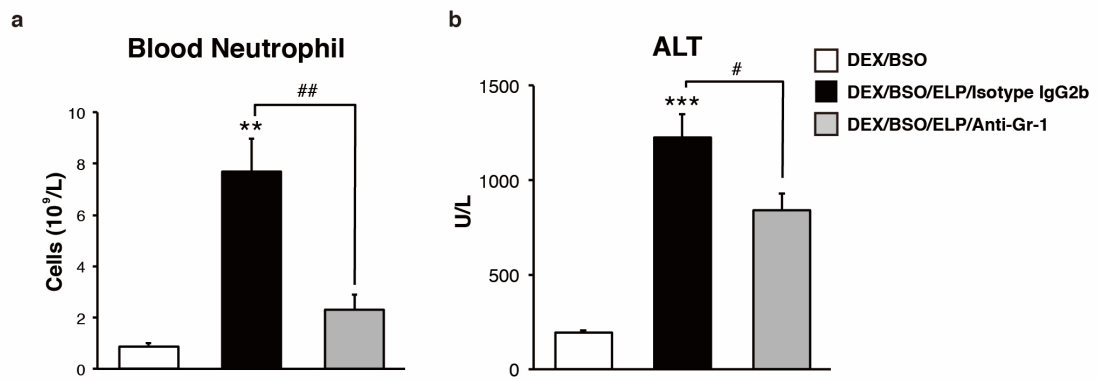


Figure. 7



## **Establishment of a mouse model of enalapril-induced liver injury and investigation of the pathogenesis**

Yuji Shirai, Shingo Oda, Sayaka Makino, Koichi Tsuneyama and Tsuyoshi Yokoi.

### **Supplementary Material and Methods**

#### **Immunoblot analysis.**

SDS-polyacrylamide gel electrophoresis and immunoblot analyses were performed according to the method described by Laemmli.<sup>1</sup> Liver microsomes were prepared according to our previous study.<sup>2</sup> The liver microsome was separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore Corporation, Billerica, MA). The membranes were probed with monoclonal goat anti-rat CYP3A2 antibody (Daiichi Pure Chemicals, Tokyo, Japan) or a mouse monoclonal anti- $\beta$ -actin antibody (Santa Cruz, CA), as well as a secondary corresponding fluorescent dye-conjugated antibody. Anti-rat CYP3A2 antibody has cross-reactivity with murine Cyp3a11 and Cyp3a13, which are the major Cyp3A enzymes in mouse liver.<sup>3</sup> An Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE) was used for detection. The relative expression level was quantified using Odyssey Application Software (version 3.0). CYP3A2 protein levels were normalized using the  $\beta$ -actin protein level.

#### **PCN, BSO and ELP administration.**

PCN, which is known to a CYP3A inducer, was suspended in corn oil (5 mg/ml) and intraperitoneally administered to male BALB/c mice (8-week-old) at a dose of 50 mg/kg/day for 4 days. BSO was intraperitoneally administered to the mice at a dose of 700 mg/kg 1 h prior to ELP administration. Twenty-four hours after the final PCN administration, ELP was orally administered to the mice at a dose of 1000 mg/kg. At 6 h after ELP administration, the

mice were sacrificed under isoflurane anesthesia, and the blood was collected from the inferior vena cava and the liver was excised from the left lateral lobe.

#### **Administration of enalaprilat in DEX/BSO-pretreated mice.**

Male BALB/c mice (8-week-old) were pretreated with DEX (60 mg/kg, i.p.) once daily for 3 days and BSO (700 mg/kg, i.p.) at 1 h prior to enalaprilat administration. Twenty-four hours after the final DEX treatment, the mice were administered enalaprilat (340 mg/kg, i.p.). Six hours after the enalaprilat administration, the mice were sacrificed. Since enalaprilat is poorly absorbed from gastrointestinal tract, we administered enalaprilat to the mice by intraperitoneal. We decided the dose of enalaprilat at 340 mg/kg to accomplish the same exposure level of enalaprilat with oral ELP (1000 mg/kg) administration, because 34% of the orally administered ELP was absorbed and then rapidly converted to enalaprilat by hepatic and intestinal enzymes in rats.<sup>4</sup>

### **Supplementary figure legends**

#### **Supplementary Figure 1** CYP3A protein levels in ELP-induced liver injury model.

The liver microsomes at 6 h after ELP administration were subjected to immunoblot analysis using anti-rat CYP3A2 antibody. The CYP3A (CYP3A11 and CYP3A13) protein expression levels were quantified and normalized to that of  $\beta$ -actin. Fold changes of CYP3A protein levels compared with the vehicle control group are shown. The data are presented as the means  $\pm$  SEM (n = 4 mice per each group). The differences compared with the vehicle control group were considered significant at  $**P < 0.01$ .

#### **Supplementary Figure 2** Effects of PCN pretreatment on ELP-induced liver injury and CYP3A protein levels.

Male BALB/c mice were pretreated with PCN (50 mg/kg, i.p.) once daily for 4 days and BSO (700 mg/kg, i.p.) at 1 h prior to ELP administration. Twenty-four hours after the final PCN treatment, the mice were administered ELP (1000 mg/kg, p.o.). At 6 h after ELP administration, the blood and the livers were collected to assess the plasma ALT levels and protein expression levels. (a) Plasma ALT levels at 6 h after ELP administration were measured. The data are presented as the means  $\pm$  SEM (n = 4 to 5 mice per each group). The differences compared with the DEX/BSO-treated group were considered significant at  $***P < 0.001$ . (b) The liver microsomes at 6 h after ELP administration were subjected to immunoblot analysis using anti-rat CYP3A2 antibody. The CYP3A protein expression levels were quantified and normalized to that of  $\beta$ -actin. Fold changes of CYP3A protein levels compared with the vehicle control group are shown. The data are presented as the means  $\pm$  SEM (n = 4 mice per each group). The differences compared with the control group were considered significant at  $*P < 0.05$ .

### **Supplementary Figure 3** Administration of enalaprilat in DEX/BSO-pretreated mice.

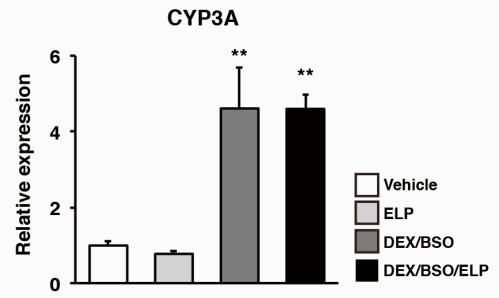
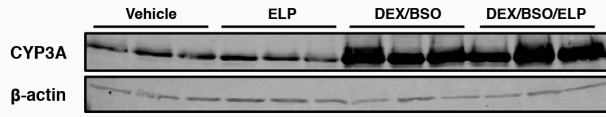
Male BALB/c mice were pretreated with DEX (60 mg/kg, i.p.) once daily for 3 days and BSO (700 mg/kg, i.p.) at 1 h prior to ELP or enalaprilat administration. Twenty-four hours after the final DEX treatment, the mice were administered ELP (1000 mg/kg, p.o.) or enalaprilat (340 mg/kg, i.p.). Plasma ALT levels at 6 h after ELP or enalaprilat administration were measured. The data are presented as the means  $\pm$  SEM (n = 4 to 5 mice per each group). N.S., not significant.

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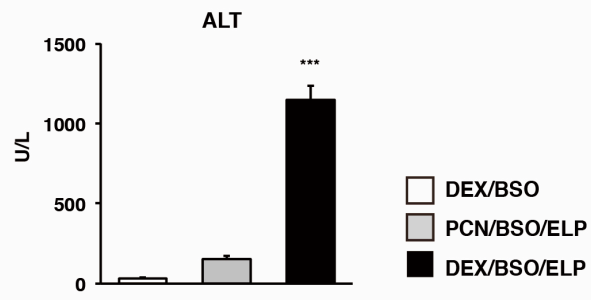


Supplemental Figure. 1

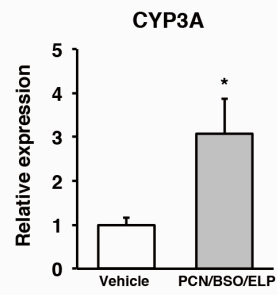
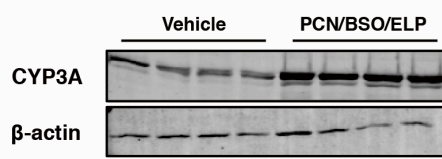


Supplemental Figure. 2

**a**



**b**



Supplemental Figure. 3

