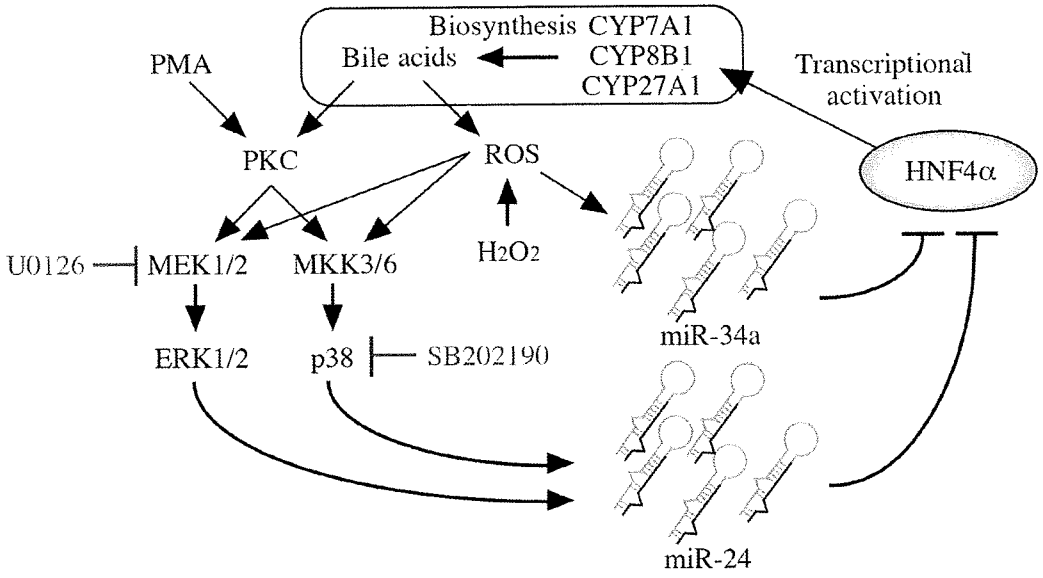

Fig. 13. **A proposal of the regulatory loop of miR-24, miR-34a and HNF4α in bile acid synthesis.** Bile acids are known to activate PKC and ROS generation, resulting in the activation of MAPK pathway. The miR-24 and miR-34a expression are induced by MAPK-dependent and -independent pathways, respectively. In turn, miR-24 and miR-34a negatively regulate the HNF4α. The down-regulation of HNF4α decreases the expression of bile acid-synthesizing enzymes CYP7A1 and CYP8B1 resulting in the decrease of bile acids.

E. 結論

miRNAs regulate the expression of target genes by binding to complementary regions of transcripts to repress their translation or mRNA degradation. The roles of miRNAs have received attention especially in the cancer field, but hardly yet in the field of pharmacokinetics. In the present study, the possibility of miRNA-dependent regulation of the nuclear receptors expression and its impact on the metabolism of endo/xenobiotics were investigated.

In the present study, it was demonstrated that the human HNF4α expression is regulated by miR-24 and miR-34a by mRNA degradation and translational repression, respectively. miRNA-dependent down-regulation of the HNF4α expression decreased its downstream genes involving in bile acid synthesis and gluconeogenesis. These miRNAs were found to be regulated by PKC/MAPK and/or ROS pathways, suggesting the presence of miRNA-mediated negative feedback regulation of bile acid synthesis. Since HNF4α has been known to control homeostasis in the liver, this regulatory

mechanism might contribute to the pathogenesis of the liver disease.

In conclusion, the post-transcriptional repression of the PXR and HNF4α expression by miRNAs was clarified. In this thesis, I could throw new insights into the molecular mechanism regulating the nuclear receptor expression and its effects on the metabolism of xeno/endobiotics.

G. 研究発表

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H. 知的財産権の出願・登録状況

該当無し

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III. 研究成果の刊行に関する一覧表

別紙４

研究成果の刊行に関する一覧表

雑誌

発表者氏名	論文タイトル名	発表誌名	巻号	ページ	出版年
Yukitaka Yoshikawa, Mayu Morita, Hiroko Hosomi, Koichi Tsuneyama, Tatsuki Fukami, Miki Nakajima and Tsuyoshi Yokoi	Knockdown of superoxide dismutase-2 enhances acetaminophen-induced hepatotoxicity in rat.	***Toxicology***	264	89-95	2009
Eisuke Kobayashi, Masanori Kobayashi, Koichi Tsuneyama, Tatsuki Fukami, Miki Nakajima and Tsuyoshi Yokoi	Halothane-induced liver injury is mediated by interleukin-17 in mice.	***Toxicological Sciences***	111	302-310	2009
Sayaka Komagata, Miki Nakajima, Shingo Takagi, Takuya Mohri and Tsuyoshi Yokoi	Human CYP24 catalyzing the inactivation of calcitriol is post-transcriptionally regulated by miR-125b.	***Molecular Pharmacology***	76	702-709	2009
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Shingo Takagi, Miki Nakajima, Katsuhiko Kida, Yu Yamaura, Tatsuki Fukami, and Tsuyoshi Yokoi	MicroRNAs regulate human hepatocytes nuclear factor 4α modulating the expression of metabolic enzymes and cell cycle.	***Journal of Biological Chemistry***	285	4415-4422	2010
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総説

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横井　毅	薬物動態と医薬品の薬効・副作用-代謝を中心として-	治療学	43	1262-1266	2009

著書

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VI. 研究成果の刊行物・別刷

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Knockdown of superoxide dismutase 2 enhances acetaminophen-induced hepatotoxicity in rat

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ABSTRACT

Drug-induced hepatotoxicity is a major problem in drug development, and oxidative stress is known as one of the causes. Superoxide dismutases (SODs) are important antioxidant enzymes against reactive oxygen species (ROS). Mitochondria are the major source of superoxide production, and SOD2 is mainly localized in mitochondria and, with other SODs, plays an important role in scavenging superoxide. Previously, we reported the establishment of an adenovirus vector with short hairpin RNA against rat SOD2 (AdSOD2-shRNA), and applied this to evaluate drug-induced cytotoxicity. In this study, infection of AdSOD2-shRNA to Fisher 344 rats resulted in a significant decrease of SOD2 mRNA, protein expression, and SOD2 enzyme activity to 28%, 35%, and 39%, respectively, 7 days after infection. Serum AST and ALT were significantly increased by single oral administration of acetaminophen (1000 mg/kg) to these SOD2-knockdown rats without fasting compared with the control adenovirus infected groups. Heme oxygenase-1 protein, known to be induced by oxidative stress, was detected in SOD2-knockdown rats administered acetaminophen. In addition, protein carbonyl and lipid peroxidation, also known to be induced by oxidative stress, were significantly increased in SOD2 knockdown rats. This is the first report of a SOD2-knockdown rat model that could be useful to evaluate the drug-induced hepatotoxicity with high sensitivity.

1. Introduction

Oxidative stress is one of the causes of drug-induced hepatotoxicity (Kaplowitz, 2005) and is known to be induced by superoxide, which is mainly generated in mitochondria. Superoxide is able to react with NO to form peroxynitrite or, after conversion to hydrogenperoxide, undergo metal-catalysed Fenton reactions to form highly reactive hydroxyl radicals (Boelsterli and Lim, 2006). Superoxide dismutases (SODs) are the first and most important line of antioxidant enzymes against reactive oxygen species (ROS) and, particularly, superoxide anion radicals (Zelko et al., 2002). At present, three distinct SOD isoforms, SOD1, SOD2, and SOD3, have been identified in mammals. SOD1, SOD2 and SOD3 are mainly localized to the cytoplasm, mitochondria and plasma, respectively (Zelko et al., 2002). In addition, SOD2 is known to be induced by a wide range of compounds, including anticancer drugs and lipopolysaccharide (Das et al., 1998; Visner et al., 1990). Meanwhile, the importance of the SOD2 function in mammalian organisms was confirmed by disruption of the SOD2 gene, which turns out to be lethal for mice due to neurodegeneration and damage to the heart (Lebovitz et al., 1996). These suggest that SOD2 plays an especially important role in the detoxification of ROS and superoxide anion radicals.

At present, a heterozygous *Sod2* knockout mouse, whose Sod2 activity is decreased by 55% in the liver, has been developed (Van Remmen et al., 1999). However, mouse is much smaller than rat, which is the most frequently used experimental animal for pharmacological and toxicological studies in the drug development process because of its body weight and ease of sampling blood or urine. A standard technique of gene knockout in rat has not been established yet. Recently, recombinant adenovirus methods are being developed and used for the purpose of gene delivery (Akai et al., 2007; Yoshikawa et al., 2009). Furthermore, a small interfering RNA strategy, which has been proven to be more specific and efficient than the full-length antisense cDNA strategy, has been established (Meister and Tuschl, 2004). In a previous study, we constructed a recombinant adenovirus expressing SOD2-short hairpin RNA (AdSOD2-shRNA) that could knockdown rat SOD2 mRNA efficiently (Yoshikawa et al., 2009). In that report, a cell model with SOD2-knockdown and CYP3A4-overexpression was constructed using AdSOD2-shRNA and AdCYP3A4, and SOD2 activity was decreased by 50% with high CYP3A4 activity 3 days after infection.

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Treatment with various drugs known to induce hepatotoxicity in this cell model demonstrated a decrease in the cell viability and an increase in the production of superoxide (Yoshikawa et al., 2009). In the present study, we established the SOD2-knockdown rat model to evaluate the drug-induced hepatotoxicity with high sensitivity.

2. Materials and methods

2.1. Materials

Acetaminophen (APAP) and glutathione (GSH) were obtained from Wako Pure Chemical Industries (Osaka, Japan). ReverTra Ace (Moloney Murine Leukemia Virus Reverse Transcriptase RNaseH Minus) was from Toyobo (Tokyo, Japan). The Adenovirus Expression Vector kit (Dual Version), RNAiso, random hexamer and SYBR Premix Ex Taq were obtained from Takara (Osaka, Japan). The QuickTiter Adenovirus Titer Immunoassay kit and protein carbonyl kit were obtained from Cell Biolabs (Tokyo, Japan). Lipofectamine 2000 was obtained from Invitrogen (Grand Island, NY). The GeneSilencer shRNA Vector kit was obtained from Gene Therapy Systems (San Diego, CA). Dulbecco's modified Eagle's medium was obtained from Nissui Pharmaceutical (Tokyo, Japan). All primers and oligonucleotides for shRNA were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Lipid peroxidation measurement kit was obtained from BIOMOL (Philadelphia, USA). Other chemicals were of analytical or the highest grade commercially available.

2.2. Animals

Male Fisher 344 rats (7 weeks old, 130–150 g) were obtained from SLC Japan (Hamamatsu, Japan). Animals were housed in a controlled environment (temperature 25 ± 1 °C, humidity 50 ± 10%, and 12 h light/12 h dark cycle) in the institutional animal facility with access to food and water *ad libitum*. Animals were acclimatized for a week before use for the experiments. Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Welfare of Japan, as approved by the Institutional Animal Care and Use Committee of Kanazawa University, Japan.

2.3. Design of short hairpin RNA

Rat SOD2 (Gene Bank™, accession code NM_017051 Gene bank) knockdown was achieved by RNA interference using the adenovirus vector-based short hairpin RNA (shRNA) approach as previously described (Yoshikawa et al., 2009). As a negative control, the oligonucleotide sequences of the shRNA target for luciferase from a GeneSilencer shRNA Vector kit were used.

2.4. Recombinant adenovirus

We generated the recombinant adenovirus vector expressing SOD2-shRNA (AdSOD2-shRNA) as previously described (Yoshikawa et al., 2009). In brief, pGSU6-GFP plasmids were recombined into the pAxcwit using the cosmid-terminal protein complex method according to the manufacturer's instructions. The recombinant adenovirus was isolated and propagated into the 293 cells. Then, an adenovirus containing shRNA of SOD2 was constructed. As a negative control, the oligonucleotide sequences of the shRNA target for luciferase from a GeneSilencer shRNA Vector kit were used (AdLuc-shRNA). The titer was determined by a QuickTiter Adenovirus Titer Immunoassay kit. The titers of AdSOD2-shRNA and AdLuc-shRNA were 6.0×10^9 pfu/mL and 1.0×10^{10} pfu/mL, respectively.

2.5. Real-time reverse transcription (RT)-PCR analysis

RNA from the hepatic cells was isolated using RNAiso. Rat SOD2 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time RT-PCR. The primer sequences used in this study were as follows: rat SOD2, 5′-GAGGCTATCAAGCGTGACTTTGG-3′ and 5′-AAGCGTGCTCCCACACATCAATC-3′; rat GAPDH, 5′-GTTACCAGGGCTGCCTTCTC-3′ and 5′-GGGTTTCCCGTTGATGACC-3′. For the reverse transcription process, total RNA (2 μg) and 150 ng of random hexamer were mixed and incubated at 70 °C for 10 min. RNA solution was added to a reaction mixture containing 100 units of ReverTra Ace, reaction buffer, and 0.5 mM dNTPs in a final volume of 40 μL. The reaction mixture was incubated at 30 °C for 10 min, 42 °C for 1 h, and heated at 98 °C for 10 min to inactivate the enzyme. The real-time RT-PCR was performed using the Smart Cycler (Cepheid, Sunnyvale, CA). The PCR mixture contained 1 μL of template cDNA, SYBR Premix Ex Taq solution, and 10 pmol of sense and antisense primers. The PCR condition for GAPDH and SOD2 were as follows: after an initial denaturation at 95 °C for 30 s, the amplification was performed by denaturation at 94 °C for 4 s, annealing and extension at 64 °C for 20 s for 45 cycles. Amplified products were monitored directly by measuring the increase of the dye intensity of the SYBR Green I (Molecular Probes, Eugene, OR) that binds to double strand DNA amplified by PCR.

2.6. Western blot analysis

In the SOD2, HO-1 or sulfotransferase (SULT) 1 protein measurement, 10 μg of denatured mitochondrial (SOD2) or cytosol (HO-1 and SULT1) protein were loaded per lane, separated on 15% (SOD2 and HO-1) or 7.5% (SULT1) SDS-polyacrylamide gels and transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilon-P; Millipore). These specific proteins were detected by rabbit anti-human SOD2 polyclonal antibody, cross-reacting to rat SOD2 (sc-30080; Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-human HO-1 polyclonal antibody, cross-reacting to rat HO-1 (sc-1796; Santa Cruz Biotechnology), and rabbit anti-human SULT1 polyclonal antibody, cross-reacting to rat SULT1 (sc-32928; Santa Cruz Biotechnology, Santa Cruz, CA) at a dilution of 1:200. The protein bands were developed by biotinylated second antibody-peroxidase reaction. The quantitative analysis of protein expression was performed using ImageQuant TL Image Analysis software (Amersham Biosciences).

2.7. SOD2 activity

The enzyme activity of SOD2 was measured in 1 μg of protein using a kit (Cayman Chemical, MI, USA). The method utilizes tetrazolium salt to quantify superoxide radicals generated by xanthine oxidase and hypoxanthine. The standard curve was generated using a quality controlled SOD standard included with the kit. SOD2 activity was determined by performing the assay in the presence of potassium cyanide to inhibit SOD1, and thus measuring the residual SOD2 activity.

2.8. GSH level

Livers (100 mg) were homogenized with ice-cold 5% sulfosalicylic acid and centrifuged at 8000 × *g* at 4 °C for 10 min. The GSH concentration in the supernatant was measured as described previously (Tietze, 1969).

2.9. Adenovirus infection and APAP administration in rats

Seven days after single intravenous injection of AdSOD2-shRNA or AdLuc-shRNA at 2.0×10^{11} pfu/body, the rats were orally administered APAP suspended in 0.5% carboxymethylcellulose (0, 300, 1000 mg/kg body weight). Blood samples were collected at 0, 1, 2, 3, and 12 h after the APAP treatment. Twenty-four hours after the administration of APAP, serum samples were collected for assessment of the transaminase levels and for APAP metabolite analysis. The liver was fixed in buffered neutral 10% formalin. The fixed samples were embedded in paraffin and sectioned at a thickness of 2 μm and stained with hematoxylin-eosin for microscopic examination. Rat liver cytosol and microsomes were prepared as described previously (Tabata et al., 2004). In all experiments, the rats were not treated by fasting prior to the APAP treatment or sacrifice.

2.10. Determination of plasma concentrations of APAP and its metabolites

The plasma concentrations of APAP and its metabolites were measured using high performance liquid chromatography (HPLC) as follows. Plasma (50 μL) was mixed with an equivalent aliquot of acetonitrile containing 200 pmol *m*-aminophenol as an internal standard. After extraction and centrifugation, the resulting supernatant was evaporated under nitrogen. The residue was diluted with the mobile phase (2% methanol–50 mM sodium acetate) before being injected into HPLC. APAP and its metabolites, APAP-glucuronide, APAP-sulfate, and APAP-mercapturate, were separated in a Mightysil RP-18 column (4.6 × 150 mm; 5 μm; Kanto Chemical, Tokyo, Japan). The retention times of APAP, APAP-glucuronide, APAP-sulfate, APAP-mercapturate, and *m*-aminophenol were 7.60, 2.75, 5.00, 10.50, and 4.20 min, respectively. APAP and the metabolites, eluted with the mobile phase at a flow rate of 1.0 mL/min, were monitored at 248 nm.

2.11. Protein carbonyl measurements

The protein carbonyl was measured in 1 μg of protein using a kit. In brief, protein samples were added to the 96 well Protein Binding Plate, and incubated at 37 °C for 3 h. After rinsed with PBS, dinitrophenylhydrorazine (DNPH) working solution was added, and incubated for 45 min at room temperature in the dark. After rinsed with PBS/ethanol (1:1, v/v), blocking solution was added, and incubated for 1 h. After rinsed with wash buffer, anti-DNP antibody was added, and incubated for 1 h. After rinsed with wash buffer, secondary antibody was added, and incubated for 1 h. After rinsed with wash buffer, substrate buffer was added, and incubated for 10 min, and then absorbance at 405 nm was measured.

2.12. Lipid peroxidation measurement

The lipid peroxidation was measured in 10 mg of protein using a kit. In brief, for each reaction, 10 μL of probucol and 640 μL of diluted R1 reagent (1:3 of methanol:N-methyl-2-phenylindole) were added, and mixed with 150 μL of 12 M HCl. Each reaction was incubated at 45 °C for 60 min and centrifuged at 10,000 × *g* for 10 min. The supernatant was taken and used to measure malondialdehyde (MDA) formation at 586 nm. MDA data were normalized versus the protein concentration.

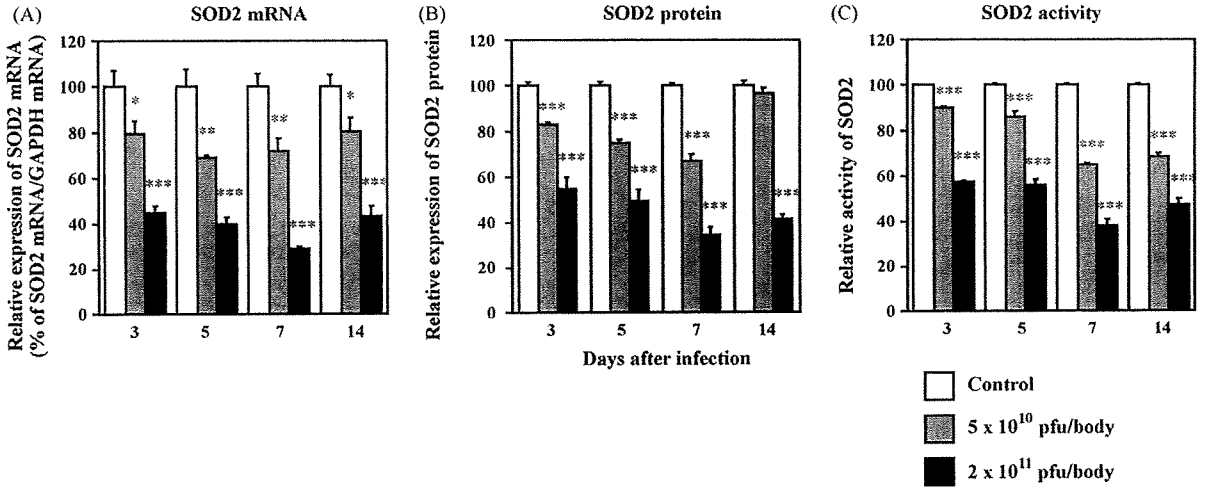

Fig. 1. Effects of adenovirus infection on hepatic SOD2 mRNA (A), protein (B), and activity (C). SOD2 mRNA (A), protein (B), and enzyme activity (C) were determined in rats infected with AdLuc-shRNA (control, 2.0×10^{11} pfu/body or AdSOD2-shRNA (5.0×10^{10} pfu/body and 2.0×10^{11} pfu/body). SOD2 protein was quantified by immunoblotting as described in Section 2. The control SOD2 enzyme activity was 12.85 ± 0.06 (U/mg protein). Data are mean ± SD ($n = 3$). $^*P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with AdLuc-shRNA infected rats.

2.13. Statistical analysis

Statistical analyses were performed with a GraphPad Instat version 2.0 computer program (GraphPad Software, San Diego, CA) by Student *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. Effects of adenovirus infection on hepatic SOD2

To investigate the most efficient condition of AdSOD2-shRNA infection, Fisher-344 rats were infected with AdSOD2-shRNA at 5.0×10^{10} or 2.0×10^{11} pfu/body for 3, 5, 7, and 14 days (Fig. 1). SOD2 mRNA, protein expression, and enzyme activity began to decrease 3 days after infection, and 72%, 65%, and 61% decreases were achieved 7 days after infection in the 2.0×10^{11} pfu/body infected group, respectively. Fourteen days after infection of AdSOD2-shRNA, SOD2 mRNA, protein expression, and enzyme activity had recovered a little. These results suggested that 7 days infection would be the most efficient condition for knocking down SOD2. In addition, serum AST and ALT were not increased in any group (Supplementary Fig. 1). These results suggested that 7 days infection at 2.0×10^{11} pfu/body would be an appropriate condition for AdSOD2-shRNA infection.

3.2. Hepatotoxic effect of APAP in adenovirus-infected rats

To determine whether APAP-induced hepatotoxicity was potentiated by the suppression of hepatic SOD2, rats were tail vein-injected once with 2.0×10^{11} pfu/body AdSOD2-shRNA or AdLuc-shRNA. After 7 days, APAP was orally administered without previous fasting treatment. The serum AST and ALT levels are shown in Fig. 2A. Twenty-four hours after APAP administration, the 300 mg/kg treated groups did not demonstrate hepatotoxicity. In contrast, the AdSOD2-shRNA infected rats treated with 1000 mg/kg APAP demonstrated a significant increase of AST (114.25 ± 40.91 U/L) and ALT (51.79 ± 16.11 U/L) compared with AdLuc-shRNA infected rats. The AdLuc-shRNA rats administered 1000 mg/kg APAP did not demonstrate hepatotoxicity. The results of the histological examination in 1000 mg/kg APAP-administered rats are shown in Fig. 2B. Remarkable hepatic necrosis, especially around the central vein, and lymphocyte infiltrations were observed in AdSOD2-shRNA infected rats given 1000 mg/kg APAP, consistent with the elevation of AST and ALT. There were no histological changes in the other groups.

3.3. Changes of the plasma concentrations of APAP and its metabolites in adenovirus infected rats

Changes in the plasma concentration of APAP and its metabolites are shown in Fig. 3. For APAP and APAP-sulfate, the maximum plasma concentration was observed 1 h after APAP administration. In the AdSOD2-shRNA infected group, the time to peak concentration of APAP-glucuronide was earlier than that of the AdLuc-shRNA infected group. On the other hand, APAP-sulfate, a major detoxification product in rats generated directly from APAP, was decreased. For APAP-mercapturate, the maximum plasma concentration was observed 3 h after APAP administration in rats infected with AdSOD2-shRNA, and was significantly decreased compared with the AdLuc-shRNA infected group.

3.4. Change of expression of HO-1 protein and GSH concentration in APAP-administered rats infected with AdSOD2-shRNA

To investigate the mechanism of APAP-induced hepatotoxicity in AdSOD2-shRNA infected rats, HO-1 protein, known to be induced by oxidative stress, was measured. Superoxide itself is not able to be measured *in vivo*, thus HO-1 protein was measured as an alternative method to evaluate APAP-induced oxidative stress. The HO-1 protein band was observed only in AdSOD2-shRNA infected rats given 1000 mg/kg APAP (Fig. 4A), suggesting that APAP-induced hepatotoxicity was caused by oxidative stress. The GSH concentrations in AdSOD2-shRNA infected rats were significantly increased compared with the AdLuc-shRNA infected control rats (Fig. 4B). In contrast, the GSH concentrations in AdSOD2-shRNA infected rats given 1000 mg/kg APAP were significantly decreased compared with the AdLuc-shRNA infected rats (Fig. 4B).

3.5. Change of protein carbonyl and lipid peroxidation in APAP-administered rats infected with AdSOD2-shRNA

HO-1 was known to be induced by oxidative stress, but also induced by hypoxia. Thus, we measured protein carbonyl and lipid peroxidation in mitochondria and serum to reveal that APAP-induced hepatotoxicity in SOD2 knockdown rats was caused by

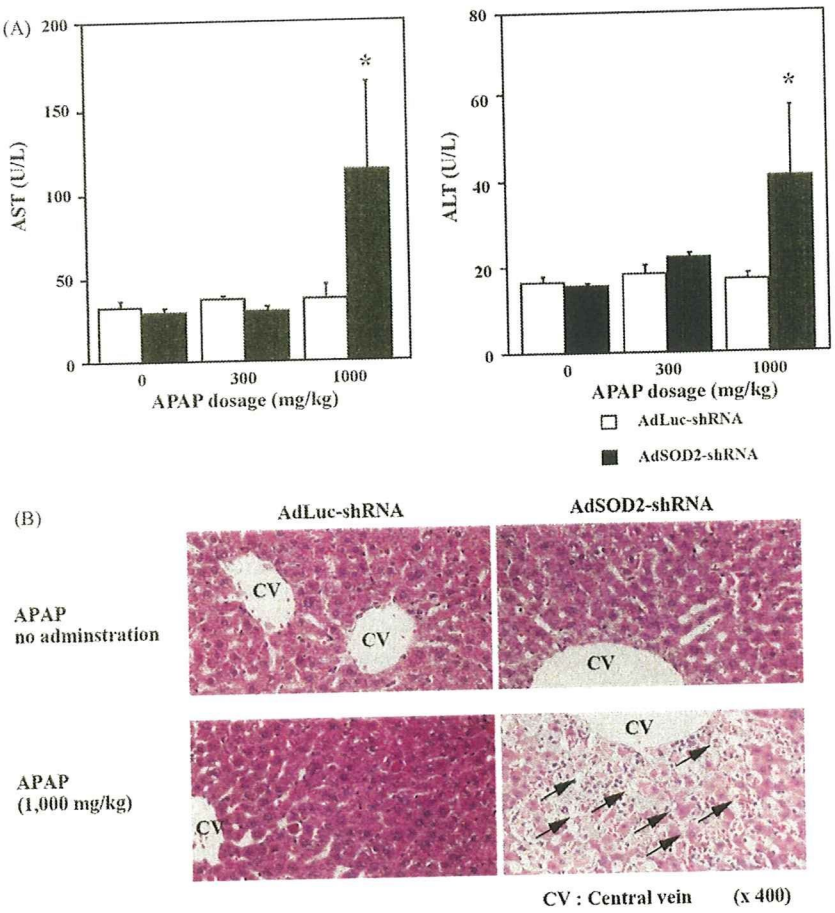

Fig. 2. Hepatotoxic effect of APAP in adenovirus-infected rats. APAP was orally administered without previous fasting. After 24 h, serum AST and ALT were measured (A). Hematoxylin-eosin staining was performed in sections of rat liver. Hepatic necrosis and lymphocyte infiltrations indicated by arrows were observed only in AdSOD2-shRNA infected rats given 1000 mg/kg APAP (B). Data are mean ± SD ($n = 5$). *$P < 0.05$ compared with AdLuc-shRNA infected rats.

oxidative stress more clearly. In both mitochondria and serum, protein carbonyl and lipid peroxidation concentrations in AdSOD2-shRNA infected rats given 1000 mg/kg APAP were significantly increased compared with the AdLuc-shRNA infected rats (Fig. 5), indicating that APAP-induced hepatotoxicity was caused by oxidative stress.

4. Discussion

In our previous study, a cell model with SOD2-knockdown and CYP3A4-overexpression was constructed using an adenovirus expressing SOD2-short hairpin RNA (AdSOD2-shRNA) and AdCYP3A4, which decreased SOD2 activity by 50% 3 days after infection with high CYP3A4 activity (Yoshikawa et al., 2009). Treatment with various drugs known to induce hepatotoxicity to this cell model demonstrated decreases in the cell viability and increases in the production of superoxide. In the present study, the SOD2 knockdown rat model was established using AdSOD2-shRNA, which decreased the SOD2 activity by 60% 7 days after infection (Fig. 1). In addition, acetaminophen (APAP)-induced hepatotoxicity was demonstrated in AdSOD2-shRNA infected rats (Fig. 2).

We successfully produced a SOD2-knockdown rat model by means of adenovirus-mediated RNA interference technology in order to detect drug-induced hepatotoxicity with high sensitivity. A previous report described that over 2.0×10^{11} pfu/body infection of an adenovirus caused adenovirus derived hepatotoxicity (Akai et al., 2007). There were no differences in AST and ALT between PBS-injected rats (AST: 31.98 ± 3.12, ALT: 16.20 ± 1.12) and adenovirus-infected rats. Therefore, we infected AdLuc-shRNA or AdSOD2-shRNA at 2.0×10^{11} pfu/body, which resulted in no hepatotoxicity. In AdSOD2-shRNA infected rats, SOD2 activity was decreased by 60% in rat liver. This decrease in the level of SOD2 is a little greater than that in heterozygous *Sod2* knockout mice (55% decrease). Therefore, we determined that a single injection of AdSOD2-shRNA (2.0×10^{11} pfu/body) was the proper condition for testing the drug-induced hepatotoxicity in this study.

In previous APAP-induced hepatotoxicity studies, in most cases rats were fasted for a half or 1 day before drug administration (Merrick et al., 2006; Kim et al., 2006; Pessayre et al., 1980). In the present study, in order to clarify the involvement of SOD2 knockdown, the rats were not fasted throughout the experiment. Previous reports demonstrated that fasting caused an approximately 50% decrease of GSH in liver. Moreover, the CYP2E1 activity is significantly increased (8- to 9-fold) by fasting (Hu et al., 1995; Jaeschke and Wendel, 1985). APAP is metabolized to *N*-acetyl-*p*-benzo-quinone imine, mainly by CYP2E1, and thus fasting would cause an overestimation of APAP-induced hepatotoxicity. As an *in vivo* hepatotoxicity screening system, a single oral administration dose of APAP at 300 and 1000 mg/kg to normal rats in a fasting condition was reported by a Pfizer group (Kikkawa et al., 2006), and there was no increase of ALT and AST at 300 mg/kg at 24 h after p.o. administration, although there was a potent increase of ALT (209.0 ± 252.3) and AST (573.3 ± 490.1) at 1000 mg/kg in normal rats. The same single oral administration of APAP (1000 mg/kg) in a

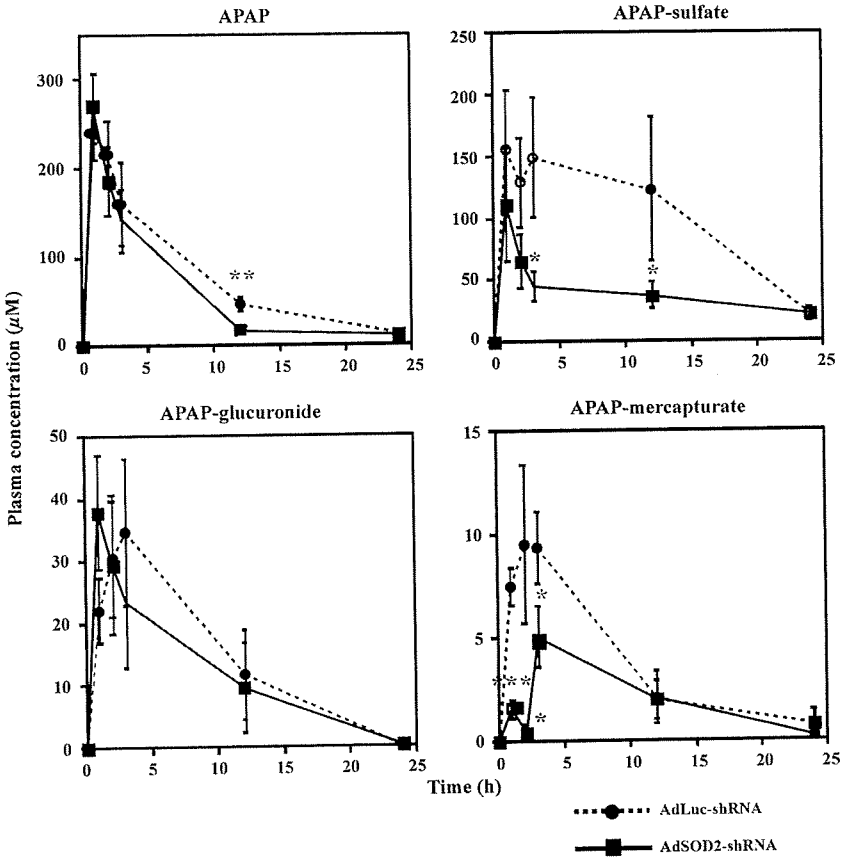

Fig. 3. Changes of the plasma concentrations of APAP and its metabolites in rats infected with the adenovirus. Rats were administered APAP (1000 mg/kg, p.o.). Data are mean ± SD ($n = 5$). $^{*}P < 0.05$, $^{**}P < 0.01$, and $^{***}P < 0.001$ compared with AdLuc-shRNA infected rats given 1000 mg/kg APAP.

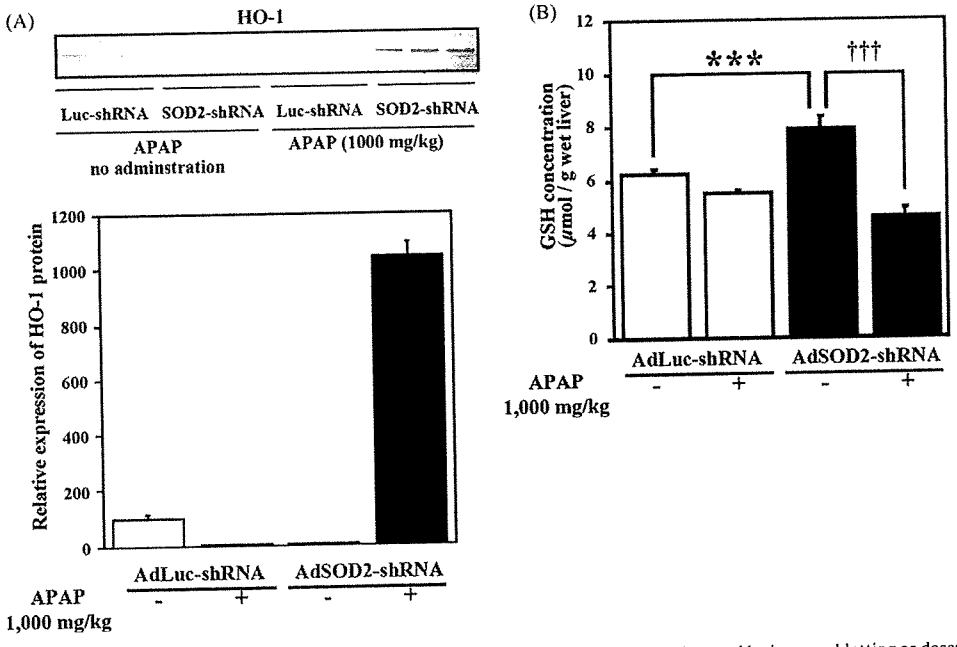

Fig. 4. Changes of expression of hepatic HO-1 protein (A) and glutathione concentration (B). HO-1 protein was detected by immunoblotting as described in Section 2 (A). In the GSH concentration measurement, rat liver was excised at 24 h after administration of saline or APAP (B). $^{***}P < 0.001$ compared with AdLuc-shRNA infected rats given saline, and $^{\dagger\dagger\dagger}P < 0.001$ compared with AdSOD2-shRNA infected rats given saline.

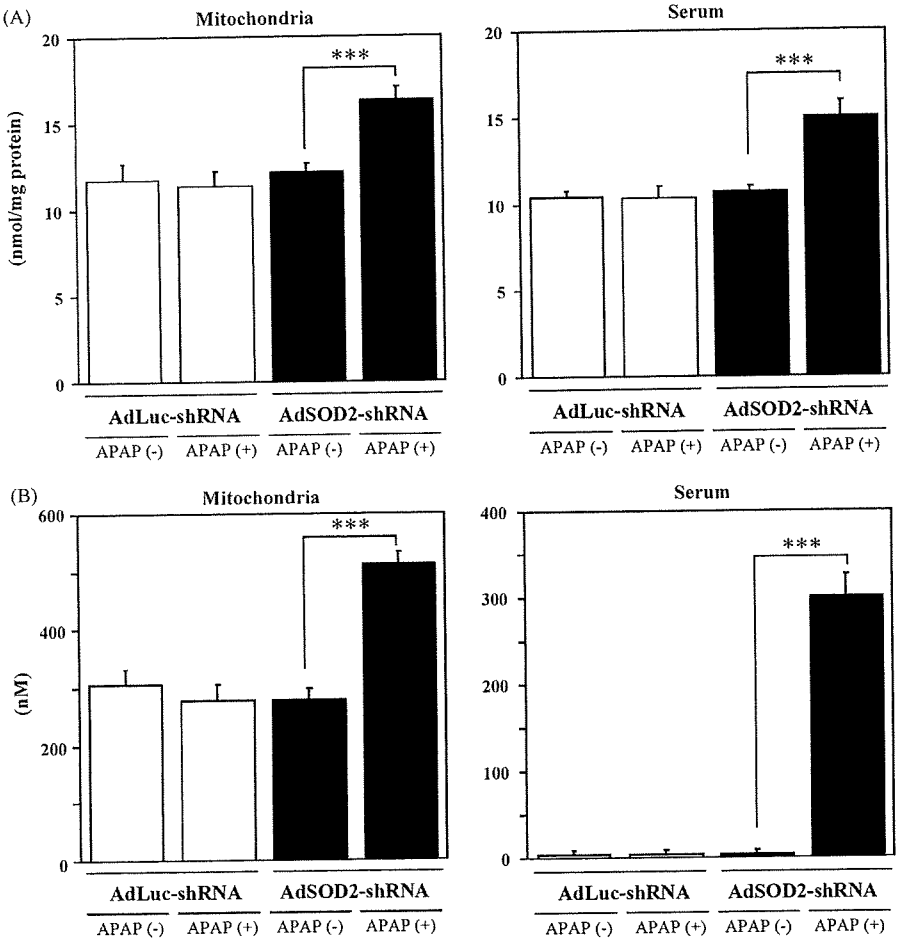

Fig. 5. Changes of protein carbonyl (A) and lipid peroxidation (B). Rats were administered APAP (1000 mg/kg, p.o.). Protein carbonyl (A) and lipid peroxidation (B) in mitochondria and serum were measured 24 h after administration of saline or APAP. Data are mean ± SD ($n = 3$). ***$P < 0.001$ compared with AdLuc-shRNA infected rats given 1000 mg/kg APAP.

fasting condition was also adopted by the National Toxicogenomics project in Japan as a screening system, and a significant increase of ALT and AST was shown at 24 h after treatment (Morishita et al., 2006). Based on these lines of considerations, we performed the experiments without fasting treatment.

Infection of AdSOD2-shRNA caused a significant decrease of APAP-sulfate in the rat plasma compared with AdLuc-shRNA infected control rats (Fig. 3), suggesting that SOD2 knockdown caused a suppression of the sulfotransferase (SULT) activity. In SOD2 knockdown rat, SULT1 expression was significantly decreased (Supplementary Fig. 2), thus the concentration of APAP-sulfate was decreased. In our previous report using adenovirus vector with short hairpin RNA against rat γ-glutamylcysteine synthetase, the concentration of APAP-sulfate was also decreased (Akai et al., 2007), thus adenovirus-mediated suppression in SULT1 protein might be occurred. However, the mechanism remains unknown. Furthermore, infection of AdSOD2-shRNA caused a significant decrease of APAP-mercapturate in the plasma. The GSH concentration was increased in AdSOD2-shRNA infected rats (Fig. 4), thus the plasma concentration of APAP-mercapturate should be increased, however, the opposite result was obtained, suggesting that the other metabolites of APAP resulted in compensatory increases, however, it was difficult to identify the whole metabolites. This result also remains to be clarified.

In the present study, APAP-induced hepatotoxicity was caused by knocking down SOD2 in rats. The mechanism of APAP-induced hepatotoxicity is very complex, and many reports have been published (McConnachie et al., 2007; Ganey et al., 2007; Gunawan et al., 2006; Lei et al., 2006). In one of these papers, APAP-induced hepatotoxicity was reported not to be occurred in *Sod1* knockout mice (Lei et al., 2006). In our study, there was no difference in SOD1 expression between AdLuc-shRNA and AdSOD2-shRNA infected group (data not shown), suggesting that SOD2 knockdown was not affected on SOD1 expression. Thus, SOD1 might be not involved in APAP-induced hepatotoxicity in SOD2 knockdown rat. Actually, it is difficult to evaluate APAP-induced hepatotoxicity only by SOD2 knockdown, as many factors are thought to be involved in the toxicity. However, it became clear that SOD2 is partly involved in APAP-induced hepatotoxicity *in vivo* in rat. In the near future, by using AdSOD2-shRNA infected rats, we will evaluate the hepatotoxicity of other drugs that induced cytotoxicity in SOD2 knockdown- and CYP3A4 overexpressing-cells in our previous report (Yoshikawa et al., 2009).

In conclusion, we firstly established SOD2-knockdown rats. This rat model could be useful as a highly sensitive drug-induced hepatotoxicity test for drug candidates in preclinical drug development.

Conflict of interest

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

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tox.2009.07.017.

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Halothane-Induced Liver Injury is Mediated by Interleukin-17 in Mice

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Drug-induced liver injury is a major problem in drug development and clinical drug therapy. In most cases the mechanisms are still unknown, thus, it is difficult to predict or prevent these reactions. It has been known that halothane, an inhaled anesthetic, induces liver injury. To investigate the mechanisms of halothane-induced liver injury, we used a recently established mouse model of liver injury. The expression of transcription factors and cytokines specific for Th1 and Th2 (helper T cells), respectively, were compared between BALB/c and C57BL/6 mice. The mRNA expression ratios of mouse T-bet(a Th1-specific transcription factor)/GATA-binding protein (GATA-3, a Th2-specific transcription factor) and interferon γ/interleukin (IL)-10 were lower in BALB/c mice compared with C57BL/6 mice, suggesting that a typical Th1 or Th2-dominant response could not be distinguished in halothane-induced liver injury. We observed increases of the plasma IL-17 level and hepatic macrophage inflammatory protein 2 expression in halothane-administrated BALB/c mice, as well as neutrophil infiltration. Neutralization of IL-17 suppressed the hepatotoxic effect of halothane. Administration of recombinant IL-17 (1 μg per mouse, single ip) to the halothane-treated mice resulted in a remarkable increase of alanine and aspartate aminotransferases. In conclusion, we demonstrated that IL-17 is involved in the halothane-induced liver injury.

Key Words: cytokine; MIP-2; neutrophils; helper T cells; prostaglandin E_1.

Drug-induced liver injury is the most frequent reason for the withdrawal of an approved drug from the market and for failures in drug development in pharmaceutical companies. Because of significant adverse drug reactions associated with hepatotoxicity, several drugs have been removed from the pharmaceutical market, including bromfenac, ebrotidine, and troglitazone (Holt and Ju, 2006). In most cases, the mechanisms of the hepatotoxicity are unknown and predictive experimental animal models are lacking.

Halothane, an inhaled anesthetic, causes asymptomatic increases of plasma transaminases in approximately 20% of patients and life-threatening fulminant hepatitis is induced in a small percentage of patients (Ray and Drummond, 1991). Halothane is metabolized to trifluoroacetyl radicals by cytochrome P450 2E1 and covalent binding to target macromolecules (Eliasson *et al.*, 1998). Although trifluoroacetylated-protein in the liver was believed to cause the liver injury, interstrain, and intrastrain differences in metabolism were not correlated with susceptibility in guinea pigs, suggesting that the inherent ability of metabolizing halothane cannot completely account for the mechanism of halothane-induced liver injury (Farrell *et al.*, 1996; Lind *et al.*, 1989).

Recently, a new animal model of halothane-induced liver injury was established which demonstrated the existence of strain differences between BALB/c and C57BL/6 mice (You *et al.*, 2006). However, no significant difference in either the patterns or levels of hepatic trifluoroacetylated-protein adducts formed in these two strains was observed.

Helper T cells (Th cells) are an important regulator of acquired immunity. Th cells are subdivided into Th1, Th2, and Th17 subsets by their unique production of cytokines and characteristic transcription factors (Kidd, 2003; Zhu and Paul, 2008). Th1 and Th2 direct different immune response pathway (Kidd, 2003). Th1 responses drive cellular immunity to kill intracellular pathogens and overactivation of Th1 cells induces some autoimmune diseases. On the other hand, Th2 responses drive humoral immunity and cause allergic inflammatory diseases including asthma (Zhu and Paul, 2008). C57BL/6 and BALB/c mice develop predominantly Th1 and Th2 responses, respectively (Knight *et al.*, 2007; Mizuhara *et al.*, 1998; Tanaka *et al.*, 1996). Mouse T box expressed in T cells (T-bet) and GATA-binding protein (GATA-3) are two major T helper–specific transcription factors that regulate the expression of Th1 or Th2 cytokine genes and play a crucial role in T-helper cell differentiation. Interferon-γ (IFN-γ) is a major cytokine of Th1 cells, whereas, interleukin-10 (IL-10) is a cytokine mainly synthesized by Th2 cells that inhibits IFN-γ expression in Th1 cells (Fiorentino *et al.*, 1989).

Th17 is a newly defined Th-cell subset which mainly produces IL-17 and plays critical roles in the protection against microbial challenges and the induction of autoimmune diseases. IL-17 can induce many inflammatory cytokines and

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