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CYP2C9-Mediated Metabolic Activation of Losartan Detected by a Highly Sensitive Cell-Based Screening Assay[S]

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ABSTRACT:

Drug-induced hepatotoxicity is a major problem in drug development, and reactive metabolites generated by cytochrome P450s are suggested to be one of the causes. CYP2C9 is one of the major enzymes in hepatic drug metabolism. In the present study, we developed a highly sensitive cell-based screening system for CYP2C9-mediated metabolic activation using an adenovirus vector expressing CYP2C9 (AdCYP2C9). Human hepatocarcinoma HepG2 cells infected with our constructed AdCYP2C9 for 2 days at multiplicity of infection 10 showed significantly higher diclofenac 4′-hydroxylase activity than human hepatocytes. AdCYP2C9-infected cells were treated with several hepatotoxic drugs, resulting in a significant increase in cytotoxicity by treatment with losartan, benzbromarone, and tienilic acid. Metabolic activation of losartan by CYP2C9 has never been reported, although the metabolic activations of benzbromarone and tienilic acid have been reported. To identify the reactive metabolites of losartan, the semicarbazide adducts of losartan were investigated by liquid chromatography-tandem mass spectrometry. Two CYP2C9-specific semicarbazide adducts of losartan (S1 and S2) were detected. S2 adduct formation suggested that a reactive metabolite was produced from the aldehyde metabolite E3179, but a possible metabolite from S1 adduct formation was not produced via E3179. In conclusion, a highly sensitive cell-based assay to evaluate CYP2C9-mediated metabolic activation was established, and we found for the first time that CYP2C9 is involved in the metabolic activation of losartan. This cell-based assay system would be useful for evaluating drug-induced cytotoxicity caused by human CYP2C9.

Introduction

Drug-induced hepatotoxicity is a serious problem in drug development and clinical practice. In the United States, it accounts for more than 50% of cases of acute liver failure, and more than 600 drugs have been associated with hepatotoxicity (Lee, 2003; Park et al., 2005). That is why some drugs that were launched on the market were later withdrawn. Therefore, the prediction of drug-induced hepatotoxicity before clinical trials is important in drug development, and multiple cell-based assays have been developed for evaluation of drug-induced hepatotoxicity (Greer et al., 2010). Sometimes, drug-induced hepatotoxicity is associated with reactive metabolites produced by drug-metabolizing enzymes (Guengerich, 2008). However, species differences in drug-metabolizing enzymes or other factors between humans and laboratory animals are a major problem in predicting the hepatotoxicity.

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Cytochrome P450 (P450) enzymes are the most studied drug-metabolizing enzymes, accounting for ~75% of the metabolism of clinical drugs (Guengerich, 2008). Among them, CYP3A4 is the predominant isoform expressed in human liver, accounting for up to 60% of the total hepatic P450 protein and responsible for more than 50% of drug metabolism (Guengerich, 2008). To date, many researchers have tried to predict drug-induced hepatotoxicity in vitro using human hepatocarcinoma HepG2 cells, but the low expression levels of P450 enzymes in HepG2 cells may be responsible for the fact that 30% of the compounds were falsely classified as nontoxic (Rodríguez-Antona et al., 2002; Hewitt and Hewitt, 2004). Useful in vitro cell-based assays have been established with HepG2 cells, leading to improved evaluation of drug-induced cytotoxicity. For example, our previous study showed that benzodiazepines such as flunitrazepam and nimetazepam were metabolically activated by CYP3A4 by coincubation with HepG2 cells and CYP3A4 Supersomes (Mizuno et al., 2009). Vignati et al. (2005) demonstrated that various hepatotoxic drugs such as flutamide and troglitazone were activated by CYP3A4 using HepG2 cells transiently transfected with CYP3A4. Thus, the activation of hepatotoxic drugs by CYP3A4 has been well evaluated, but the contribution of other P450 enzymes remains to be evaluated. CYP2C is the second most highly expressed P450 subfamily in human liver, and CYP2C9 is the most highly expressed isoform in this family

ABBREVIATIONS: P450, cytochrome P450; Nrf2, nuclear factor-E2 p-45-related factor; GFP, green fluorescent protein; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; siRNA, small interfering RNA; HPLC, high-performance liquid chromatography; WST-8, 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium monosodium salt; LC, liquid chromatography; MS/MS, tandem mass spectrometry; LCMS-IT-TOF, liquid chromatography ion trap time-of-flight mass spectrometry; MOI, multiplicity of infection; BSO, buthionine sulfoximine; ALT, alanine aminotransferase; FLU-1, 4-nitro-3-(trifluoromethyl)phenylamine.

(Edwards et al., 1998). CYP2C9 is responsible for the metabolism of various pharmaceutical drugs and appears to be partially involved in the generation of reactive metabolites, as is CYP3A4 (Li, 2002). For example, benzbromarone is metabolized via 6-hydroxybenzbromarone to a catechol by CYP2C9, followed by the oxidization of the catechol to a reactive *ortho*-quinone metabolite (McDonald and Rettie, 2007). Tienilic acid is metabolized to reactive intermediates, the thiophene sulfoxide or the thiophene epoxide, by CYP2C9 (Koenigs et al., 1999). In recent studies, we developed useful in vitro cell-based assays using adenovirus to sensitively evaluate the involvement of CYP3A4 and superoxide dismutase 2 in drug-induced cytotoxicity (Yoshikawa et al., 2009; Hosomi et al., 2010). In the present study, a highly sensitive cytotoxicity assay system for CYP2C9-mediated metabolic activation was established in a similar way, and the drug-induced cytotoxicity was evaluated with the established assay system. Drugs investigated in this study were hepatotoxic drugs that are known to be CYP2C9 substrates (flutamide, fluvastatin, losartan, terbinafine, valproic acid, and zolpidem) and those that are known to be metabolically activated by CYP2C9 (benzbromarone and tienilic acid). As a result, we found for the first time that the cytotoxicity of losartan was enhanced by CYP2C9 and then performed additional studies to identify the structures of the reactive metabolites.

Materials and Methods

Chemicals and Reagents. Diclofenac, fluvastatin, and tienilic acid were obtained from Wako Pure Chemicals (Osaka, Japan). Losartan and terbinafine were obtained from LKT Laboratories (St. Paul, MN). Benzbromarone, flutamide, valproic acid, and zolpidem were obtained from Sigma-Aldrich (St. Louis, MO). Candesartan, eprosartan, irbesartan, telmisartan, and valsartan were obtained from Toronto Research Chemicals (Ontario, ON, Canada). Olmesartan was kindly provided by Daiichi-Sankyo (Tokyo, Japan). 4′-Hydroxydiclofenac and human CYP2C9 and CYP3A4 Supersomes (recombinant cDNA-expressed P450 enzymes prepared from a baculovirus insect cell system) were purchased from BD Gentest (Woburn, MA). The Adenovirus Expression Vector Kit (Dual Version) and adenovirus genome DNA-TPC were obtained from Takara Bio (Shiga, Japan). The QuickTiter Adenovirus Titer Immunoassay Kit was from Cell Biolabs (Tokyo, Japan). Stealth Select RNAi for Nrf2 (accession number NM_006164) and Stealth RNAi Negative Control Medium GC Duplex #2 were obtained from Invitrogen (Carlsbad, CA). Dulbecco's modified Eagle's medium was from Nissui Pharmaceutical (Tokyo, Japan). Restriction enzymes were from New England Biolabs (Ipswich, MA) and Takara Bio. All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). Other chemicals were of analytical or the highest grade commercially available.

Cell Culture. Human embryonic kidney 293 cells and human hepatocarcinoma HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). The 293 and HepG2 cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen), 3% glutamine, 16% sodium bicarbonate, and 0.1 mM nonessential amino acids (Invitrogen) in a 5% CO_2 atmosphere at 37°C. Cells were infected with the adenovirus in medium containing 5% fetal bovine serum.

Recombinant Adenovirus. A recombinant adenovirus expressing CYP2C9 (AdCYP2C9) was constructed using the cosmid-terminal protein complex method according to the manufacturer's instructions. CYP2C9 cDNA prepared by reverse transcription-polymerase chain reaction using total RNA from human liver obtained at autopsy was inserted into the SwaI site of the pAxcwtit vector. The use of human liver was approved by the ethics committees of Kanazawa University (Kanazawa, Japan) and Iwate Medical University (Morioka, Japan). The nucleotide sequences of CYP2C9 were confirmed by DNA sequence analysis (Long-Read Tower DNA sequencer; GE Healthcare, Little Chalfont, Buckinghamshire, UK). This vector and the parental adenovirus DNA terminal protein complex were cotransfected into 293 cells by Lipofectamine 2000 (Invitrogen). The recombinant adenovirus was isolated and propagated into the 293 cells. In a similar way, the recombinant adenovirus vector expressing a green fluorescence protein (GFP) was generated in the previous study (Hosomi et al., 2010). Viral titers were determined by a QuickTiter Adenovirus Titer Immunoassay Kit. The titers of AdCYP2C9 and AdGFP were 8.6×10^8 and 2.1×10^8 plaque-forming units/ml, respectively.

Immunoblot Analyses of Human CYP2C9 and Nrf2. SDS-polyacrylamide gel electrophoresis and immunoblot analyses of human CYP2C9, Nrf2, and GAPDH were performed. For human CYP2C9, total cell homogenates from adenovirus-infected HepG2 cells (5 μg) were separated on 7.5% polyacrylamide gels and electrotransferred onto a polyvinylidene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The membrane was probed with a polyclonal rabbit anti-human CYP2C9 antibody (Daiichi Pure Chemicals, Tokyo, Japan). Biotinylated anti-rabbit IgG and a VECTASTAIN ABC Kit (Vector Laboratories, Burlingame, CA) were used for diaminobenzidine staining. For human Nrf2, total cell homogenates from siRNA-transfected and adenovirus-infected HepG2 cells (25 μg) were separated on 7.5% polyacrylamide gels and electrotransferred onto a polyvinylidene difluoride membrane, Immobilon-P. The membrane was probed with polyclonal rabbit anti-human Nrf2 antibody (Santa Cruz Biotechnology, Inc., San Diego, CA), and the corresponding fluorescent dye-conjugated second antibody and an Odyssey infrared imaging system (LI-COR Biosciences, Lincoln, NE) were used for detection. For human GAPDH, SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to H. Hosomi, T. Fukami, A. Iwamura, M. Nakajima, and T. Yokoi (manuscript submitted for publication).

Diclofenac 4′-Hydroxylase Activity. HepG2 cells (3×10^5 cells/well) were seeded in 12-well plates. After a 24-h incubation, cells were infected with AdCYP2C9 or AdGFP for 1, 2, 3, or 5 days. Then, after a 1-h incubation with 100 μM diclofenac, the medium was subjected to high-performance liquid chromatography (HPLC) to measure the concentration of 4′-hydroxydiclofenac, a metabolite of diclofenac catalyzed by CYP2C9. The HPLC analysis was performed using an L-2130 pump (Hitachi, Tokyo, Japan), an L-2200 autosampler (Hitachi), and a D-2500 Chromato-Integrator (Hitachi) equipped with a Mightysil RP-18 C18 GP column (5-μm particle size, 4.6 mm i.d. × 150 mm; Kanto Chemical, Tokyo, Japan). The eluent was monitored at 280 nm. The mobile phase was 35% acetonitrile containing 20 mM sodium perchlorate (pH 2.5). The flow rate was 1.0 ml/min. The column temperature was 35°C. The retention times of 4′-hydroxydiclofenac and diclofenac were 8.1 and 22.8 min, respectively. The quantification of 4′-hydroxydiclofenac was performed by comparing the HPLC peak height with that of an authentic standard. The limit of quantification in the reaction mixture for 4′-hydroxydiclofenac was 250 nM with a coefficient of variation of <2%.

Cytotoxicity Assay. Nrf2 is known to regulate cytoprotective genes such as glutathione transferase, heme oxygenase-1, NAD(P)H:quinine oxidoreductase, superoxide dismutase, and UDP-glucuronosyltransferase (Copple et al., 2008). Our recent study demonstrated that drug-induced cytotoxicity could be detected with high sensitivity by the knockdown of Nrf2 in HepG2 cells (H. Hosomi, T. Fukami, A. Iwamura, M. Nakajima, and T. Yokoi, manuscript submitted for publication). Likewise, knockdown of Nrf2 was performed by siRNA transfection in this study. HepG2 cells were transfected with Stealth Select RNAi for Nrf2 (siNrf2) and Stealth RNAi Negative Control Medium GC Duplex #2 (siScramble) by Lipofectamine RNAiMAX Reagent (Invitrogen). According to the manufacturer's protocol, RNAi duplex-Lipofectamine RNAiMAX complexes were prepared and added to each well before the HepG2 cells were seeded (1.0×10^4 cells/well). The concentrations of siNrf2 and siScramble were 10 nM. After 24-h incubation, the cells were infected with AdCYP2C9 or AdGFP. Forty-eight hours after infection, the cells were treated with benzbromarone, tienilic acid, flutamide, fluvastatin, terbinafine, valproic acid, zolpidem, or sartans (candesartan, eprosartan, irbesartan, losartan, olmesartan, telmisartan, or valsartan) for 24 h. After incubation with the drugs, cell viability was quantified by 2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2, 4-disulfophenyl)-2*H*-tetrazolium monosodium salt (WST-8) and ATP assays according to the manufacturer's protocol. The WST-8 assay, which is a modified 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium assay, was performed using a Cell Counting Kit-8 (CCK-8 kit; Wako Pure Chemicals). After incubation with the drugs for 24 h, CCK-8 reagent was added and the absorbance of WST-8 formazan was measured at 450 nm. The ATP assay was performed using a CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, WI). After incubation with the drugs for 24 h, CellTiter-Glo Reagent was added, and the generation of a luminescent signal

was recorded by using a 1420 ARVO MX luminometer (PerkinElmer Life and Analytical Sciences–Wallac Oy, Turku, Finland).

Detection of Semicarbazide Adducts. A typical reaction mixture (final volume of 0.5 ml) contained 100 nM human CYP2C9 or CYP3A4 Supersomes, 50 mM potassium phosphate buffer (pH 7.4), 1 mM NADPH, 10 mM semicarbazide, and 20 μM [^{14}C]losartan. The final concentration of ethanol in the reaction mixture was less than 1%. Incubation was performed at 37°C for 60 min and terminated by addition of 2 ml of ice-cold methanol. After centrifugation at 15,000*g*, the supernatant was subjected to liquid chromatography (LC)-tandem mass spectrometry (MS/MS) (4000 QTRAP; Applied Biosystems, Foster City, CA). An Agilent 1200 (Agilent Technologies, Santa Clara, CA) was used as the liquid chromatograph with an Inertsil ODS-3V column (5-μm particle size, 4.6 mm i.d. × 250 mm; GL Science, Inc., Tokyo, Japan). The column temperature was 40°C. The mobile phase was 10 mM ammonium acetate buffer (A) and acetonitrile (B). The conditions for elution were as follows: 5 to 45% B (0–5 min), 45 to 70% B (5–55 min), 70 to 100% B (55–60 min), and 5% B (60.01–70 min). Linear gradients were used for all solvent changes. The flow rate was 1 ml/min. The liquid chromatograph was connected to a 4000 QTRAP mass spectrometer operated by the enhanced product ion under the positive mode. The turbo gas was maintained at 450°C. Air was used as the nebulizing and turbo gas at 50 psi. Nitrogen was used as the curtain gas at 30 psi. The declustering potential and collision energy were 50 V and 20 V, respectively. The *m/z* 150 to 500 was scanned at the precursor ion (*m/z* 494.2; semicarbazide adducts of losartan hydroxide).

Identification of Semicarbazide Adducts. A liquid chromatography ion trap time-of-flight mass spectrometry (LCMS-IT-TOF) system (Shimadzu, Kyoto, Japan) was used to identify the structures of the semicarbazide adducts of the losartan hydroxide. The incubation mixture was the same as described above. After centrifugation at 15,000*g* for 5 min, the supernatant was subjected to LCMS-IT-TOF using an Inertsil ODS-3 analytical column (5-μm particle size, 4.6 mm i.d. × 250 mm). The LC conditions were the same as described earlier.

Statistical Methods. Data are expressed as means ± S.D. Statistical significance between the two groups was determined by a two-tailed Student's *t* test. $P < 0.05$ was considered statistically significant.

Results

MOI- and Time-Dependent Changes of Diclofenac 4′-Hydroxylase Activity and CYP2C9 Protein Level. To investigate the optimum multiplicity of infection (MOI), HepG2 cells were infected with AdCYP2C9 at MOI 0, 2.5, 5, 10, or 20 for 2 days. Diclofenac 4′-hydroxylase activity and CYP2C9 protein level were measured (Fig. 1). The activity and CYP2C9 protein level were increased MOI dependently in AdCYP2C9-infected cells, whereas they were not detected in AdGFP-infected cells at MOI 20. The highest activity and protein level were observed in cells infected with AdCYP2C9 at MOI 20, but the cells were slightly damaged (data not shown). At MOI 10, diclofenac 4′-hydroxylase activity was 0.957 ± 0.070 nmol/min/mg protein, a value that was higher than those in human hepatocytes reported in other reports (Supplemental Table 1). With HepG2 cells infected with AdCYP2C9 at MOI 10 for 1, 2, 3, or 5 days, the highest activity was observed after a 2-day infection, although the protein levels appeared to be similar after 2- to 5-day infections (Fig. 1B). From these results, AdCYP2C9 infection to HepG2 cells was performed at MOI 10 for 2 days in the subsequent experiments.

Effect of SiNrf2 on Nrf2 Protein Expression in Adenovirus-Infected HepG2 Cells. Our recent study demonstrated that CYP3A4-induced cytotoxicities of several drugs such as acetaminophen and flutamide were sensitively detected by Nrf2 knockdown (H. Hosomi, T. Fukami, A Iwamura, M. Nakajima, and T. Yokoi, manuscript submitted for publication). This study also used HepG2 cells transfected with siNrf2. Nrf2 protein expression in HepG2 cells was efficiently decreased by transfection of siNrf2 (26.8 ± 1.1 and 27.1 ± 2.0%, respectively), and the effect of siNrf2 was not affected by CYP2C9 overexpression (Fig. 1C).

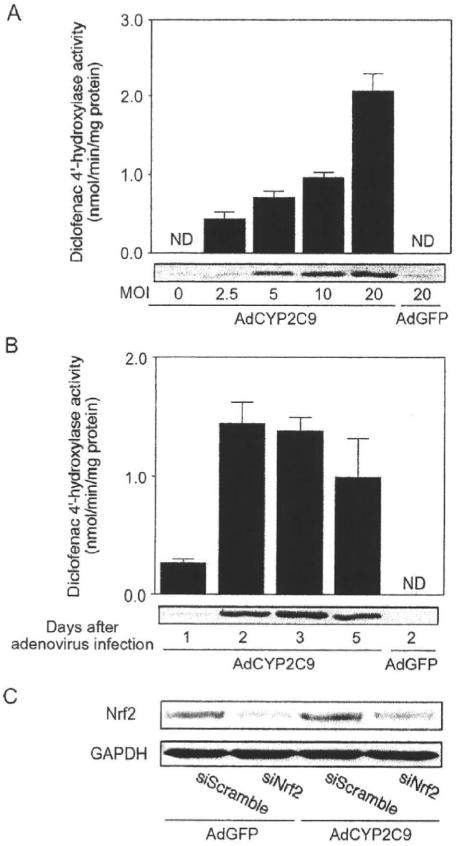

FIG. 1. MOI-dependent (A) and time-dependent (B) changes of diclofenac 4′-hydroxylase activity and CYP2C9 protein level in adenovirus (AdCYP2C9 or AdGFP)-infected HepG2 cells. HepG2 cells were infected with adenovirus for 2 days (A) or at MOI 10 (B). Diclofenac 4′-hydroxylase activity was measured as described under *Materials and Methods*. The CYP2C9 protein level was analyzed by immunoblotting using total cell homogenates from adenovirus-infected HepG2 cells, and the representative bands are demonstrated. Data are means ± S.D. (n = 3). C, Nrf2 protein level in HepG2 cells transfected with siScramble or siNrf2. HepG2 cells were infected with adenovirus (AdCYP2C9 or AdGFP) at MOI 10 for 2 days after a 24-h incubation with 10 nM siRNA. The relative band intensity of Nrf2 was normalized with the band intensity of GAPDH. Data are means ± S.D. (n = 3). **, $P < 0.01$ compared with AdGFP-infected groups transfected with siScramble. ND, not detected.

CYP2C9-Induced Cytotoxicity in HepG2 Cells Transfected with siNrf2. To investigate the CYP2C9-mediated metabolic activation of eight hepatotoxic drugs (benzbromarone, flutamide, fluvastatin, losartan, terbinafine, tienilic acid, valproic acid, and zolpidem), HepG2 cells infected with AdCYP2C9 at MOI 10 for 2 days were treated with drugs for 24 h. As a negative control, AdGFP was infected at MOI 10. To improve the sensitivity, HepG2 cells were transfected with siNrf2 24 h before adenovirus infection. Cytotoxicity was evaluated by WST-8 and ATP assays (Figs. 2 and 3). In the WST-8 assay, the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-infected cells by treatment with benzbromarone (10–40 μM), tienilic acid (100 and 200 μM), and losartan (25–100 μM) (Fig. 2). On the other hand, the viabilities of AdCYP2C9-infected cells were not different from those of AdGFP-infected cells by treatment with flutamide, fluvastatin, terbinafine, valproic acid, and zolpidem, except when treated with 100 μM fluvastatin. The ATP assay revealed a result similar to that of the WST-8 assay in that the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-infected cells by treatment with the benzbromarone (10–40 μM), tienilic acid

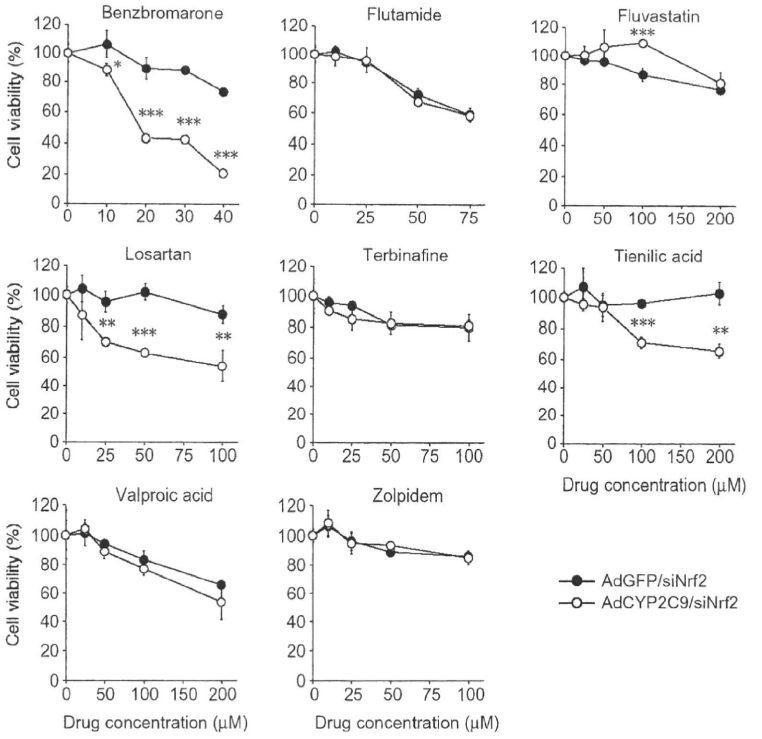

FIG. 2. CYP2C9-induced cytotoxicity in HepG2 cells transfected with siNrf2 (WST-8 assay). HepG2 cells were infected with adenovirus at MOI 10 for 2 days after a 24-h incubation with 10 nM siNrf2. Cell viability was measured by WST-8 assay after a 24-h treatment with the test drugs. Cell viability is expressed as a percentage of cells without drug treatment. Data are means ± S.D. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with AdGFP-infected groups.

(50–200 μM), and losartan (10–100 μM) (Fig. 3). These results suggested that the benzbromarone-, tienilic acid-, and losartan-induced cytotoxicities are caused by the metabolic activation of CYP2C9.

CYP2C9-Induced Cytotoxicity in HepG2 Cells Transfected with siScramble. To investigate whether Nrf2-associated cytoprotective genes were involved in the benzbromarone-, tienilic acid-, and losartan-induced cytotoxicities mediated by CYP2C9, the cytotoxicity was evaluated with HepG2 cells transfected with siScramble instead of siNrf2 (Fig. 4). Terbinafine was used as a negative control. With the drugs except terbinafine, the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-

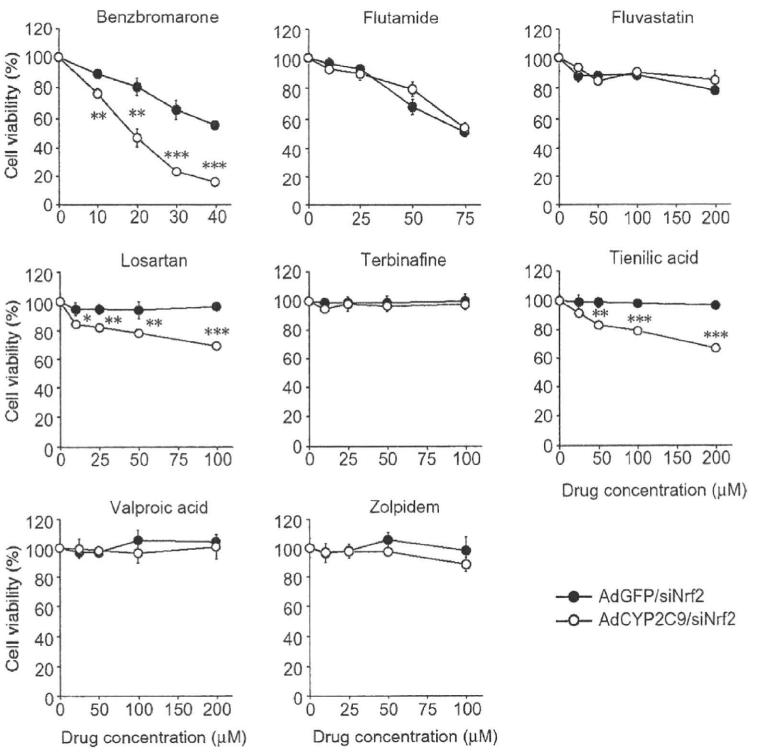

FIG. 3. CYP2C9-induced cytotoxicity in HepG2 cells transfected with siNrf2 (ATP assay). HepG2 cells were infected with adenovirus at MOI 10 for 2 days after a 24-h incubation with 10 nM siNrf2. Cell viability was measured by ATP assay after a 24-h treatment with the test drugs. Cell viability is expressed as a percentage of cells without drug treatment. Data are means ± S.D. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with AdGFP-infected groups.

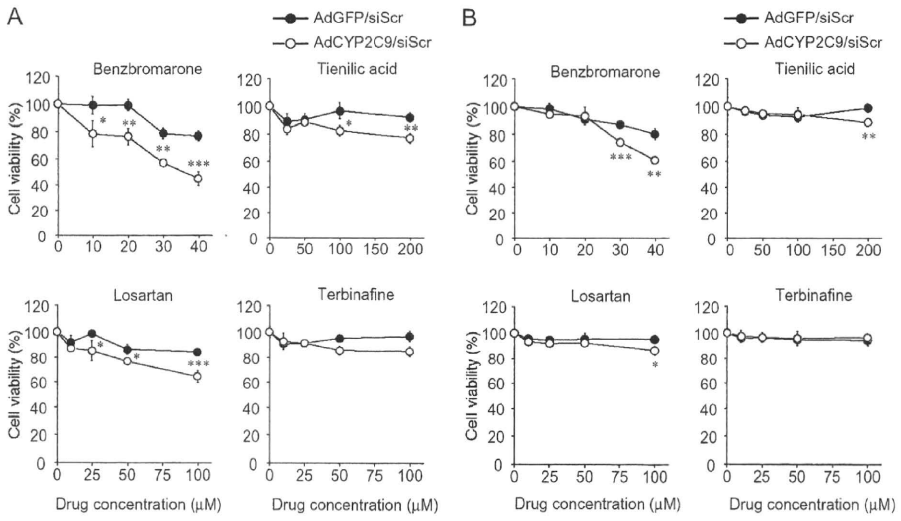

FIG. 4. CYP2C9-induced cytotoxicity in HepG2 cells transfected with siScramble. HepG2 cells were infected with adenovirus at MOI 10 for 2 days after a 24-h transfection with 10 nM siScramble. Cell viability was measured by the WST-8 assay (A) and the ATP assay (B) after a 24-h treatment with the test drugs. Cell viability is expressed as a percentage of cells without drug treatment. Data are means ± S.D. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$, compared with AdGFP-infected groups.

infected cells, but the differences in the viabilities between AdGFP- and AdCYP2C9-infected cells transfected with siScramble were less than those of cells transfected with siNrf2.

Comparison of CYP2C9-Mediated Cytotoxicity between Losartan and Various Sartans. Because the cell-based assay system revealed that the losartan-induced cytotoxicity involved metabolic activation by CYP2C9, it was conceivable that other sartans with similar structures were also metabolically activated by CYP2C9. The viabilities of AdCYP2C9-infected cells were investigated by treatment with various sartans such as eprosartan, candesartan, irbesartan, olmesartan, telmisartan, and valsartan. However, no sartans other than losartan affected the cell viabilities (Fig. 5). Thus, among members of the sartan family, only losartan is associated with CYP2C9-mediated cytotoxicity.

Detection of Semicarbazide Adducts of Losartan. The semicarbazide adducts of losartan were investigated by the positive ion mode of LC-MS/MS. It was reported that CYP3A4 is involved in the metabolism of losartan (Stearns et al., 1995). However, no cytotoxicity of losartan was induced when the cells were infected with AdCYP3A4 constructed previously (Hosomi et al., 2010) instead of AdCYP2C9 (data not shown). Therefore, to detect adducts specifically generated by CYP2C9, losartan was incubated with CYP2C9 or CYP3A4 (negative control) Supersomes. As shown in Fig. 6, three semicarbazide adducts of losartan (S1, S2, and S3) were detected in the presence of CYP2C9 Supersomes by a precursor ion scan at *m/z* 494.2 ($[M + H]^+$). Because S3 was also detected when incubated with the CYP3A4 Supersomes, S3 was considered not to be involved in the CYP2C9-mediated cytotoxicity. Therefore, the subsequent study of S3 was not performed.

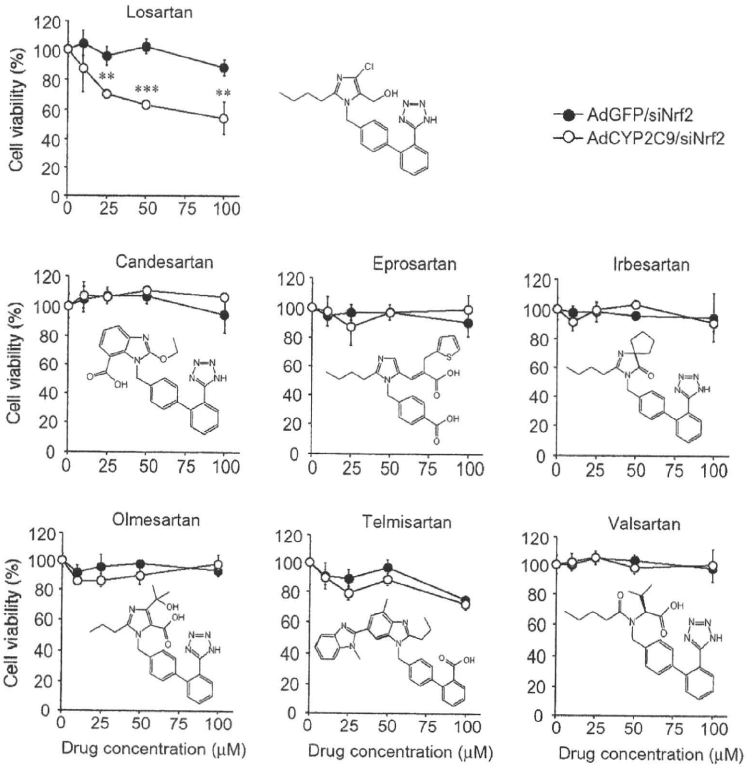

FIG. 5. Comparison of CYP2C9-mediated cytotoxicity between losartan and various sartans. HepG2 cells were infected with adenovirus at MOI 10 for 2 days after a 24-h transfection with 10 nM siNrf2. Cell viability was measured by the WST-8 assay after a 24-h treatment with the drugs. Cell viability is expressed as a percentage of cells without drug treatment. Data are means ± S.D. ($n = 3$). *, $P < 0.05$; **, $P < 0.01$, compared with AdGFP-infected groups.

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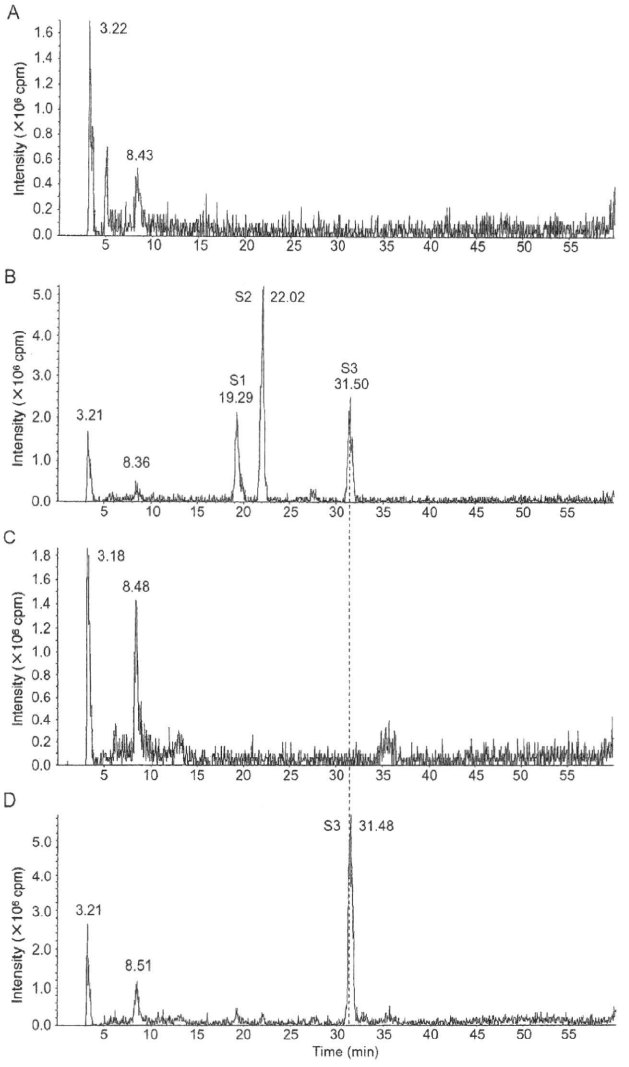

FIG. 6. Ion chromatograms from LC-MS/MS analysis of the semicarbazide adducts of losartan at *m/z* 494.2 ($[M + H]^+$). A, CYP2C9 Supersomes without semicarbazide. B, CYP2C9 Supersomes with semicarbazide. C, CYP3A4 Supersomes without semicarbazide. D, CYP3A4 Supersomes with semicarbazide. Incubation and LC-MS/MS conditions were as described under *Materials and Methods*.

Identification of Semicarbazide Adducts of Losartan. The structures of S1 and S2 were estimated by the positive ion mode of LCMS-IT-TOF (Fig. 7). The product ion mass spectrum of losartan exhibited a major fragment ion at *m/z* 405.1513 ($C_{22}H_{22}N_6Cl$) (Fig. 7A). The fragment ion at *m/z* 405.1513 was $[M + H - 18]^+$, indicating the losses of H_2O from alcohol group of losartan. The product ion mass spectrum of S1 exhibited two major fragment ions at *m/z* 476.1606 ($C_{23}H_{23}N_9OCl$) and *m/z* 459.1312 ($C_{23}H_2ON_8OCl$). The fragment ions at *m/z* 476.1606 and *m/z* 459.1312 were $[M + H - 18]^+$ and $[M + H - 35]^+$, indicating the losses of H_2O and NH_3 and H_2O, respectively. On the other hand, the product ion mass spectrum of S2 exhibited fragment ions at *m/z* 477.1477. The fragment ions at *m/z* 477.1477 were $[M + H - 17]^+$, indicating the loss of NH_3 from semicarbazide. Furthermore, the fragment ion at *m/z* 207.08 given from all three precursor ions indicated no conjugation with the biphenyl or tetrazole ring. The possible structures of S1 and S2 are shown in Fig. 7, B and C. *m/z* 207.08 indicated no conjugation with the biphenyl or tetrazole ring, and $[M + H - 18]^+$ indicated the losses of H_2O from alcohol group of losartan. Therefore, these two fragment

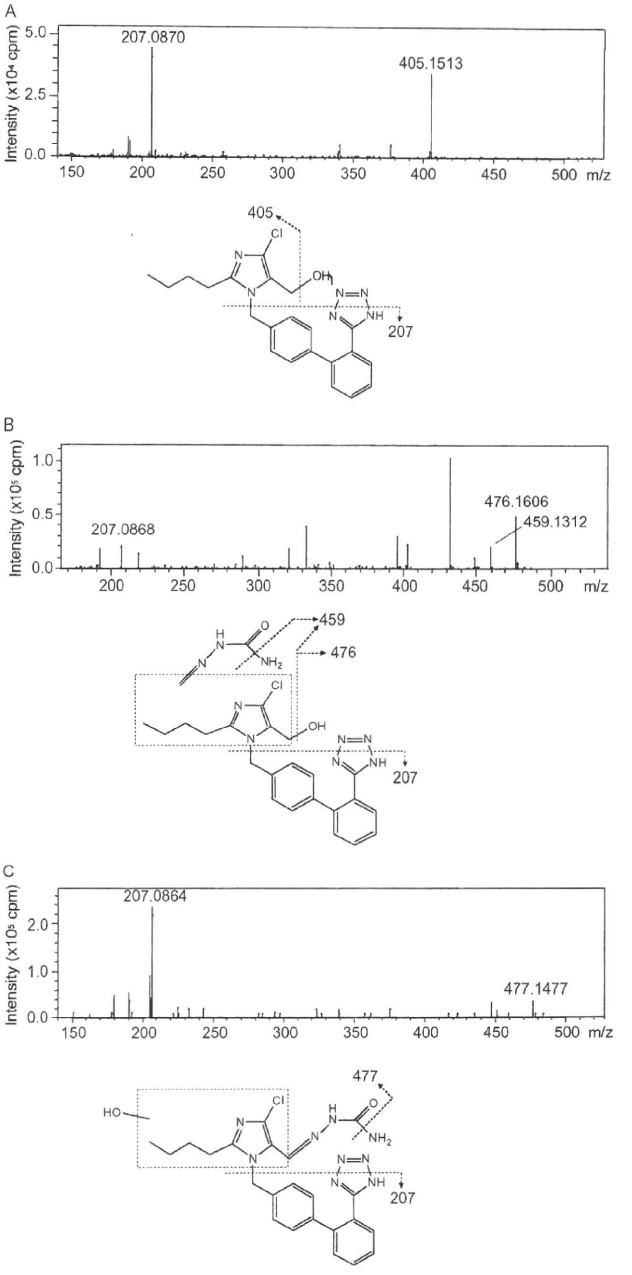

FIG. 7. Predicted structures of semicarbazide adducts of losartan and MS/MS spectra of the product ion obtained by collision-induced dissociation of (A) losartan at *m/z* 423.2 ($[M + H]^+$) and (B) S1 and (C) S2 at *m/z* 494.2 ($[M + H]^+$). The precursor ion at *m/z* 423.2 is semicarbazide adducts of losartan hydroxide. These spectra were scanned using LCMS-IT-TOF. Incubation and LCMS-IT-TOF conditions were as described under *Materials and Methods*.

ions detected in both losartan and S1 suggested that semicarbazide conjugates with another position, that is, somewhere in the imidazole ring or the adjacent butyl side chain. In contrast, $[M + H - 17]^+$ instead of $[M + H - 18]^+$ given from S2 suggested that a reactive metabolite conjugated with semicarbazide is a hydroxylated form of E3179, an aldehyde metabolite of losartan.

Discussion

In this study, we constructed an in vitro cell-based assay system to evaluate the hepatotoxicity mediated by CYP2C9 and performed a

cytotoxicity assay for drugs that have been known to cause hepatotoxicity. Benzbromarone and tienilic acid are converted to reactive metabolites by CYP2C9. In addition, six other hepatotoxic drugs, whose reactive metabolites generated by CYP2C9 have not been identified although CYP2C9 is involved in their metabolism, were evaluated by our cell-based assay system. According to O'Brien et al. (2006), the cytotoxicity assay was performed within the drug concentration of 30 times the maximal efficacious concentration or 100 μM. We found that the viabilities of AdCYP2C9-infected cells were significantly decreased compared with those of AdGFP-infected cells by treatments with benzbromarone, tienilic acid, and losartan, suggesting that the hepatotoxicity induced by these drugs involves metabolic activation by CYP2C9. Benzbromarone is metabolized via 6-hydroxybenzbromarone to the catechol by CYP2C9, followed by the oxidization of the catechol to a reactive *ortho*-quinone metabolite (McDonald and Rettie, 2007). Tienilic acid is metabolized via the sulfoxide to 5-hydroxytienilic acid by CYP2C9. This sulfoxide can form a covalent bond with CYP2C9 or other proteins. In rat, administration of tienilic acid in combination with the glutathione biosynthesis inhibitor, buthionine sulfoximine (BSO), induced a marked elevation of the serum alanine aminotransferase (ALT) level, but no increase in the serum ALT activity was observed in the presence of the P450 inhibitor, 1-aminobenzotoriazole (Nishiya et al., 2008). Thus, the mechanisms for the metabolic activation of these drugs have been well examined. However, to our knowledge, cell-based assays for assessment of the metabolic activations of these drugs have not been performed. The results obtained in our cell-based assay system were in agreement with several reports that CYP2C9 is involved in the metabolic activations of benzbromarone and tienilic acid. However, there have been no reports of the involvement of CYP2C9 in the cytotoxicity of losartan. It has been reported that losartan could form protein or glutathione adducts by incubation with human liver microsomes and/or human hepatocytes, suggesting the metabolic activation of losartan (Gan et al., 2009; Usui et al., 2009). The present study demonstrated for the first time that CYP2C9 was responsible for the metabolic activation of losartan. The concentrations at which the metabolic activation of losartan was observed were much higher than those in plasma in clinical practice. To predict the involvement of CYP2C9 in losartan-induced toxicity, the combination of our established cell-based assay with other studies is needed.

Sartans have generally been used as safe drugs in clinical practice, but there have been various reports of losartan-induced hepatotoxicity, which is categorized as hepatocellular injury (Tabak et al., 2002; Chang and Schiano, 2007). In some case reports, a rechallenge to losartan after ALT normalization caused hepatotoxicities again (Bosch, 1997; Tabak et al., 2002). However, the contribution of immunological factors to losartan-induced hepatotoxicity was unknown. Losartan is metabolized to the carboxylic acid metabolite E3174, which is pharmacologically more active than the parent compound, via the aldehyde metabolite E3179, which is an intermediate in the oxidation of losartan. These biotransformations are catalyzed by CYP2C9 and CYP3A4. In addition to this pathway, the monohydroxylation of the butyl side chain is also catalyzed by CYP2C9 (Stearns et al., 1995). The viability of HepG2 cells was not decreased by treatment of E3179 and E3174 (Supplemental Fig. 1), suggesting that they may not show cytotoxicity. In this study, two CYP2C9-specific semicarbazide adducts of losartan (S1 and S2) were detected (Figs. 6 and 7). From the fragment ions of S1 and S2, it was suggested that S2 was produced via E3179, but S1 was not (Fig. 8). The cytotoxicity of losartan induced by CYP2C9 was attenuated by the treatment with semicarbazide (Supplemental Fig. 2). Therefore, the possible reactive metabolites from S1 and S2 might be involved in the cytotoxicity. Furthermore, no significant decreases in cell viabilities were observed by treatment with various sartans (irbesartan, valsartan, candesartan, olmesartan, telmisartan, and eprosartan) other than losartan. Taken together, these results suggested that the side chains or a chloro group besides the imidazole ring that is unique to losartan is important for the losartan-induced cytotoxicity mediated by CYP2C9.

The CYP2C9-induced cytotoxicities of benzbromarone, tienilic acid, and losartan were enhanced by Nrf2 knockdown, suggesting that the genes regulated by Nrf2 are associated with detoxification of their cytotoxicities. In our recent study, CYP3A4-induced cytotoxicities of several drugs such as acetaminophen and flutamide were sensitively detected by Nrf2 knockdown (H. Hosomi, T. Fukami, A. Iwamura, M. Nakajima, and T. Yokoi, manuscript submitted for publication). In addition, it was demonstrated that *nrf2*(−/−) mice are more vulnerable to acetaminophen-induced liver injury, due in part to lower cellular thiol levels and decreased expression of detoxification enzymes (Enomoto et al., 2001). Thus, Nrf2 is considered to play a quite important role in the detoxification of hepatotoxic drugs. Among the genes regulated by Nrf2, there are various genes involved in glutathione synthesis, such as the glutamate cysteine ligase catalytic subunit, the glutamate cysteine ligase regulatory subunit, and glutathione synthetase (Copple et al., 2008). Glutathione is an important intracellular peptide that detoxifies reactive metabolites by conjugation (Lu, 1999). In fact, reactive *ortho*-quinone metabolites of benzbromarone generated by CYP2C9 can be trapped with glutathione (McDonald and Rettie, 2007). In addition, the presence of glutathione markedly decreased the level of covalent binding of tienilic acid to microsomal proteins (Bonierbale et al., 1999). From these backgrounds, we considered whether the cytotoxicity of losartan could be clearly observed in a cytotoxicity assay with HepG2 cells transfected with siRNA for γ-glutamylcysteine synthetase heavy chain or treated with BSO instead of being transfected with siNrf2. However, no significant decreases in cell viabilities were observed by either transfection of siRNA for γ-glutamylcysteine synthetase heavy chain or treatment with BSO (data not shown). These results suggested that glutathione conjugation was not required for the detoxification of losartan-induced cytotoxicity but that other detoxification enzymes regulated by Nrf2 would be involved. Therefore, semicarbazide was used as a trapping agent for the reactive metabolites of losartan in this study. Semicarbazide is a hard nucleophile, which will preferentially react with hard electrophiles, such as aldehydes (Chauret et al., 1995). Indeed, the cytotoxicity of losartan induced by CYP2C9 was attenuated by treatment with semicarbazide (Supplemental Fig. 2). Thus, it is conceivable that reactive metabolites trapped by semicarbazide are involved in the CYP2C9-induced cytotoxicity of losartan.

In the present study, CYP2C9-mediated metabolic activation was not observed with flutamide, fluvastatin, terbinafine, valproic acid, and zolpidem, which are suspected to be associated with hepatotoxicity (Karsenti et al., 1999; Thole et al., 2004; Chang and Schiano, 2007). Flutamide is hydrolyzed to 4-nitro-3-(trifluoromethyl)phenylamine (FLU-1), which is further metabolized to *N*-hydroxy FLU-1, which can cause hepatotoxicity in rat (Ohbuchi et al., 2009). The N-hydroxylation of FLU-1 is catalyzed by CYP2C9 as well as by CYP3A4 (Goda et al., 2006). In this study, flutamide-induced cytotoxicity could not be detected. One possibility is that the intracellular concentration of FLU-1 was low because of the low flutamide hydrolase activity in HepG2 cells, although it was not measured. Terbinafine is known to be metabolized by a wide range of P450 enzymes including CYP2C9, primarily through N-demethylation, deamination, and hydroxylation (Vickers et al., 1999). Among its metabolites, 7,7-dimethylhept-2-ene-4-ynal was considered to play a role in the pathogenesis of hepatotoxicity (Iverson and Uetrecht, 2001), but this

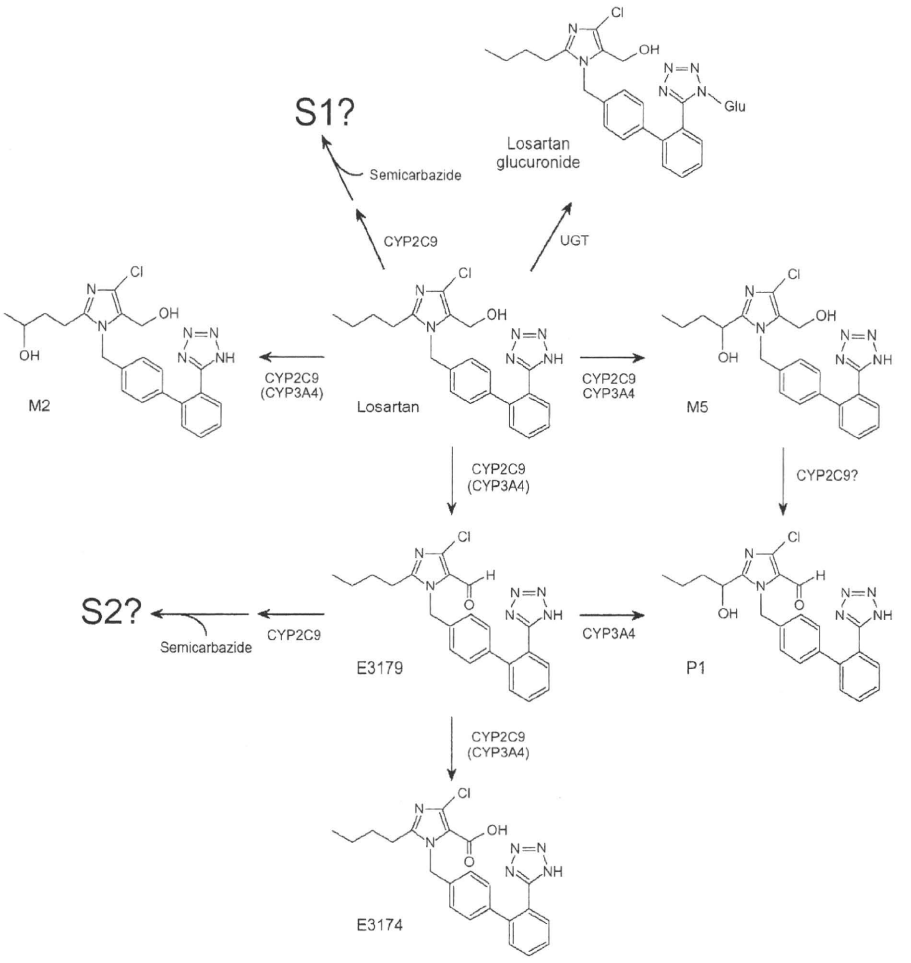

FIG. 8. Proposed metabolic pathways of losartan. UGT, UDP-glucuronosyltransferase.

metabolite is generated by CYP3A4, not by CYP2C9. That is why the cell viability was not affected by treatment with terbinafine in AdCYP2C9-infected cells. For the hepatotoxicity caused by valproic acid, the involvement of its reactive metabolites such as 4-ene-valproic acid and 2,4-diene-valproic acid was suggested (Baillie, 1988; Kassahun et al., 1991; Tang et al., 1995). CYP2C9 played a role in the formation of 4-ene-valproic acid (Sadeque et al., 1997), but no involvement of CYP2C9 in the cytotoxicity of valproic acid was observed in this study. It was reported that valproic acid produced a high level of covalent binding in rat liver after oral administration, although it did not bind to microsomal protein in vitro (Leone et al., 2007). Other factors as well as CYP2C9 might be responsible for the hepatotoxicity of valproic acid. The cytotoxicities of fluvastatin and zolpidem induced by CYP2C9 were not detected in the present study. Until now, the mechanisms of their cytotoxicities have been unknown. The present study suggested low involvement of CYP2C9 in their cytotoxicities, although it is responsible for the metabolism of these drugs (Fischer et al., 1999; Von Moltke et al., 1999).

In conclusion, we constructed a highly sensitive cell-based assay system to evaluate CYP2C9-mediated cytotoxicity and found for the first time that CYP2C9 is involved in the metabolic activation of losartan. This cell-based assay system would be useful in evaluating drug-induced cytotoxicity caused by human CYP2C9.

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Authorship Contributions

Participated in research design: Iwamura, Fukami, Nakajima, and Yokoi.
Conducted experiments: Iwamura and Hosomi.
Contributed new reagents or analytic tools: Iwamura and Hosomi.
Performed data analysis: Iwamura and Fukami.
Wrote or contributed to the writing of the manuscript: Iwamura, Fukami, and Yokoi.

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CYP2C9-mediated Metabolic Activation of Losartan Detected by A Highly Sensitive Cell-based Screening Assay

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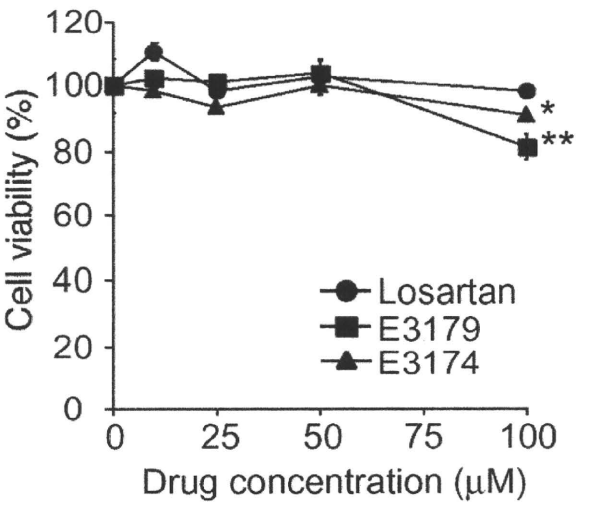
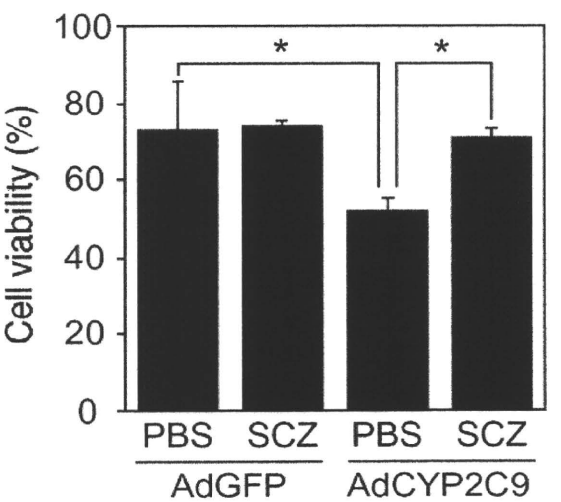
Drug Metab Dispos

Supplemental Table 1. Diclofenac 4'-hydroxylase activity in cultured human hepatocytes in previous studies.

Number of donors	Diclofenac concentration	Activity or Vmax[a]	References
	μM	*pmol/min/mg total cellular protein*	
5	100	32 ± 16	Zhang et al. (2006) *Drug Metab Dispos* **34**: 734-737.
-	200	149 ± 35	Bort et al. (1999) *Toxicol In Vitro* **13**: 633-638.
5	300	49 ± 10	Donato et al. (2006) *Drug Metab Dispos* **34**: 1556-1562.
-	300	317 ± 73	Gomez-Lechon (2001) *Eur J Biochem* **268**: 1448-1459.
-	3 - 1000	73[a]	Donato et al. (2004) *Drug Metab Dispos* **32**: 699-706.



Supplemental Figure 1. Cytotoxicity of losartan and its metabolites (E3179 and E3174) in HepG2 cells. Drugs were treated for 24 hr. Data are mean ± SD (n = 3). *$P < 0.05$ and **$P < 0.01$ compared with losartan-treated groups.



Supplemental Figure 2. The effects of trapping by semicarbazide (SCZ) on the metabolic activation of losartan by CYP2C9. HepG2 cells were treated with 100 μM losartan and 10 mM SCZ (or 1% PBS) for 24 hr. Data are mean ± SD (n = 3). *$P < 0.05$.

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Estradiol and progesterone modulate halothane-induced liver injury in mice

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ABSTRACT

Drug-induced liver injury (DILI) is one of the major problems in drug development and clinical drug therapy. In general, it is believed that women exhibit worse outcomes from DILI than men. It is known that halothane (HAL), an inhaled anesthetic, rarely induces severe liver injury. The risk factors for severe HAL-induced liver injury (HILI) are female sex, genetics and adult age. To investigate the underlying mechanism by which women are more susceptible to HILI, we focused on two major female sex hormones, estradiol (E2) and progesterone (Prog). In this study, we first found that pretreatment of mice with E2 attenuated HILI, whereas pretreatment with Prog exacerbated HILI. E2 and Prog had no effects on the degree of metabolic activation, the ratio of GSH/GSSG or oxidative stress in the liver. We observed higher numbers of neutrophils infiltrated into the liver and increased hepatic mRNA levels of proinflammatory cytokines, tumor necrosis factor (TNF) α, interleukin (IL)-1β and IL-6 and chemokines, CXCL1 and CXCL2 by pretreatment with Prog, whereas E2 pretreatment resulted in the opposite effects. These results suggest that E2 and Prog play a critical role in HILI via immune-related responses and female sex hormone balance might represent a risk factor for HILI.

1. Introduction

The occurrence of drug-induced liver injury (DILI) can be a major problem in all phases of clinical drug development. In most cases, the mechanisms of hepatotoxicity are unknown, but it is likely to arise from complex interactions among drugs, genetic, age, gender, disease and environmental factors (Chalasani and Björnsson, 2010; Li, 2002). In general, women are more susceptible to DILI than men. Seventy-eight percent of DILI cases occur in women, and a significantly greater number of women show hepatocellular DILI than men (Ostapowicz et al., 2002; De Valle et al., 2006; Björnsson and Olsson, 2005). In contrast, some reports described that there was no significant gender difference in the incident rates of DILI (Lucena et al., 2009; Andrade et al., 2005). However, it was also reported that patients with severe DILI who underwent liver transplantation in the US were more frequently women (76%) and that nearly 90% of patients with fluminant liver injury from DILI were women. Thus, women appear to be at greater risk of developing severe DILI (Lucena et al., 2009; Andrade et al., 2005; Russo et al., 2004), but it is not clear why women exhibit the worst outcomes in DILI.

Women elicit more vigorous cellular and humoral immune reactions, and suffer in greater numbers from autoimmune disease than men (Ansar et al., 1985; Ostensen, 1999). There is also evidence that the immune system is regulated by the circulating levels of sex steroid hormones, estradiol (E2), progesterone (Prog), and testosterone (Grossman, 1985). It was also reported that E2 inhibits proinflammatory cytokine production by murine peritoneal macrophages and mononuclear cells in vitro, whereas Prog may counteract this effect of E2 (Huang et al., 2008; Yuan et al., 2008). However, there has been little information concerning the involvement of female sex hormones in DILI.

Halothane (HAL) is an inhaled anesthetic that causes asymptomatic increases of plasma transaminases in approximately 20% of patients and severe liver injury in a small percentage of patients (Ray and Drummond, 1991). Risk factors for severe halothane-induced liver injury (HILI) are female sex, adult age, genetics, and multiple halothane exposures (Inman and Mushin, 1974; Cousins et al., 1989). HAL is metabolized to trifluoroacetyl (TFA)-chloride by cytochrome P450 (CYP) 2E1 and covalent binding to proteins and lipids. It has been suggested that TFA-adduct or halothane-modified macromolecules may be an initiating event for an immune response (Bourdi et al., 2001; Njoku et al., 1997; Gut et al., 1993). Recent studies indicate the mechanism of HILI involves immune responses such as neutrophils, IL-17 and natural killer cells (You et al., 2006; Kobayashi et al., 2009; Cheng et al., 2010).

Recently, a new mouse model of HILI was established and gender differences in the degree of HILI were suggested (You et al.,

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2006). In this study, we investigated whether two major female sex hormones, E2 and Prog, play a functional role in HILI.

2. Materials and methods

2.1. Materials

HAL was purchased from Takeda (Osaka, Japan) and isoflurane (Iso) was from Abott Japan (Tokyo, Japan). 17β-Estradiol (E2) and Prog were from Sigma–Aldrich (St. Louis, MO). ICI182,780 (ICI) was from TOCRIS bioscience (Ellisville, MO) and RU486 (RU) was from Tokyo Kasei (Tokyo, Japan). Rabbit polyclonal antibody against myeloperoxidase (MPO) was from DAKO (Carpinteria, CA). All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All other chemicals were of the highest grade commercially available.

2.2. Mice and HAL-administration

Female BALB/cCrSlc mice (8 weeks old, 18–21 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 before use for the experiments (Sugaya et al., 2000). E2 and Prog pretreatment methods were modified from those of a previous report that the serum hormone levels were almost the same or slightly higher than those during late pregnancy in rodent, although the mice used in the present study were not ovariectomized since we intend to perform the present studies under the autologus condition and to mimic the condition of late pregnancy. Mice were pretreated with E2 (0.3 μg/mouse, *s.c.*) or Prog (0.3 mg/mouse, *s.c.*) for 7 days followed by HAL administration (15 or 30 mmol/kg, dissolved in olive oil 2 mL/20 g of body weight, *i.p.*) 1.5 h after the last E2 or Prog treatment. Mice were pretreated with the receptor antagonist, ICI (Estrogen receptor (ER) antagonist, 50 μg/mouse, *s.c.*) or RU (Progesterone receptor (PR) antagonist, 50 μg/mouse, *s.c.*) 0.5 h before the E2 or Prog treatment, respectively. In the Isoflurane (Iso) administration experiment, Iso was administered instead of HAL. Mice were sacrificed and the plasma and the liver were collected 3, 6 and 24 h after the HAL administration. The liver was fixed in buffered neutral 10% formalin and used for immunohistochemical staining. The degree of liver injury was assessed by hematoxylin–eosin (H&E) staining and the plasma aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels were determined using Fuji Dri-Chem 4000V (Fuji Film Med. Co., Tokyo, Japan). The neutrophil infiltration was assessed by immunostaining for MPO as previously described (Kumada et al., 2004). Animal maintenance and treatment were conducted in accordance with the National Institutes of Health Guide for Animal Care and Use Committee of Kanazawa University, Japan.

2.3. Measurement of plasma E2, Prog and IL-17 levels

Plasma E2 and Prog were measured by enzyme immunoassay (EIA) using Estradiol-17β (serum/plasma) EIA kit and Progesterone EIA kit from Assay Designs Inc. (Ann Arbor, MI) according to the manufacturer's instructions. The plasma IL-17 level was measured by ELISA using a Ready-SET-GO! Mouse Interleukin-17A (IL-17A) kit from eBioscience (San Diego, CA) according to the manufacturer's instructions.

2.4. Immunoblot analysis

SDS-polyacrylamide gel electrophoresis and immunoblot analysis were perfomed. Mouse liver homogenates (30 μg) were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobion-P (Millipore Corporation, Billerica, MA). The membranes were probed with goat anti-rat Cyp2e1 (1:10,000, Daiichi Pure Chemicals, Tokyo, Japan), anti-TFA antisera (1:1000, kindly provided by Dr. Lance Pohl, National Institutes of Health, Bethesda, MD) and rabbit anti-human glyceraldehyde-3-phoshate dehydrogenase (GAPDH) polyclonal antibodies (1:100, IMAGENEX, San Diego, CA) and the corresponding fluorescent dye-conjugated second antibody (1:5000) and an Odyssey Infrared Imaging System (LI-COR Biosciences, Lincoln, NE) were used for detection. The relative expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare, Buckinghamshire, UK).

2.5. Glutathione assay

Mouse liver was homogenized with a glass homogenizer on ice-cold 5% sulfosalicylic acid and centrifuged at 8000 × *g* at 4 °C for 10 min. Total GSH and GSSG concentration in the supernant were measured as described previously (Tietze, 1969; Griffith, 1980). GSH was calculated from the difference between the total GSH and GSSG concentration.

Table 1
Sequence of primers used for real-time RT-PCR analyses in this study.

Target	Primer	Sequence
TNFα	FP	5′-TGT CTC AGC CTC TTC TCA TTC C-3′
	RP	5′-TGA GGG TCT GGG CCA TAG AAC-3′
IL-1β	FP	5′-GTT GAC GGA CCC CAA AAG AT-3′
	RP	5′-CAC ACA CCA GCA GGT TAT CA-3′
IL-6	FP	5′-CCA TAG CTA CCT GGA GTA CA-3′
	RP	5′-GGA AAT TGG GGT AGG AAG GA-3′
CXCL1	FP	5′-GAT TCA CCT CAA GAA CAT CCA GAG-3′
	RP	5′-GAA GCC AGC GTT CAC CAG AC-3′
CXCL2	FP	5′-AAG TTT GCC TTG ACC CTG AAG-3′
	RP	5′-ATC AGG TAC GAT CCA GGC TTC-3′
ICAM-1	FP	5′-CAA GGA GAT CAC ATT CAC GG-3′
	RP	5′-CTT CCA GGG AGC AAA ACA AC -3′

FP, forward primer; RP, reverse primer.

2.6. Protein carbonyl content measurement

The protein carbonyl was measured in whole liver homogenate using a Protein Carbonyl kit (Cell biolabs, Tokyo, Japan). The assay was performed according to the manufacturer's instructions.

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

RNA from mouse liver was isolated using RNAiso according to the manufacturer's instructions. Tumor necrosis factor α (TNFα), Interleukin (IL)-1β, IL-6, Chemokine (C-X-C motif) ligand 1, 2 (CXCL1, CXCL2), intercellular adhesion molecule-1 (ICAM-1), and Gapdh were quantified by real-time RT-PCR. The primer sequences used in this study are shown in Table 1. The reverse transcription process and real-time RT-PCR were performed as described previously (Kobayashi et al., 2009).

2.8. Statistical analysis

Data are presented as mean ± SD. Comparison of 2 groups was made with an unpaired, two-tailed Student's *t*-test. Comparison of multiple groups was made with ANOVA followed by Dunnett or Tukey test. A value of $P < 0.05$ was considered statistically significant.

3. Results

3.1. The effects of E2 and Prog on halothane-induced liver injury

To investigate the role of the female sex hormones on HILI, female BALB/c mice pretreated with E2 or Prog were administered with HAL at a dose of 30 or 15 mmol/kg, respectively. The plasma E2 levels were 87.72 ± 15.98 pg/mL in mice 24 h after the last E2 pretreatment and 42.39 ± 18.85 pg/mL in mice pretreated with vehicle. The plasma Prog levels was 80.43 ± 33.25 ng/mL in mice 24 h after the last Prog pretreatment and 29.24 ± 14.77 ng/mL in mice pretreated with vehicle. Plasma transaminase levels were not changed 3 and 6 h after the HAL administration (data not shown). In addition, E2 or Prog pretreatment only did not affect the plasma transaminase levels 3 and 6 h after the HAL administration (data not shown). At 24 h after the HAL administration, E2 pretreatment significantly decreased the plasma transaminase levels compared with HAL (30 mg/kg) alone and Prog pretreatment caused a remarkable increase of the plasma transaminase levels compared to HAL (15 mg/kg) alone. These effects were significantly inhibited by pretreatment with ICI (ER antagonist) or RU (PR antagonist) in E2 or Prog pretreated groups, respectively (Fig. 1A). Histopathological changes demonstrated that E2 pretreatment decreased hepatocyte degeneration and damage lesions, which were enhanced by Prog pretreatment. In addition, immunohistochemical analyses with anti-MPO antibody demonstrated that E2 pretreatment decreased the numbers of MPO-positive cells infiltrated in liver at 24 h after HAL administration, whereas Prog pretreatment increased (Fig. 1B). From these results, pretreatment of mice with E2 attenuated HILI, whereas pretreatment of mice with Prog exacerbated HILI and these effects were likely mediated via each hormone receptor. Iso,

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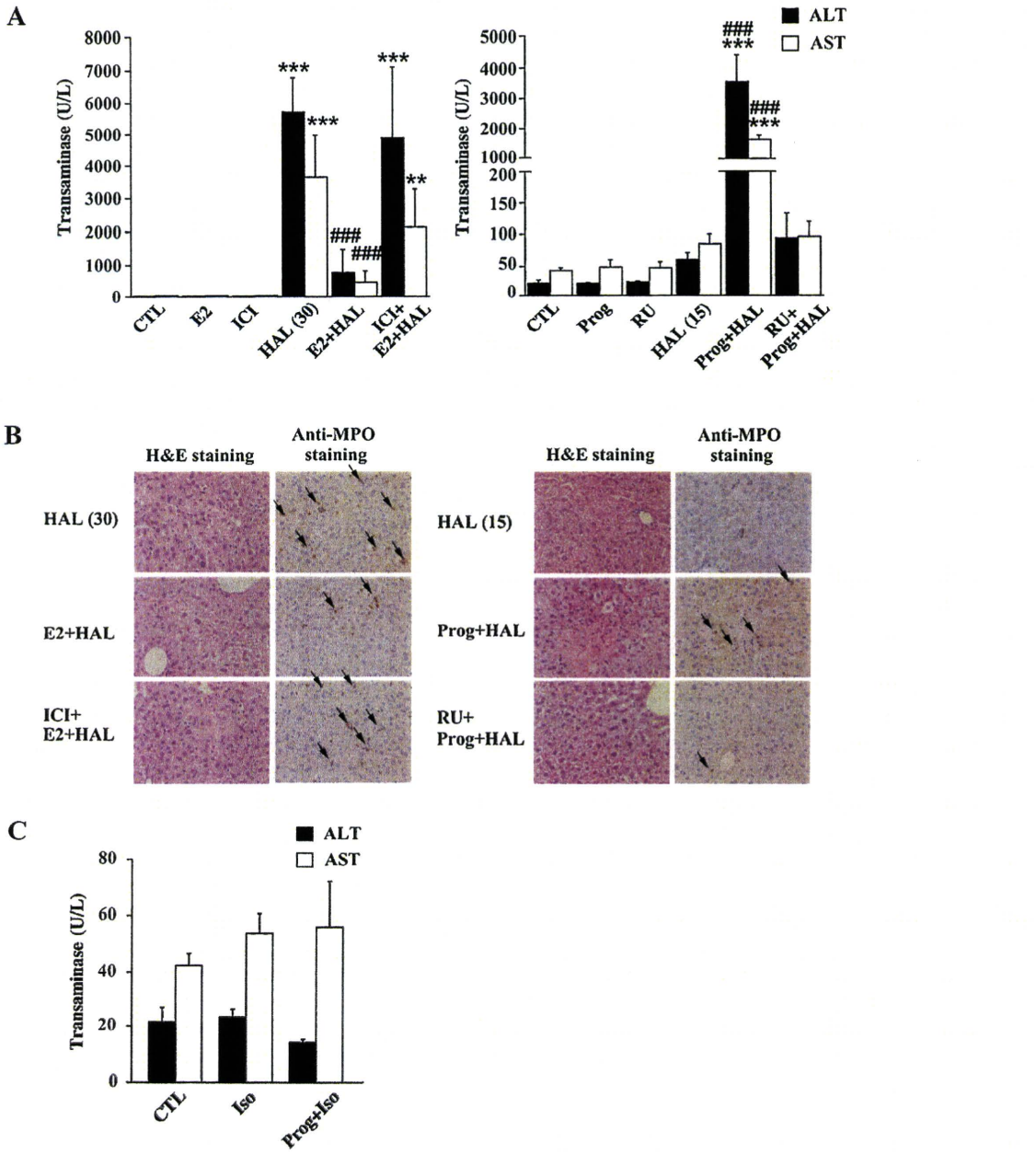

Fig. 1. Effects of E2 or Prog administration in HAL-induced liver injury. Mice (female, 8-week old) were pretreated with E2 (0.3 μg/mouse, *s.c.*), Prog (0.3 mg/mouse, *s.c.*) or vehicle (CTL: olive oil) for 7 days followed by HAL administration (15 or 30 mmol/kg, *i.p.*) or Iso administration (15 mmol/kg, *i.p.*) 1.5 h after the last treatment of E2 or Prog. In experiments using antagonist, mice were treated with ICI (50 μg/mouse, *s.c.*) or RU (50 μg/mouse, *s.c.*) 0.5 h prior to the treatment with E2 or Prog, respectively for 7 days. Twenty-four hours after the HAL or Iso administration, serum samples were collected for assessment of the transaminase levels (A and C). Liver tissue sections were stained with H&E or immunostained with anti-MPO antibody (B). Arrows indicate MPO-positive cells. The data are mean ± SD of 4–5 mice. $^{**}P < 0.01$ and $^{***}P < 0.001$, campared with CTL. $^{\#\#\#}P < 0.001$, compared with only HAL-administered mice.

structurally and pharmacologically similar to HAL, is less hepatotoxic than HAL (Njoku et al., 1997). In female BALB/c mice, the transaminase levels were not increased at doses up to 30 mmol/kg of Iso (data not shown). In addition, Prog pretreatment showed no effects on the serum transaminase levels at 24 h after the Iso administration (Fig. 1C).

3.2. E2 and Prog had no effect on the metabolic activation of halothane or on the oxidative stress

HAL is metabolized by CYP2E1 to reactive metabolites, trifluoroacetyl radicals, which bind a number of hepatic proteins in human, rats and mice (Bourdi et al., 2001; Njoku et al., 1997; Gut et al., 1993). To investigate whether the pretreatment with E2 or Prog affected the metabolic activation of HAL, immunoblotting was performed using anti-TFA polyclonal antibody and goat anti rat Cyp2e1 antibody. No difference was found in either the profiles or levels of TFA–protein adducts formed in the liver of mice pretreated with E2 or Prog 24 h after the HAL administration (Fig. 2A). In addition, the expression levels of Cyp2e1 protein in E2- or Prog-pretreated mouse liver also showed no changes (Fig. 2B). GSH plays a protective role against HILI in guinea pig (Lind et al., 1992). To investigate whether GSH and oxidative stress were modulated by pretreatment with E2 or Prog, the ratio of GSH/GSSG and

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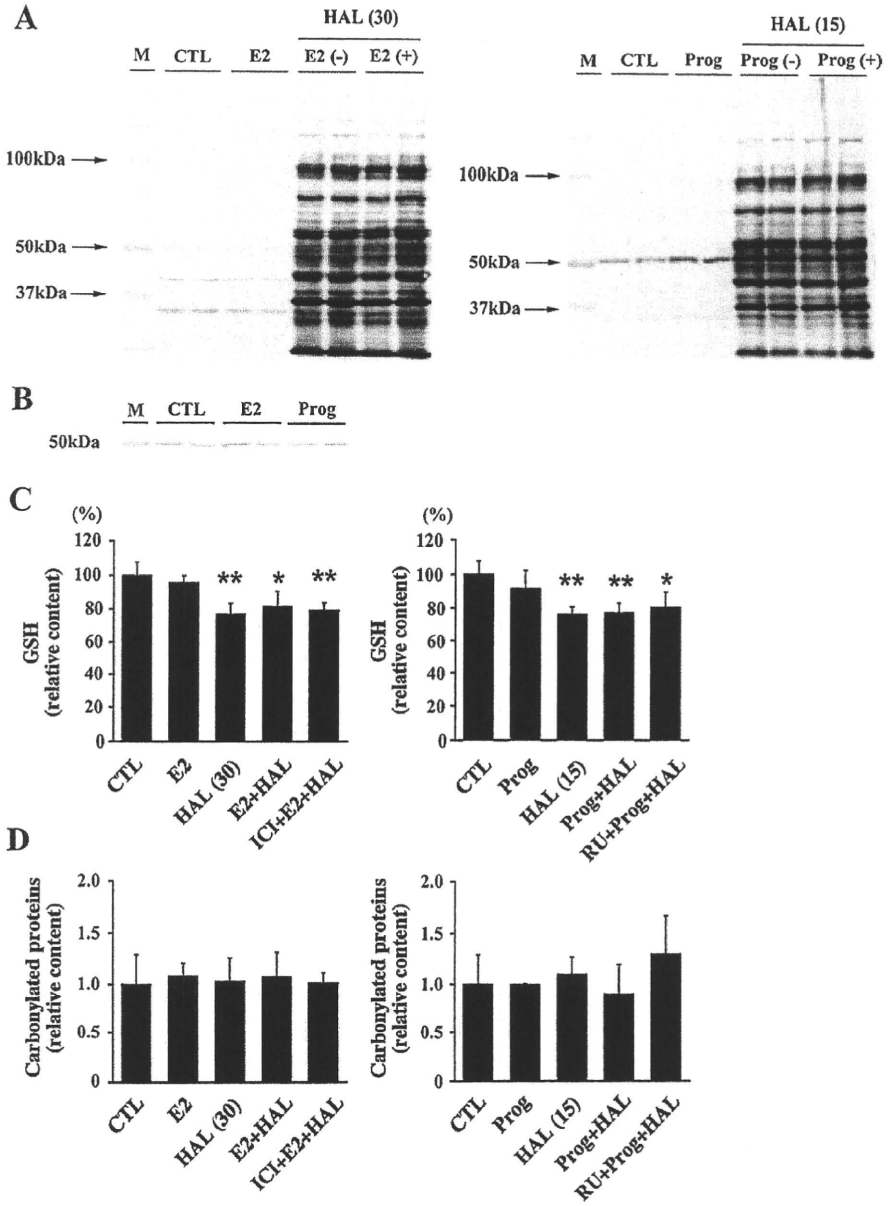

Fig. 2. Effects of E2 or Prog administration on the degree of metabolic activation, the ratio of GSH/GSSG and oxidative stress in the liver in HAL-induced liver injury. Experimental conditions for animal treatments were the same as those in Fig. 1. Twenty-four hours after the HAL administration, liver samples were collected. Immunoblot of TFA–protein adducts (A), Cyp2e1 protein expression (B) and Gapdh, a lording control, were performed using whole liver homogenate. Each lane shows an individual mouse (30 μg/lane). The ratio of GSH/GSSG (C) and carbonylated proteins (D) in whole liver homogenate were measured. The data are mean ± SD of 4 mice. $^{*}P < 0.05$ and $^{**}P < 0.01$, campared with CTL. M: molecular weight marker.

carbonylated proteins, a biomarker of oxidative stress, in the liver were measured. The ratio of GSH/GSSG of the HAL-administered groups were significantly decreased to 50–70% of the control group, but there was no effect on the ratio of GSH/GSSG in E2- or Prog-pretreated mouse liver (Fig. 2C). In addition, the levels of carbonylated proteins were also not altered (Fig. 2D). These results indicated that E2 and Prog had no effect on the metabolic activation of HAL or on the oxidative stress.

3.3. E2 and Prog modulated the immune responses in halothane-induced liver injury

In many cases, inflammation reactions and immune-related cells play a crucial role in DILI. In HILI, proinflammatory cytokines and neutrophils play important roles in the propagation of tissue damage (You et al., 2006; Kobayashi et al., 2009; Cheng et al., 2010). To investigate whether E2 or Prog pretreatment affect the production of inflammatory cytokines (TNFα, IL-1β and IL-6), potent neutrophil chemotactic chemokines, CXCL1 and CXCL2, and ICAM-1, which are important in the transendothelial migration of neutrophils, hepatic mRNA was measured. E2 pretreatment alone did not affect the production of proinflammatory cytokines, chemokines and ICAM-1 (Fig. 3). The expression of TNFα, IL-1β and IL-6 were significantly increased compared with CTL at indicated time after the HAL administration (Fig. 3A). Similarly, CXCL1 and ICAM-1 were significantly increased compared with CTL at 3 and 6 h after HAL administration and CXCL2 was markedly increased at 24 h after the HAL administration (Fig. 3B). Moreover, E2 pretreat-

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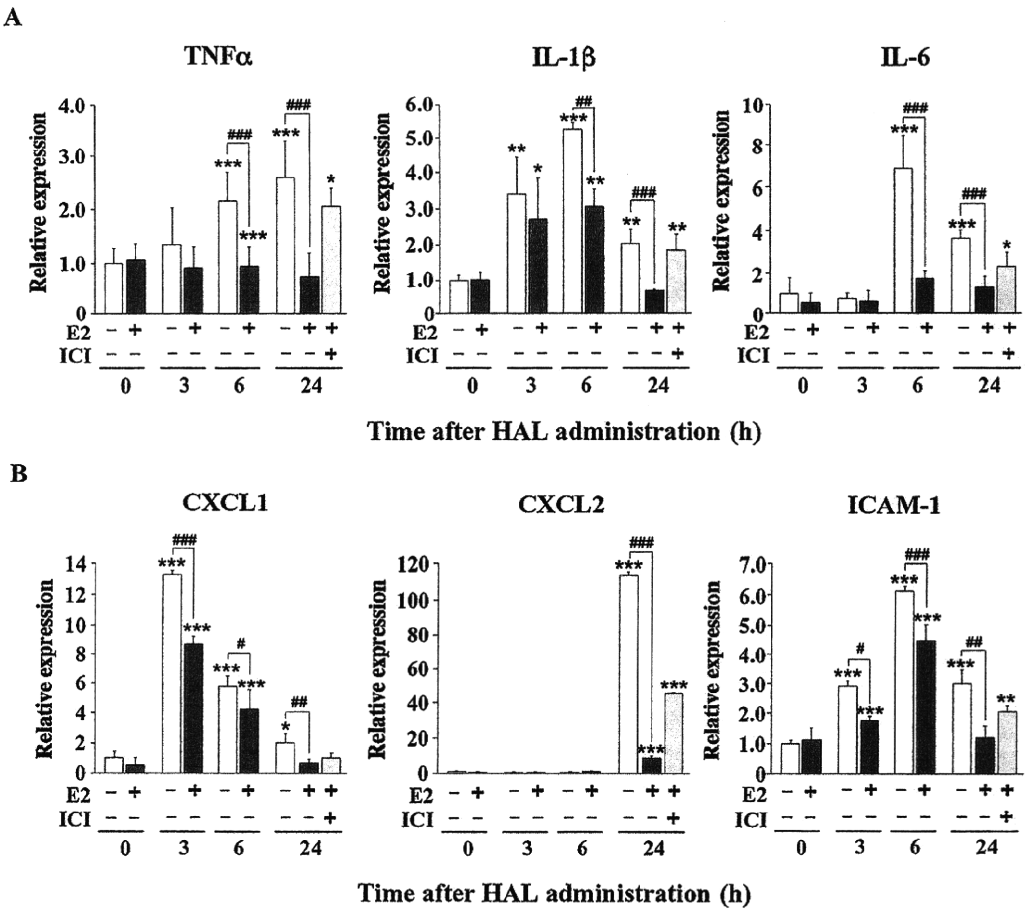

Fig. 3. Time-dependent changes of immune responses by E2 pretreatment in HAL-administered mice. Experimental conditions for animal treatments were the same as those in Fig. 1. Relative expression of hepatic mRNA was measured for proinflammatory cytokines (A) and chemokines and ICAM-1 related to neutrophil infiltration (B) in the E2-pretreated mouse liver 0, 3, 6 and 24 h after the HAL administration. Expression of hepatic mRNA was normalized to Gapdh mRNA. The data are mean ± SD of 4–5 mice. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with CTL. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, compared with only HAL-administered mice.

ment significantly decreased inflammatory mediators compared with HAL alone. These effects of E2 were blocked by ICI administration (Fig. 3).

Interestingly, Prog pretreatment alone significantly upregulated CXCL1, whereas proinflammatory cytokines, CXCL2 and ICAM-1 did not change (Fig. 4). Prog pretreatment significantly increased the expression of TNFα, IL-1β and IL-6 at indicated time after the HAL administration (Fig. 4A). Similarly, Prog pretreatment significantly increased CXCL1, CXCL2 and ICAM-1 at indicated time after the HAL administration (Fig. 4B). These effects of Prog were blocked by RU administration (Fig. 4). These results suggested that E2 and Prog played a crucial role in HILI by modulating the hepatic inflammation.

3.4. E2 and Prog modulated the production of IL-17 in halothane-induced liver injury

We previously demonstrated that IL-17 is involved in HILI (Kobayashi et al., 2009). To investigate whether E2 or Prog pretreatment affect the production of IL-17, plasma IL-17 level was measured. The IL-17 level was significantly increased only 24 h after the HAL (30 mmol/kg) administration, but not HAL (15 mmol/kg) administration (Fig. 5). E2 or Prog pretreatment alone did not affect the IL-17 levels. As with the expression of inflammatory mediators (Figs. 3 and 4), E2 pretreatment significantly decreased and Prog pretreatment significantly increased the plasma IL-17 levels (Fig. 5). These effects of E2 or Prog were blocked by ICI or RU administration, respectively (Fig. 5).

4. Discussion

Generally, women exhibit worse outcomes from liver injury than men (Lucena et al., 2009; Andrade et al., 2005; Russo et al., 2004). In this study, we focused on two major female sex hormones, E2 and Prog. The circulating levels of E2 and Prog fluctuate as a result of the reproductive phase and pregnancy in women (Wood et al., 2007; Barkley et al., 1979). E2 reduces the severity of various types of liver injuries such as ischemia-reperfusion, trauma-hemorrhage and acetaminophen, but there is little information about the role of Prog in liver injury (Yokoyama et al., 2005; Chandrasekaran et al., 2011; Shimizu et al., 2008). It has been reported that immune-based diseases may have exacerbations during the reproductive phase (Ansar et al., 1985; Ostensen, 1999). Since the activation of immune cells plays an important role in HILI (You et al., 2006; Kobayashi et al., 2009; Cheng et al., 2010), we hypothesized that female sex hormones would affect HILI.

To investigate whether two female sex hormones affect HILI, mice were pretreated with E2 or Prog for 7 days followed by HAL administration. In this study, the E2 and Prog concentrations were

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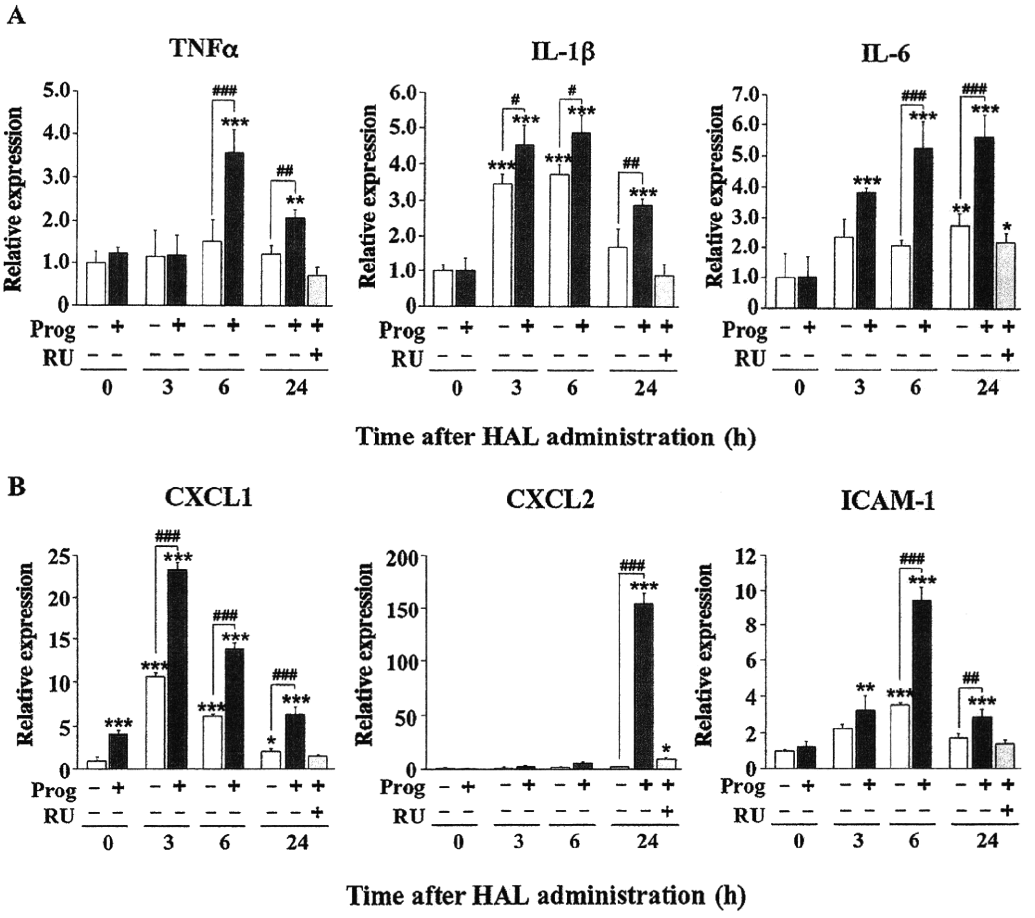

Fig. 4. Time-dependent changes of immune responses by Prog pretreatment in HAL-administered mice. Experimental conditions for animal treatments were the same as those in Fig. 1. Relative expression of hepatic mRNA was measured for proinflammatory cytokines (A) and chemokines and ICAM-1 related to neutrophil infiltration (B) in the Prog-pretreated mouse liver 0, 3, 6 and 24 h after the HAL administration. Expression of hepatic mRNA was normalized to Gapdh mRNA. The data are mean ± SD of 4–5 mice. $^{*}P<0.05$, $^{**}P<0.01$ and $^{***}P<0.001$, compared with CTL. $^{\#}P<0.05$, $^{\#\#}P<0.01$ and $^{\#\#\#}P<0.001$, compared with only HAL-administered mice.

higher in E2 or Prog-pretreated mice than the vehicle treated mice. The plasma E2 levels were 87.72 ± 15.98 pg/mL in mice pretreated with E2 and the plasma Prog levels were 80.43 ± 33.25 ng/mL in mice pretreated with Prog. In general, E2 and Prog secretion increased to maximum during the late pregnancy, plasma E2 level of 60–120 pg/mL and plasma Prog level of 60–120 ng/mL, respectively (Barkley et al., 1979). Therefore, mice pretreated with E2 or Prog in the present study was almost the same as those during

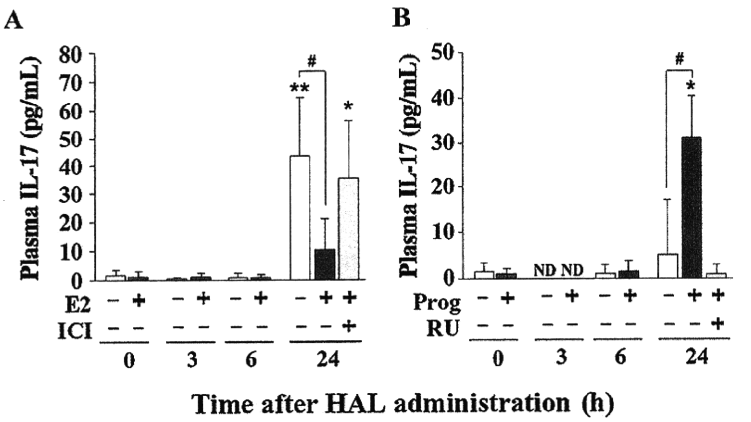

Fig. 5. Time-dependent changes of plasma IL-17 by E2 or Prog pretreatment in HAL-administered mice. Experimental conditions for animal treatments were the same as those in Fig. 1. Plasma IL-17 level was measured in mice pretreated with E2 or Prog 0, 3, 6 and 24 h after the HAL administration (E2; 30 mmol/kg, Prog; 15 mmol/kg). The data are mean ± SD of 4–5 mice. $^{*}P<0.05$ and $^{**}P<0.01$, compared with CTL. $^{\#}P<0.05$, compared with only HAL-administered mice. ND: not detected.

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late pregnancy. The transaminase levels and hepatic damage in HILI were attenuated by E2 pretreatment and exacerbated by Prog pretreatment at 24 h but not at 3 and 6 h after the HAL administration (Fig. 1). Neutrophils play a crucial role in the pathology of HILI (You et al., 2006; Kobayashi et al., 2009; Cheng et al., 2010). In this study, MPO-positive cells that infiltrated in the liver were decreased by E2 pretreatment and increased by Prog pretreatment suggesting that E2 and Prog may modulate the degree of HILI via neutrophils. Iso is widely used clinically and shows less hepatotoxicity than HAL (Njoku et al., 1997). No changes in transaminases were observed in mice administered Iso alone. Moreover, Prog pretreatment did not affect the transaminase levels in Iso-administered mice (Fig. 1C). These results indicated that Prog exacerbates the severity of liver injury rather than causing the liver injury. These results may relate to the fact that women exhibit worse outcomes in DILI (Lucena et al., 2009; Andrade et al., 2005; Russo et al., 2004). Moreover, these results indicated that the balance of E2 and Prog changed as a result of the reproductive phase and pregnancy in women affect the severity of DILI.

It has been suggested that TFA–protein adducts caused mild hepatotoxicity and initiated immune reactions (Bourdi et al., 2001; Njoku et al., 1997; Gut et al., 1993). E2 and Prog pretreatment did not affect TFA–protein adduct formation and Cyp2e1 expression (Fig. 2A and B). It was also reported that GSH-depleted guinea pigs exhibited a significant enhancement of HILI (Lind et al., 1992). Recently, it was reported that E2 attenuated APAP-induced liver injury by inhibiting oxidative stress (Chandrasekaran et al., 2011). However, in the present study E2 and Prog pretreatment had no effect on the depletion of the ratio of GSH/GSSG and protein carbonyl contents after HAL administration (Fig. 2C and D). These results indicated that the effects of E2 or Prog may not be involved in the metabolic activation of HAL and oxidative stress. Thus, it is suggested that E2 and Prog are related to another pathway in the pathogenesis of HILI.

Activated immune cells contribute to enhancing liver injury by releasing proinflammatory cytokines and chemokines to further hepatic inflammation, which determines the extent of liver injury (Kaplowitz, 2005; Adams et al., 2010). It has been demonstrated that proinflammatory cytokines, such as TNFα, IL-1β and IL-6, were decreased by E2 pretreatment and enhanced by Prog pretreatment in HILI. In addition, CXC chemokines (CXCL1 and CXCL2) and ICAM-1, which play an important role in neutrophil infiltration, were decreased by E2 pretreatment and enhanced by Prog pretreatment (Figs. 3 and 4). It is reported that transaminase levels achieved peak at 24 h after the HAL administration (You et al., 2006; Kobayashi et al., 2009). Although transaminase levels were not increased at earlier time point (3 or 6 h), the expression levels of TNFα, IL-1β, IL-6, CXCL1 and ICAM-1 were modulated by E2 and Prog pretreatment at earlier time point after HAL administration (Figs. 3 and 4). These results suggested that E2 and Prog affect the severity of HILI by modulating the production of proinflammatory cytokines and chemokines. CXC chemokines and ICAM-1 mRNA expression levels were correlated with neutrophil infiltration and accumulation in the histopathology of HILI (Figs. 1, 3 and 4). CXC chemokines are considered to attract predominantly neutrophils to the liver under stress conditions and the neutrophils undergo adhesion to hepatocytes via hepatocyte ICAM-1 (Ramaiah and Jaeschke, 2007). Furthermore, the roles of neutrophils have been demonstrated in a variety of liver diseases (Ramaiah and Jaeschke, 2007; Li et al., 2004; Frink et al., 2007). It is suggested that these mediators are related to neutrophil infiltration into the liver in HILI.

Interestingly, CXCL1 is increased by Prog pretreatment. CXCL1 not only mediates neutrophil infiltration, but also causes hepatotoxicity effects itself, which lead to massive liver necrosis in preinjured liver (Ramaiah and Jaeschke, 2007; Li et al., 2004; Frink et al., 2007; Stefanovic et al., 2005). In addition, previous studies have demonstrated the important role of CXCL1 in liver injury such as trauma-hemorrhage, endotoxemia and CCl_4-induced liver injury (Shimizu et al., 2008; Ramaiah and Jaeschke, 2007; Li et al., 2004; Frink et al., 2007; Stefanovic et al., 2005). It was also reported that recombinant CXCL1 had no effect in normal liver (Stefanovic et al., 2005). In accordance with these findings, Prog pretreatment had no hepatotoxic effect in normal mice and exacerbated liver injury by HAL administration but not by Iso administration (Fig. 1). We previously reported that IL-17 is involved in HILI (Kobayashi et al., 2009). In this study, changes of IL-17 were correlated with transaminases after HAL administration. E2 or Prog pretreatment alone do not affect the levels of IL-17 (Figs. 1 and 5). Although IL-17 does not initiate an immune response, IL-17 is able to amplify immune response of the early stage (Maione et al., 2009). IL-17-induced immune response was significantly reduced in mice treated with anti-CXCL1 antibody (Maione et al., 2009). Therefore, CXCL1 might play an important role in the progression of HILI by Prog pretreatment in the early stage of immune response.

Previous studies reported that E2 and Prog modulate proinflammatory cytokine production by murine peritoneal macrophages and human mononuclear cells in vitro. These reports also demonstrated that the hormonal effects were mediated by hormonal receptors (Huang et al., 2008; Yuan et al., 2008). In addition, E2 decreased the production of CXCL1 from KC via ER following trauma-hemorrhage (Shimizu et al., 2008). KCs act as the major source of proinflammatory cytokines (TNFα, IL-1β and IL-6) and CXC chemokines under severe stress and various liver injuries (Kaplowitz, 2005; Adams et al., 2010; Laskin, 1990; Mosher et al., 2001). The effects of E2 and Prog on HILI and immune responses were blocked by ICI or RU administration, respectively (Figs. 1, 3 and 4) indicating that the effects of E2 and Prog on HILI could be mediated via ER and PR, respectively. Thus, it might be thought that E2 and Prog affect immune cells responding to E2 and Prog via each receptor, such as monocytes or macrophages, which might result in modulation the immune reaction in HILI.

In conclusions, this study indicated that E2 attenuated and Prog exacerbated the severity of HILI via immune-related reactions. This is the first report that E2 and Prog modulated the severity of HILI. It is also indicated that the E2/Prog hormone balance might represent a risk factor for HILI and that female sex hormones might have a role in one of the mechanism of sex differences in HILI.

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Conflict of interest

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

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Development of A Highly Sensitive Cytotoxicity Assay System for CYP3A4-mediated Metabolic Activation

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This manuscript consists of 23 pages of text, 4 figures, and 40 references.

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Abbreviation: CCK-8, Cell Counting kit-8; CYP, Cytochrome P450; DMEM, Dulbecco's modified eagle's medium; GCLC, glutamate-cysteine ligase, catalytic subunit; GCLM, glutamate-cysteine ligase, modifier subunit; GSH, glutathione; 3HAA, 3-hydroxyacetanilide; HO-1, heme oxygenase-1; MOI, multiplicity of infection; NQO1, NADPH-quinone oxidoreductase; RT, reverse transcription; PCR, polymerase chain reaction; TBF-A, 7,7-dimethylhept-2-ene-4-ynal