Mechanism of Exacerbative Effect of Progesterone on Drug-Induced Liver Injury

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Received August 23, 2011; accepted November 22, 2011

Drug-induced liver injury (DILI) is a major safety concern in drug development and clinical drug therapy. However, the underlying mechanism of DILI is little known. It is generally believed that women exhibit worse outcomes from DILI than men. Recently, we found that pretreatment of mice with estradiol attenuated halothane (HAL)-induced liver injury, whereas pretreatment with progesterone exacerbated it in female mice. To investigate the mechanism of sex difference of DILI, we focused on progesterone in this study. We found the exacerbating effect of progesterone in thioacetamide (TA), α-naphthylisothiocyanate, and dicloxacillin-induced liver injury only in female mice. Higher number of myeloperoxidase-positive mononuclear cells infiltrated into the liver and increased levels of Chemokine (C-X-C motif) ligand 1 and 2 (CXCL1 and CXCL2) and intercellular adhesion molecule-1 in the liver were observed. Interestingly, CXCL1 was slightly increased by progesterone pretreatment alone. Progesterone pretreatment increased the extracellular signal-regulated kinase (ERK) phosphorylation in HAL-induced liver injury. Pretreatment with U0126 (ERK inhibitor) significantly suppressed the exacerbating effect of progesterone and the expression of inflammatory mediators. In addition, pretreatment with gadolinium chloride (GdCl3; inhibitor of Kupffer cells) significantly suppressed the exacerbating effect of progesterone pretreatment and the expression of inflammatory mediators. Moreover, post-treatment of RU486 (progesterone receptor antagonist) 1 h after the HAL or TA administration ameliorated the HAL- or TA-induced liver injury, respectively, in female mice. In conclusion, progesterone exacerbated the immune-mediated hepatoxic responses in DILI via Kupffer cells and ERK pathway. The inhibition of progesterone receptor and decrease of the immune response may have important therapeutic implications in DILI.

Key Words: drug-induced liver injury; CXCL1; sex difference; Kupffer cell; progesterone receptor antagonist.

Drug-induced liver injury (DILI) is the most frequent reason for the withdrawal of an approved drug from the market and for failures in drug development in pharmaceutical companies. In most cases, the mechanisms of hepatotoxicity are not elucidated, but it is likely to arise from complex interactions among drug properties, daily dose, genetic variations, age, sex, diseases, and environmental factors (Chalasani and Björnsson, 2010; Li, 2002). In general, women are more susceptible to liver injury by therapeutic drugs than men. Seventy-four percent of all acute liver failure cases are women (Miller, 2001). It has been reported that 78% of DILI cases are in women and a significantly greater number of women show DILI than men (Björnsson and Olsson, 2005; DeValle et al., 2006; Ostapowicz et al., 2002). Although some reports described that female sex is not a predisposing factor for DILI, it was also reported that patients with severe DILI who underwent liver transplantation were more frequently women (76%) and that nearly 90% of patients with fulminant liver injury from DILI were women (Andrade et al., 2005; Lucena et al., 2009; Russo et al., 2004). From these lines of study, women appear to be at greater risk of developing severe liver injury, but it is not clear why women exhibit the worst outcomes from liver injury.

It has been reported that 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). Moreover, immune-mediated diseases in women may be exacerbated during the reproductive phase (Ansar et al., 1985; Ostensen, 1999). Circulating levels of estradiol (E2) and progesterone fluctuate as a result of the reproductive phase and pregnancy in females (Barkley et al., 1979; Wood et al., 2007). There is evidence that the immune system is regulated by circulating level of sex steroid hormones, E2, progesterone, and testosterone (Grossman, 1985). It was also reported that E2 decreased and progesterone increased the production of proinflammatory cytokines in oxidative stress-stimulated murine peritoneal macrophage and human mononuclear cells and their receptor antagonists, ICI 182,780 and RU486, blocked these effects, respectively (Huang et al., 2008; Yuan et al., 2008). Recently, there have been many reports that immune reactions may have a critical role in DILI and that hepatic inflammation determines the
extent of liver injury (Adams et al., 2010; Deng et al., 2009; Holt and Ju, 2006). However, there has been little information concerning the involvement of female sex hormones in DILI. There are some reports that E2-attenuated liver injury caused by ischemia-reperfusion, trauma-hemorrhage, and acetaminophen (APAP) (Chandrasekaran et al., 2011; Shimizu et al., 2008; Yokoyama et al., 2005), but there is little information about the effect of progesterone in liver injury.

We recently reported that the progesterone pretreatment exacerbated the immune-mediated hepatotoxic responses in halothane (HAL)-induced liver injury in female mice (Toyoda et al., 2011). In this study, we investigated the underlying mechanism of the progesterone-induced exacerbation of DILI using a mouse model.

MATERIALS AND METHODS

Materials. HAL was purchased from Takeda Yakuhin (Osaka, Japan) and Isoflurane (ISO) was from Abbott Japan (Tokyo, Japan). Progesterone, gadolinium chloride (GdCl3), and dicycloxaclin (DCX) were purchased from Sigma-Aldrich (St Louis, MO). Mifepristone (RU486) and 9-naphthylisothiocyanate (ANIT) were from Tokyo Kasei (Tokyo, Japan). U0126, SB203580, and thioacetamide (TA) were from Wako Pure Chemical Industries (Osaka, Japan). SP600125 was from Calbiochem (Los Angeles, CA). ICI 182,780 (IC) was from TOCRIS Bioscience (Ellisville, MO). Fuji Dri-Chem slides of GPT/ALT-PII and GOT/AST-PII to measure alanine aminotransferase (ALT)/glutamic pyruvic transaminase (GPT) and aspartate aminotransferase (AST)/glutamic oxaloacetic transaminase (GOT), respectively, were from Fuji Film Med. Co. (Tokyo, Japan). Rabbit polyclonal antibody against mouse myeloperoxidase (MPO) was from DAKO (Carpinteria, CA). Rat polyclonal antibody against F4/80 was from U.K.-Serotec (Oxford, U.K.). The monoclonal antibodies of anti-Thr202/Tyr204 phosphorylated extracellular signal-regulated kinase (ERK) 1/2, anti-Thr180/Tyr182 phosphorylated p38 mitogen-activated protein (MAP) kinase, and anti-Thr183/Tyr185 phosphorylated c-Jun N-terminal kinase (JNK) 1/2 were purchased from Cell Signaling Technology (Beverly, MA). The monoclonal antibodies against ERK1/2 and JNK1/2 and the polyclonal antibody against p38 MAP kinase were also from Cell Signaling Technology. All primers were commercially synthesized at Hokkaido System Sciences (Sapporo, Japan). All other chemicals were of the highest grade commercially available.

Animals. Female BALB/cCrSlc mice (8 weeks old, 20–25 g) were obtained from SLC Japan (Shizuoka, 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 performed 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.

Administration of hepatotoxic compounds in progesterone-pretreated mice. The progesterone pretreatment methods were described previously (Toyoda et al., 2011). In brief, female mice were pretreated with progesterone (0.3 mg/mouse, sc) for 7 days followed by the administration of HAL (15 or 30 mmol/kg, ip), TA (50 mg/kg, ip), ANIT (80 mg/kg, po), DCX (600 mg/kg, ip), or ISO (15 mmol/kg, ip) 1.5 h after the last treatment of progesterone. In the ANIT experiments, the mice were fasted for 15 h prior to the ANIT administration. Six hours after DCX administration and 24 h after HAL, TA, ANIT, or ISO administration, the mice were sacrificed, and the plasma and the liver were collected. 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 AST and ALT levels were determined using Fuji Dri-Chem 4000V (Fuji Film Med. Co.). The mononuclear cells infiltration was assessed by immunostaining for MPO as previously described (Kumada et al., 2004).

Administration of HAL in U0126- or GdCl3-pretreated mice. Mice were pretreated with progesterone for 7 days. In experiments using ERK inhibitor, mice were treated with U0126 (ERK inhibitor, 10 mg/kg, ip) 1 h before the HAL administration (30 mmol/kg, ip). In experiments using an inhibitor of Kupffer cells, mice were treated with GdCl3 (10 mg/kg, iv) 24 and 48 h before the HAL administration (30 mmol/kg, ip). Twenty-four hours after the HAL administration, the mice were sacrificed. It was reported that a 40–61% reduction of the number of Kupffer cells in the mouse liver tissue occurred when treated with GdCl3 in this method (Mosher et al., 2001).

Administration of RU486 and HAL in mice. Mice were pretreated with RU486 (progesterone receptor antagonist, 50 μg/mouse, sc) for 7 days followed by HAL administration (30 mmol/kg, ip) 1.5 h after the last RU486 treatment, according to the method described previously (Toyoda et al., 2011). In the experiments of postadministration of RU486, mice were administered RU486 (1 mg/kg, iv) 1 h after the HAL administration (30 mmol/kg, ip). Twenty-four hours after the HAL administration, the mice were sacrificed.

Real-time reverse transcription PCR analysis. RNA from mouse liver was isolated using RNAiso according to the manufacturer’s instructions. Tumor necrosis factor α (TNFα), Chemokine (C-X-C motif) ligand 1 and 2 (CXCL1 and CXCL2), intercellular adhesion molecule-1 (ICAM-1), and glycerolaldehyde-3-phosphate dehydrogenase (GAPDH) were quantified by real-time reverse transcription (RT)PCR. The primer sequences used in this study are shown in Table 1. The RT process and real-time PCR were performed as described previously (Kobayashi et al., 2009).

Enzyme-linked immunosorbent assay. The CXC chemokines, CXCL1 and CXCL2, in plasma were measured by Quantikine Mouse CXCL1/KC ELISA and Quantikine Mouse CXCL2/MIP-2 ELISA (R&D Systems, Minneapolis, MN), respectively, according to the manufacturer’s instructions.

Immunoblot analysis. SDS-polyacrylamide gel electrophoresis and immunoblot analysis were performed according to Laemmli (1970). Whole liver homogenates (50 μg) were separated on 10% polyacrylamide gels and electrotransferred onto polyvinylidene difluoride membrane, Immobilon-P (Millipore Corporation, Billerica, MA). The membranes were probed with the monoclonal antibodies of anti-ERK1/2, anti-JNK1/2, anti-p38 MAP kinase, anti-Thr202/Tyr204 phosphorylated ERK1/2, anti-Thr183/Tyr185 phosphorylated JNK1/2, and anti-Thr180/Tyr182 phosphorylated p38 MAP kinase and the corresponding fluorescent dye–conjugated second antibody. An Odyssey Infrared Imaging system (LI-COR Biosciences, Lincoln, NE) was used for the detection. The relative expression level was quantified using ImageQuant TL Image Analysis software (GE Healthcare, Little Chalfont, Buckinghamshire, U.K.).
**RESULTS**

**Effect of Progesterone Pretreatment on the Time-Dependent Changes of Plasma Transaminase Levels in HAL-Induced Liver Injury**

To investigate the effects of progesterone pretreatment on the time-dependent changes of plasma transaminase levels in HAL-induced liver injury, mice pretreated with progesterone (0.3 mg/mouse, sc) were administered HAL (15 mmol/kg, ip), which resulted in a significant increase of the ALT and AST levels at 24 and 36 h after the HAL administration in female mice but not in male mice (Fig. 1A and Supplementary fig. 1A).

**Effect of Progesterone Pretreatment on the Time-Dependent Changes of CXC Chemokines in HAL-Induced Liver Injury**

To investigate whether the changes in liver injury in mice pretreated with progesterone after HAL administration resulted in increases of chemokines, we measured the hepatic messenger RNA (mRNA) expression and serum protein levels of CXCL1 and CXCL2. The hepatic CXCL1 and CXCL2 mRNA levels were markedly increased at 3 and 24 h after HAL administration in progesterone-pretreated mice, respectively (Fig. 1B). Interestingly, progesterone pretreatment alone increased CXCL1 mRNA (4.1-fold) and serum protein (2.3-fold) (0 h point in Figs. 1B and C). CXCL1 expression in response to HAL administration peaked at an earlier time point compared with CXCL2 expression. The time-dependent changes of the mRNA and protein levels were similar in CXCL1 and CXCL2. Thus, changes of mRNA expression were mainly followed in the subsequent experiments.

Among male mice, there was no marked difference in the mRNA expression levels of CXCL1 and CXCL2 after HAL administration in progesterone-pretreated mice compared with vehicle-pretreated mice (Supplementary fig. 1B). As with HAL-induced liver injury, the time-dependent changes of the transaminase levels and mRNA levels of CXCL1 and CXCL2 were similar to those associated with TA-induced liver injury (Supplementary fig. 2).

**Effects of Progesterone Pretreatment on Various Hepatotoxic Compound-Induced Liver Injury**

To investigate the effects of progesterone pretreatment on various compounds, TA, ANIT, DCX, or ISO were administered to the progesterone-pretreated mice. Female mice pretreated with progesterone showed significantly increased ALT and AST levels after the administration of TA, ANIT, or DCX, but not ISO, compared with vehicle-pretreated mice (Fig. 2A). However, male mice showed no effects on the transaminase levels by the administration of TA or ANIT as well as HAL (Supplementary fig. 3). Histopathological changes demonstrated that progesterone pretreatment enhanced TA- or ANIT-induced hepatocyte degeneration and damage. In addition, immunohistochemical analyses with anti-MPO antibody demonstrated that progesterone pretreatment increased the number of MPO-positive cells infiltrated in liver at 24 h after TA or ANIT administration (Fig. 2B). There was no change in either the histopathology findings or the number of MPO-positive cells in the liver of mice pretreated with progesterone alone. As with HAL-induced liver injury, hepatic mRNA level of CXCL1 and CXCL2 was increased significantly by progesterone pretreatment after TA, ANIT, and DCX administration compared with vehicle-pretreated mice (Fig. 2C).

**Effects of Progesterone Pretreatment on Activation of MAP Kinase-Signaling Pathway in HAL-Induced Liver Injury**

MAP kinases, including ERK1/2, p38 MAP kinase, and JNK1/2, are important components for many intracellular signaling pathways. The phosphorylation of MAP kinases, which are required for the enzyme activity, activates signaling cascades, the downstream effects of which have been linked to the regulation of the inflammatory responses (DeFranco et al., 1998). To clarify the role of the MAP kinase-signaling pathway in the liver after the HAL administration, the phosphorylation of ERK1/2, p38 MAP kinase, and JNK1/2 in liver were assessed by immunoblot analyses. Progesterone pretreatment alone significantly increased the phosphorylation of ERK in female mice but not in male mice (Fig. 3 and Supplementary fig. 4). The phosphorylation of ERK was also significantly increased in female mice by the HAL administration compared with control (Fig. 3). Progesterone pretreatment and HAL administration had no effect of the phosphorylation of p38 MAP kinase and JNK1/2 in female and male mice (data not shown).

**Effects of ERK Pathway on Progesterone Pretreatment–Induced Exacerbation of HAL-Induced Liver Injury**

To investigate whether the activation of the ERK pathway is involved in the progesterone pretreatment–induced exacerbation of liver injury, mice pretreated with progesterone were treated with an ERK inhibitor U0126, followed by the administration of
HAL. U0126 pretreatment decreased of the progesterone-induced phosphorylation of ERK by 50% (Fig. 4A). Although U0126 pretreatment alone did not affect the liver injury induced by HAL alone, U0126 pretreatment significantly decreased the progesterone-induced exacerbation of the HAL-induced liver injury (Fig. 4B). Notably, the CXCL1 mRNA levels increased by progesterone pretreatment alone were significantly decreased by U0126 treatment. Furthermore, U0126 pretreatment significantly decreased the CXCL1, CXCL2, and ICAM-1 mRNA levels increased by progesterone pretreatment after HAL administration (Fig. 4C).

Involvement of Kupffer Cells on Progesterone Pretreatment–Induced Exacerbation of HAL-Induced Liver Injury in Female Mice

Kupffer cells act as a major source of proinflammatory cytokines and CXC chemokines. To determine whether Kupffer
Cells are involved in the exacerbation of liver injury, mice pretreated with progesterone were treated with GdCl₃, an inhibitor of the Kupffer cell function, followed by the administration of HAL. Although GdCl₃ treatment did not affect the liver injury induced by HAL alone, GdCl₃ pretreatment significantly decreased the progesterone-induced exacerbation of the liver injury.

**FIG. 2.** Effects of progesterone pretreatment on various hepatotoxic compound–induced liver injury. Female mice were pretreated with progesterone (0.3 mg/mouse, sc) for 7 days followed by TA (50 mg/kg, ip), ANIT (80 mg/kg, po), DCX (600 mg/kg, ip), or ISO (15 mmol/kg, ip) administration 1.5 h after the last treatment of progesterone. Six hours after the administration of DCX and 24 h after the administration of TA, ANIT, or ISO, plasma and liver samples were collected for assessment of the transaminase levels (A). Liver tissue sections were stained with H&E or immunostained with anti-MPO antibody (B). Relative expression of hepatic mRNA was measured for CXCL1 and CXCL2 and was normalized to GAPDH mRNA (C). The data are mean ± SD of four mice. *p < 0.05, **p < 0.01, and ***p < 0.001, compared with CTL (control).
HAL-induced liver injury (Fig. 5A). The GdCl3 pretreatment alone did not affect the mRNA levels of CXCL1, CXCL2, and ICAM-1. The increased levels of CXCL1 mRNA by progesterone pretreatment alone were significantly decreased by GdCl3 treatment. Furthermore, GdCl3 pretreatment significantly decreased the CXCL1, CXCL2, and ICAM-1 mRNA levels in mouse liver pretreated with progesterone after HAL administration (Fig. 5B).

To confirm the effect of GdCl3 pretreatment, the number of Kupffer cells was evaluated using F4/80 antibody-staining method and counted number of the F4/80-positive cells microscopically. As shown in Supplementary fig. 5, the numbers of F4/80-positive cells were significantly decreased by GdCl3 pretreatment to 31 and 29% in vehicle-pretreated control mice and in progesterone-pretreated mice, respectively.

Effects of Progesterone on RAW264.7 Cells

To determine whether Kupffer cells are involved in the production of CXCL1 by progesterone treatment, mouse macrophage cell line RAW264.7 cells were treated with progesterone and measured for the expression of CXCL1 mRNA levels. The CXCL1 mRNA levels were significantly increased by progesterone exposure. The increased expression of CXCL1 mRNA was inhibited by cotreatment of RU486 (Fig. 6A). In addition, the increased expression of CXCL1 mRNA was inhibited by cotreatment of U0126 with progesterone but not by ICI, SP600125, or SB203580 (Fig. 6B).

Pretreatment of RU486 Ameliorates HAL-Induced Liver Injury in Female Mice

Since progesterone pretreatment exacerbated the liver injury mediated the activation of immune response, we hypothesized that progesterone receptor antagonist would ameliorate liver injury. To investigate the effect of progesterone receptor antagonist against DILI, mice pretreated with RU486 for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last treatment of RU486. RU486 pretreatment alone had no effect on the transaminase levels, but RU486 pretreatment significantly decreased the transaminase levels and mRNA levels of CXCL1, CXCL2, ICAM-1, and TNFα (Fig. 7).

Post-Administration of RU486 Ameliorates HAL-Induced Liver Injury

From a therapeutic point of view, a more clinically relevant approach is to treat RU486 after HAL administration. Therefore, mice were administered RU486 after the HAL administration (30 mmol/kg, ip). As with the pretreatment experiments, posttreatment of RU486 significantly decreased the transaminase levels and mRNA levels of CXCL1, CXCL2, ICAM-1, and TNFα in female mice (Fig. 8). Similarly, posttreatment of RU486 decreased the transaminase levels after TA administration in female mice (Supplementary fig. 6).

DISCUSSION

Progesterone, one of the female sex hormones, plays an important role in the female reproductive function. There is also evidence that the immune system is regulated by the circulating levels of sex hormones (Grossman, 1985). Our previous report demonstrated that progesterone pretreatment exacerbated HAL-induced liver injury, whereas E2 pretreatment resulted in the opposite effect in female mice (Toyoda et al., 2011). It was also demonstrated that HAL-induced liver injury was exacerbated in female mice in estrus, during which the plasma concentration of progesterone is elevated, and ovariectomized mice showed significantly suppressed HAL-induced liver injury (Dugan et al., 2011). These reports suggested that progesterone has an important effect in DILI, therefore, we put the focus on the mechanism of the progesterone-induced exacerbating effect of liver injury in this study.

To investigate the effect of progesterone in DILI, female BALB/c mice pretreated with progesterone were administered with hepatotoxicant. In this study, the plasma progesterone level was 80.4 ± 33.3 ng/ml in mice 24 h after the last progesterone pretreatment and 29.2 ± 14.8 ng/ml in mice pretreated with vehicle. In general, progesterone secretion increased to the maximum plasma progesterone level of 60–120 ng/ml during late pregnancy (Barkley et al., 1979). Thus, the serum progesterone levels of mice pretreated with progesterone in the present study was almost the same as during late pregnancy.
The transaminase levels, hepatic tissue damage, and mononuclear cells infiltration in HAL-, TA-, ANIT- and DCX-induced liver injury were exacerbated by progesterone pretreatment (Figs. 1 and 2). Progesterone pretreatment alone did not increase the transaminase levels in nontreated mice and did not affect the transaminase levels in ISO-administered mice. ISO is structurally and pharmacology similar to HAL but less hepatotoxic, indicating that the progesterone pretreatment exacerbated the severity of liver injury in female mice. Higher numbers of mononuclear cells infiltrated in the liver of mice pretreated with progesterone after hepatotoxic compound administration (Fig. 2B). In addition, it was demonstrated that the mRNA levels of CXCL1, CXCL2, and ICAM-1 were correlated with the infiltration and accumulation of MPO-positive cells. Most of MPO-positive cells were considered as neutrophils because of their nuclear morphology in the liver histopathology in this study. Neutrophils have an important role in various types of liver injury (Ramaiah and Jaeschke, 2007). 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. In this study, CXC chemokines, CXCL1 and CXCL2, were markedly increased after HAL administration in progesterone-pretreated female mice but not in male mice (Fig. 1 and Supplementary fig. 1). Interestingly, progesterone pretreatment alone increased CXCL1 and CXCL1 was quickly and significantly increased after the subsequent administration of hepatotoxic compounds. It was demonstrated that liver injury after carbon tetrachloride administration was exacerbated by injection of recombinant CXCL1, but injection of recombinant CXCL1 did not affect it in normal mice (Stefanovic et al., 2005). In accordance with this report, increased expression of CXCL1 by the progesterone pretreatment demonstrated no hepatotoxic effect in normal mice, but progesterone pretreatment exacerbated liver injury.

FIG. 4. Role of ERK pathway on the effect of progesterone pretreatment in HAL-induced liver injury. Female mice pretreated with progesterone for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last progesterone treatment, then U0126 (10 mg/kg, ip) was administered 1 h before the HAL administration. Whole liver homogenate was collected 3 h (A) and 24 h (B and C) after the HAL administration. Immunoblot of ERK proteins in whole liver homogenates collected 3 h after the U0126 administration was performed and quantified (A). Each lane shows an individual mouse (50 μg/lane). Plasma and liver samples were collected 24 h after the HAL administration to assess the transaminase levels (B) and expression of hepatic mRNA levels of chemokines and ICAM-1 (C). Expression of hepatic mRNA was normalized to GAPDH mRNA. The data are mean ± SD of 5–8 mice. *p < 0.05, **p < 0.01, and ***p < 0.001, compared with CTL. †††p < 0.001, compared with progesterone pretreatment alone. ###p < 0.001, compared with HAL-administered mice pretreated with progesterone.
in mice after hepatotoxicants administration, but not ISO administration, indicating that the increased expression of CXCL1 mediated the activation of immune responses after the hepatotoxicant administration.

Progesterone pretreatment method used in this study did not increase mRNA levels of CXCL1 and subsequent increase of transaminase levels after hepatotoxicants administration in male mice (Supplementary figs. 1 and 3). In human, autoimmune diseases, such as multiple sclerosis, were much higher incidence in women. Some reports indicated that male sex hormones, testosterone, have immunosuppressive effects, which may partly account for the sex difference of autoimmune diseases (Fijak et al., 2011; Gold and Voskuhl, 2009). In mice, serum testosterone levels was 6.9 ± 2.3 ng/ml in male mice and < 0.1 ng/ml in female mice (Bo¨sl et al., 2001). It is thought that testosterone may partly suppress immune activation such as increased expression of CXCL1 by progesterone pretreatment in male mice. It was also reported that liver injury after carbon tetrachloride administration was exacerbated by injection of recombinant CXCL1 in male mice (Stefanovic et al., 2005). Therefore, if CXCL1 is upregulated by modifying the progesterone pretreatment method, the liver injury might be exacerbated in male mice. However, further study of the precise mechanism by which male mice are not responsive to progesterone is needed.

It is suggested that the human serum level of Glo-α, the homolog of CXCL1, is correlated with female sex hormones (Kanda et al., 1997). It is well known that women have greater susceptibility to alcoholic liver injury than men. In alcoholic liver injury, Glo-α was significantly increased in human (Maltby et al., 1996). In addition, female rats fed an ethanol diet showed significantly increased ALT- and cytokine-induced neutrophil chemoattractant (CINC)-1 mRNA, the homolog of CXCL1, after lipopolysaccharide injection (Yamada et al., 1999). This report also demonstrated that gonadectomy totally abolished the sex difference of the CINC-1 mRNA expression. From these
mRNA expression in a progesterone-pretreated mouse macrophage cell line, RAW264.7 (Fig. 6). Thus, the ERK pathway in Kupffer cells may have an important role in the exacerbation of liver DILI by progesterone. Kupffer cells act as a major source of proinflammatory cytokines and CXC chemokines under severe stress and various types of liver injury (Adams et al., 2010; Kaplowitz, 2005; Laskin, 1990; Mosher et al., 2001). Progesterone increased the production of proinflammatory cytokines in monocyte and macrophages via progesterone receptor (Huang et al., 2008; Yuan et al., 2008). In the present study, the inhibition of Kupffer cells by GdCl3 did not affect the liver injury and immune response induced by HAL alone but significantly decreased the progesterone-induced exacerbation of HAL-induced liver injury and immune responses (Fig. 5). Moreover, the inhibition of Kupffer cells significantly decreased the CXCL1 mRNA expression increased by progesterone alone. Although it was reported that Kupffer cells do not contribute to HAL-induced liver injury, cotreatment with poly (I:C), ligand of TLR3, exacerbated the HAL-induced liver injury by activation of Kupffer cells (Cheng et al., 2009, 2010; Dugan et al., 2011). Considering these findings, progesterone led exacerbation of HAL-induced liver injury by activation of Kupffer cells and increased expression of CXCL1 in this study. In addition, GdCl3 attenuated progesterone-induced exacerbation of liver injury and expression of CXCL1.

In the present study, both pretreatment and posttreatment RU486, a potent progesterone receptor antagonist, significantly suppressed HAL- or TA-induced liver injury and immune responses (Figs. 7 and 8 and Supplementary fig. 6). Because progesterone affected the immune responses mediated by Kupffer cells, it is conceivable that RU486 also affects the immune responses mediated by Kupffer cells. The progesterone receptor is expressed on immune cells, natural killer cells, leukocytes as well as Kupffer cells (Gilliver, 2010). Therefore, the mechanism for the suppression of liver injury by RU486 may be due to the effect on immune cells. Moreover, RU486 is also a glucocorticoids (GCs) receptor antagonist. Recently, it was reported that GCs play a role in DILI and pretreatment with RU486 attenuated HAL- and APAP-induced liver injury (Masson et al., 2010). This report also showed that RU486 effects were diminished in adrenalectomized male mice. Thus, they concluded that RU486 play a pathologic role mediated via GC receptor. In general, GCs are thought to have a salutary effect on immune-mediated disease due to their immunosuppressive effects (Prais et al., 2006). Masson et al. (2010) also indicated that pretreatment with RU486 exacerbated carbon tetrachloride- and concanavalin A-induced liver injury in male mice. Recent report indicated that estrus cycle and female sex hormones affected HAL-induced liver injury (Dugan et al., 2011; Toyoda et al., 2011). Therefore, RU486 may affect the liver injury via progesterone receptor, but we could not determine whether GCs may be involved in the RU486 effect in this study. Further studies are necessary to investigate the precise mechanism of the RU486, but the inhibition of
progesterone receptor and decrease of the immune response may have important therapeutic implications in severe liver injury.

In summary, we demonstrated that progesterone exacerbates the severity of liver injury mediated the activation of immune responses after the administration of hepatotoxicant. The mechanism of the exacerbation by progesterone appears to involve immune responses such as the production of the CXC chemokines and neutrophils infiltration via the activation of ERK pathway and Kupffer cells. Moreover, progesterone receptor antagonist administration suppressed the severity of

FIG. 7. Effects of RU486 pretreatment on HAL-induced liver injury. Female mice pretreated with RU486 (50 μg/mouse, sc) for 7 days were administered HAL (30 mmol/kg, ip) 1.5 h after the last treatment of RU486. Plasma and liver samples were collected for assessment of the transaminase levels (A) and the expression of hepatic mRNA levels (B) 24 h after the HAL administration. Expression of hepatic mRNA was normalized to GAPDH mRNA. The data are mean ± SD of 3–5 mice. *p < 0.05, **p < 0.01, and ***p < 0.001, compared with CTL. ##p < 0.01 and ###p < 0.001, compared with only HAL administered mice.
DILI, which suggests the potential clinical application of progesterone receptor antagonist in immune-mediated responses in DILI.

SUPPLEMENTARY DATA

Supplementary data are available online at http://toxsci.oxfordjournals.org/.

ACKNOWLEDGMENTS

We thank Mr Brent Bell for reviewing the manuscript. No conflicts of interest were declared.

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