Biochemical Pharmacology 80 (2010) 533–539

Contents lists available at ScienceDirect

Biochemical Pharmacology

journal homepage: www.elsevier.com/locate/biochempharm

The effect of dimerumic acid on LPS-induced downregulation of Mrp2 in the rat

Kentaro Yano [a], Shuichi Sekine [a], Kanako Nemoto [a], Toru Fuwa [b], Toshiharu Horie [a,*]

[a] Laboratory of Biopharmaceutics, Graduate School of Pharmaceutical Sciences, Chiba University, Inohana 1-8-1, Chuo-ku, Chiba 260-8675, Japan
[b] Research and Development Division, Wakunaga Pharmaceutical company, Osaka, Japan

ARTICLE INFO

Article history:
Received 11 March 2010
Accepted 30 April 2010

Keywords:
Dimerumic acid
Cholestasis
Internalization
Oxidative stress
Mrp2

ABSTRACT

Oxidative stress is known to be a common feature of cholestatic syndrome. Lipopolysaccharide (LPS) induces cholestasis, causing multidrug resistance-associated protein 2 (Mrp2) downregulation in two different ways: early retrieval from the canalicular membrane and the latter event of reduced mRNA expression. However, the triggering factor for LPS-induced cholestasis is not fully understood.

In this study, we examined the effect of dimerumic acid (DMA), an antioxidant and traditional Chinese medicine, on endotoxin-induced Mrp2 downregulation in rat liver. At 3 h following LPS injection (4 mg/kg body weight), canalicular Mrp2 localization was disrupted without changing the expression of Mrp2 protein or the integrity of tight junctions in the liver. Pretreatment with DMA (12 mg/kg body weight) counteracted LPS-induced subcellular distribution, and decreased the bile flow rate and biliary glutathione (GSH) excretion. At 12 h following LPS injection, Mrp2 protein and mRNA expression were significantly decreased by 58% and 7%, respectively. In contrast, pretreatment with DMA did not have any effect on the decreased Mrp2 expression and biliary excretion of GSH induced by LPS exposure. Taken together, our data clearly indicate that LPS-induced short-term rapid retrieval of Mrp2 from the canalicular surface resulted from LPS-induced oxidative stress, while the long-term transcriptional regulation of Mrp2 expression did not depend on the intracellular redox status.

1. Introduction

Bile flow is mainly regulated by two canalicular transporters. The bile salt export pump (Bsep/Abcb11) and multidrug resistance-associated protein 2 (Mrp2/Abcc2) are involved in bile salt-dependent and -independent bile flow formation, respectively. Canalicular secretion of several amphiphilic organic anions, including bilirubin glucuronides, glutathione (GSH) and its conjugates is mediated by Mrp2, a conjugate export pump encoded by the *mrp2* gene [1]. In the liver, downregulation of these canalicular transporters leads to cholestasis, a pathological condition manifested by downregulation of various hepatobiliary transporting proteins. The transporter expression is regulated on a post-transcriptional and transcriptional basis in normal subjects, with important implications for the pathogenesis of cholestatic syndromes [2]. Post-transcriptional regulation includes rapid retrieval of the transporter from the canalicular membrane of hepatocytes and its translocation into the cytosol in putative vesicles under the influence of lipopolysaccharide (LPS) [3], hyperosmolarity [4,5], phalloidin [6], bile acid [7], and oxidative stress [8,9]. On the other hand, transcriptional regulation includes *mrp2* gene expression, which is affected by LPS [2], bile duct ligation or a variety of drugs [10]. LPS, a bacterial endotoxin, is a potent inflammatory agent and causes cholestasis, accompanied by post-transcriptional (short-term basis) and transcriptional downregulation (long-term basis) of various hepatobiliary transporters (e.g. Mrp2). The triggering factor of LPS-induced cholestasis with Mrp2 downregulation is not yet fully understood. We have previously revealed that Mrp2 was rapidly internalized under ethacrynic acid (EA)-induced acute oxidative stress in the perfused rat liver [8]. In patients with chronic hepatic failure (primary biliary cirrhosis and hepatitis C virus infection) and chronic cholestatic disorder, disrupted canalicular localization of Mrp2 without changing its mRNA expression was observed [11,12]. Moreover, oxidative stress markers are thought to be closely related to chronic cholestatic disorder in these liver failure patients. In these patients, choleretic drugs (tauroursodeoxycholate and genipin) are prescribed in order to improve cholestatic jaundice with disrupted canalicular Mrp2 localization [7,13]. These observations strongly suggest that not only the mRNA expression of canalicular transporters, but also their localization, which might be triggered by oxidative stress, were equally important in human

Abbreviations: Bsep, bile salt export pump; Mrp2, multidrug resistance-associated protein 2; GSH, glutathione; LPS, lipopolysaccharide; EA, ethacrynic acid; DMA, dimerumic acid; ALT, alanine aminotransferase; NO, nitric oxide; CrM, crude membrane; ZO-1, zonal occuldin-1; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; P-gp, P-glycoprotein.

* Corresponding author. Tel.: +81 43 226 2886; fax: +81 43 226 2886.
E-mail address: horieto@p.chiba-u.ac.jp (T. Horie).

0006-2952/$ – see front matter © 2010 Elsevier Inc. All rights reserved.
doi:10.1016/j.bcp.2010.04.036

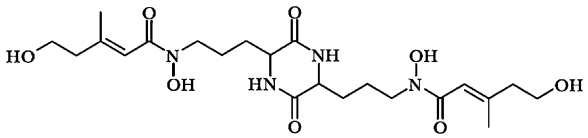

Fig. 1. Chemical structure of dimerumic acid.

cholestatic liver failure. From this point of view, it is suggested that antioxidants could prevent chronic hepatic failure with chronic cholestatic disorder.

Dimerumic acid (DMA), whose structure is shown in Fig. 1, has been reported as a fermented product of *Monascus anka* and *Monascus pilosus*. Genus *Monascus* has been traditionally used for the preparation of fermented foods and food additives in Asia; moreover, it is utilized as a traditional Chinese medicine. DMA has a strong antioxidative effect *in vitro* and a protective effect on carbon tetrachloride-induced liver injury in mice [14].

Here, we propose the hypothesis that DMA might have a protective effect on the LPS-induced downregulation of Mrp2. Therefore, we examined the effect of DMA on the LPS-induced downregulation of Mrp2 in both the short- and long-term.

2. Materials and methods

2.1. Chemicals

LPS, 3-fluorotyrosine, *o*-phthalaldehyde, 2-mercaptoethanol and GSH were obtained from Wako Pure Chemical Industries (Osaka, Japan). Rabbit anti-Mrp2 antiserum was raised against the 12-amino-acid sequence at the carboxyl terminus of rat Mrp2 [15]. All other chemicals and solvents were of analytical grade.

2.2. Isolation of dimerumic acid from M. purpureus

The mold *M. purpureus* was cultured at 30 °C for 3 days in a yeast extract medium supplemented with glucose and peptone. Subsequently, the cultured medium was separated using polystyrene–divinylbenzene resin (Diaion HP-20, Mitsubishi Chemical Industries) with ethanol. After concentration using an evaporator, the extract was dissolved in water and further separated using an HP-20 column with 50% methanol. Eluate was evaporated and dissolved in acetonitrile (10%, v/v). After centrifugation (1,500 × *g* for 10 min), the supernatant was evaporated and powdered DMA extract was obtained. Purity of the DMA was measured using an HPLC method. A TSKgel-80Tm column (4.6 mm inner diameter × 150 mm; GL Sciences, Tokyo, Japan) was used with a mobile phase (A H_2O:phosphates = 1000:1, B H_2O:acetonitrile:phosphates = 500:500:1) at a flow rate of 1.0 mL/min. A UV detector was used and operated at wavelength 260 nm.

2.3. In vivo experimental procedures

Male Wistar rats weighing 170–220 g (6–7 weeks old; Japan SLC, Shizuoka, Japan) were used throughout the experiments. The rats were given food and water, housed under a 12 h light/dark cycle, and acclimatized for at least 1 week before experimental use. All of the animals were treated humanely in accordance with the guidelines issued by the National Institutes of Health and all procedures described below were approved by the animal care committee of Chiba University. The rats were injected intravenously with 4 mg/kg LPS (dissolved in saline). In some experiments, we set the DMA dosing regimen (12 mg/kg i.v. at 1 and 15 h before the LPS treatment), following a previous report regarding carbon tetrachloride-induced hepatic oxidative stress [14]. After the treatments, the bile duct was cannulated with PE-10 tube (Natume, Tokyo, Japan) to collect bile and cumulative bile was collected for 20 min in rat anesthetized with pentobarbital. Subsequently after the experiment, venous blood samples were collected, and the respective liver was removed from the deeply anesthetized rat and then snap frozen in liquid nitrogen.

2.4. Assessment of hepatotoxicity

Serum alanine aminotransferase (ALT) activities and nitric oxide (NO) levels were measured as markers of LPS-induced hepatotoxicity. Assays were performed following the methods described by Katsuki et al. [16] with some modifications. NO in venous blood was measured as its metabolites (NO_2^- + NO_3^-) using the Griess reaction as previously described [17].

2.5. Measurement of GSH content in collected homogenate and bile samples

GSH contents in liver homogenates and bile were determined using the HPLC method described by Keller and Menzel [18] with some modifications. Treated liver homogenate and bile were mixed with 0.5 mL solution consisting of 5% metaphosphoric acid and 0.1% EDTA (2:7, v/v). The sample was centrifuged (16,000 × *g*, 5 min) and supernatant (0.5 mL) mixed with 3-fluorotyrosine as an internal standard followed by filtration through a 0.45 μm syringe filter (Millex-LH; Millipore, Bedford, MA). HPLC was performed as described previously [18]. Briefly, an Inertsil ODS column (4.6 mm inner diameter × 250 mm; GL Sciences, Tokyo, Japan) was used with a mobile phase (0.1% trifluoroacetic acid:methanol = 20:1) at a flow rate of 1.0 mL/min. The eluate from the column was mixed with a solution containing 18.6 mM *o*-phthalaldehyde and 17.1 mM 2-mercaptoethanol in 100 mM carbonate buffer (pH 10.5), which was delivered at a rate of 0.2 mL/min. The mixture was then passed through a stainless-steel coil at 70 °C to facilitate derivatization. A fluorescence detector was used and operated at an excitation wavelength of 355 nm and an emission wavelength of 425 nm. The concentration of GSH was calculated with reference to a standard GSH sample.

2.6. Isolation of liver crude membrane (CrM) and Western blot analysis

Hepatic crude membrane fraction, which contains the canalicular membrane, was prepared as described previously with some modifications [19]. Briefly, the liver specimens (2 g) were homogenized using a glass–glass homogenizer. The homogenate was centrifuged (1,500 × *g*, 15 min) and the resulting pellet suspended in a 2.5-fold volume of 70% (w/w) sucrose. Subsequently, the suspension was overlaid with 44% (w/w) sucrose and 36.5% (w/w) sucrose and was centrifuged (10,000 × *g*, 90 min). The CrM fraction was obtained from interface between the 44% and 36.5% sucrose, and this suspension fraction was stored at −80 °C. CrM fractions (2.5 μg protein) and homogenates (30 μg protein) were subjected to immunoblot analysis as described previously [8,20].

2.7. Immunohistochemical detection of Mrp2

Frozen tissue embedded in TissueTek OCT compound (Sakura Finetechnical, Tokyo, Japan) was used to prepare 4 μm thick slices at −25 °C, which were then fixed in acetone at room temperature for 10 min. Sections were stored at −80 °C until immunohistochemical staining. Sections on the glass slide were rehydrated in PBS for 30 min and then incubated for 1 h with a mixture of primary antibodies and monoclonal antibody for Mrp2 (M2III5) (Abcam, Cambridge, UK) (1:50) and anti-ZO-1 (zonal occuldin-1;

Abcam) (1:50) diluted in 0.1% BSA/PBS. After rinsing and washing with PBS, the slices were incubated in the dark for 1 h with a combination of secondary antibodies Alexa Fluor 546 (1:100)/FITC (1:100) (Invitrogen, CA, USA) diluted in 0.1% BSA/PBS. The samples were analyzed using a confocal laser scanning microscope, LSM510 type (Carl Zeiss, Jena, Germany).

2.8. Semiquantitative real-time RT-PCR

Total mRNA was prepared from rat liver using RNA-Solv reagent (Omega Bio-Tek, Doraville, GA). Reverse transcription was performed using 1 μg of total RNA from a Takara RNA PCR kit ver. 3.0 (Takara Bio, Shiga, Japan). Real-time PCR was performed to quantify the Mrp2 mRNA expression (forward primer: 5′-CTT GTG GGC TTT GTT CTG TCC-3′, reverse primer: 5′-GAG GCA ACA TCT ATC CCA TCA03′) relative to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) using the qPCR Master Mix for SYBR Green 1 (Eurogentec, Seraing, Belgium). Real-time RT-PCR amplification was determined using an ABI Prism 7000 system (Applied Biosystems, Foster City, CA).

2.9. Statistical analysis

The experimental groups were compared using a Newman–Keuls multiple comparisons test to determine significant differences between the group means.

3. Results

3.1. Protection of rat liver from LPS-induced liver injury by DMA treatment

As previously reported, administration of LPS (4 mg/kg) to male Wistar rats induced an increase in the ALT leakage into the serum 12 h after LPS treatment (190.3 ± 20.5 IU/L). However, no significant elevation of ALT leakage was observed 3 h after the treatment (35.9 ± 5.9 IU/L; Table 1). On the other hand, this serum ALT leakage was significantly suppressed by the pretreatment with DMA (12 mg/kg) (41.7 ± 6.6 IU/L; Table 1). Serum NO level continuously increased after the LPS injection, while this increase of serum NO release was suppressed to about half of the LPS treatment response by pretreatment with DMA (Table 1).

3.2. Effect of DMA on the LPS-induced hepatic GSH decrease

LPS is known to induce oxidative stress in rat liver. We have previously described that DMA could have a strong antioxidative effect on salicylic acid and *tert*-butyl hydroperoxide-induced oxidative stress [21]. The antioxidative effect of DMA on LPS-induced oxidative stress was evaluated. Intrahepatic GSH content was not altered by the DMA pretreatment. The intracellular reduced form of GSH was significantly decreased (49.5 ± 7.6% of control) by 3 h after LPS treatment. On the other hand, hepatic GSH content recovered to a control value (136.1 ± 28.6% of control) after 12 h LPS treatment. In addition, the decreased intrahepatic GSH content induced by the LPS was completely replenished (102.0 ± 8.1% of control) by pretreatment with DMA (Fig. 2).

Table 1
Effect of DMA on LPS-induced hepatic injury.

	ALT (IU/L)		$NO_3^- + NO_2^-$ (nmol/mL)	
	3 h	12 h	3 h	12 h
Control	19.1 ± 2.3	17.0 ± 1.8	3.7 ± 0.7	8.6 ± 0.8
DMA	29.3 ± 1.7	20.0 ± 3.9	3.9 ± 0.9	3.6 ± 2.3
LPS	35.8 ± 5.9	190.3 ± 20.5**	88.4 ± 3.0**	423.4 ± 48.0**
DMA + LPS	38.5 ± 7.4	41.7 ± 6.6##	31.6 ± 11.0##	152.3 ± 17.0##

Rats pretreated with DMA (12 mg/kg) or saline, 1 h and 15 h before LPS (4 mg/kg) administration, were killed at 0, 3, and 12 h after the LPS treatment. The results are given as the mean ± S.E.M. of three rats.
** $p < 0.01$ compared with control.
$p < 0.01$ compared with the LPS-treated group.

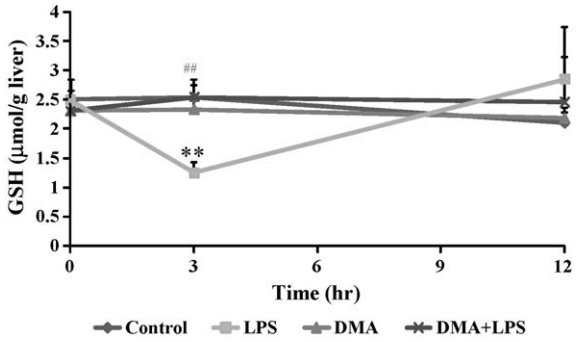

Fig. 2. The effect of DMA on LPS-induced hepatic GSH decrease. Rats pretreated with DMA (12 mg/kg) or saline, 1 and 15 h before LPS (4 mg/kg) administration, killed at indicated time points, and liver specimens were removed. Intrahepatic GSH content was measured by HPLC analysis using *o*-phthalaldehyde postlabeling followed by fluorometric detection. The results are given as the mean ± S.E.M. of three rats. $^{**}p < 0.01$ compared with control. $^{\#\#}p < 0.01$ compared with LPS-treated group.

3.3. Effect of DMA on the short-term LPS-induced cholestasis and downregulation of Mrp2 function

Because both GSH and its oxidized form are known to be good substrates of Mrp2, the biliary excretion of GSH is often estimated as an index of Mrp2 activity [22]. LPS treatment for 3 h induced a decrease in cumulative bile flow (41.9 ± 13.0% of control) and biliary excretion of GSH into bile (15.7 ± 3.3% of control). On the other hand, LPS-induced decreased bile flow rate and biliary excretion of GSH could be suppressed by DMA pretreatment (79.8 ± 4.2% and 62.2 ± 10.7% of control, respectively; Fig. 3A and B). These data imply that suppression of LPS-induced intracellular GSH decrease restores the bile flow rate and the function of Mrp2 to the control levels.

3.4. Effect of DMA on the LPS-induced early retrieval of Mrp2 localization

Endotoxin treatment leads to an impaired excretion of Mrp2 substrates into bile [2,23,24]. This may be due to an early (approximately 3 h) retrieval of Mrp2 from the canalicular membrane, whereas downregulation of Mrp2 mRNA is a later event (12–24 h) [19]. We have previously demonstrated that canalicular Mrp2 localization was reversibly and strictly regulated by intracellular GSH content [25]. Therefore, we first tried to confirm the effect of DMA on the LPS-induced early retrieval of Mrp2 from the canalicular membrane with both quantitative (immunoblotting) and qualitative (immunohistochemical) analysis. As shown in Fig. 4, Mrp2 staining (red) in the canalicular space was merged with the tight junctional protein ZO-1 (green) in saline-treated control liver. On the other hand, subcellular Mrp2 localization was observed after LPS treatment without changing the canalicular localization of ZO-1 (Fig. 4B). DMA pretreatment also suppressed the LPS-induced altered localization of Mrp2 in the canalicular membrane space (Fig. 4C). Moreover, the expression of Mrp2 in fractionated CrM was decreased to about half of the saline-treatment-induced expression (68.4 ± 4.7% of control) without changing the total Mrp2 expression in the obtained homogenate fraction. Moreover, pretreatment with DMA could suppress the LPS-induced decreased Mrp2 expression in the membrane fraction (106.6 ± 6.4% of control; Fig. 5A). On the other hand, mRNA expression of Mrp2 in the liver was not significantly decreased by

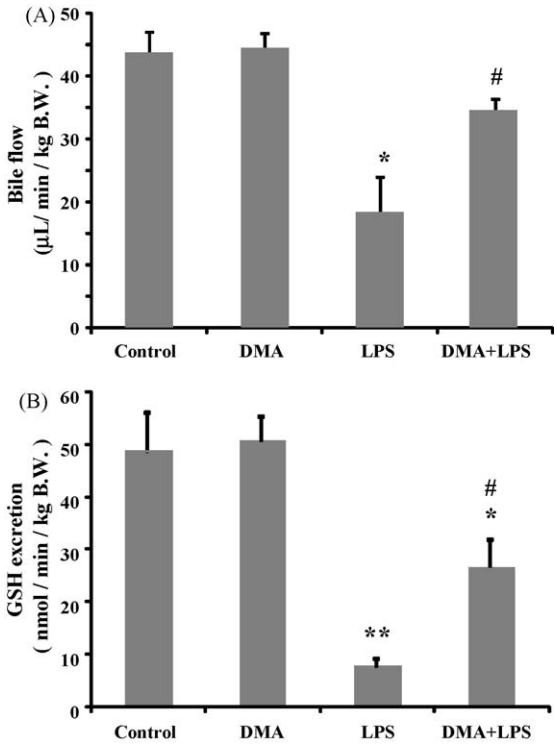

Fig. 3. The effect of DMA on LPS-induced reduction of bile flow and biliary excretion of excretion at 3 h after LPS treatment. Rats were pretreated with DMA (12 mg/kg) or saline, 1 and 15 h before LPS (4 mg/kg) administration, and their bile ducts cannulated at 3 h after LPS treatment. Bile samples were collected for 20 min, and bile flow was measured gravimetrically in tared tubes (A). Collected bile samples were used for the determination of GSH excretion (B). Results are given as the mean ± S.E.M. of three rats. $^{*}p < 0.05$ compared with control. $^{**}p < 0.01$ compared with control. $^{\#}p < 0.05$ compared with LPS-treated group.

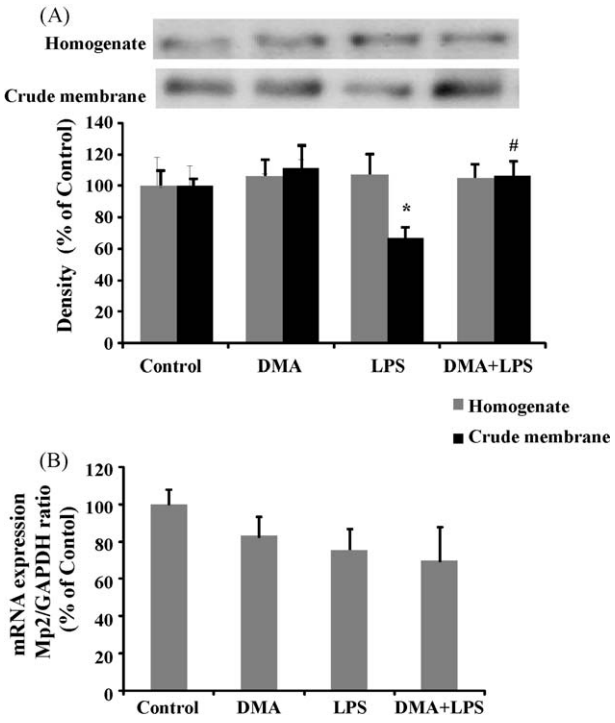

Fig. 5. Effect of DMA and LPS on Mrp2 protein (A) and mRNA (B) expression at 3 h after LPS treatment. Rats were pretreated with DMA (12 mg/kg) or saline, 1 h and 15 h before LPS (4 mg/kg) administration, killed at 3 h, and liver specimens were collected. Liver homogenate (30 μg protein/lane) and crude membrane fraction (2.5 μg protein/lane) prepared from treated-rat livers were subjected to western blotting analysis and a densitometric analysis was conducted (A). Mrp2 mRNA level for treated-rat liver was measured using real-time RT-PCR. The value was normalized using the GAPDH mRNA level (B). The results are given as the mean ± S.E.M. of three rats. $^{*}p < 0.05$ compared with controls. $^{\#}p < 0.05$ compared with LPS-treated group.

any treatment (Fig. 5B). These data suggested that LPS-induced rapid retrieval of Mrp2 localization was triggered by oxidative stress, accompanied by an intracellular GSH decrease.

3.5. Effect of DMA on bile flow and Mrp2 function in the long-term effect of LPS

After long-term (12–24 h) exposure to endotoxin treatment, Mrp2 protein expression in the liver was decreased, accompanied by the transcriptional downregulation of Mrp2 mRNA expression. However, the effect of LPS on the bile flow rate was inconsistent among the models of LPS treatment, whereas the excretion of Mrp2 substrates into bile was decreased by the LPS exposure [2,24]. Therefore, we have examined the effect of LPS and DMA on the bile flow and Mrp2 function 12 h after LPS administration. LPS treatment induced a decrease of biliary excretion of GSH into the bile (34.1 ± 17.2% of control) at 12 h. However, pretreatment by DMA could not significantly suppress the LPS-induced decrease of GSH excretion (Fig. 6B). On the other hand, LPS had no effect on the bile flow rate (93.8 ± 9.7% of control; Fig. 6A). These data imply that LPS could have some suppressive effect on the Mrp2-mediated GSH excretion into bile, but has no effect on the bile flow rate, as previously reported [23].

3.6. Effect of DMA on LPS-induced long-term downregulation of Mrp2 expression

It has been reported that mRNA expression of Mrp2 is downregulated by LPS treatment for more than 12 h [26]. It has also been reported that treatment with proinflammatory cytokines TNF-α, IL-1β and IL-6 leads to suppression of Mrp2 expression at the transcriptional level via activation of the inflammatory nuclear factor-κB. At 12 h after LPS treatment, Mrp2 protein and mRNA

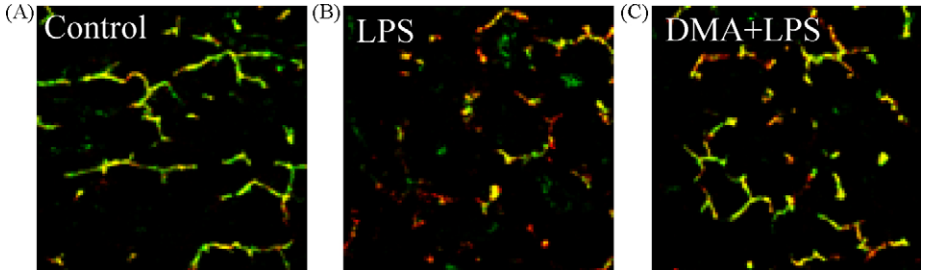

Fig. 4. The effect of DMA on the altered localization of Mrp2 induced by LPS. Rat liver slices fixed with acetone subjected to immunofluorescence staining with M2III5 and anti-ZO-1 antibodies. Confocal microscopy analysis of Mrp2 (red) and Zo-1 (green) was performed for sections from animals treated with LPS for 3 h after DMA pretreatment. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

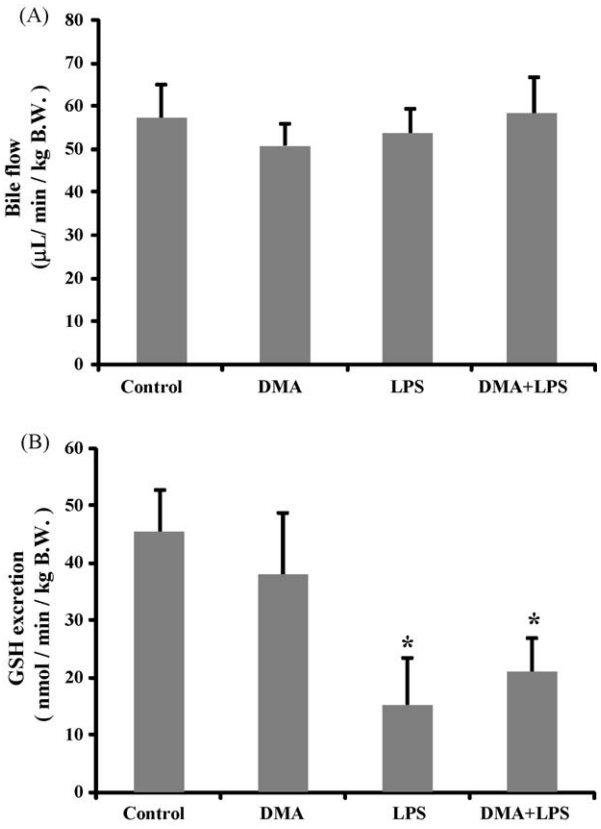

Fig. 6. The effect of DMA on the LPS-induced reduction of bile flow and biliary excretion of GSH at 12 h after LPS treatment. Rats were pretreated with DMA (12 mg/kg) or saline, 1 and 15 h before LPS (4 mg/kg) administration, and their bile ducts cannulated at 12 h after LPS treatment. Bile flow (A) and biliary excretion of GSH (B) were estimated as described in the legend to Fig. 3. Results are given as the mean ± S.E.M. of three rats. $^{*}p < 0.05$ compared with control.

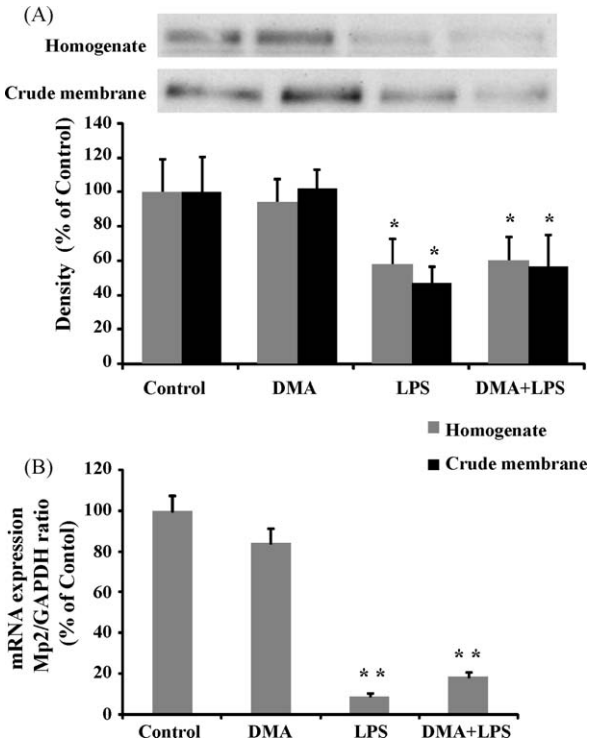

Fig. 7. Effect of DMA and LPS on Mrp2 protein (A) and mRNA (B) expression at 12 h after LPS treatment. Rats were pretreated with DMA (12 mg/kg) or saline, 1 and 15 h before LPS (4 mg/kg) injection, killed at 12 h, and liver specimens were collected. Liver homogenate (30 μg protein/lane) and crude membrane fraction (2.5 μg protein/lane) prepared from treated-rat livers were subjected to western blotting analysis and a densitometric analysis was conducted (A). Mrp2 mRNA level of treated-rat liver was measured by real-time RT-PCR. The value was normalized using the GAPDH mRNA level (B). The results are given as the mean ± S.E.M. of three rats. $^{*}p < 0.05$ compared with control. $^{\#}p < 0.05$ compared with the LPS-treated group.

expression were significantly decreased to 58.1 ± 15.5% and 6.6 ± 1.7% of controls, respectively (Fig. 7). Moreover, Mrp2 expression in the canalicular membrane also decreased to a value comparable with that following the LPS treatment (53.2 ± 10.5%; Fig. 7A). In contrast, DMA pretreatment did not have a significant suppressive effect on the decreased Mrp2 mRNA and protein expression in liver homogenate and canalicular membrane fraction induced by LPS exposure (Fig. 7). These data suggested that Mrp2 localization was not altered, whereas canalicular Mrp2 expression was significantly decreased 12 h after LPS treatment. Moreover, pretreatment with DMA could suppress neither the downregulated Mrp2 protein nor mRNA expression induced by LPS treatment.

4. Discussion

We have previously stated that because both GSH itself and glutathione conjugates are substrates of Mrp2, rapid down-regulation of Mrp2 under GSH-depleting conditions seems a favorable feedback mechanism for hepatocytes to retain their intracellular GSH [27]. In this study, we have demonstrated the effect of the antioxidative effect of DMA on cholestasis induced by LPS through oxidative stress and inflammatory responses.

It has been accepted that the static expression of Mrp2 on the canalicular surface, and its dynamic insertion and internalization processes, are of great importance because the steady-state expression level directly depends on these turnover rates. Moreover, canalicular Mrp2 localization was reversibly changed by various conditions, including osmolarity [4,5,28], estradiol 17β glucuronide [29–31], and redox status [25]. In this study, LPS-induced GSH decrease induced the rapid retrieval of Mrp2 by 3 h after LPS injection. However, Mrp2 localization was not changed when the intracellular GSH content was replenished by 12 h after LPS treatment, whereas Mrp2 expression in homogenates and the canalicular membrane were equally decreased. This observation strongly suggested that there is a co-relationship between intracellular GSH content and Mrp2 localization, as we have previously reported [25].

The affected pericanalicular actin cytoskeleton structure appears to be one of the common features of cholestasis [32]. Phalloidin, an inhibitor of F-actin depolymerization, induces internalization of not only Mrp2, but also P-glycoprotein (P-gp) because of the overall dysfunction of pericanalicular F-actin organization [6]. We have previously demonstrated that EA-induced selective activation of novel protein kinase C isoforms (δ and ε) leads to Mrp2-selective internalization without altered Bsep and P-gp internalization [20]. However, the pericanalicular cytoskeletal structure is not disrupted in this oxidative stress condition [25]. In line with these observations, we have found that the localization of one of the other biliary efflux transporters, Bsep, was not altered in this experimental model (unpublished data). Furthermore, the expression of the canalicular marker protein DPPIV (dipeptidyl peptidase IV) in the CrM fraction (data not shown) and the integrity of ZO-1 in the canalicular space (Fig. 4) were not changed in this experimental model. These data suggest that LPS-induced cholestasis accompanied by Mrp2 internalization is not caused by a disruption of pericanalicular cytoskeletal structure, but by a precise signaling pathway, leading to PKC activation, as we have previously reported [2].

In hepatitis, it has been reported that Mrp2 mRNA expression is downregulated by endotoxin and cytokines [33]. Moreover,

LPS-induced downregulated Mrp2 expression was inhibited by pretreatment with dexamethasone, an immunosuppressive agent. In this study, we confirmed that the LPS-induced downregulation of Mrp2 function was probably due to a decrease in Mrp2 mRNA and protein expression, and not because of a decrease in Mrp2 localization in the canalicular membrane 12 h after the LPS treatment. This downregulation induced by 12 h LPS treatment was not suppressed by the DMA pretreatment. These data suggest that the LPS-induced downregulation of Mrp2 expression 12 h after LPS treatment is not dependent on oxidative stress, but on proinflammatory cytokines, as previously described [34].

Canalicular GSH secretion provides the major driving force for bile-acid-independent bile flow. Among several canalicular transporters, GSH secretion is largely dependent on Mrp2. In line with this, bile flow in Mrp2-deficient rats (Eisai hyperbilirubinemic rats) is decreased to about a half of that found in normal rats [35]. At 3 h after LPS treatment, both the intracellular concentration ($49.5 \pm 7.6\%$ of control; Fig. 2) and biliary secretion ($15.7 \pm 3.3\%$ of control; Fig. 3) of GSH are decreased. Because Mrp2 expression in the canalicular membrane was decreased to $68.4 \pm 4.7\%$ of control (Fig. 5), the decreased biliary secretion of GSH was thought to be dependent on not only the decreased Mrp2 localization, but also the decreased GSH content in the liver. It has been reported that bile flow rate is not altered by LPS treatment for more than 12 h [23], while another report indicated that LPS induced a decrease of bile flow [24,34]. However, the biliary excretion of Mrp2 substrates into bile was significantly decreased in these two opposing reports [23,24,34]. In this study, bile flow rate was not changed by 12 h after LPS treatment, whereas Mrp2-mediated biliary GSH excretion was decreased in a similar manner to that described previously [23,24,34]. From these observations, the compensatory mechanism for sustaining the decrease in biliary-excreted substrates seemed to be upregulated in the 12 h after LPS treatment. However, the underlying mechanism remains unclear.

Oxidative stress is involved in the pathogenesis and progression of liver diseases, such as alcoholic liver disease and biliary cirrhosis [36]. During this process, the expression of biliary efflux transporters is affected [12,37,38] and, through this mechanism, the importance of the intracellular GSH content in regulating Mrp2 internalization was suggested [8,9]; however, whether the leading cholestatic model including Mrp2 internalization induced by LPS also depends on intracellular redox status has not yet been elucidated. Importantly, LPS-induced rapid retrieval of Mrp2 from the canalicular surface seen in rat was also observed in freshly isolated human liver slices. Surprisingly, however, LPS-induced long-term downregulation of Mrp2 mRNA expression was not observed in human liver slices [39]. From these points of view, antioxidative agents, such as DMA, may be good mediators against chronic liver failure accompanied by cholestasis in humans.

In conclusion, our series of studies have demonstrated that LPS-induced short-term rapid retrieval of Mrp2 from the canalicular surface resulted from a decrease of intracellular GSH content, while the long-term transcriptional regulation of Mrp2 expression was not affected by intracellular redox status.

Acknowledgments

This work was supported by a Grant-in-Aid for Scientific Research (A) (21249003) and a Grant-in-Aid for Young Scientists (B) (21790141) from the Ministry of Education, Culture, Sports, Science and Technology, Japan.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2010.04.036.

References

[1] Jedlitschky G, Leier I, Buchholz U, Hummel-Eisenbeiss J, Burchell B, Keppler D. ATP-dependent transport of bilirubin glucuronides by the multidrug resistance protein MRP1 and its hepatocyte canalicular isoform MRP2. Biochem J 1997;327:305–10.
[2] Kubitz R, Wettstein M, Warskulat U, Haussinger D. Regulation of the multidrug resistance protein 2 in the rat liver by lipopolysaccharide and dexamethasone. Gastroenterology 1999;116:401–10.
[3] Kubitz R, Warskulat U, Schmitt M, Haussinger D. Dexamethasone- and osmolarity-dependent expression of the multidrug-resistance protein 2 in cultured rat hepatocytes. Biochem J 1999;340(Pt 3):585–91.
[4] Schmitt M, Kubitz R, Lizun S, Wettstein M, Haussinger D. Regulation of the dynamic localization of the rat bsep gene-encoded bile salt export pump by anisoosmolarity. Hepatology 2001;33:509–18.
[5] Kubitz R, D'Urso D, Keppler D, Haussinger D. Osmodependent dynamic localization of the multidrug resistance protein 2 in the rat hepatocyte canalicular membrane. Gastroenterology 1997;113:1438–42.
[6] Rost D, Kartenbeck J, Keppler D. Changes in the localization of the rat canalicular conjugate export pump mrp2 in phalloidin-induced cholestasis. Hepatology 1999;29:814–21.
[7] Beuers U, Bilzer M, Chittattu A, Kullak-Ublick GA, Keppler D, Paumgartner G, et al. Tauroursodeoxycholic acid inserts the apical conjugate export pump, mrp2, into canalicular membranes and stimulates organic anion secretion by protein kinase c-dependent mechanisms in cholestatic rat liver. Hepatology 2001;33:1206–16.
[8] Ji B, Ito K, Sekine S, Tajima A, Horie T. Ethacrynic-acid-induced glutathione depletion and oxidative stress in normal and mrp2-deficient rat liver. Free Radic Biol Med 2004;37:1718–29.
[9] Schmitt M, Kubitz R, Wettstein M, vom Dahl S, Haussinger D. Retrieval of the mrp2 gene encoded conjugate export pump from the canalicular membrane contributes to cholestasis induced by tert-butyl hydroperoxide and chlorodinitrobenzene. Biol Chem 2000;381:487–95.
[10] Kauffmann HM, Keppler D, Kartenbeck J, Schrenk D. Induction of cmrp/cmoat gene expression by cisplatin, 2-acetylaminofluorene, or cycloheximide in rat hepatocytes. Hepatology 1997;26:980–5.
[11] Kojima H, Nies AT, Konig J, Hagmann W, Spring H, Uemura M, et al. Changes in the expression and localization of hepatocellular transporters and radixin in primary biliary cirrhosis. J Hepatol 2003;39:693–702.
[12] Zollner G, Fickert P, Zenz R, Fuchsbichler A, Stumptner C, Kenner L, et al. Hepatobiliary transporter expression in percutaneous liver biopsies of patients with cholestatic liver diseases. Hepatology 2001;33:633–46.
[13] Okada K, Shoda J, Kano M, Suzuki S, Ohtake N, Yamamoto M, et al. Inchinkoto, a herbal medicine, and its ingredients dually exert mrp2/mrp2-mediated choleresis and nrf2-mediated antioxidative action in rat livers. Am J Physiol Gastrointest Liver Physiol 2007;292:G1450–63.
[14] Aniya Y, Ohtani II, Higa T, Miyagi C, Gibo H, Shimabukuro M, et al. Dimerumic acid as an antioxidant of the mold, monascus anka. Free Radic Biol Med 2000;28:999–1004.
[15] Akita H, Suzuki H, Ito K, Kinoshita S, Sato N, Takikawa H, et al. Characterization of bile acid transport mediated by multidrug resistance associated protein 2 and bile salt export pump. Biochim Biophys Acta 2001;1511:7–16.
[16] Katsuki H, Kanayuki H, Yoshida T, Kawano C, Tanaka S. The determination of alpha-ketoglutaric acid by 2,4-dinitrophenylhydrazine; salting-out extraction method. Anal Biochem 1961;2:421–32.
[17] Ding AH, Nathan CF, Stuehr DJ. Release of reactive nitrogen intermediates and reactive oxygen intermediates from mouse peritoneal macrophages. Comparison of activating cytokines and evidence for independent production. J Immunol 1988;141:2407–12.
[18] Keller DA, Menzel DB. Picomole analysis of glutathione, glutathione disulfide, glutathione s-sulfonate, and cysteine s-sulfonate by high-performance liquid chromatography. Anal Biochem 1985;151:418–23.
[19] Vos TA, Hooiveld GJ, Koning H, Childs S, Meijer DK, Moshage H, et al. Up-regulation of the multidrug resistance genes, mrp1 and mdr1b, and down-regulation of the organic anion transporter, mrp2, and the bile salt transporter, spgp, in endotoxemic rat liver. Hepatology 1998;28:1637–44.
[20] Sekine S, Ito K, Horie T. Oxidative stress and mrp2 internalization. Free Radic Biol Med 2006;40:2166–74.
[21] Yamashiro J, Shiraishi S, Fuwa T, Horie T. Dimerumic acid protected oxidative stress-induced cytotoxicity in isolated rat hepatocytes. Cell Biol Toxicol 2008;24:283–90.
[22] Yang B, Hill CE. Nifedipine modulation of biliary gsh and gssg/conjugate efflux in normal and regenerating rat liver. Am J Physiol Gastrointest Liver Physiol 2001;281:G85–94.
[23] Hojo M, Sano N, Takikawa H. Effects of lipopolysaccharide on the biliary excretion of bile acids and organic anions in rats. J Gastroenterol Hepatol 2003;18:815–21.
[24] Muhlfeld A, Kubitz R, Dransfeld O, Haussinger D, Wettstein M. Taurine supplementation induces multidrug resistance protein 2 and bile salt export pump expression in rats and prevents endotoxin-induced cholestasis. Arch Biochem Biophys 2003;413:32–40.
[25] Sekine S, Ito K, Horie T. Canalicular mrp2 localization is reversibly regulated by the intracellular redox status. Am J Physiol Gastrointest Liver Physiol 2008;295:G1035–41.
[26] Cherrington NJ, Slitt AL, Li N, Klaassen CD. Lipopolysaccharide-mediated regulation of hepatic transporter mrna levels in rats. Drug Metab Dispos 2004;32:734–41.

[27] Ji B, Ito K, Horie T. Multidrug resistance-associated protein 2 (mrp2) enhances 4-hydroxynonenal-induced toxicity in Madin–Darby canine kidney II cells. Chem Res Toxicol 2004;17:158–64.
[28] Dombrowski F, Kubitz R, Chittattu A, Wettstein M, Saha N, Haussinger D. Electron-microscopic demonstration of multidrug resistance protein 2 (mrp2) retrieval from the canalicular membrane in response to hyperosmolarity and lipopolysaccharide. Biochem J 2000;348(Pt 1):183–8.
[29] Mottino AD, Hoffman T, Crocenzi FA, Sanchez Pozzi EJ, Roma MG, Vore M. Disruption of function and localization of tight junctional structures and mrp2 in sustained estradiol-17beta-d-glucuronide-induced cholestasis. Am J Physiol Gastrointest Liver Physiol 2007;293:G391–402.
[30] Mottino AD, Crocenzi FA, Pozzi EJ, Veggi LM, Roma MG, Vore M. Role of microtubules in estradiol-17beta-d-glucuronide-induced alteration of canalicular mrp2 localization and activity. Am J Physiol Gastrointest Liver Physiol 2005;288:G327–36.
[31] Mottino AD, Cao J, Veggi LM, Crocenzi F, Roma MG, Vore M. Altered localization and activity of canalicular mrp2 in estradiol-17beta-d-glucuronide-induced cholestasis. Hepatology 2002;35:1409–19.
[32] Thibault N, Claude JR, Ballet F. Actin filament alteration as a potential marker for cholestasis: a study in isolated rat hepatocyte couplets. Toxicology 1992;73:269–79.
[33] Hartmann G, Cheung AK, Piquette-Miller M. Inflammatory cytokines, but not bile acids, regulate expression of murine hepatic anion transporters in endotoxemia. J Pharmacol Exp Ther 2002;303:273–81.
[34] Nakamura J, Nishida T, Hayashi K, Kawada N, Ueshima S, Sugiyama Y, et al. Kupffer cell-mediated down regulation of rat hepatic cmoat/mrp2 gene expression. Biochem Biophys Res Commun 1999;255:143–9.
[35] Ito K, Koresawa T, Nakano K, Horie T. Mrp2 is involved in benzylpenicillin-induced choleresis. Am J Physiol Gastrointest Liver Physiol 2004;287: G42–9.
[36] Bjorneboe A, Bjorneboe GE. Antioxidant status and alcohol-related diseases. Alcohol 1993;28:111–6.
[37] Ros JE, Libbrecht L, Geuken M, Jansen PL, Roskams TA. High expression of mdr1, mrp1, and mrp3 in the hepatic progenitor cell compartment and hepatocytes in severe human liver disease. J Pathol 2003;200:553–60.
[38] Kullak-Ublick GA, Baretton GB, Oswald M, Renner EL, Paumgartner G, Beuers U. Expression of the hepatocyte canalicular multidrug resistance protein (mrp2) in primary biliary cirrhosis. Hepatol Res 2002;23:78–82.
[39] Elferink MG, Olinga P, Draaisma AL, Merema MT, Faber KN, Slooff MJ, et al. Lps-induced downregulation of mrp2 and bsep in human liver is due to a posttranscriptional process. Am J Physiol Gastrointest Liver Physiol 2004;287:G1008–16.