Ursodeoxycholic acid stimulates Nrf2-mediated hepatocellular transport, detoxification, and antioxidative stress systems in mice

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Combined analysis of the clinical trials of UDCA for primary biliary cirrhosis, a chronic cholestatic liver disease characterized by a progressive destruction of bile ducts, has indicated that UDCA improves survival free of liver transplantation (24). Thus the therapeutic efficacy of UDCA in various types of cholestatic liver diseases is currently accepted (25). Suggested mechanisms of UDCA action, which are supported by experimental evidence, include stimulation of hepatobiliary excretory function and/or detoxification and cytoprotection (23).

Cholestasis leads to hepatic and systemic accumulation of potentially toxic biliary compounds. Hepatic accumulation of hydrophobic bile acids induces oxidative stress by stimulating the generation of reactive oxygen species in hepatocytes (33). Evidence of oxidative stress-induced liver injury has been found in rats receiving intravenously infused hydrophobic bile acids (32) and in bile duct-ligated rats (37). It has recently been suggested that the cytoprotective mechanisms of UDCA against cholestasis may be mediated by protection of hepatocytes against oxidative stress (23). Pretreatment with UDCA prevented hydrogen peroxide-induced injury by increasing γ-glutamylcysteine synthetase (γ-Gcs) mRNA, and consequently, levels of reduced glutathione (GSH) (17, 29). GSH levels were higher in liver specimens perfused with UDCA than those perfused with taurocholic acid (27).

One transcription factor that serves as a cellular sensor for oxidative stress is termed nuclear factor-E2-related factor-2 (Nrf2). Nrf2 is sequestered in the cytosol by Kelch-like Ech-associated protein (Keap1). However, during oxidative challenge, modification of Keap1 sulfhydryl groups results in release and nuclear translocation of Nrf2 (43). Nrf2 is essential for antioxidant responsive element/electrophile-responsive element (ARE/EpRE)-mediated gene induction of detoxifying enzymes (8) and antioxidative stress genes, e.g., GSH (7), and can transcriptionally activate several enzymes involved in cellular protection (4, 8, 43). Because oxidative stress to hepatocytes could be expected to induce efflux pathways along with phase I detoxification enzymes and phase II conjugating

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URSODEOXYCHOLIC ACID (UDCA) improves clinical and biochemical indexes in a variety of cholestatic liver diseases (23).

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enzymes in hepatocytes, and because many phase I detoxifying enzymes are induced via Nrf2, it could be hypothesized that multidrug resistance-associated protein (Mrp) family members are inducible by Nrf2 activation. Supporting this hypothesis, the recent studies of our group (14, 20) and another group (1, 13) have shown that the Nrf2 activators induce Mrp2 and other Mrp members in rodent livers.

Intrinsic hepatoctellar adaptive induction of transporters and antioxidative stress action in various types of cholestatic liver diseases are too weak to prevent ongoing liver injury (5, 38). Therefore, stimulation and restoration of Mrp expression and function and induction of potent detoxification and antioxidative stress systems by Nrf2 activation may be an important target for specific pharmacotherapeutic interventions against the progression of biliary cirrhosis induced by chronic cholestasis.

In this study, we aimed at determining whether 1) Mrp family members and other plasma membrane transporters are induced by the constitutive activation of Nrf2 in Keap1 gene-knockdown (Keap1-kd) mice, 2) UDCA activates Nrf2 in vitro and in vivo experiments, and 3) UDCA induces Mrps, detoxifying enzymes, and antioxidative stress genes via the Nrf2-transcriptional pathway by comparing their induction in UDCA-treated wild-type (WT) and Nrf2 gene-null (Nrf2-null) mice.

MATERIALS AND METHODS

Cell cultures. RL34 cells (Health Science Research Resources Bank, Osaka, Japan) were cultured in Dulbecco’s modified Eagle’s medium (DMEM, Sigma-Aldrich, St. Louis, MO) containing 10% fetal bovine serum (Invitrogen Japan, Tokyo, Japan). The cells were incubated with or without 50 μM diethyl maleate (DEM), with 100 and 300 μM UDCA, or taurine-conjugated UDCA (T-UDCA) for 6 and 12 h in a humidified 5% CO2 atmosphere at 37°C. Nuclear protein fractions of the cells were prepared by use of a Pierce NE-Per kit (Rockford, IL). Nrf2 levels in the fractions were determined by immunoblotting using a MAb against Nrf2, which had been developed in our laboratories (7).

Animals. Keap1 gene-knockout (Keap1-ko) mice, which were previously generated at the Center for Tsukuba Advanced Research Alliance and Institute of Basic Medical Science, the University of Tsukuba, were found to die before weaning because of the hyperkeratinotic lesions in the esophagus and forestomach, leading to obstruction of the upper digestive tract (21). Therefore, pair-matched littermate WT and Keap1-kd mice on a C57/B6 background (Taguchi K, Yamamoto M, unpublished observations), similar to Keap1-kd mice (21), in which cytoplasmic protein Keap1, a repressive factor of Nrf2, is markedly reduced and thereby results in constitutive activation of Nrf2, were engineered and bred at the Center. Pair-matched littermate WT and Nrf2-null (8) on an Institute for Cancer Research (ICR) background were also engineered and bred at the Center. Chimeric mice with humanized livers, which were developed in the laboratories of Life Science Research Laboratory, PhoenixBio (Hiroshima, Japan), were used to assess the pharmacological responses of human hepatocytes. Livers of urokinase-type tissue plasminogen activator transgenic severe combined immunodeficient (SCID) mice were treated with an anti-human complement drug and were then partially repopulated with human hepatocytes as described previously (36). The human hepatocytes repopulated in the mouse liver express human liver proteins, including cytokinin 8/18 (CK8/18), various cytochrome P450 subtypes, human albumin, and plasma membrane transporters (20, 31). All experiments were conducted according to the institution’s guidelines for the care and use of laboratory animals in research. The experiments were performed under protocols approved by the Institutional Animal Care and Use Committees of University of Tsukuba, Mitsubishi Tanabe Pharma Corporation, and PhoenixBio, respectively. UDCA was mixed at 1% (wt/wt) in standard laboratory chow (CRF-1; Oriental Yeast, Tokyo, Japan) in reference to the previous studies (38) and fed to the mice for 7 days.

Immunoblot analysis. Liver total homogenates and nuclear extract fractions were prepared as previously described (20, 30). Protein levels of transporters, detoxifying enzymes, and antioxidative stress genes in mouse livers were determined by using polyclonal antibodies (pAbs) raised against Mrp2, Mrp3, Bsep, γ-Gcs, and Ho-1 and MAbs raised against Mrp4 and Nqo1 (Abcam, Cambridge, UK) according to recently detailed methodology (20, 30). Nrf2 levels in nuclear protein fractions were determined by using the MAb raised against Nrf2 as described before (7). The membranes were stripped and reprobed again with an antibody (Ab) raised against actin or an Ab against lamin. Immunoreactive bands were densitometrically quantified and normalized to the amounts of actin or lamin present in each specimen and then averaged. For the humanized mice livers, Nrf2 levels in nuclear protein fractions were determined by using a pAb raised against Nrf2 (H-300, Santa Cruz Biotechnology, Santa Cruz, CA).

Transporter protein levels were determined by using MAbs raised against MRPs, MRP2 (M-JH-6, Alexis Biochemicals, Lausen, Switzerland) and MRP4 (Abcam) and a pAb of MRP3 that had been raised against a mouse-binding protein fusion protein containing the 147 amino acids corresponding to 836-983 of the deduced human MRP3 amino acid sequence (9). The membranes were reprobed with a MAb raised against human cytokeratin 8/18 (CK8/18) (Santa Cruz Biotechnology, Santa Cruz, CA).

Immunohistochemical localization of transporters in the liver. For studies on immunohistochemical localizations of Mrp2, Mrp3, Mrp4, and Bsep in the mouse livers, liver specimens (each ~50 mg) were frozen in Freon/liquid nitrogen, embedded in optimal cutting temperature compound (Miles, Elkhart, IN), cut into 6-μm-thick sections, and mounted on slides. The tissue sections were immunostained with each MAb or pAb by using previously detailed methodologies (20, 30). Quantification of the data of localization of the transporters in the liver tissue sections was performed by image analysis as previously described (30).

Real-time quantitative PCR. Total RNA was isolated from tissue specimens by use of ISOGEN (Nippon Gene, Tokyo, Japan). Steady-state mRNA levels in the specimens were determined by real-time quantitative PCR using previously detailed methodologies (20). Primers and probes used for this study were designed by use of Primer Express (Applied Biosystems, Foster City, CA) and are summarized in Table 1. In the PCR analysis using the nonspecific intercalating dye SYBR-green, the specificity of amplification for each gene was verified by melting curve and agarose gel analyses. Data were normalized to the amounts of GAPDH present in each specimen and then averaged.

ChIP. Hepa1c1c7 cells (American Type Culture Collection, Manassas, VA) were cultured in 15-cm dishes containing DMEM with 10% fetal bovine serum. Cells at 40% confluence were transfected with 15 μg of a Ntcp-pS9 expression vector per dish in Fugene transfection reagent (Roche Applied Science, Indianapolis, IN) for 30 h (Ntcp-pS9 vector was kindly provided by Dr. Guo G, University of Kansas Medical Center). At 24 h after the start of transfection, Ntcp-transfected Hepa1c1c7 cells were treated with DMSO (vehicle) or 450 μM UDCA for 6 h and then assayed for Nrf2 binding to ARE response elements. Chromatin immunoprecipitation assays (ChIP) assays were performed as previously described (14, 18). Immunoprecipitations were performed by using either rabbit IgG or pAb against Nrf2 (H-300). A 1:20 dilution of the nonimmunoprecipitated DNA (input) was analyzed along with IgG or anti-Nrf2 Ab precipitated DNA. PCR was performed with 35 cycles for Mrp2, 38 cycles for Mrp3 and Mrp4, 36 cycles for Nqo1 and lactose dehydrogenase (LDH), and Nqo1 and LDH genes were analyzed as positive and negative controls, respectively (18, 27). The primers for ARE amplification are listed as follows: Mrp2, forward 5′-CAGGGCTTT-GGAGAAGTGTAA-3′, reverse 5′-GGAAGCAGATGTATTTAGGA-

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Table 1. Primer and probe sets

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Fluorogenic probes for TaqMan RT-PCR are 5’ FAM- and 3’ TAMRA-labeled. *Real-time polymerase chain reaction using the SYBRgreen assay.

GCAGG-3’; Mrp3, forward 5’-AAAGGTCTCTTGTTCTTACTCCGT GG-3’, reverse 5’-TGGCAGCAGTCCTCCCGCTAGTG-3’; Mrp4, forward 5’-CTTGAGGCTGGGACTTTGAGT-3’; reverse 5’-ACTGACGACAGTGTTGTAAGCTGTCCG-3’. To distinguish the bands of desired PCR products from those of nonspecific products, the bands on electrophoresis were sequenced to confirm their specificity for Mrp2, Mrp3, and Mrp4 AREs.

**Results**

Effects of constitutive activation of Nrf2 on hepatocellular transporters in the mouse liver. Similar to Keap1-ko mice (21), nuclear levels of Nrf2 were markedly increased in livers of Keap1-kd mice compared with the levels in livers of WT mice (Fig. 1A). The results confirmed constitutive activation of Nrf2 in Keap1-kd mice. As shown in Figs. 1B and 2A, upon constitutive activation of Nrf2, mRNA and protein levels of Mrp2, Mrp3, and Mrp4 were significantly increased in livers of Keap1-kd mice compared with the corresponding levels in livers of WT mice. However, bile salt export pump (Bsep) mRNA and protein levels in livers were not different between Keap1-kd and WT mice. For other transporters, whereas the organic anion transporting polypeptide (Oatp) 1a1 mRNA level was significantly decreased in livers of Keap1-kd mice, multidrug resistance P-glycoprotein 2 (Mdr2), Na+-taurocholate cotransporting polypeptide (Ntcp) and Oatp1a2 mRNA levels were not changed in the livers (Fig. 1B).

To investigate whether Nrf2 regulates classical nuclear receptors, the mRNA levels of AhR, CAR, FXR, Lh1-1, and PXR were determined. As shown in Fig. 1C, there was found to be only a significant induction of CAR mRNA in livers of Keap1-kd mice.

The immunostaining of Mrp2 was more intensive and more diffuse in the bile canaliculi of liver tissue sections of Keap1-kd mice than in those of WT mice (Fig. 2B). Consistent with the marked induction of Mrp3 and Mrp4 protein (Fig. 2A), the immunostainings of Mrp3 and Mrp4 in liver tissue sections showed a remarkable difference between Keap1-kd and WT mice (Fig. 2B). Intense staining of Mrp3 was observed in the plasma membranes of liver tissue sections of Keap1-kd mice compared with the staining in WT mice. The staining pattern did not show any zonal fashion in the liver tissue sections studied. In contrast to Mrp3, hepatic Mrp4 was expressed in a zonal fashion (Fig. 2B). Low-power magnification showed that in Keap1-kd mice staining of Mrp4 was increased dramatically from negligible in zone 1 (pericentral) regions to intense staining in zone 3 (periportal) regions. Moreover, high-power magnification revealed that staining of Mrp4 was observed in the plasma membranes, the reported localization of Mrp4 in the human liver (26). However, the immunostaining of Bsep was not significantly different between Keap1-kd and WT mice.

In vitro and in vivo effects of UDCA on activation of Nrf2. Rat hepatocytes RL34 cells were treated with DEM (50 μM), UDCA (100, 300 μM), or T-UDCA (100, 300 μM) at different points of time (6 and 12 h). Nuclear protein was extracted from the cells and the nuclear level of Nrf2 was assessed. Treatment with both UDCA and T-UDCA increases nuclear Nrf2 levels (Fig. 3A). From a therapeutic point of view, it should be noted that in hepatocytes UDCA and T-UDCA did induce more potent Nrf2 activation in a concentration-dependent fashion.

Next, the ChiP assays were performed (Fig. 3B). Nqo1 (positive control) has a known ARE in the 5’ flanking sequence that can bind Nrf2, whereas LDH (negative control) does not contain an ARE in the promoter sequence. As expected, Nrf2 binding was detected in the Nqo1 promoter, weakly in the vehicle-treated lane, and much more strongly after UDCA treatment (Fig. 3B), whereas Nrf2 binding was not observed with the LDH promoter fragment, suggesting Nrf2-specific immunoprecipitation of ARE regions. For Mrp2, binding of Nrf2 to the ARE was observed with both vehicle and UDCA-
treated cells. For Mrp3, the ARE binds Nrf2, indicating modest binding in vehicle-treated cells that was increased after UDCA administration. For Mrp4, binding of Nrf2 to the ARE was observed only with UDCA-treated cells.

Similarly, in vivo treatment with UDCA induced increases in the nuclear levels of Nrf2 in WT mouse livers but not in Nrf2-null mouse livers (Fig. 4A). Nuclear Nrf2 levels in the livers of UDCA-treated WT mice were about three times those in vehicle-treated WT mice.

Bile acid concentration in the liver. Figure 4B shows the concentrations of total bile acids and major bile acid species in liver tissue specimens from WT and Nrf2-null mice. The concentration of DCA was significantly increased in livers of vehicle-treated Nrf2-null mice (Nrf2-null Vehicle) compared with that in vehicle-treated WT mice (WT Vehicle). UDCA treatment significantly increased the concentrations of total bile acids, β-muricholic acid, and UDCA in livers of both WT (WT UDCA) and Nrf2-null mice (Nrf2-null UDCA), but the levels were not significantly different between the two groups. On the other hand, the concentrations of CA and DCA in livers were markedly decreased in both WT and Nrf2-null mice.

Effects of UDCA on expression of hepatocellular transporters and nuclear receptors in the mouse liver. As shown in Fig. 5A, the mRNA levels of Mrp2, Mrp3, Mrp4, and Oatp1a2 were significantly increased in livers of WT UDCA, compared with the corresponding levels in WT Vehicle. Conversely, in Nrf2-
null UDCA, the treatment did not increase the level of Mrp2 compared with the level in Nrf2-null Vehicle. The basal levels of Mrp3 and Mrp4 were significantly lower in Nrf2-null Vehicle than in WT Vehicle mice, and UDCA-induced increases in the levels of Mrp3 and Mrp4 were also significantly less in Nrf2-null UDCA than in WT UDCA mice. For other transporters, i.e., Bsep, Mdr2, and Ntcp, UDCA treatment did not cause any significant increases in their mRNA levels in either WT or Nrf2-null mice.

To further test whether UDCA regulates classical nuclear receptors, the mRNA levels of AhR, CAR, FXR, Lrh-1, and PXR were determined (Fig. 5B). There was a significant induction of Lrh-1 and PXR expression in livers of WT UDCA but not in Nrf2-null UDCA mice. For AhR mRNA levels, there was a significant induction in livers of both WT UDCA and Nrf2-null UDCA mice. The basal mRNA levels of CAR and FXR were significantly lower in Nrf2-null Vehicle than in WT Vehicle mice, and UDCA treatment did not induce any signifi-
UDCA Stimulates Nrf2-Mediated Hepatic Transport

The immunoblot analysis, in parallel to the mRNA levels (Fig. 6A), UDCA treatment induced a significant increase in Mrp2 protein level in WT UDCA, but not in Nrf2-null UDCA mice (Fig. 6A). For Mrp3 and Mrp4, UDCA treatment induced marked increases in hepatic protein levels in livers of WT UDCA mice. However, in Nrf2-null mice, the basal protein levels of Mrp3 and Mrp4 were markedly lower than in WT Vehicle mice, and UDCA-induced Mrp3 and Mrp4 expression in Nrf2-null UDCA mice was also significantly less than that in WT UDCA mice. Bsep protein levels did not differ significantly either between WT and Nrf2-null mice regardless of the treatment.

The immunohistochemical localizations of Mrp2, Mrp3, Mrp4, and Bsep in liver tissue sections were studied (Fig. 6B). Immunostaining of Mrp2 was more intensive and more diffuse in the bile canaliculi of liver tissue sections of WT UDCA mice than in those of WT Vehicle mice. Image analysis showed a significant increase in the protein level of Mrp2 in liver tissue sections of WT UDCA mice. The same effect was also found, to a lesser extent, in liver tissue sections of Nrf2-null UDCA mice.

Consistent with the marked induction of Mrp3 and Mrp4 protein by UDCA treatment (Fig. 6A), immunostainings of Mrp3 and Mrp4 in liver tissue sections showed remarkable differences between WT Vehicle and UDCA mice (Fig. 6B). Similar to Keap1-kd mice, intense staining of Mrp3 was observed in the plasma membranes of liver tissue sections of WT UDCA mice compared with the staining in WT Vehicle mice. The staining pattern did not show any zonal fashion in the liver tissue sections studied. Image analysis showed a significant increase in the protein level of Mrp3 protein in liver tissue sections of WT UDCA mice.

In contrast to Mrp3, hepatic Mrp4 was expressed in a zonal fashion (Fig. 5B). The results were quite similar to those of Keap1-kd mice (Fig. 2B). Low-power magnification showed that in WT UDCA mice staining of Mrp4 was increased dramatically from negligible in zone 1 (periportal) regions to intense staining in zone 3 (pericentral) regions. Image analysis showed a significant increase in the protein level of Mrp4 in liver tissue sections of WT UDCA mice. Consistent with the markedly reduced basal protein levels of Mrp3 and Mrp4, almost no staining was observed in liver tissue sections of Nrf2-null Vehicle mice (Fig. 6B) and their induction by UDCA treatment was only weakly observed in Nrf2-null UDCA mice.

The immunostaining of Bsep was more intensive and more diffuse in the bile canaliculi of liver tissue sections of both WT UDCA and Nrf2-null UDCA mice than in those of WT Vehicle and Nrf2-null Vehicle mice (Fig. 6B). The UDCA effect on immunohistochemical localization of Bsep was found to be quite similar to the effect on Mrp2 localization.

Effects of UDCA on expression levels of detoxifying enzymes and antioxidative stress genes in the mouse liver. UDCA treatment upregulated the mRNA expression of the hepatic detoxifying enzymes, Nqo1, Gsta2, and Ugt1a1 in livers of both WT UDCA and Nrf2-null UDCA mice (Fig. 7A), with corresponding increases in protein levels of Nqo1 in livers of both WT UDCA and Nrf2-null UDCA mice (Fig. 7B). Among hepatic detoxifying enzymes studies, the mRNA levels of Nqo1 and Gsta2 in Nrf2-null Vehicle and Nrf2-null UDCA mice showed a significant increase in the protein level of Mrp3 protein in liver tissue sections of WT UDCA mice.
mice were significant lower than in those of WT Vehicle and WT UDCA mice, respectively. Protein levels of Nqo1 were markedly lower in livers of Nrf2-null Vehicle than in those of WT Vehicle mice, and UDCA-induced increases in their levels were also significantly diminished in livers of Nrf2-null UDCA than in livers of WT UDCA mice.

In addition, UDCA treatment upregulated the mRNA and protein expression of the antioxidative stress genes, γ-Gcs and Prx1, in livers of both WT UDCA and Nrf2-null UDCA mice (Fig. 7), yet no induction of heme oxygenase-1 (Ho-1) expression was observed. The mRNA levels of γ-Gcs in both untreated and UDCA-treated conditions were significantly lower in livers of Nrf2-null Vehicle than in those of WT Vehicle mice, with corresponding decreases in protein expression observed as well.

**Effects of UDCA on expression level of human Nrf2, MRP2, MRP3, and MRP4 in the chimeric mice with humanized livers.** To investigate the effects of UDCA on human livers, chimeric mice with humanized livers (the replacement index of the human hepatocytes more than 80%) were fed diets containing...
UDCA at 1% (wt/wt) for 7 days. Nuclear levels of Nrf2 were increased in livers of UDCA-treated chimeric mice compared with the levels in those of vehicle-treated mice. The results confirmed Nrf2 activation by UDCA treatment. Immunoblot analysis revealed that MRP2, MRP3, and MRP4 protein levels were significantly increased in the human livers of UDCA-treated chimeric mice compared with the levels in those of vehicle-treated mice (Fig. 8).

**DISCUSSION**

The results of this study and another recent study by our laboratory (14) have shown that hepatic expression of Mrp family members, phase I detoxifying enzymes, phase II conjugating enzymes and antioxidative stress genes are markedly upregulated by potent Nrf2 activation in models of Keap1-kd mice (Figs. 1 and 2), in glutamate cysteine ligase hepatocyte-specific Gclc-null mice, and in mice with Nrf2 chemically activated (by oltipraz or butylated hydroxyanisole treatment). In Keap1-kd mice with bile duct ligation (obstructive cholestasis), hepatocellular transport, along with a potent expression of Mrp efflux pumps, was stimulated, which in turn resulted in the decreased serum bilirubin level (unpublished observation).

From a clinical perspective, therefore, our interest has been focused on a cytoprotective role of Nrf2 activation by drugs and/or agents against hepatocellular injury associated with cholestatic liver diseases.

For bile acids, in vitro experiments have recently shown that some species including UDCA activate Nrf2 and provoke adaptive defense responses at the emergence of oxidative stress (35). Following the results of in vitro experiments of UDCA-induced Nrf2 activation in this study (Fig. 3), we had a chance to perform in vivo experiments using Nrf2-null mice in this study and determined whether UDCA induces Mrp family members, detoxifying enzymes, and antioxidative stress genes via the Nrf2-transcriptional pathway. In response to UDCA treatment in vivo, Nrf2 plays an important role in upregulating hepatic expression of Mrp2, Mrp3, and Mrp4. Regarding the UDCA effect on Mrp2 expression, whereas the treatment increased the mRNA and protein levels in mouse livers (Figs. 5 and 6), the density of Mrp2 in the bile canaliculi was significantly increased and the area of bile canaliculi showing Mrp2 staining was also significantly expanded in the liver tissue sections of UDCA-treated mice (Fig. 6). This seems to demonstrate that Nrf2 can alter not only the expression of...
Mrp2 but also the cellular localization. Posttranslational modification has been shown to be important in the regulation of Mrp2 (11, 22). Such a mechanism for Nrf2 would seem unique but could occur because of a slowing of Mrp2 turnover.

It should be noted that the expression of inducible efflux transporters for organic anions and bile acids, Mrp3 and Mrp4, were upregulated in the livers of UDCA-treated mice (Figs. 5 and 6). Nrf2 is important not only for the induction of Mrp3 and Mrp4 in the livers but also their constitutive levels. Their protein amount and immunohistochemical expression levels in the livers of Nrf2-null mice are barely detectable (Fig. 6). Thus, Nrf2 is important for both the constitutive levels of Mrp family members and their induction in livers, and as has been demonstrated for phase-II genes, Nrf2 activation can alter basal, inducible or basal and inducible expression of Mrp family members. Moreover, Mrp family members in mouse liver seem to be coordinately regulated by Nrf2 and classical nuclear receptors such as AhR, CAR, PXR, and PPARs (13). The increased transcription level of Mrp3 in livers of Nrf2-null UDCA mice may be associated with the induction of AhR mRNA (Fig. 5). However, the unchanged expression levels of CAR, PXR, and FXR in the livers cannot be responsible for the observation if the expression levels, rather than the ligand availability, are limiting.

Potent induction of Mrp3 and Mrp4 proteins in rat livers with obstructive cholestasis in the setting of decreased expression and function of Mrp2 had been interpreted as an attempt to minimize cytotoxicities to the hepatobiliary system as cholestatic liver injury progresses (6, 10, 34). As investigated by the recent studies on the effects of obstructive cholestasis in
Fig. 7. **A**: steady-state mRNA levels of detoxifying enzymes (Nqo1, Gsta2, Ugt1a1) and antioxidative stress genes (γ-Gcs, Ho-1, Prx I) in livers of vehicle-treated WT (WT Vehicle), UDCA-treated WT (WT UDCA), vehicle-treated Nrf2-null (Nrf2-null Vehicle), and UDCA-treated Nrf2-null mice (Nrf2-null UDCA). Numbers in parentheses are numbers of animals in each group. **B**: immunoblot analysis of detoxifying enzyme (Nqo1) and antioxidative stress genes (γ-Gcs, Ho-1, Prx I) in livers of WT Vehicle, WT UDCA, Nrf2-null Vehicle, and Nrf2-null UDCA mice. Bar graphs show quantitation of optical density of the immunoblots. Numbers in parentheses are numbers of animals in each group. †P < 0.01, significantly different from the group of WT Vehicle mice; ‡P < 0.01, significantly different from the group of WT UDCA mice; Brackets *P < 0.05, **P < 0.01, significantly different between the 2 groups.
mice with targeted disruption of Mrp3 (3, 42) and those with targeted disruption of Mrp4 (16), hepatic Mrp3 is a preferential efflux pump for bilirubin conjugates, whereas Mrp4 is a preferential pump for bile acid conjugates. Both Mrp3 and Mrp4 play a protective role in the adaptive response to obstructive cholestatic liver injury. Here, this study has demonstrated that UDCA markedly upregulates hepatic Mrp3 and Mrp4 in part via the Nrf2 transcriptional pathway along with the upregulation of Mrp2 expression. Besides UDCA, some nuclear receptor ligands (13, 41) and other Nrf2 activators (14, 20) may also be involved in the regulation of transcriptional levels of Mrp3 and Mrp4. Moreover, UDCA and other bile acids act as FXR ligands (12) and UDCA represses a transcriptional factor NF-κB (19). Therefore, it should be noted that UDCA-induced alternative Nrf2-independent pathways might exist, potentially mediating some of the phenomena observed in this study.

Following the study on stimulation of hepatobiliary transport and detoxification systems by UDCA in humans (15), experiments using the chimeric mice were performed to study the effects of UDCA on the expression levels of human MRP2, MRP3, and MRP4. Analogous to mice, the expression levels of MRP2, MRP3, and MRP4 proteins were found to be significantly upregulated in the humanized livers of UDCA-treated mice (Fig. 8). These findings indicate that the effect of UDCA on induction of these MRP family members are traced in humanized livers of chimeric mice. Similarly in human livers, UDCA may stimulate Nrf2-mediated hepatocellular transport.

In terms of the mechanism by which Nrf2 regulates the expression of Mrp family members, we have recently reported that several ARE/EpRE core elements can be found in the promoter regions of Mrp2, Mrp3, and Mrp4 (14), besides a previous study (39). The presence of these ARE/EpRE elements, thus, gives further evidence that Nrf2 can directly interact with cis-acting elements in the promoter regions of Mrp2, Mrp3, and Mrp4, and some of these elements show positive Nrf2 binding in vitro assays (14). Also in this study, the ChIP assays have shown that UDCA results in increased

Fig. 9. Schematic summary of the effects of UDCA via the Nrf2 transcriptional pathway on coordinated stimulation of hepatobiliary transport, detoxification and antioxidative stress systems in mouse liver. UDCA upregulates 1) the efflux pumps Mrp2, Mrp3, and Mrp4; 2) the detoxifying enzyme Nqo1; and 3) the antioxidative stress gene γ-Gcs in the liver. ROS, reactive oxygen species; Bili, bilirubin; Bili-Glc, bilirubin glucuronide; BA, bile acid; GSH, glutathione; PL, phospholipid.
binding of Nrf2 to the ARE/EpRE elements in the promoter regions of the Mrp2, Mrp3, and Mrp4 (Fig. 3).

In response to UDCA treatment, Nrf2 also plays an important role in upregulating hepatic expression of detoxifying enzymes, i.e., Nqo1 and Gsta2 and that of antioxidative stress genes, i.e., γ-GCs via the Nrf2 transcriptional pathway (Fig. 6). A number of studies have demonstrated cytoprotective effects of UDCA on cholestatic livers possibly through the stabilizing generation system of GSH, an important endogenous antioxidative stress system of GSH, an important endogenous antioxidative stress system (2, 27), and the resultant increase in GSH synthesis has already been demonstrated in rats (27). Thus the intrinsic induction of GSH-mediated antioxidant defense may be potentiated by UDCA treatment via the Nrf2 transcriptional pathway.

In summary, the results of this study suggest that 1) activation of Nrf2 regulatory pathway serves to stimulate coordinated induction of hepatic efflux transporters, i.e., Mrp2, Mrp3, and Mrp4; 2) UDCA treatment releases Nrf2 from Keap1 repression in livers, where it translocates into the nucleus; 3) the UDCA-induced activation of Nrf2 results in potent induction of these efflux transporters, detoxifying enzymes, i.e., Nqo1, and antioxidative stress genes, i.e., γ-GCs in the livers (Fig. 9); 4) UDCA exerts coordinate regulation via the Nrf2 transcriptional pathway of hepatobiliary transport, detoxification, and antioxidative stress systems; and 5) that the effect of UDCA on Mrp family members are traced in humanized livers of chimeric mice. Thus the properties of UDCA encourage further research to establish the rationale for therapeutic options in cholestatic hepatobiliary diseases.

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