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

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Endotoxin-induced cholestasis is mainly caused by an impaired canalicular secretion. Mrp2, the canalicular multispecific organic anion transporter, is strongly down-regulated in this situation, and canalicular bile salt secretion is also reduced. We hypothesized that other adenosine triphosphate–binding cassette (ABC) transporters may compensate for the decreased transport activity to protect the cell from cytokine-induced oxidative damage. Therefore, we examined the expression of ABC-transport proteins in membrane fractions of whole liver and of isolated hepatocytes of endotoxin-treated rats and performed reverse-transcriptase polymerase chain reaction (RT-PCR) on mRNA isolated from these livers. In addition, the localization of these transporters was examined using confocal scanning laser microscopy. By 6 hours after endotoxin administration, we found a clear increase of mrp1 mRNA and protein, whereas mrp2 mRNA and protein were decreased. This was confirmed in isolated hepatocytes. In addition, *mdr1b* mRNA was strongly increased, whereas *mdr1a* and *mdr2* mRNA did not change significantly. Both the mRNA and protein levels of the sister of P-glycoprotein (spgp), the recently cloned bile salt transporter, decreased. After endotoxin treatment, the normally sharply delineated canalicular staining of mrp2 and spgp had changed to a fuzzy pattern, suggesting localization in a subapical compartment. We conclude that endotoxin-induced cholestasis is caused by decreased mrp2 and spgp levels, as well as an abnormal localization of these proteins. The simultaneous up-regulation of mrp1 and mdr1b may confer resistance to hepatocytes against cytokine-induced metabolic stress. (HEPATOLOGY 1998;28:1637-1644.)

Excretion of a large variety of endogenous and exogenous compounds from hepatocytes into bile is an adenosine triphosphate (ATP)-dependent process, predominantly performed by members of the P-glycoprotein (Pgp) subfamily and the multidrug-resistance protein (MRP) subfamily of the ATP-binding cassette (ABC) protein superfamily.[1,2]

At least four members of the Pgp subfamily are located at the canalicular membrane of rodent liver: mdr1a, mdr1b, mdr2, and spgp. In normal rodent liver, mdr1a and mdr1b are present at low levels. Overexpression of mouse *mdr1a/mdr1b* confers multidrug resistance against a broad variety of natural product drugs.[3,4] The main physiological function of these multidrug-resistance proteins is presumably the transport of bulky amphiphilic compounds, such as cationic drugs, hydrophobic peptides, steroids, and atypical glycolipids, across the canalicular membrane.[1,4,5] The expression of mdr2 in normal rodent liver is high. This transporter functions as a flippase that translocates phosphatidylcholine across the membrane.[6] From pig liver, Childs et al. cloned part of another member of the Pgp subfamily: the sister gene of Pgp (*spgp*).[7] Most recently, Gerloff et al. cloned the full-length cDNA of spgp from rat liver.[8] They provided evidence that spgp, which is exclusively present in the liver, most likely is the major canalicular bile salt export pump of mammalian liver.

So far, the presence of four members of the mrp subfamily has been demonstrated in liver.[9,10] MRP1/mrp1 is present at very low levels at the lateral membrane of the hepatocyte.[11,12] The human MRP1 and its murine homologue, mrp1, have been shown to confer multidrug resistance to natural product drugs.[13,14] Moreover, MRP1 is able to transport glutathione disulfide[15] and glutathione (GSH) *S*-conjugates including the lipid peroxidation products, leukotriene C_4[16,17] and 4-hydroxynonenal S-GSH.[18] The recently cloned *mrp1* homologue, *mrp2*,[19,20] is strongly expressed at the canalicular

Abbreviations: ATP, adenosine triphosphate; Pgp, P-glycoprotein; MRP, multidrug-resistance protein; ABC, ATP-binding cassette; MDR, multidrug resistance; spgp, product of the sister gene of Pgp; GSH, glutathione; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor α; NF-κB, nuclear factor-κB; RT-PCR, reverse-transcriptase polymerase chain reaction; CSLM, confocal scanning laser microscopy; PBS, phosphate-buffered saline; dppIV, dipeptidyl peptidase IV; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

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membrane and functions as the canalicular multispecific organic anion transporter.[21,22] Kool et al. recently demonstrated the presence of two other *MRP1* homologues, *MRP3* and *MRP5*, in human liver.[10] The function and subcellular localization of these proteins are still unknown.

Cholestasis caused by the action of lipopolysaccharide (LPS) seems to be mainly the result of a decreased bile salt–independent bile flow, which is most probably caused by down-regulation of the transport capacity for mrp2 substrates (polyanionic organic conjugates) and GSH.[23-25] Recently, down-regulation of mrp2 during endotoxemia has been reported that may explain the defective anion secretion found after endotoxin treatment.[26] Secretion of monovalent bile salts is also impaired during endotoxemia,[23,27-29] suggesting down-regulation of the bile salt transporter, but this has not yet been studied.

LPS-induced changes in hepatocytes are mainly caused by the action of tumor necrosis factor α (TNF-α), and possibly interleukin-1, interleukin-6, and other cytokines. TNF-α binding to its receptor activates a complex intracellular signaling pathway that includes translocation of the transcription factor, nuclear factor-κB (NF-κB) to the nucleus and the production of reactive oxygen species.[30] We have hypothesized that transporters such as mrp1 and mrp2 play a role in the GSH-dependent cellular response to oxidative stress.[1] As a result of the decreased mrp2 activity during endotoxemia, a significant increase of liver damage by products of cytokine-induced oxidative stress would be expected. However, cholestasis and sepsis usually occur without much hepatocellular damage. Indeed, we observed only a minimal increase of aminotransferase levels during endotoxemia.[31] These observations suggest that the hepatocyte may compensate for the mrp2 down-regulation by up-regulation of other transport systems.

The aim of the present study was to evaluate the expression of the presently known rat liver ABC-transporters during endotoxemia using specific antibodies against mrp1, mrp2, and spgp, and semiquantitative reverse-transcriptase polymerase chain reaction (RT-PCR) for *mrp1*, *mrp2*, *mdr1a*, *mdr1b*, *mdr2*, and *spgp*. We also investigated the subcellular localization of these transporters in normal and endotoxemic rat liver using confocal scanning laser microscopy (CSLM).

MATERIALS AND METHODS

Animals. Specified pathogen-free male Wistar rats (220-300 g) were purchased from Harlan-CPB, Zeist, the Netherlands. They were kept under routine laboratory conditions at the Central Animal Laboratory of the University of Groningen. The rats received standard laboratory chow and had free access to food and water. The study as presented was approved by the Local Committee for Care and Use of Laboratory Animals.

Experimental Design. Rats were injected intraperitoneally with 5 mg/kg endotoxin (LPS, *Escherichia coli*, serotype 0127:B8, Sigma, St. Louis, MO) or the same volume of phosphate-buffered saline (PBS) (control group, n = 6 for each experimental group). In the experimental groups, no mortality caused by endotoxin administration was observed. At indicated time points, the rats were anesthetized with pentobarbital (60 mg/kg intraperitoneally). The livers were perfused with PBS, removed, cut into small pieces, snap-frozen in liquid nitrogen, and stored at −80°C until used for isolation of crude membranes and RNA. For isolation of hepatocytes, rats were injected intraperitoneally with 2 mg/kg LPS (Sigma) or PBS, and rat hepatocytes were isolated 15 hours later by two-step collagenase perfusion as described previously.[32]

Antibodies. Specific polyclonal antibodies against mrp1 (k5) were raised by immunizing rabbits with the peptide, RGLFYSMAKDAGLV, located at the carboxy terminal of human MRP1. This antibody does not cross-react with mrp2, as described by Roelofsen et al.[12] In addition, no cross-reactivity with rat mrp3 was found (Roelofsen et al., February 1997, unpublished data). Polyclonal antibodies against mrp2 (k4) were raised by immunizing rabbits with the peptide, AKEAGIENVNHTEL, located at the carboxy terminal of rat mrp2. On Western blot, the mrp2 antibody specifically recognizes a protein of approximately 180 kd in enriched canalicular membrane fractions from normal rat liver. This protein is absent in the canalicular membrane fractions from mrp2-deficient (TR^-) rats. A polyclonal antibody against spgp (k12) was raised by immunizing rabbits with the peptide, KGAYYKLVTTGAPIS, from the carboxy terminal of the pig spgp sequence.[7] This peptide differs in one amino acid with the recently published rat spgp[8]; a threonine (underlined) in pig spgp is substituted by an isoleucine in rat spgp. The spgp sequence exhibits no homology with mrp1, mrp2, mdr1a, mdr1b, or mdr2 sequences. On Western blot, the spgp antibody recognizes a protein of approximately 150 kd in canalicular membrane fractions from rat liver, but not in the basolateral enriched fraction. No specific signals were seen in membrane subfractions from cell lines overexpressing MRP1 or MDR1 (data not shown). Mouse monoclonal antibody C219 (Signet Laboratories Inc., Dedham, MA) was used to detect all Pgps. Mouse monoclonal antibody against dipeptidyl peptidase IV (dppIV/CD26) was purchased from Endogen (Woburn, MA).

Isolation of Crude Membranes From Liver Homogenates. Crude membranes were isolated according to Meier and Boyer,[33] with some modifications. In brief, for each sample, 2 g of frozen rat liver was thawed in 5 mL cold 1 mmol/L $NaHCO_3$/complete protease inhibitor cocktail (1 tablet/50 mL; Boehringer Mannheim GmbH, Mannheim, Germany) and homogenized with a Polytron (Kinematica GmbH, Luzern, Switzerland) and a loose Dounce homogenizer (five strokes up and down). The homogenate was further diluted in ice-cold 1 mmol/L $NaHCO_3$/0.1 mmol/L phenylmethylsulfonyl fluoride (PMSF) and centrifuged at 1,200*g* for 15 minutes at 4°C. The crude nuclear pellet was resuspended in 2.5 volumes of 70% (wt/wt) sucrose. The homogenate was poured into a SW28 rotor tube (Beckman Instruments Inc., Palo Alto, CA) and overlaid with 10 mL 44% and 10 mL 36.5 % (wt/wt) sucrose. The tube was centrifuged at 90,000*g* for 90 minutes at 4°C. The crude membrane fraction from the 36.5% to 44% interface was centrifuged in 1 mmol/L $NaHCO_3$/0.1 mmol/L PMSF at 7,800*g* for 15 minutes at 4°C. The pellet was resuspended in 500 μL 1 mmol/L $NaHCO_3$/protease inhibitor by repeated (15 times in and out) suctioning through a 25-gauge needle. The sample was stored at −80°C until further use.

Isolation of Crude Membranes From Isolated Hepatocytes. Freshly isolated hepatocytes were washed in Krebs buffer (118 mmol/L NaCl, 5 mmol/L KCl, 1.1 mmol/L $MgSO_4$, 2.5 mmol/L $CaCl_2$, 1.2 mmol/L KH_2PO_4, 25 mmol/L $NaHCO_3$, 10 mmol/L D(+)glucose, 10 mmol/L HEPES, 1% bovine serum albumin [pH 7.42]) and pelleted at 500*g* for 8 minutes. For permeabilization, cells were stirred in 35 mL 1 mmol/L $NaHCO_3$/0.1 mmol/L PMSF for 1 hour at 4°C. After centrifugation of the homogenate at 90,000*g* for 30 minutes at 4°C, the pellet was resuspended in 40 mL 250 mmol/L sucrose/0.1 mmol/L PMSF and homogenized 50 times with a tight Dounce homogenizer. Per liver, three SW28 tubes were loaded with 15 mL 38% sucrose and 15 mL hepatocyte homogenate, respectively. After centrifugation at 90,000*g* for 90 minutes at 4°C, the 38% fraction was washed in 1 mmol/L $NaHCO_3$/0.1 mmol/L PMSF and centrifuged at 47,000*g* for 30 minutes at 4°C. Pellets were resuspended in 250 mmol/L sucrose/protein inhibitor by 15 times in-and-out suctioning through a 25-gauge needle and stored at −80°C until further use.

Western Blot Analysis. The protein concentrations in membrane fractions were determined with the DC Protein Assay from Bio-Rad (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as standard. Fifty micrograms of membrane proteins was fractionated on a 7.5% sodium dodecyl sulfate–polyacrylamide gel electro-

phoresis (SDS-PAGE) gel and transferred to nitrocellulose (Amersham International plc, Buckinghamshire, England), using a tankblotting system according to the manufacturer's instructions (Bio-Rad Laboratories). For dppIV detection, samples were boiled in sample buffer for 5 minutes before loading on SDS-PAGE. BDH molecular-weight standards (42,700-200,000 molecular-weight range; BDH Ltd., Dorset, England) were used as marker proteins. The blots were stained with Ponceau S-solution (0.1% Ponceau S [wt/vol] in 5% acetic acid [vol/vol]; Sigma) to confirm similar protein concentrations in every lane. The blots were incubated with the first antibody diluted in PBS containing 4% SKIM milk (Fluka BioChemica, Buchs, Switzerland) and 0.1% polyoxyethylene sorbitan monolaurate (Tween-20, Sigma), washed in PBS/0.1% Tween-20, subsequently incubated with horseradish peroxidase–labeled swine anti-rabbit IgG or rabbit anti-mouse IgG diluted in PBS/4% SKIM milk/0.1% Tween-20 (dilution 1:2,000; DAKO A/S, Glostrup, Denmark), and finally developed using Pierce SuperSignal Chemiluminescent Substrate Luminol/Enhancer (Pierce, Rockford, IL).

RT-PCR. Total RNA was isolated from frozen rat liver and isolated hepatocytes using TRIzol Reagent (Gibco Laboratories, Grand Island, NY) according to the manufacturer's instructions. Subsequently, mRNA was isolated using the Oligotex mRNA mini-kit (Qiagen GmbH, Hilden, Germany). Single-stranded cDNA was synthesized from 2.5 μg mRNA using 0.5 nmol random primers (Pharmacia, Uppsala, Sweden) and 50 U AMV Reverse-Transcriptase (Promega, Madison, WI) in a buffer containing 50 mmol/L Tris-HCl, 50 mmol/L KCl, 10 mmol/L dithiothreitol, 10 mmol/L $MgCl_2$ (Promega), 50 U RNA guard (Pharmacia), and 1.25 mmol/L of each dNTP (Pharmacia) in a total volume of 100 μL. RT was performed for 10 minutes at 25°C and for 1 hour at 50°C, and the samples were subsequently heated for 5 minutes at 95°C to terminate the RT reaction. With the cDNA obtained, a PCR reaction was performed using 4 μL of the RT reaction mix. To this, 2.5 U Taq polymerase (Pharmacia), 50 pmol sense, and 50 pmol antisense primer were added. The final reaction volume was 50 μL. The tubes were incubated in a Gene Amp PCR system 2400 (Perkin-Elmer, Norwalk, CT) at 95°C for 5 minutes to denature the primers and cDNA. The cycling program was 95°C for 30 seconds, 54/56°C for 30 seconds, 72°C for 30 seconds, and for 5 minutes in the last cycle, and comprised 20 to 33 cycles. For each primer set, an increasing number of PCR cycles with otherwise fixed conditions was performed to determine the optimal number of cycles to be used. This was determined to be halfway through the exponential phase. The numbers of cycles were 20 for spgp, 22 for glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and mrp2, 26 for mdr2, 29 for mdr1b, and 33 for mdr1a and mrp1. For every PCR reaction, GAPDH was used as internal control. Primer sequences and resulting PCR products are listed in Table 1. Because of the high homology between mrp1 and mrp3, we sequenced the mrp1 PCR product. Sequence analysis confirmed the specificity of the PCR primers for mrp1. In each experiment, water was used as a negative control. Ten microliters of PCR product was loaded on a 2.5% agarose gel and stained with ethidium bromide.

CSLM. Frozen rat liver sections were stained with C219 (dilution, 1:100), k12 (dilution, 1:250), or k4 (dilution, 1:500), washed, and subsequently incubated with fluorescein isothiocyanate–labeled goat anti-mouse or sheep anti-rabbit immunoglobulin G or tetramethylrhodamine isothiocyanate–labeled goat anti-mouse or goat anti-rabbit immunoglobulin G (all from Sigma). Images were taken with a CSLM (True Confocal Scanner 4D, Leica, Heidelberg, Germany) equipped with an argon/krypton laser and coupled to a Leitz DM IRB inverted microscope (Leica). Double-labeled images were taken sequentially at 488 and 562 nm to avoid bleed-through into the other channel.

TABLE 1. Primers Used for the Analysis of ABC-Transporter Gene Expression by RT-PCR

Primers	Sense and Antisense	PCR Product (bp)	Reference
mrp1	5′-TTCTAGTGTTGGACGAGGCT-3′	227	34
	5′-TGGCCATGCTATAGAAGACG-3′		
mrp2	5′-ACCTTCCACGTAGTGATCCT-3′	1084	19
	5′-GATTTCCCAGAGCCTACAGT-3′		
mdr1a	5′-GATGGAATTGATAATGTGGACA-3′	351	35
	5′-AAGGATCAGGAACAATAAA-3′		
mdr1b	5′-GAAATAATGCTTATGAATCCCAAAG-3′	325	35
	5′-GGTTTCATGGTCGTCGTCTCTTGA-3′		
mdr2	5′-AAGAATTTGAAGTTGAGCTAAGTGA-3′	143	35
	5′-TGGTTTCCACATCCAGCCTAT-3′		
spgp	5′-GAGGTTACTTAATAGCCTACG-3′	413	36
	5′-CATCTATCATCACAGTTCCC-3′		
GAPDH	5′-CCATCACCATCTTCCAGGAG-3′	576	37
	5′-CCTGCTTCACCACCTTCTTG-3′		

RESULTS

Western Blot Analysis. To study the expression of different hepatic ABC-transport proteins during endotoxemia, specific polyclonal antibodies were raised against mrp1, mrp2, and spgp, and their protein levels were examined by immunoblot analysis. Results are shown in Fig. 1. For the sake of clarity, only immunoblots of crude membrane preparations of each time point of one representative experiment are shown. Similar results were observed with membrane preparations from other rat liver samples (n = 4 per time point).

During endotoxemia, a clear increase of mrp1 protein in crude membranes of LPS-treated rat liver was seen (Fig. 1). This increase was already noticeable 6 hours after LPS and was still present after 48 hours. In contrast, a strong decrease of mrp2 protein was observed. This decrease of the mrp2 protein levels was observed as early as 15 hours after LPS, and 24 to 48 hours after LPS, mrp2 was undetectable. In addition to members of the mrp subfamily, we also examined the protein levels of members of the Pgp subfamily. This was performed by using the monoclonal antibody, C219, which recognizes a conserved epitope in all known members of the Pgp subfamily.[7] The C219 staining of crude membranes decreased during endotoxemia (Fig. 1). Using a specific antibody against spgp, a similar decrease was observed. The crude membrane fractions showed a similar staining of dppIV, a major canalicular membrane protein, indicating that the recovery of crude membranes during the isolation procedure was approximately the same.

mRNA Analysis by RT-PCR. We chose RT-PCR to investigate the regulation of the ABC-transporter genes, because Northern blotting is not sensitive enough to detect mrp1, mdr1a, and mdr1b in normal rat liver. Furthermore, the specificity of the PCR reactions can be confirmed by sequencing the PCR products. We developed RT-PCR assays using rat-specific PCR primers against mrp1, mrp2, mdr1a, mdr1b, mdr2, and spgp to analyze mRNA levels of the respective transporter genes. In Fig. 2, RT-PCR products are shown from a typical experiment of RNA samples from control rat liver and livers 6, 15, 24, and 48 hours after LPS treatment. The results were similar for the other mRNA samples examined (n = 4 per time point).

Mrp1 mRNA was increased 6 hours after LPS administration and remained increased for at least 48 hours, whereas mrp2 mRNA completely disappeared 6 hours after LPS and returned after 48 hours (Fig. 2). In the Pgp subfamily, the mRNA levels of mdr1a and mdr2 did not change significantly

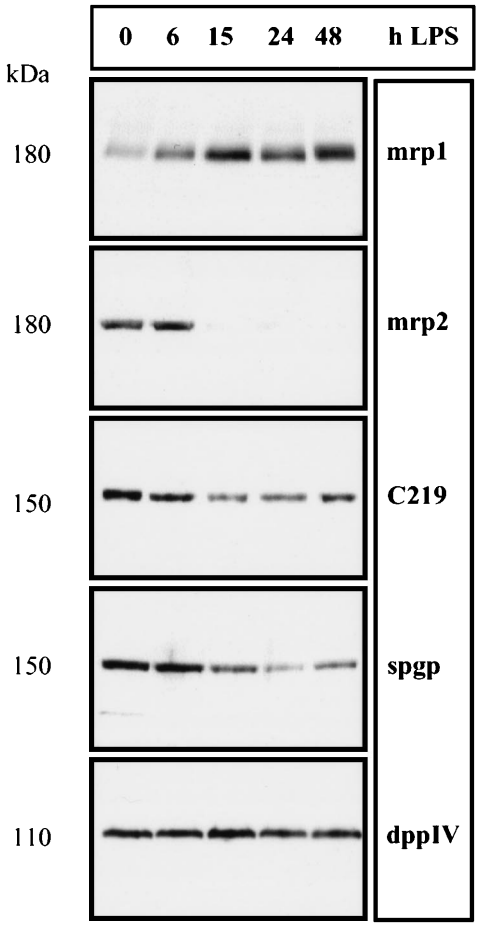

FIG. 1. Mrp1, mrp2, C219, spgp, and dppIV levels in crude membrane fractions of endotoxin-treated rat liver. Fifty micrograms of crude membranes isolated from livers of control rats or rats 6, 15, 24, or 48 hours after LPS administration was separated on a 7.5% SDS-PAGE gel and transferred to nitrocellulose. Mrp1, mrp2, C219 (detecting all Pgps), spgp, and dppIV were visualized using the corresponding antibodies, as indicated on the *right side* of each blot. Time after LPS administration is indicated *above* the figure; apparent molecular weights are on the *left side* of each blot. Representative experiment of n = 4 per time point.

during endotoxemia. Compared with mrp2, spgp showed a moderately decreased mRNA expression. A clear increase of mdr1b mRNA was observed from 6 hours until at least 48 hours after LPS treatment (Fig. 2). Levels of GAPDH were not influenced by LPS treatment.

mRNA and Protein Levels of Mrp1 and Mrp2 in Isolated Hepatocytes. In spite of the detection of mrp1 in crude membrane fractions of total rat liver (Fig. 1), we were not able to detect the protein in frozen liver sections from LPS-treated rats (CSLM). This is most likely a result of the relatively low expression of mrp1 in rat liver hepatocytes. To prove that the mrp1 up-regulation takes place in hepatocytes, we isolated hepatocytes from normal and LPS-treated rats (2 mg/kg, 15 hours) and analyzed the mRNA and protein levels of mrp1 and mrp2. Also in isolated hepatocytes, mrp1 protein (Fig. 3) as well as mrp1 mRNA (Fig. 4) were increased, whereas mrp2 protein (Fig. 3) and mrp2 mRNA (Fig. 4) were decreased. Moreover, mRNA levels of mdr1a and mdr2 did not change, whereas mdr1b mRNA increased and spgp mRNA decreased (Fig. 4). On Western blot, spgp and C219 signal decreased, whereas dppIV signal did not change (Fig. 3). These results are comparable with the results obtained from total rat liver (Figs. 1 and 2).

Localization Studies Using CSLM. CSLM was used to assess the localization of hepatic ABC-transport proteins during endotoxemia. The staining of spgp, C219 (detecting all Pgps), and mrp2 was examined in frozen liver sections of control rats and rats 6 hours after LPS administration.

Comparison of the spgp and C219 labeling showed a clear colocalization at the canalicular membrane of normal rat liver (Fig. 5A and 5C). The same pattern was observed when mrp2 staining was compared with C219 (not shown). However, by 6 hours after LPS, the staining of spgp and C219 became irregular and "fuzzy," as shown by double-labeling (Fig. 5B and 5D). Likewise, mrp2 staining was normal in control (Fig. 5E) and irregular in LPS-treated rat liver (Fig. 5F). When detectable, this "fuzzy" staining was also present at later time points after LPS (not shown).

DISCUSSION

In the present study, we have demonstrated that mrp1 and mdr1b expression are increased in the early phase of endotoxemia (6 hours after LPS), whereas mrp2 expression is decreased. In addition, at 15 hours after LPS treatment, the expression of the recently cloned bile salt transporter, spgp,[8] is also decreased. These results are the first indications of increased expression of the two multidrug-resistance genes, *mrp1* and *mdr1b*, in hepatocytes of LPS-treated animals. Furthermore, the decreased levels of spgp may explain the

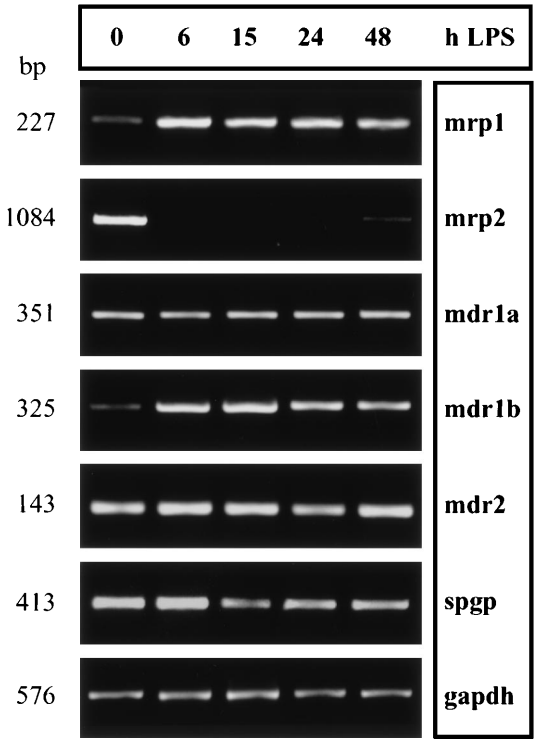

FIG. 2. Regulation of mrp1, mrp2, mdr1a, mdr1b, mdr2, spgp, and GAPDH mRNA expression during endotoxemia. RT-PCR products of total rat liver RNA after LPS administration. Mrp1, mrp2, mdr1a, mdr1b, mdr2, spgp, and GAPDH PCR products were separated on a 2.5% agarose gel. PCR products are indicated on the *right side* of each gel; corresponding base pair lengths are on the *left side*; and time after LPS administration is *above* the figure. Representative experiment of n = 4 per time point.

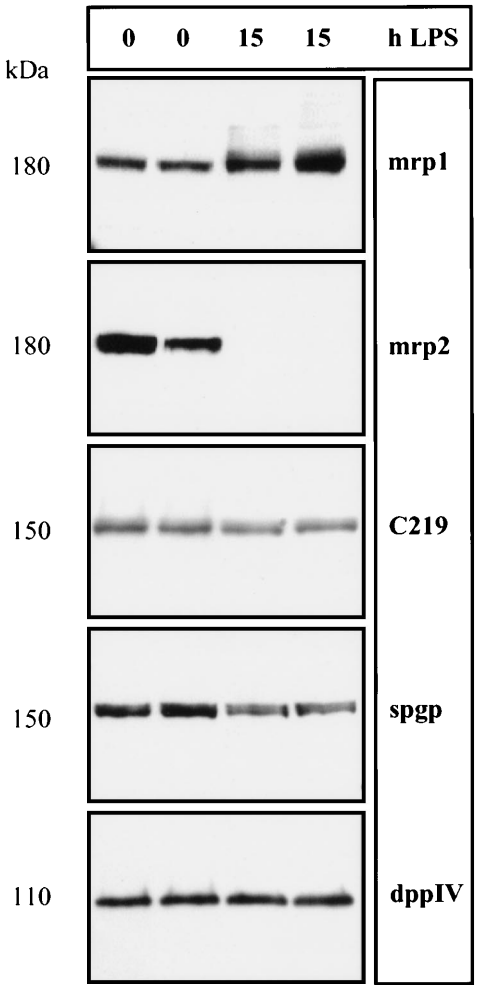

FIG. 3. Mrp1, mrp2, C219, spgp, and dppIV levels in isolated hepatocytes during endotoxemia. Western blot of crude membranes (50 μg) from isolated hepatocytes of control rats or rats 15 hours after LPS administration.

reported decreased transport of bile salts after endotoxin treatment.[23,27-29]

It is known that LPS-induced changes in hepatocytes are mainly mediated by TNF-α.[38] The transcription factor NF-κB has been recognized as a major modulator of gene regulation induced by TNF-α and other cytokines. A κB-element (mdr-κB) has recently been identified in the promoter of the rat *mdr1b* gene.[39] Thus, on the basis of our results and data from the literature, we speculate that TNF-α binding to hepatocytes results in the rapid translocation of NF-κB to the nucleus, where it is in part responsible for up-regulation of transporter genes such as *mdr1b* and *mrp1*. Interestingly, as demonstrated recently, TNF-α induces *MRP1* in human colon carcinoma cells,[40] and TNF-α induces *mdr1* in a rat hepatoma cell line.[41] The finding that mrp1 induction in cultured hepatocytes takes place at the mid G_1-phase of the cell cycle[42] further supports our finding, because cytokines such as TNF-α and interleukin-6 are known to initiate a proliferation trigger in hepatocytes *in vivo*.[43-46]

We hypothesize that ABC-transporters like MDR1 and MRP1 and their rodent homologues function as an aspecific protection system to maintain cellular membrane integrity by extruding cytotoxic compounds such as lipid peroxidation products. Furthermore, MRP1/mrp1 may contribute to the regulation of the intracellular redox state by transporting glutathione disulfide.[15] In addition, MRP1/mrp1 has been demonstrated to transport the GSH *S*-conjugate of the major lipid peroxidation product, 4-hydroxynonenal.[18]

In contrast to increased mrp1 and mdr1b levels, we observed an almost complete disappearance of mrp2 and a moderate decrease of spgp on mRNA as well as protein levels. A decreased canalicular expression of mrp2 is in agreement with the observed reduction in transport of mrp2 substrates into bile after LPS treatment.[23-25,29,47] Trauner et al.[26] recently described down-regulation of mrp2 mRNA and protein 18 hours after LPS. We can add to this that mrp2 mRNA was undetectable as early as 6 hours after LPS and started to return to normal levels 48 hours later.

GSH is an important cytosolic factor for protection of cells against reactive oxygen species and toxic electrophiles resulting from peroxidation reactions. It has been suggested that MRP2 is involved in canalicular secretion of GSH,[1,48] the molecular driving force of bile salt–independent bile flow. If this is the case, the rapid down-regulation of mrp2 during sepsis may prevent secretion of GSH into bile. Indeed, after LPS administration, decreased biliary GSH output and decreased GSH concentrations in bile have been reported.[47] Interestingly, MRP1 may also transport GSH.[49] Thus, up-regulation of lateral mrp1 may result in an increased GSH secretion into the blood. Extracellular GSH may serve as a scavenger for reactive oxygen species released by activated neutrophils.

In our hands, spgp and Pgps levels showed a comparable decrease during endotoxemia, whereas Trauner et al.[26] described an unchanged signal when the C219 antibody was

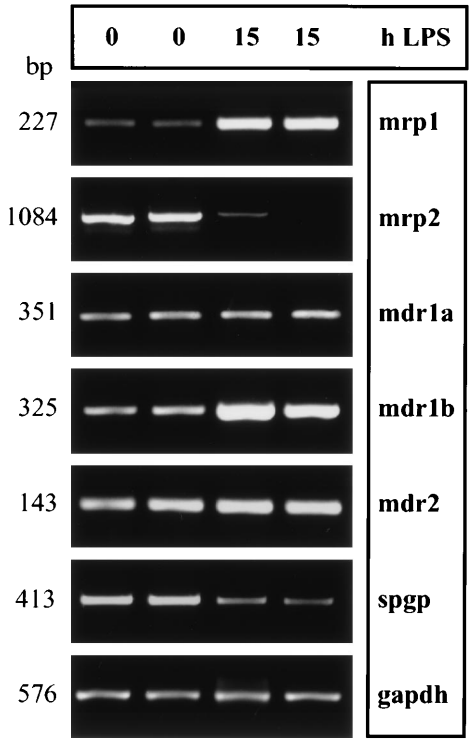

FIG. 4. Regulation of mrp1, mrp2, mdr1a, mdr1b, mdr2, spgp, and GAPDH mRNA expression in isolated hepatocytes during endotoxemia. RT-PCR products of RNA from isolated hepatocytes of control rats or rats 15 hours after LPS administration.

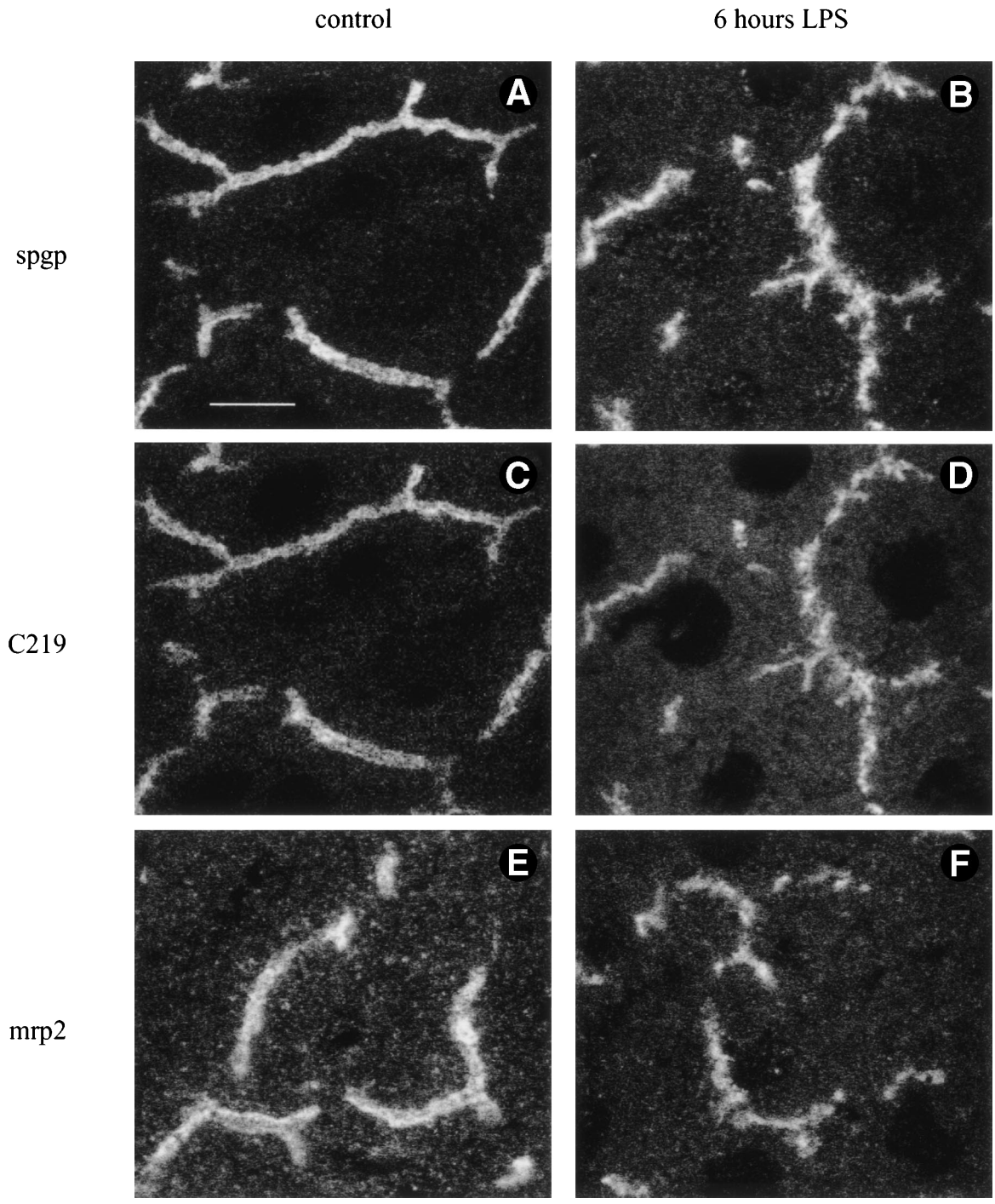

FIG. 5. Localization of spgp, C219, and mrp2 in control rat liver and rat liver 6 hours after LPS treatment using CSLM. A canalicular colocalization of spgp (A) and C219 (C) is seen in frozen rat liver sections of control rats. Six hours after LPS, the localization of spgp (B) and C219 (D) appears irregular and fuzzy. In addition, the localization of mrp2 is normal in control rat liver (E) and changed to a fuzzy pattern in rat livers 6 hours after LPS (F). Typical staining patterns of n = 3 per group. *Bar* = 10 μm.

used. In normal rodent liver, spgp and mdr2 are present at high levels, whereas the expression of mdr1a and mdr1b are very low. Therefore, the signal detected by C219 in normal liver will predominantly represent spgp and mdr2. The decrease seen with the C219 antibody after LPS treatment will therefore result from the combination of a down-regulated spgp, an increased mdr1b, and unchanged mdr1a and mdr2. Our finding that spgp is decreased after LPS treatment may explain the reported reduced bile salt transport after LPS administration.[23,28,29,50]

Not only a variation of the amount of protein can lead to an altered canalicular transport; it is obvious that the subcellular localization of the protein is also important. Trauner et al.[26] reported on a punctuate staining of mrp2 in LPS-treated rat livers 18 hours after LPS. In their study, this punctuate pattern was not observed with C219 or an antibody against

ecto-ATPase/cell CAM105. By 6 hours after LPS treatment, we found a fuzzy staining pattern in frozen liver sections of endotoxemic rats for mrp2, spgp, and Pgps (C219). The origin of the fuzzy staining could be a result of localization of transporters in a subapical vesicular compartment, as speculated by Trauner for mrp2.[26]

In summary, we conclude that LPS-induced cholestasis is the result of a down-regulation of mrp2- and spgp-expression, as well as an altered localization of the transporters. The expression of the multidrug-resistance genes, *mrp1* and *mdr1b*, is strongly increased. By conferring resistance to hepatocytes, these transporters likely play a role in the cellular response against cytokine-induced stress.

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REFERENCES

1. Müller M, Jansen PLM. Molecular aspects of hepatobiliary transport. Am J Physiol 1997;272:G1285-G1303.
2. Oude Elferink RPJ, Meijer DKF, Kuipers F, Jansen PLM, Groen AK, Groothuis GMM. Hepatobiliary secretion of organic compounds; molecular mechanisms of membrane transport. Biochim Biophys Acta 1995;1241: 215-268.
3. Gottesman MM, Hrycyna CA, Schoenlein PV, Germann UA, Pastan I. Genetic analysis of the multidrug transporter. Annu Rev Genet 1995;29: 607-649.
4. Silverman JA, Thorgeirsson SS. Regulation and function of the multidrug resistance genes in liver. Prog Liver Dis 1995;13:101-123.
5. van Helvoort A, Smith AJ, Sprong H, Fritzsche I, Schinkel AH, Borst P, van Meer G. MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 p-glycoprotein specially translocates phosphatidylcholine. Cell 1996;87:507-517.
6. Oude Elferink RPJ, Tytgat GNJ, Groen AK. The role of mdr2 P-glycoprotein in hepatobiliary lipid transport. FASEB J 1997;11:19-28.
7. Childs S, Yeh RL, Georges E, Ling V. Identification of a sister gene to P-glycoprotein. Cancer Res 1995;55:2029-2034.
8. Gerloff T, Stieger B, Hagenbuch B, Madon J, Landmann L, Roth J, Hofmann AF, et al. The sister of P-glycoprotein represents the canalicular bile salt export pump of mammalian liver. J Biol Chem 1998;273:10046-10050.
9. Müller M, Roelofsen H, Jansen PLM. Secretion of organic anions by hepatocytes: involvement of homologues of the multidrug resistance protein. Semin Liver Dis 1996;16:211-220.
10. Kool M, de Haas M, Scheffer GL, Scheper RJ, van Eijk MJT, Juijn JA, Baas F, et al. Analysis of expression of *cMOAT (MRP2)*, *MRP3*, *MRP4*, and *MRP5*, homologues of the multidrug resistance-associated protein gene *(MRP1)*, in human cancer cell lines. Cancer Res 1997;57:3537-3547.
11. Kartenbeck J, Leuschner U, Mayer R, Keppler D. Absence of the canalicular isoform of the *MRP* gene-encoded conjugate export pump from the hepatocytes in Dubin-Johnson syndrome. HEPATOLOGY 1996;23: 1061-1066.
12. Roelofsen H, Vos TA, Schippers IJ, Kuipers F, Koning H, Moshage H, Jansen PLM, et al. Increased levels of the multidrug resistance protein in lateral membranes of proliferating hepatocyte-derived cells. Gastroenterology 1997;112:511-521.
13. Cole SPC, Sparks KE, Fraser K, Loe DW, Grant CE, Wilson GM, Deeley RG. Pharmacological characterization of multidrug resistant MRP-transfected human tumor cells. Cancer Res 1994;54:5902-5910.
14. Stride BD, Valdimarsson G, Gerlach JH, Wilson GM, Cole SPC, Deeley RG. Structure and expression of the messenger RNA encoding the murine multidrug resistance protein, an ATP-binding cassette transporter. Mol Pharmacol 1996;49:962-971.
15. Leier I, Jedlitschky G, Buchholz U, Center MS, Cole SPC, Deeley RG, Keppler D. ATP-dependent glutathione disulphide transport mediated by the *MRP* gene-encoded conjugate export pump. Biochem J 1996;314: 433-437.
16. Leier I, Jedlitschky G, Buchholz U, Cole SPC, Deeley RG, Keppler D. The *MRP* gene encodes an ATP-dependent export pump for leukotriene C_4 and structurally related conjugates. J Biol Chem 1994;269:27807-27810.
17. Müller M, Meijer C, Zaman GJR, Borst P, Scheper RJ, Mulder NH, de Vries EGE, et al. Overexpression of the gene encoding the multidrug resistance-associated protein results in increased ATP-dependent glutathione S-conjugate transport. Proc Natl Acad Sci U S A 1994;91:13033-13037.
18. Renes J, de Vries EGE, Nienhuis EF, Jansen PLM, Müller M. Transport of the glutathione S-conjugate of 4-hydroxynonenal and of hydrophobic cationic drugs by the multidrug resistance protein MRP1 [Abstract]. Proc Am Assoc Cancer Res 1997;38:392.
19. Paulusma CC, Bosma PJ, Zaman GJR, Bakker CTM, Otter M, Scheffer GL, Scheper RJ, et al. Congenital jaundice in rats with a mutation in a multidrug resistance-associated protein gene. Science 1996;271:1126-1128.
20. Büchler M, König J, Brom M, Kartenbeck J, Spring H, Horie T, Keppler D. cDNA cloning of the hepatocyte canalicular isoform of the multidrug resistance protein, cMrp, reveals a novel conjugate export pump deficient in hyperbilirubinemic mutant rats. J Biol Chem 1996;271:15091-15098.
21. Evers R, Kool M, van Deemter L, Janssen H, Calafat J, Oomen LCJM, Paulusma CC, et al. Drug export activity of the human canalicular multispecific organic anion transporter in polarized kidney MDCK cells expressing *cMOAT (MRP2)* cDNA. J Clin Invest 1998;101:1310-1319.
22. Ito K, Suzuki H, Hirohashi T, Kume K, Shimizu T, Sugiyama Y. Functional analysis of a canalicular multispecific organic anion transporter cloned from rat liver. J Biol Chem 1998;273:1684-1688.
23. Roelofsen H, Van der Veere CN, Ottenhoff R, Schoemaker B, Jansen PLM, Oude Elferink RPJ. Decreased bilirubin transport in the perfused liver of endotoxemic rats. Gastroenterology 1994;107:1075-1084.
24. Wettstein M, Noé B, Häussinger D. Metabolism of cysteinyl leukotrienes in the perfused rat liver: the influence of endotoxin pretreatment and the cellular hydration state. HEPATOLOGY 1995;22:235-240.
25. Roelofsen H, Schoemaker B, Bakker C, Ottenhoff R, Jansen PLM, Oude Elferink RPJ. Impaired hepatocanalicular organic anion transport in endotoxemic rats. Am J Physiol 1995;269:G427-G434.
26. Trauner M, Arrese M, Soroka CJ, Ananthanarayanan M, Koeppel TA, Schlosser SF, Suchy FJ, et al. The rat canalicular conjugate export pump (mrp2) is down-regulated in intrahepatic and obstructive cholestasis. Gastroenterology 1997;113:255-264.
27. Green RM, Beier D, Gollan JL. Regulation of hepatocyte bile salt transporters by endotoxin and inflammatory cytokines in rodents. Gastroenterology 1996;111:193-198.
28. Moseley RH, Wang W, Takeda H, Lown K, Shick L, Ananthanarayanan M, Suchy FJ. Effect of endotoxin on bile acid transport in rat liver: a potential model for sepsis-associated cholestasis. Am J Physiol 1996;271: G137-G146.
29. Bolder U, Ton-Nu HT, Schteingart CD, Frick E, Hofmann AF. Hepatocyte transport of bile acids and organic anions in endotoxemic rats: impaired uptake and secretion. Gastroenterology 1997;112:214-225.
30. Fernández-Checa JC, Kaplowitz N, Garcia-Ruiz C, Colell A, Miranda M, Marí M, Ardite E, et al. GSH transport in mitochondria: defense against TNF-induced oxidative stress and alcohol-induced defect. Am J Physiol 1997;273:G7-G17.
31. Vos TA, Gouw ASH, Klok PA, Havinga R, van Goor H, Huitema S, Roelofsen H, et al. Differential effects of nitric oxide synthase inhibitors on endotoxin-induced liver damage in rats. Gastroenterology 1997;113: 1323-1333.
32. Moshage H, Casini A, Lieber CS. Acetaldehyde selectively stimulates collagen production in cultured rat liver fat-storing cells but not in hepatocytes. HEPATOLOGY 1990;12:511-518.
33. Meier PJ, Boyer JL. Preparation of basolateral (sinusoidal) and canalicular plasma membrane vesicles for the study of hepatic transport processes. Methods Enzymol 1990;192:534-545.
34. Mayer R, Kartenbeck J, Büchler M, Jedlitschky G, Leier I, Keppler D. Expression of the *MRP* gene-encoded conjugate export pump in liver and its selective absence from the canalicular membrane in transport-deficient mutant hepatocytes. J Cell Biol 1995;131:137-150.
35. Zhang F, Riley J, Gant TW. Use of internally controlled reverse transcriptase-polymerase chain reaction for absolute quantitation of individual multidrug resistant gene transcripts in tissue samples. Electrophoresis 1996;17:255-260.
36. Childs S, Yeh RL, Hui D, Ling V. Taxol resistance mediated by transfection of the liver-specific sister gene of P-glycoprotein. Cancer Res 1998;58:4160-4167.
37. Fort P, Marty L, Piechaczyk M, el Sabrouty S, Dani C, Jeanteur P, Blanchard JM. Various rat adult tissues express only one major mRNA species from the glyceraldehyde-3-phosphate-dehydrogenase multigenic family. Nucleic Acids Res 1985;13:1431-1442.
38. Hewett JA, Roth RA. Hepatic and extrahepatic pathobiology of bacterial lipopolysaccharides. Pharmacol Rev 1993;45:381-411.

39. Zhou G, Kuo MT. NF-κB–mediated induction of *mdr1b* expression by insulin in rat hepatoma cells. J Biol Chem 1997;272:15174-15183.
40. Stein U, Walther W, Laurencot CM, Scheffer GL, Scheper RJ, Shoemaker RH. Tumor necrosis factor-α and expression of the multidrug resistance–associated genes LRP and MRP. J Natl Cancer Inst 1997;89:807-813.
41. Chapekar MS, Huggett AC, Cheng C. Dexamethasone prevents the growth inhibitory effects of recombinant tumor necrosis factor in a rat hepatoma cell line Reuber-RC-3: an association with the changes in the messenger RNA levels for multidrug resistance gene. Biochem Biophys Res Commun 1991;181:1524-1531.
42. Roelofsen H, Havinga R, Jansen PLM, Müller M. Differential regulation of two glutathione-*S*-conjugate transporters mrp1 and mrp2 during the initial phase of cell proliferation in hepatocytes [Abstract]. HEPATOLOGY 1996;24:258A.
43. Laskin DL, Rodriguez del Valle M, Heck DE, Hwang S, Ohnishi ST, Durham SK, Goller NL, et al. Hepatic nitric oxide production following acute endotoxemia in rats is mediated by increased inducible nitric oxide synthase gene expression. HEPATOLOGY 1995;22:223-234.
44. Ohira H, Miyata M, Kuroda M, Takagi T, Tojo J, Ochiai H, Kokubun M, et al. Interleukin-6 induces proliferation of rat hepatocytes *in vivo*. J Hepatol 1996;25:941-947.
45. Feingold KR, Barker ME, Jones AL, Grunfeld C. Localization of tumor necrosis factor–stimulated DNA synthesis in the liver. HEPATOLOGY 1991;13:773-779.
46. Auer KL, Contessa J, Brenz-Verca S, Pirola L, Rusconi S, Cooper G, Abo A, et al. The Ras/Rac1/Cdc42/SEK/JNK/c-Jun cascade is a key pathway by which agonists stimulate DNA synthesis in primary cultures of rat hepatocytes. Mol Biol Cell 1998;9:561-573.
47. Trauner M, Nathanson MH, Rydberg SA, Koeppel TA, Gartung C, Sessa WC, Boyer JL. Endotoxin impairs biliary glutathione and HCO^-_3 excretion and blocks the choleretic effect of nitric oxide in rat liver. HEPATOLOGY 1997;25:1184-1191.
48. Paulusma CC, van Geer M, Heijn M, Evers R, Ottenhoff R, Oude Elferink RPJ. The canalicular multispecific organic anion transporter mediates transport of reduced glutathione [Abstract]. HEPATOLOGY 1997;26:292A.
49. Zaman GJR, Lankelma J, van Tellingen O, Beijnen J, Dekker H, Paulusma C, Oude Elferink RPJ, et al. Role of glutathione in the export of compounds from cells by the multidrug-resistance–associated protein. Proc Natl Acad Sci U S A 1995;92:7690-7694.
50. Whiting JF, Green RM, Rosenbluth AB, Gollan JL. Tumor necrosis factor-alpha decreases hepatocyte bile salt uptake and mediates endotoxin-induced cholestasis. HEPATOLOGY 1995;22:1273-1278.