Increase in renal glutathione in cholestatic liver disease is due to a direct effect of bile acids

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1Department of Internal Medicine III, Medical Faculty, Aachen University of Technology, D-52057 Aachen, Germany; and 2Department of Medicine, Division of Gastroenterology and Hepatology, Karolinska Institute, Huddinge University Hospital, S-14186 Stockholm, Sweden

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PURUCKER, Edmund, Hanns-Ulrich Marschall, Andreas Geier, Carsten Gartung, and Siegfried Matern. Increase in renal glutathione in cholestatic liver disease is due to a direct effect of bile acids. Am J Physiol Renal Physiol 283: F1281–F1289, 2002.—Hepatic synthesis and plasma levels of glutathione are markedly decreased in chronic liver disease. Because glutathione turnover is highest in kidneys, we examined whether changes in kidney glutathione occur in chronic cholestasis and whether they are related to kidney dysfunction in liver disease. Kidney and plasma GSH and GSSG were measured 1) in bile duct-ligated (BDL) rats; 2) in healthy rats after bile acid loading to mimic cholestasis; and 3) after irreversible inhibition of glutathione synthetase with buthionine-sulfoximine (BSO), where glutathione consumption, urinary volume, and sodium excretion were also estimated. In addition, γ-glutamylcysteine synthetase (γ-GCS) mRNA, protein, and enzymatic specific activity were measured in kidney tissue after BDL. After BDL, kidney GSH and GSSG increased within hours by 67 and 66%, respectively. The increases were not related to plasma glutathione, which decreased below control values. Intravenous bile acid loading caused identical increases in GSH and GSSG as occurred after BDL, when glycine- or taurine-conjugated dihydroxy bile acids were administered. Glutathione consumption, as estimated after blocking of de novo synthesis with BSO, was significantly increased after BDL (127 vs. 44 nmol·g−1·min−1). γ-GCS mRNA and enzymatic specific activity were significantly reduced 5 days after BDL, whereas protein concentrations did not change. The urinary sodium concentration was 70% lower in BDL than in control rats. Depletion of renal glutathione normalized sodium excretion by increasing urinary sodium concentration and urinary volume. The increase in kidney glutathione after BDL seems to be mediated by an increase in plasma bile acids and is critically related to sodium retention. The increase in GSH consumption despite reduced γ-GCS activity indicates a decreased GSH turnover tentatively due to reduced renal GSH efflux by competition with organic anions at membrane transport proteins.

Bile duct ligation; sodium excretion; glutathione depletion; γ-glutamylcysteine synthetase

GLUTATHIONE (γ-glutamyl-cysteinyl-glycine) is a multi-functional tripeptide that is present in every mammalian cell. Highest concentrations are found in the liver, kidney, and other parenchymal organs. Glutathione participates in a wide range of redox and conjugation reactions and has regulatory functions (6, 14, 32). Decreased hepatic output and plasma concentrations of glutathione are common features of liver disease in humans (4) and in experimental animals (29). Decreased glutathione enhances the susceptibility of the liver to oxidative damage (e.g., acetaminophen toxicity). According to the concept of interorgan transfer of glutathione (7), the kidney content of glutathione depends on the transfer of glutathione from the liver to the kidney because only the liver can synthesize the key constituent of glutathione, cysteine. This transfer is of particular importance in the fasting state, when dietary cysteine is lacking (25, 26). Changes in hepatic and plasma concentrations of glutathione should therefore influence kidney glutathione. Because kidneys exhibit the highest organ-specific turnover rates for glutathione (17, 26), decreased renal glutathione content should be expected in chronic liver disease. However, continuously increasing levels of kidney GSH and GSSG were found in experimental carbon tetrachloride-induced cirrhosis in rats, and this increase paralleled the deterioration of liver function (29). It is not known whether these changes were caused by a direct toxic effect of carbon tetrachloride on the kidneys. Therefore, the following experiments were done to clarify 1) whether an increase in kidney glutathione occurs in other forms of chronic liver disease, 2) whether bile acids influence renal glutathione metabolism, and 3) what impact changes in glutathione levels have on renal function.

METHODS

Surgery and Sampling

Male Wistar rats [220–250 g body wt (BW)] were held in groups of two in Macrolon cages and fed ad libitum with a

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standard chow (Altromin, Lage, Germany) and free access to water. The animals were randomly assigned to a bile duct ligation (BDL) or sham-operated group. All rats were anesthetized for surgery and blood or tissue sampling with pentobarbital sodium (40 mg/kg body wt ip). The common bile duct was exposed through a 1-cm-long right paramedian incision. In animals designated as BDL, the common bile duct was ligated twice with silk and cut in between. Ligations were positioned 1 cm distal to the duodenum to avoid ligation of parabiliary pancreatic ductuli. No ligation was performed in sham operations. To circumvent postoperative or diurnal variations in kidney glutathione, each BDL group was compared with a sham-operated group. Each group consisted of five animals.

Blood for plasma glutathione was drawn rapidly (6–8 ml within 45 s) from the aorta abdominis under complete anesthesia 15–20 min after injection of pentobarbital sodium. Tissue samples were removed immediately from the cranial half of the left kidney.

Bile Acid-Loading Experiments

Rats were injected into the dorsal penile vein with a crude mixture of primary and secondary bile acids from ox bile (mixed bile acids, tauroglycocholic acid; Sigma, Deisenhofen, Germany) dissolved in Ringer solution containing (in mM) 147 Na+, 4 K+, 2.3 Ca²⁺, and 155.5 Cl⁻. The actual concentration of 50 mg of crude bile acids dissolved in 50 ml water was 34 ± 2.8 mM bile acids, consisting of glycodeoxycholic acid or tauroaminated cholic (76.7 ± 2.2 (SD)%), deoxycholic (18.9 ± 2.6%), and chenodeoxycholic acids (4.4 ± 0.5%; n = 3).

Kidney GSH and GSSG were determined at 1, 2, 4, 5, 6, 8, and 12 h after injection of 50 mg/kg BW of the bile acid mixture from ox bile and at 5 h after doses of 20, 50, 100, and 200 mg/kg BW, respectively. Control rats were injected with equal volumes of Ringer solution.

To identify the effect of particular bile acids, glycodeoxycholic acid or taurocholate amides of primary and secondary bile acids were injected in doses of 35 μmol/kg BW, which equals the injected amount of total bile acids in the crude ox bile mixture. The bile acids tested were glycodeoxycholic (GCA), taurocholic (TCA), glycine- (GCDCA), and alanine- (GCA) conjugates. Kidney GSH and GSSG were determined 4 h after injection.

Glutathione Consumption/Depletion

Glutathione consumption was assessed by irreversible inhibition of glutathione synthetase (22) using DL-buthionine-[S,R]-sulfoximine (BSO; Sigma). Five days after BDL or sham operation, animals received a single intraperitoneal dose of 2 mmol BSO/kg BW. The concentrations of renal and hepatic glutathione were determined at 15, 30, 60, and 180 min after injection.

To assess the metabolic effect of glutathione depletion on bile acid metabolism and kidney function, urine was collected for 24 h before (on day 4 after BDL or sham operation) and after glutathione depletion by BSO (on day 5). For this experiment, two doses of 2 mmol/kg BSO were injected intraperitoneally at 8:00 AM and 8:00 PM on day 5 after surgery. Because the effect of BSO on GSH catalase using NADPH as a coenzyme to regenerate GSH, which reacts again ("catalytic assay"). The formation of TNB is followed spectrophotometrically. GSSG is then reduced enzymatically by glutathione reductase using DTNB to yield GSSG and 2-nitro-5-thiobenzoic acid (TNB).

All studies were performed in accordance with required standards of the local animal welfare committee.

Analytic Procedures

Glutathione. GSH and GSSG levels were determined according to Griffith (11). GSH reacts nonenzymatically with DTNB to yield GSSG and 2-nitro-5-thiobenzoic acid (TNB). GSSG is then reduced enzymatically by glutathione reductase using NADPH as a coenzyme to regenerate GSH, which reacts again ("catalytic assay"). The formation of TNB is followed spectrophotometrically. GSSG is determined after incubation of samples with 2-vinylpyridine, which derivatizes GSH. To prevent the rapid breakdown of GSH and GSSG by γ-glutamyl transpeptidase (γ-GT), it was important to chill the samples in an ice-cold water bath immediately after dissection (28). Kidney specimens were then rapidly homogenized in 5 ml ice-cold 5% 5-sulfosalicylic acid using an Ultraturrax blender (Jahnke & Kunkel, Staufen, Germany). Despite cooling, 5–10% of plasma GSH is lost every 5 min. Thus plasma samples were stabilized exactly 4 min after vein puncture by adding 10% 5-sulfosalicylic acid.

Bile acids. Materials, procedures, and equipment for the analysis of bile acid profiles in plasma and urine by gas chromatography-mass spectrometry were the same as described previously (19). In short, Sep-Pak C₁₈ cartridges (Waters, Milford, MA) were used for the quantitative extraction of bile acids from urine and from 1 ml of plasma, the latter diluted 10-fold with 0.5 M triethylammonium sulfate, pH 7.4, and heated for 10 min to 65°C to diminish protein adsorption. Separation into different groups of conjugates was then performed by anion-exchange chromatography on Lipidex-DEAP (Packard-Becker, Groningen, The Netherlands) after the addition of choly [¹⁴C]glycine (Amersham, Buckinghamshire, UK) as an internal standard for the monitoring of recovery and group separation. The following fractions were eluted from Lipidex-DEAP: unconjugated bile acids, glycine- or taurine-conjugated bile acids; glycine- or taurine-conjugated bile acid glucuronides; and glycine- or taurine-conjugated bile acid sulfates. The glycine and taurine moieties were hydrolyzed with cholyglycine hydrolase for 24 h at 37°C. Bile acid glucuronides and sulfates, respectively, were then hydrolyzed with the digestive juice of Helix pomatia (L’Industrie Biologique Francaise, Clichy, France) or solvolyzed with trifluoroacetic acid in water-free tetrahydrofuran, both for 1 h at 45°C. Finally, bile acids were converted to volatile methyl ester trimethylsilyl ether derivatives for gas chromatography-mass spectrometry analysis. Aliquots of samples before and after separation on Lipidex-DEAP were analyzed by fast-atom bombardment-mass spectrometry for deprotonated M-1 ions to obtain information about occurrence, successful separation, and hydrolysis of bile acid conjugates.

γ-Glutamylcysteine Synthetase

Northern blot analysis. RNA was isolated from whole kidney by standard phenol chloroform extraction procedures (5). Total mRNA (10 μg) was analyzed as previously described (9, 33) by Northern blotting with specific and constitutively expressed cDNA probes of GAPDH and rat γ-glutamylcysteine synthetase (γ-GCS) heavy chain, kindly provided by Dr. D. Eaton (University of Washington, Seattle, WA). Specific mRNA levels were detected after exposure of membranes to a PhosphorImager screen (Molecular Dynamics, Sunnyvale, CA) and quantified using ImageQuant software (Bio-Rad).

Western blot analysis. Preparation of kidney cytosol for analysis of protein mass were performed as previously de-
scribed (8). After determination that the linear range was 25–200 μg, similar amounts of protein (75 μg) were separated by SDS-PAGE and probed after transfer to blotting membranes to anti-γ-GCS peptide antiserum, kindly provided by Dr. D. Dickinson (University of Alabama at Birmingham, Birmingham, AL). Immune complexes were detected using an ECL Western blotting kit (Amercontrols, Little Chalfont, UK). Immunoreactive bands obtained by autoradiography were quantified by laser densitometry and ImageQuant software.

Activity. The enzymatic specific activity of γ-GCS was measured in cytosolic preparations of kidney tissue. The reaction mixtures contained 0.1 M Tris·HCl buffer [in mM] 150 KCl, 10 L-glutamate, 10 L-α-aminobutyrate, 20 MgCl2, 2 Na2EDTA, 0.2 NADH, and 5 Na2ATP. In a coupled enzyme procedure with lactate dehydrogenase and pyruvate kinase in the presence of 2 mM phosphoenol/pyruvate, ADP formation was measured spectrophotometrically from the decrease in NADH (31).

γ-GT

The activity of γ-GT was measured in crude kidney homogenates in 1:10 (wt/vol) 0.9% NaCl 5 days after BDL or sham operation in a photometric assay where γ-glutamyl-p-nitroaniline serves as the substrate and glycylglycine as the acceptor for the γ-glutamyl group released by the action of γ-GT. Liberated p-nitroaniline is diazotized and measured at 540 nm (21).

Recoveries

The recoveries of 125 μmol GSSG or 250 μmol GSH, added immediately before kidney tissue homogenization, were 96.6 ± 6.1%. The homogenization technique used was the same for all samples from BDL or sham-operated rats. No excess GSSG formation could be detected when GSH was added. The addition of bile acids (0.1–0.5 mM) also did not affect recoveries.

Statistics

Values are expressed as means ± SD. Analysis of variance was used to calculate differences between the sample means. When relevant, statistical tests between the groups were done using Scheffe’s test. P < 0.05 was considered significant for rejecting the hypothesis of equal expected means.

RESULTS

Kidney Glutathione After BDL

Kidney GSH and GSSG in sham-operated control rats at t = 0 h were 2.86 ± 0.24 and 0.12 ± 0.02 μmol/g wet wt (WW), respectively, and did not differ significantly from these values at 1, 3, 6, or 24 h and 5, 8, 15, 23, or 38 days.

Three hours after BDL, a significant increase in GSH to 3.48 ± 0.22 μmol/g WW (+34% compared with 2.60 ± 0.26 μmol/g WW in sham-operated controls, P < 0.01) was detected, reaching a maximum of 4.84 ± 0.51 μmol/g WW after 24 h (+67% compared with 2.89 ± 0.27 μmol/g WW in sham-operated controls P < 0.001). Thereafter, no further increase was seen (Fig. 1). The increases were smaller on days 15 and 23 (+52%) and at the end of the observation period on day 38 (+49%).

GSSG also increased significantly 1 h after BDL to 0.16 ± 0.01 μmol/g WW (+24% compared with 0.12 ± 0.01 in sham-operated controls; P < 0.01). GSSG went up to 52–62% above controls between 3 h and day 8, with no statistical difference between the groups. Later on, GSSG increased further to 81 (day 15), 108 (day 23), and 123% (day 38) above the respective controls.

Plasma Glutathione After BDL

GSH and GSSG concentrations in plasma from abdominal aorta of sham-operated controls were 10.0 ± 1.30 and 0.75 ± 0.28 μM, respectively, at t = 0 h. Compared with these values, the maximum decrease in GSH (27%) occurred 3 h after the sham operation, and the maximum increase in GSSG (+32%) occurred 6 h after the sham operation. After 24 h, the concen-

![Fig. 1. GSH (circles; top) and GSSG (squares; bottom) in kidney tissue after sham operation (open symbols) or bile duct ligation (filled symbols). Note the different scales for GSH and GSSG. Values are means ± SD (n = 5). *P < 0.01. **P < 0.001.](http://ajprenal.physiology.org/)

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trations were not different from $t = 0$ h in sham-operated controls.

In contrast, the plasma GSH concentration increased 6 h after BDL by 18% and was normalized after 24 h (Fig. 2). Comparisons between sham-operated and BDL rats revealed significant differences ($P < 0.05$) after 3 h (+31%), 6 h (+48%), and 5 days (+14%). After 8 days, GSH tended to be lower compared with sham-operated controls (-14%; not significant). Fifteen days after BDL, the arterial GSH concentration was reduced by 41% ($P < 0.05$), 45% after 23 days, and 51% at the end of the observation period ($P < 0.01$).

In BDL rats, arterial GSSG increased by 39 and 62% after 3 and 6 h, respectively (Fig. 2). These increases were significantly different ($P < 0.05$) compared with sham-operated controls. After 1, 8, and 15 days, no increase was detected. After 23 and 38 days, GSSG was 27 (not significant) and 44% ($P < 0.05$) lower than in controls, respectively.

Bile Acid Profiles

Plasma bile acids and urinary excretion of bile acids increased ~25- and 350-fold, respectively, after BDL compared with sham-operated controls (Table 1). In BDL rats, 95% of plasma and 90% of urinary bile acids were glycine or taurine conjugates. Minor amounts, between 1.4 and 6.4%, of glucuronides and sulfates were found in plasma and urine. The prevalence of these conjugates was confirmed by fast-atom bombardment-mass spectrometry. The spectra that were dominated by deprotonated $M-1$ ions at mass-to-charge ratios ($m/z$) of 498 and 514, indicating taurine-conjugated di- and trihydroxy bile acids, also showed ions at $m/z$ of 578 and 594, indicating taurine-conjugated diol and triol sulfates, and at $m/z$ of 674 and 690, indicating the respective glucuronides.

Ions indicative of other forms of conjugate were not recorded. After glutathione depletion, the urinary ex-

Table 1. Urinary excretion and plasma concentrations of unconjugated and conjugated bile acids in sham-operated and bile duct-ligated rats and changes under glutathione depletion with buthionine-sulfoximine

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<thead>
<tr>
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<th>SOP</th>
<th>SOP + GSH Depletion</th>
<th>BDL</th>
<th>BDL + GSH Depletion</th>
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<tr>
<td><strong>Urine, µmol/24 h</strong></td>
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<tr>
<td>Total</td>
<td>95 ± 18</td>
<td>214 ± 48*</td>
<td>33,382 ± 9,650</td>
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<tr>
<td>Unconjugated</td>
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<td>10 ± 1</td>
<td>51 ± 18</td>
<td>137 ± 32*</td>
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<td>Glycine/taurine conjugates</td>
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<td>173 ± 49*</td>
<td>30,627 ± 9,089</td>
<td>35,227 ± 11,326</td>
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<tr>
<td>Glucuronides</td>
<td>15 ± 5</td>
<td>15 ± 2</td>
<td>574 ± 52</td>
<td>407 ± 26*</td>
</tr>
<tr>
<td>Sulfates</td>
<td>38 ± 8</td>
<td>18 ± 3*</td>
<td>2,130 ± 359</td>
<td>1,445 ± 420*</td>
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<td><strong>Plasma, µmol/l</strong></td>
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<td>44 ± 19</td>
<td>41 ± 7</td>
<td>1,119 ± 99</td>
<td>1,525 ± 481</td>
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<tr>
<td>Unconjugated</td>
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<td>17 ± 2</td>
<td>3 ± 1</td>
<td>1 ± 1</td>
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<tr>
<td>Glycine/taurine conjugates</td>
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<td>17 ± 1</td>
<td>1,065 ± 103</td>
<td>1,478 ± 485</td>
</tr>
<tr>
<td>Glucuronides</td>
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<td>1 ± 1</td>
<td>35 ± 7</td>
<td>25 ± 8</td>
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<td>Sulfates</td>
<td>14 ± 11</td>
<td>5 ± 3</td>
<td>16 ± 1</td>
<td>16 ± 6</td>
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Values are means ± SD. SOP, sham-operated rats; BDL, bile duct-ligated rats. *$P < 0.05$. 

Figure 2. GSH (circles; top) and GSSG (squares; bottom) in plasma after sham operation (open symbols) or after bile duct ligation (filled symbols). Values are means ± SD ($n = 5$). *$P < 0.05$. **$P < 0.01$. 

Bile Acid Profiles

Plasma bile acids and urinary excretion of bile acids increased ~25- and 350-fold, respectively, after BDL compared with sham-operated controls (Table 1). In BDL rats, 95% of plasma and 90% of urinary bile acids were glycine or taurine conjugates. Minor amounts, between 1.4 and 6.4%, of glucuronides and sulfates were found in plasma and urine. The prevalence of these conjugates was confirmed by fast-atom bombardment-mass spectrometry. The spectra that were dominated by deprotonated $M-1$ ions at mass-to-charge ratios ($m/z$) of 498 and 514, indicating taurine-conjugated di- and trihydroxy bile acids, also showed ions at $m/z$ of 578 and 594, indicating taurine-conjugated diol and triol sulfates, and at $m/z$ of 674 and 690, indicating the respective glucuronides.

Ions indicative of other forms of conjugate were not recorded. After glutathione depletion, the urinary ex-
cretion of glucuronides and sulfated bile acids decreased by 29 and 32%, respectively, whereas the excretion rate of unconjugated bile acids increased. These changes were significant ($P < 0.05$). Plasma bile acids after glutathione depletion did not change significantly in BDL or sham-operated rats (Table 1).

**Bile Acid-Loading Experiments**

GSSG and GSH rapidly increased after intravenous bile acid injection, as shown for the single dose of 50 mg/kg BW of a crude mixture of bile acids from ox bile (Fig. 3). The maximum GSH increase (+50%) occurred after 4 and 5 h and the maximum GSSG increase (+31%) after 2–5 h. Higher intravenous doses of bile acids did not cause further increases in kidney GSH and GSSG at 5 h after application, as shown in experiments where bile acid doses of 20–200 mg/kg BW were injected. The GSH increases were +26 and +44% after bile acid doses of 20 and 50 mg/kg BW, respectively; thereafter, no significant further increase was observed. The GSSG increase was maximal after a dose of 20 mg/kg BW, and there was no significant further increase with higher doses (Fig. 4).

The effects of single bile acids are shown in Fig. 5. The amidates of cholic acid, GCA and TCA, had no effect on GSH but caused a 31 and 26% increase, respectively, in kidney GSSG ($P < 0.01$). Conjugates of deoxycholic acid increased both GSH by 42% ($P < 0.01$ for GDCA, $P < 0.001$ for TDCA) and GSSG by 63 and 68%, respectively ($P < 0.001$). Conjugates of chenodeoxycholic acid, GCDC and TCDC, caused GSH increases of 31 and 57% and GSSG increases of 49 and 36%, respectively. The taurine conjugate of lithocholic acid, TCA, had no effect on GSH or GSSG.

**Glutathione Consumption**

Kidney glutathione was determined before and at 15, 30, 60, and 180 min after an intraperitoneal injection of BSO that was administered on **day 5** after BDL or sham operation. Kidney glutathione was $5.00 \pm 0.66$ mmol/g WW in animals after BDL, which was 73% higher than in sham-operated controls. GSH rapidly decreased to $1.3 \pm 0.4$ mmol/g in both groups within 30 min after BSO application. The initial glutathione consumption was 127 nmol·min$^{-1}$·g$^{-1}$ in cholestatic rats and 44 nmol·min$^{-1}$·g$^{-1}$ in controls (Fig. 6).
The urinary sodium concentration after BSO increased by 64% compared with sham-operated controls (33 ± 0.7 mmol·kg⁻¹·day⁻¹; controls, 57.1 ± 2 mmol/l; P < 0.01). Thus net sodium excretion after BDL was reduced by 51% (BDL, 3.0 ± 0.6 mmol·kg⁻¹·day⁻¹; controls, 6.1 ± 0.7 mmol·kg⁻¹·day⁻¹; P < 0.01).

After glutathione depletion with BSO, urinary volume further increased to 79 ± 9 ml·kg⁻¹·day⁻¹ (P < 0.01) in BDL rats and, to a lesser extent, in sham-operated controls (40 ± 3 ml·kg⁻¹·day⁻¹; P < 0.05). The urinary sodium concentration after BSO increased to 100 ± 15 mmol/l in BDL rats (P < 0.01), resulting in a normalization of sodium excretion to 7.8 ± 0.6 mmol·kg⁻¹·day⁻¹, which was equal to control values after BSO (8.1 ± 1.2 mmol·kg⁻¹·day⁻¹).

γ-GCS

The effect of BDL for up to 5 days on steady-state mRNA levels of γ-GCS is shown in Fig. 8. Densitometric analysis of autoradiographs showed a decreased density of 72 ± 38% after 1 day. The value in controls was 100 ± 27% (P = 0.18, not significant). Five days after BDL, the density had further and significantly decreased to 42 ± 24% (P < 0.01).

The effect of BDL on protein mass is shown in Fig. 9. No significant changes were observed 1 and 5 days after BDL.

The enzymatic specific activity of γ-GCS in kidney tissue of sham-operated animals was 546 ± 180 nmol·g⁻¹·min⁻¹. One and five days after BDL, γ-GCS activity decreased significantly to 283 ± 116 (~48%) and 315 ± 57 (~42%) nmol·g⁻¹·min⁻¹, respectively (Fig. 10).}

γ-GT

The activity of γ-GT in kidney tissue of sham-operated animals was 21.9 ± 3.32 U/g WW, and 5 days after BDL it was 18.5 ± 2.18 U/g (n = 5, not significant).

DISCUSSION

Cholestasis has a strong impact on renal glutathione metabolism. We found that kidney GSH and GSSG rapidly increased after BDL. These changes already occurred within 6 h after BDL and largely persisted until the end of the observation period of 38 days. At this time, complete secondary biliary cirrhosis with ascites is anticipated with decreased liver and plasma glutathione (30).

Elevated kidney glutathione in chronic liver disease was first described in carbon tetrachloride-induced cirrhosis in rats (29). In that model, kidney glutathione increased by 13% and 44% after 15 and 20 wk of treatment, respectively, when complete cirrhosis had developed. The reproducibility of these changes in our study with chronic BDL cholestasis makes direct toxic effects of carbon tetrachloride on the kidneys unlikely.

The physiological hepatic turnover of GSH and GSSG in the adult rat is characterized by a transport rate into bile of 14.8 ± 1.1 and 2.3 ± 0.2 nmol·min⁻¹·100 g⁻¹ (17). In cholestasis in general, an increased sinusoidal hepatic efflux of the tripeptide occurs. After BDL, the canalicular block also results in an increased junctional permeability, allowing biliary glutathione to reflux from bile into plasma.

Kidney glutathione is de novo synthesized in the cytosol of each cell (6). The constituent amino acids are mainly derived from the degradation of glutathione at the external surface of the luminal and basolateral membranes of the proximal tubule by the activity of γ-GT and dipeptidase (1). As increased availability of
glutathione is able to raise the level in the kidney (32), changes due to cholestasis might therefore be secondary to elevated plasma glutathione that might also be degraded more slowly, by inhibition of γ-GT activity when amidated bile acids reach levels as occurred in our study (2).

However, we did not find larger amounts of glutathione in arterial plasma after BDL. In fact, only modest

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<th>Protein</th>
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<tr>
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<td></td>
<td>74</td>
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<tr>
<td>day 5</td>
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Fig. 8. Effect of bile duct ligation (BDL) on steady-state mRNA levels of γ-glutamylcysteine synthetase (γ-GCS). Total RNA was isolated from rat kidney (n = 5) as described in METHODS. mRNA levels of γ-GCS and GAPDH were analyzed by Northern blotting using specific 32P-labeled cDNA probes. A: representative autoradiographs 1 and 5 days after BDL. B: densitometric analysis of mRNA expression quantified by Phosphor-Imaging. Values are means ± SD expressed as percentage of control values. *P < 0.05.

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Fig. 9. Effect of BDL on γ-GCS protein mass. A: representative immunoblots are shown for days 1 and 5 after BDL. Molecular weight (MW) markers are given in kDa. B: densitometric analysis of protein levels. γ-GCS protein mass was quantified by laser densitometry and expressed as percentage of control values. Values are means ± SD (not significant).
amounts of excess GSH and GSSG were detected in the first 6 h after BDL, and control values were reestablished after 24 h, indicating a downregulation of hepatic glutathione synthesis. Thus the observed rapid increase in plasma glutathione does not explain the lasting changes in kidney glutathione after BDL.

The increase in kidney glutathione was also too rapid to be explained by severe liver dysfunction. Therefore, we speculated that biliary compounds could induce increases in kidney glutathione. These rise rapidly in plasma of rats after BDL. Our hypothesis that bile acids were responsible for the observed changes was confirmed in bile acid-loading experiments. The injection of a crude bile acid mixture resulted in a rapid increase in glutathione identical to that observed after BDL. Bile acid-loading experiments using individual bile acids revealed significant GSH increases after all glycine or taurine conjugates tested, with the exception of taurolithocholic acid, which is a nonidentical compound. GSH increased significantly as well but only after the injection of the conjugates of chenodeoxy and deoxycholic acids. The injection of cholic acid conjugates did not elevate GSH, which is probably due to the higher hepatocellular uptake, thus higher plasma clearance, of these compounds that are most abundant in noncholestatic rodents (15).

The observed increase in kidney glutathione could result from increased synthesis through stimulation of γ-GCS, decreased catabolism, decreased consumption, or diminished turnover by hindered cellular export. Increased synthesis was ruled out by showing that mRNA expression and enzymatic specific activity of γ-GCS were significantly reduced 5 days after BDL. Protein concentrations estimated by Western blot analysis tended to decrease, but this did not reach statistical significance. Decreased catabolism was unlikely by measuring equal γ-GT activities in kidneys of rats with and without BDL. Finally, we found an increased glutathione consumption after de novo synthesis was blocked. Thus the increase in kidney glutathione is probably due to diminished cellular export of the unchanged tripeptide GSH. This is supported by the following observations.

In cholestasis, renal membrane transport proteins are regulated in a manner that the excretion of biliary compounds such as bilirubin and bile acid conjugates is facilitated (18). The main transporter for these compounds is multidrug resistance-associated protein 2 (MRP2), which is also responsible for the excretion of GSH (18). However, the upregulation of MRP2 in cholestasis is obviously not sufficient to export both biliary compounds and glutathione. GSH synthesis is reduced by nonallosteric feedback inhibition of GSH that competes with the substrate at the L-glutamate binding site of γ-GCS (13). This feedback inhibition was obviously very effective. One would have expected that the oxidative damage that occurs in proximal kidney tubules under cholestasis (3) should actually stimulate glutathione synthesis (16).

In the healthy kidney, GSH is continuously exported from kidney cells and cleaved by γ-GT, and there is a reuptake of constituent amino acids into the cells for resynthesis of GSH (1). This cycle is thought to protect the membranes from oxidative damage. Oxidative stress leads to an increase in the cellular redox state that is reflected by the GSSG/GSH ratio (4.6 ± 0.6% of kidney glutathione is in the oxidized form in control animals). A normal GSSG/GSH ratio is critical for the determination of protein structure, regulation of enzyme activity, and regulation and binding of transcriptional factors (6, 14). To restore a normal GSSG/GSH ratio, excess GSSG is either exported from the cell or intracellular GSH increases (6, 27). In our experiments, kidney tissue GSSG increased as soon as 1 h after BDL. This increase was immediately followed by a pronounced GSH increase. Thus the GSSG/GSH ratio was not different or even lower in BDL compared with sham-operated rats and increased only at the end of the observation period.

We also gained information about the urinary excretion of bile acid conjugates related to glutathione metabolism. After BDL, the amount of sulfated bile acids in urine increased ~50-fold. As glutathione is a key source for sulphydryl groups, the increased glutathione consumption after BDL may be related to increased sulfation of bile acids (10). The decreased excretion of sulfated bile acid after glutathione depletion supports this assumption. We also found a significantly reduced excretion of bile acid glucuronides under this condition, which is in accordance with decreased activity of the bile acid-transporting organic anion transporter 1, which is glutathione driven (14, 34).

Finally, we observed changes in the sodium balance. After glutathione depletion in BDL rats, sodium excretion increased and hyperdiuresis was augmented. This may be related to the action of nitric oxide for the following reasons. Similar changes in renal function were observed after inhibition of nitric oxide synthetase in cirrhotic rats (20), and it has been shown that glutathione depletion inhibits nitric oxide production by reducing inducible nitric oxide synthetase gene expression (24). Interestingly, bile acids induce nitric oxide synthesis.
oxide production in vascular endothelial cells (23). Thus a speculative sequence leading to sodium retention after BDL would be a bile acid-induced increase in kidney glutathione as a first step. The second step would be increased synthesis of nitric oxide, leading to a reduction of renal perfusion pressure and sodium retention.

In conclusion, the increase in kidney glutathione seems to be a common feature in cholestatic liver disease in the rat. Hindered cellular export of glutathione is one of the likely pathomechanism. Elevated bile acid conjugates trigger these changes. The increase in kidney glutathione is related to sodium retention.

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