Nifedipine modulation of biliary GSH and GSSG/conjugate efflux in normal and regenerating rat liver

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Yang, Bo, and Ceredwyn E. Hill. Nifedipine modulation of biliary GSH and GSSG/conjugate efflux in normal and regenerating rat liver. Am J Physiol Gastrointest Liver Physiol 281: G85–G94, 2001.—Canalicular glutathione secretion provides the major driving force for bile acid-independent bile flow (BAIF), although the pathways involved are not established. The hypothesis that GSH efflux proceeds by a route functionally distinct from the high-affinity, low-capacity, mrp2-mediated pathway was tested by using perfused rat liver and three cholestatic compounds that modify biliary secretion of GSH (the dihydropyridine nifedipine and organic anion probenecid) or GSSG [sodium nitroprusside (SNP)]. Whereas nifedipine (30 μM) stimulated GSH secretion and blocked SNP-stimulated GSSG efflux and choleretic, SNP (1 mM) was ineffective against nifedipine-stimulated GSH efflux or BAIF, suggesting that most GSSG exits through a GSH-inhibitable path independent of high-affinity GSSG/glutathione conjugate transport. Three observations support this proposal. SNP, but not nifedipine, significantly inhibited bromosulfophthalein (BSP, 1 μM) excretion. Probenecid (1 mM) blocked resting or nifedipine-stimulated GSH secretion but only weakly inhibited BSP excretion. Glutathione, but not BSP, efflux capacity was reduced following partial hepatectomy. We suggest GSH efflux is mediated by a high-capacity organic anion pathway capable of GSSG transport when its high-affinity route is saturated.

Canalicular GSH transport is not as clearly defined as that for its oxidized form, although it provides ~80% of the biliary glutathione pool. A yeast homolog of MRP2 has been shown to transport GSH, leading to the suggestion that the hepatic isoform may also be capable of such activity (27). The evidence for this is persuasive, albeit indirect. First, cells engineered to over-express MRP2 also have much greater GSH transport capability than native cells (26, 34); second, the natural mrp2 mutation in TR− rats results in greatly diminished GSH secretion (16, 25); third, endotoxin exposure reduces both immunoreactive mrp2 in the canalicular membrane and total glutathione secretory rate (33, 35); and fourth, chronic feeding of mice with mrp2-inducing chemicals leads to increased biliary secretion of GSH (37). Conversely, GSH transport in canalicular membranes of normal and TR− rats is not significantly different (7), and biliary secretion of GSH is depressed in partially hepatectomized rats in the absence of changes in mrp2 expression or bilirubin excretion (8, 15, 36). To add to the complexity, there is some evidence that hepatic GSH is transported by both high-affinity and low-affinity processes (3). Some reports suggest that both processes are mediated by mrp2 (26, 34). In any case, functional differentiation between GSSG/glutathione conjugate efflux and GSH secretion in the normal rat liver has not been demonstrated.

Bile acid-independent choleretic is also induced by other compounds synthesized by the liver such as nitric oxide (NO). The choleretic caused by exogenous, perfused NO donors is coupled to enhanced GSSG but not GSH secretion (14, 32). In both wild-type and TR− rats (that do not secrete GSSG in response to NO), NO exposure roughly doubles the tissue levels of GSSG without significantly affecting the total glutathione pool (32). Thus high levels of NO appear to induce an oxidative stress response, which may be buffered by mrp2-mediated excretion of GSSG and subsequent stimulation of transport away from the hepatocyte. The effect of acute exposure to NO donors on GSSG secretion and bile acid-independent bile flow in the regenerating liver has not been reported.

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Here we show that the two choleretic compounds nifedipine and probenecid have opposite effects on biliary GSH secretion in the perfused rat liver. We used these compounds, along with the NO donor sodium nitroprusside (SNP), to investigate the processes responsible for the secretion of GSH and GSSG/glutathione conjugates in the intact perfused rat liver. The results suggest that GSH is transported by a mechanism that can also move GSSG, the latter at lower relative affinity and under conditions in which the high-affinity GSSG transporter cMOAT/mrp2 is saturated. Thus the GSH pathway would provide an overflow route for excess GSSG during conditions leading to increased NO production (hepatic inflammation), and L-type Ca\(^{2+}\) channel blockers such as nifedipine may attenuate injury by maintaining intra- and/or extracellular GSH levels.

**MATERIALS AND METHODS**

**Animals and materials.** Male Sprague-Dawley rats weighing 200–225 g (Charles River Laboratories, Montreal, PQ) were maintained on a 12:12-h light/dark cycle with access to rat chow and water ad libitum according to the regulations of the Animal Care Committee of Canada. Nifedipine was purchased from Research Biochemicals, and glutathione reductase and NADPH were from Roche Biochemicals (Montreal, PQ); all other chemicals were from Sigma Chemical (St. Louis, MO) or British Drug Houses (Toronto, ON).

**Liver perfusions.** Livers were perfused via the portal vein with Krebs-Henseleit bicarbonate-buffered (KH) saline using a non-recirculated, flow-constant perfusion system as described previously (13). KH saline was warmed to 37°C, saturated with 95%/5% (vol/vol) O\(_2/\)CO\(_2\), and perfused at 4–5 ml min\(^{-1}\) g liver\(^{-1}\). Tissue viability was assessed throughout each perfusion by monitoring portal pressure (2.53 ± 0.13 cmH\(_2\)O/g liver; \(n = 40\)). Bile samples were collected over consecutive 3- or 5-min intervals from a cannula placed in the common bile duct, and bile volume was determined assuming a density of 1 g/ml. Samples, stored on ice, were immediately assayed for GSH and within 6 h for total glutathione. Nifedipine was dissolved in DMSO and diluted (1:1,000) into the perfusate. The perfusate concentration of nifedipine and DMSO were 30 \(\mu\)M and 0.1% (vol/vol), respectively. DMSO at this concentration had no effect on portal pressure or bile flow. Probenecid was dissolved in 0.1 M Tris, pH 8.0, before being diluted (1:400) to the KH saline. Tris alone (0.25 mM) had no effect on glutathione secretion, bile flow, or portal pressure. Neither nifedipine, probenecid, nor SNP infusion had any effect on portal pressure. Livers were perfused for at least 20 min before introduction of test substances into the KH saline.

**Partial hepatectomy and perfusion in situ.** Animals were anesthetized with an intraperitoneal injection of ketamine/xylazine (90/10 mg/kg body wt) following administration of an analgesic (buprenorphine, 0.03 mg/kg ip). Two-thirds partial hepatectomy was performed as described (11). Sham operations involved all procedures, with the exception of externalization and removal of the median and left lateral lobes. Perfusions were carried out as described above, except that the perfusate flow rate was reduced to account for the decreased liver mass. Under these conditions, portal pressure was 5.88 ± 0.14 cmH\(_2\)O/g liver (\(n = 11\)). From body and liver weight data collected before these experiments we calculated perfusion flow rates, and after each we determined that flow was 4.49 ± 0.18 ml min\(^{-1}\) g liver\(^{-1}\).

**Glutathione assay.** GSH was measured in bile samples immediately following collection by diluting (1:200) into ice-cold 0.1 M 4,4'-dipyridylidisulfide (DPS) in 0.1 M sodium phosphate buffer (pH 7.4) and monitoring the absorbance at 324 nm. After correcting for absorbance due to other non-sulphydryl constituents in bile (parallel samples were assayed in the absence of DPS) and comparing optical density against cysteine standards, the rate of secretion of reduced thiol was calculated. Total glutathione (GSH + GSSG) was routinely assayed in bile samples by using the enzyme recycling method and was expressed as GSH equivalents (9). To check the reliability of the DPS assay, in selected experiments bile was deproteinized by collection into 6% sulfosalicylic acid (SSA) in the absence (GSH + GSSG) or presence (GSSG) of 2-vinylpyridine followed by processing through the glutathione reductase recycling assay (9). In the latter experiments, acivicin (20 \(\mu\)mol/kg body wt) was instilled in a retrograde fashion over 1 min into the common bile duct, which was occluded for an additional minute before cannulation to inhibit ectopic \(\gamma\)-glutamyl transpeptidase (33). In rats 24 h after partial hepatectomy, the amount and volume of acivicin were reduced by two-thirds to reflect the decrease in liver mass. Tissue levels of glutathione were determined from liver samples removed at the termination of perfusion. Samples were immediately frozen in liquid N\(_2\) and then homogenized in 5 volumes of 6% SSA, neutralized, and assayed for GSH and total glutathione using the DPS and reductase assays, respectively.

**Bromosulfophthalein analysis.** Bile samples were diluted (1:200) in 0.1 N NaOH, and the absorbance at 580 nm was recorded and quantified against known standards.

**Statistical analysis.** Data are presented as means ± SD of at least three perfusions for each condition. Student’s t-test was used to compare pairs of samples, and P values <0.05 were considered significant.

**RESULTS**

**Differential modulation of canalicular GSH, GSSG, and bromosulfophthalein efflux by nifedipine and NO suggests the presence of a conjugate-independent GSH transport pathway.** Although it is already established that nifedipine is choleretic at doses expected to be seen in the clinical setting (12, 28), the mechanism responsible for this choleresis is not known. Figure 1A shows that 30 \(\mu\)M nifedipine caused a peak stimulation of bile flow of 1.6-fold, from 1.1 ± 0.1 \(\mu\)mol min\(^{-1}\) g liver\(^{-1}\) under basal conditions to 1.8 ± 0.3 \(\mu\)mol min\(^{-1}\) g liver\(^{-1}\) (\(n = 4\)) in the isolated perfused rat liver. This choleresis is paralleled by an increase in the total glutathione secretory rate (Fig. 1A), from 5.1 ± 0.4 nmol min\(^{-1}\) g liver\(^{-1}\) under basal conditions to 18.3 ± 3.4 nmol min\(^{-1}\) g liver\(^{-1}\) at 32.5 min of perfusion. The increase in total glutathione could be accounted for by an increase in GSH secretion from 3.0 ± 1.1 to 15.8 ± 4.5 nmol min\(^{-1}\) g liver\(^{-1}\) (Fig. 1A), suggesting that a major fraction of the choleretic potential of nifedipine results from its stimulation of GSH secretion. Conversely, the NO donor SNP is choleretic as a result of its stimulation of GSH excretion without affecting GSH efflux (Fig. 1B), as has already been reported for SNP and other NO donors (14, 32). Furthermore, GSSG appeared in bile at more than double the rate of nifedipine-induced GSH efflux. These distinct effects of nifedipine and SNP on GSH and GSSG secretion were
exploited to determine whether these are functionally competitive processes or whether they are mediated by independent pathways.

In the majority of our experiments, we used DPS as a rapid method to trap GSH. Additionally, we did not routinely inhibit canalicular γ-glutamyl transpeptidase activity or acidify bile samples. To determine whether our approach significantly affected the outcome of the glutathione assays, we performed the experiments illustrated in Fig. 1 following retrograde infusion of acivicin to inhibit ectopic transpeptidase and collection of bile into SSA to inhibit GSH oxidation. Figure 2 shows the basal, SNP-, and nifedipine-stimulated bile flow rates and secretion of total glutathione and GSH following acivicin/acidification exposure compared with non-pretreated samples. Mean basal rates of total glutathione and GSH secretion but not bile flow were significantly higher in the pretreated liver and bile samples. In contrast, nifedipine- or SNP-stimulated bile flow and, respectively, GSH or total glutathione secretion were not significantly affected by the retrograde infusion of acivicin and subsequent acidification. These results confirm an earlier report showing that acivicin had no significant effect on SNP-induced glutathione secretion in bile samples collected into acid (33). Therefore, for the remainder of the experiments in which the normal rat liver was used, the acivicin infusion and bile acidification steps were omitted, and the DPS assay was used for rapid monitoring of GSH secretion.

To determine whether GSH efflux is mediated by a GSSG transporter, experiments were designed to identify the existence of mutual competition between the efflux of SNP- and nifedipine-stimulated GSSG and GSH, respectively. When SNP was infused after the secretory response to nifedipine had reached a steady state, a small increase in mean choleresis was observed, although this was not significant at any time point (Fig. 3). No significant change in GSH or additional GSSG excretion was observed, indicating that SNP-induced GSSG release is completely inhibited by nifedipine, whereas GSH efflux induced by the latter is not significantly affected by SNP.

To determine whether nifedipine- or SNP-stimulated GSH or GSSG secretion is mediated by the high-affinity, low-capacity cMOAT/mrp2 route, bromosulfo-

Fig. 1. Effects of nifedipine or sodium nitroprusside (SNP) on bile flow and biliary glutathione secretion. Livers were perfused in the absence (open symbols; n = 3) or presence (solid symbols; n = 4) of either 30 μM nifedipine between 20 and 70 min (A) or 1 mM SNP between 35 and 55 min (B). Mean (± SD) rates of bile flow, total glutathione (GSH + GSSG), and reduced glutathione (GSH) efflux are plotted.

Fig. 2. Effect of acivicin/acidification pretreatment on biliary glutathione fractions in nifedipine- and SNP-perfused rat liver. Livers were perfused in the absence of acivicin (open bars) or following retrograde infusion of acivicin (20 μmol/kg) (hatched bars) with either 1 mM SNP (A) or 30 μM nifedipine (NIF; B) between 35 and 55 min. Bile samples were collected into ice-cold 4,4’-dipyridyl disulfide (DPS; − acivicin) or sulfosalicylic acid (SSA; + acivicin) and assayed for total (SNP-treated livers) or reduced (NIF-treated livers) glutathione. Mean (± SD; n = 4 for each condition) rates of bile flow and total (GSSG + GSH) or reduced (GSH) glutathione secretion under basal (at 32.5 min of perfusion) or stimulated (52.5 min) conditions were plotted.
phthalein (BSP) excretion was monitored in the absence or presence of nifedipine or SNP. BSP was infused at 1 mM, which is 30-fold less than the apparent Michaelis-Menten constant ($K_m$) for high-affinity, ATP-dependent efflux of this compound in isolated canalicular membranes (22). BSP efflux was significantly inhibited by SNP but not by nifedipine (Fig. 4A). As summarized in Table 1, SNP blocked 55.3±9.8% of BSP excretion, whereas nifedipine did not significantly alter BSP efflux. Furthermore, nifedipine pretreatment did not affect the SNP inhibition of BSP excretion (Fig. 4B; see Table 1). These results suggest that GSH secretion stimulated by nifedipine is not mediated by mrp2, whereas SNP and BSP generate glutathione conjugates that are transported through mrp2.

Probenecid block of basal and nifedipine-stimulated GSH secretion but not BSP excretion suggests independent efflux pathways for GSH and BSP. The organic anion probenecid is excreted into bile as the unchanged anion and its glucuronic acid conjugate in a 1:2 ratio (10). Figure 5 shows that 1 mM probenecid is choleretic without inhibiting tracer (1 mM) BSP excretion. The amount of BSP excreted between 41 and 62 min changed from $3.3 ± 0.3 \text{nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ ($n = 3$) under basal conditions (Table 1) to $2.9 ± 0.2 \text{nmol} \cdot \text{g}^{-1} \cdot \text{min}^{-1}$ ($n = 3$) in the presence of probenecid. This minimal effect on BSP excretion (as was also seen in response to nifedipine) suggested that probenecid efflux occurs by a pathway separate from the high-affinity mrp2 route and therefore might competitively inhibit nifedipine-induced GSH secretion. Furthermore, if GSH and organic anions are transported by the same organic anion transporter, probenecid should inhibit basal GSH secretion.

When 1 mM probenecid was perfused between 35 and 85 min (Fig. 6A) it caused a reversible choleretic that was accompanied by a 76.0% decrease in GSH release, suggesting that excretion of this organic anion directly competes with GSH efflux. The choleresis was not due to enhanced GSSG efflux (not shown), and therefore it was likely a result of the excretion and biliary accumulation of probenecid and its glucuronic acid conjugate. Further support for a common GSH/probenecid transporter was gained from experiments in which probenecid and nifedipine (30 μM) were alternately perfused and choleresis and GSH secretion were measured (Fig. 6). Whether probenecid was perfused first or during steady-state stimulation of GSH secretion by nifedipine, the nifedipine-induced GSH efflux was inhibited by 75.7%, suggesting competitive interaction at the level of the GSH transport pathway.
in steady-state levels of tissue glutathione. NO increases GSSG efflux in the isolated, perfused rat liver as a consequence of oxidative stress-induced accumulation of GSSG (see Ref. 32 and references therein). Table 2 shows that 15 min after cessation of SNP perfusion, tissue glutathione levels are not significantly different from control tissue. To determine whether nifedipine in the absence or presence of SNP or probenecid affected GSH transport activity as a result of perturbation of cellular glutathione pools, tissue levels of GSH and total glutathione were measured at the end of each group of perfusions. Table 2 shows that total glutathione, determined by an enzymatic assay, and GSH, determined by an acid-soluble thiol trapping technique, were not significantly different under any of the conditions used in this study. These results indicate that the acute increase in GSH or GSSG efflux in response to nifedipine or SNP, respectively, reflects small changes in flux through the glutathione pool that are compensated for by a mass action response at the transporter level (i.e., efflux is not rate limiting). Consequently, the cellular GSH/GSSG steady state is maintained. The lack of a significant increase in tissue GSH consequent to inhibition of canalicular efflux by probenecid suggests, again, that flux through the tissue glutathione pool changes to maintain a steady state.

**Table 1. Summary of bile flow and glutathione and bromosulfophthalein efflux under basal and test conditions**

<table>
<thead>
<tr>
<th>Condition</th>
<th>Bile Flow, μl·g⁻¹·min⁻¹</th>
<th>GSH, nmol·g⁻¹·min⁻¹</th>
<th>GSH + GSSG, nmol·g⁻¹·min⁻¹</th>
<th>BSP, nmol·g⁻¹·min⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>1.1 ± 0.1(4)</td>
<td>2.7 ± 0.8(3)</td>
<td>4.1 ± 0.2(3)</td>
<td>3.3 ± 0.3(3)</td>
</tr>
<tr>
<td>SNP</td>
<td>1.5 ± 0.1(7)*</td>
<td>3.6 ± 0.5(4)</td>
<td>29 ± 0.9(3)*</td>
<td>1.5 ± 0.1(3)*</td>
</tr>
<tr>
<td>NIF</td>
<td>1.7 ± 0.2(4)*</td>
<td>17 ± 2.1(4)*</td>
<td>17 ± 3.6(4)*</td>
<td>3.1 ± 0.2(4)*</td>
</tr>
<tr>
<td>NIF + SNP</td>
<td>2.0 ± 0.1(6)*†</td>
<td>13 ± 2.0(4)†</td>
<td>18 ± 2.3(4)†</td>
<td>1.9 ± 0.1(3)*</td>
</tr>
</tbody>
</table>

Values are means ± SD of no. of experiments in parentheses. Data from Figs. 1 and 3 were used to calculate the averaged rates of bile flow and glutathione efflux between 35 and 55 min in the absence (Basal) or presence of 1 mM sodium nitroprusside (SNP), 30 μM nifedipine (NIF), or nifedipine and SNP (NIF + SNP). Bromosulfophthalein (BSP) efflux data (Fig. 4) were averaged between 41 and 62 min for basal, SNP, or NIF and between 56 and 71 min for the NIF + SNP experiments. *P < 0.05 vs. basal. †P > 0.05 vs. nifedipine.
Short-term regeneration is associated with decreased glutathione secretion but not BSP efflux. One day after partial hepatectomy, the regenerating liver is characterized by a twofold increase in tissue GSH and a 50% reduction in canalicular GSH secretion in the absence of significant changes in mrp2 expression or activity (15, 35), indicating that the canalicular GSH efflux pathway is depressed preferentially over mrp2. We extended these observations to the sham-operated or partially hepatectomized liver perfused in the absence of bile salts (i.e., depressed bile flow was not compensated for by bile acid perfusion or an intact circulation). Figure 7 demonstrates that BSP excretion in the regenerating rat liver is not significantly different between sham-operated and partially hepatectomized animals. These results extend the earlier reports by specifically demonstrating that high-affinity, low-capacity (mrp2) activity is not perturbed following partial hepatectomy.

Our results also confirm earlier reports (15) that regeneration is accompanied by decreased GSH secretion and bile flow (Fig. 8, 20- to 35-min intervals). As summarized in Table 3, mean basal bile production, GSH, and total glutathione secretion were decreased significantly by 24, 43, and 57%, respectively, in regenerating livers compared with sham-operated animals. The effect of nifedipine or SNP on bile flow and glutathione secretion was also monitored in regenerating livers. The time courses are illustrated in Fig. 8, and the mean rates of GSSG or GSH released per gram liver in the presence of nifedipine or SNP were plotted.

Fig. 8. Effect of PH on nifedipine- or SNP-stimulated glutathione secretion and bile flow. Livers 24 h after PH (solid symbols; n = 4/group) or sham operation (open symbols; n = 4/group) were perfused in the presence of 30 μM nifedipine (A) or 1 mM SNP (B) between 35 and 55 min. Mean (± SD) rates of bile flow (top) and efflux of GSH (A, bottom) or GSH + GSSG (B, bottom) were plotted.

Table 2. Effects of different treatments on tissue levels of GSH and total glutathione

<table>
<thead>
<tr>
<th>Treatment</th>
<th>GSH + GSSG, μmol/g</th>
<th>GSH, μmol/g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Basal</td>
<td>3.55 ± 0.53(3)</td>
<td>4.36 ± 0.21(3)</td>
</tr>
<tr>
<td>Nifedipine</td>
<td>4.06 ± 0.68(6)</td>
<td>4.71 ± 0.58(6)</td>
</tr>
<tr>
<td>SNP</td>
<td>3.55 ± 0.52(7)</td>
<td>4.34 ± 0.36(7)</td>
</tr>
<tr>
<td>Nifedipine + SNP</td>
<td>4.80 ± 0.70(4)</td>
<td>4.03 ± 0.33(4)</td>
</tr>
<tr>
<td>Probenecid</td>
<td>5.48 ± 0.26(3)</td>
<td>4.13 ± 0.14(3)</td>
</tr>
</tbody>
</table>

Values are means ± SD of no. of experiments given in parentheses. Livers were perfused in the absence or presence of 30 μM nifedipine between 20 and 70 min and either 1 mM SNP or 1 mM probenecid between 35 and 55 min. Tissue samples were snap frozen in liquid nitrogen at 70 min perfusion. When SNP and nifedipine were tested, nifedipine was perfused between 20 and 70 min and SNP between 35 and 55 min.
Pathways of Canalicular GSH and GSSG Transport

Table 3. Effect of partial hepatectomy on SNP- and nifedipine-induced glutathione efflux and choleresis

<table>
<thead>
<tr>
<th></th>
<th>Sham</th>
<th>PH</th>
<th>%Change</th>
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</thead>
<tbody>
<tr>
<td><strong>Basal</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bile Flow</td>
<td>1.3±0.1(9)</td>
<td>1.0±0.07(9)*</td>
<td>-24</td>
</tr>
<tr>
<td>GSH</td>
<td>5.3±0.9(5)</td>
<td>3.0±1.1(4)*</td>
<td>-43</td>
</tr>
<tr>
<td>GSH+GSSG</td>
<td>6.6±1.1(4)</td>
<td>2.8±0.5(5)*</td>
<td>-57</td>
</tr>
<tr>
<td>+ SNP</td>
<td>1.8±0.1(4)</td>
<td>1.2±0.1(4)*</td>
<td>-35</td>
</tr>
<tr>
<td>Bile Flow</td>
<td>35.8±1.9(4)</td>
<td>17.2±1.6(5)*</td>
<td>-52</td>
</tr>
<tr>
<td>GSH+GSSG</td>
<td>1.6±0.1(5)</td>
<td>1.1±0.1(4)*</td>
<td>-30</td>
</tr>
<tr>
<td>GSH</td>
<td>8.0±1.3(5)</td>
<td>4.4±1.2(4)*</td>
<td>-54</td>
</tr>
</tbody>
</table>

Values are means ± SD of no. of livers sampled given in parentheses. Rates of bile produced and glutathione appearing in bile were calculated from the time courses in Fig. 8. Basal parameters were averaged between 20 and 35 min, and responses to SNP (1 mM) or nifedipine (30 μM) were averaged between 35 and 55 min and expressed as microliter bile or microgram GSH or GSH + GSSG per gram per minute. %Change is partial hepatectomy (PH) value as fraction of sham; underlined, italicized numbers denote fractional stimulation above corresponding basal levels. *P < 0.05 vs. sham.

Lastly, we assessed the potential effect of partial hepatectomy on biliary glutathione stability, since canalicular γ-glutamyl transpeptidase activity has been shown to increase in other models of impaired bile acid-independent flow (33). Figure 9 shows the rates of SNP-stimulated bile flow and total glutathione efflux in livers from regenerating vs. sham-operated animals. Livers were perfused and bile samples were collected in the absence or presence of acivicin and SSA. The latter treatment decreased bile flow under basal and SNP-stimulated conditions in the partially hepatoclemized livers but did not significantly affect the sham-operated animals. Similar trends were observed for total glutathione secretion, suggesting that retrograde infusion of acivicin into the bile duct of the regenerating liver compromises bile flow and inhibits glutathione secretion, possibly as a result of backflow through the weakened paracellular junctions of the regenerating liver. We conclude that the results obtained in the absence of acivicin/acidification are likely more representative of bile acid-independent bile flow in the isolated, perfused rat liver 24 h after partial hepatectomy.

**DISCUSSION**

In this study, we demonstrate that the L-type calcium channel blocker nifedipine and the anion transport inhibitor probenecid are choleretic in spite of their abilities, respectively, to stimulate or block the secretion of reduced glutathione into the biliary lumen. We used these compounds, combined with a concentration of SNP already established to stimulate near-maximum canalicular GSSG secretion (32) and tracer amounts of a glutathione conjugate-generating substance (BSP), to study the functional relationship between GSSG/conjugate and GSH transport in the intact, nominally bile acid-free, perfused liver. This was pursued because although GSH secretion provides a significant driving force for canalicular bile acid-independent bile formation (5), the molecular processes are not yet understood, and the physiological significance of those that have been reported from studies in vitro is not established.

Our results support the following functional model of glutathione secretion. This model predicts two major routes for GSH, GSSG, and glutathione conjugate efflux across the canalicular membrane. Under basal conditions, GSSG/conjugates are transported by a high-affinity but low-capacity route that is GSH insensitive and mediated by mrp2 (17). Under conditions in which cytosolic GSSG concentration is increased (e.g., oxidative stress and increased NO generation), the low-capacity route becomes saturated and excess GSSG overflows through the GSH transporter, operationally defined as cgsht (see Ref. 4). This model also serves as the major conduit for GSH efflux. It has a relatively high capacity and higher affinity for GSH and unconjugated organic anions than oxidized glutathione and glutathione conjugates. This model, and the results presented herein, provide functional evidence supporting some of the pathways described in canalicular membrane vesicles and demonstrate that specific drugs can independently modulate these routes. The model will be discussed in terms of the existing studies in vitro and the data reported here.

The model proposes that the major route for GSH efflux is a high-capacity path also capable of transporting organic anions and, at lower affinity, glutathione...

![Fig. 9. Effect of acivicin/acidification pretreatment on basal and SNP-stimulated total biliary glutathione secretion in sham-operated and PH rat liver. Livers were perfused in the absence of acivicin (open bars) or following retrograde infusion of acivicin (20 μmol/kg) (hatched bars) (n = 6 for each group) with 1 mM SNP between 35 and 55 min. Bile samples were collected between 20 and 35 min (Basal), 35 and 55 min (+SNP), and 55 and 70 min (Recovery) into DPS (−acivicin) or SSA (+acivicin) before assay for total glutathione. Mean (± SD) rates of bile flow and GSH + GSSG efflux were plotted.](http://ajpgi.physiology.org/.../10.22033.4)
disulfide and glutathione conjugates. Studies using canalicular membranes reported that GSH secretion occurs via ATP-independent and potential-sensitive high ($K_m \sim 0.25\text{mM}$) and low ($K_m \sim 17\text{mM}$) affinity components, the latter having 25 times the capacity of the former (3, 7). The high-affinity path is inhibited by glutathione conjugates (3), whereas that for low affinity secretion is sensitive to millimolar concentrations of organic anions (3, 7). However, the contribution of the high-affinity route to GSH secretion in the intact liver is minor since organic anions, but not their conjugates or GSSG, inhibit biliary GSH secretion in the perfused liver (2, 18). Similarly, we found that GSH efflux under basal conditions or in the presence of nifedipine was inhibited by the organic anion probenecid but not significantly affected by the GSSG secretagog SNP. Probenecid was choleretic, as reported earlier, as a result of its concentrative efflux into the biliary lumen as the glucuronic acid conjugate and the unchanged organic anion in a roughly 2:1 ratio (10). Thus organic anion/glucuronyl conjugate excretion, as represented by probenecid, directly competes with GSH efflux. Our results also suggest that probenecid is not a strong inhibitor of cMOAT/mrp2.

Two different models of canalicular GSH efflux have been proposed to account for the high and low affinity processes demonstrated in canalicular membranes. One, based on radiation inactivation studies, suggests that two different transport proteins are involved (20), whereas the alternative, based on correlation of GSH transport and mrp2 expression, suggests that mrp2 alone can account for both pathways (26). Support for the latter comes from the report that mrp2 overexpression results in increased ATP-independent, low affinity GSH flux (34), thus potentially accounting for the energy-independent transport seen in canalicular membranes. However, these data also show that mrp2-mediated GSH flux is significantly cis- and trans-inhibited by, respectively, physiological levels of glutathione conjugates (micromolar) and GSH (millimolar) (34). Therefore, mrp2 would account for only a small fraction of GSH efflux in vivo. In view of this data, the increased rates of GSH efflux in mrp2 overexpression models, or lack of release in mrp2-deficient livers of TR<sup>−</sup> rats, cannot be adequately explained as resulting from mrp2-mediated high capacity GSH efflux. It would therefore be of interest to extend the observations presented here and to determine whether nifedipine can stimulate GSH efflux in mrp2-deficient rats.

The correlation between mrp2 expression and GSH transport may yet be explained by a functional relationship between GSH and GSSG release mechanisms such that the glutathione redox balance is maintained. This is supported by the observation that although the total glutathione level is doubled in TR<sup>−</sup> rat liver compared with controls, the fraction of GSSG is not significantly different (32). Thus, without a functioning transporter for the release of GSSG, an intact GSH transporter, if present, would be inactive. In this case, nifedipine would not be expected to significantly stimulate GSH efflux in mrp2-deficient animals. The role of mrp2 in GSH efflux will be equivocal until purified, reconstituted preparations of this transporter are analyzed.

The model also predicts that GSSG or glutathione conjugates at high relative concentrations are released through the high-capacity GSH pathway. In favor of this is the block of near-maximal SNP-stimulated glutathione efflux by nifedipine (Fig. 3) and the minimal effect of nifedipine on efflux of tracer amounts of BSP (Fig. 4), which is mainly secreted as its glutathione conjugate through mrp2 (23, 30). The present results extend observations in canalicular vesicles showing that both GSH (1 mM) and organic anions cis-inhibited the low-affinity, ATP-independent uptake of both glutathione conjugates and GSH (3, 6).

Lastly, the model predicts that, under basal conditions, GSSG is transported mainly by a GSH-insensitive, low-capacity route mediated by mrp2. Since GSSG is transported against its concentration gradient into the canaliculus, efflux must be an energy-requiring process. ATP-dependent conjugate or GSSG transport in canalicular membranes occurs through a high-affinity, GSH-insensitive process (1, 3, 22), with properties similar to mrp2-mediated flux (17). We monitored the activity of this transporter by following the excretion of tracer concentrations of the glutathione conjugate of BSP (BSP-SG). Whereas GSSG (from SNP) blocked the major fraction of BSP excretion (Fig. 4), GSH (via nifedipine) and organic anions (probenecid) were much less effective in inhibiting BSP appearance in bile (Figs. 4 and 5). Since ~26% of nifedipine is excreted into the bile as its glucuronyl conjugate and the remainder mainly as carboxylate metabolites (29), the minor inhibition of BSP excretion by nifedipine and probenecid could result from competition with excretion of their conjugates through mrp2.

As an independent test of the model of GSH and GSSG/conjugate secretory pathways, we followed BSP and glutathione efflux in the regenerating liver perfused in the absence of added bile salts. Bile acid-independent bile flow, extrapolated from flow vs. bile salt secretion curves (38), and GSH secretion (15) are decreased in the early stages of regeneration as confirmed here, even though tissue glutathione is increased (15, 31). Furthermore, mrp2 activity and expression, as assessed by Northern and Western blot and functional studies (bilirubin excretion), are not significantly affected by partial hepatectomy (8, 35). Thus the low-affinity, high-capacity glutathione transport process, rather than the high-affinity, low-capacity, ATP-dependent path, appears to be selectively depressed during this period. Our model predicts, then, that tracer BSP excretion should not be affected, whereas GSH and GSSG, secreted by nifedipine and saturating amounts of SNP, respectively, should be depressed. In support of this, both basal and nifedipine-induced GSH secretion as well as SNP-augmented GSSG efflux were all decreased at 24 h after partial hepatectomy (Fig. 8; Table 3), whereas tracer BSP excretion was not significantly different between sham-operated and hepatectomized animals (Fig. 7).
Conversely, the percent stimulation of efflux above basal levels by nifedipine or SNP was not significantly different between sham-operated and regenerating livers (Table 3). These results suggest that, although the residual low affinity, high capacity transporters are decreased in number (as opposed to the BSP transporters), their functional activity is not modified. In combination, these results provide further evidence for the existence of a glutathione transporter distinct from mRP2. Additionally, the GSH transporter appears to be more sensitive to conditions in which polarized functions are compromised, such as regeneration.

In summary, we have identified a new GSH secretagog and used this to define the major physiological transport processes taken by GSH and GSSG/glutathione in their transfer into the biliary compartment. These results support the proposal that two distinct processes are responsible for GSSG/conjugate and GSH/organic anion secretion. In addition, we have provided evidence for a reinterpretation of the mechanism responsible for the cytoprotective effects of calcium channel blockers.

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