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Research Paper

4-Hydroxyacetophenone-Induced Choleresis in Rats is Mediated by the Mrp2-Dependent Biliary Secretion of Its Glucuronide Conjugate

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Purpose. The present study examined the underlying mechanism by which 4-hydroxyacetophenone (4-HA), a bioactive compound found in several medicinal herbs, exerts its potent stimulatory effects on hepatic bile secretion.
Methods. Bile flow, and biliary excretion of 4-HA, its metabolites, and inorganic electrolytes was examined in both normal Wistar rats and in TR^- Wistar rats that have a congenital defect in the multidrug resistance-associated protein-2, Mrp2/Abcc2. The effects of 4-HA were also examined in animals treated with buthionine sulfoximine to decrease hepatic glutathione (GSH) levels.
Results. In normal rats, 4-HA dramatically increased bile flow rate, whereas it failed to exert a choleretic effect in TR^- rats. This choleresis was not explained by increased biliary output of Na^+, K^+, Cl^- or HCO_3^-, or by increased biliary GSH excretion. Depletion of hepatic GSH with buthionine sulfoximine had no effect on the 4-HA-induced choleresis. HPLC analysis revealed that a single major compound was present in bile, namely.4-hydroxyacetophenone-4-*O*-β-glucuronide, and that the parent compound was not detected in bile. Biliary excretion of the glucuronide was directly correlated with the increases in bile flow. In contrast to normal rats, this 4-HA metabolite was not present in bile of TR^- rats.
Conclusions. These results demonstrate that the major biliary metabolite of 4-HA in rats is the 4-*O*-β-glucuronide, a compound that is secreted into bile at high concentrations, and may thus account in large part for the choleretic effects of 4-HA. Transport of this metabolite across the canalicular membrane into bile requires expression of the Mrp2 transport protein.

KEY WORDS: electrolyte; glucuronide conjugate; hepatic bile secretion; Mrp2/Abcc2; 4-hydroxyacetophenone.

INTRODUCTION

Hepatic bile formation plays a vital role in the elimination of both endogenous and exogenous compounds, including xenobiotics and toxic substances. Impairment of this process leads to retention of these compounds and to many harmful conditions. In addition to causing direct cell damage, the accumulated compounds may interact with nuclear receptors and other transcription factors, thus altering expression of their target genes and leading to other pathological conditions (1). Hence, compounds that are able to promote choleresis and facilitate the elimination of toxic compounds may have therapeutic potential to alleviate liver disorders.

Bile is secreted by hepatocytes into bile canaliculi. Canalicular bile formation is regarded as an osmotic water flow generated in response to active solute transport. Bile flow is classified into two components: one is related to bile acid output which is characterized by a correlation between bile acid secretion and bile flow, and the second is a bile-acid independent flow (BAIF), in which bile flow is determined by the output of non-bile acid solutes, including reduced glutathione (GSH) and other organic solutes, as well as inorganic electrolytes. The bile-acid dependent fraction (BADF) accounts for 40–50% of canalicular bile flow in rats, and the rest is BAIF. Biliary excretion of GSH and HCO_3^- has been considered as primary driving forces for BAIF (2–4). BAIF may also be generated by active biliary secretion of foreign organic molecules or their metabolites (5–8). Inorganic electrolytes (Na^+, Cl^- and K^+) function largely as counter-ions that are passively secreted from hepatocytes or/and through tight junctions down their electrochemical gradients.

Recent studies have shown that hydroxyacetophenones are potent stimulants of bile flow, and have described a

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ABBREVIATIONS: BADF, bile acid dependent flow; BAIF, bile acid independent flow; BSO, buthionine sulfoximine; GSH, reduced glutathione; Mrp2, multidrug resistance-associated protein-2; 4-HA, 4-hydroxyacetophenone.

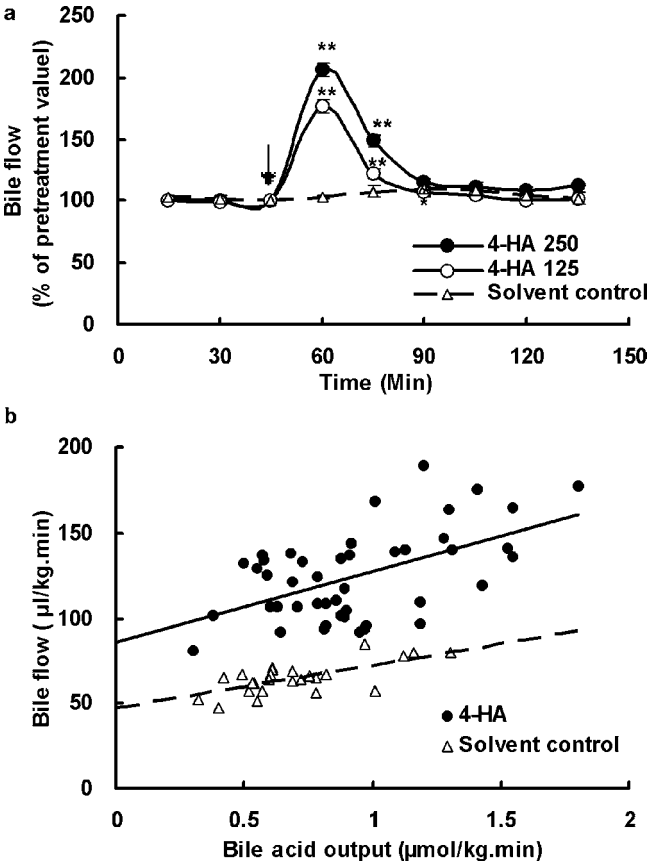

Fig. 1. Effect of 4-HA on bile flow in normal Wistar rats. (a) Choleretic activity of 125 (*open circle*) and 250 (*filled circle*) µmol/kg body weight of 4-HA or solvent control (*open triangle*). (b) Relationship between bile flow and bile acid output. Bile samples were collected for 15 min after intraduodenal injection of 4-HA (*filled circle*); $y=41.95x+85.29$, $r^2=0.2972$, $p<0.005$, or solvent control (*open triangle*); $y=25.37x+47.01$, $r^2=0.4785$, $p<0.005$. Values are means ± SEM from five to ten animals. $^{**}p < 0.005$ and $^{*}p<0.05$ significantly different from individual control at the corresponding time.

relationship between the structures of hydroxyacetophenones and their choleretic activities (9). The acetophenone with a single hydroxyl group at the 4-position (4-hydroxyacetophenone; 4-HA) exhibited a potent choleresis with low lithogenic index (9). This compound has been reported as an active ingredient in plants used in indigenous medicines of several Asian countries, including *Aster batangensis* (10), barley tea (11), and *Artemisia capillare* (12). *A. capillare* is a component of Chinese medicines that are used to enhance bilirubin clearance in newborns (12). However, the choleretic mechanism of 4-HA has not yet been identified, and is the focus of the present study.

MATERIALS AND METHODS

Chemicals

4-HA was purchased from Fluka Chemie AG (Buchs, Switzerland). 3α-Hydroxysteroid dehydrogenase, β-NAD, β-NADPH, 5,5′-dithiobis (2-nitrobenzoic acid), GSH, and GSSG reductase were purchased from the Sigma Aldrich (St. Louis, MO, USA). All the other reagents were commercially obtained and are of analytical grade.

Animal Treatments and Bile Sample Collections

Male Wistar rats weighing 220–290 g (7–8 weeks) were obtained from the National Animal Center, Bangkok, Thailand and were maintained on a standard diet and water *ad libitum*. A colony of TR^- rats is maintained at the University of Rochester School of Medicine Animal Facility, and studies with these animals were performed at the University of Rochester. Overnight fasted animals were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and prepared for collection of bile samples as previously described (9). A tracheostomy was performed to facilitate breathing. The common bile duct and femoral vein were cannulated using polyethylene (PE) tubing for collection of secreted bile and

Table I. Effect of 4-HA on Biliary Concentrations of Inorganic Electrolytes (Na^+, K^+, Cl^- and HCO_3^-)

	BFR (µl/min.kg)	Na^+ (mM)	K^+ (mM)	Cl^- (mM)	HCO_3^- (mM)
Solvent control					
Before	65.8±2.5	150.5±0.3	5.78±0.3	95±2.5	22.4±3.6
After	67.6±3.3	152.5±0.3	5.46±0.2	94±2.8	24.4±3.3
4-HA 125 µmol/kg					
Before	69.4±2.5	150.8±0.6	4.71±0.2	93.6±1	28.2±0.6
After	118.0±3.9**	147.2±0.6**	4.82±0.2	86.9±0.8**	26.4±0.7**
4-HA 250 µmol/kg					
Before	68.5±1.3	152.1±0.9	4.52±0.2	95.4±1.4	28.2±1.0
After	140.5±3.9**	148.2±0.8**	4.93±0.2**	86.3±1.5**	26.3±1.0
UDCA 50 µmol/kg					
Before	70.1±6.3	150.7±0.3	5.85±0.6	89.7±1.3	26.5±1.6
After	85.4±6.3**	155.7±0.9*	6.42±0.6*	84.7±1.3*	28.9±1.4*
UDCA 100 µmol/kg					
Before	67.1±5.1	151.0±0.5	5.15±0.5	90.7±0.8	27.2±0.4
After	88.3±2.1*	153.3±1.33*	5.37±0.4*	84.7±1.8*	30.9±0.7*

Bile samples were collected for 15 min after intraduodenal injection of 4-HA (125 or 250 µmol/kg body weight) and after UDCA injection into the portal vein (50 or 100 µmol/kg body weight). Values are means ± SEM from 10–11 animals.
$^{*}p<0.05$ significantly different from individual control before administration.
$^{**}p<0.005$ significantly different from individual control before administration.

for 0.9% saline infusion at the rate of 1.2 ml/h by using an infusion pump. The body temperature of animals was maintained at 37±0.5°C with a heating lamp to prevent hypothermic alterations of bile flow. The experimental protocols were approved by the Institutional Animal Care and Use Committees.

Bile samples were collected at 15-min intervals into preweighed tubes. After collection of three control samples, 4-HA dissolved in a solvent mixture (dimethyl sulfoxide: ethanol: water, at 25:15:60) was injected intraduodenally at various doses. Control rats received a similar volume of solvent (0.5 ml). The effects of ursodeoxycholic acid (UDCA), administered as a single injection into the duodenum, was examined as a positive control. UDCA was dissolved in dimethyl sulfoxide: ethanol: water (25:15:60). Buthionine sulfoximine (BSO), 6 mmol/kg body weight, i.p., was given 4 h before 4-HA administration to examine the effect of GSH depletion on the 4-HA choleresis. Bile flow rate was determined by gravimetry, assuming a bile density of 1.0 g/ml. The biliary excretion rate was calculated as the product between bile flow and biliary concentration.

Analysis of Biliary Components

Bile acid concentrations were determined using the 3α-hydroxysteroid dehydrogenase procedure (13). Biliary electrolytes including Na^+, K^+, Cl^- and HCO_3^- concentrations were determined using ion selective electrodes (DADE Dimension RxL, Dade Behring Holdings, USA). Bile samples were collected under mineral oil to prevent volatization of CO_2. The electrolyte operating range was 50–200 mmol/l for Na^+, 1–10 mmol/l for K^+, 50–200 mmol/l for Cl^- and 5–45 mmol/l for total CO_2.

Biliary and hepatic content of total glutathione was determined using the enzymatic methods of Tietze (14) and Griffith (15). Bile was collected under 500 µl of 6% sulfosalicylic acid to precipitate protein and prevent autooxidation. Samples were centrifuged at 4°C for 10 min to precipitate the denatured proteins, and the supernatant was stored at −70°C until assayed by spectrophotometry. Livers samples were placed in tared tubes containing ice-chilled 5% perchloric acid with 1 mM EDTA, and minced. Livers were homogenized on ice and centrifuged at 7,500×*g* at 4°C for

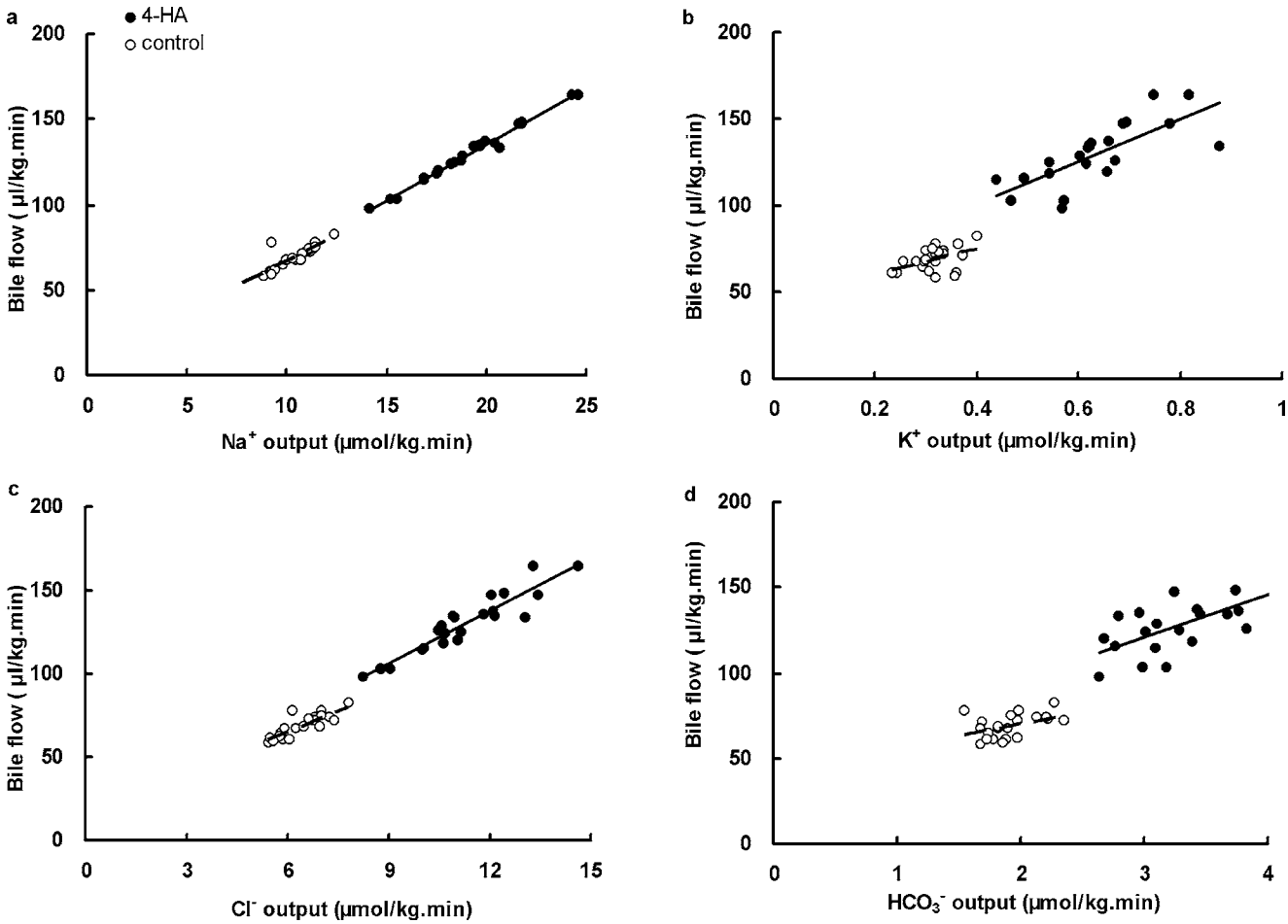

Fig. 2. Relationship between bile flow and electrolyte output. Bile samples were collected for 15 min after intraduodenal injection of 4-HA (*filled circle*) or solvent control (*open circle*). Bile samples were analyzed for correlation between bile blow and output of Na^+ (a); $y=5.78x+9.06$, $r^2=0.6973$, $p<0.005$ (control), $y=6.56x+4.08$, $r^2=0.9871$, $p<0.005$ (4-HA), K^+ (b); $y=76.25x+44.89$, $r^2=0.2219$, $p=0.0269$ (control), $y=122.3x+52.65$, $r^2=0.5578$, $p<0.0001$ (4-HA), Cl^- (c); $y=8.32x+15.18$, $r^2=0.6798$, $p<0.0001$ (control), $y=10.5x+11.81$, $r^2=0.8707$, $p<0.005$ (4-HA), HCO_3^- (d); $y=14.08x+42.17$, $r^2=0.2128$, $p=0.0307$ (control), $y=25.02x+45.48$, $r^2=0.5954$, $p<0.0001$ (4-HA).

5 min. Supernatant was removed and samples were diluted further for measurement of hepatic glutathione content.

Isolation and Structure Elucidation of Biliary Components

Bile samples collected for 15 min after administration of 4-HA were diluted with 20 volume of absolute ethanol, boiled for 30 min and left overnight. Thereafter, they were filtered through Whatman no. 1 paper. The filtrates were dried using a speed vacuum, then redissolved with 5% acetic acid in methanol (mobile phase). For HPLC analysis, a Waters 600 system controller and pump, a Waters 996 diode array detector, and a Waters manual injector (Waters Associates, Milford, MA, USA) were employed. Separation and quantization was achieved by using μ-Bondapak C18, 10 μm, 3.9 × 300 mm column (Waters Associates), a mobile phase of 5% acetic acid in methanol, a flow rate of 1.0 ml/min, and a wavelength of 264 nm. The HPLC peak corresponding to the single major metabolite (retention time 8–10 min) was collected and the solvent was removed by co-evaporation with n-butanol under reduced pressure. The nuclear magnetic resonance (NMR) spectra were recorded on a Bruker AVANCE 400 FT-NMR spectrometer (Faellanden, Switzerland). The residual nondeuterated dimethyl sulfoxide-d_6 signal at 2.49 ppm was used as reference for ^{1}H-NMR spectra. High-resolution fast atom bombardment mass spectra were measured with a Finnigan MAT 90 instrument (Bremen, Germany). The amount of 4-hydroxyacetophenone-4-*O*-β-glucuronide in bile was determined spectrophotometrically at a wavelength of 264 nm. Standards were prepared from 4-hydroxyacetophenone-4-*O*-β-glucuronide isolated by HPLC from bile samples collected from rats treated with 4-HA.

Statistical Analyses

All data are expressed as mean ± SEM. Data obtained after treatment was compared with their respective controls before treatment using ANOVA, followed by Student–Newman–Keul's test. The relation between bile flow and biliary excretion rate was examined by linear regression analysis. Statistical significance was considered at $p<0.05$.

RESULTS

Dose Response Effect of 4-HA on Bile Secretion

In normal Wistar rats, 4-HA produced an immediate and dramatic increase in bile flow. In the first 15-min collection interval after 4-HA administration, bile flow was increased to 176±6 and 206±5% of control by 4-HA doses of 125 and 250 μmol/kg, respectively. However, the choleresis was transient, and bile flow rate rapidly returned to baseline levels (Fig. 1).

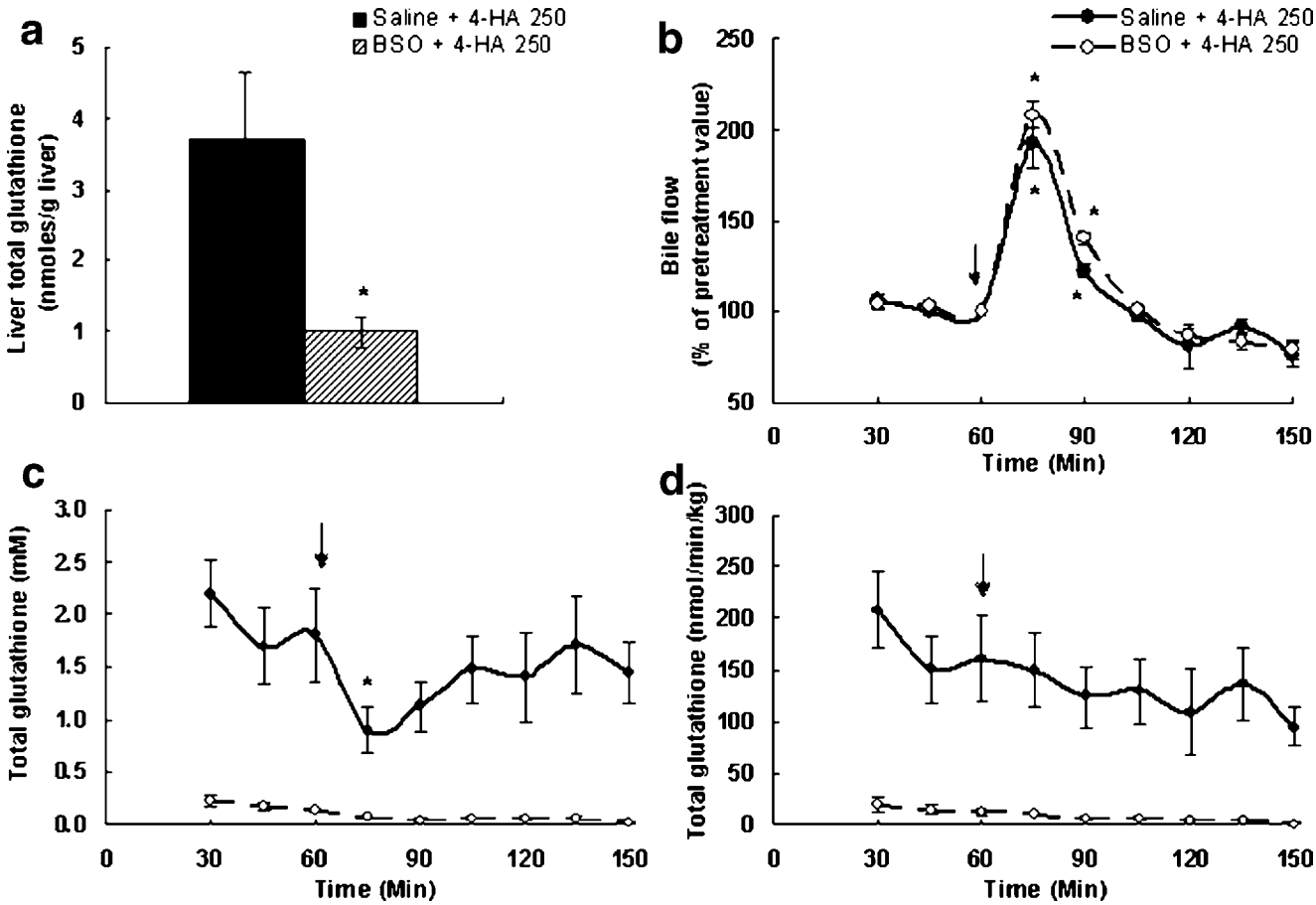

Fig. 3. The 4-HA-induced choleresis does not depend on GSH. Saline (*filled circle*) or BSO (*open circle*), 6 mmol/kg BW, i.p., was injected 4 h before 4-HA administration. Liver was removed at the end of each experiment and analyzed for total glutathione (a). Bile flow (b), and total glutathione concentration (c) and output (d) were measured. *$p<0.05$ significantly different from individual control (b).

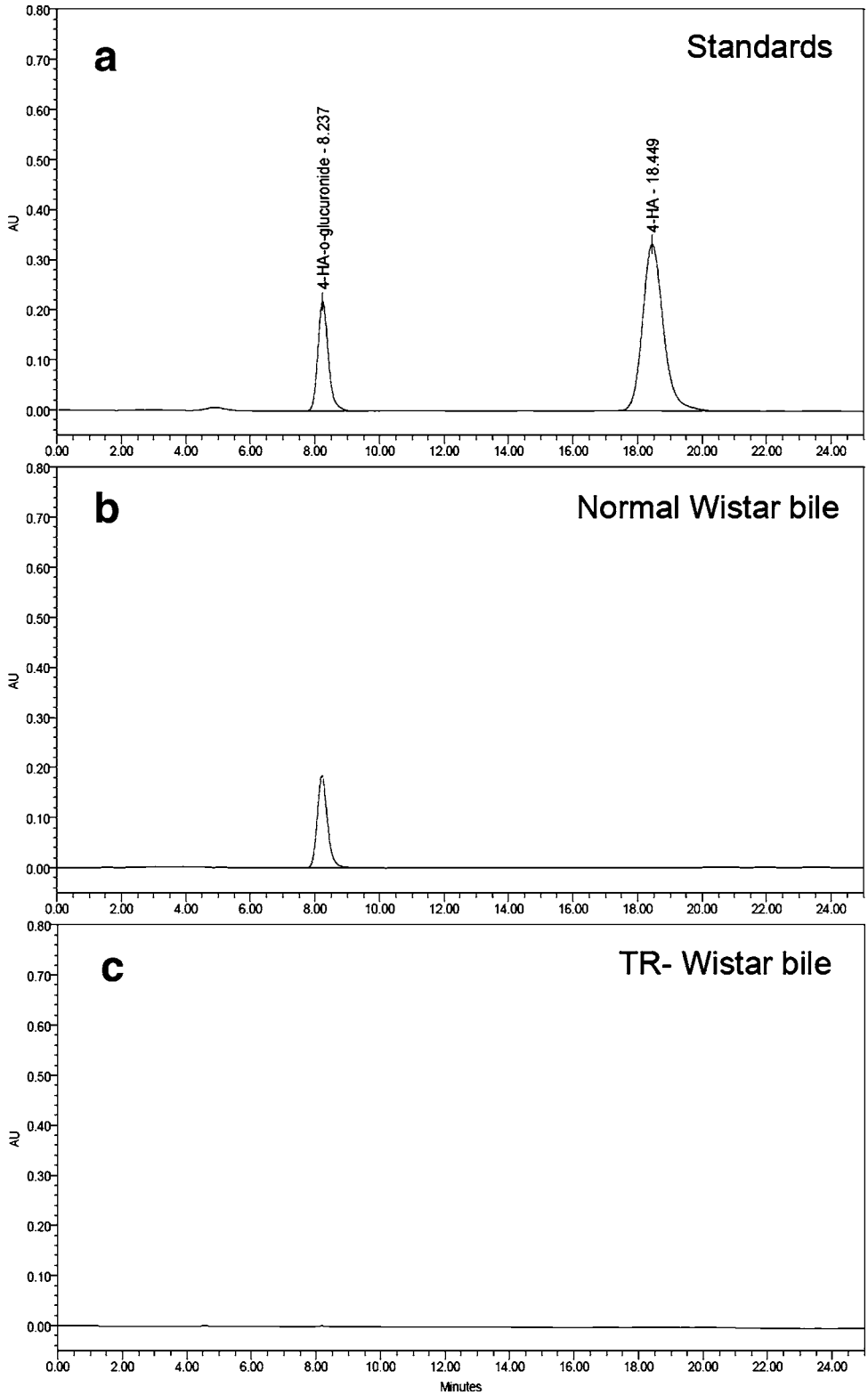

Fig. 4. HPLC analysis of possible 4-HA metabolites in bile. (a) Retention times of a standard solution containing 4-hydroxyacetophenone-4-*O*-β-glucuronide and 4-HA. (b) Bile was found to contain one major metabolite in Wistar rats treated with 4-HA, and this compound was subsequently identified as 4-hydroxyacetophenone-4-*O*-glucuronide. (c) Neither 4-HA nor any metabolites were detected in bile from TR^{-} rats treated with 4-HA.

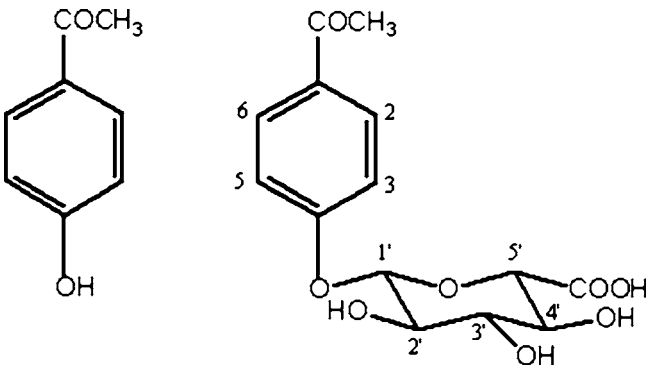

Fig. 5. Structures of 4-hydroxyacetophenone (4-HA) (*left*), and 4-hydroxyacetophenone-4-*O*-β-glucuronide (*right*).

Concurrent with the increased bile flow, bile acid concentration was decreased. As illustrated in Fig. 1, the slope of the line that defines the relation between bile flow and bile acid output in the 4-HA-treated rats was similar to that of control. These observations indicate that the increase in bile flow is due to an increase in BAIF.

Effect of 4-HA on Biliary Electrolyte and GSH Excretion

To examine which osmotically active solute(s) may be responsible for the increase in BAIF, biliary levels of Na^+, K^+, Cl^- and HCO_3^- and GSH were analyzed. As shown in Table I, 4-HA slightly reduced the biliary concentrations of Na^+, Cl^-, HCO_3^- and increased the concentration of K^+. When bile flow was increased, the outputs of these inorganic electrolytes were increased. UDCA, which elicits a choleresis that is associated with an increase in HCO_3^- and a decrease in Cl^- concentration (16) was also examined for comparison. After UDCA treatment, the choleresis was associated with an elevation of HCO_3^- and a reduction of Cl^- concentrations in bile (Table I). Linear regression analyses of bile flow rates and electrolyte outputs are shown in Fig. 2. In both control and 4-HA treated animals, bile flow rates were positively correlated with electrolyte outputs; however, all of the electrolyte outputs showed similar changes, suggesting that they are not the prime determinants for drawing water into bile after 4-HA administration.

Because GSH is a key osmotically active solute contributing to BAIF (2,3), studies were performed to examine whether GSH is involved in the 4-HA-induced choleresis. GSH levels in bile were measured in both control animals and in animals pre-treated with BSO to deplete hepatic GSH levels. Although BSO produced a significant depletion of hepatic GSH content (Fig. 3), it had no effect on the 4-HA-induced choleresis (Fig. 3). Moreover, total GSH output into bile was not changed significantly after 4-HA administration (Fig. 3), indicating that GSH is not involved in the 4-HA-induced choleresis.

Biliary Excretion of 4-HA and Its Metabolites

To examine whether the choleresis may be attributed to the biliary excretion of 4-HA itself or of a metabolite, HPLC was used to detect and quantify these compounds in bile. Bile from normal Wistar rat that received 4-HA revealed the presence of a single major new peak that eluted with a retention time of 8–10 min (Fig. 4). No parent compound was detected in bile (the parent compound eluted at a retention time of 17–19 min; Fig. 4). NMR and mass spectral analysis were used to determine the structure of this compound. The compound showed a pseudomolecular ion $[M\text{-}H]^-$ at m/z 311.0768 in the negative ion high-resolution fast atom bombardment (HR-FAB) mass spectrum, compatible with a molecular formula of $C_{14}H_{16}O_8$. The ^{1}H-NMR spectral data were consistent with a 4-hydroxyacetophenone moiety, based on the three-proton singlet of the acetyl group at δ 2.51, the proton doublet (J=8.7 Hz) of H-3 and H-5 at δ 7.10, and the two-proton doublet (J=8.7 Hz) of H-2 and H-6 at δ 7.92. The presence of a glucuronide moiety was suggested by a doublet signal at δ 5.18 corresponding to the anomeric proton, H-1′, and broad triplet signals of H-2′, H-3′ and H-4′ at δ 3.30, 3.26 and 3.35 with apparent coupling constants of 8.5–8.7 Hz. The relatively large coupling constant (7.0 Hz) of H-1′ indicates a β-orientation of the sugar acid residue. Thus, these data indicate that this biliary constituent is 4-hydroxyacetophenone-4-*O*-β-glucuronide (Fig. 5, right).

Of significance, this 4-HA metabolite was present in bile in relatively high concentrations (up to 13 mM; Fig. 6). The amount of 4-HA administered correlated well with and the amount of the metabolite in bile, and a significant positive correlation between bile flow rate and the amount of the secreted metabolite was observed (Fig. 6). The excreted bile volume per micromole of 4-hydroxyacetophenone-4-*O*-β-glucuronide was 34 μl (Fig. 6), which is comparable to that of bile acids (Fig. 1). Hence, the choleretic effect of 4-HA may be explained in large part by the excretion of this metabolite into bile.

4-HA Fails to Induce a Choleresis in Mrp2-Deficient Rats

In contrast to the normal Wistar rats, 4-HA failed to produce a choleresis in TR^- rats that lack the canalicular Mrp2 transport protein (Fig. 7). In addition, 4-hydroxyacetophenone-4-*O*-β-glucuronide was not detected in bile from TR^- rats

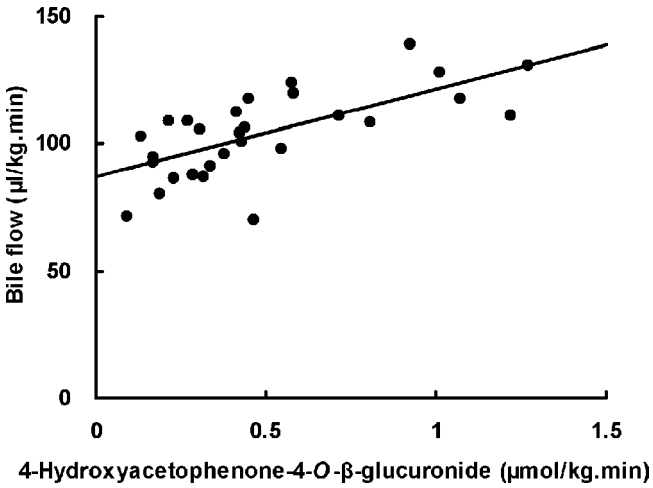

Fig. 6. Relationship between bile flow and 4-hydroxyacetophenone-4-*O*-β-glucuronide excretion rate after 4-HA administration. Bile samples were collected and analyzed for 4-hydroxyacetophenone-4-*O*-β-glucuronide output by HPLC as described in materials and methods, y=34.48x+86.89, r^2=0.4583, p<0.005.

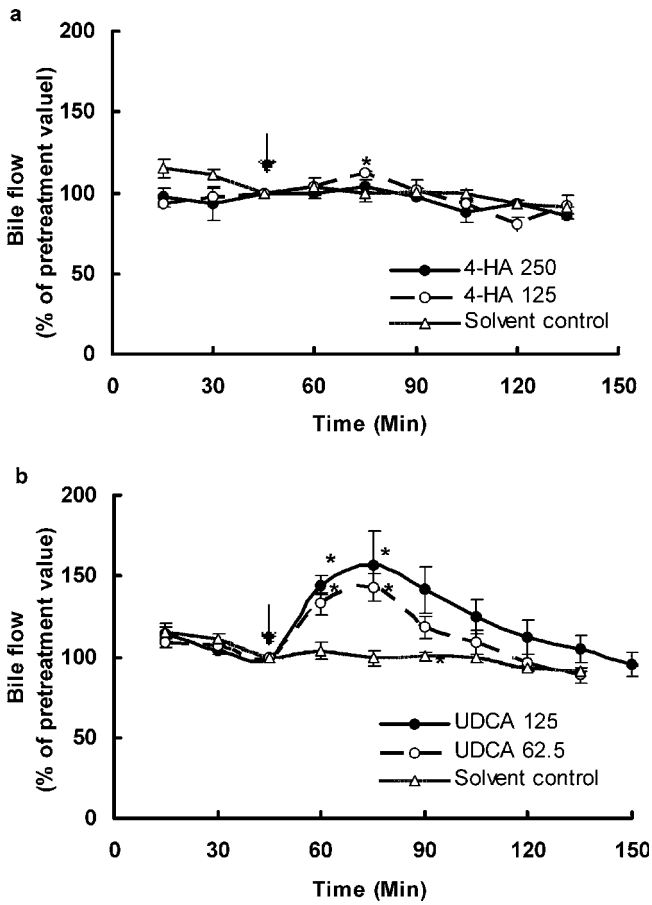

Fig. 7. Lack of choleretic effect of 4-HA in TR^- Wistar rats. (a) Animals treated with 125 (*open circle*), 250 (*filled circle*) μmol/kg body weight of 4-HA or solvent control (*open triangle*). (b) Animals treated with 62.5 (*open circle*), 125 (*filled circle*) μmol/kg body weight of UDCA or solvent control (*open triangle*). *$p<0.05$ significantly different from pretreatment value at the corresponding time.

(Fig. 4). As expected, TR^- animals treated with UDCA demonstrated a robust choleresis (Fig. 7). These results indicate that Mrp2-mediated transport is required for the choleresis, and that 4-hydroxyacetophenone-4-*O*-β-glucuronide is a likely substrate for Mrp2.

DISCUSSION

4-HA is a naturally occurring bioactive compound found in several plants. It exerts potent stimulatory effects on hepatic bile secretion, and may thus be a useful compound to treat a variety of conditions associated with impaired bile secretion (9). The present study identifies the mechanism for this choleretic effect by showing that the major biliary metabolite of 4-HA in rats is the 4-*O*-β-glucuronide, a compound that is secreted into bile at high concentrations. Canalicular transport of the glucuronide requires the presence of Mrp2, and the concentrative accumulation of this metabolite in bile accounts in large part for the choleretic effect.

Canalicular bile formation is an osmotic process, in which water and electrolytes enter bile canaliculi in response to the active transport of solutes into this space. The major solutes that drive bile secretion are bile acids (i.e., BADF); however non-bile acid solutes, including reduced glutathione (GSH) and other organic solutes, including many foreign chemicals, also drive bile secretion. The biliary excretion of GSH (2,3), and HCO_3^- (4) have been widely considered as the major solutes for BAIF. After giving 4-HA, the concentrations of Na^+, K^+, Cl^- and HCO_3^- were only slightly modified, and were close to those in blood plasma. There was also no change in biliary GSH excretion. As bile flow was increased after receiving 4-HA, the outputs of Na^+, K^+, Cl^- and HCO^-_3 were all increased in a similar manner and were positively correlated with the increase of bile secretion. UDCA, which stimulates BAIF mainly by promoting biliary output of HCO_3^- in exchange with Cl^- at canalicular and ductular membranes (16), was examined for comparison. A single injection of UDCA produced an increase in biliary HCO^-_3 concentration and a decrease in Cl^- concentration from control, and these results differ from those of 4-HA showing a decrease in biliary HCO_3^- concentration. Although 4-HA choleresis was associated with increases in biliary output of inorganic electrolytes, these do not appear to be the primary stimulus for the choleresis. GSH, another osmotically active solute contributing to BAIF (2,3), also did not contribute to the choleretic activity 4-HA. Bile flow was still increased after 4-HA administration, even when GSH levels in the liver were depleted to 30% of control. In addition, the 4-HA-induced choleresis occurred with only a small increase in bile acid output, confirming previous results that the effect of 4-HA is largely on BAIF (9). On the other hand, as demonstrated by the present data, 4-hydroxyacetophenone-4-*O*-β-glucuronide was secreted into bile in concentrations up to 13 mM, and the excretion of this metabolite directly correlated with the increase in bile flow. 4-HA exhibits low water solubility, and thus it is anticipated that it would need to be modified in the liver in order to facilitate its membrane transport and excretion from the cell. The excreted bile volume per micromole of 4-hydroxyacetophenone-4-*O*-β-glucuronide in bile was 34 μl (Fig. 6), which is similar to that of bile acids (Fig. 1), and thus may completely explain the choleresis. In contrast, the parent compound was not detected in bile.

To test whether Mrp2, the major canalicular transport protein for secretion of glucuronide conjugates into bile is involved in the secretion of 4-hydroxyacetophenone-4-*O*-β-glucuronide, studies were carried out in TR^- rats that lack functional Mrp2 transport activity. TR^- rats failed to demonstrate a choleresis, and no 4-hydroxyacetophenone-4-*O*-β-glucuronide was detected in their bile. These results support the hypothesis that the Mrp2-mediated excretion of 4-hydroxyacetophenone-4-*O*-β-glucuronide generates the osmotic driving force responsible for the enhanced bile flow observed after 4-HA administration. Because a number of plants contain 4-HA (10–12,17) and several polyphenol can be degraded into 4-HA (18,19), the present findings on the effect of 4-HA may explain some of the pharmacological effects of these plant-derived products.

In conclusion, the choleretic effect of 4-HA may be explained by the osmotic activity of a 4-HA metabolite that is secreted into bile in high concentrations by an Mrp2-dependent mechanism. An understanding of the mechanisms by which bioactive compounds in medicinal plants, including

4-HA, stimulate biliary excretion is of interest with respect to possible therapeutic interventions in liver dysfunction, and for developing new drugs.

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