Phloracetophenone-induced choleresis in rats is mediated through Mrp2

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Canalicular bile flow is formed from the excretion of bile acids (bile acid-dependent flow, BADF), and a bile acid-independent component (BAIF) that results from the excretion of glutathione (GSH) and other organic and inorganic solutes (2, 20, 36). The biliary excretion of these osmotically active compounds, which also include metabolic by-products and xenobiotics, is the major driving force for the passive movement of fluid and electrolytes across the hepatocyte epithelium and the tight junctions between these cells (4). The secretion of bile is a vital function for eliminating endogenous and exogenous compounds, which often undergo biotransformation by phase I and II hepatic enzymes before their excretion into bile.

The therapeutic potential of a number of these compounds is dependent on this metabolic pathway. For example, genipin, an intestinal bacterial metabolite of geniposide which is the major active compound in Inchino-to, is an herbal medicine used in China and Japan for treatment of jaundice. Genipin has been shown to have potent choleretic activity (32) and is conjugated with glucuronide in the liver before excretion. Genipin stimulates the insertion of the multidrug-resistance-associated protein 2 (Mrp2) into the bile canalicular membrane and thereby increases the biliary excretion of GSH and BAIF (31).

Ursodeoxycholic acid, an anticholestatic drug, prevents the cholestatic effects of ethinylestradiol by inhibiting both the activity and expression of the hepatic microsomal enzyme involved in ethinylestradiol glucuronidation (29). This in vivo effect appears to be mediated by the major metabolite of ursodeoxycholic acid, tauroursodeoxycholate, which inhibits ethinylestradiol glucuronidation noncompetitively in vitro (29).

Phloracetophenone, or 2,4,6-trihydroxyacetophenone (THA), the aglycone component of phloracetophenone glucoside, is a naturally occurring compound obtained from the rhizome of Curcuma comosa (Family Zingiberaceae) (24). When administered via the duodenum to rats, THA stimulates bile flow and enhances the biliary excretion of bile acids, leading to a lowering in the plasma levels of cholesterol (25, 26). Thus THA may have potential as a therapeutic drug for the treatment of cholestasis and prevention of gallstones (3, 22). However, the underlying mechanism by which THA enhances bile flow remains unknown. In the present study, we utilized the isolated perfused rat liver (IPRL) to examine the effects of THA on bile flow and defined the possible pathways that may account for its choleretic effects. The IPRL model was employed because it facilitates study of the direct action of compounds on the liver without the confounding effects of blood flow or extrahepatic tissue involvement (21). Our findings indicate that the choleretic effect of THA is primarily dependent on Mrp2 and that THA may have therapeutic potential to attenuate E2-17G-induced cholestasis.

MATERIALS AND METHODS

Chemicals. THA was purchased from Fluka Chemie (Buchs, Switzerland). Taurocholate (TC), estradiol-17β-D-glucuronide (E2-17G), glutathione reductase, the reduced form of glutathione (GSH), the reduced form of β-nicotinamide adenine dinucleotide phosphate, tetrasodium (β-NADPH), DTNB, and sulfobromophthalein (BSP)
were purchased from Sigma Chemical (St. Louis, MO), Phenol-3,6-dibromomphthalane disulfonate (DBSP) was from Société de Recherches Biologiques (Paris, France). Bile acid reagents A and B for bile acid determination were from Trinity Biotech (Wicklow, Ireland). All other chemicals were of analytical grade.

**IPRL studies.** Male Sprague-Dawley rats (190–200 g) were obtained from Charles River Laboratories (Wilmington, MA), and all experimental protocols were conducted under the National Institutes of Health guidelines and were approved by The Yale Animal Care and Use Committee.

Rat livers were isolated and perfused in the Cellular and Molecular Physiology Core of the Yale Liver Center as described previously (6). Following pentobarbital sodium anesthesia (50 mg/kg body wt ip) the bile duct and portal vein were cannulated with PE-10 tubing (Becton Dickinson Primary Care Diagnostics, Sparks, MD) and a 14-gauge Teflon intravascular catheter (Mckesson General Medical, Cheshire, CT), respectively. The liver was perfused immediately at a rate of 30 ml/min with oxygenated (95% O₂-5% CO₂) Krebs-Ringer bicarbonate (KRB) buffer (pH 7.4) containing 0.1% glucose and maintained at 37 ± 0.5°C. After cannulation of the inferior vena cava, the liver was transferred into a temperature-controlled chamber and then perfused at a constant flow rate of 40 ml/min with oxygenated KRB buffer (4.4–5.7 ml·min⁻¹·g liver⁻¹). KRB buffer was gassed continuously with a humidified mixture of 95% O₂-5% CO₂ and maintained at 37 ± 0.5°C by monitoring the temperature with a thermistor probe (Yellow Springs Instrument, Yellow Springs, OH) inserted between the lobes of the liver. The viability of the liver was determined by continuously monitoring perfusion pressure and O₂ consumption throughout the course of experiment. At the end of the experiment, Trypan blue was injected into the liver to evaluate the uniformity of perfusion from the distribution of the staining dye.

All IPRL studies utilized a single-pass system with oxygenated (95% O₂-5% CO₂) KRB (pH 7.4). Following a 15-min period of equilibration, THA was infused and bile flow and biliary excretion of bile acid and total glutathione were assessed. THA was dissolved in solvent (DMSO-absolute ethanol-KRB, 25:15:60) and was infused into the perfusate at a rate of 1, 2, or 4 μmol/min (25, 50, and 100 μM, respectively) in a stepwise fashion at 20-min intervals. Bile samples were collected at 5-min intervals into preweighed tubes throughout the experiment. Six percent sulfosalicylic acid was added when GSH was determined. The volume of bile was determined gravimetrically and the consumption of O₂ was determined. The experiment was performed by determining the distribution of the staining dye.

Analytical methods. Total bile acid excretion was determined by using a diagnostic bile acid reagent kit (Trinity Biotech) that is based on 3α-hydroxysteroid dehydogenase enzymatic method (17). Total glutathione in bile was measured immediately from bile samples collected in preweighed tubes containing 6% sulfosalicylic acid to minimize oxidation of GSH and was determined spectrophotometrically by the enzymatic recycling procedure of Tietze (33) as modified by Griffith (8). Concentration of BSP and DBSP in bile were measured spectrophotometrically at the absorption maximum (580 nm) after appropriate dilution with 0.1 N NaOH.

Statistical analysis. All data are expressed as means ± SE. The differences in pairs of samples between control and THA-treated group in IPRL were compared by Student’s paired t-test. For comparing more than two groups, the significance of the difference among groups was analyzed by ANOVA, followed by Student-Newman-Keuls test. Values of P < 0.05 were considered to be significantly different.

**RESULTS**

Effect of THA on BFR. The choleretic effect of THA infusion into the IPRL was characterized by utilizing a stepwise infusion of THA (1, 2, and 4 μmol/min). This caused a dose-dependent increase in BFR that attained a steady state at 10–15 min after start of the infusion (Fig. 1). THA at a dose of 1 μmol/min caused an immediate increase in bile flow and maintained BFR at 1.8 ± 0.1 l/min (g liver⁻¹; n = 4) compared with 1.0 ± 0.1 l/min (g liver⁻¹) under basal conditions. THA at 2 μmol/min further increased BFR to a maximum level of 2 ± 0.2 l/min (g liver⁻¹), whereas THA at 4 μmol/min did not induce any further increase in BFR. In control animals, BFR slightly declined with time. Thus THA directly stimulated bile flow in a dose-dependent manner but reached a maximum effect.

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Effect of THA on biliary secretion of bile acids and total glutathione (GSH + GSSG). To examine the determinants of bile secretion, the effect of THA on the two major endogenous osmotically active solutes, bile acids and glutathione, was determined. In the isolated perfused liver, the concentration of biliary bile acids in control animals rapidly declined with time and reached a minimal level of 0.9 ± 0.2 mM after 30–35 min of perfusion, as shown in Fig. 2A. The THA-infused livers showed a similar decline in the concentration of bile acids to that of the control. Fifteen minutes after THA infusion, bile acid concentration significantly decreased to 0.4 ± 0.04 mM (P < 0.05) (Fig. 2A). However, the total output of biliary bile acids was not significantly different from that of control (Fig. 2B). THA also markedly decreased both the concentration and total amount of excretion of biliary glutathione (GSH + GSSG), in contrast to control animals where glutathione levels were maintained throughout the course of the experiment (Fig. 2, C and D). THA, at a dose of 1 μmol/min, significantly reduced the concentration of biliary total glutathione to 67.3 ± 5.1% of control values within 10 min before higher doses of THA were infused and further declines were noted during the remaining course of the experiment (Fig. 2C). Biliary GSH excretion rates also decreased significantly after THA infusions at a dose of 4 μmol/min by 45.0 ± 7.9% compared with controls (Fig. 2D). However, during the first 5 min, THA transiently increased the rate of excretion of glutathione to 130.2 ± 4.8% of control whereas the GSH concentration was not significantly altered. This effect was most likely a “washout” phenomenon.

Effect of THA on BSP excretion. The multidrug resistance-associated protein 2 (Mrp2) is responsible for the biliary excretion of many glucuronide, sulfate, and glutathione S-conjugates, as well as GSH itself (7). Because THA infusions inhibited the excretion of GSH, Mrp2 was presumed to be a candidate for THA excretion. Therefore we examined the effect of THA on the excretion of BSP and DBSP, two typical substrates of the Mrp2 canalicular membrane transporter. As shown in Fig. 3A, infusion of BSP, at a rate of 0.04 μmol/min, did not alter BFR, which was steadily maintained at 1.1 ± 0.1 g liver⁻¹ min⁻¹. In contrast, simultaneous infusions of THA (40 μmol/min) caused an immediate and marked approximately twofold increase in BFR within the first 5 min of infusion that subsequently declined with time (Fig. 3A). Concurrent with the increase in BFR, both the concentration and excretion rate of BSP in bile were markedly decreased.
Biliary BSP concentration was significantly decreased from 4.8 ± 0.2 mM in control to 2.6 ± 0.6 mM 10 min after THA infusions. These findings suggest that THA may compete with BSP for excretion into bile. The small increase in BSP excretion in the first 10 min and the decrease in biliary BSP excretion that persisted for 10 min after THA infusion was stopped is likely due to the effect of the increase and decrease in bile flow, respectively, on previously secreted BSP residing within the bile canaliculi and biliary tree, i.e., a washout phenomenon (Fig. 3C).

(Fig. 3, B and C). Biliary BSP concentration was significantly decreased from 4.8 ± 0.2 mM in control to 2.6 ± 0.6 mM 10 min after THA infusions. These findings suggest that THA may compete with BSP for excretion into bile. The small increase in BSP excretion in the first 10 min and the decrease in biliary BSP excretion that persisted for ~10 min after THA

Fig. 3. Effect of THA on bile flow rate (A), biliary sulfobromophthalein (BSP) concentration (B), and BSP excretion rate (C) in the isolated perfused rat liver. BSP at a dose of 0.04 μmol/min was continuously infused into the liver throughout the course of the experiment. THA was added into the perfusion medium for coperoxion at a constant rate of 40 μmol/min at the indicated time point. Values are means ± SE from 4 animals. *P < 0.05 and **P < 0.01, significant difference from control at the corresponding time.

Fig. 4. Effect of THA on bile flow rate (A), biliary dibromosulfobromophthalein (DBSP) concentration (B), and DBSP excretion rate (C) in the isolated perfused rat liver. DBSP at a dose of 0.04 μmol/min was continuously infused into the liver throughout the course of the experiment. THA was added into the perfusion medium for coperoxion at a constant rate of 40 μmol/min at indicated time point. Values are means ± SE from 4 animals. *P < 0.05 and **P < 0.01, significant difference from control at the corresponding time.
Effect of THA on biliary DBSP excretion. Because BSP is conjugated with glutathione, we studied the effect of THA on the excretion of DBSP, a nonmetabolizable analog of BSP that is readily excreted into bile but does not require conjugation as does BSP (5, 9, 14). As seen in Fig. 4A, DBSP infusion did not alter basal BFR at the dose used. Simultaneous infusion of THA with DBSP also caused a rapid increase in BFR from 0.9 ± 0.1 to 1.4 ± 0.2 μl·min⁻¹·g liver⁻¹. However, the increase was not statistically different from the control and it declined with time. THA caused an immediate decrease in both biliary concentration and excretion of DBSP (Fig. 4, B and C). Note that the onset of inhibition of THA on biliary DBSP excretion was more rapid and the effect greater than observed on BSP excretion. Together these findings suggest that THA inhibits the bile excretory phase rather than the preceding intracellular step of phase II conjugation of BSP.

Effect of THA on immunofluorescent localizations of Mrp2, Bsep, and ZO-1 in the liver. Since THA infusions acutely induced a transient increase in BFR, this short-term action of THA might result from the canalicular insertion of submembranous vesicles containing canalicular transport proteins. To examine this possibility, Bsep and Mrp2 proteins in control and THA-treated livers were examined by fluorescent confocal microscopy. As shown in Fig. 5, Bsep and Mrp2 are mainly localized on the canalicual membrane in control livers and no significant changes in the expression or localization of these two transporters were observed after THA was infused at 1, 2, 4, or 8 μmol/min. The possibility that THA increased the permeability of tight junctions was also evaluated. However, the structure and localization of the tight junction protein ZO-1 was unaltered by THA at either a low or high dose (1 and 8 μmol/min) compared with control livers (Fig. 6).

Effect of THA on BFR in TR<sup>−</sup> mutant rat. To directly determine whether Mrp2 is the putative canalicular transporter for THA and responsible for its choleretic activity, the choleretic effect of THA was examined in TR<sup>−</sup> mutant rats, genetically deficient in Mrp2 transporter (13), by using a bile fistula. As shown in Fig. 7, choleretic activity of THA at doses of 125 and 250 μmol/kg body wt is not observed in the TR<sup>−</sup> mutant rat, whereas in normal rats THA at a dose of 250 μmol/kg body wt markedly increased BFR to 197.3 ± 13.4% of control. These studies unequivocally establish that Mrp2 is required for the choleretic activity of THA.

Effect of THA on estradiol-17β-<i>D</i>-glucuronide (E<sub>2</sub>-17G) induced cholestasis. Finally we examined the therapeutic potential of THA by determining whether THA could reduce the cholestatic effects of the steroid E<sub>2</sub>-17G. TC at a dose of 0.5 μmol/min was infused into the IPRL to supplement endogenous bile acid throughout the experiment. As shown in Fig. 8, TC significantly increased BFR by ~190% of the initial BFR (P < 0.05). A single injection of E<sub>2</sub>-17G rapidly decreased BFR from 191.2 ± 22.7 to 52.6 ± 15.5% of the initial BFR, reaching a minimum value at 10 min after the E<sub>2</sub>-17G injection. Thereafter, BFR gradually returned toward the TC-stimulated level. In contrast, THA infusions partially prevented the E<sub>2</sub>-17G induced cholestasis. In addition, the decrease in BFR induced by E<sub>2</sub>-17G was delayed in time. Moreover, recovery of BFR in THA-infused liver occurred faster, returning to control values 20 min after E<sub>2</sub>-17G administration (Fig. 8). E<sub>2</sub>-17G administration rapidly decreased biliary bile acid concentration and excretion in control animals (Fig. 9, A and B). However, after reaching minimum values at 10–15 min, they returned toward normal levels. With THA infusions, the concentration of bile acids continued to be maintained at low levels compared
with E2-17G administration alone \((P < 0.05)\) (15.5 ± 1.4 mM with and 24.8 ± 2.0 mM without THA infusion) (Fig. 9A). However, the transient decrease in bile acid excretion in the THA-infused livers was comparable to controls without THA infusion (Fig. 9B). E2-17G had no effect on the total concentration of glutathione in bile of control animals (Fig. 9C). However, when THA was infused with E2-17G, a marked decrease in biliary total glutathione concentration was observed from 103.0 ± 6.3% in the control group to 27.5 ± 7.0% of the pretreatment value (before E2-17G administration), resulting in a transient decrease in excretion of total glutathione in bile to 9.4 ± 3.1% of the pretreatment value. This was significantly different from the TC-control value, which was 39.3 ± 5.8% of pretreatment value at 15 min after THA infusion \((P < 0.05)\) (Fig. 9D).

**DISCUSSION**

The present study confirms the potent choleretic effect of THA using the IPRL and provides new findings concerning the mechanism by which this choleresis occurs. First, THA infusions resulted in an immediate dose-dependent increase in bile secretion that reached saturation at the higher infusions rate. The latter effect is characteristic of a carrier-mediated transport maximum (Fig. 1). Second, there was no effect on bile acid excretion in these experiments so that the bile salt-independent fraction was stimulated. Prior unpublished studies in the IPRL during bile acid infusions also failed to demonstrate any effect of THA on bile acid excretion. Third, THA selectively inhibited the biliary excretion of BSP and DBSP, both substrates for Mrp2. Together these findings suggest that the choleretic effect of THA was determined by the excretion of osmotically active solutes via Mrp2. Moreover, the choleretic effect of THA did not influence the subcellular localization of the transporter nor its targeting from its submembranous intracellular compartment into the apical membrane, suggesting that this posttranscriptional modification of Mrp2 expression was probably not involved.

In earlier studies in the bile fistula rat, THA increased both BAIF and BADF (23, 26). However, its mechanism of action was unclear, particularly whether THA had direct effects on the liver or acted indirectly via signaling molecules derived from the intestine. The present study, using the IPRL, clearly demonstrates that THA mediates its choleretic effect by a direct effect on the liver. It is also evident that THA increases BFR without stimulating biliary bile acid or glutathione secre-

**Fig. 6.** Effect of THA on immunofluorescent localization of zonula occludens 1 protein (ZO-1). Confocal microscopy analysis of ZO-1 was performed in livers that were infused with vehicle or THA (1 and 8 \(\mu\text{mol/min}\)). \(A\) and \(B\): images visualized at different areas of the liver with the same magnification. \(C\): image acquired at high magnification. Bar = 10 \(\mu\text{M}.\)

**Fig. 7.** Effect of THA on bile flow rate in TR\(^{-}\) and normal rat. A single intraduodenal injection of THA at the doses of 125 and 250 \(\mu\text{mol/kg body wt}\) was administered at 45 min. Values are means ± SE from 4 animals. **\(P < 0.01\), significant difference from control at the corresponding time.

**Fig. 8.** Effect of THA on estradiol-17β-β-glucuronide (E2-17G)-induced cholestasis. Taurocholate (TC) at a dose of 0.5 \(\mu\text{mol/min}\) was continuously infused to supplement the endogenous bile acid in the liver starting from 15 min to the end of the experiment. THA was added into the perfusion medium for coperfusion at a constant rate of 40 \(\mu\text{mol/min}\) from 45 to 90 min of perfusion. E2-17G at a dose of 1 \(\mu\text{mol/100 g body wt}\) was administered as a bolus injection into the liver immediately before the start of THA infusion. Values are means ± SE from 4 animals. \(P < 0.05\) and **\(P < 0.01\), significant difference from control at the corresponding time.

**Fig. 9A.**
The lack of an effect of THA on bile acid excretion in the present study does not agree with the earlier finding in the bile fistula rat where THA enhanced bile acid excretion and subsequently lowered plasma cholesterol (23, 26). These earlier findings in the bile fistula rat might be due to the washout of bile acids excreted into the biliary tree before the administration of THA.

Since THA did not directly stimulate the biliary excretion of bile acids and glutathione, the two major determinants of bile flow, the mechanism by which THA increases BFR in the isolated liver is most likely due to the excretion of osmotically active solutes of the biotransformed THA. Indeed, two glucuronide metabolites of THA have been identified (S. Khambong, A. Suksamrarn, and P. Piyachaturawat, unpublished observation). This finding is consistent with an earlier report that the glucuronide conjugation of phloracetophenone is the major biliary metabolite (11, 16). Mrp2, the multispecific resistance-associated protein 2, plays an important role in the biliary excretion of a number of endogenous and exogenous compounds including glucuronide, glutathione, and sulfates conjugates (7). In our earlier study on the choleretic effect of 4-monohydroxyacetophenone (a THA analog), there was a clear parallel increase in bile flow and metabolites in bile. Moreover, 4-monohydroxyacetophenone failed to exert a choleretic effect in TR rats where the excretion of metabolites was absent (16). By analogy, the metabolites of THA should also be excreted in bile and provide the osmotic force for stimulating bile flow.

Because THA infusions markedly reduced the excretion of free glutathione, it was not clear whether THA was conjugated with GSH before being excreted and/or competed with GSH transport via the canalicular transporter, Mrp2. The rate-limited effects of THA on BFR also could be explained by such a competitive effect or by a direct inhibitory effect on the transport mechanism. Since the biliary excretion of BSP, which largely is dependent on prior conjugation with GSH (5, 9), was also abruptly inhibited by THA coinfusion, this concern was heightened. To clarify whether THA was interfering with GSH conjugation or excretion, we examined the excretion of DBSP, a dibrominated analog of BSP that does not require conjugation with GSH or other biotransformation before excretion in bile (14). The finding that THA also abruptly inhibited biliary DBSP excretion indicates that THA’s inhibitory effects are at the level of the canalicular transporter. This conclusion is also consistent with the findings that preinfusions of DBSP, but not BSP, markedly suppressed the stimulating effect of THA on BFR and that THA inhibited DBSP excretion earlier than BSP. Together these findings suggest that THA competed with these other Mrp2 substrates at the transporter’s substrate binding site or the transport sites.

Although the biliary excretion of osmotically active metabolites is the most likely explanation for THA’s choleretic effects, we cannot exclude the possibility that the compound(s) might directly stimulate the activity of Mrp2 and/or the insertion of the transporter from intracellular compartments to the canalicular membrane (15). However, confocal immunofluorescence microscopy indicated that THA had no detectable effect on the subcellular localization of either the canalicular bile salt export pump (Bsep) or Mrp2 even when infused with the highest dose (Fig. 7). Thus it seems unlikely that THA stimulated insertion of vesicles containing new transporters to the canalicular membrane. This conclusion is also supported by our earlier study showing that THA does not increase the hepatobiliary excretion of horseradish peroxidase, a marker of exocytosis (35).
Tight junction permeability can be regulated by changes in osmotic and hydrostatic pressure (28) created by osmotic gradients across the tight junction (30, 34). However, neither low or high choleretic doses of THA altered the structure and localization of the tight junction protein ZO-1 (Fig. 5). This finding is also consistent with our prior studies that indicated that THA does not stimulate the paracellular pathway in rat liver when marked by horseradish peroxidase (35). Thus the choleretic effects of THA are unlikely to be mediated by increasing the permeability of the paracellular pathway.

Finally, we examined the therapeutic potential of THA in a model of cholestasis induced by E2-17G. E2-17G is an endogenous estrogen metabolite that induces an acute, dose-dependent, and completely reversible cholestasis in the rat (18, 19). Although the mechanism by which E2-17G induces cholestasis is incompletely understood, Mrp2 mediated E2-17G excretion into bile is required for its cholestatic effect (12). Since THA has a high choleretic activity and also interacts with Mrp2, we anticipated that THA might attenuate E2-17G-induced cholestasis, presumably by competing with E2-17G for excretion via Mrp2 as shown for BSP and DBSP. As illustrated in Figs. 8 and 9, the findings support this hypothesis. THA infusions in IPRLs receiving a constant infusion of TC, attenuated the cholestatic effects of E2-17G on bile flow although there was no difference in bile acid excretion between THA and the controls. As seen in prior studies, THA rapidly reduced the biliary excretion of GSH, suggesting again that competition for excretion of Mrp2 substrates accounted for attenuation of E2-17G’s acute cholestatic effect. Thus THA might be an effective anicholestatic agent by competing with binding of the toxic agent to, or excretion by, Mrp2. THA has also been shown to have a protective effect on ethyinlestradiol-induced cholestasis in the rat. Gastroenterology 65: 651–657, 1973.

In summary, we have described a potent choleretic effect of the herbal compound THA in an isolated rat liver perfusion system. THA stimulates bile flow by the saturable excretion of osmotically active solutes into bile via the canalicular transporter, Mrp2. THA’s choleretic effect occurs without alteration of the distribution and localization of canalicular transporters Mrp2 and Bsep or the tight junction protein ZO-1. Its anticholestatic effect appears to be mediated by competing with the excretion of Mrp2 substrates. THA might be an effective and safe candidate for further development as an anticholestatic therapeutic agent.

REFERENCES


