STUDIES ON INCREASED BILE FORMATION PRODUCED BY POLYOXYBENZENES IN RATS

Shigeharu TANAYAMA and Yoshio KANAI

Medicinal Research Laboratories, Central Research Division, Takeda Chemical Industries Ltd., Juso, Yodogawa-ku, Osaka 532, Japan

Accepted September 13, 1976

Abstract—Mechanism of hypercholeretic effect of three polyoxybenzenes, 2,4,6-tri-hydroxypropiophenone (THPP), 4-methylumbelliferone (4-MU) and 3-(2',4',5'-triethoxybenzoyl)propionic acid (AA-149), was studied in rats. Biliary clearance of 14C-labeled erythritol indicated that all the choleretics increased canalicular bile production. AA-149 excreted in the bile chiefly as the glucuronide provided no significant osmotic drive for bile formation. Both the biliary bile acid concentration and total biliary excretion of bile acids were lower in the rats treated with THPP, 4-MU or AA-149 than in control rats. These choleretics were also effective in the isolated rat liver perfusion system, in which concentration and output of biliary bile acids were low. Thus, it was unlikely that bile acids were involved in the hypercholeresis induced by the choleretics. THPP, 4-MU and AA-149 increased biliary excretion of sodium both in the biliary-cannulated rats and in the isolated rat liver perfusion system. It was concluded that the hypercholeresis induced by THPP, 4-MU and AA-149 was due to an enhanced formation of the bile acid-independent fraction of canalicular origin, probably mediated by the active transfer of sodium into the canaliculi.

The spasmolytic and choleretic actions of some polyoxybenzenes, such as 2,4,6-tri-hydroxypropiophenone (THPP) (1, 2), 4-methylumbelliferone (4-MU) (3, 4) and 3-(2',4',5'-triethoxybenzoyl)propionic acid (AA-149) (5, 6), are well established. The mechanisms of these two actions of the compounds are, however, not well understood, although the spasmolytic action of THPP in the biliary system is reported to be due partially to the inhibition of catechol-O-methyltransferase activity (7).

The purpose of the present study was to investigate various characteristics of the increase in bile flow, such as quantitating canalicular and ductular bile flow and composition of the bile in the rats treated with THPP, 4-MU or AA-149, in an attempt to determine the mechanism by which these choleretics increase bile flow. Choleretic effect of the compounds was also investigated using isolated perfused rat livers.
MATERIALS AND METHODS

Animals and treatments: Male Sprague-Dawley rats (JCL-SD, CLEA Japan, Inc., Tokyo) weighing 200 to 300 g were used throughout. THPP (Cospanon®, Eisai Co., Ltd., Tokyo), 4-MU (Crodimone®, Japan Roussel KK, Tokyo) and AA-149 (synthesized by Drs. T. Murata et al. (5) of Medicinal Research Laboratories of this Division) were administered at a dose of 100 mg/kg i.v., unless otherwise noted. Control rats were given identical volumes of saline (5 ml/kg). \( ^{14} \text{C-} \text{AA-149} \) (labeled at the benzoyl carbon, 12.0 \( \mu \text{Ci/mg} \)), synthesized by Drs. N. Hayashi et al. (8) of the Radioisotope Laboratory of this Division, was also used.

Surgical procedure: Rats were anesthetized with pentobarbital sodium 50 mg/kg i.p. The common bile duct was surgically exposed by a midline abdominal incision and cannulated with PE-10 tubing just below the bifurcation of the common bile duct, in order to avoid contamination by pancreatic juice. The rectal temperature of the anesthetized rat was maintained at 37°C with a heat lamp in conjunction with a temperature regulator to prevent hypothermic alteration in bile flow (9).

Liver perfusion experiments: This preparation has been fully described in our previous report (10). Isolated rat livers were perfused with a recirculating perfusate (60 ml) which consisted of 1 part of homologous blood diluted with 3 parts of Krebs-Ringer bicarbonate buffer, containing 0.1% of glucose and 0.2% of gelatin. After addition of the choleretics to the perfusate, bile flow rates were periodically measured.

Biliary erythritol excretion: \( ^{14} \text{C(U)-Erythritol} \) (3.6 mCi/mmol) was obtained from the Radiochemical Centre (Amersham) and administered as a single i.v. injection of 5 \( \mu \text{Ci} \) in a volume of 0.1 ml of saline to rats in which both renal pedicles had been tied. A 2-hr period was then allowed for the equilibration, after which the common bile duct was cannulated. Biliary clearance of the labeled solute was measured on the first 30 min-bile sample of the rat given the choleretics, blood samples being taken at the midpoint of the bile collection. In the liver perfusion experiments, \( ^{14} \text{C-erythritol} \) was added to the perfusate 30 min before addition of the choleretics.

Analytical methods: The radioactivity in bile and plasma was measured with a liquid scintillation counter, Aloka model LSC-502 (Japan Radiation and Medical Electronics Ltd., Tokyo) with automatic quenching monitor using a dioxane-phosphor mixture (11). Biliary bile acid concentrations were determined by an enzymatic method using hydroxy steroid dehydrogenase (12), from Pseudomonous testosteroni (Sigma Chemical Co., St. Louis, grade II, ATC 11996), as follows: enzyme solution; 500 \( \mu \text{l} \) (0.25 \( \alpha \) unit), 0.1 M hydrazine hydrate; 200 \( \mu \text{l} \), 6.25 mM \( \beta \)-NAD (from Yeast, Sigma Chemical Co., St. Louis, grade III); 200 \( \mu \text{l} \), 0.1 M sodium pyrophosphate; 500 \( \mu \text{l} \). An aliquot of 10 \( \mu \text{l} \) of a sodium taurocholate (from ox bile, Sigma Chemical Co., St. Louis, crude) standard in methanol or bile diluted with 3 volumes of 0.1 M pyrophosphate was added. After shaking, the mixture was allowed to incubate for 1 hr at a room temperature of 25°C and then the extinction of the solution was measured against a reagent blank. The extinction of the correspondingly diluted bile from control rats or rats treated with the choleretics was subtracted from the measured
extinction as described above. We determined whether or not choleretics or metabolites excreted in bile altered the results of bile acid measurements. Spectrophotometric measurements were performed in quartz microcells (light path: 1 cm) at 340 nm using a Hitachi spectrophotometer model 101 (Hitachi Seisakusho, Tokyo).

Sodium and potassium concentrations in bile were measured with a Hitachi Flame Photometer 205D (Hitachi Seisakusho, Tokyo) and osmolality was measured in a Fiske osmometer.

RESULTS

Bile flow

Fig. 2 depicts the bile flow after i.v. injection of THPP, 4-MU and AA-149 in rats. These three choleretics all significantly increased the bile flow, the effect of AA-149 being long-lasting as compared with that of THPP and 4-MU. Based on these results, all measurements described below were performed using the first 30 min-bile sample after administration of the choleretics.

![Fig. 2. Hypercholeresis induced by intravenous THPP, 4-MU and AA-149 in rats. Dose of the choleretics was 100 mg/kg. Data are expressed as the mean±S.D. Number of rats is shown in parentheses. P versus saline control: * <0.05, ** <0.01, *** <0.001.]

Canalicular bile flow

As erythritol enters the bile only at the canaliculi and is not reabsorbed in the ductules, it can be used as a measure of canalicular bile flow (13–16). The bile/plasma ratio of erythritol was not significantly different between control rats and rats treated with THPP, 4-MU or AA-149 (Table 1). Therefore, the increase in the bile flow seen in rats treated with the choleretics appears to be due to an increase in canalicular production rather than a change in ductular secretion or reabsorption of fluid.
TABLE 1. Biliary erythritol excretion after intravenous administration of THPP, 4-MU and AA-149 in rats

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bile:plasma conc. ratio of erythritol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline  (7)</td>
<td>1.11±0.07</td>
</tr>
<tr>
<td>THPP (5)</td>
<td>1.01±0.17</td>
</tr>
<tr>
<td>4-MU (5)</td>
<td>1.12±0.19</td>
</tr>
<tr>
<td>AA-149 (7)</td>
<td>1.13±0.19</td>
</tr>
</tbody>
</table>

Dose of the choleretic was 100 mg/kg. Data are expressed as the mean ± S.D. Number of rats is shown in parentheses.

**Biliary excretion of the choleretics**

The increase in bile flow induced by the choleretics could be due to an osmotic choleresis produced by the excretion of the drugs and/or metabolites into bile. In fact, 4-MU (17) and AA-149 (Y. Kanai et al., unpublished observation) proved to be excreted into the rat bile chiefly as glucuronides. Therefore, to determine if this is indeed the mechanism by which these choleretics increase bile flow, the relation between bile flow and biliary excretion of 14C was studied using 14C-AA-149. Rats were injected with 50 to 200 mg/kg i.v. of the labeled drug and the increment of bile flow was plotted against the biliary excretion of 14C (Fig. 3). If we assume that the osmotic activity of AA-149 is not so different from that of its glucuronide, AA-149 must have the osmotic activity of 10.3 mOsm/mM when the compound is the only factor responsible for the hypercholeresis,

![Fig. 3. Relationship between AA-149-induced hypercholeresis and biliary 14C excretion in rats. Rats were given 150 to 200 mg/kg of 14C-AA-149 i.v. and the increment of bile flow was plotted against the biliary excretion of 14C.](image)

TABLE 2. Effect of AA-149 or metabolite(s) excreted in bile on AA-149-induced hypercholeresis in rats

<table>
<thead>
<tr>
<th>Osmolality of bile (mOsm)</th>
<th>Biliary concen. of 14C (mM)</th>
<th>Osmolality of biliary 14C (mOsm/mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Expected (a)</td>
</tr>
<tr>
<td>303</td>
<td>29.4</td>
<td>10.3 (100%)</td>
</tr>
</tbody>
</table>

a, Pooled 1 hr-bile from five rats given 14C-AA-149 (100 mg/kg) i.v. b, Inversion of the slope of the regression line shown in Fig. 3, which indicates the concentration of AA-149 or its glucuronide in the increment of fluid produced in response to AA-149 and its glucuronide. c, Osmolality of bile (a) was divided by biliary concentration of 14C (b). d, Osmolality of sodium salt of AA-149.
as calculated from the slope of the regression line shown in Fig. 3 and the osmolality of the bile of rats treated with $^{14}$C-AA-149 (Table 2). The estimated activity of AA-149 was, however, only 1.3 mOsm/mM, indicating that AA-149 and/or its glucuronide provide only 12.8% of the osmotic drive for bile formation (Table 2).

**Bile composition**

It is generally accepted that canalicular bile production both in animals and man is due to bile acid-dependent and bile acid-independent fractions, and the latter is postulated to be mediated, at least partly, by an active sodium transport from hepatocytes to canaliculi (15, 18-20). Table 3 demonstrates the biliary excretion of bile acids and electrolytes after administration of THPP, 4-MU and AA-149 in rats. The concentration of bile acids was significantly lower in rats treated with these choleretics than in control rats and there was also a trend towards a decrease in the total bile acid output in the treated rats. The concentration of sodium was significantly higher in rats treated with THPP and 4-MU than in the controls, but no difference was noted between control and AA-149-treated rats. Hepatic output of sodium in bile was, however, appreciably increased in rats treated with the choleretics, since bile flow was increased about 150 to 170% in these groups. The potassium concentration in the bile of rats treated with three choleretics was slightly but significantly lower than that of controls.

**Table 3. Biliary excretion of bile acids and electrolytes after intravenous administration of THPP, 4-MU and AA-149 in rats**

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bile acid output</th>
<th>Biliary concn. of sodium</th>
<th>Biliary concn. of potassium</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(nmol/ml)</td>
<td>(nmol/min/g liver)</td>
<td>(mEq/L)</td>
</tr>
<tr>
<td>Saline</td>
<td>35.2 ± 6.8</td>
<td>77.5 ± 24.2</td>
<td>168 ± 7</td>
</tr>
<tr>
<td>THPP</td>
<td>16.0 ± 21***</td>
<td>52.6 ± 22.4a</td>
<td>182 ± 3***</td>
</tr>
<tr>
<td>4-MU</td>
<td>19.3 ± 7.0***</td>
<td>59.9 ± 20.8</td>
<td>181 ± 6***</td>
</tr>
<tr>
<td>AA-149</td>
<td>16.4 ± 6.9***</td>
<td>59.5 ± 25.1</td>
<td>168 ± 9</td>
</tr>
</tbody>
</table>

Dose of the choleretics was 100 mg/kg. Data are expressed as the mean ± S.D. Number of rats is shown in parentheses. P versus saline control: *<0.05, **<0.01, ***<0.001.

To obtain additional information on the hypercholeresis induced by THPP, 4-MU and AA-149, bile flow and biliary excretion of erythritol and electrolytes were measured using an isolated rat liver perfusion system. In this system, the bile acid excretion is minute compared with that in biliary-cannulated rats and therefore participation of bile acids to the hypercholeretic effect of the drugs could be excluded. Three choleretics used were also effective in this system (Fig. 4) and essentially the same results were obtained on the biliary excretion of erythritol and electrolytes as those in biliary-cannulated rats (Table 4). The biliary concentration of bile acids in control experiments was only 2.3% (0.81 μmol/ml bile) of that in biliary-cannulated control rats (35.2 μmol/ml bile, Table 3) and upon addition of THPP, 4-MU and AA-149 to the perfusate, no significant change was noted in the total
FIG. 4. Choleretic effect of THPP, 4-MU and AA-149 in the isolated rat liver perfusion system. Data are the mean±S.D. of three experiments. Initial concentration of the choleretics was 300 μg/ml of the perfusate.

TABLE 4 Effect of THPP, 4-MU and AA-149 on biliary excretion of erythritol and electrolytes in the isolated rat liver perfusion system

<table>
<thead>
<tr>
<th>Drug</th>
<th>Bile/plasma concn. ratio of erythritol(10)</th>
<th>Biliary concn. of sodium (mEq/L)</th>
<th>Biliary concn. of potassium (mEq/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.05±0.04</td>
<td>153</td>
<td>7.4</td>
</tr>
<tr>
<td>THPP</td>
<td>1.05±0.06</td>
<td>149</td>
<td>7.1</td>
</tr>
<tr>
<td>4-MU</td>
<td>1.02±0.04</td>
<td>150</td>
<td>7.2</td>
</tr>
<tr>
<td>AA-149</td>
<td>1.00±0.02</td>
<td>139</td>
<td>6.8</td>
</tr>
</tbody>
</table>

a, Samples used were the first 30 min-bile specimens obtained after addition of the choleretics to the perfusate in Fig. 4. Measurements were made on the pooled bile from three livers in each experiment, unless otherwise noted. b, Mean±S.D. (n=3).

These results suggest that the increase in canalicular bile formation by THPP, 4-MU and AA-149 was due to an increase in the hepatic secretion of sodium rather than that of bile acids.

DISCUSSION

It is well established that the primary site of bile formation is at the canaliculi and that the bile is later modified in the ductules and ducts by a net secretion or reabsorption of fluid. The dominant mechanism for the initiation of canalicular bile formation is considered to be the active secretion of bile acids into the canaliculi. This secretion creates an osmotic gradient which results in the passive transfer of fluid and electrolytes (21-23). It has also been recently reported that there is a bile acid-independent fraction of canalicular bile.
production both in animals (15, 18, 19) and man (20).

THPP, 4-MU and AA-149 increased bile flow in rats by stimulating the canalicular bile formation (Fig. 2 & Table 1). The hypercholeresis induced by these choleretics was not accompanied by an increase in bile acid excretion (Table 3). The hypercholeresis therefore appears to be due either to 1) an increasing effect on the osmotic activity of bile acids or 2) the stimulation of a bile acid-independent canalicular bile formation. The former mechanism appears to be of little significance since the choleretics also increased bile flow in the isolated perfused rat liver, in which the biliary concentration of bile acids was low and therefore the osmotic pressure of bile acids would not serve as an explanation for the increased flow in the treated rat livers (Fig. 4).

Of interest is the finding that the biliary excretion of sodium is significantly higher in treated rats than in controls (Table 3). Recent studies on bile formation suggest that the active transfer of sodium into the canaliculi appears to play a role in the formation of a bile acid-independent bile fraction of canalicular origin. Namely, this fraction has been reported to be inhibited by ouabain, ethacrynic acid and amiloride in rabbits (19) and by scillaren in the isolated perfused rat liver (18), these four compounds being the inhibitor of Na⁺, K⁺-activated adenosine triphosphatase activity. In addition, cyclic AMP reportedly increases transport of sodium out of the cells (24, 25), and stimulates choleresis in dogs by increasing the bile acid-independent bile production (26). Cyclic AMP increases the bile acid-independent canalicular bile production also in the isolated perfused rat liver, the hypercholeresis being accompanied by an increase in the biliary excretion of sodium (S. Tanayama, unpublished observation). Studies on the role played by Na⁺, K⁺-activated adenosine triphosphatase and/or cyclic AMP in the hypercholeresis by THPP, 4-MU and AA-149 are in progress in this laboratory using the isolated rat liver perfusion system.

REFERENCES