Manganese-Bilirubin Effect on Cholesterol Accumulation in Rat Bile Canalicular Membranes

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Manganese-bilirubin (Mn-BR)-induced cholestasis in rats is associated with altered lipid composition of various hepatic subcellular fractions. Increased bile canalicular (BCM) cholesterol content in Mn-BR cholestasis and the intracellular source of the accumulating cholesterol were investigated. To label the total hepatic cholesterol pool, male Sprague-Dawley rats were given ip 3H-cholesterol, followed 18 h later by 2-14C-mevalonic acid (a precursor of cholesterol synthesis). To induce cholestasis, manganese (Mn, 4.5 mg/kg) and bilirubin (BR, 25 mg/kg) were injected iv; animals were killed 30 min after BR injection; canalicular and sinusoidal membranes, microsomes, mitochondria, and cytosol were isolated. Total cholesterol content of each fraction was determined by spectrophotometric techniques as well as radiolabeled techniques. In Mn-BR cholestasis, the total cholesterol concentrations of BCM and cytosol were significantly increased. Also, the contribution of 14C-labeled cholesterol (newly synthesized cholesterol) was enhanced in all isolated cellular fractions. The results are consistent with the hypothesis that accumulation of newly synthesized cholesterol in BCM is involved in Mn-BR cholestasis. An enhanced rate of synthesis of cholesterol, however, does not appear to be the causal event, as the activity of HMG-CoA reductase (rate-limiting enzyme in cholesterol synthesis), assessed in vitro, was decreased following Mn-BR treatment. Treatment with the Mn-BR combination may affect other aspects of intracellular cholesterol dynamics.

Key Words: cholestasis; membrane; cholesterol; manganese; bilirubin

Intrahepatic cholestasis is generally defined as impaired secretion of bile via the hepatobiliary system without anatomic obstruction (Feuer and DiFonzo, 1992). In humans, intrahepatic cholestasis results most often as an adverse consequence of drug therapy, and a wide array of drugs are known to induce cholestasis (Maxwell and Williams, 1973; Plaa and Priestly, 1976; Sherlock, 1970). The list of agents (steroid hormones, bile acids, drugs, and other chemicals) associated with cholestasis is continually expanding (Roy et al., 1989; Tanner, 1986; Verhamme et al., 1989; Vore, 1987; Zimmerman, 1998).

In hospitals due to jaundice, the major clinical manifestation of cholestasis, 2–5% of the cases are estimated to be drug-induced adverse effects (Feuer and DiFonzo, 1992; Sturgill and Lambert, 1997). Therefore, the need to understand mechanisms involved in this condition is important.

Among the different models of experimentally induced intrahepatic cholestasis in the rat, the one utilizing the combination of manganese and bilirubin (Mn-BR) has been the choice of our laboratory because the morphological changes observed (disappearance of microvilli and dilatation of canalicular lumen) resemble those seen in human cholestasis (de Lamirande et al., 1982; Steiner et al., 1965; Witzleben, 1972). The Mn-BR model involves two substances (manganese and bilirubin) that are not cholestatic when given alone but produce cholestasis when given sequentially (de Lamirande and Plaa, 1979).

Over the years, a number of hypotheses have emerged from the various experimental models as possible mechanisms leading to intrahepatic cholestasis: impaired sinusoidal membrane uptake (Schwarz et al., 1977), functionally inactive enzymes (Samuels and Carey, 1978), impairment of cytoskeletal function (Tuchweber et al., 1986), and altered canalicular membrane function (de Lamirande et al., 1981). More recently, our studies reported that bile canalicular membranes (BCM) are thought to be the more likely site of action for the development of Mn-BR cholestasis (Duguay et al., 1998; Plaa et al., 1998). Therefore, a special interest arose to elucidate how BCM alterations are involved in this phenomenon. As the cholesterol content of biologic membranes influences their physical, morphologic, and physiologic characteristics (Sandermann, 1978; Spector and Yorek, 1985; Yeagle, 1991), increases in membrane cholesterol content, alteration in cholesterol/phospholipid ratio, and/or changes in membrane fluidity have been investigated and proposed as contributing pathogenic factors in Mn-BR cholestasis (Duguay et al., 1998; Plaa et al., 1982, 1998).

Previously, we indicated that Mn-BR injections could alter BCM cholesterol content and that this increase was accompanied by altered BCM fluidity (Duguay et al., 1998). The purpose of the present study is to characterize the source of the cholesterol that accumulates in BCM after Mn-BR and to further extend our knowledge on Mn-BR cholestasis. The
findings indicate that altered intracellular cholesterol dynamics are likely involved in Mn-BR cholestasis.

**MATERIALS AND METHODS**

**Animals.** Male Sprague-Dawley rats (200–250g) were purchased from Charles River Inc. (St-Constant, Québec) and maintained on Charles River rat chow and water *ad libitum*. The experiments were performed after a 3-day acclimation period.

**Cholestasis.** Animals were anesthetized with urethane (1 g/kg ip) and catheters were placed in the femoral vein (PE-50). To induce decreased bile flow, freshly prepared aqueous solutions were used. Monohydrated manganese sulfate (MnSO₄·H₂O) was dissolved in saline (0.9% NaCl) and injected iv at a dosage of 4.5 mg Mn/kg; 15 min later, bilirubin (Sigma Chemical Co. St. Louis, MO), dissolved in a solution of 0.5% NaCl and 0.5% Na₂CO₃ per 100 ml, was injected over a 2-min period at a dosage of 25 mg BR/kg. As previously described, maximum decrease in bile flow occurred 30 min after BR (Duguay and Plaa, 1997); animals were then killed and the liver rapidly removed: liver cell plasma membranes (canalicular and sinusoidal), microsomes, mitochondria, and cytosol were prepared according to techniques described elsewhere (de Lamirande et al., 1982; Roy et al., 1989).

**Membrane.** Livers were perfused via the portal vein with ice-cold physiological saline, buffered (pH 7.4) with 1 mM NaHCO₃, rapidly excised, weighed, and homogenized with a Polytron homogenizer for approximately 5–10 sec. The homogenate was mixed with 1 mM NaHCO₃, buffer to a total volume of 10 ml/g of liver puree and centrifuged at 50 x g for 5 min. The supernatant was removed and centrifuged at 4°C, 1700 x g for 10 min (3700 rpm, SS-34 rotor, Sorval RC2-B refrigerated centrifuge). The supernatant fraction was removed and placed on ice for further isolation of microsomes, mitochondria, and cytosol. The pellet was resuspended in NaHCO₃ buffer and centrifuged for 10 min at 1700 x g. The resulting pellets were mixed with a sucrose solution of 1.26 density and placed into ultracentrifuge tubes containing 4 ml NaHCO₃ buffer and 7 ml 1.18-density sucrose solution. The ultracentrifugation duration was set at 1 h at 73,000 x g (28,500 rpm, Beckman L centrifuge). The bile canalicular (BCM)-enriched fraction was observed at the upper 1.18–1.16 density interface; the plasma sinusoidal (PM)-enriched fraction was observed at the lower 1.26 density interface. Both fractions were separated using a 5-ml syringe equipped with a long needle. They were diluted in NaHCO₃-CaCl₂ buffer and centrifuged for 20 min at 9000 x g (8500 rpm, SS-34 rotor, Sorval RC2-B refrigerated centrifuge). This step was repeated twice to assure adequate purification. Microsomes, mitochondria, and cytosol were isolated from the first supernatant. Centrifugation (10 min at 10,000 rpm, Beckman L centrifuge) separated mitochondria from microsomes and cytosol. The resulting supernatant was further centrifuged for 15 min at 15,000 rpm; the cytosol was directly separated from microsomes in the pellet and frozen at −40°C. The pellet was washed and centrifuged (15 min, 15,000 rpm, Beckman L centrifuge) twice with 11.5% Tris-HCl buffered at pH 7.4. Mitochondria were washed with the NaHCO₃-CaCl₂ buffer and centrifuged for 10 min at 10,000 rpm.

**Lipids.** To label the hepatic cholesterol pool, the rats were injected ip with 25 μCi/100 g (1,2-3H)-cholesterol (New England Nuclear, Montréal, specific activity 50 Ci/mmol). Eighteen hours later, during the cholestatic challenge, they received iv 10 μCi/100 g (2-14C)-mevalonic acid (New England Nuclear, Montréal, specific activity 55 mCi/mmol) in 7.5% albumin/saline solution to label newly synthesized cholesterol. Forty-five minutes later, after (2-14C)-mevalonic acid (30 min after BR injection), membranes were prepared, and total lipids were extracted with chloroform/methanol (2:1) according to the technique described by Sperry and Webb's (1950). The organic phase was dried under nitrogen and resolubilized with 1 ml acetone:ethanol (1:1). The pH was adjusted to 7 with acetic acid (1:10). Cholesterol was precipitated by digestion (5% solution in 50% ethanol). Eighteen hours later, a centrifugation was performed (15 min, 5000 x g, 24°C, Sorval centrifuge) and the supernatant discarded. The precipitate was washed with diethyl ether and centrifuged 15 min at 24°C, at 5000 x g (Sorval centrifuge). The precipitate was resolubilized with chloroform/methanol (2:1) and dried under nitrogen, and the total radioactivity was counted with a Beckman scintillation counter. The specific activity of H- and 14C-labeled cholesterol was determined. The specific activity of H is equivalent in the various fractions, as the distribution of the label over the intracellular cholesterol pools is homogeneous (Yousef et al., 1984).

**Enzymes.** The protein contents of all fractions were determined by the method of Lowry et al. (1951), using crystalline bovine serum albumin as the standard. Standard procedures to evaluate membrane enrichment with enzymatic markers were used as described elsewhere (de Lamirande et al., 1981; Tuchweber et al., 1986). Results indicated that the fractionation technique yielded subcellular fractions suitable for the purpose of these investigations. In a separate experiment, the effect of Mn-BR treatment on the activity of hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase was assessed. The rats were treated with the Mn-BR combination as described above; 30 min after BR administration, the animals were killed, the livers excised, and microsomes prepared. The *in vitro* assay of HMG-CoA reductase activity in microsomes was performed according to Bertolotti et al. (1995).

**Statistics.** The data are expressed as means ± SE. Comparisons between groups were submitted to a one-way analysis of variance (ANOVA) and multiple comparisons were evaluated according to Fisher’s protected least significant difference (PLSD) test. In all analyses, p < 0.05 was set as the criterion of significance.

**RESULTS**

Table 1 describes the protein concentration of homogenate, bile canalicular- and sinusoidal-enriched plasma membranes, microsomes, mitochondria, and cytosol obtained from control, Mn, BR, and Mn-BR groups. Mn or BR, when given alone, had no statistically significant effect on the protein concentrations of the various isolated fractions when compared to controls. Also, the cholestasis induced by Mn-BR had no effect on the protein concentrations of all subcellular fractions. The protein contents of the various isolated subcellular fractions are similar to those previously obtained and fall within the wide range reported by other investigators (de Lamirande et al., 1981; Yousef et al., 1984).

Table 2 summarizes the cholesterol concentration of the
TABLE 2

Cholesterol Concentrations of Hepatic Subcellular Fractions
Isolated from Control, Manganese, Bilirubin, and
Manganese-Bilirubin Cholestatic Rats

<table>
<thead>
<tr>
<th>Cellular fractions</th>
<th>Control</th>
<th>Manganese</th>
<th>Bilirubin</th>
<th>Mn-BR cholestasis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>0.172 ± 0.05</td>
<td>0.223 ± 0.03</td>
<td>0.128 ± 0.02</td>
<td>0.185 ± 0.02</td>
</tr>
<tr>
<td>BCM</td>
<td>1.24 ± 0.33</td>
<td>1.00 ± 0.18</td>
<td>1.80 ± 0.14</td>
<td>2.35 ± 0.30*</td>
</tr>
<tr>
<td>PM</td>
<td>0.548 ± 0.08</td>
<td>0.597 ± 0.03</td>
<td>0.536 ± 0.16</td>
<td>0.733 ± 0.05</td>
</tr>
<tr>
<td>Microsomes</td>
<td>0.304 ± 0.04</td>
<td>0.215 ± 0.03</td>
<td>0.228 ± 0.02</td>
<td>0.196 ± 0.02*</td>
</tr>
<tr>
<td>Mitochondria</td>
<td>0.161 ± 0.03</td>
<td>0.111 ± 0.01</td>
<td>0.117 ± 0.01</td>
<td>0.139 ± 0.04</td>
</tr>
<tr>
<td>Cytosol</td>
<td>0.039 ± 0.01</td>
<td>0.058 ± 0.02</td>
<td>0.088 ± 0.01</td>
<td>0.159 ± 0.04*</td>
</tr>
</tbody>
</table>

Note. BCM, bile canalicular plasma membranes; PM, sinusoidal plasma membranes. Values are expressed in μmol cholesterol/mg protein and are the mean ± SE for six animals.

*Value is significantly different from control (p < 0.05).

Various subcellular fractions isolated in controls, Mn, BR, or Mn-BR groups. With Mn or BR given alone, no significant changes were observed; the cholesterol concentrations were similar to controls in all the subcellular fractions. Mn-BR treatment, however, significantly increased the cholesterol concentrations of the BCM and cytosol. In BCM, the cholesterol content increased significantly by about 2-fold (1.24 to 2.35 μmol cholesterol/mg protein); in cytosol, a comparable, statistically significant, 4-fold increase was obtained (0.04 to 0.16 μmol/mg protein). In contrast, the cholesterol concentration of microsomes showed a statistically significant 35% decrease compared to controls (0.30 to 0.19 μmol/mg protein). PM and mitochondrial cholesterol concentrations were unaffected.

Figures 1 and 2 depict 3H- and 14C-labeled cholesterol contributions to the total cholesterol concentration (Table 2) in each isolated fraction after control, Mn, BR, or Mn-BR treatments. In control rats, the contribution of 3H-labeled cholesterol (Fig. 1) was generally superior to the contribution of 14C-labeled cholesterol (Fig. 2), indicating that the cholesterol content resulted mostly from intracellular cholesterol pools rather than from newly synthesized cholesterol. With Mn or BR given alone, the relative contribution of 3H-labeled and 14C-labeled cholesterol to the total cholesterol pool of each isolated fraction resembled that obtained in control rats; the contribution of 3H-cholesterol to the total cholesterol concentration of each fraction was greater than the contribution of 14C-cholesterol. With the cholestatic Mn-BR combination, however, the relative contribution of 14C-cholesterol in each fraction was increased substantially compared to controls (Fig. 2). After Mn-BR administration, the total cholesterol concentration in BCM (2.35 ± 0.30 μmol cholesterol/mg protein, Table 2) nearly doubled, and 1.68 ± 0.20 μmol/mg protein (about 70%, Figure 2) came from newly synthesized cholesterol, compared to 3H-labeled cholesterol pools. In controls, however, from a total BCM cholesterol concentration of 1.24 ± 0.33 μmol/mg protein (Table 2), only 0.39 ± 0.10 μmol/mg protein (about 30%, Figure 2) came from newly synthesized material. Also, in all the other isolated subcellular fractions, Mn-BR administration significantly increased the contribution of newly synthesized cholesterol compared to the control group (Fig. 2). After Mn-BR, the 3H-cholesterol concentrations in microsomes and mitochondria were significantly smaller than the values obtained in the control group (Figure 1). Therefore, incorporation of cholesterol resulting from...
newly synthesized cholesterol (\(^{14}\)C-labeled cholesterol) appears to be more important in the cholestatic state than the incorporation of cholesterol derived from previously formed pools (\(^{3}\)H-labeled cholesterol).

HMG-CoA reductase is a microsomal enzyme that catalyses the reduction of HMG-CoA to mevalonate, which is the rate-limiting step of cholesterol synthesis (Bertolotti et al., 1995). Measurement of the activity of this enzyme provides a means of assessing if enhanced cholesterol synthesis is implicated in Mn-BR-induced cholestasis. The activity in vitro of HMG-CoA reductase in liver microsomes prepared from six rats treated in vivo with the Mn-BR cholestatic regimen was compared to that of six other rats treated only with the vehicles. The assays were performed in a time frame coinciding with that used for the hepatic cholesterol measurements previously described. The mean activity in vitro of the enzyme in control animals was 200.7 \pm 26.4 pmol/mg protein/min, whereas the activity in Mn-BR–treated rats was 91.3 \pm 12.9 pmol/mg protein/min. Thus, the Mn-BR combination treatment resulted in a statistically significant (t-test, \(p < 0.05\)) decrease of 54\% in HMG-CoA reductase activity in vitro activity 30 min after completion of the treatment in vivo.

**DISCUSSION**

Mn-BR-induced cholestasis can affect the molecular organization of BCM, resulting in impaired biliary secretory function. The results of the present study (Table 2) confirm that cholesterol can accumulate in BCM during cholestasis (Duguay et al., 1998; Vore, 1987; Yousef and Tuchweber, 1984). Indeed, after Mn-BR treatment, the cholesterol content of BCM is increased by about 2-fold compared to controls. This increase is also seen in cytosol, where a statistically significant increase was obtained compared to control animals. In PM and mitochondria isolated after Mn-BR administration, however, an alteration in cholesterol was not seen. In microsomes, a significant decrease in cholesterol content was observed.

The main objective of the present study was to seek the source of the cholesterol that accumulates in BCM and cytosol during Mn-BR cholestasis and to determine if this cholesterol arises from intracellular cholesterol pools or is newly synthesized (issues from de novo cholesterol synthesis). The results of the present study demonstrate (Figs. 1 and 2) that in non-cholestatic states (control, Mn, or BR) the contribution of \(^{3}\)H-labeled cholesterol resulting from intracellular pools to the total cholesterol content of the various isolated fractions is greater than the contribution of cholesterol synthesized from the \(^{14}\)C-precursor. With the Mn-BR cholestatic treatment, however, the contribution of newly synthesized \(^{14}\)C-labeled cholesterol to the total cholesterol concentration in homogenate, BCM, PM, microsomes, mitochondria, or cytosol is significantly enhanced. Also, the contribution of \(^{3}\)H-labeled cholesterol to the total cholesterol content of microsomes or mitochondria after Mn-BR is significantly decreased. Thus, in all subcellular fractions, Mn-BR treatment favors the contribution of newly synthesized cholesterol rather than the contribution of intracellular preformed cholesterol pools.

From our data (Table 1, Fig. 2), we can calculate the amount of newly synthesized cholesterol that accumulates in BCM; this value is 394 nmol cholesterol/g liver. The amount of cholesterol that would be excreted in 30 min (the time between BR administration and termination of the experiment) can also be calculated. Assuming a biliary secretion rate of 1 nmol cholesterol/min/g liver, this value comes to 30 nmol cholesterol. The amount of cholesterol accumulating in BCM markedly exceeds the estimated amount of synthesized cholesterol destined for biliary excretion. This quantitative estimation of the relative cholesterol excretion versus cholesterol accumulation allows us to postulate, therefore, that even if blockage of bile flow could result in accumulation of newly synthesized cholesterol destined for biliary secretion, the amount appears small compared to the actual cholesterol accumulation observed in the BCM.

Newly synthesized cholesterol accumulation in BCM seems to be an important component in the pathogenesis of Mn-BR–induced cholestasis. The data obtained for evaluating HMG-CoA reductase activity in vitro, however, indicate the increased accumulation of newly synthesized cholesterol in the various isolated subcellular fractions did not arise from enhanced cholesterol synthesis, as the activity of HMG-CoA reductase, the rate-limiting enzyme in cholesterol synthesis was not increased compared to controls. Because there is no evidence of an increased rate of cholesterol synthesis after Mn-BR treatment, other explanations for why Mn-BR treatment appears to favor the accumulation of newly synthesized cholesterol should be sought.

Biliary lipid secretion involves the exocytosis of cholesterol- and phospholipid-containing vesicles (Crawford et al., 1995). Although the exact vesicular mechanism has not been revealed, the following sequence of events for biliary lipid secretion has been proposed: cytosolic delivery of lipid vesicles to the internal hemileaflet of the canalicular membrane; transfer from the internal to the external hemileaflet of the canalicular membrane; and formation and detachment of unilamelar vesicles from the canalicular membrane (Kunze and Rüstow, 1993). In Mn-BR cholestasis, Mn and BR are both taken up by BCM (Aytote and Plaa, 1985), supporting the notion that a complex between both substances could be formed. Furthermore, the structural integrity of the membrane is affected and morphologic alterations such as distended bile canaliculi, lack of microvilli, and enlarged pericanalicular ectoplasm result from Mn-BR treatment (de Lamirande et al., 1982). These biochemical and morphologic alterations could also modify normal \(^{14}\)C-labeled cholesterol vesicle secretion into bile and result in \(^{14}\)C-labeled cholesterol retention in BCM and in organelles. However, as previously mentioned, we estimate that the newly synthesized cholesterol destined for biliary secretion is only a small contributing component of the total accumulated cholesterol.
terol observed in the various isolated fractions. Therefore, blockage of cholesterol destined for biliary secretion and further retention of this cholesterol in the hepatocyte cannot account for all the accumulated cholesterol.

Although movement of cholesterol into bile is a well-described process (Crawford et al., 1995), mechanisms by which cholesterol is distributed in the cell are still poorly understood. Nevertheless, it is thought that cholesterol can be transported from its site of synthesis to plasma membranes by several distinct mechanisms. Some evidence indicates that intracellular cholesterol trafficking could occur by vesicular means (Reinhart et al., 1987), while other studies render it conceivable that a lipoprotein-like particle between source and target membranes in the cell may be involved (Kaplan and Simoni, 1985). Still, processes responsible for the delivery of cholesterol, from its site of synthesis or from intracellular cholesterol pools, to plasma membranes are not completely elucidated and remain unclear (DeGrella and Simoni, 1982; Lange and Matthies, 1984; McIntyre and Sleight, 1994; Reinhart, 1990).

Urbani and Simoni (1990) reported that newly synthesized cholesterol bypasses the Golgi apparatus and is routed directly to the plasma membrane. These data suggest newly synthesized cholesterol can be transported to the plasma membrane by a novel pathway significantly different from other recognized pathways that deliver lipids or proteins to target organelles (Urbani and Simoni, 1990). To complete the trafficking process towards plasma membranes, the transport of newly synthesized cholesterol and that of intracellular cholesterol may occur via different mechanisms.

Yousef et al. (1984) showed that cholesterol was incorporated in BCM after cholestatic injections of lithocholic acid and that cholesterol accumulation in BCM in vitro was markedly enhanced if cytosolic proteins were added to the incubations. Also, pretreatment with an inhibitor of protein synthesis (cycloheximide) abolished cholesterol accumulation and prevented the cholestasis induced by lithocholic acid (Yousef et al., 1983). Unaltered protein synthesis, as an important contributing component in the pathogenesis of experimentally induced cholestasis, has been demonstrated with two other rat models of cholestasis, tauroliothocholic acid and Mn-BR injection (Dahlström-King and Plaa, 1989). Thus, one can speculate that protein synthesis is involved in cholesterol dynamics.

In the present study, Mn-BR treatment appears to favor \(^{14}\)C-labeled cholesterol in all subcellular fractions. Perhaps Mn-BR treatment interferes with intracellular trafficking of existing cholesterol pools but not that of newly synthesized cholesterol. Clearly, the effects of Mn-BR treatment on hepatobiliary cholesterol dynamics should be investigated in greater detail. As drug-induced cholestasis is becoming more prevalent with the occurrence of multidrug therapy, the need to clarify mechanisms involved in cholestasis is important. To better characterize the role of cholesterol in Mn-BR cholestasis, further studies are warranted.

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