High plasma cholesterol in drug-induced cholestasis is associated with enhanced hepatic cholesterol synthesis

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The compound \(\alpha\)-naphthylisothiocyanate (ANIT) has been used to induce experimental cholestasis in a variety of laboratory animals (4). In rats, a single dose of ANIT (100 mg/kg) administered by gavage results in a transient and fully reversible intrahepatic cholestasis that is accompanied by remarkable alterations to the plasma constituents (7). Specifically, ANIT treatment results in markedly elevated levels of plasma FC and PL, the appearance of Lp-X-like vesicles, elevations in plasma apoA-I and apoE, and a pronounced shift of apoE-containing particles into the LDL density range. These effects peak 48 h after ANIT administration and return to normal after 168 h. Thus ANIT-induced experimental cholestasis appears to be a useful nonsurgical approach to study the metabolism of abnormal cholestatic lipoproteins. Here we report the effects of ANIT on the metabolism of cholesterol in treated mice. The data demonstrate that ANIT treatment results in significant modification of the activities of enzymes involved in the synthesis and degradation of cholesterol in the liver. The alteration of lipid metabolism in the plasma of ANIT-treated mice also appears to evolve differently and more severely compared with that in ANIT-treated rats.

MATERIALS AND METHODS

Animals. Female C57BL/6 mice (10 wk old) were purchased from Charles River Laboratories. Mice were fed standard laboratory food, given water ad libitum, and housed under a 12:12-h light-dark photoperiod. Food was withdrawn 8–10 h before ANIT treatment. Mice were lightly anesthetized by intraperitoneal administration of 2% ketamine/0.4% xylazine at a dosage of 1–1.5 ml/kg and then given 100 mg/kg ANIT (Sigma Chemical) in a 10 mg/ml corn oil (Mazola) bolus by gavage. The control group received a volume of corn oil that was appropriate for the weight of each mouse. At the indicated experimental time points, fasted mice were deeply anesthetized by intraperitoneal injection of 2% ketamine/0.4% xylazine at a dosage of 2–3 ml/kg, and blood was collected from the descending aorta into tubes containing Na2EDTA, thimerosal, aprotinin, and NaN3 to final respective concentrations of 0.1, 0.005, 0.001, and 0.02%.

Mouse livers and gallbladders were excised at necropsy. Liver pieces were washed in saline, weighed, and then quick frozen in liquid nitrogen until analysis. Gallbladders were quickly spun to the bottom of a 1.5-ml microcentrifuge tube, and the bile was collected with a glass capillary and then quick frozen in liquid nitrogen until analysis.

Lipid and lipoprotein analyses. Liver lipids were extracted from homogenized liver sections by the method of Folch et al. (16). Plasma, density gradient fractions, and liver lipid mass
and compositional analysis were performed by gas chromatography (28). Bile acid content of plasma and bile as well as bile cholesterol and PL were measured using commercial diagnostic kits (Boehringer Mannheim, Sigma Chemical, and Wako Pure Chemical Industries). Pooled plasma from control and ANIT-treated mice was subjected to density gradient ultracentrifugation and fractionated manually into 400-µl fractions as previously described (7). The protein concentration of density gradient fractions was measured by the bicinchoninic assay (Pierce) modified by the addition of 0.25% deoxycholate (Sigma Chemical). The apolipoprotein composition of isolated density gradient fractions was analyzed by SDS-PAGE on 5–19% gradient gels as previously described (7).

Enzyme activity assays. Microsomes were prepared from livers of control and ANIT-treated mice as described previously (5). Cyp7 activity in isolated microsomes was measured by isotope incorporation using β-cyclodextrin encapsulated [3H]cholesterol as a substrate (1, 29) and by an HPLC-based method that uses endogenous microsomal cholesterol as a substrate (5, 6). Hydroxymethylglutaryl CoA reductase (HMGR) activity in the isolated microsomes was measured by following the formation of [14C]mevalonate from [14C]hydroxymethylglutaryl CoA (23). The radiolabeled products generated by the enzyme assays were separated by thin-layer chromatography and then quantitated using a Fuji BAS1000 PhosphorImager. Exogenous lecithin:cholesterol acyl transferase (LCAT) activity was measured by the method of Sparks et al. (43) using recombinant high-density lipoprotein (HDL) particles (80:10:0:1; 1,2-di[(cis-9-octodecanoyl)-sn-glycero-3-phosphocholine:F:C:Ce:human apo A-I). Aspartate aminotransferase and alanine aminotransferase activities in mouse plasma were measured using commercial diagnostic kits (Boehringer Mannheim).

RNA analyses. Total RNA from mouse livers was purified according to standard procedures (8). Complementary DNA was synthesized from total liver RNA (1 µg/reaction) using Superscript reverse transcriptase (Life Technologies) and random hexamers as primers. The CDNA for mouse cyp7 and mouse glyceraldehyde-3-phosphate dehydrogenase were amplified in vitro using Taq polymerase and primer pairs specific for each of the mRNA species. Amplification products were visualized by ethidium bromide staining of agarose gels after electrophoresis.

Histology. Liver samples were collected at necropsy and preserved in 10% neutral buffered Formalin. Preserved tissue samples were processed routinely, embedded in paraffin, sectioned at 5 µm, and then stained with hematoxylin and eosin using standard techniques.

RESULTS

Bile acids, bilirubin, and liver-specific enzymes in the plasma of ANIT-treated mice. To verify the induction of cholestasis in ANIT-treated mice, plasma collected 48 h after treatment was analyzed for markers of hepatic damage. Compared with controls, bilirubin levels in the plasma of ANIT-treated mice were increased by 11.2-fold (Table 1). The activities of alanine aminotransferase and aspartate aminotransferase in plasma of ANIT-treated mice were also increased by 7.4- and 4.3-fold, respectively. Finally, the concentration of bile acids in the plasma of ANIT-treated mice was increased by 263-fold. These features are consistent with the changes that have been previously documented to occur in the plasma of ANIT-treated rats (7). Unlike the ANIT-treated rat, ANIT-treated mice were LCAT deficient 48 h after ANIT treatment (Table 1) and had a 5.2-fold reduction in exogenous LCAT activity when compared with controls.

Plasma lipids and lipoproteins. The FC and PL mass in the plasma of ANIT-treated mice were increased 17.3- and 10.4-fold, respectively, at 48 h after treatment (Fig. 1). This result is similar to what has been previously documented in ANIT-treated rats (7). By 168 h after treatment, FC and PL levels were 38.9- and 13.4-fold greater, respectively, than control values. Thus the PL level began to plateau by 48 h, whereas FC levels continued to rise significantly. This response differs from that observed in rats where both FC and PL levels began to decrease after 48 h, reaching near-

Table 1. Plasma markers of liver damage and LCAT activity

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>BileAcids, µM</th>
<th>Bilirubin, µM</th>
<th>AST, IU</th>
<th>ALT, IU</th>
<th>LCAT, nmol CE formed·mL⁻¹·h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>11.9 ± 3.5</td>
<td>5.2 ± 1.7</td>
<td>26.6 ± 6.1</td>
<td>1.5 ± 0.5</td>
<td>10.3 ± 0.5</td>
</tr>
<tr>
<td>A48</td>
<td>3.152 ± 1.400*</td>
<td>58.7 ± 18.4*</td>
<td>197.3 ± 64.0*</td>
<td>6.4 ± 1.7*</td>
<td>2.0 ± 0.4*</td>
</tr>
</tbody>
</table>

Values are means ± SE. Plasma separated from blood collected 48 h after treatment of mice with corn oil (control, n = 4) or α-naphthylisothiocyanate (ANIT; A48, n = 5) were tested as described in MATERIALS AND METHODS. AST, aspartate aminotransferase; ALT, alanine aminotransferase; LCAT, lecithin:cholesterol acyl transferase; CE, cholesterol esters. *P < 0.05 vs. control values.

Fig. 1. Plasma lipid concentration after α-naphthylisothiocyanate (ANIT) administration. Values are means ± SD (or range of mean where n = 2) (0 h, n = 3; 24 h, n = 2; 48 h, n = 10; 72 h, n = 3; 168 h, n = 3). FC, free cholesterol; CE, cholesteryl esters; PL, phospholipids; TG, triacylglycerol.
Differences were evaluated using Student’s t-test (21) and do not include carbons derived from cholesterol. Differences were evaluated using Student’s t-test. *P < 0.01, †P < 0.0005, ‡P < 0.0001 vs. control values.

Table 2. Molecular species of phospholipids in the plasma

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>C-34, mg/dl</th>
<th>C-36, mg/dl</th>
<th>C-38, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>27.2 ± 3.9</td>
<td>33.6 ± 4.5</td>
<td>22.2 ± 4.5</td>
</tr>
<tr>
<td>A48</td>
<td>44.1 ± 69.3t(16.2)</td>
<td>367.8 ± 54.6t(10.9)</td>
<td>176.6 ± 30.1t(7.9)</td>
</tr>
<tr>
<td>A168</td>
<td>638.9 ± 256.3t(23.5)</td>
<td>432.9 ± 171.6t(12.9)</td>
<td>163.8 ± 70.2t(7.4)</td>
</tr>
</tbody>
</table>

Values are means ± SD. Numbers in parentheses show the fold change from control values. Phospholipid species in plasma samples were collected 48 h after treatment of mice with corn oil (control, n = 6) or ANIT (A48, n = 10) and 168 h after treatment with ANIT (A168, n = 3) were determined as described in MATERIALS AND METHODS. C-34, C-36, and C-38 refer to total number of carbons from the 2 fatty acyl moieties and do not include the 3 carbons in glycerol backbone. The likely major fatty acid combinations represented by C-34, C-36, and C-38 are C-16/18, C-18/18, and C-18/20, respectively. Differences were evaluated using Student’s t-test. *P < 0.005, †P < 0.0005, ‡P < 0.0001 vs. control values.

Table 3. Molecular species of cholesteryl esters in the plasma

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>C-16, mg/dl</th>
<th>C-18, mg/dl</th>
<th>C-20, mg/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>14.0 ± 1.2</td>
<td>42.5 ± 7.1</td>
<td>29.0 ± 1.9</td>
</tr>
<tr>
<td>A48</td>
<td>21.9 ± 6.3t(1.6)</td>
<td>28.8 ± 3.9t(−0.32)</td>
<td>7.4 ± 2.4t(−0.74)</td>
</tr>
<tr>
<td>A168</td>
<td>57.2 ± 9.2t(4.1)</td>
<td>45.3 ± 0.5(1.07)</td>
<td>3.2 ± 3.2t(−0.89)</td>
</tr>
</tbody>
</table>

Values are means ± SD. Numbers in parentheses show the fold change from control values. Cholesteryl ester species in plasma samples collected 48 h after treatment of mice with corn oil (control, n = 6) or ANIT (A48, n = 10) and 168 h after treatment with ANIT (A168, n = 3) were determined by gas chromatographic total lipid profiling (21) and do not include carbons derived from cholesterol. Differences were evaluated using Student’s t-test. *P < 0.01, †P < 0.0005, ‡P < 0.0001 vs. control values.

Fig. 2. Lipoprotein density profile and lipid distribution in pooled plasma collected from mice at 48 h after treatment with corn oil (control, n = 6) or with ANIT (B; n = 10). Only fractions 1–24 are shown because fractions 25–30 were devoid of lipid. Lp, lipoprotein; VLDL, very-low-density lipoprotein; IDL, intermediate-density lipoprotein; HDL, high-density lipoprotein.
plasma of ANIT-treated mice, but apo B100 was associated with more buoyant particles, whereas apo B48 was associated with denser particles (Fig. 3). The distribution of apo A-I in ANIT-treated mouse HDL fractions was similar to that of the control mouse (fractions 12–22, Fig. 3), although the total mass of apo A-I in the lipoprotein fraction of ANIT-treated mouse plasma was decreased by ~35%. The increase in both apo E and apo A-IV in the less-dense HDL fractions (fractions 12–15, Fig. 3) may indicate the decreased uptake of apo E-containing HDL by the liver.

Gallbladder bile lipids. Unlike rats, mice accumulate bile in the gallbladder. ANIT treatment did not abolish gallbladder content. In fact, the volume of bile in the gallbladders of ANIT-treated mice was two- to fourfold greater than in the controls (data not shown). Bile collected from the gallbladders of ANIT-treated mice had reduced bile lipid concentrations (Table 4). At 48 h, there was a greater reduction in PL and total bile acid concentrations (62 and 86% decrease, respectively) compared with cholesterol concentration (30% decrease). The volume of bile in the gallbladder of ANIT-treated mice at 168 h was substantially reduced and did not allow for reliable compositional analysis.

Table 4. Composition of gallbladder bile

<table>
<thead>
<tr>
<th>Treatment Group</th>
<th>Cholesterol, µM</th>
<th>Phospholipids, µM</th>
<th>Total Bile Acids, µM</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>1.8 ± 0.1</td>
<td>21.8 ± 1.2</td>
<td>2.024 ± 223</td>
</tr>
<tr>
<td>A48</td>
<td>1.2 ± 0.1*</td>
<td>7.7 ± 1.2†</td>
<td>293 ± 15†</td>
</tr>
</tbody>
</table>

Values are means ± SD of duplicate determinations. Equal volumes of bile collected 48 h after treatment with corn oil (control, n = 6) or ANIT (A48, n = 10) were pooled before analysis. Phospholipid values represent phosphatidylcholine. Differences were evaluated using Student's t-test. *P < 0.05, †P < 0.01 vs. control values.

Hepatic lipids. Lipid composition analysis did not show significant changes in total cholesterol content in the liver of ANIT-treated mice (Table 5). The hepatic PL level was comparable to controls at 48 h after treatment but was slightly reduced after 168 h. However, the TG level was doubled at 48 h (Table 5). At 168 h, the hepatic TG level of ANIT-treated mice was less than that in the controls. Molecular species analysis of CE and PL did not reveal any significant changes resulting from ANIT treatment (data not shown).

Hepatic cholesterol and bile acid metabolism. The activities of HMGR and cyp7 were determined to study the basis for the altered levels of cholesterol and bile acids. Measurement of cyp7 activity beginning from the time of ANIT treatment revealed a ~75% decrease in cyp7 enzyme activity at 24 h that persisted until at least 72 h (Fig. 4A). The reduction in cyp7 activity was accompanied by a decrease in cyp7 mRNA to undetectable levels at 48–96 h after ANIT treatment (Fig. 4B). The reduction in cyp7 activity was confirmed in another experiment by following the conversion of endogenous cholesterol to 7α-hydroxycholesterol (Fig. 5). Interestingly, the cyp7 activity increased 6.4-fold over the...
control values at 168 h after treatment. This increase in cyp7 activity corresponded with the increase in the cyp7 mRNA abundance (data not shown). HMGR activity in liver microsomes of ANIT-treated mice was enhanced sixfold relative to controls at 48 h after treatment (Fig. 5). By 168 h after the treatment, the HMGR activity stimulated by the ANIT treatment decreased but to a level that was still 2.3-fold higher than the controls. The rise in plasma FC level may therefore be due to the combined effects of increased hepatic cholesterol biosynthesis, impaired bile acid synthesis and biliary cholesterol secretion, and decreased CE formation in the plasma.

Liver histopathology. Liver samples from control and ANIT-treated mice were subjected to histopathological analysis. Liver samples from the control group did not reveal any significant abnormal features. However, sections made of the livers collected 48 h after ANIT treatment revealed multiple focal areas of cell death that appeared to be located randomly within the liver parenchyma (Fig. 6A). There were no obvious signs of cell death in the area adjacent to either the portal triads or the central veins. No inflammation was apparent around the areas of damage in the parenchyma, but inflammatory cell infiltration in the periportal areas was evident. In addition, there was proliferation of fibrocytic cells particularly around the bile ducts (Fig. 6B). An increased number of mitotic figures were also evident throughout the liver. The cytoplasm of hepatocytes in the liver of ANIT-treated mice taken at 48 h after treatment often contained multiple small vacuoles. Surprisingly, the changes were largely resolved at 168 h after ANIT treatment. The vacuoles, which may have contained TG, that were evident at 48 h after treatment were no longer apparent in liver samples collected at 168 h after treatment. The reactive fibroplasia that was prominent in the periportal region had entirely disappeared. Small clusters of neutrophils were occasionally present, and the region surrounding these areas was essentially microscopically normal.

**DISCUSSION**

The elevation of plasma FC and PL concentrations and the appearance of abnormal vesicular lipoprotein particles known as Lp-X are often prominent features in human cholestasis (18). Experimental cholestasis in animals can be induced by surgical ligation of the common bile duct or administration of drugs such as ethinyl estradiol, chlorpromazine, and ANIT (33). A single dose of ANIT administered to rats by gavage induces transient and fully reversible changes to plasma lipids and lipoproteins that are strikingly similar to those associated with cholestatic liver disease (7). In this study, experimental cholestasis was induced in mice by the administration of ANIT to investigate the metabolic basis underlying the changes in plasma lipid levels.

Administration of ANIT to mice at a dose equivalent to that given to rats resulted in alterations to the
plasma compartment that were consistent with human cholestasis (18), experimental cholestasis in the rat (4, 7, 15, 22), and human LCAT deficiency (20, 40, 46). Analysis of plasma, liver, and bile lipids indicated that the modification of lipid levels in these three compartments was distinct. FC and PL levels became elevated in the plasma of ANIT-treated mice, and the rise was progressive over the entire experimental time period (0–168 h). This response differs from ANIT-treated rats with respect to apo A-I levels after ANIT treatment may be directly related to changes in LCAT activity, either through increased apo A-I catabolism or decreased apo A-I and/or mature HDL production. As in the ANIT rat, apo E was dramatically increased in the LDL density range. Elevated plasma apo E in human liver disease (9) and LCAT deficiency (20) has been previously observed and associated with large discoidal HDL particles. Whether apo E in the ANIT mouse is associated with HDL or Lp-X was not determined in this study. The level of apo A-IV associated with the LDL density range of plasma from ANIT-treated mice also was increased, and this is consistent with that previously observed in ANIT-treated rats (7). The reason for this remains unknown, but based on its location, we speculate that apo A-IV may have a strong affinity for the PL-rich lipoproteins in cholestatic plasma.

The activity of HMGR in hepatic microsomes of ANIT-treated mice was increased substantially during the early phase of the response. Considering that HMGR catalyzes the rate-limiting step in the biosynthesis of cholesterol (3), it appears that ANIT-induced intrahepatic cholestasis occurs under conditions where liver cholesterol synthesis is stimulated, and this is consistent with that observed in rats with extrahepatic cholestasis (11). Osono et al. (31) estimated that hepatic synthesis accounts for ~22% of the total cholesterol...
The importance of the mdr2 P-glycoprotein, the protein responsible for the translocation of phosphatidylcholine across the canalicular membrane (42), in the formation of Lp-X was recently investigated (32). Lp-X was not found in the plasma of mdr2-deficient mice after bile duct ligation, suggesting that mdr2 function is necessary for Lp-X formation. In addition, experiments using mice with only one functional allele of the Mdr2 gene and transgenic mice expressing the human MDR3 (homolog of Mdr2) at different levels demonstrated that the amount of Lp-X that appears in the plasma after bile duct ligation is proportional to mdr2 activity in canalicular membranes (32). The abundance of the mdr2 mRNA is increased by cholic acid feeding (17). Thus, during cholestasis, the expression of the Mdr2 gene may actually be stimulated in response to increased intracellular bile acid concentration.

The PL-to-FC ratio of gallbladder bile taken from mice at 48 h after treatment was considerably greater than the PL-to-FC ratio of lipoproteins in the LDL density range of plasma from the same mice. Increased tight junction permeability is postulated to be responsible for the leakage of biliary constituents into the plasma compartment during ANIT-induced cholestasis (27). It was previously suggested that nascent Lp-X particles initially form within the bile canaliculi, then are transferred through liver parenchymal cells to the space of Disse, thereby gaining access to the plasma compartment. It is interesting to note that the bile secreted by rat livers perfused with various species of bile acids has nearly a fixed PL-to-FC molar ratio of 12:1 (2), whereas the PL-to-FC molar ratio of Lp-X in plasma is nearly equal (7, 39). Because PL vesicles are known to be efficient acceptors of cellular cholesterol (see Ref. 48 for review), the nascent cholesterol-poor vesicles secreted by the ANIT mouse liver may promote the efficient efflux of cholesterol from hepatic cell membranes and subsequently accumulate in the plasma. The continuous extraction of cholesterol from hepatic cellular membranes would explain the stimulation of cholesterol synthesis in the liver.

A transient rise of TG that peaked at 24 h after treatment was evident in the plasma of ANIT-treated mice. Previously, Felker et al. (14) reported that the perfusate of bile duct-ligated rat liver contained LDL particles that were abnormally enriched in TG. The production of these particles may represent the very early stages of aberrant lipoprotein metabolism after the induction of cholestasis. The TG level in the plasma of ANIT-treated mice was considerably greater than in controls. A transient rise of TG that peaked at 24 h after treatment was evident in the plasma of ANIT-treated mice. Previously, Felker et al. (14) reported that the perfusate of bile duct-ligated rat liver contained LDL particles that were abnormally enriched in TG. The production of these particles may represent the very early stages of aberrant lipoprotein metabolism after the induction of cholestasis. The TG level in the plasma of ANIT-treated mice decreased to near-normal levels in the early stages of aberrant lipoprotein metabolism after the induction of cholestasis. The TG level in the plasma of ANIT-treated mice decreased to near-normal levels. A transient rise of TG that peaked at 24 h after treatment was evident in the plasma of ANIT-treated mice. Previously, Felker et al. (14) reported that the perfusate of bile duct-ligated rat liver contained LDL particles that were abnormally enriched in TG. The production of these particles may represent the very early stages of aberrant lipoprotein metabolism after the induction of cholestasis. The TG level in the plasma of ANIT-treated mice decreased to near-normal levels in the early stages of aberrant lipoprotein metabolism after the induction of cholestasis. The TG level in the plasma of ANIT-treated mice decreased to near-normal levels.
loss of hepatocytes and hyperplasia of cells surrounding the bile ducts were observed. Injury to hepatocytes and cholangiohepatitis has been documented previously in ANIT-treated rats (10, 19, 26). However, the exact basis for ANIT hepatotoxicity in vivo remains to be defined (30). Liver samples taken from ANIT-treated mice during the latter phase of the response were surprisingly histologically normal and in marked contrast to the remarkable pathological changes evident during the early phase.

In summary, we investigated the metabolism of lipids in mice with drug-induced cholestasis. The ANIT-treated mouse clearly elaborates the aberrant metabolism of plasma lipids and lipoproteins associated with cholestasis and human LCAT deficiency. It is also apparent that the response of mice to ANIT treatment is complex and produces a variety of sequelae. With respect to the changes in lipid metabolism, the rise in intrahepatic bile acid concentration after induction of cholestasis appears to stimulate the synthesis of biliary PL, a response that likely represents a mechanism to mitigate the cytotoxic effects of bile acids. The accumulation of biliary PL in the plasma is accompanied by the rise in plasma lipids and lipoproteins associated with cholestasis. The high density lipoprotein particle size distribution in subjects with obstructive jaundice. J. Lipid Res. 29: 121–135, 1988.


