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α-Naphthylisothiocyanate (ANIT) Induced Cholestasis in Rats

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In order to distinguish the disorder of bile acid and cholesterol metabolism in α-naphthylisothiocyanate (ANIT)-induced cholestasis, we examined changes in bile acid levels and compositions in bile, serum, feces and urine, as well as cholesterol levels in bile, serum, liver and feces in Wistar male rats (10–13 weeks) after a single oral administration of 100 mg/kg of ANIT. The bile flow and the biliary secretions of cholesterol, phospholipids and bile acids markedly decreased on days 1 and 2 but increased over the normal values on day 4 and then returned to the normal ranges. The fecal excretion of bile acids decreased after the treatment and remained low by day 4 but markedly increased thereafter. The urinary excretion of bile acids changed almost in parallel with serum bile acid level, increasing to 37 mg/day on day 2, 28 mg/day on days 3–4 but to a trace on days 5–6. The urinary bile acids on day 2 mainly consisted of cholic acid while those on days 3–4 and biliary bile acids on day 4 were mostly β-muricholic acid. The serum cholesterol level markedly increased maximally on day 2 and decreased thereafter. The fecal excretion of sterols, cholesterol and coprostanol, decreased on days 1–2 but rather increased thereafter. These data suggest that the cholestasis induced by ANIT is very similar to that in bile duct ligated rats for a short period but not to those ligated for long periods. In addition, the present data suggest that the bile acid independent bile flow is impaired and the daily synthesis of bile acids, especially β-muricholic acid, is increased in the ANIT induced cholestasis.

Key words: bile secretion; biliary lipids; fecal bile acids and sterols; α-naphthylisothiocyanate; serum lipids; urinary bile acids

α-Naphthylisothiocyanate (ANIT) is a compound which causes cholestasis, hepatocellular and biliary epithelial cell necrosis, bile duct obstruction and biliary epithelial cell hyperplasia in rats (Plaa and Priestly, 1976). The changes in biliary epithelial cells are reported to be comparable to that induced by bile duct ligation (Kossor et al., 1995).

Previously, we examined the changes in bile acid metabolism after bile duct ligation in rats (Kinugasa et al., 1981, Takita et al., 1988) and those after the release of bile duct ligation (Fujio et al., 1989). A striking change in bile duct ligated rats was a marked increase in β-muricholic acid with a concomitant decrease of cholic acid. The change was normalized within a week after the release of ligation regardless of the duration of ligation, but the changes in bile flow and biliary bile acid secretion were different according to the duration. Release after a 1-week ligation showed a temporal rebound increase in both bile flow and biliary bile acid secretion, but release after a 2-week ligation caused a continuous hypersecretion of the bile without any rebound increase in biliary bile acid secretion (Fujio et al., 1989).

In the present experiments, we examined the changes in bile flow and bile acid metabolism after ANIT administration until cholestasis was completely recovered in rats.

Abbreviations: ANIT, α-naphthylisothiocyanate; bsep, bile salt export pump; GLC, gas-liquid chromatography; mdr2, multidrug resistance 2; mrp2, multidrug resistance associated protein 2

Materials and Methods

ANIT obtained from Nakarai Pure-Chemicals (Tokyo, Japan) was dissolved in corn oil at a concentration of 100 mg/mL, and a single dose of ANIT (100 mg/kg) was administered orally to rats. This dosage was selected based on the report of Kossor et al. (1995) who examined the effect of various doses of ANIT (25–150 mg/kg) in rats and showed that a dose of 100 mg/kg induced a modest cholestasis at 48 h after treatment. Control rats received 1 mL/kg of corn oil orally.

Animal treatments

Wistar strain male rats weighing about 300 g (10–13 weeks old) were kept in an air-conditioned room (25 ± 1˚C, 50–60% humidity) lighted 12 h a day (8:00–20:00) and maintained on a commercial stock diet (Japan CLEA CA-1, Tokyo, Japan).

In the first experiment, rats were individually caged and daily urine specimens were collected for 10 days after the administration of ANIT. Daily urine volume, diet intake and water intake were recorded. In the second experiment, rats were individually caged and sacrificed 2, 4, 6, 8 and 10 days after the administration of ANIT to collect bile, blood and liver specimens. Two-day feces specimens were collected prior to sacrifice as described previously (Uchida et al., 1977). On the day of sacrifice, the rats were cannulated of their bile duct with PE-10 polyethylene tubings in order to collect the bile for 30 min under sodium pentobarbital anesthesia (50 mg/kg, intraperitoneally). During the time of bile collection, the rectal temperature was maintained at 36–37˚C using an electric warm plate. The blood was then withdrawn by heart puncture and the liver was removed for lipid determination. Bile was collected between 9:00–11:00 to avoid any variation due to the circadian rhythm.

Serum and liver lipid determination

The serum was separated by centrifugation at 3,000 rpm for 15 min after allowing the blood to stand for at least 30 min at room temperature. A portion of the largest lobe of the liver (*lobus sinistra externa*) was homogenized with 9 volumes of water. The serum and the liver homogenate were extracted in 9 volumes of ethanol by refluxing for 20 min at 90–95˚C. Total cholesterol was determined with portions of the serum and liver lipid extracts as previously reported (Uchida et al., 1965). Phospholipids were determined by the method of Gomori (1942).

Biliary lipid determination

The bile was added to 20 volumes of ethanol, brought to boil once for several minutes, cooled down to room temperature, and then filtered. An aliquot of the filtrate was evaporated to dryness under a mild stream of nitrogen and the residue was hydrolyzed at 120˚C for 6 h in 1.25 M sodium hydroxide solution. Sterols were extracted with diethyl ether, the reaction mixture was acidified with 2 M hydrochloric acid and then the bile acids were extracted with diethyl ether. The bile acids were methylated with freshly prepared diazomethane and then trifluoroacetylated with trifluoroacetic anhydride. The bile acid derivatives were quantified by gas-liquid chromatography (GLC) with a 1% QF-1 column and cholesterol with a 1% SE-30 column (Uchida et al., 1970, 1977). Biliary phospholipids were determined by the methods of Gomori (1942).

Urinary and serum bile acid determination

The urine was applied to an Amberlite XAD-2 column and bile acids were eluted with methanol according to the method of Makino et al. (1974). The eluate was evaporated to dryness and the residue was dissolved in 30 mL of acetone-ethanol (9:1) containing 0.01 volume of 2 M hydrochloric acid. The solution was left at room temperature for 2 to 3 days to complete solvolysis. The reaction mixture was then evaporated to dryness and the residue was subjected to alkaline hydrolysis. After extraction with diethyl ether, the bile acids were methylated and trifluoroacetylated, and analyzed by GLC as mentioned above.

Serum bile acids were isolated by the use of an Amberlyst A-26 column (Sandberg et al.,

Table 1. Changes in body weight, diet intake and water intake after α-naphthylisothiocyanate (ANIT) administration in rats

	Days after ANIT administration						
	0	1	2	4	6	8	10
Body weight (g)	338 ± 3.2	315 ± 3.8*	304 ± 2.3*	312 ± 1.5*	318 ± 5.2	332 ± 4.1	342 ± 2.7
Diet intake (g/day)	22 ± 1.0	5 ± 0.8*	3 ± 1.3*	14 ± 2.7*	22 ± 1.0	26 ± 1.4	—
Water intake (mL/day)	30 ± 4.5	5 ± 1.6*	18 ± 4.4	33 ± 2.8	34 ± 5.3	39 ± 3.5	—

Mean ± SE in 5 rats.
—, not determined.
* Statistically significant compared with day 0 ($P < 0.05$).

1965) and quantified by GLC after alkaline hydrolysis without any procedure for solvolysis.

Fecal sterol and bile acid determination

The feces were dried and powdered, according to the procedures described previously (Uchida et al., 1977). An aliquot of the pulverized feces was extracted with 20 volumes of boiling ethanol for 1 h and filtered after cooling down to room temperature. The extraction procedures were repeated three times. The combined filtrate was evaporated to dryness under reduced pressure, and the residue was hydrolyzed at 120˚C for 6 h in 1.25 M sodium hydroxide solution. Sterols were extracted with diethyl ether, the reaction mixture was acidified with 2 M hydrochloric acid and then bile acids were extracted with diethyl ether. The methylated and trifluoroacetylated bile acids were quantified by GLC with a 1% QF-1 column and sterols with a 1% SE-30 column (Uchida et al., 1977; Kinugasa et al., 1981). The extraction efficiency of sterols and bile acids from feces was over 92.5% (Kinugasa et al., 1981).

Statistical analysis

Data are expressed as mean ± SE. The difference between the means of variables was calculated

Table 2. Changes in urinary bile acids after α-naphthylisothiocyanate (ANIT) administration in rats

	Days after ANIT administration						
	0	1	2	3–4‡	5–6‡	7–8‡	9–10‡
Urine volume (mL/day)	12 ± 1.8	15 ± 1.2*	11 ± 2.0	15 ± 3.3	18 ± 2.2	19 ± 2.1*	20 ± 2.1*
Urinary bile acids (mg/day)	0.01 ± 0.00	2.01 ± 0.56*	37.29 ± 3.74*	28.07 ± 3.10*	0.09 ± 0.01*	0.07 ± 0.01*	0.05 ± 0.01*
Bile acid composition (%)							
Lithocholic acid	31 ± 4.7	< 1	< 1	< 1	17 ± 5.0	6 ± 0.8*	11 ± 3.3*
Chenodeoxycholic acid	3 ± 2.2	< 1	1 ± 0.0	2 ± 0.5	< 1	ND	ND
α-Muricholic acid	2 ± 1.7	3 ± 0.4	1 ± 0.1	3 ± 0.2	2 ± 0.6	3 ± 1.0	3 ± 1.2
β-Muricholic acid	4 ± 0.6	16 ± 5.8	16 ± 1.1*	46 ± 3.4*	16 ± 2.4*	7 ± 2.0	9 ± 2.5
ω-Muricholic acid	8 ± 0.6	3 ± 0.4	3 ± 0.2	3 ± 0.9	7 ± 0.8	4 ± 0.8	7 ± 0.7
Ursodeoxycholic acid	1 ± 0.8	< 1	< 1	3 ± 0.6	< 1	ND	ND
Hyodeoxycholic acid	< 1	< 1	< 1	1 ± 0.3	ND	ND	ND
Deoxycholic acid	ND	< 1	< 1	< 1	< 1	ND	< 1
Cholic acid	47 ± 6.4	55 ± 7.0	62 ± 0.9	31 ± 2.5	42 ± 7.6	73 ± 1.9	63 ± 6.6
Peak 8†	ND	15 ± 5.4	9 ± 0.8	7 ± 0.8	3 ± 1.2	4 ± 0.8	4 ± 1.2
Others	3 ± 1.0	6 ± 0.6	6 ± 0.2	4 ± 0.3	13 ± 3.3	< 1	4 ± 0.7

Mean ± SE in 5 rats.
ND, not detectable.
† Unidentified.
‡ Samples of 2-day urine specimens were combined.
* Statistically significant compared with day 0 ($P < 0.05$).

Table 3. Changes in feces dry weight and fecal sterols and bile acids after α-naphthylisothiocyanate (ANIT) administration in rats

	Days after ANIT administration					
	0	1–2	3–4	5–6	7–8	9–10
Feces dry weight (g/day)	4.5 ± 0.39	1.3 ± 0.18*	3.6 ± 0.53	4.6 ± 0.22	5.4 ± 0.27	5.7 ± 0.16
Fecal total sterols (mg/day)	7.49 ± 0.71	1.98 ± 0.27*	13.98 ± 2.58*	12.72 ± 1.22*	11.24 ± 0.42*	9.78 ± 1.06
Sterol composition (%)						
Coprostanol	54 ± 5.8	46 ± 7.7	57 ± 9.4	50 ± 7.7	50 ± 6.3	56 ± 7.0
Cholesterol	47 ± 4.5	54 ± 11.0	43 ± 9.2	50 ± 11.0	50 ± 2.7	44 ± 5.1
Fecal bile acids (mg/day)	8.17 ± 0.68	2.56 ± 0.34*	1.61 ± 0.25*	14.73 ± 1.28*	12.98 ± 1.47*	11.26 ± 0.69*
Bile acid composition (%)						
Lithocholic acid	10 ± 0.5	7 ± 1.0	26 ± 3.0*	7 ± 1.7	7 ± 1.0	7 ± 0.7
β-Muricholic acid	6 ± 0.6	15 ± 5.9	13 ± 1.4*	16 ± 3.2*	10 ± 1.9	8 ± 1.3
ω-Muricholic acid	29 ± 5.2	20 ± 2.0	18 ± 3.8	49 ± 5.3*	47 ± 3.3*	46 ± 4.2*
Hyodeoxycholic acid	22 ± 5.5	22 ± 5.3	7 ± 2.6*	2 ± 0.4*	4 ± 1.2*	6 ± 1.9*
Deoxycholic acid	26 ± 1.4	28 ± 2.6	18 ± 1.2*	17 ± 1.4*	24 ± 1.5	26 ± 0.7
Peak 8† and others	8 ± 0.4	9 ± 1.5	18 ± 1.2*	10 ± 1.3	9 ± 0.8	7 ± 0.6

Mean ± SE in 5 rats.
† Unidentified.
* Statistically significant compared with day 0 ($P < 0.05$).

by Student's *t*-test. Values of $P < 0.05$ were considered to be statistically significant.

Results

As shown in Table 1, the body weight decreased and showed the lowest value on day 2 after ANIT administration and recovered gradually thereafter. Diet and water intake decreased markedly on the first day but diet intake recovered to the initial level by day 6 and the water intake by day 4.

Urine volume increased on day 1, returned to the initial level on day 2, and slightly increased thereafter. The urinary excretion of bile acids markedly increased on day 2 and probably on day 3, and then rapidly decreased. The major component in urinary bile acids was cholic acid in the control rats, but β-muricholic acid increased after ANIT administration and became the major component on days 3–4. Cholic acid rather decreased on days 3–4, but gradually increased thereafter in accordance with the decrease of β-muricholic acid (Table 2).

The changes in the feces dry weight, and fecal sterol and bile acid excretions are shown in Table 3. The feces dry weight decreased on days 1–2 but was restored after days 5–6. The fecal sterol excretion decreased once on days 1–2, but markedly increased on days 3–4 and then gradually decreased. The composition ratios of coprostanol and cholesterol were almost identical in all the groups. The fecal bile acid excretion decreased after ANIT administration to the lowest level on days 3–4, but in turn markedly increased on days 5–6 attaining almost twice the level of the control rats, and gradually declined thereafter. Deoxycholic acid comprised about 25% in the control rats but decreased on days 3–4 and 5–6, and was then restored to the initial level. Instead, β-muricholic acid and ω-muricholic acid increased while deoxycholic acid decreased. Hyodeoxycholic acid decreased after days 3–4, and remained low thereafter, while ω-muricholic acid increased and kept high levels after days 5–6.

Table 4 shows the changes in the bile flow and biliary lipid secretions after ANIT administration. The bile flow markedly decreased, almost to 1/10 that of the initial level, on days 1 and 2, but increased showing higher levels than the initial level on days 4 and 10. Biliary bile acid secretion also decreased on days 1 and 2, increased on day 4 and was restored to the control level after day 6. The biliary cholesterol

Table 4. Changes in bile flow and biliary lipid secretions after α-naphthylisothiocyanate (ANIT) administration in rats

		Days after ANIT administration					
		0	1	2	4	6	10
Bile flow	(mL/h)	1.43 ± 0.02	0.12 ± 0.02*	0.12 ± 0.04*	1.83 ± 0.11*	1.23 ± 0.17	1.60 ± 0.05*
Total cholesterol	(mg/h)	0.20 ± 0.01	0.05 ± 0.00	0.05 ± 0.00	0.43 ± 0.05*	0.18 ± 0.03	0.19 ± 0.01
Phospholipids	(mg/h)	5.03 ± 0.39	0.27 ± 0.07*	0.24 ± 0.06*	20.22 ± 3.74*	6.74 ± 0.90	7.53 ± 0.81*
Bile acids	(mg/h)	12.90 ± 0.82	0.37 ± 0.26*	0.52 ± 0.16*	34.49 ± 5.32*	12.61 ± 1.37	16.72 ± 1.49

Mean ± SE in 5 rats.
* Statistically significant compared with day 0 ($P < 0.05$).

and phospholipid secretions changed almost in parallel with the bile acid secretion.

Striking changes in the biliary bile acid composition were an increase of β-muricholic acid and a concomitant decrease of cholic acid on days 2 and 4. The compositions of the other bile acids were not changed significantly (Table 5).

As shown in Table 6, the serum bile acid concentration markedly increased on day 1 and maximally on day 2 (about 30-fold) but abruptly declined on day 4 almost attaining the control level. A decrease in cholic acid and an increase in β-muricholic acid were also noted on days 2 and 4. Chenodeoxycholic acid showed high values on day 0 and day 4, but the reason for this large variation was not clear. Such a high value for chenodeoxycholic acid was detected neither in the urine, feces nor bile.

The correlations between biliary bile acid secretion and bile flow, biliary bile acid secretion and biliary cholesterol secretion, and biliary bile acid secretion and biliary phospholipid secretion are shown in Fig. 1. As shown in Fig. 1A, a linear regression line was obtained between the bile acid secretion and bile flow in the region where the bile acid secretion was over 10 mg/h, by which the bile acid dependent bile flow was calculated to be 0.018 mL/mg and the bile acid independent bile flow to be 1.25 mL/h. However, in the region where the bile acid secretion was less than 10 mg/h, the spots were under the regression line.

On the contrary, the cholesterol and phospholipid secretions were linearly related to the bile acid secretion in all regions. It was calculated from these relationships that the cholesterol/bile acids ratio in the bile was 0.012 and the phospholipids/bile acids ratio was 0.27 on a

Table 5. Changes in biliary bile acid composition after α-naphthylisothiocyanate (ANIT) administration in rats

Composition	Days after ANIT administration					
(%)	0	1	2	4	6	10
Lithocholic acid	1 ± 0.1	< 1	<1	1 ± 0.0	< 1	< 1
Chenodeoxycholic acid	3 ± 0.8	1 ± 0.4	1 ± 0.2*	5 ± 0.9	2 ± 0.2	3 ± 0.5
α-Muricholic acid	3 ± 0.5	2 ± 0.6	2 ± 0.1	4 ± 0.5	3 ± 0.4	3 ± 0.3
β-Muricholic acid	24 ± 4.5	25 ± 10.3	48 ± 7.3*	42 ± 5.3*	27 ± 7.0	22 ± 4.6
ω-Muricholic acid	1 ± 0.1	2 ± 0.2	1 ± 0.1	< 1	1 ± 0.1	1 ± 0.2
Hyodeoxycholic acid	2 ± 0.4	4 ± 0.7	1 ± 0.2	1 ± 0.2	1 ± 0.4	2 ± 0.4
Deoxycholic acid	2 ± 0.1	1 ± 0.1*	1 ± 0.1*	1 ± 0.3*	3 ± 0.3	4 ± 0.3*
Cholic acid	41 ± 0.8	38 ± 9.6	31 ± 6.8	21 ± 1.6*	42 ± 4.4	45 ± 2.1
Peak 8†	14 ± 2.4	16 ± 2.1	9 ± 1.5	16 ± 4.3	13 ± 3.7	13 ± 2.7
Others	9 ± 0.6	12 ± 0.9*	8 ± 0.7	9 ± 1.4	8 ± 1.6	8 ± 1.0

Mean ± SE in 5 rats.
† Unidentified.
* Statistically significant compared with day 0 ($P < 0.05$).

Table 6. Changes in serum bile acids after α-naphthylisothiocyanate (ANIT) administration in rats

	Days after ANIT administration					
	0	1	2	4	6	10
Bile acids (μg/mL)	4.4	64.4	124.0	6.6	5.2	3.8
Composition (%)						
Lithocholic acid	ND	ND	ND	ND	ND	ND
Chenodeoxycholic acid	16.9	1.4	4.4	18.5	6.0	6.1
α-Muricholic acid	ND	0.7	1.4	5.0	1.5	1.6
β-Muricholic acid	—	6.0	56.9	20.5	5.0	—
Hyodeoxycholic acid	2.1	4.6	0.7	1.9	2.4	8.6
Peak 8†	1.4	1.8	0.5	ND	0.9	ND
Cholic acid	72.2	75.7	31.7	45.1	69.3	68.2
Deoxycholic acid	2.5	2.1	0.1	1.7	4.2	4.1
Others	5.0	7.6	4.2	7.4	10.8	11.4

Samples of 5 rats were combined before analysis.
—, included in cholic acid.
ND, not detectable.
† Unidentified.

molar basis. Therefore, the molar ratio of cholesterol and phospholipids in the bile was about 1:22.

The changes in the serum and liver lipid levels are given in Table 7. The serum cholesterol and phospholipid levels significantly increased on day 1 and further increased on day 2, but gradually decreased thereafter and returned to control levels on day 10. The cholesterol/phospholipids ratio remained almost constant but rather increased after day 4. The serum triglyceride level slightly decreased after ANIT administration. The liver weight per 100 g body weight rather increased after day 2, reflecting decreases in body weight. The liver cholesterol level slightly increased after ANIT treatment but the phospholipid levels remained unchanged. The liver triglyceride level decreased on day 2 but only slightly on days 6 and 10.

Discussion

The present experiments not only demonstrated that ANIT caused cholestasis in rats, but also clarified the changes in cholesterol and bile acid levels in the bile, serum, liver, urine and feces in relation to cholestasis. The bile flow was inhibited, less than 10% of the control, for at least 2 days after a single oral administration of 100 mg/kg of ANIT, but showed hypersecretion on day 4 and then returned to the control level. The biliary secretions of bile acids, cholesterol and phospholipids changed almost in parallel with the bile flow. These changes are almost consistent with previous reports (Maeyama, 1977, Fukumoto et al., 1980, Lock et al., 1982).

In the previous experiment, we examined changes in bile flow and biliary bile acid secretion after release of the bile duct ligation in rats (Fujio et al., 1989). The release after a short period of obstruction restored normal bile flow but release after a long period of obstruction did not. When the ligation was released by 1 week, bile flow and biliary bile acid secretion increased once over the control levels and were then restored to the normal ranges. When the ligation was released after 2 weeks, the bile flow markedly increased and remained high thereafter, while the biliary bile acid secretion gradually increased to the normal range. The present data in the ANIT treated rats coincide with the pattern observed after a short period ligation.

As shown in Fig. 1A, the spots in the region where the bile acid secretion was less than 10 mg/h were under the regression line. The bile acid dependent bile flow is provided by the biliary bile acid composition and is increased by non-micellar bile acids such as oxo-bile acids (Loria et al., 1989). ANIT treatment caused changes in bile acid composition, an increase of β-muricholic acid with a concomitant decrease of cholic acid, but showed no remarkable increase of oxo-bile acids (Table 5). Therefore, it