Altered Bile Acid Metabolism in Nonobese, Spontaneously Diabetic (NOD) Mice

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SUMMARY

Cholesterol and bile acid metabolism was examined in nonobese, spontaneously diabetic (NOD) female mice before and after the development of diabetes. After the development of glucosuria, the plasma and liver cholesterol levels, gallbladder bile weight after 5-h fasting, biliary cholesterol, phospholipid and bile acid concentrations, the lithogenic index, the pool size of bile acids, and fecal sterol excretion markedly increased, but fecal bile acid excretion and fractional turnover rates for the cholic acid and chenodeoxycholic acid groups decreased. The distribution percentage of bile acids in the small intestine did not change, but it increased in the gallbladder and decreased in the large intestine. One striking finding was a change in the bile acid composition: increases were recorded in cholic and deoxycholic acids while decreases occurred in bile acids derived from chenodeoxycholic acid, such as β-muricholic and ursodeoxycholic acids in the bile and α-muricholic, β-muricholic, ω-muricholic, and hyodeoxycholic acids in the feces. Therefore, the cholic acid group/chenodeoxycholic acid group (CA/CDCA) ratio increased in the bile, feces, and small and large intestines after the development of diabetes. These changes were very similar to those observed in alloxan-treated mice, suggesting that the changes found in NOD mice are caused by insulin deficiency.

DIABETES 1985; 34:79–83.

Makino and Tochino[1] established a nonobese, diabetic mouse (NOD) strain that abruptly develops diabetes with signs of polyuria, polydipsia, glucosuria, and hyperglycemia after maturation. The incidence differs with the sex: 60–80% in females and about 10% in males when compared at around 20 wk of age.[2] This diabetes is characterized by infiltration of lymphocytes into the islets of Langerhans, conspicuous reduction in the number of β-cells and the size of islets, and insulin deficiency.[2,3]

The cholesterol and bile acid metabolism is markedly altered in insulin-deficient, diabetic animals such as alloxan- or streptozocin-treated rats.[4–7] In alloxan-diabetic mice, plasma and liver cholesterol levels increase, biliary cholesterol, phospholipid and bile acid concentrations, and the pool size of bile acids greatly increase, and the bile acid composition significantly changes; cholic acid increases with a concomitant decrease in β-muricholic acid.[8]

In the present experiments, we examined plasma and liver lipid levels, biliary and fecal excretion of bile acids, and the pool size and distribution of bile acids in NOD female mice before and after the development of diabetes in an effort to elucidate the characteristics of this NOD mouse strain.

MATERIALS AND METHODS

Animals. NOD female mice bred in our laboratories (Shionogi Aburahi Laboratories, Shiga, Japan) were individually housed in metabolic cages at the age of 16 wk and kept in an air-conditioned room (25 ± 1°C, 50–60% humidity) lighted 12 h/day (8:00 a.m. to 8:00 p.m.) with free access to chow diet (Japan Clea, CA-1) and tap water. The urinary glucose was checked every day except Saturday and Sunday. The animals were divided into two groups, one that was killed just before diabetes development at the age of 19 wk, and another that was killed 3 wk after the onset of diabetes (a mean age of 22 wk). Feces were collected for two days before killing.

Before autopsy, the animals were made to fast for 5 h and the gallbladder was removed under sodium methylhexabital (sodium 5-(1-cyclohexen-1-yl)-1,5-dimethylbarbiturate) anesthesia (125 mg/kg, i.p.). Next, blood was withdrawn from the abdominal aorta with a heparinized syringe and the liver and small and large intestines, with their contents, were removed.

Biliary lipid determination. The gallbladder was crushed in 20 ml ethanol with a glass rod and biliary lipids were extracted by refluxing for 10 min at 80–90°C. After filtration,

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Received for publication 14 April 1984 and in revised form 30 June 1984.

TABLE 1
Blood glucose level and plasma and liver lipid levels before and after the development of glucosuria in NOD female mice

	Before development	After development
No. of mice	6	6
Body weight (g)	25.1 ± 0.30*	23.5 ± 1.16
Blood glucose (mg/dl)	203 ± 12.2	555 ± 9.2†
Plasma cholesterol (mg/dl)	57 ± 3.4	124 ± 14.9†
Plasma phospholipids (mg/dl)	216 ± 13.4	352 ± 29.2†
Plasma C/P ratio	0.27 ± 0.022	0.35 ± 0.014†
Liver wt (g)	1.24 ± 0.045	1.47 ± 0.067†
Liver wt (g/10 g body wt)	0.49 ± 0.017	0.63 ± 0.001†
Liver cholesterol (mg/g)	6.15 ± 0.045	8.71 ± 0.545†
Liver phospholipids (mg/g)	33.3 ± 3.07	20.3 ± 4.35†

*Mean ± SEM.
†Statistically significant ($P < 0.05$).

a portion was evaporated to dryness under a stream of nitrogen and the residue was hydrolyzed in 4 ml of 1.25 N sodium hydroxide solution at 120°C for 6 h. Cholesterol was extracted with diethyl ether and then the bile acids were extracted with diethyl ether after the mixture had been acidified with 2 N hydrochloric acid solution.[9] Cholesterol was determined by GLC with a 1% SE-30 column.[10] Bile acids were converted into methylester trifluoroacetate derivatives and determined by GLC with a 1.5% QF-1 and a 1.5% AN-600 column.[10] Phospholipids were determined by the method of Gomori.[11] Cholesterol and taurocholic acid added to the bile were quantitatively recovered by the present procedures. The recovery of phospholipids was not examined. In the present experiments, the gallbladder bile was analyzed together with gallbladder tissue, but the weight of one sample of gallbladder tissue was about 1 mg or less when examined after the bile had been washed out with saline. The mean cholesterol content in one sample of gallbladder tissue was 2.62 μg and the phospholipid content was 39.4 μg, which did not seem to affect the values of biliary lipids. Lithogenic indexes were calculated by the formula of Thomas and Hofmann.[12]

Fecal sterol and bile acid determination. Fecal sterols and bile acids were determined as reported previously.[9,10,13] Briefly, dried and powdered feces were extracted with absolute ethanol and petroleum ether and hydrolyzed in 1.25 N sodium hydroxide solution at 120°C for 6 h. After extraction of the sterols with 2.5 vol of diethyl ether three times, the hydrolysate was acidified to pH 2 or below with 2 N hydrochloric acid, and bile acids were extracted with 2.5 vol of diethyl ether three times. The sterols and bile acids were quantified by GLC with a 1% SE-30 column and a 1.5% QF-1 column, respectively.

To determine the extraction efficiency of sterols and bile acids from feces, a dose of 1 μCi/68 μg/mouse of cholesterol-4-^{14}C (Amersham Japan Co., Tokyo, Japan) was administered once orally to male mice and the feces were collected daily for 1 wk. The feces were extracted by procedures described previously.[9,10,13] Portions of the feces before ethanol extraction and the residues after ethanol extraction were combusted using a Packard TRI-CARB Sample Oxidizer Model B 0306 and the radioactivities were determined. The ethanol extract was evaporated to dryness and hydrolyzed in 1.25 N sodium hydroxide solution at 120°C for 6 h, then the sterols and bile acids were extracted with ethyl ether. The radioactivities in the ethyl ether extracts were also determined by the combustion method. The extraction efficiency with ethanol was 95% and the efficiency after the whole procedure was 87%.

Tissue bile acid determination. The small and large intestines with their contents were homogenized with distilled water and lyophilized. The lyophilized preparations were extracted three times with 100 ml of absolute ethanol by refluxing for 1 h at 85–90°C. The filtrates were combined and evaporated to dryness under reduced pressure. The residue for the large intestine was dissolved in 10–15 ml of 70% methanol and neutral lipids were removed by extraction with an equal volume of n-hexane. The 70% methanol layer was evaporated to dryness. The residues were dissolved in 1.25 N sodium hydroxide solution, hydrolyzed at 120°C for 6 h, and acidified with 2 N hydrochloric acid. Next, the bile acids were extracted with diethyl ether and quantified by GLC with 1.5% QF-1 and 1.5% AN-600 columns. The extraction efficiency of the intestinal bile acids was not examined but, since that for the fecal bile acids was over 90%,[13] similar values could be expected for the procedures.

Plasma and liver lipid determination. Blood was centrifuged at 3000 rpm (1800 × *g*) for 15 min to separate the plasma. About 1 g of the largest lobe of the liver (*lobus sinistra externa*) was excised and homogenized with 9 vol of ice-chilled water using an Ultra-Turrax TP 18-10 (IKE-WERK, Janke and Kunkel KG, FRG). The plasma and the liver homogenate were extracted with 10 vol of ethanol by refluxing for 20 min at 90–95°C. Portions of the extracts were

TABLE 2
Gallbladder bile and biliary lipid levels before and after the development of glucosuria in NOD female mice

	Before development	After development
No. of mice	6	6
Bile weight (mg)	14.4 ± 2.98*	35.8 ± 7.22†
Cholesterol (mg/g)	1.44 ± 0.161	3.92 ± 0.397†
Phospholipids (mg/g)	22.20 ± 4.695	35.35 ± 2.293
Bile acids (mg/g)	41.74 ± 6.073	69.50 ± 7.170†
Cholesterol (mole %)	2.9 ± 0.33	4.5 ± 0.53†
Phospholipids (mole %)	22.0 ± 6.45	19.7 ± 1.39
Bile acids (mole %)	75.1 ± 6.48	75.9 ± 1.64
Lithogenic index	0.33 ± 0.033	0.42 ± 0.027†

*Mean ± SEM.
†Statistically significant ($P < 0.05$).

TABLE 3
Biliary bile acid composition before and after the development of glucosuria in NOD female mice

	Before development	After development
No. of mice	6	6
Cholic acid group (%)	55.2 ± 2.20*	69.2 ± 1.68‡
Cholic acid	45.8 ± 1.98	56.0 ± 2.01‡
Deoxycholic acid	6.8 ± 1.32	11.9 ± 1.91‡
3α,12α-Dihydroxy-7-oxo-	2.6 ± 0.54	1.4 ± 0.23
Chenodeoxycholic acid group (%)	41.7 ± 2.26	28.5 ± 1.89‡
Chenodeoxycholic acid	3.1 ± 0.23	2.0 ± 0.44
Lithocholic acid	0.4 ± 0.06	0.3 ± 0.05
Hyodeoxycholic acid	2.0 ± 0.12	1.2 ± 0.26
Ursodeoxycholic acid	6.8 ± 0.95	3.8 ± 0.48‡
α-Muricholic acid	4.1 ± 0.71	4.1 ± 0.68
β-Muricholic acid	22.2 ± 1.78	14.3 ± 0.60‡
ω-Muricholic acid	3.4 ± 0.91	3.1 ± 0.68
3α-Hydroxy-7-oxo-	<0.1	<0.1
3α-Hydroxy-6-oxo-	<0.1	0.1 ± 0.04
Others† (%)	2.9 ± 0.44	3.1 ± 0.30
CA/CDCA ratio	1.35 ± 0.128	2.49 ± 0.205‡

*Mean ± SEM.
†Others comprise unidentified peaks with RRT 0.74 and 0.85.
‡Statistically significant ($P < 0.05$).

hydrolyzed in 15% potassium hydroxide:50% ethanol solution at 75–80°C for 20 min and cholesterol was extracted three times with 2.5 vol of petroleum ether. The cholesterol was quantified by GLC with a 1% SE-30 column. Phospholipids were determined from the ethanol extracts by the method of Gomori.[11] The recovery of plasma and liver cholesterol was almost 100%, but that for phospholipids was not examined.

Blood and urinary glucose determination. Blood and urinary glucose levels were determined using a Blood Sugar-GOD-Perid-Test (Boehringer Mannheim, FRG).

Pool size of bile acids. The pool size of bile acids was obtained by totaling the amounts in the bile and in the small and large intestines.[14]

Statistical analysis. The results are expressed as the mean ± SEM. Student's *t*-test was used to determine statistical significance.

RESULTS

Blood glucose level and plasma and liver lipid levels. Table 1 shows the blood glucose level and the plasma and liver cholesterol and phospholipid levels before and after the development of diabetes in NOD female mice. After the onset of diabetes, the blood glucose level, plasma cholesterol and phospholipid levels, plasma cholesterol/phospholipid ratio, liver weight, and liver cholesterol level markedly increased, but the liver phospholipid level decreased.

Biliary lipid levels. The gallbladder bile weight after 5 h of fasting markedly increased after the development of diabetes, and the biliary cholesterol, phospholipid, and bile acid concentrations also increased (Table 2). The increase in the cholesterol concentration was most significant, and the cholesterol molar concentration ratio (mole %) and the lithogenic index increased.

The biliary bile acid composition, with each individual bile acid being expressed as a percentage of the total, also changed after the development of diabetes (Table 3). Cholic and deoxycholic acids increased, while ursodeoxycholic and β-muricholic acids decreased, resulting in an increase in the cholic acid group/chenodeoxycholic acid group (CA/CDCA) ratio.

Diet intake, feces weight, and fecal sterols and bile acids. The daily diet and water intakes greatly increased and the feces dry weight also increased (Table 4). The coprostanol and cholesterol excretion and, thus, the total sterol excretion, increased, but the total bile acid excretion decreased, with

TABLE 4
Diet and water intakes and fecal sterol and bile acid excretions before and after the development of glucosuria in NOD female mice

	Before development	After development
No. of mice	6	6
Diet intake (g/day)	4.3 ± 0.17*	8.8 ± 0.46†
Water intake (g/day)	8.3 ± 0.80	46.3 ± 3.46†
Feces dry wt (g/day)	1.53 ± 0.035	2.31 ± 0.167†
Total sterols (mg/day)	2.93 ± 0.205	7.11 ± 0.619†
Coprostanol (mg/day)	0.13 ± 0.037	1.15 ± 0.341†
Cholesterol (mg/day)	2.80 ± 0.194	5.96 ± 0.550†
Total bile acids (mg/day)	2.25 ± 0.092	1.59 ± 0.163†
Cholic acid group (mg/day)	0.68 ± 0.033	0.85 ± 0.076
Chenodeoxycholic acid group (mg/day)	1.52 ± 0.076	0.70 ± 0.087†
CA/CDCA ratio	0.45 ± 0.025	1.25 ± 0.084†

*Mean ± SEM.
†Statistically significant ($P < 0.05$).

TABLE 5
Fecal bile acid composition before and after the development of glucosuria in NOD female mice

	Before development	After development
No. of mice	6	6
Cholic acid group (%)		
Cholic acid	1.8 ± 0.44*	2.5 ± 1.06
Deoxycholic acid	23.6 ± 1.56	41.5 ± 4.40‡
Ursocholic acid	2.2 ± 0.62	0.6 ± 0.00
3α,12α-Dihydroxy-7-oxo-	5.3 ± 0.00	10.1 ± 1.38‡
Chenodeoxycholic acid group (%)		
Lithocholic acid	5.8 ± 0.44	6.3 ± 0.88
Hyodeoxycholic acid	2.7 ± 0.44	1.3 ± 0.00‡
α-Muricholic acid	4.4 ± 0.00	1.9 ± 0.00‡
β-Muricholic acid	14.7 ± 2.53	8.2 ± 1.70‡
ω-Muricholic acid	38.2 ± 3.78	25.2 ± 2.58‡
3α-Hydroxy-6-oxo-	0.4 ± 0.00	0.6 ± 0.00
Others† (%)	1.8 ± 0.00	2.5 ± 0.00‡

*Mean ± SEM.
†Others comprise unidentified peaks with RRT 0.74 and 0.85.
‡Statistically significant ($P < 0.05$).

the cholic acid group not changing but the chenodeoxycholic acid group decreasing to cause an increase in the CA/CDCA ratio.

Table 5 shows the fecal bile acid composition. In mouse feces, ω-muricholic acid and deoxycholic acid, formed by intestinal bacteria from β-muricholic acid and cholic acid, respectively, are major constituents. After the development of diabetes, decreases were registered in ω-muricholic, β-muricholic, α-muricholic, and hyodeoxycholic acids, but increases in deoxycholic and 3α,12α-dihydroxy-7-oxo-bile acids derived from cholic acid were seen.

Pool size, fractional turnover rate, and distribution of bile acids. As shown in Table 6, the pool size increased twofold or more after the development of diabetes and the increase was mainly due to increases of cholic acid and its related bile acids (cholic acid group). Therefore, the CA/CDCA ratio increased. The fractional turnover rate for the chenodeoxycholic acid group was higher than that for the cholic acid group in NOD mice, and both the values for the cholic acid and chenodeoxycholic acid groups markedly decreased after the development of diabetes. About 80% of the total bile acids were found in the small intestine before the development of diabetes, and this value did not change after its development. The percentage in the gallbladder markedly increased while that in the large intestine decreased after diabetes development.

DISCUSSION

The present experiments clearly demonstrated that blood glucose level, plasma and liver cholesterol levels, biliary lipid concentrations (especially cholesterol concentration), and the pool size of bile acids markedly increased after the development of diabetes in NOD female mice. These changes occurred only 3 wk after glucosuria was first observed and were very similar to those observed in alloxan-diabetic mice.[8] The diet and water intakes and the liver weight also increased, while the body weight did not change during the 3 wk.

Among the changes in bile acid metabolism, the most striking finding was the change in the bile acid composition. Cholic and deoxycholic acids increased and ursodeoxycholic and β-muricholic acids decreased; thus, the CA/CDCA ratio increased. Similar changes were found in the fecal bile acid composition. Since bile acids are transformed by intestinal bacteria, the bile acid composition in the feces was somewhat different from that in the bile, but decreases

TABLE 6
Pool size, fractional turnover rate, and distribution of bile acids before and after the development of glucosuria in NOD female mice

	Before development	After development
No. of mice	6	6
Pool size (mg/mouse)	7.56 ± 0.24*	17.11 ± 1.04†
Cholic acid group	4.23 ± 0.23	11.16 ± 0.83†
Chenodeoxycholic acid group	3.26 ± 0.09	5.79 ± 0.42†
Others	0.08 ± 0.01	0.17 ± 0.01†
CA/CDCA ratio	1.30 ± 0.08	1.97 ± 0.17†
Fractional turnover rate		
Cholic acid group	0.164 ± 0.011	0.069 ± 0.004†
Chenodeoxycholic acid group	0.473 ± 0.035	0.126 ± 0.018†
Distribution of bile acids (mg/mouse)		
Bile	0.67 ± 0.20 (9%)	2.80 ± 0.76 (16%)
Small intestine	5.97 ± 0.36 (79%)	13.90 ± 0.95 (81%)†
Large intestine	0.96 ± 0.17 (13%)	0.41 ± 0.07 (2%)†

Percentage of the total pool size is shown in parentheses.
*Mean ± SEM.
†Statistically significant ($P < 0.05$).

were observed in the bile acids derived from chenodeoxycholic acid, such as α-muricholic, β-muricholic, ω-muricholic, and hyodeoxycholic acids, resulting in an increase in the CA/CDCA ratio. The reason for the increase in the CA/CDCA ratio is not known, but Nervi et al.[5] have suggested that insulin plays an important role in the regulation of bile acid synthesis.

The changes found in NOD mice (increases in serum and liver cholesterol levels, biliary lipid concentrations, pool size, and the CA/CDCA ratio) are very similar to those found in alloxan-treated mice.[8] This suggests that the changes in NOD mice are caused by insulin deficiency. In the present experiment, the NOD mice were killed 3 wk after glucosuria was first observed, because most could not live more than 1 mo after its onset without insulin therapy.[2]

Bile acids are mainly absorbed by an active transport mechanism confined to the lower ileum and by passive nonionic diffusion, which occurs at all levels of the gastrointestinal tract, but active transport is considered to be more dominant than passive diffusion.[15] Lack and Weiner[16] demonstrated that more cholic acid was transported by active transport than chenodeoxycholic acid, and Caspary[17] reported that active transport of bile acids was increased greatly in streptozocin-diabetic rats in in vitro experiments. These phenomena, if they occur in vivo, can explain the mechanism by which the pool size and the CA/CDCA ratio increase in diabetic animals. The fractional turnover rate for the chenodeoxycholic acid group was higher than that for the cholic acid group in our NOD mice, suggesting that absorption of the cholic acid group is higher than that of the chenodeoxycholic acid group. Cholic acid is the major constituent in the cholic acid group while β-muricholic acid is predominant in the chenodeoxycholic acid group in the bile acid pool of mice. Therefore, cholic acid may be absorbed more efficiently than β-muricholic acid. The fractional turnover rates for both the cholic acid and chenodeoxycholic acid groups markedly decreased after the development of diabetes. This suggests that the absorption of bile acids increases in diabetes, confirming the observations of Caspary.[17] The decrease of the fractional turnover rate in the chenodeoxycholic acid group seemed to be larger than that in the cholic acid group.

Makino et al.[2] previously reported marked hypercholesterolemia in NOD mice after the development of diabetes; this was confirmed by the present experiment. The mechanisms for the hypercholesterolemia and the cholesterol accumulation in the liver are not yet known, but the increase in the pool size of bile acids, especially of cholic acid, may be a contributing cause, since cholic acid enhances cholesterol absorption much more than chenodeoxycholic acid or ursodeoxycholic acid.[18] Similar data demonstrating the prominent effect of cholic acid on cholesterol absorption have also been reported for rats.[19,20] Cholic acid, when fed to rats with cholesterol, markedly increased serum and liver cholesterol levels, but chenodeoxycholic acid and its related bile acids (viz. lithocholic, hyodeoxycholic, and ursodeoxycholic acids) showed almost no change.[21,22] In addition, our diet contained about 0.1% cholesterol and the diabetic animals ate more than the nondiabetics. Therefore, cholesterol absorption may have increased after the development of diabetes, resulting in increases in the plasma and liver cholesterol levels. On the other hand, Nakayama et al.[23] have demonstrated that hepatic cholesterol synthesis decreases but intestinal synthesis increases in streptozocin-diabetic rats. Although it is not yet known how endogenously synthesized cholesterol contributes to plasma and liver cholesterol levels in mice, the factor(s) should be related to the development of hypercholesterolemia and cholesterol accumulation in the liver after the development of diabetes.

ACKNOWLEDGMENTS

The authors thank H. Takase and Y. Nomura of Shionogi Research Laboratories for their help and discussions. We also thank M. Katayama for preparing the manuscript.

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