ALTERED BILE ACID METABOLISM IN ALLOXAN DIABETIC RATS

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Abstract—Changes of cholesterol, phospholipid, triglyceride or bile acid levels in serum, liver, bile and feces after the treatment with alloxan were examined in Wistar strain male rats. Serum cholesterol, phospholipid and triglyceride levels and liver cholesterol level markedly increased but liver phospholipid and triglyceride levels remained unchanged. The lipid levels in serum very low density and low density lipoproteins were elevated but those in high density lipoprotein were not. Bile flow was not changed but biliary secretion of cholesterol, phospholipid and bile acids markedly increased. Among the biliary bile acid components, cholic acid markedly increased but the amount of chenodeoxycholic acid was similar to that of normal rats. Fecal excretion of deoxycholic acid increased but that of lithocholic and hyodeoxycholic acids decreased, and α-, β- and α-muricholic acids did not change, thus, the total amount of fecal bile acids remained unchanged. Hepatic cholesterol synthesis was markedly depressed, while cholesterol 7α-hydroxylase activity did not change and cytochrome P-450 content was elevated by about 40%. From such evidence, it was apparent that synthesis of cholic acid increased while that of chenodeoxycholic acid decreased and the total amount of bile acids synthesized did not change in the diabetic rats. Furthermore, marked increase of the pool size of cholic acid and hepatic secretion of cholic acid stimulated the absorption of lipids and produced a hyperlipidemia in the diabetic rats.

Serum cholesterol and triglyceride levels in diabetics increase markedly and this increase is related to a high incidence of atherosclerosis (1, 2) and cholesterol gallstones (3). Diabetic rats, treated with alloxan or streptozotocin, also developed marked hyperlipidemia (4-10) with a higher incidence of cardiovascular lesions when fed a high cholesterol diet (7). The mechanism by which marked hyperlipidemia is produced in diabetic rats is not fully understood, but Bierman et al. (6) have postulated that the intake of dietary fat is related to the manifestation of hyperlipidemia, and Nervi et al. (9) found an increase in biliary secretion and daily synthesis of bile acids in alloxan diabetic rats and concluded that hypercholesterolemia is due to an increase in cholesterol absorption. We examined cholesterol or bile acid level in serum, liver, bile and feces of alloxan diabetic rats in an attempt to elucidate the mechanism underlying hypercholesterolemia.

MATERIALS AND METHODS

Male Wistar rats weighing approximately 300 g were kept in an air-conditioned room (25 ± 1°C, 50-60% humidity) lighted 12 hr a day (08:00 to 20:00) and maintained on a com-
ceral balanced stock diet (Japan CLEA CA-1, Tokyo, Japan). The composition of the
diet was as follows; protein 25.5%, lipids 4.0%, carbohydate 53.5%, fiber 4.0%, ash
7.0% and water 6.0%. The content of cholesterol was 0.04-0.05%. The diet also contained
phytosterols such as campesterol and β-sitosterol but the amounts were considerably less
than that of cholesterol. After fasting overnight, the rats were given 40 mg/kg of alloxan
monohydrate iv. One week after the injection, the blood glucose level was determined by
the glucose-oxidase method (Glucostat Reagent Kit, Worthington Biochemical Corp.
Freehold, N.J., U.S.A.) and the rats with a level of 300 to 350 mg/100 ml were chosen for
the experiments. Four to 5 weeks after the treatment with alloxan the rats were individually
caged and feces were collected for two days before sacrifice as described previously (11).
The rats were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the bile duct
was cannulated with PE-10 polyethylene tubing to collect the initial bile for exactly 30 min.
Next, blood was withdrawn by heart puncture and the liver was removed. The liver was
homogenized with nine volumes of physiological saline. Bile was collected between 09:00
and 10:00 in the morning.

**Lipid determination:** Serum was separated by centrifugation at 3000 rpm for 15 min
after the blood had been left 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 nine volumes
of ice-chilled physiological saline utilizing a ULTRA-TURRAX TP 18–10 (IKE-WERK,
Janke & Kunkel KG, West Germany). Serum and liver homogenates were extracted in
10 volumes of ethanol by refluxing for 20 min at 90–95°C. Total cholesterol levels were
determined with portions of the lipid extracts as previously reported (12, 13). Phospholipids
were determined by the method of Gomori (14) and triglycerides by that of Fletcher (15).

Bile was extracted with ethanol; one volume of bile was poured into 20 volumes of
ethanol, boiled for about 5 minutes, and filtered through Toyo Filter Paper No. 2 (Toyo
Roshi Co. Ltd., Tokyo, Japan) after cooling to room temperature. An aliquot of the
filtrate was evaporated to dryness under a stream of nitrogen, and the residue was hydrolyzed
in 2 to 4 ml of 1.25 N sodium hydroxide solution at 120°C for 6 hr. Sterols were extracted
with ethyl ether, and then bile acids after acidifying with 2 N hydrochloric acid solution (11).
Sterols were determined spectrophotometrically (16) and phospholipids were determined
by the method of Gomori (14). Bile acids were methylated with freshly prepared diazo-
methane and then trifluoroacetylated with trifluoroacetic anhydride. The bile acid derivatives
were quantified by gas liquid chromatography utilizing a Shimadzu Gas Chromatograph
Model GC-4BPF (Shimadzu Co. Ltd., Kyoto, Japan) equipped with a hydrogen flame
ionization detector (16). A 1.5 m × 4 mm i.d. glass column packed with 3% QF-1 on 60–80
mesh Chromosorb W was used. The operation temperature was 235°C for the column
and 290°C for the detector. When sterols were analyzed by gas liquid chromatography,
a column packed with 1.5% SE-30 on 60–80 mesh Chromosorb W was used at 230°C.

Fecal sterols and bile acids were determined as reported previously (11). Briefly,
dried and powdered feces were extracted with absolute ethanol and hydrolyzed in sodium
hydroxide solution at 120°C under pressure; the sterols and bile acids were then quantified
by gas liquid chromatography. Fecal sterols comprised cholesterol, coprostanol, \( \Delta^7 \)-cholestenol and phytosterols such as campesterol, \( \beta \)-sitosterol and their metabolites (11). In the present experiment, \( \Delta^7 \)-cholestenol and phytosterols were not included in the fecal sterols.

**Serum lipoprotein lipid analysis:** Ultracentrifugation of serum was performed according to the method described by Havel et al. (17) with separation into four fractions; chylomicron \( (d<1.006) \), very low density lipoprotein \( (VLDL, 1.006<d<1.019) \), low density lipoprotein \( (LDL, 1.019<d<1.063) \) and high density lipoprotein \( (HDL, d>1.063) \). Each fraction was extracted with chloroform-methanol \( (2:1) \) (18) for the determination of lipids.

**In vitro cholesterol synthesis using liver slices:** A 0.3 g sample of liver slices about 0.5 mm thick was incubated in 3 ml Krebs-Ringer phosphate buffer \( (pH 7.4) \) containing 2 \( \mu \)Ci \( (20 \mu \)mole) of sodium acetate-\( 1^{14}C \) \( (55.3 \)mCi/m mole, Schwarz/Mann, New York, U.S.A.) at \( 37^\circ C \) for 3 hr with air as the gas phase. The incorporation rate of sodium acetate-\( 1^{14}C \) into digitonin-precipitable sterols was determined by procedures described previously (19).

**Other determinations:** Cholesterol 7a-hydroxylase activity was determined by the isotope incorporation method (20) based on the method of Mitropoulos and Balasubramanian (21). Cytochrome P-450 in liver homogenate was determined by dithionite-difference spectroscopy of CO-pretreated samples (22). Statistical significance was estimated by Student’s \( t \) test.

### RESULTS

Serum and liver lipid levels in the control and alloxan diabetic rats are given in Table 1. Body weight slightly decreased and blood glucose level markedly increased in the diabetic rats. Serum cholesterol, phospholipid and triglyceride levels in the diabetic rats were significantly higher than those in the control rats. As increase in the serum phospholipid level was less than that of the cholesterol increase, the cholesterol/phospholipid (C/P) ratio increased in the diabetic rats. Both liver weight and cholesterol level increased in the

| Table 1. Blood glucose and serum and liver lipids in control and alloxan diabetic rats |
|-----------------------------------------------|
| **No. of rats** | **Control** | **Alloxan** |
|-----------------|-------------|-------------|
| Body weight final (g) | \( 344 \pm 7.1 \) \( ^{a} \) | \( 310 \pm 31.8 \) \( ^{b} \) |
| Blood glucose (mg/100 ml) | \( 106 \pm 3.3 \) | \( 374 \pm 16.4 \) * |
| Serum cholesterol (mg/100 ml) | \( 84 \pm 7.7 \) | \( 171 \pm 30.1 \) * |
| phosopholipid (mg/100 ml) | \( 138 \pm 10.1 \) | \( 180 \pm 17.4 \) |
| triglyceride (mg/100 ml) | \( 90 \pm 16.0 \) | \( 156 \pm 46.8 \) |
| C/P ratio | \( 0.60 \pm 0.020 \) | \( 0.91 \pm 0.087 \) * |
| Liver weight (g/100 g BW) | \( 3.57 \pm 0.145 \) | \( 4.09 \pm 0.087 \) * |
| cholesterol (mg/g) | \( 3.44 \pm 0.415 \) | \( 5.02 \pm 0.544 \) * |
| phospholipid (mg/g) | \( 39.9 \pm 1.15 \) | \( 43.5 \pm 1.31 \) |
| triglyceride (mg/g) | \( 6.73 \pm 0.599 \) | \( 8.47 \pm 1.878 \) |

\( ^{a} \) Mean \( \pm \) S.E.  *Statistically significant against control \( (P<0.05) \).
diabetic rats but phospholipid and triglyceride levels remained unchanged. The amount of lipids in serum VLDL and LDL fractions markedly increased in the diabetic rats but the amount of these lipids in HDL did not change (Table 2). The amount of lipids in LDL was higher than that in VLDL but the increase ratio was larger in VLDL.

Bile flow and biliary secretion of cholesterol, phospholipid and bile acids are given in Table 3. Bile flow in the diabetic rats was similar to that in the controls but biliary lipids in the diabetic rats increased greatly; the amount of cholesterol was 1.5 times, phospholipid 3 times, and bile acids 2.3 times that of the control group, respectively. Among the biliary bile acids, cholic and deoxycholic acids markedly increased but chenodeoxycholic acid and its secondary bile acids such as α-muricholic or hyodeoxycholic acid decreased. The term “others” in the table includes ketonic bile acids and unidentified peaks considered to be bile acids.

Contrary to the increase in biliary secretion of bile acids, no significant increase in their fecal excretion was found in the diabetic rats as shown in Table 4. Although diet intake and the mass of feces were larger in the diabetic rats, daily excretion of bile acids was similar to that in the control rats. In the fecal bile acid composition, deoxycholic acid markedly increased, while lithocholic and hyodeoxycholic acids decreased and α-, β- and ω-muricholic

### Table 2. Lipoprotein lipids in control and alloxan diabetic rats

| Lipoprotein | Control | Alloxan |
|-------------|---------|---------|
| Cholesterol | μg/ml   | μg/ml   |
| Chylomicron (d < 1.006) | 8 (1) | 19 (1) |
| VLDL (1.006 < d < 1.019) | 59 (9) | 504 (26) |
| LDL (1.019 < d < 1.063) | 207 (32) | 1015 (51) |
| HDL (d > 1.063) | 366 (57) | 439 (22) |
| Phospholipid | μg/ml |
| Triglyceride | μg/ml |

Contrary to the increase in biliary secretion of bile acids, no significant increase in their fecal excretion was found in the diabetic rats as shown in Table 4. Although diet intake and the mass of feces were larger in the diabetic rats, daily excretion of bile acids was similar to that in the control rats. In the fecal bile acid composition, deoxycholic acid markedly increased, while lithocholic and hyodeoxycholic acids decreased and α-, β- and ω-muricholic

### Table 3. Bile flow and biliary lipid secretion in control and alloxan diabetic rats

| Bile flow (ml/hr/rat) | Control | Alloxan |
|-----------------------|---------|---------|
| Cholesterol (mg/hr/rat) | 1.33 ± 0.086 * | 1.33 ± 0.082 * |
| Phospholipid (mg/hr/rat) | 0.26 ± 0.018 | 0.39 ± 0.040 * |
| Bile acids (mg/hr/rat) | 4.49 ± 0.433 | 14.22 ± 0.850 * |
| Lithocholic (mg/hr/rat) | 17.93 ± 1.415 | 41.38 ± 4.640 * |
| Deoxycholic (mg/hr/rat) | 0.10 ± 0.013 | 0.06 ± 0.011 |
| α-Muricholic (mg/hr/rat) | 0.62 ± 0.111 | 1.98 ± 0.424 * |
| Chenodeoxycholic (mg/hr/rat) | 0.15 ± 0.040 | 0.16 ± 0.021 |
| Hyodeoxycholic (mg/hr/rat) | 0.94 ± 0.134 | 0.65 ± 0.087 |
| Cholic (mg/hr/rat) | 0.66 ± 0.139 | 0.06 ± 0.043 * |
| Others (mg/hr/rat) | 11.35 ± 0.917 | 35.01 ± 4.102 * |
| Others (mg/hr/rat) | 4.11 ± 0.951 | 3.46 ± 0.676 |

a) Mean ± S.E.  *Statistically significant against control (P < 0.05).
acids did not change in the diabetic rats. Fecal excretion of sterols did not change, and the ratios of coprostanol and cholesterol were not significantly different between the control and diabetic rats.

**TABLE 4. Fecal excretion of sterols and bile acids in control and alloxan diabetic rats**

|                      | Control     | Alloxan     |
|----------------------|-------------|-------------|
| No. of rats          | 8           | 8           |
| Diet intake (g/day/rat) | 20.0 ± 0.37* | 33.2 ± 2.87* |
| Feces (g/day/rat)    | 4.40 ± 0.121 | 9.54 ± 0.508* |
| Sterols (mg/day/rat) | 11.88 ± 1.103 | 9.52 ± 2.160 |
| Coprostanol (mg/day/rat) | 5.78 ± 0.631 | 4.25 ± 1.126 |
| Cholesterol (mg/day/rat) | 6.10 ± 0.562 | 5.27 ± 1.130 |
| Bile acids (mg/day/rat) | 11.15 ± 0.700 | 12.74 ± 2.041 |
| Lithocholic (mg/day/rat) | 1.11 ± 0.128  | 0.67 ± 0.083* |
| Deoxycholic (mg/day/rat) | 3.00 ± 0.264  | 7.03 ± 1.462* |
| α-Muricholic (mg/day/rat) | 0.19 ± 0.035  | 0.24 ± 0.038 |
| Hyodeoxycholic (mg/day/rat) | 3.21 ± 0.359 | 0.77 ± 0.353* |
| β-Muricholic (mg/day/rat) | 1.52 ± 0.342  | 1.12 ± 0.349 |
| αα-Muricholic (mg/day/rat) | 1.60 ± 0.215  | 1.99 ± 0.441 |
| Others (mg/day/rat)    | 0.53 ± 0.078  | 0.92 ± 0.629  |

a) Mean ± S.E.  
*Statistically significant against control (P<0.05).

**TABLE 5. Fractional synthetic rates of cholic and chenodeoxycholic acids in control and alloxan diabetic rats**

|                      | Control     | Alloxan     |
|----------------------|-------------|-------------|
| No. of rats          | 8           | 8           |
| Cholic acid (mg/day/rat) | 3.00 ± 0.264* | 7.03 ± 1.462* |
| Chenodeoxycholic acid (mg/day/rat) | 7.62 ± 0.429  | 4.78 ± 0.970* |
| Others (mg/day/rat)    | 0.53 ± 0.078  | 0.92 ± 0.629  |
| Total (mg/day/rat)     | 11.15 ± 0.700 | 12.74 ± 2.041 |

a) Mean ± S.E.  
*Statistically significant against control (P<0.05).

**TABLE 6. Cholesterol synthesis, cholesterol 7α-hydroxylase activity and cytochrome P-450 content in the liver from control and alloxan diabetic rats**

|                      | Control     | Alloxan     |
|----------------------|-------------|-------------|
| 1) Cholesterol synthesis |             |             |
| 3 hr incorporation (dpm/3 hr/g liver) | 7727 ± 14* (3) | 748 ± 266* (3) |
| 2) Cholesterol 7α-hydroxylase Activity (n moles/hr/g liver) | 26.2 ± 2.32* (4) | 21.0 ± 2.72* (4) |
| 3) Cytochrome P-450 content |             |             |
| Content (n moles/g liver) | 44.0 ± 3.58* (8) | 60.0 ± 5.65* (8) |

a) Mean ± S.E.  
( ) No. of rats.  
*Statistically significant against control (P<0.05).

acids did not change in the diabetic rats. Fecal excretion of sterols did not change, and the ratios of coprostanol and cholesterol were not significantly different between the control and diabetic rats.

The fractional synthetic rates of cholic and chenodeoxycholic acids in both control and diabetic rats were calculated from the data of Table 4 on the assumption that deoxycholic acids did not change in the diabetic rats. Fecal excretion of sterols did not change, and the ratios of coprostanol and cholesterol were not significantly different between the control and diabetic rats.
acid was formed from cholic acid, and lithocholic, hyodeoxycholic, α, β- and ω-muricholic acids from chenodeoxycholic acid, though some components remained unidentified. The results are shown in Table 5. It was inferred from the calculation that the synthesis of cholic acid increased but that of chenodeoxycholic acid decreased, resulting in no change in the total amount of bile acids in the diabetic rats.

Hepatic activity to synthesize cholesterol decreased greatly but cholesterol 7α-hydroxylase activity was not significantly altered and cytochrome P-450 content increased (Table 6) in the diabetic rats. When individual values for cytochrome P-450 content and cholesterol level in the liver were plotted, a positive correlation was noted and such is shown in Fig. 1. It would thus appear that the increase of cytochrome P-450 occurred in parallel with the increase of cholesterol level.

**DISCUSSION**

A marked increase in serum lipids was found in alloxan diabetic rats and such is in good accordance with previous reports (4–10). Liver cholesterol level also increased in the diabetic rats but to a lesser extent than the increase in the serum. The increase of serum lipids was mainly due to the increase of VLDL and LDL. Bar-On et al. (10) noted a marked increase of all lipoprotein fractions in streptozotocin diabetic rats, however, no such increase in HDL lipids was evident in our animals. In addition, the increase of VLDL and LDL cholesterol was smaller in their rats than in ours. The large ingestion of food by our rats may explain this difference since sucrose feeding produced a marked increase in VLDL and even LDL cholesterol (10). In human diabetes mellitus, the increase of LDL-cholesterol has been reported (23), but in rats, the increase of VLDL and LDL was remarkable. The increase of VLDL may be related to the decrease of lipoprotein lipase activity in the diabetic rats (24).

Cholesterol synthesis in liver slices decreased greatly in the diabetic rats (Table 6), thus confirming previous reports (25, 26). This decrease is probably the result of cholesterol accumulation in the liver. A marked increase of biliary secretion of bile acids was predominant in the diabetic rats. The increase was mainly due to the increase of cholic and deoxy-
Cholic acids. Chenodeoxycholic acid, on the contrary, decreased. Nervi et al. (9) also noted the increase of biliary excretion of bile acids and expansion of the trihydroxycholanoic acid pool in alloxan diabetic rats.

Normal rats produced no hypercholesterolemia when fed cholesterol but did so after simultaneous feeding of bile acids with cholesterol. Among the various bile acids, cholic acid was most effective in producing hypercholesterolemia while Chenodeoxycholic acid was ineffective (27, 28). Deoxycholic acid was effective but less so than was cholic acid. Thus, the hypercholesterolemia in the diabetic rats is partly due to the increase of hepatic cholic and deoxycholic acid secretion. In fact, Nervi et al. (9) found an increase of cholesterol absorption in diabetic rats. Biliary secretion of cholesterol and phospholipid also increased in the diabetic rats. Since bile acids stimulate the synthesis (29) and secretion (20-32) of these biliary lipids, the increase can be explained by the increase of bile acids. The increase of cholesterol was less but that of phospholipid was larger than the increase of bile acids. Contrary to the increase of biliary lipids, no increase of bile flow was found in the diabetic rats. According to the current concept, bile flow is regulated by bile salt-dependent (33) and -independent mechanisms (34) and the increase of biliary bile acids is expected to increase the bile flow. However, there was no increase in bile flow in the present experiments. The relationship between bile acid secretion and bile flow in both normal (closed circles) and diabetic rats (open circles) was plotted in Fig. 2. Although there was more fluctuation in the data for the diabetic rats, the slope was smaller than that for the normal rats indicating that their excretory mechanism, especially bile salt dependent bile flow, was inadequate.

In a steady state, the fecal excretion rate of bile acid is presumed to correspond to the hepatic synthetic rate of bile acids. In the present experiments, the fecal excretion of bile acids in the diabetic rats was similar to that in the control rats. In addition, hepatic cholesteryl 7a-hydroxylase activity did not increase in these rats. The results indicate that total bile acid synthesis did not increase in the diabetic rats. This conclusion, however, differs from that of Nervi et al. (9) who found an increase of daily synthesis of bile acids in alloxan diabetic rats. A portion of bile acids was excreted into the urine in our rats, but the maximum amount was 1 mg a day (Uchida et al., unpublished data). This value is too low to explain the discrepancy.
As shown in Table 5, the synthesis of cholic acid increased but that of chenodeoxycholic acid decreased without producing a change in the total amount of bile acids in the diabetic rats. Since Nervi et al. (9) came to the same conclusion based on the kinetics of trihydroxycholanoic acid, the discrepancy may be explained by the decrease of chenodeoxycholic acid synthesis. Sadahiro et al. (8) noted a decrease of bile acid synthesis in diabetic rats but used a large dosage of alloxan monohydrate (100 mg/kg) to produce the diabetic state. Such a severe condition may depress most functions in the liver including bile acid synthesis. Cytochrome P-450 content in the liver markedly increased in the diabetic rats. Since there are several microsomal hydroxylation reactions in the synthesis of bile acid from cholesterol, e.g. 7α-, 12α-, or 26-hydroxylation, involve cytochrome P-450 (35-37), and a positive correlation was found between cholesterol concentration and cytochrome P-450 content in the liver (Fig. 1), a close relationship between bile acid synthesis and hepatic cytochrome P-450 content was expected, but the present data failed to show this. The alloxan diabetic rats showed a higher content of cytochrome P-450 but no increase in bile acid synthesis. Mitropoulos and Balasubramaniam (38) examined the effects of various treatments on microsomal cholesterol 7α-hydroxylase activity and cytochrome P-450 content and showed that these activities and contents did not always change in parallel. Hepatic concentration of P-450, expressed as a function of g tissue, increased but since microsomal concentration did not increase, the increase of cytochrome P-450 was due to the proliferation of endoplasmic reticulum in the diabetic rats, and such was confirmed by electron microscopy (39). The increase of hepatic cholesterol level may be partly due to the increase of microsomes.

In conclusion, our present experiments demonstrated a considerable increase in the biliary secretion of bile acids while there was no increase in fecal excretion in alloxan diabetic rats. Cholic acid content increased remarkably, while chenodeoxycholic acid content decreased. These results suggest that the pool size of cholic acid greatly increased in the diabetic rats and this increase in hepatic cholic acid secretion enhanced cholesterol absorption thus resulting in marked hypercholesterolemia in these rats.

Ingesting a larger amount of food, the diabetic rats consumed about double the amount of dietary cholesterol as compared to normal rats. This increase may explain the hypercholesterolemia in the diabetic rats, but since the cholesterol diet failed to produce hypercholesterolemia in normal rats (11) this state in the diabetic rats is probably caused primarily by the increased synthesis cholic acid.

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