A CAUSE OF HYPERCHOLESTEROLEMIA IN ALLOXAN DIABETIC RAT

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Summary In studies on the cause of hypercholesterolemia in alloxan diabetic rats, the pool size and basal daily synthesis of conjugated bile salts were measured by the washout method and neutral sterols in the luminal contents were determined using cholestyramine. The results showed that in diabetic rats: 1) the biliary excretions of cholesterol and bile salts were significantly increased; 2) the pool size of conjugated bile salts was increased and its rate of synthesis was higher than in controls; 3) the amount of neutral sterols in the luminal contents doubled when cholesterol absorption was inhibited by administration of cholestyramine; 4) the amount of neutral sterols excreted in the feces was the same as in controls. Thus, it was concluded that hypercholesterolemia may be partly caused by an increased rate of intestinal absorption of cholesterol, derived mainly from sloughed off epithelial cells and bile, and due to facilitated micellar formation by the increased amount of bile salts in the intestine.

Hepatic cholesterol synthesis in diabetic rats has been reported to be increased (1), normal (2), or decreased (3). Recently, we found that these discrepant results were due to differences in the dietary conditions used. Cholesterol synthesis in the liver of alloxan diabetic rats is maintained at a subnormal level when the diet had a high content of simple sugar (sucrose, glucose, or fructose) without fat, but is severely impaired with diets of all other compositions (4), including laboratory chow (3, 5). In general, alloxan diabetic rats show an increased level of serum cholesterol irrespective of the dietary conditions. Therefore, the hypercholesterolemia observed in diabetic rats seems to be caused by some other factor(s) than the rate of hepatic cholesterol synthesis.

Two factors have been suggested as causes of hypercholesterolemia in diabetic

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rats: a decreased turnover time of plasma cholesterol (6) and a decreased rate of conversion of cholesterol to cholic acid (7). However, in discrepancy with the latter possibility, NERVI et al. (8) showed that the rate of daily synthesis of bile salts was significantly increased in diabetic rats, with increases in the pool size of bile salts. They also demonstrated that the turnover of trihydroxycholanic acid fraction of bile salts was decreased and the rate of intestinal absorption of cholesterol was significantly increased. From these findings they suggested that hypercholesterolemia may be partly due to an increased rate of cholesterol absorption.

In studies on hypercholesterolemia in diabetic rats we used a quite different approach from NERVI et al. (8) but reached a similar conclusion to theirs.

MATERIALS AND METHODS

Animals and diets. Male Sprague-Dawley rats, initially weighing about 180 g, were used. Diabetes was induced as described in a previous paper (4). The animals were kept in individual cages and given a diet and water ad libitum for 2 weeks. The diet consisted of 20% casein, 45% corn starch, 23% sucrose, 5% oil (soybean oil: cod liver oil, 4:1, v/v), 4% salt mixture,* 1% vitamin mixture,* 0.15% choline-Cl, and 1.85% cellulose powder.

For measurement of the sterol content of the lumen, rats were allowed free access to the diet and water from 20.00 hr to 14.00 hr on the following day for 14 days, and on day 15 they were given a diet containing 5% cholestyramine at 20.00 hr, and were killed exactly 6 hr later.

Collection of bile. Animals were anesthetised with Nembutal and the abdomen was opened by a midline incision from below the xyphisternum. A polyethylene tube (OD 0.97 mm, ID 0.58 mm, INTRAMEDIC PE50) was inserted into the upper part of the common bile duct. The free end of the tube was passed out of the animal through the midscapular region and connected to a small test tube on the back of the rat, as shown in Fig. 1. Cholesterol-4-14C (1.75 ƒÊCi) was then rapidly injected into the femoral vein. The rat was allowed free access to a diet and water from the time that it recovered from anesthesia. The experiment was started at 10.00 hr and the bile was collected in a weighed test tube every 2 hr for over 22 hr. The amount of bile was calculated by the change in weight of the test tube. The bile was kept frozen until analyzed.

Preparation of cholesterol-4-14C for injection. Cholesterol-4-14C was purchased from New England Nuclear and treated by the method of MEIER et al. (9) before use for injection.

Determination of conjugated bile salts and cholesterol in bile. Conjugated bile salts and cholesterol in bile were extracted by the method of SHIODA et al. (10); the individual conjugated bile salts were separated by thin-layer chromatography.

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Fig. 1. Apparatus for collecting bile in unrestrained rats.

and determined spectrophotometrically by the method of SJÖVALL (11); the total amount of conjugated bile salts was then calculated as the sum of the amount of individual bile salts. Free bile salts were not determined in this experiment because they were present in only trace amount in rat bile. An aliquot of the aqueous phase obtained by the method of SHIODA et al. (10) was used for determination of radioactivity in a liquid scintillation spectrometer (Aloka, Model 601). The scintillation fluid was composed of 4 g PPO and 100 mg dimethyl-POPOP dissolved in 1 liter of toluene. A correction was made for quenching by the external standardization method.

Determination of sterols precipitated by digitonin (DPS) in the luminal contents and feces. Feces were collected for two days before rats were given the diet containing 5% cholestyramine. The feces were moistened with water and homogenized in 10 volumes of chloroform–methanol (1:1, v/v) with a Potter-Elvehjem glass homogenizer and then treated by the method of GUSTAFSSON et al. (12).

The rats were given the diet containing cholestyramine at 20.00 hr and sacrificed 6 hr later. The peritoneum was opened and the small intestine was tied with thread at the pylorus and the ileocecal valve to prevent loss of the luminal contents. The entire small intestine was then rapidly removed and the luminal contents were washed out with 50 ml of saline and evaporated to dryness. Neutral sterols in the luminal contents were extracted and precipitated as digitonides using the same method as for feces.

Determination of cholesterol, DPS, and blood glucose. Cholesterol in bile extracts and serum and DPS in feces and luminal contents were measured by the method of ZAK et al. (13). Blood glucose was assayed by the SOMOGYI-NELSON method (14).

RESULTS

The plasma cholesterol level of diabetic rats was higher than that of controls, but the total cholesterol content of the liver was the same as in controls (Table 1).
Table 1. Body weight and concentration of total cholesterol in the plasma and liver in control and alloxan diabetic rats with a biliary diversion.

|       | $n$ | Body weight (g) | Cholesterol |
|-------|-----|-----------------|-------------|
|       |     |                 | Plasma (mg/dl) | Liver (mg/g) |
| Control | 6   | 262±15.7        | 81±7.4      | 2.5±0.24    |
| Diabetic | 6   | 193±30.4        | 108±10.5    | 2.4±0.52    |
| Statistical significance | | | $p<0.01$ | NS |

Figure 2 shows the washout pattern of conjugated bile salts and cholesterol in the bile for 22 hr after creation of the biliary diversion. The rate of biliary secretion was fairly constant throughout the experiment in both diabetic and control animals, but the volume of bile was significantly less ($p<0.05$) in the diabetic rats. The concentration of conjugated bile salts excreted in 12 hr first decreased to a minimum and then gradually increased during the experiment, indicating increased synthesis of conjugated bile salts in the liver due to emptying of the pool. The minimal concentration of conjugated bile salts represents the amount of basal hepatic synthesis and basal daily synthesis can be calculated from this value. The pool size of conjugated bile salts can also be calculated by subtracting the contribution due to basal hepatic synthesis from the total amount excreted by the time.
the minimal secretion was reached. These calculated values are shown in Table 2.

In diabetic rats, the pool size and basal daily synthesis of conjugated bile salts increased markedly: the basal daily synthesis, expressed per 100 g body weight, was $30.9 \pm 5.1 \mu$moles in the controls and $58.4 \pm 2.7 \mu$moles in the diabetics; expressed per rat it was 1.4 times higher in diabetic rats than in controls.

The changes in specific radioactivity of cholesterol and conjugated bile salts excreted in the bile are shown in Fig. 3; as diabetic rats gained far less weight than control rats but were injected with the same amount of labeled cholesterol, the specific radioactivity of cholesterol in their bile was higher than in controls.

| Pool size       | Basal synthesis               |
|-----------------|-------------------------------|
|                 | Pool size                    | Basal synthesis               |
|                 | $n$  | $\mu$moles | $\mu$moles/100 gBW | $\mu$moles/day/100 gBW |
| Control         | 6   | 149 $\pm$ 43.7 | 57 $\pm$ 18.8 | 30.9 $\pm$ 5.10 |
| Diabetic        | 6   | 419 $\pm$ 101.1 | 229 $\pm$ 40.0 | 58.4 $\pm$ 2.70 |
| Statistical significance | $p < 0.001$ | $p < 0.001$ |

means $\pm$ S.D.

Fig. 3. Specific activities of bile cholesterol and bile salts (conjugated) in control and alloxan diabetic rats with a biliary diversion.
However, the slope of the regression line was less steep in diabetic rats, indicating a decreased rate of either hepatic cholesterol synthesis or its excretion. As is seen in Fig. 2, the rate of cholesterol excretion in the bile increased in diabetic rats. Thus a slower decline of specific-activity of $^{14}$C-cholesterol in diabetic rat shown in Fig. 3 should be explained as due to a decreased hepatic synthesis of cholesterol. The specific radioactivity of conjugated bile salts in diabetic rats fell significantly between 6.00 and 16.00 hr, and then increased to over the control value.

Table 3 shows that the amounts of neutral sterols excreted in the feces in two days by the two groups were similar. When intestinal absorption of neutral sterols was inhibited by administration of cholestyramine, the amounts of neutral sterols accumulated in the luminal contents in 6 hr were $2.34\pm0.53$ mg in the controls and $4.81\pm1.10$ mg in diabetic rats. In diabetic rats the weight of the small intestine per rat increased to 65.6% more than that in control animals.

**DISCUSSION**

In studies on the mechanism of hypercholesterolemia in alloxan diabetic rats, SADAHIRO *et al.* (7) showed that after intravenous injection of labeled cholesterol the cumulative radioactivity of bile salts obtained from a biliary diversion in diabetic rats is less than in controls and concluded that this may be due to a decreased rate of conversion of cholesterol to bile salts. On the other hand, NERVI *et al.* (8) reported that the biliary excretions of cholesterol and bile salts and the daily rate of synthesis of bile salts were significantly increased in diabetic rats. Our results obtained by the washout method (15), showing that diabetic rats have a much bigger pool of bile salts than controls (Fig. 2, Table 2), are in agreement with those of NERVI *et al.* (8) obtained by the method of isotope dilution using sodium-taurocholate-24-14C.

The pool of bile salts may be defined as the total mass of bile salts in the enterohepatic circulation; about 85% of the total bile salts are in the intestinal lumen, 10% in the intestinal wall and the rest in the liver. The decreased specific radioactivity of conjugated bile salts excreted by diabetic rats from 6 to 16 hr

| Table 3. Effect of cholestyramine on digitonin-precipitable sterols (DPS) in the lumen of the small intestine. |
|---------------------------------------------------------------|
|                        | Control      | Diabetic     | Statistical significance |
|---|-----------------|--------------|------------------------|
| n | 4               | 3            |                        |
| Body weight (g)       | 306±14       | 196±23       |                        |
| Wet weight of small intestine (g) | 6.4±1.1     | 10.6±1.0     | $p < 0.001$            |
| Amount of DPS         |               |              |                        |
| intestinal lumen (mg) | 2.34±0.53    | 4.81±1.10    | $p < 0.001$            |
| feces (mg/day)        | 4.59±1.01    | 4.74±0.66    | NS                     |
| means±S.D.             |              |              |                        |
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after creation of the biliary diversion (Fig. 3) also suggests that the pool of bile salts increases, since more bile salts are present and absorbed from the intestinal lumen in diabetic rats, so that the newly synthesized conjugated bile salts in the liver are more diluted by absorbed bile salts than in controls.

Previously, we reported (16) enlargement of the small intestine of diabetic rats due to the mucosal hyperplasia with some hypertrophy. The small intestine is known to be a site of very active cholesterol synthesis (17) and this is not impaired in the diabetic state (18). Moreover, it has been reported that the rates of epithelial cell migration and of sloughing off of epithelial cells in the small intestine increase in diabetic rats (19).

Thus, there must be more cholesterol derived from epithelial cells in the intestinal lumen of diabetic rats than in that of controls. Cholesterol from the bile and ingested food, which is somewhat greater in quantity in diabetic rats, may also contribute to the increased cholesterol content of the lumen. In fact, the amount of neutral sterols in the lumen doubled in diabetics, when cholesterol absorption was inhibited by administration of cholestyramine (Table 3), although the amount of neutral sterols excreted in the feces in rats not treated with cholestyramine were similar in diabetic and control rats. These findings indicate that cholesterol absorption is greater in diabetic rats than in controls.

We conclude from this study that hypercholesterolemia is partly due to an increased rate of intestinal absorption of endogenous cholesterol; the cholesterol is derived mainly from sloughed off epithelial cells and from the bile, and the absorption is facilitated by increased micelle formation, because the amount of bile salts in the small intestine is increased.

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