SEX DIFFERENCE IN THE DEVELOPMENT OF FATTY LIVER BY OROTIC ACID

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Abstract - Effects of orotic acid on liver lipid accumulation and incorporation of methionine [methyl-14C] into liver phosphatidylcholine and protein, and into serum beta-lipoprotein were studied. Male and female rats of Wistar strain were fed a semisynthetic diet supplemented with 1 per cent orotic acid for 7 days. Feeding of orotic acid induced a marked fatty liver in female rats, but not in males. In female rats, radioactivity in liver phosphatidylcholine was significantly decreased by orotic acid, and that in liver protein was slightly decreased. In male rats, incorporation of methionine [methyl-14C] into liver phosphatidylcholine and protein was unchanged between the control and the rats fed orotic acid. Radioactivity in serum beta-lipoprotein was decreased to a greater extent in female rats than in males. These results suggest that sex difference in the development of fatty liver may be due to the difference in the effect of orotic acid on liver phosphatidylcholine biosynthesis.

In weaning rats, orotic acid is well known to induce a marked fatty infiltration of the liver, and a reduction of serum beta-lipoprotein when it is fed in a 1 per cent semisynthetic diet (1-6). Sidransky (7) demonstrated that fatty liver induced by orotic acid in adult rats was greater in female rats than that in males, as in ethionine-induced fatty liver (8-10), and suggested that, since males significantly developed fatty liver like females after orchietomy and administration of androgen to castrated rats protected lipid accumulation, this sex difference has been attributed to a function of androgen.

Fatty infiltration of the liver appears to result from an inhibition of synthetic or release of liver beta-lipoprotein by orotic acid (11-14). However, it is little known how the release of lipids out of the liver can be inhibited. It would seem that phospholipid may an important role in the release of lipoprotein. Phospholipid molecule, which has both polar and nonpolar sites, seem to couple conveniently with neutral lipids and proteins. In fact, it has been found that the combination of phospholipid with apoprotein is the first step in the synthesis of high density lipoprotein (15) and later, neutral lipids are assumed to be bound to phospholipid-protein matrix (16). Beta-lipoprotein may be synthesized through similar steps.

Accordingly, it seemed of interest to determine whether or not phospholipid synthesis is impaired by ingestion of orotic acid. In the present study, sex difference in the incorporation of methionine [methyl-14C] into liver phosphatidylcholine and protein, and into serum beta-lipoprotein in vivo was examined.

MATERIALS AND METHODS

Male and female albino rats of Wistar strain weighing from 180 to 210 g were fed a
semisynthetic diet of the following composition (in per cent) (2): sucrose 72.8, corn oil 2.0, vitamin mixture 2.2, and salts 5.0. After 2 days, animals were divided into two groups. Animals in the control group were continued on a semisynthetic diet and the orotic acid-fed group was given 1 per cent of orotic acid supplemented in the semisynthetic diet for 7 days. At the end of this period, rats were administered i.p. 0.5 ml of 0.9 per cent sodium chloride solution containing 5.0 μCi of methionine [methyl-¹⁴C] (7.06 mCi/mM). Two hr after the injection, the animals were sacrificed by heart puncture under ether anesthesia.

The liver was homogenized with ice-cold 5 per cent trichloroacetic acid and centrifuged. The precipitate was resuspended and recentrifuged once. Liver lipids were then extracted once with 80 per cent ethanol, once with 100 per cent ethanol, twice with chloroform-ethanol mixture (1:1, by vol.), and once with ether. The combined extracts were evaporated to dryness and the residue was dissolved in a small amount of chloroform-ethanol mixture. Total lipids were measured by gravimetry and a portion was taken for analysis of phosphatidylcholine. Phosphatidylcholine was separated by paper chromatography according to Marinetti et al. (17), and visualized with Rhodamine 6G under ultraviolet ray. Fraction of phosphatidylcholine was cut off and eluted with chloroform-methanol-water (75:25:2, by vol.). Phosphate was estimated by the procedure of Chen et al. (18) after digestion with perchloric acid. Radioactivity of ¹⁴C-labeled phosphatidylcholine was determined by liquid scintillation counting in a toluene-phosphor solution. The counting efficiency was checked by the internal standardization method.

Nucleic acid was extracted with 10 per cent trichloroacetic acid at 90° for 15 min from insoluble residue after extraction of lipids (19). To determine liver protein, the residue was then dissolved in formic acid, an aliquot of the solution was transferred to a counting vial containing 10 ml of liquid scintillation solution consisting of 4.6 g PPO, 0.046 g of POPOP, 73.8 g of naphthalene, 350 ml of xylene, 350 ml of dioxan, and 210 ml of ethanol, and counted in a liquid scintillation spectrometer. Correction for quenching was carried out as described above. An aliquot was evaporated, the residue dissolved in 0.1 N sodium hydroxide, and the amount of protein was then determined according to the method of Lowry et al. (20) with bovine serum albumin as standard.

Serum beta-lipoprotein was separated according to precipitation method with mepsulfate (sodium salts of sulfated methyl polygalacturonate methyl glycoside) as described by Florsheim and Gonzales (21). Lipids in serum beta-lipoprotein were extracted with chloroform-methanol (2:1, by vol.). Analysis of phosphatidylcholine and protein was carried out as described above.

RESULTS

Significant sex difference were found in the liver lipid accumulation in rats fed orotic acid, as shown in Table 1. In female rats, liver lipid content in rats fed orotic acid increased by three times that of control, while in males no significant difference was found between control and orotic acid-fed rats.

The results shown in Table 2 and 3 indicate sex difference in the incorporation of me-
TABLE 1. Effect of orotic acid on the content of liver total lipids

|       | Total lipids (mg/g wet wt.) | Per cent of control |
|-------|----------------------------|--------------------|
| Male  |                           |                    |
| Control | 102 ± 8.3                | 100                |
| Orotic acid | 110 ± 11.1       | 108                |
| Female |                           |                    |
| Control | 103 ± 7.9                | 100                |
| Orotic acid | 292 ± 11.3*            | 283                |

Each value represents mean ± standard error.

* Mean significant difference from control (P < 0.01). Rats were fed a semisynthetic diet alone (Control) or supplemented with 1 per cent orotic acid (Orotic acid) for 7 days.

TABLE 2. Effect of orotic acid on the incorporation of methionine [methyl-14C] into liver phosphatidylcholine

|       | Total activity (dpm/g liver) | Per cent of control | Specific activity (dpm/pg P) | Per cent of control |
|-------|----------------------------|--------------------|-----------------------------|--------------------|
| Male  |                           |                    |                             |                    |
| Control | 55,600 ± 6,670          | 100                | 348 ± 48                   | 100                |
| Orotic acid | 42,800 ± 750         | 77.1               | 357 ± 15                   | 103                |
| Female |                           |                    |                             |                    |
| Control | 56,800 ± 5,020          | 100                | 347 ± 21                   | 100                |
| Orotic acid | 23,500 ± 3,440*        | 41.4               | 174 ± 21                   | 50.1               |

Each value represents mean ± standard error.

* Mean significant difference from control (P < 0.01).

TABLE 3. Effect of orotic acid on the incorporation of methionine [methyl-14C] into liver protein

|       | Total activity (dpm/g liver) | Per cent of control | Specific activity (dpm/mg protein) | Per cent of control |
|-------|----------------------------|--------------------|-----------------------------------|--------------------|
| Male  |                           |                    |                                   |                    |
| Control | 56,000 ± 3,120            | 100                | 372 ± 31                          | 100                |
| Orotic acid | 48,100 ± 2,530       | 86.0               | 379 ± 13                          | 102                |
| Female |                           |                    |                                   |                    |
| Control | 57,900 ± 4,810           | 100                | 461 ± 50                          | 100                |
| Orotic acid | 40,800 ± 5,600*        | 70.4               | 356 ± 35                          | 77.2               |

Each value represents mean ± standard error.

* Mean significant difference from control (P < 0.05).

TABLE 4 contains the result of the incorporation of methionine-methyl group into serum beta-lipoprotein fraction in control and the rats fed orotic acid. After ingestion of orotic acid, the depression in the incorporation of methionine [methyl-14C] into serum

thionine [methyl-14C] into liver phosphatidylcholine and protein by orotic acid administration. In female rats, the presence of 1 per cent orotic acid resulted in a marked decrease in the incorporation of methionine [methyl-14C] into liver phosphatidylcholine (Table 2). On the other hand, in males the radioactivity in liver phosphatidylcholine was almost the same between control and the group fed orotic acid. Incorporation of methionine-methyl group into liver protein in females was slightly changed by ingestion of orotic acid, while in males no alternation was observed (Table 3).

Table 4 contains the result of the incorporation of methionine-methyl group into serum beta-lipoprotein fraction in control and the rats fed orotic acid. After ingestion of orotic acid, the depression in the incorporation of methionine [methyl-14C] into serum
TABLE 4. Effect of orotic acid in the incorporation of methionine [methyl-14C] into serum beta-lipoprotein

| Beta-lipoprotein         | Total activity | Specific activity |
|--------------------------|----------------|------------------|
|                          | dpm/ml serum   | dpm/μg P         |
| Phosphatidylcholine moiety |               |                  |
| Male Control             | 1,460 ± 221 (100) | 346 ± 19 (100)   |
| Orotic acid              | 505 ± 36** (34.6)  | 259 ± 27* (75.9)  |
| Female Control           | 1,330 ± 21 (100)   | 326 ± 18 (100)   |
| Orotic acid              | 288 ± 12** (21.6)  | 232 ± 20* (71.1)  |
| Protein moiety           | dpm/ml serum     | dpm/μg protein   |
| Male Control             | 2,420 ± 384 (100) | 1,090 ± 218 (100) |
| Orotic acid              | 1,810 ± 248* (74.8) | 996 ± 41 (91.9)  |
| Female Control           | 2,610 ± 482 (100) | 1,210 ± 216 (100) |
| Orotic acid              | 855 ± 28** (32.8)  | 675 ± 6** (55.8)  |

Each value represents mean ± standard error.
Data given in parentheses represent per cent taking the value of control rats as 100 per cent.
* Mean significant difference from control (P<0.05).
** Mean significant difference from control (P<0.01).

beta-lipoprotein fraction was also greater in female rats than that in males. In both female and male rats, the total activity of phosphatidylcholine in serum beta-lipoprotein was significantly decreased by ingestion of orotic acid, but the depression in females was greater than that in males. However, the decrease in specific activity of phosphatidylcholine moiety was not different between male and female rats. A more distinct sex difference was found in the incorporation of methionine [methyl-14C] into protein moiety in serum beta-lipoprotein. Female rats fed orotic acid showed a significant decrease in the incorporation of methionine [methyl-14C] into protein moiety, while, in male rats, no alternation was observed in the specific activity of protein moiety but the total activity decreased by ingestion of orotic acid.

Thus, after ingestion of orotic acid, sex difference was observed in the liver lipid accumulation and the incorporation of methionine [methyl-14C] into liver phosphatidylcho-

TABLE 5. Effect of orotic acid on the incorporation of acetate [1-14C] into liver lipids

| Total lipids         | Radioactivity (dpm/g liver × 10^3) | Per cent of control |
|----------------------|-------------------------------------|---------------------|
| Control              | 876 ± 41.6                          | 100                 |
| Orotic acid          | 1,210 ± 69.0*                       | 138                 |
| Phosphatidylcholine  | Control                             | 224 ± 10.9          | 100                 |
|                      | Orotic acid                         | 227 ± 6.9           | 101                 |

Each value represents mean ± standard error.
* Mean significant difference from control (P<0.05).
After dietary treatment, female rats were given 20 μCi of sodium acetate [1-14C] (49 mCi/mM) i.p. and were sacrificed 6 hr later. Liver lipids were extracted and determined, as described in “Materials and Methods”.
line and protein, and into serum beta-lipoprotein. Fatty infiltration in the liver and the depression of methionine-methyl group into liver phosphatidylcholine was especially greater in female rats. Accordingly, in the preliminary experiment, the incorporation of sodium acetate \([1-^{14}C]\) into liver total lipids and phosphatidylcholine was examined in female rats. However, the radioactivity in liver phosphatidylcholine was almost the same in control rats, and that of total lipids was only slightly increased by ingestion of orotic acid (Table 5).

**DISCUSSION**

Previously, Sidransky reported that female rats fed orotic acid developed a significantly greater degree of liver lipid accumulation than males (7). Similar results were obtained in the present study which showed that orotic acid induced a significant liver lipid accumulation in females, but not in males. Moreover, our results demonstrated that there was a sex difference in the incorporation of methionine \([\text{methyl-}^{14}\text{C}]\) into liver phosphatidylcholine and protein, and into serum beta-lipoprotein. However, it is not clear whether this sex difference is mediated by androgen or estrogen. Sidransky suggested that, in the study on castrated rats, sex difference in the rats fed orotic acid may be due to the level of androgen in male rats, though, in his data, liver lipid content was decreased by the administration of estradiol to castrated rats.

There are two pathways for the \textit{de novo} biosynthesis of phosphatidylcholine in the liver; one involving direct phosphorylation of choline and subsequent incorporation into phosphatidylcholine via a CDP-choline intermediate (22), and a second route involving methylation of phosphatidylethanolamine (23). In addition, it is known that methylation of phosphatidylethanolamine is a major pathway in the biosynthesis of phosphatidylcholine in female rats (24). As shown in Table 2, this pathway is markedly inhibited in female rats fed orotic acid, but not in males. These results are probably due to the depression of acid-soluble adenine nucleotide in the liver (5, 25), through an effect on S-adenosylmethionine. In the case of fatty liver induced by choline-deficient diet, phosphatidylcholine biosynthesis via CDP-choline mediated pathway was inhibited, while the pathway of the methylation of phosphatidylethanolamine was stimulated (26).

Numerous investigators suggest that a decreased release of beta-lipoprotein from the liver may be the underlying mechanism for fatty liver production (11–14). This hypothesis is also supported by the results of the incorporation of methionine \([\text{methyl-}^{14}\text{C}]\) into serum beta-lipoprotein (Table 3). The sequence of biochemical event leading to the synthesis and release of serum beta-lipoprotein is still obscure in detail. It appears, however, that it must involve at least four major steps; (1) synthesis of neutral lipids and phospholipid, (2) synthesis of apoprotein, (3) conjugation of various moieties, and (4) release of lipoprotein into the blood stream. The present work and that of others (4, 27) have shown that protein synthesis in the liver was little or not affected and the synthesis of liver neutral lipid was increased by the ingestion of orotic acid. Moreover, Roheim et al. found that the rats fed orotic acid have normal apoprotein of beta-lipoprotein (28). These observations differ from those on fatty liver induced by ethionine (29, 30), puromycin (31,
and carbon tetrachloride (33, 34), which also inhibit the release of lipoprotein from the liver. Biosynthesis of phosphatidylcholine in the liver was significantly depressed by the ingestion of orotic acid (Table 2). Phospholipid seems to play a specific role in lipoprotein, especially by coupling with neutral lipid and protein moiety, since its molecule has both polar and nonpolar sites.

Accordingly, our study suggests that the inhibition of phosphatidylcholine biosynthesis in the rats fed orotic acid results in the depression of lipoprotein synthesis and consequently induces lipid accumulation in the liver. Further, it seems that sex difference in fatty liver induced by orotic acid is due to the degree of inhibition of methylation of phosphatidylethanolamine.

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