Effects of Refeeding Diets on Emeriamine-Induced Fatty Liver in Fasting Rats

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Summary We recently reported that fatty liver and hypertriglyceridemia are easily induced by the administration of an inhibitor of fatty acid oxidation (emeriamine; (R)-3-amino-4-trimethylaminobutyric acid) to fasting rats, and that these conditions are not accompanied by the increased de novo synthesis of fatty acid [J. Nutr. Sci. Vitaminol., 42, 111–120, (1996)]. To study whether emeriamine-induced fatty liver is affected by nutrients during recovery from fatty acid oxidation inhibition, we fed rats with either a high-carbohydrate (HCHO) diet or a high-fat (HFAT) diet. Rats fed an HCHO diet following the administration of emeriamine showed a marked decrease in serum and hepatic triglycerides, and a marked increase in hepatic glycogen. The lower levels of serum and hepatic triglycerides were accompanied by decreased activities of the NADPH-generating enzymes such as malic enzyme and glucose-6-phosphate dehydrogenase. By contrast, rats fed an HFAT diet showed less significant changes in hepatic triglyceride and glycogen levels. These results suggest a reciprocal relationship between the triglyceride level and glycogen accumulation caused by HCHO diet during recovery from emeriamine.

Key Words dietary composition, emeriamine, fatty acid, glycogen, fatty liver, high-carbohydrate diet, high-fat diet, triglyceride

In the liver, de novo synthesized fatty acids and free fatty acids (FFA) mobilized from peripheral adipose tissue are either catabolized to ketone bodies and carbon dioxide through β-oxidation or esterified to lipoproteins and then secreted (I). These fatty acid uses ultimately regulate hepatic and plasma triglyceride levels.

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Inhibitors of fatty acid oxidation have been useful for investigating the relationship between fatty acid oxidation and lipid levels in the liver under various nutritional conditions (2). Emeriamine [(R)-3-amino-4-trimethylaminobutyric acid], a derivative of a carnitine analogue from the fungus Emericella quadrilineata, inhibits long-chain fatty acid oxidation; its inhibitory mechanism is thought to be mediated by the specific inhibition of carnitine palmitoyltransferase (3, 4).

We recently reported that fatty liver and hypertriglyceridemia are easily induced by the administration of emeriamine to fasting rats; these conditions are not induced by the administration of emeriamine to feeding rats (5). Therefore, it appears that decreasing the rate of fatty acid oxidation by specifically inhibiting carnitine palmitoyltransferase affects the hepatic and plasma triglyceride levels. Little is known, however, about the effect of nutrients on recovery from fatty acid oxidation inhibition. The present study investigates the effects of a high-carbohydrate diet and a high-fat diet on recovery from fatty acid oxidation inhibition due to emeriamine.

MATERIALS AND METHODS

Animals. Male Wistar rats (5–6 weeks old) were fed standard laboratory chow ad libitum for seven days as they adjusted to the new environment. For Experiment 1, rats weighing 230–245 g were fasted for 48 h and then divided into two groups. The first group received saline (2.5 ml/kg body weight) administered orally; the second group received emeriamine (10 mg emeriamine/2.5 ml saline/kg body weight) administered orally. Eight hours later, the rats were decapitated.

For Experiment 2, rats weighing 175–195 g were fasted for 48 h and then divided into two groups. The first group received saline and the second group received emeriamine as described above. To determine serum triglyceride levels on a time course, blood was drawn from the tail vein at the end of the 48 h fast and 8 h after the administration of saline or emeriamine. Both groups of rats were fasted for another 24 h, then fed a fat-free high-carbohydrate diet (HCHO diet: casein, 20%; sucrose, 10%; corn starch, 64.5%; mineral mixture, 4%; vitamin mixture, 1%; choline chloride, 0.2%; DL-methionine, 0.3%) for two days.

For Experiment 3, rats weighing 155–175 g were fasted for 48 h and then divided into two groups. The first group received saline and the second group received emeriamine as described previously. To determine serum triglyceride levels on a time course, blood was drawn from the tail vein at the end of the 48 h fast and 8 h after the administration of saline or emeriamine. Both groups of rats were fasted for another 24 h, then fed a high-fat diet (HFAT diet: casein, 20%; sucrose, 10%; corn starch, 34.5%; soybean oil, 30%; mineral mixture, 4%; vitamin mixture, 1%; choline chloride, 0.2%; DL-methionine, 0.3%) for two days. The rats in both Experiments 2 and 3 were deprived of food for 6 h before decapitation. After sacrificing the livers were immediately removed, frozen in liquid nitrogen and stored at −30°C pending lipid, glycogen and protein analyses.

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Enzyme assay. The livers of rats from Experiments 2 and 3 were immediately removed and homogenized in a Teflon homogenizer with 10 volumes of 0.25 M sucrose solution containing 5 mM Tris-HCl buffer (pH 7.4) and 0.1 mM EDTA. The homogenate was centrifuged at 8,000 g for 20 min, and the supernatant was further centrifuged at 10,500 g for 60 min. The resulting supernatant was used as the source of glucose-6-phosphate dehydrogenase [EC 1.1.1.49] and malic enzyme [EC 1.1.1.40]. The activities of these enzymes were determined photometrically as described previously (5). The enzyme activities were measured as mU/mg protein, where 1 mU was defined as the formation of 1 μmol of NADPH per minute for glucose-6-phosphate dehydrogenase and malic enzyme.

Lipid analysis. Hepatic lipids were extracted and purified according to the method of Folch et al. (6). Hepatic triglyceride, phospholipid and cholesterol levels were determined colorimetrically as described previously (5). Serum triglyceride, phospholipid, cholesterol and FFA levels were determined using an enzymatic assay kit (Wako Pure Chemicals Co., Osaka).

Glycogen analysis. Livers were homogenized with four volumes of distilled water at 4°C. Glycogen in the liver homogenate was hydrolyzed in 2.25 N H2SO4 for 2 h in a boiling water bath. The sample was then neutralized with ten volumes of 0.4 M potassium phosphate buffer (pH 7.4) (7). After centrifugation of the acid hydrolysate at 16,000 g for 20 min, an aliquot of the clear supernatant was tested for glucose using an enzymatic assay kit (Wako Pure Chemicals Co., Osaka). The glycogen content was calculated by subtracting the glucose content remaining in the acid hydrolysate from the glucose content measured in the liver sample without acid hydrolysis.

Other measurements. The protein concentration of the liver homogenate was determined by the biuret reaction (8), and the protein concentration of the cytosol fraction was determined by the method of Lowry et al. (9). The data were statistically evaluated using Student’s t-test and the value of p < 0.01 was considered significant.

RESULTS

Table 1 shows the effects of emeriamine on hepatic lipids after fasting for 48 h (Experiment 1) and subsequent feeding with an HCHO diet (Experiment 2) or an HFAT diet (Experiment 3). In Experiments 2 and 3, the food intake of the emeriamine group was less than that of the saline group. Yet in all three experiments, the liver weight of the emeriamine group was greater than that of saline group. Livers from the emeriamine group in Experiment 1 exhibited fatty liver during necroscopy, and their hepatic triglyceride content had increased approximately 8-fold from that of the saline group as described previously (5). Livers from the emeriamine group in Experiment 2, in which rats were fed an HCHO diet, were very aqueous and fragile, and their hepatic triglyceride and phospholipid levels were significantly decreased. In particular, their hepatic tri-
Table 1. Effects of emeriamine on hepatic lipids after fasting and subsequent feeding.

| Groups                  | Fasted-saline | Fasted-emeriamine |
|-------------------------|---------------|------------------|
| Experiment 1 (Fasted)   |               |                  |
| Liver weight (g)        | 5.8±0.5       | 6.8±0.1*         |
| Triglyceride (mg/g liver) | 12±1         | 95±12**         |
| Phospholipid (mg/g liver) | 36±2         | 34±2             |
| Cholesterol (mg/g liver) | 3.6±0.2      | 3.5±0.3         |
| Experiment 2 (Fed with HCHO diet after fasting) |  |                  |
| Food intake (g/day)     | 22±3          | 18±1*            |
| Liver weight (g)        | 11.0±0.8      | 14.1±1.7*        |
| Triglyceride (mg/g liver) | 86±11        | 26±8**          |
| Phospholipid (mg/g liver) | 25±2         | 20±2*           |
| Cholesterol (mg/g liver) | 3.4±0.2      | 2.8±0.5         |
| Experiment 3 (Fed with HFAT diet after fasting) |  |                  |
| Food intake (g/day)     | 17±2          | 15±1*            |
| Liver weight (g)        | 8.9±1.0       | 10.7±0.9*        |
| Triglyceride (mg/g liver) | 57±15        | 57±23            |
| Phospholipid (mg/g liver) | 25±1         | 19±2**          |
| Cholesterol (mg/g liver) | 2.9±0.2      | 3.0±0.1         |

Values are M±SD of four rats in Experiment 1 and of five rats (saline group) or eight rats (emeriamine group) in Experiment 2 and Experiment 3. The statistical significance of differences was compared between the saline group and the emeriamine group of each experiment, and was calculated using Student's t-test (*p<0.01, **p<0.001).

The effect of emeriamine on the serum triglyceride level over time is shown in Fig. 1. It is clear that serum triglyceride concentrations tripled 8h after the administration of emeriamine (Fig. 1-A and B). However, subsequent feeding with either the HCHO diet (Fig. 1-A) or the HFAT diet (Fig. 1-B) reduced the elevated levels; the reduction was more pronounced in response to the HCHO diet than to the HFAT diet.

Table 2 shows the levels of serum phospholipid, cholesterol and FFA following subsequent feeding with either the HCHO diet or the HFAT diet. When rats were fed an HCHO diet, the FFA level of the emeriamine group was lower than that of the saline group. No significant differences in serum phospholipid or cholesterol levels were observed between the two groups. When rats were fed an HFAT diet, the plasma cholesterol level of the emeriamine group was lower than that of the saline group. No significant differences in serum FFA or phospholipid levels were observed between the two groups.

Table 3 shows the activities of the NADPH-producing enzymes, malic enzyme.
Fig. 1. Effect of emeriamine on serum triglyceride levels over time with subsequent feeding with either an HCHO diet (Fig. 1-A) or an HFAT diet (Fig. 1-B). F, rats fasted 48 h; FSE, 8 h from administration of saline (○) or emeriamine (●) to fasted rats; FSR, 48 h from subsequent feeding with an HCHO diet (Fig. 1-A) or an HFAT diet (Fig. 1-B). Values are M±SD of five rats (saline group) or eight rats (emeriamine group). The statistical significance of differences was compared between the saline group and the emeriamine group, and was calculated by Student's t-test (*p < 0.01).

Table 2. Effects of emeriamine on serum lipids after fasting and subsequent feeding.

| Groups                  | Fasted-saline | Fasted-emeriamine |
|-------------------------|---------------|-------------------|
| Experiment 2 (Fed with HCHO diet after fasting) |               |                   |
| Phospholipids (mg/dl)   | 154±22        | 120±20            |
| Cholesterol (mg/dl)     | 68±6          | 64±9              |
| Free fatty acids (mEq/liter) | 0.74±0.16   | 0.50±0.08*        |
| Experiment 3 (Fed with HFAT diet after fasting) |               |                   |
| Phospholipids (mg/dl)   | 187±35        | 193±66            |
| Cholesterol (mg/dl)     | 106±9         | 76±14*            |
| Free fatty acids (mEq/liter) | 0.29±0.03   | 0.27±0.01         |

Values are M±SD of five rats (saline group) or eight rats (emeriamine group). The statistical significance of differences was compared between the saline group and the emeriamine group, and was calculated by Student’s t-test (*p < 0.01).

and glucose-6-phosphate dehydrogenase, following subsequent feeding with either the HCHO diet or the HFAT diet. When rats were fed an HCHO diet, the activities of malic enzyme and glucose-6-phosphate dehydrogenase were significantly lower in the emeriamine group than in the saline group. When rats were fed an HFAT diet, the activity of glucose-6-phosphate dehydrogenase was significantly lower in the emeriamine group than in the saline group.

Hepatic glycogen and protein concentrations are shown in Table 4. In Experiment 1, hepatic glycogen levels were very low in both groups of rats.
Table 3. Effects of emeriamine on the activity of hepatic malic enzyme and glucose-6-phosphate dehydrogenase after fasting and subsequent feeding.

| Groups                     | Malic enzyme (mU/mg cytosol protein) | Glucose-6-phosphate dehydrogenase |
|----------------------------|-------------------------------------|----------------------------------|
| Experiment 2 (Fed with HCHO diet after fasting) |                                      |                                  |
| Fasted-saline              | 103±12                              | 224±29                           |
| Fasted-emeriamine          | 54±18**                             | 76±37**                          |
| Experiment 3 (Fed with HFAT diet after fasting) |                                      |                                  |
| Fasted-saline              | 69±8                                | 148±33                           |
| Fasted-emeriamine          | 61±9                                | 64±28**                          |

1 mU is defined as the formation of 1 μmol of NADPH per minute for glucose-6-phosphate dehydrogenase and malic enzyme. Values are M±SD of five rats (saline group) and eight rats (emeriamine group). The statistical significance of differences was compared between the saline group and the emeriamine group, and was calculated using Student’s t-test (*p<0.01, **p<0.001).

Table 4. Effects of emeriamine on hepatic glycogen and protein after fasting and subsequent feeding.

| Groups                     | Fasted-saline (mg/g liver) | Fasted-emeriamine |
|----------------------------|----------------------------|-------------------|
| Experiment 1 (Fasted)      |                            |                   |
| Glycogen                   | 1.2±0.8                    | 0.2±0.1           |
| Protein                    | 196±11                     | 168±8*            |
| Experiment 2 (Fed with HCHO diet after fasting) |                            |                   |
| Glycogen                   | 35±17                      | 97±16**           |
| Protein                    | 197±21                     | 150±10**          |
| Experiment 3 (Fed with HFAT diet after fasting) |                            |                   |
| Glycogen                   | 17±1                       | 20±2              |
| Protein                    | 172±14                     | 140±13*           |

Values are M±SD of five rats (saline group) and eight rats (emeriamine group). The statistical significance of differences was compared between the saline group and the emeriamine group of each experiment, and was calculated using Student’s t-test (*p<0.01, **p<0.001).

However, when rats were subsequently fed either the HCHO diet or the HFAT diet, hepatic glycogen levels rapidly recovered, increasing more in the emeriamine group than in the saline group. In particular, when rats were fed an HCHO diet, their hepatic glycogen levels increased abnormally, 2.7-fold as compared to that of the saline group. In all three experiments, the hepatic protein concentration was significantly lower in the emeriamine group than in the saline group.

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DISCUSSION

Lipid metabolism may be investigated using various inhibitors of fatty acid synthesis and oxidation. Several reports suggest that there is a reciprocal relationship between the rates of ketogenesis and triglyceride secretion \((1, 2, 10-14)\). The inhibition of fatty acid synthesis in rat liver by 5-tetradecyloxy-2-furoic acid \((10, 11)\) or 4-amino-5-ethyl-3-thiophenecarboxylic acid \((12)\) concurrently stimulated ketogenesis and reduced the secretion of triglyceride. Conversely, increased secretion of hepatic triglyceride was observed following the inhibition of long-chain fatty acid oxidation by perfusing rat liver with 2-tetradecylglycidic acid \((13)\) and 3-mercaptopropionic acid \((14)\). As reported previously \((5)\), the \textit{in vivo} effects of emeriamine on triglyceride metabolism were dramatic. Eight hours following the oral administration of emeriamine to rats fasted for 48 h, plasma and liver triglyceride levels were markedly elevated without enhanced lipogenesis. However, the effects of emeriamine are both prevented and reversed by feeding \((5)\). Similar results were reported by Jenkins and Griffith \((15, 16)\): mice given authentic DL-aminocarnitine or acetyl-DL-aminocarnitine and then fasted for 24 h exhibited gross hepatic and renal steatosis, which were reversed upon subsequent feeding. These results suggest that the availability of FFA in the liver following fasting determines hepatic and plasma triglyceride levels.

However, the induction of fatty liver by fasting for 24 h and its improvement by subsequent feeding has been observed without chemical compounds causing the disruption of lipid metabolism \((17, 18)\). Fatty liver induced by fasting in suncus is thought to result from impaired triglyceride secretion due to the failure of lipoprotein assembly.

In this study, as the liver weights of fasted rats recovered upon subsequent feeding with either an HCHO diet or an HFAT diet, the triglyceride levels of the saline groups markedly increased. Conversely, the triglyceride levels of the emeriamine groups decreased upon subsequent feeding with either diet (Table 1); the decrease was more pronounced in response to the HCHO diet than to the HFAT diet.

When lipid levels per whole liver were compared between the saline and emeriamine groups in all three experiments, the only significant difference observed was the triglyceride levels before and after feeding with an HCHO diet. The hepatic triglyceride level of the emeriamine group increased to nine times that of the saline group, but then decreased to one-third that of the saline group upon subsequent feeding with an HCHO diet. It is assumed that emeriamine depressed the lipogenesis, regardless of subsequent feeding. This assumption is supported by the decreased plasma triglyceride levels and the low enzyme activities of the hepatic NADPH-producing enzymes, glucose-6-phosphate dehydrogenase and malic enzyme, in the emeriamine group, even after subsequent feeding with an HCHO diet.

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Conversely, the hepatic glycogen concentration was significantly elevated in the emeriamine group upon subsequent feeding with either an HCHO diet or an HFAT diet. An especially marked increase in glycogen was observed with the HCHO diet. This abnormal glycogen concentration may be one reason for the aqueous and fragile livers found in the emeriamine group. It is not clear, from this study, whether the increased glycogen content was the result of elevated glycogen synthesis or of impaired glycogenolysis. It is likely, however, that the utilization of fatty acid is preferred to that of glucose when recovering from the effect of emeriamine once the inhibition of fatty acid oxidation has ceased.

Changes in the FFA metabolism have been reported to affect the hepatic carbohydrate metabolism. When emeriamine is administered orally to fasted, diabetic and nondiabetic rats, dose-dependent hypoglyceridemia and antiketogenic activities are observed (3,4,19). In addition, by similarly inhibiting hepatic fatty acid oxidation, phenylalkyloxirane carboxylic acids (20,21) and etomoxir (ethyl-2-[6-(4-chlorophenoxy)-hexyl]oxirane-2-carboxylate) (22,23) can also reduce the plasma glucose and ketone body levels and increase the plasma FFA levels in diabetic and nondiabetic rats. It has been suggested that the hypoglycemic potency of these agents is due to their ability to suppress hepatic gluconeogenesis in fasted animals (21). Therefore, the inhibitory effects of emeriamine may be developed for potential therapeutic use in treating diabetes. Emeriamine may be less promising for the treatment of acute diabetes, however, because it also induces metabolic disturbances such as the marked elevation of plasma and hepatic triglyceride levels, as shown previously in rats (5), and the marked deposition of glycogen when fasting is followed by consumption of an HCHO diet, as shown in this study. The exact mechanism of this glycogen deposition and the long-term effects of emeriamine on biological parameters remain to be explored.

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