Effects of Fatty Liver Induced by Excess Orotic Acid on B-Group Vitamin Concentrations of Liver, Blood, and Urine in Rats

Katsumi SHIBATA, Nobuya MORITA, Tomoyo KAWAMURA, Ai TSUJI and Tsutomu FUKUWATARI

Department of Nutrition, School of Human Cultures, The University of Shiga Prefecture, 2500 Hassakacho, Hikone, Shiga 522–8533, Japan

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Summary Fatty liver is caused when rats are given orotic acid of the pyrimidine base in large quantities. The lack of B-group vitamins suppresses the biosynthesis of fatty acids. We investigated how orotic acid-induced fatty liver affects the concentrations of liver, blood, and urine B-group vitamins in rats. The vitamin B₆ and B₁₂ concentrations of liver, blood, and urine were not affected by orotic acid-induced fatty liver. Vitamin B₂ was measured only in the urine, but was unchanged. The liver, blood, and urine concentrations of niacin and its metabolites fell dramatically. Niacin and its metabolites in the liver, blood, and urine were affected as expected. Although the concentrations of vitamin B₁, pantothenic acid, folate, and biotin in liver and blood were decreased by orotic acid-induced fatty liver, these urinary excretion amounts showed a specific pattern toward increase. Generally, as for the typical urinary excretion of B-group vitamins, these are excreted when the body is saturated. However, the ability to sustain vitamin B₁, pantothenic acid, folate, and biotin decreased in fatty liver, which is hypothesized as a specific phenomenon. This metabolic response might occur to prevent an abnormally increased biosynthesis of fatty acids by orotic acid.

Key Words orotic acid, fatty liver, vitamins, rat

Orotic acid is a precursor of pyridine nucleotides (1), which have growth-promoting activity for rats in low dosages (0.005% in a diet) (2). Conversely, orotic acid produces fatty livers with a perportal distribution of triglycerides in high dosage (1% in a diet) (3–5). This phenomenon is not accompanied by other serious pathological disturbances, is readily reversible, and can be prevented by supplementation of the diet with adenine (4, 5). These reports indicate that orotic acid-induced fatty livers are entirely different from other types of fatty livers reported in the literature so far. The accumulation of triglycerides in the liver following orotic acid administration is reported to result from an inhibition of the synthesis and release of hepatic β-lipoprotein (6) and from an inhibition of oxidation of fatty acids (7).

Other than fatty livers, orotic acid administration is reported to elicit many effects: the liver concentrations of vitamin B₁, vitamin B₂, and niacin were lower in orotic acid-fed animals compared with those not given orotic acid (5); the concentration of liver adenine is decreased to some extent (8, 9) with a concomitant increase in the concentration of uridine 5'-monophosphate (UMP) (8); and liver pyridine nucleotide is also decreased (5, 8–10) by significantly reducing the conversion of tryptophan to niacin in rats (11).

For other B-group vitamins and fat deposition, a vitamin B₆ deficiency induces fat deposition (12) and affects the fatty acid profile (13). Vitamin B₁ prevents obesity in OLETF rats (14). A vitamin B₂ deficiency develops some syndromes of abnormal fatty acid oxidation (15). Pantothenic acid deficiency produces fat accumulation and repletion of pantothenic acid diminishes the fat deposition (16). Folate deficiency changes the gene expression of fatty acid metabolism (17). Biotin deficiency induces an abnormality in fatty acid metabolism (18). Thus, it is clear that B-group vitamin deficiencies disrupt lipid metabolism (19). These reports seem to indicate some relationship between orotic acid-induced fatty livers and B-group vitamin concentrations in the liver.

In the present experiment, we investigated the effects of fatty liver induced by the administration of excess orotic acid on the concentrations of B-group vitamins in the liver, blood, and urine in rats.

MATERIALS AND METHODS

Vitamin-free milk casein, sucrose, and L-methionine were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil was purchased from Ajinomoto Co., Inc. (Tokyo, Japan). Gelatinized cornstarch, a mineral mixture (AIN-93G mineral mixture) (20), and a vitamin mixture (nicotinic acid-free AIN-93 vitamin mixture containing 25% choline bitartrate) (20) were obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan).

Thiamin hydrochloride (C₁₂H₁₇CN₄OS.HCl, molecular weight [MW]= 337.27), riboflavin (C₁₇H₂₀N₂O₆, MW = 376.37), pyridoxine hydrochloride (C₆H₁₁NO₁-HCl, MW = 205.63), cyanocobalamin (C₆₃H₉₀CoN₁₄O₁₄P, MW = 1,355.40), nicotinamide (C₁₃H₁₂N₂O₆, MW = 212.13), calcium pantothenate (C₁₉H₁₈N₂O₁₀-Ca, MW = 476.54), folic acid (C₁₉H₁₅N₇O₆, MW = 441.40), and D(+)-biotin
(C10H16N2O3S, MW=244.31) were purchased from Wako Pure Chemical Industries. 4-Pyridoxic acid (4-PIC) (C9H9NO4, MW=183.16) was made by ICN Pharmaceuticals (Costa Mesa, CA) and obtained through Wako Pure Chemical Industries.

N1-Methyl nicotinamide (N-MNA) chloride (C7H9N2O4HCl, MW=152.15) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N1-Methyl-2-pyridone-5-carboxamide (2-Py) (C7H8N2O2, MW=159.61) was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). N1-Methyl-4-pyridone-3-carboxamide (4-Py) (C7H8N2O2, MW=152.15) were synthesized by the methods of Pullman and Colowick (21) and Shibata et al. (22), respectively. All other chemicals used were of the highest purity available from commercial sources.

**Animals and treatment.**

Ethical approval of the study protocol: The care and treatment of experimental animals conformed to the guidelines set by the University of Shiga Prefecture (Shiga, Japan) for the ethical treatment of laboratory animals. The room was maintained at about 22°C with a humidity of about 60%. A 12-h light–dark cycle (06:00–18:00/18:00–06:00) was used.

Animals and diets: Male Wistar rats (3 wk old with a body weight of around 37 g) were obtained from CLEA Japan Inc., Tokyo, and immediately kept in individual rat metabolic cages (CT-10: CLEA Japan). The rats were then divided into three groups. The first group was given free access to a nutritionally complete diet (gold standard group), the second group was fed a diet supplemented with 1% orotic acid (orotic acid group), and the third group was pair-fed with the orotic acid group (pair-fed control group) (Table 1) for 20 d. Body weights and food intakes were measured daily at around 09:00, and food and water were renewed daily. Twenty-four-hour urine samples were collected on the last day of the experiment (09:00 on day 20–09:00 on day 21) in amber bottles containing 1 mL of 1 mol/L HCl, and then stored at -25°C until use. Rats were sacrificed at about 09:00 on day 21 and blood was taken from the carotid artery, and livers were taken and weighed.

Measurement of vitamins: B-group vitamins such as vitamin B1, vitamin B2, vitamin B6 and its catabolite (4-pyridoxic acid=4-PIC), vitamin B12, nicotinamide and its catabolites (N1-methylnicotinamide=MNA, N1-methyl-2-pyridone-5-carboxamide=2-Py, and N1-methyl-4-pyridone-3-carboxamide=4-Py), pantothenic acid, folate, and biotin in liver, blood, and urine were precisely determined (23).

In brief, vitamin B1 in liver (thiamin+thiamin monophosphate+thiamin diphosphate), whole blood (thiamin+thiamin monophosphate+thiamin diphosphate), and urine (thiamin) was reacted with BrCN and the product thiocrome was measured by HPLC. Vitamin B2 (riboflavin) in urine was directly measured by...
HPLC. Vitamin B6 in liver (pyridoxine+pyridoxal+pyridoxamine phosphate+PLP+pyridoxamine phosphate) was measured by microbioassay using *Saccharomyces cerevisiae* ATCC 9080. PLP in plasma was measured by HPLC. 4-PIC in urine was measured by HPLC. Vitamin B12 in liver, plasma, and urine was measured by microbioassay using *Lactobacillus leichmanii* ATCC 7830. Niacin in liver (Nam+NAD+NAdp), whole blood (Nam+NAD+NAdp), and urine (Nam+MNA+2-Py+4-Py) was measured by HPLC. Pantothenic acid in liver (pantothenic acid+CoA and its derivatives), plasma (pantothenic acid), and urine (pantothenic acid) was measured by microbioassay using *Lactobacillus plantarum* ATCC 8014. Folate in liver (monoglutamatated folate+polyglutamated folate), plasma (monoglutamatated folate+polyglutamated folate), and urine (monoglutamatated folate) was measured by microbioassay using *Lactobacillus rhamnosus* ATCC 27773. Biotin in liver (free form of biotin+bound form of biotin), plasma (free form of biotin+bound form of biotin), and urine (free form of biotin) was measured by microbioassay using *Lactobacillus plantarum* ATCC 8014.

**Measurement of lipid.** The concentration of total lipid in liver was measured by the method of Folch et al. (24).

**Statistical analysis.** Differences between pair-fed control and orotic acid groups were analyzed by Student’s *t*-tests. The gold standard group was arranged and used to show reference values. A value of *p*<0.05 was considered significant. Graph Pad Prism 5.0 (Graph Pad Software, San Diego, CA) was used for all analyses.

**RESULTS**

**Food intakes and body weight gains**

As is known, when excess orotic acid diet is fed, food intake is lower compared with the group with free access to a nutritionally complete diet (9, 10). Thus, we compared food intakes between the orotic acid and the pair-fed control groups. Total food intakes in the orotic acid group were 245.3±5.8 g/20 d, and in the pair-fed group fed the complete diet were 240.2±0.3 g/20 d. The food intake of the group of rats with free access to a nutritionally complete diet was 261.4±0.9 g/20 d. The group with free access to the complete diet was used for reference values and as a gold standard. The body weight gains on the last day of the experimental period of 20 d among rats fed the orotic acid diet were lower than those of the group pair-fed the complete diet (103.4±2.7 g vs 115.9±1.7 g; *p*<0.001). The body weight gain in the gold standard group was 130.8±3.1 g.

**Liver concentrations of B-group vitamins**

Administration of excess orotic acid induced fatty liver and enlargement (Table 2). Fatty liver induced by excess orotic acid administration lowered the concentrations of vitamin B1, niacin, pantothenic acid, folate, and biotin in terms of g of liver compared with those in the pair-fed control group (Table 2). However, for the comparison in terms of liver, the difference in the amount
of folate between the pair-fed and orotic acid groups disappeared.

**Blood concentrations of B-group vitamins**

Fatty liver lowered the blood concentrations of vitamin B₁, PLP, niacin, pantothenic acid, folate, and biotin compared with those of the pair-fed control groups (Table 3).

**Urine concentrations of B-group vitamins**

Urinary excretion amounts of vitamin B₁, pantothenic acid, folate, and biotin were higher in the orotic acid group than in the pair-fed control group (Table 4). Conversely, the urinary excretion of the sum of Nam and its catabolites, such as MNA, 2-Py, and 4-Py, was lower in the orotic acid group than in the pair-fed control group (Table 4). The urinary excretion amounts of vitamin B₂, vitamin B₆ (measured as 4-PIC), and vitamin B₁₂ were not different between groups (Table 4).

**DISCUSSION**

The most interesting event following excess administration of orotic acid is that the liver accumulates a lot of triglycerides (5); this effect is completely prevented and recovered by further administration of dietary adenine (4, 8), which is induced by an inhibition of the synthesis and release of hepatic β-lipoprotein (6) and by an inhibition of the biosynthesis of adenine nucleotides (9). The de novo synthesis of ATP via inosine 5'-monophosphate needs 5-phosphoribosyl-1-pyrophosphate (PRPP), amino acids such as glutamine, glycine, and aspartic acid, and vitamins such as folate and niacin.

Orotic acid is a precursor of pyrimidine nucleotides, which react to form orotate 5'-monophosphate (OMP) in the presence of PRPP, which is then metabolized to uridine 5'-monophosphate (UMP). All pyrimidine nucleotides are synthesized from UMP. In contrast, pyrimidine bases can be completely catabolized into CO₂ in mammals via uracil, unlike purine bases. Orotic acid can be catabolized into CO₂ via UMP and uracil, but not directly. Therefore, when orotic acid is catabolized into CO₂, an extraordinary amount of PRPP is consumed because the synthesis of UMP from orotic acid needs PRPP. However, the reaction of uridine to uracil releases ribose, and the ribose is then reused to make ribose-5-phosphate and PRPP. Thus, for the complete degradation of orotic acid, ribose-5-phosphate might not be consumed theoretically, but the ATP consumption should be accelerated. Von Euler et al. (9) reported that a large amount of UMP but not orotic acid accumulated in orotic acid-induced fatty livers. Thus, excess administration of orotic acid reduces adenine nucleotides (6) and total pyridine nucleotides concentrations (5, 8–10) because the reaction of ribose-5-phosphate to PRPP needs ATP and the biosynthesis of ATP and NAD from precursors such as adenine, nicotinamide, nicotinic acid, and quinolinic acid need PRPP. The complete degradation pathway of orotic acid and the biosynthesis of PRPP from glucose-6-phosphate are shown in Fig. 1. Vitamin B₆ and niacin are involved in the pathway.

In the present experiment, we investigated how orotic acid administration affected the vitamin concentrations in the liver, blood, and urine excretion in rats. As was previously reported (5, 8–10), the concentrations of niacin in the liver, blood and urine were lower in the orotic acid group than in the pair-fed control group. In the present experiment, a nicotinic acid-free diet was used, so that the Nam and its catabolites, such as MNA, 2-Py, and 4-Py, originated only from tryptophan. The urinary excretion amounts of the sum (Nam + MNA + 2-Py + 4-Py) were 150 nmol/daily urine in the orotic acid group and 1,000 nmol/daily urine in the pair-fed control group. Although this finding was already reported by us (10), the conversion ratio of tryptophan to Nam was dramatically lower in the orotic acid group than in the pair-fed control group in the present experiment. A target site of orotic acid is reported to be the reaction of 3-hydroxanthranilic acid to α-amino-β-carboxymuconate-ε-semialdehyde (ACMS), which is catalyzed by 3-hydroxanthranilic acid 3,4-dioxygenase (10). However, the precise mechanism has not been elucidated.

A PLP enzyme, β-alanine-pyruvate transaminase, is involved in the degradation pathway of orotic acid. The concentration of vitamin B₆ in the liver and the urinary excretion amount of 4-pyridoxic acid (4-PIC), a major catabolite of vitamin B₆, were not affected by orotic acid administration; however, concentrations in the blood were lower in the orotic acid group than in the control group. The reaction of pyridoxal to 4-PIC normally occurs in the liver and the 4-PIC is also normally transported to the blood and then eliminated in the urine. Conversely, the transport of the active form of vitamin B₆ from the liver to the blood would be suppressed by orotic acid administration because of a higher demand for β-alanine-pyruvate transaminase.

Although vitamin B₁ is not directly involved in the complete degradation pathway of orotic acid, it is involved in the metabolism of ribose-5-phosphate. When a requirement of PRPP in cells is lower and that of NADPH is higher, ribose-5-phosphate is re-converted into glucose-6-phosphate by the catalysis of a thiamin diphosphate-dependent enzyme, transketolase, and other enzymes. The biosynthesis of fatty acids from acetate and acetyl-CoA needs much of the NADPH. Creasey et al. (5) reported that the addition of orotic acid in vitro to liver slices of normal rats caused a stimulation of the incorporation of ¹⁴C-labelled acetate into fatty acids. De novo synthesis of fatty acids occurs in the livers of rats fed orotic acid. In the biosynthesis, acetyl-CoA carboxylase is the limiting enzyme, which is a biotin-dependent enzyme. Pantothenic acid occupies an integral part of CoA. The concentrations of vitamin B₁ in liver and blood were lower in the orotic acid group than in the pair-fed control, while the urinary excretion amount was converse. Similar findings were observed with biotin and pantothenic acid. Marchetti and Puddu (25), and Sarma and Sidransky (26) have reported that the amounts of biotin in the livers of orotic acid-fed rats were lower than in the controls. The lower amounts of vitamin B₁ and biotin in liver indicated that the elimination of vitamin B₁ and biotin was accelerated because of an increase in the de novo synthesis of fatty acids. Our
previous data show that the increase in urinary excretion amounts of B-group vitamins generally means surplus intake of vitamins (23, 27, 28). But, in the present experiment, such a consideration would not apply because the concentrations of vitamin B1, biotin, and pantothenic acid in liver and blood were decreased. Although the following consideration might be curious, it is possible that the accelerated elimination of vitamin B1, biotin, and pantothenic acid suppresses the abnormal increase in the de novo fatty acid synthesis by orotic acid. Such strange phenomena might be basically dependent on the amount of the thiamin (vitamin B1) transporter THTR (29), biotin transporter sodium-dependent multivitamin transporter SMVT (30), and pantothenic acid transporter SMVT (31) in tissues. Thus, orotic acid-induced fatty liver could affect the expressions of such transports.

A lot of ATP is consumed in catabolizing orotic acid to CO2. Folate is involved in the de novo synthesis of purine nucleotides such as ATP and GTP in

Fig. 1. Catabolic pathway of orotic acid. (1) Glucose-6-phosphate dehydrogenase [EC 1.1.1.49], (2) 6-phosphogluconolactonase [EC 1.1.1.3], (3) 6-phosphogluconate dehydrogenase [EC 1.1.1.44], (4) ribulose-5-phosphate isomerase [EC 5.3.1.6], (5) 5-phosphoribosyl-1-pyrophosphate synthetase [EC 2.7.6.1], (6) orotate phosphoribosyltransferase [EC 2.4.2.10], (7) orotate 5′-mononucleotide decarboxylase [EC 4.1.1.23], (8) 5′-nucleotidase [EC 3.1.3.5], (9) uridine nucleosidase [EC 3.2.2.3], (10) dihydouracil dehydrogenase [EC 1.3.1.1], (11) 5,6-dihydrouracil amidohydrolase [EC 3.5.2.2], (12) β-ureidopropionase [EC 3.5.1.6], (13) β-alanine-pyruvate transaminase [EC 2.6.1.18], (14) malonate semialdehyde dehydrogenase [EC 1.2.1.18], (15) ribokinase [EC 2.7.1.15].
the reactions of $5'$-phosphoribosylglycinamide$\rightarrow 5'$-
phosphoribosyl-N-formylglycinamide (catalyzed by for-
myltransferase) and $5'$-phosphoribosyl-4-carboxamide-
$5$-aminomimidazole$\rightarrow 5'$-phosphoribosyl-4-carboxamide-
$5$-formamimimidazole (catalyzed by formyltransferase).
The concentration of folate in the liver was lower in the
orotic acid group than in the pair-fed control, but the
amount of folate per liver and the blood concentrations
of folate were almost the same between the two groups.
The urinary excretion of folate was higher in the orotic
acid group than in the pair-fed control. These findings
indicated a trend toward metabolic control might be
suppressive of ATP synthesis through the decrease in
folate concentration in the cells. Similar phenomena
were observed with pantothenic acid, folate, and biotin;
however, the blood concentrations of these three vita-
mins were not different.

The concentrations of vitamin B$_{12}$ in the liver and
blood, and the urinary excretion were not affected by
orotic acid administration. For vitamin B$_{2}$, the data for
urine were obtained and the excretion was not affected
by orotic acid administration.

In conclusion, the ability to sustain vitamin B$_{1}$, pan-
tothenic acid, folate, and biotin decreased in fatty liver,
which was a very specific phenomenon. This specific
phenomenon might occur to prevent an abnormally
increased biosynthesis of fatty acids by orotic acid,
which we hypothesize is a metabolic response to recover
normal metabolic conditions.

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**Author contributions**

K.S. designed the study, and drafted the manuscript.
N.M., T.K., A.T., and T.F. performed the experiments, and
all authors reviewed the manuscript and helped in the
study design.

**Competing interests**

The authors disclose no potential conflicts of interest.

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