Effects of Fatty Liver Induced by Niacin-free Diet with Orotic Acid on the Metabolism of Tryptophan to Niacin in Rats

Tsutomu FUKUWATARI¹, Yuko MORIKAWA, Etsuro SUGIMOTO, and Katsumi SHIBATA

Laboratory of Food Science and Nutrition, Department of Life Style Studies, School of Human Cultures, The University of Shiga Prefecture, 2500 Hassakacho, Hikone, Shiga, 522-8533, Japan

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The effects of dietary orotic acid on the metabolism of tryptophan to niacin in weaning rats was investigated. The rats were fed with a niacin-free, 20% casein diet containing 0% (control diet) or 1% orotic acid diet (test diet) for 29 d. Retardation of growth, development of fatty liver, and enlargement of liver were observed in the test group in comparison with the control group. The concentrations of NAD and NADP in liver significantly decreased, while these in blood did not decrease compared to the control group. The formation of the upper metabolites of tryptophan to niacin such as anthranilic acid, kynurenic acid, and 3-hydroxyanthranilic acid were not affected, but the quinolinic acid and beyond, such as nicotinamide, N¹-methylnicotinamide, N¹-methyl-2-pyridone-5-carboxamide, and N¹-methyl-4-pyridone-3-carboxamide, were significantly reduced by the administration of orotic acid. Therefore, the conversion ratio of tryptophan to niacin significantly decreased in the test group in comparison with the control group.

Key words: orotic acid; tryptophan; niacin; NAD; fatty liver

Fatty liver has been induced by drinking much of alcohol,¹ obesity,² diabetes,² and so on, and the prevention of fatty liver is very important for guarding against degenerative diseases. Chronic administration of ethanol to experimental animals has been reported to disturb the metabolism of NAD.³ Mammals including humans can de novo synthesize NAD from tryptophan and the supply from tryptophan accounts for over a half of the total niacin supply in humans.⁴ Therefore, the decreased conversion ratio of tryptophan to niacin was a severe influence on the systemic metabolism since over 500 enzymes need niacin coenzymes. Shibata et al.⁵ have been reported that the conversion ratio was lower in diabetic rats than in normal rats. Furthermore, Shibata and Onodera⁶ have been reported that a large amount of fat administration, especially oil including with polyunsaturated fatty acids, significantly increased the conversion ratio. From these reports, it might be probable that the metabolism of tryptophan to niacin is disturbed in fatty liver.

Orotic acid is an intermediate in the pyrimidine nucleotide synthetic pathway. The administration of large amounts of orotic acid induces fatty liver⁷ and liver injuries.⁸ Furthermore, it has been reported that increased triglyceride synthesis mediated by changes in liver phosphatidate phosphohydrolase activity was involved in the hepatic triglyceride accumulation induced by orotic acid administration and that this change was markedly suppressed by dietary n-3 fatty acids.⁹ Goto et al.¹⁰ also have been reported that dehydroepiandrosterone, as well as clofibrate, may prevent orotic-acid-induced fatty liver.

Accumulation of lipid within hepatocytes, or steatosis, often occurs after the ingestion of alcohol and certain drugs, including corticosteroids, tetracycline, and some nonsteroidal disorders and diseases with hepatic involvement.¹¹ Microvesicular steatosis is identified by the presence of multiple small lipid droplets in hepatic sections and appears to be secondary to mitochondrial injury (which results in impaired β-oxidation of fatty acids and impaired respiration leading to ATP depletion) and/or to decreased synthesis of lipoproteins by the liver.¹²-¹⁴ Although early steatosis may be reversible, continued hepatotoxic injury may lead to fibrosis and eventual cirrhosis. In patients with cirrhosis of the liver, it has been reported that urinary excretion of N¹-methylnicotinamide (MNA), which is a main catabolite of NAD, twofold increase in comparison with normal subjects.¹⁵ Therefore, fatty liver might disturb the catabolism of NAD. The catabolites are excreted into urine and, therefore, the urinary

¹ To whom correspondence should be addressed. Phone: +81-749-28-8454; Fax: +81-749-28-8601; E-mail: fukkie@shc.usp.ac.jp

Abbreviations: PRPP, 5-phosphoribosyl-1-pyrophosphate; NIA, nicotinic acid; Nam, nicotinamide; MNA, N¹-methylnicotinamide; 2-Py, N¹-methyl-2-pyridone-5-carboxamide; 4-Py, N¹-methyl-4-pyridone-3-carboxamide; ANA, anthranilic acid; KA, kynurenic acid; XA, xanthurenic acid; 3-HA, 3-hydroxyanthranilic acid; QA, quinolinic acid; NaMN, nicotinic acid mononucleotide; ACMS, α-amino-β-carboxyamyconate-carboxylase; ACMSD, α-amino-β-carboxyamyconate-carboxylase deacetylase; QPRT, quinolinate phosphoribosyltransferase.
catabolites would be useful indices of fatty liver and/or cirrhosis of the liver.

5-phosphoribosyl-1-pyrophosphate (PRPP) is an essential cosubstrate for the biosyntheses of purine, pyrimidine, and pyridine nucleotides. As the concentration of PRPP in rat liver was reported to be about 15 μM, PRPP would be a limiting substrate for phosphoribosylation of purine, pyrimidine, and pyridine bases. In fact, exposure to orotic acid results in an imbalance in nucleotide pools characterized by an increase in uridine nucleotides and a decrease in adenosine nucleotides. An inhibitory effect of orotic acid on DNA synthesis was further supported by an observation of decreased incorporation of [3H]deoxyadenosine into DNA and a lower rate of cellular proliferation. It has been suggested that the phenomenon is due to the competition between orotic acid and purine bases for PRPP. The biosynthesis of pyridine nucleotides from nicotinic acid (NiA), nicotinamide (Nam), and quinolinic acid (QA) also needs PRPP. Rajalakshmi et al. and von Euler et al. reported that the addition of 1% orotic acid to a diet decreased the level of pyridine nucleotides in rat liver. To gain an insight into the mechanism of this decrease and the effects of fatty liver on the metabolism of tryptophan to niacin, Shibata et al. have reported the effects of dietary orotic acid on the levels of liver and blood NAD in rats, however, at that time, the catabolic metabolites could not be measured correctly. Now, we can measure these metabolites. Accordingly, we re-investigated precisely the effects of dietary orotic acid on the metabolism of tryptophan to niacin in the weaning rats was studied.

Materials and Methods

Chemicals. NAD⁺ and NADP⁺ were purchased from Sigma Chemical Company (St. Louis, MO, USA). Vitamin-free milk casein, sucrose, L-methionine, nicotinamide, and anthranilic acid were purchased from Wako Pure Chemical Industries (Osaka, Japan). Kynurenine sulfite, kynurenic acid, 3-hydroxyanthranilic acid, and N⁴-methylnicotinamide were purchased from Tokyo Kasei Kogyo (Tokyo, Japan). N¹-methyl-2-pyridone-5-carboxamide and N¹-methyl-4-pyridone-5-carboxamide were synthesized by the methods of Pullman and Colowick and of Shibata et al., respectively. Gelatinized cornstarch and corn oil were purchased from Nichiden Kagaku (Tokyo, Japan) and Ajinomoto (Tokyo, Japan), respectively. The mineral and vitamin mixtures were obtained from Oriental Yeast Kogyo (Tokyo, Japan), all the other chemicals used being of the highest purity available from commercial sources.

Animals. The care and treatment of the experimental animals conformed to The University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals.

Male rats of the Wistar strain (3 weeks old with a body weight of around 37 g) were obtained from Clea Japan (Tokyo, Japan) and immediately placed in individual metabolic cages (CT-10; Clea Japan). They were then divided into two groups and fed ad libitum for 29 d, one group with a niacin-free, 20% casein diet, and the other with the same diet + 1.0% orotic acid (Table 1). Niacin-free diets were used because all of niacin and its metabolites come from only tryptophan.

The room temperature was maintained at around 22°C and about 60% humidity, and a 12-hr light (06:00-18:00)/12-hr dark (18:00-06:00) cycle was maintained. Body weight and food intake were measured daily at around 10:00. Urine samples (24-h; 10:00-10:00) were periodically (day 14, day 21, and day 29) collected in amber bottles containing 1 ml of 1 M HCl, and were stored at −25°C until needed. The rats were killed by decapitation at around 10:00 on the last day (day 29) of the experiment and a 20-μl sample of blood was taken from the carotid artery and treated as described in the literature for measuring NAD (NAD⁺ + NADH) and NADP (NADP⁺ + NADPH). The liver of each animal was removed, and a portion (approximately 0.2 g) was

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**Table 1. Composition of the Diets**

<table>
<thead>
<tr>
<th>Ingredients</th>
<th>Control diet</th>
<th>Orotic acid diet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(NiA-free, 20% casein diet)</td>
<td>(control diet + orotic acid)</td>
</tr>
<tr>
<td></td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Milk casein</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>L-Methionine</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Gelatinized-cornstarch</td>
<td>45.9</td>
<td>45.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>22.9</td>
<td>22.6</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Mineral mixture</td>
<td>5.0</td>
<td>5.0</td>
</tr>
<tr>
<td>Vitamin mixture (NiA-free)</td>
<td>1.0</td>
<td>1.0</td>
</tr>
<tr>
<td>Orotic acid</td>
<td>0</td>
<td>1.0</td>
</tr>
</tbody>
</table>

immediately treated as described in the literature to measure NAD and NADP. Another portion of the liver (approximately 1 g) was treated as described in the literature to measure the enzyme activities involved in the metabolism of tryptophan to niacin.

Analyses. The contents of NAD (NAD+ + NADH) and NADP (NADP+ + NADPH) were measured by the colorimetric method of Shibata and Murata and Shibata and Tanaka, respectively. To calculate the conversion ratio of tryptophan to niacin, the urinary contents of Nam and of metabolites N'-methylnicotinamide, N'-methyl-2-pyridone-5-carboxamide, and N'-methylene4-pyridone-5-carboxamide were measured. This method does not take account of the content of Nam in the body weight gain, and the value does not, therefore, represent the net conversion ratio. The conversion ratio was calculated as the sum of the urinary excretions of nicotinamide + N'-methylnicotinamide + N'-methyl-2-pyridone-5-carboxamide + N'-methylene4-pyridone-5-carboxamide (mmol/day) × 100/tryptophan intake during urine collection (mmol/day). The contents of nicotinamide, N'-methyl-2-pyridone-5-carboxamide, and N'-methylene4-pyridone-5-carboxamide in the urine were simultaneously measured by the HPLC method of Shibata et al., while the content of N'-methylnicotinamide in the urine was measured by the HPLC method of Shibata.

The contents of kynurenic acid, xanthurenic acid, anthranilic acid, 3-hydroxyanthranilic acid, and quinolinic acid in the urine were measured by HPLC.

Tryptophan dioxygenase (EC 1.13.11.11), kynureninase (EC 3.7.1.3), kynurenine aminotransferase (EC 2.6.1.7), 3-hydroxyanthranilic acid oxygenase (EC 1.13.11.6), α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (EC 4.1.1.45), quinolinate phosphoribosyltransferase (EC 2.4.2.19), and NAD+ synthetase (EC 6.3.1.5) were measured as described in the literature.

The activity of NAD+-splitting enzymes was measured as follows. The reaction mixture (500 μl) comprised 50 μl of 500 mM KH2PO4-K2HPO4 buffer (pH 7.0), 100 μl of 5 mM NAD+, 340 μl of water and 10 μl of liver homogenate. Incubation was started by adding liver homogenate, and done at 37°C for 10 min. The reaction was stopped by heating in a 90°C water bath for 1.5 min. After cooling on ice, the mixture was centrifuged and the supernatant used for measurement of NAD+. In this case, much NAD+ exists in the supernatant, and NAD+ can be measured by the increase in absorbance of 340 nm using alcohol dehydrogenase. The activity of NAD+-splitting enzymes is the sum of all enzyme activities that can split NAD+; for example, poly(ADP-ribose) polymerase (EC 2.4.2.30), NAD+ nucleosidase (NAD+ glycohydrolase, EC 3.2.2.5), and mono ADP-ribosyltransferase.

The content of total lipid in liver was measured by the method of Folch et al.

Results

Body weight gain, food intake, and liver weight
Weaning rats were fed with a diet containing a large amount of orotic acid in order to induce fatty liver. The food intake was lower in the orotic acid group than in the control group from day 15 onwards and the body weight gain was also lower from day 15 onwards as shown in Figure 1. Table 2 shows the food efficiency ratio and liver weight. The food efficiency ratio was not affected by feeding the orotic acid diet. The orotic acid administration induced the liver weight gain in comparison with the control. The

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**Fig. 1.** Effects of Dietary Orotic Acid on Body Weight Gain (A) and Food Intake (B).

○, Control; ●, Orotic acid. Values are means ± SEM (n = 5); *Significant difference from the control group at p < 0.05 on the same day, calculated by Student’s t test.
The contents of NAD and NADP in liver and blood

Table 3 shows the comparison of the concentration of NAD and NADP in liver and blood between the control and orotic acid groups. The concentrations of NAD and NADP in liver were lower in the orotic acid group than in the control group, while these concentrations were not observed to be different between the two groups in blood.

Urinary excretion of the upper metabolites of tryptophan oxidation pathway

Figure 2 shows the influence of orotic acid-induced fatty liver on the urinary excretion of the upper part of the metabolites of the tryptophan-niacin. Urinary outputs of these metabolites, anthranilic acid (AnA), kynurenic acid (KA), 3-hydroxyanthranilic acid (3-HA), and xanthurenic acid (XA) were expressed in terms of nmol/g of food, since food intakes were not the same among the groups. AnA, KA, and 3-HA were almost the same between the two groups, while only XA excretion was significantly lower in the orotic acid group than in the control group.

Urinary excretion of quinolinic acid, niacin and its metabolites

Figures 3 and 4 show the influence of orotic acid-induced fatty liver on the urinary excretion of the lower part of the metabolites of the Trp-niacin. These metabolites, QA, Nam, MNA, N^6^-methyl-2-pyridone-5-carboxamide (2-Py), and N^1^-methyl-4-pyridone-5-carboxamide (4-Py) were significantly lower in the orotic acid group than in the control.

Conversion ratio of tryptophan to niacin

Figure 5-A shows the SUM, which is the total amount of Nam + MNA + 2-Py + 4-Py, and Fig. 5-B the conversion ratio of tryptophan to niacin, which is calculated as follows; SUM (mol/daily urine)/tryptophan intake during the urine sample collection (mol/day) × 100. This value in the fatty liver rats was about 1/4 of the control.

Enzyme activities involved in the metabolism of tryptophan to niacin in liver

Many of the enzymes involved in the tryptophan to niacin pathway, tryptophan dioxygenase (TDO), kynureninase, kynurenine aminotransferase, 3-HA oxygenase, NAD⁺ synthetase, and NAD-splitting enzymes, were lower in the orotic acid group than in the control group, as shown in Table 4. The activities of quinolinic phosphorybosyl transferase (QPRT) and α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD) were not affected by the administration of orotic acid.

Discussion

The retardation of body weight gain, enlargement of liver, and accumulation of fat in liver caused by feeding orotic acid diet that have already been reported were also observed. We also found a decrease in NAD and NADP concentrations in the liver (Table 3) as reported previously. However, in this experiment, the blood concentrations of NAD and NADP were not reduced by feeding the test diet (Table 3). The blood NAD content can be a relevant biomarker of niacin status, and is affected by niacin nutrition, malaria, and exercise, but the blood NADP content is kept at a certain level, except after moderate exercise. It is unclear why the blood NAD level was not consistent with the liver NAD level.

The urinary excretions of the upper metabolites of tryptophan to niacin, such as AnA, KA, and 3-HA were not observed to be different between the two groups (Fig. 2), while the XA excretion was significantly reduced by the administration of orotic acid (Fig. 2-C). Kynurenine oxidoglutarate aminotransferase metabolizes kynurenine and 3-hydroxy kynurenine into KA and XA, respectively. Although KA and XA are metabolized by the same enzyme, it is unclear why only XA was reduced by orotic acid.
administration. The amounts of kynurenine and 3-hydroxy kynurenine may be affected by orotic-acid-induced fatty liver.

The excretion of QA, a key intermediate in the conversion pathway of tryptophan to niacin, was significantly reduced by the administration of orotic acid (Fig. 3). The excretion of the catabolic metabolites of NAD, such as Nam, MNA, 2-Py, and 4-Py were all reduced by the administration of orotic acid (Fig. 4). Therefore, the excretion of the sum of Nam, MNA, 2-Py, and 4-Py decreased in the test group (Fig. 5-A) and the conversion ratio of tryptophan to niacin was also significantly lower in the test group than in the control group.

The conversion of tryptophan to niacin occurs mainly in the liver. The activities concerned with the pathway in liver were measured. The activities of TDO, kynureninase, kynurenine aminotransferase, 3-HA oxygenase, and NAD$^+$ synthetase were significantly lower in the test group than in the control group (Table 4), while the activities of ACMSD and QPRT were not different between the two groups (Table 4). The latter two enzymes are believed to be critical in the conversion of tryptophan to niacin; ACMSD catalyzes the reaction of $\alpha$-amino-$\beta$-carboxymuconate-$\epsilon$-semialdehyde (ACMS)-$\epsilon$-aminomuconate-$\epsilon$-semialdehyde (AMS), and QPRT catalyzes the reaction of QA$\rightarrow$nicotinic acid mononucleotide (NaMN). ACMS is a very unstable intermediate and spontaneously cyclizes to QA, if ACMSD wakes ACMS, the produced QA is then

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**Fig. 2.** Effects of Dietary Orotic Acid on the Urinary Excretion of AnA (A), KA (B), XA (C), and 3-HA (D).

○, Control; ■, Orotic Acid. Values are means ± SEM (n = 5); *Significant difference from the control group at $p < 0.05$ on the same day, calculated by Student’s $t$ test.

**Fig. 3.** Effects of Dietary Orotic Acid on the Urinary Excretion of QA.

○, Control; ■, Orotic Acid. Values are means ± SEM (n = 5); *Significant difference from the control group at $p < 0.05$ on the same day, calculated by Student’s $t$ test.
Effects of Fatty Liver Induced by Niacin-free Diet with Orotic Acid on the Metabolism of Tryptophan to Niacin

Fig. 4. Effects of Dietary Orotic Acid on the Urinary Excretion of Nam (A), MNA (B), 2-Py (C), and 4-Py (D).
O, Control; ●, Orotic Acid. Values are means ± SEM (n = 5); *Significant difference from the control group at p < 0.05 on the same day, calculated by Student's t test.

Fig. 5. Effects of Dietary Orotic Acid on the Urinary Excretion of SUM (A) and the Conversion Ratio of Tryptophan to Niacin (B).
O, Control; ●, Orotic Acid. Values are means ± SEM (n = 5); *Significant difference from the control group at p < 0.05 on the same day, calculated by Student's t test.

converted to NaMN in the presence of PRPP. In this connection, AMS is metabolized into acetyl-CoA.
The liver QPRT activity is reduced only by feeding of adenine resulting in renal failure, and the liver ACMSD activity is affected by dietary protein levels, the composition of fatty acids in fats, diet, diabetes, and some hormones. In this study, administration of orotic acid did not influence the activities of QPRT or ACMSD. The activity of NAD-splitting enzymes was lower in the test group than in

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Table 4. Effect of the Administration of Orotic Acid on the Enzyme Activities Involved in the Tryptophan-niacin Pathway in Liver

<table>
<thead>
<tr>
<th>Protein concentration (mg/g liver)</th>
<th>Control</th>
<th>Orotic acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>TDO</td>
<td>1.99±0.17</td>
<td>0.79±0.07*</td>
</tr>
<tr>
<td>Kynureninase</td>
<td>1.70±0.60</td>
<td>0.71±0.06*</td>
</tr>
<tr>
<td>Kynureneaminotransferase</td>
<td>0.97±0.12</td>
<td>0.51±0.04*</td>
</tr>
<tr>
<td>3-4A oxygenase</td>
<td>501±21</td>
<td>270±41*</td>
</tr>
<tr>
<td>ACMSD</td>
<td>1.0±0.1</td>
<td>1.1±0.1</td>
</tr>
<tr>
<td>QPRT</td>
<td>0.82±0.02</td>
<td>0.73±0.06*</td>
</tr>
<tr>
<td>NAD+ synthetase</td>
<td>0.97±0.07</td>
<td>0.49±0.02*</td>
</tr>
<tr>
<td>NAD-degrading enzyme</td>
<td>25.41±2.02</td>
<td>18.52±4.43*</td>
</tr>
</tbody>
</table>

Values are means ± SEM of μmol/hr/g of wet weight (n=5); *Significant difference from the control group at p<0.05, calculated by Student’s t test.

the control group (Table 4), but the physiological meaning is not known.

As described in the section of introduction, PRPP is an essential co-substrate for the biosynthesis of purine, pyrimidine, and pyridine nucleotides. The biosynthesis of pyridine nucleotides from NaA, Nam, and QA also needs PRPP. If severe competition occurs among purine, pyrimidine, and pyridine bases, urinary excretion of QA must be increased by the administration of orotic acid. But, such a phenomenon was not observed (Fig. 3). It may be that QA administration affects the reactions of 3-HA→ACMS→QA because urinary excretions of QA and beyond were significantly reduced by the administration of orotic acid.

The effects of orotic acid on the reduced conversion ratio of tryptophan to niacin must be indirect. A very recent report from our laboratory showed that the effects of some compounds on the conversion ratio of tryptophan to niacin can be predicted from the data on urinary excretion of QA. It has been reported that the dietary fatty acid composition significantly affects the conversion ratio; polyunsaturated fatty acids increased the conversion, while, saturated fatty acids did not affect it. Although we do not know what kinds of fatty acids increase in the fatty liver induced by orotic acid, some fatty acids inhibiting the conversion might be accumulated in fatty liver induced by orotic acid. Three enzymes are involved in the fate of QA: 3-HA oxygenase (3-HA→ACMS→QA), but ACMS→QA is a non-enzymatic reaction, ACMSD (ACMS→AMS), and QPRT (QA→NaMN). Only the activity of 3-HA oxygenase was significantly lower in the test group than in the control group (Table 4). This enzyme activity is extremely high in comparison with other enzymes involved in the conversion pathway (see Table 4). Although the reason why it is so high is unclear, it must be a very important enzyme in the pathway.

In conclusion, administration of orotic acid reduced the conversion ratio of tryptophan to niacin. This effect might not be direct and was not a result of competition for PRPP between orotic acid and QA. It is possible that the reducing effect of orotic acid on the conversion of tryptophan to niacin is attributed to the accumulation of some specific fat that inhibits the reactions of 3-HA→ACMS→QA; probably due to reduction of the activity of 3-HA oxygenase.

Acknowledgment

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References


