Growth-promoting Activity of Pyrazinoic Acid, a Putative Active Compound of Antituberculosis Drug Pyrazinamide, in Niacin-deficient Rats through the Inhibition of ACMSD Activity

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We have recently reported that the antituberculosis drug, pyrazinamide (PZA), caused a significant increase in the conversion ratio of tryptophan to niacin in rats. In the present work, we investigated whether or not pyrazinoic acid (POA), a putative metabolite of PZA, increased the conversion ratio of tryptophan to niacin in rats. Weaning rats were fed with a niacin-free and tryptophan-limited diet (negative control diet), or with the negative control diet supplemented with 0.003% nicotinic acid (positive control diet) or 1% POA (test diet) for 27 days. The growth rate was almost same between the groups fed on the positive control diet and the test diet. Dietary POA significantly increased the conversion ratio of tryptophan to niacin. Although POA did not directly inhibit the activity of a-amino-β-carboxymuconate-ε-semialdehyde decarboxylase (ACMSD), the rate-limiting enzyme in the tryptophan-niacin pathway, liver ACMSD activity was only not detected in the test diet group. These results suggest that a derivative of POA metabolized by rats inhibited the ACMSD activity.

Key words: pyrazinamide; pyrazinoic acid; tryptophan metabolism; nicotinamide; tryptophan-niacin conversion ratio

The fact in the late 1940s that nicotinamide (Nam) was active against tubercle bacilli in animal models was found by accident, and subsequent synthesis of analogues of Nam led to the identification of pyrazinamide (PZA) as the most effective derivative against Mycobacterium tuberculosis. PZA is an important component of the current 6-month course of tuberculosis chemotherapy. This therapy, which includes isoniazid, rifampin, PZA and ethambutol, is recommended by the World Health Organization for treating all tuberculosis patients. The susceptibility of M. tuberculosis to PZA is correlated with the presence of a single enzyme with nicotinamide and pyrazinamidase. Strains of M. tuberculosis that are resistant to PZA are defective in PZA activity. Scorpio and Zhang have cloned the M. tuberculosis pyrazinamidase gene (pncA) and showed that the mutation of pncA is a major factor of PZA resistance in M. tuberculosis. It is therefore considered that PZA needs to be activated by bacterial nicotinamide-pyrazinamidase into pyrazinoic acid (POA) in bacterial cells. Although POA is a putative active compound of PZA, POA is not directly used to treat tuberculosis patients, because the bactericidal activity of POA, when given orally to mice infected with M. tuberculosis, was found to be not as significant as that of PZA, presumably due to poor absorption through the gastrointestinal tract.

Unlike most antibacterial agents, PZA, despite its remarkable in vivo activity, had no activity against Mycobacterium tuberculosis in vitro in a normal culture medium. This phenomenon means that the derivatives of PZA and POA that are metabolized by the host cells in humans and experimental animals must be the true active agents. In this connection, we have already investigated the effects of PZA on the Nam metabolism in rats to elucidate the mechanism for the PZA action. We have reported that PZA had growth-promoting activity in weaning rats fed with an nicotinic acid (NiA)-free and Trp-limited diet by increasing the conversion ratio of Trp to niacin; PZA increased the formation of Nam and NAD. However, Hayakawa et al. have reported that POA was a competitive inhibitor of nicotinic acid phosphoribosyltransferase (NDPT), which is a rate-limiting enzyme in the biosynthesis of NAD from NiA. On the contrary, Nasu et al. have reported that an intraperitoneal injection of POA to rats

Abbreviations: PZA, pyrazinamide; POA, pyrazinoic acid; NiA, nicotinic acid; Nam, nicotinamide; NMN, nicotinamide mononucleotide; Trp, L-tryptophan; QA, quinolinic acid; MNA, N1-methylnicotinamide; 2-Py, N1-methyl-2-pyridone-5-carboxamide; 4-Py, N1-methyl-4-pyridone-5-carboxamide; KA, kynurenic acid; XA, xanthurenic acid; ACMS, a-amino-β-carboxymuconate-ε-semialdehyde; ACMSD, a-amino-β-carboxymuconate-ε-semialdehyde decarboxylase; NPRT, nicotinic acid phosphoribosyltransferase; QPRT, quinolinic acid phosphoribosyltransferase
resulted in an increase of the hepatic NAD content by about 2-fold in the control animals by the inhibition of α-amino-β-carboxyymuconate-ε-semialdehyde decarboxylase (ACMSD) activity. We elucidated in the present study the effect of POA on the metabolism of Trp to niacin in rats.

Materials and Methods

Chemicals. NAD⁺ and NADP⁺ were purchased from Sigma Chemical Company (St. Louis, MO, USA). Vitamin-free milk casein, sucrose, 1-methionine, Nam and quinolinic acid (QA) were purchased from Wako Pure Chemical Industries (Osaka, Japan). Kynurenic acid (KA), xanthurenic acid (XA) and N⁴-methyl-2-pyridone-5-carboxamide (2-Py) and N⁴-methyl-4-pyridone-5-carboxamide (4-Py) were respectively synthesized by the methods of Pullman and Colowick¹⁴ and of Shibata et al.⁰¹. Gelatinized cornstarch and corn oil were respectively purchased from Nichiden Kagaku (Tokyo, Japan) and Ajinomoto (Tokyo, Japan). 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 with 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 40 g) were obtained from Clea Japan (Tokyo, Japan) and immediately placed in individual metabolic cages (CT-10; Clea Japan). They were then divided into three groups and respectively fed ad libitum for 27 days with an NiA-free and Trp-limited diet as the negative control, the negative control diet supplemented with 0.003% NiA as the positive control, and the negative control diet with 1.0% POA as the test diet (Table 1).

The room temperature was maintained at around 20°C and about 60% humidity, and a 12-hr light/12-hr dark cycle was maintained. The body weight and food intake were measured daily at around 10:00. Urine samples (24-hr; 10:00 a.m.-10:00 a.m.) were periodically 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 a.m. on the last day of the experiment, and a 20-μl sample of blood was taken from the carotid artery of each animal and treated as described in the literature¹⁶ for measuring NAD (NAD⁺ + NADH) and NADP (NADP⁺ + NADPH). To measure NAD and NADP, the liver or kidneys of each animal were dissected, and a portion (approximately 0.2 g) was immediately treated as described in the literature.¹⁷ To measure the activities of quinolinic acid phosphoribosyltransferase (QPRT) and ACMSD, a portion (approximately 1 g) of the liver or kidneys was immediately homogenized with a Teflon-glass homogenizer in five volumes of a cold 50 mM KH₂PO₄-K₂HPO₄ buffer (pH 7.0). One part of each homogenate was centrifuged at 105,000 × g for 20 min, and the resulting supernatants were used as enzyme sources.

Analyses. The contents of NAD (NAD⁺ + NADH) and NADP (NADP⁺ + NADPH) were measured by the calorimetric method of Shibata and Murata¹⁶ and Shibata and Tanaka,¹⁸ respectively. To determine the conversion ratio of Trp to niacin, the urinary contents of Nam and of catabolic metabolites MNA, 2-Py, and 4-Py were each 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 excretion of Nam + MNA + 2-Py + 4-Py (mmol/day) × 100 / Trp intake during urine collection (mmol/day). The contents of Nam, 2-Py, and 4-Py in the urine were simultaneously measured by the HPLC method of Shibata et al.,¹⁵ while the content of MNA in the urine was measured by the HPLC method of Shibata.¹⁹

The contents of KA²⁰ and XA²¹ in the urine were measured by HPLC methods. ACMSD (EC 4.1.1.45)²² and QPRT (EC 2.4.2.19)²³ were measured as described in the literature.

### Table 1. Composition of the Diets

<table>
<thead>
<tr>
<th></th>
<th>Negative control diet (NiA-free, Trp-limited diet)</th>
<th>Positive control diet (0.003% NiA-containing, Trp-limited diet)</th>
<th>Test diet (Negative control diet + 1% POA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ingredient</td>
<td>(%)</td>
<td>(%)</td>
<td>(%)</td>
</tr>
<tr>
<td>Milk casein</td>
<td>9</td>
<td>9</td>
<td>9</td>
</tr>
<tr>
<td>Glycine</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>l-Threonine</td>
<td>0.078</td>
<td>0.078</td>
<td>0.078</td>
</tr>
<tr>
<td>l-Cystine</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Sucrose</td>
<td>74.722</td>
<td>74.719</td>
<td>73.722</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
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<td>Corn starch</td>
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<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mixture¹</td>
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<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin mixture¹</td>
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<td>1</td>
<td>1</td>
</tr>
<tr>
<td>NiA</td>
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<td>0.003</td>
<td>0</td>
</tr>
<tr>
<td>POA</td>
<td>0</td>
<td>0</td>
<td>1</td>
</tr>
</tbody>
</table>

Results

Effect of POA on the body weight gain and food intake

Weaning rats were fed with an NiA-free and Trp-limited diet (negative control diet), the negative control diet supplemented with 0.003% NiA (positive control diet) or the negative control diet with 1.0% POA (test diet) for 27 days. The growth rate and daily food intake were almost the same between the positive control and test diet groups, and were significantly higher than those of the negative control group (Fig. 1). The growth-promoting activity of POA was clearly observed.

Effect of POA on the metabolism of Trp to niacin

The urinary output of kynureninic acid (KA) and xanthurenic acid (XA) per gram of food intake are shown in Fig. 2. The output of both metabolites was significantly higher in the positive control and test diet groups than in the negative control group. The output of KA and XA was almost the same between the positive control and test groups. These findings mean that niacin deficiency caused a decrease in flux on the upper part of the Trp-niacin pathway; niacin deficiency itself decreases the conversion ratio of Trp to niacin.

Figure 3 shows the urinary output of quinolinic acid (QA). The formation of QA was much higher in the test diet group (110-fold) than in the negative and positive controls.

Figure 4 shows the urinary output of Nam, N1-methylnicotinamide (MNA), N1-methyl-2-pyridone-5-carboxamide (2-Py) and N1-methyl-4-pyridone-5-carboxamide (4-Py), the sum (Nam+MNA+2-Py+4-Py), and the conversion ratio of Trp to niacin (sum (μmol/day)/Trp intake during urine collection (μmol/day)×100). All of these metabolites in the negative control and the test groups were derived only from the Trp, whereas those in the positive control group were derived from Trp and NiA. Therefore, the conversion ratio of Trp to niacin in the positive control group was not precisely clarified (see Fig. 4-F). The formation of Nam (11-fold), MNA (130-fold), 2-Py (30-fold), and 4-Py (50-fold) was significantly higher in the test diet group than in the
negative control group. Therefore, the sum and the conversion ratio of Trp to niacin were both 50-fold higher in the test group than in the negative control group.

**Effect of POA on the NAD and NADP contents in liver and blood**

Table 2 shows the NAD and NADP contents in the liver and blood. The contents of NAD in the liver and blood were naturally higher in the test group than in the negative control group, and almost the same as those in the positive control group.

The NADP content in the liver was higher in the test and the positive control groups than in the negative control group, whereas the content in the blood

<table>
<thead>
<tr>
<th></th>
<th>Negative control diet (NiA-free, Trp-limited diet)</th>
<th>Positive control diet (0.003% NiA-containing, Trp-limited diet)</th>
<th>Test diet (Negative control diet + 1% POA)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>NAD</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (nmol/g)</td>
<td>427.6 ± 29.7ᵃ</td>
<td>786.4 ± 32.2ᵇ</td>
<td>851.3 ± 37.6ᵇ</td>
</tr>
<tr>
<td>Blood (nmol/ml)</td>
<td>50.6 ± 2.6ᵃ</td>
<td>72.4 ± 3.2ᵇ</td>
<td>76.3 ± 3.2ᵇ</td>
</tr>
<tr>
<td><strong>NADP</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver (nmol/g)</td>
<td>246.0 ± 18.7ᵃ</td>
<td>347.5 ± 37.0ᵇ</td>
<td>393.5 ± 36.6ᵇ</td>
</tr>
<tr>
<td>Blood (nmol/ml)</td>
<td>8.7 ± 0.3ᵃ</td>
<td>10.5 ± 0.7ᵇ</td>
<td>11.1 ± 0.3ᵇ</td>
</tr>
</tbody>
</table>

Each value is the mean ± SEM for 5 rats; values with different superscript letters on the last day are statistically different at p < 0.05 by the Student-Neuman-Keuels multiple-comparison test.
was almost the same among the three groups. It has been reported that the NADP level in the blood seldom changes.\textsuperscript{[11,24]}

**Effect of POA on the key enzyme activities involved in the metabolism of Trp to niacin**

Table 3 shows the effects of POA on the ACMSD and quinolinic phosphoribosyltransferase (QPRT) activities in the liver. These enzyme activities between the negative and positive control groups were almost the same, whereas no ACMSD activity in the test group could be detected.

No ACMSD activity in the kidneys of the test group could be detected as shown in Table 3, although ACMSD activity in both the control groups was detected. The ACMSD activity is generally 10-fold higher in the kidney than in the liver.\textsuperscript{[25]} A similar result was obtained in this study.

To further assess if POA directly inhibited ACMSD activity, we next determined the inhibitory effect of POA on the ACMSD activity in rat liver. The rat liver ACMSD activity was measured in the absence or presence of 1 mM POA; 1 mM POA did not affect the ACMSD activity in rat liver (Table 4).

**Discussion**

We have previously reported that PZA increased the conversion ratio of Trp to niacin,\textsuperscript{[9,10,11]} and that the growth of weaning rats fed with a niacin-deficient diet was significantly promoted by adding PZA.\textsuperscript{[9]} In the present study, dietary POA also promoted the growth of weaning rats and increased the conversion ratio of tryptophan to niacin. The addition of 0.1% PZA to the niacin-deficient diet increased the sum of the urinary excretion of the Nam metabolites, Nam, MNA, 2-Py and 4-Py, 30-fold,\textsuperscript{[9]} while 1.0% POA increased the level 50-fold. Although it is considered that POA is poorly absorbed in the gastrointestinal tract,\textsuperscript{[3]} the marked increase in the sum of urinary excretion by POA feeding might have resulted from the intake of high dose of POA. Our results clearly demonstrate, however, that dietary POA increased the conversion ratio of Trp to niacin.

Trp-niacin metabolism follows two pathways, the Trp-\(\alpha\)-amino-\(\beta\)-carboxymuconate-\(\epsilon\)-semialdehyde (ACMS) pathway and ACMS-niacin pathway, since the Trp-niacin pathway branches at ACMS into the ACMS-acetyl CoA and ACMS-niacin pathways. ACMS metabolized to \(\alpha\)-amino muconate-\(\epsilon\)-semialdehyde is led to the ACMS-acetyl CoA pathway by ACMSD, while ACMS is spontaneously cyclized to QA.\textsuperscript{[26]} It is considered that QA formation and QA metabolism are the important regulatory events on the Trp-niacin pathway, and that ACMSD and QPRT are the rate-limiting enzymes on the Trp-niacin pathway.\textsuperscript{[27,28,29,30]} In the present study, the urinary excretion of the metabolites, KA and XA, on the Trp-ACMS pathway was not affected by POA feeding, whereas POA feeding increased the metabolites, QA, Nam, 2-Py and 4-Py, on the ACMS-niacin pathway. In addition, the liver QPRT activity was not affected by feeding POA, whereas no liver or kidney ACMSD activity was detected in the rats fed with POA (Table 3), suggesting an effect of dietary POA on the ACMSD activity. Although the kidney ACMSD activity is about 10-fold higher than that in the liver, the role of the kidneys in the metabolism of Trp to niacin is considered to be extremely light, since the decrease of kidney ACMSD activity induced by adenine administration does not contribute the formation of niacin from Trp.\textsuperscript{[23]} Dietory POA affected the ACMSD activity, whereas POA did not directly inhibit the activity (Table 4). Shin et al. have reported that a heat-treated liver homogenate from PZA-fed rats showed inhibitory activity toward ACMSD.\textsuperscript{[32]} Taking these facts together, it is suggested that the ACMSD activity rather than the expression of ACMSD was inhibited by POA metabolites.

Although PZA is a very important drug in tuberculosis therapy, its mode of action has never been identified. Zimhony et al. have recently reported that PZA inhibited the eukaryotic-like \textit{fasl} gene (encod-
The Working Site of Dietary POA on the Trp to Niacin Pathway.

In the present experiment, the diets fed to the negative control and test (1% added POA) groups did not contain niacin, so that all niacin and its metabolites were derived from dietary Trp, while the diet fed to the positive control group contained 0.003% NiA. No activity of liver ACMSD could be detected in the test group so that the formation of QA increased, because the reaction of ACMS → QA is non-enzymic. The subsequent enzymatic reactions were accelerated since each substrate concentration was increased and would have reached the optimum conditions. The pathway from Trp to niacin exists only in the liver, which can also synthesize NAD from Nam and NiA. On the contrary, in non-hepatic tissues, only Nam, which is distributed from the liver, is the precursor of NAD. When NiA was supplied from the diets, NiA was synthesized to NAD in the liver, NAD degradation to Nam, and Nam was distributed to the non-hepatic tissues. Abbreviations: N-FK, N-formylkynurenine; 3-HK, 3-hydroxykynurenine; 3-FA, 3-hydroxyanthranilic acid; AMS, a-aminocarboxymuconate-£-semialdehyde; NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide; NMN, nicotinamide mononucleotide.

...ing fatty acid synthetase I, FASI) in \textit{M. tuberculosis}, in correlation with its PZA susceptibility.\textsuperscript{33} The mycobacterial FASI generate C\textsubscript{16} from acetyl-CoA in correlation with its PZA susceptibility.\textsuperscript{33} The acid,\textsuperscript{34} and mycolic acid is a key component of the...


