Fasting Enhances p-Cresol Production in the Rat Intestinal Tract

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Abstract: p-Cresol is a metabolite of aromatic amino acid metabolism produced by intestinal microflora, and its formation is influenced by intestinal conditions. Fasting drastically changes intestinal conditions. However, the effect of fasting on p-cresol production is unclear. In this study, serum and cecal p-cresol levels were determined in non-fasted rats and in rats fasting for either 12 or 18 h. Serum p-cresol increased significantly with 12-h fasting (3.44 ± 2.15 nmol/ml; P<0.05) and 18-h fasting (5.40 ± 2.20; P<0.001) as compared to the level in the non-fasted rats (1.02 ± 0.50). Cecal p-cresol levels of the 12-h fasted (272.6 ± 313.2 nmol/cecum) and 18-h fasted rats (436.6 ± 190.8; P<0.01) were higher than those in non-fasted rats (27.1 ± 21.9). The total cecal protein in content did not change with 18-h fasting. However, the cecal protein concentration increased significantly with fasting (P<0.001), and correlated closely with total cecal p-cresol contents (P<0.001). These results indicate that fasting enhances p-cresol production in the rat cecum, resulting in accumulation of serum p-cresol. We presume that the increase in p-cresol produced by fasting is related to the enhancement of bacterial nitrogen metabolism via an increased concentration of endogenous protein in the cecum.

Key words: fasting, microflora metabolite, p-cresol, rat

Introduction

In the intestinal tracts of humans and animals, putrefactive substances are produced, which have harmful effects on the body. p-Cresol, an aromatic amino acid metabolite produced by intestinal microflora [7], is one such substance. p-Cresol has been implicated in uremia [14] and immunodeficiency in end-stage renal disease [8, 21], and it is also suspected of playing a role in colon and skin cancers [2, 3]. Effects of p-cresol on several biochemical, biological and physiological functions have been reported [20].

p-Cresol production is influenced by various factors in the intestinal environment (e.g., tyrosine intakes, the intestinal microflora, high protein intake and gastric acid suppression therapy) [1, 2, 5, 9]. Fasting is also a factor that changes the intestinal environment. Fasting increases the pH of the intestinal tract [11], prolongs
intestinal transit time and decreases the elimination of bile pigments [12]. Nonetheless, it is unclear how fasting influences \( p \)-cresol production.

In the present study, we investigated the influence of fasting on \( p \)-cresol production in rats by comparing its production in non-fasted rats with that in rats fasting for either 12 or 18 h. Intestinal putrefactive substances (\( p \)-cresol, ammonia and indole) in the cecum, as well as \( p \)-cresol and ammonia levels in blood, were determined.

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**Materials and Methods**

**Animals**

Specific-pathogen-free male Wistar rats were obtained from Charles River Japan Inc. (Yokohama, Japan) at 6 weeks of age. Animals were kept under standard conditions and housed individually in cages in an air-conditioned room at a temperature of 20–26°C, a 12-h light / dark cycle (lights on from 08:00 to 20:00), 40–60% humidity and air ventilation 10–15 times/h. All experimental procedures were carried out according to the guidelines for the ethical treatment of laboratory animals at Yakult Central Institute for Microbiological Research.

Twenty-three rats were fed a commercial diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan) for 6 days. The rats were then divided into three groups with a similar mean body weight, and were fed an experimental diet consisting of 200 g casein, 150 g corn starch, 50 g cellulose powder, 50 g corn oil, 3 g \( DL \)-methionine, 2 g choline bitartrate, 500 g sucrose, 35 g mineral mixture (AIN-76 formulation) and 10 g vitamin mixture (AIN-76 formulation) per kg of diet. Body weights were 230.9–250.8 g at the time of grouping. Food was given freely except during the fasting periods. Water was given freely during the experimental period.

**Fasting**

Nine days after grouping, the rats in the 12-h fasting group (n=8) and in the 18-h fasting group (n=8) were deprived of food from 21:00 to 09:00 and from 15:00 to 09:00, respectively. The rats in the non-fasting group (n=7) were allowed free access to food until the sampling of blood and cecum.

**Sampling of blood and cecum**

Ten days after grouping, sampling of blood and cecum was performed at 09:00. Rats were anaesthetized by a single interperitoneal injection of pentobarbital sodium. Blood was obtained from the abdominal aorta. Blood samples were collected in two polypropylene tubes containing ethylenediamine tetraacetate, dipotassium (EDTA-2K) to prevent coagulation but were not treated by anticoagulant for serum.

After exsanguination of arterial blood, the cecum was excised and dissected from the fat and mesentery, and weighed to determine total weight. pH of the cecal contents was measured directly with a compact pH meter (TPX-90i; Toko Chemical Laboratories Co., Ltd., Tokyo, Japan). After collection of the cecal contents in a polypropylene tube, the cecal tissue was washed with saline, blotted and weighed. The weight of the cecal contents was calculated by subtracting cecal tissue weight from the total cecal weight.

**Measurement of ammonia, indole, protein and \( p \)-cresol**

The cecal contents were diluted 10-fold with 0.1 M phosphate buffer (pH 5.5) and homogenized. Ammonia and indole were assayed immediately after sample preparation. Blood and cecal ammonia and indole were assayed using the Ammonia-Test Wako (Wako Pure Chemical Institute, Ltd., Osaka) and the color reaction with \( p \)-dimethylaminobenzaldehyde, respectively [13]. Cecal protein in contents was measured by the BCA protein assay using a commercial kit (Pierce Biotechnology, Inc. USA) [17]. Serum and cecal contents were stored at –80°C prior to measurement of \( p \)-cresol.

\( p \)-Cresol in serum and cecal contents was measured by the modified HPLC method described by Niwa [15]. For the determination of total (free and protein-bound) \( p \)-cresol, serum and cecal contents were hydrolyzed (100°C, 1 h) with concentrated HCl, and saturated with NaCl. \( p \)-Isopropylphenol was added as an internal standard. \( p \)-Cresol was extracted with ethyl acetate.

The chromatographic system consisted of an autosampler (GL-7420), a pump (GL-7410), a fluorescence detector (GL-7453), a column oven (GL-7430) and data analysis software (EZChrom Elite). The system was purchased from GL Science Inc., Tokyo, Japan. Organic layers (10 μl) were injected onto a 5-μm ODS (C18) column, 4.6 mm (i.d.) × 150 mm (F-411; Shodex®, Tokyo, Japan). The mobile phase of distilled water/acetonitrile (70/30) was delivered at a flow rate of 1.0 ml/min at ambient temperature. \( p \)-Cresol was detected.
EFFECT OF FASTING ON P-CRESOL PRODUCTION

by fluorescence with excitation at 260 nm and emission at 305 nm and quantified based on peak area. Concentrations in the samples were calculated from calibration curves (0.25–50 nmol/ml).

Biochemical and hemotological analysis in blood

Serum samples were analyzed by clinical analyzer 7170 (HITACHI Ltd., Tokyo, Japan). Serum concentrations of total protein (TP), glucose and urea nitrogen were determined by standard methods. Serum albumin concentration (Alb) was calculated based on TP and %Alb obtained by an electrophoretic method (REP Automated Electrophoresis, Helena Laboratories Corporation, Saitama, Japan).

Blood samples treated with EDTA-2K were analyzed by an automated hematology analyzer XT-2000iv with software suitable for analyzing rat samples (SYSMEX CORPORATION, Kobe, Japan). The following parameters were determined: counts for erythrocytes (RBC), hemoglobin (HGB) and hematocrit (HCT).

Statistical analysis

Data are expressed as means ± SD. Mean values were compared between the non-fasting group and the 12-h fasting or 18-h fasting groups using Dunnett’s test with SAS version 5.0. Correlation analysis was performed using Spearman’s rank correlation with SPSS Version 11.

Results

Food intake and body weight

Food intakes of the non-fasting, 12-h fasting and 18-h fasting groups during the 24-h period until sampling were 24.7 ± 3.3, 7.5 ± 0.8 and 1.7 ± 0.7 g, respectively. Body weights of the 12-h fasting and 18-h fasting groups at the sampling time point were 283.7 ± 14.3 (P<0.05) and 283.8 ± 9.2 g (P<0.05), respectively, and were significantly lower than that of the non-fasting group (304.9 ± 20.3 g).

Cecal content weight, cecal pH and cecal protein

Cecal content weight in the 12-h fasting and 18-h fasting group decreased significantly (P<0.001) compared to that in the non-fasting group. Relative to the non-fasting group, cecal pH increased significantly with 12-h fasting (P<0.05), but not with 18-h fasting. Total cecal protein in contents did not differ significantly between the non-fasting and 18-h fasting groups. However, cecal protein concentration in contents increased significantly with fasting (P<0.001) (Table 1).

Cecal p-cresol, ammonia and indole

The total cecal p-cresol content increased significantly with 18-h fasting (P<0.01), while total cecal ammonia contents decreased significantly (P<0.05). The cecal ammonia concentration did not differ significantly between the non-fasting and fasting groups. The total cecal indole content of the 18-h fasting group was significantly higher than that of the non-fasting group (P<0.05) (Table 1).

Correlation between cecal p-cresol and protein

Total cecal p-cresol in contents correlated significantly with protein concentration in cecum contents (P<0.001), but not with total cecal protein in contents (Fig. 1). Rank correlation coefficients between the total amount of cecal p-cresol and protein concentration in cecum contents and total amount of cecal protein were 0.664 and −0.257, respectively.

Biochemical and hemotological analysis in blood

Serum p-cresol increased with fasting in a time-dependent manner. Blood ammonia did not differ significantly between the non-fasting and fasting groups. While serum urea nitrogen and glucose decreased significantly with fasting (P<0.001), RBC, HGB, HCT, serum total protein and serum albumin did not (Table 2).

Discussion

Because p-cresol production increases upon ingestion of a high tyrosine or protein diet [1, 5] we expected that cecal p-cresol would decrease during fasting, when there is a decreased supply of dietary protein to the lower intestinal tract. Unexpectedly, serum and cecal p-cresol were found to increase significantly with fasting.

Cecal content weight decreased with fasting. Though the total cecal protein in contents did not change with 18-h fasting, the concentration of cecal protein in contents increased in a manner dependent on fasting time (Table 1). In normal rats, digestion and absorption of dietary protein occur predominantly in the small intestine. Protein found in the colon is almost entirely
Endogenous proteins are composed of secreted digestive enzymes, mucus, and shed epithelial cells [4].

Cummings et al. reported that suppression of p-cresol production by the intake of dietary fiber may be due to dilution of colonic contents and a decrease in intestinal transit time [5]. In the present study, total amount of cecal p-cresol correlated significantly with protein concentration in cecum contents (Fig. 1). Fasting suppressed the supply of indigestible dietary components to the cecum. It is possible that this increase in cecal protein concentration in contents with fasting is

Table 1. Cecal conditions, p-cresol, ammonia and indole in fasted rats

<table>
<thead>
<tr>
<th>Parameter (Unit)</th>
<th>Non-fasting a</th>
<th>12-h fasting b</th>
<th>Sig. d</th>
<th>18-h fasting c</th>
<th>Sig. d</th>
</tr>
</thead>
<tbody>
<tr>
<td>Contents weight (g)</td>
<td>3.00 ± 0.30 a</td>
<td>1.74 ± 0.54 ***</td>
<td>1.76 ± 0.45 ***</td>
<td>1.76 ± 0.45 ***</td>
<td></td>
</tr>
<tr>
<td>pH</td>
<td>6.62 ± 0.09</td>
<td>6.83 ± 0.16 *</td>
<td>6.75 ± 0.17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg/g)</td>
<td>14.9 ± 0.7</td>
<td>19.6 ± 2.9 ***</td>
<td>20.7 ± 2.1 ***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Protein (mg/contents)</td>
<td>44.8 ± 4.7</td>
<td>33.0 ± 7.7 **</td>
<td>36.0 ± 8.4 ***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Cresol (nmol/g)</td>
<td>8.7 ± 6.3</td>
<td>160.3 ± 162.2</td>
<td>259.9 ± 147.7 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>p-Cresol (nmol/contents)</td>
<td>27.1 ± 21.9</td>
<td>272.6 ± 313.2</td>
<td>436.6 ± 190.8 **</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonia (µmol/g)</td>
<td>13.4 ± 3.9</td>
<td>13.2 ± 2.2</td>
<td>16.1 ± 2.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonia (µmol/contents)</td>
<td>40.1 ± 12.0</td>
<td>22.3 ± 5.4 **</td>
<td>27.6 ± 6.8 *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indole (µmol/g)</td>
<td>0.21 ± 0.04</td>
<td>0.47 ± 0.19 *</td>
<td>0.66 ± 0.20 ***</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Indole (µmol/contents)</td>
<td>0.64 ± 0.10</td>
<td>0.79 ± 0.35</td>
<td>1.16 ± 0.45 *</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Values are means ± SD. a) Food was given freely (n=7). b) Rats fasted from 21:00 to 09:00 (n=8). c) Rats fasted from 15:00 to 09:00 (n=8). d) Significantly different from the non-fasting group (*, **, *** correspond to P<0.05, 0.01, and 0.001, respectively, Dunnett’s test).

Fig. 1. Rank correlation between protein concentration in cecum contents (A) and total cecal protein in contents (B) with total amount of cecal p-cresol. ● Non-fasted rats (n=7), ▲ 12-h fasted rats (n=8), and ■ 18-h fasted rats (n=8). P value by Spearman’s rank correlation.

dogenous protein [6]. Endogenous proteins are composed of secreted digestive enzymes, mucus, and shed epithelial cells [4].

Cummings et al. reported that suppression of p-cresol production by the intake of dietary fiber may be due to dilution of colonic contents and a decrease in intestinal transit time [5]. In the present study, total amount of cecal p-cresol correlated significantly with protein concentration in cecum contents (Fig. 1). Fasting suppressed the supply of indigestible dietary components to the cecum. It is possible that this increase in cecal protein concentration in contents with fasting is
caused by a lack of delivery of indigestible components contained in the diet, such as cellulose powder, to the cecum. It has also been reported that fasting prolongs the intestinal transit time in rats [12]. We suggest that an increase in cecal protein concentration in contents and prolongation of intestinal transit time enhanced the production of cecal \( p \)-cresol with fasting.

Low pH and high carbohydrate have been shown, by in vitro experiments using human intestinal contents, to markedly reduce aromatic amino acid metabolism [16]. In the present study, using a purified diet, cecal pH did not change in the cohort fasted for 18 h. Cecal pH in animals fed a purified diet has been shown to be higher than in those fed a non-purified diet [23]. On the other hand, carbohydrate that bacteria can utilize in the cecum probably decreases with fasting. As a result, bacteria may utilize endogenous protein instead of carbohydrate, thereby possibly enhancing \( p \)-cresol production. In fact, indole, which is a tryptophan metabolite produced by intestinal microflora, also increased markedly with fasting (Table 1).

In a previous study by Illman et al., in which a non-purified diet was fed to rats (Male Wistar rats, mean non-fasted body weight: 312 g) under conditions similar to those in our experiment [11], cecal content weights in the non-fasted and 24-h fasted rats were \( 3.80 \pm 0.19 \) and \( 2.24 \pm 0.31 \) g (mean \( \pm \) SE), and cecal pH values were \( 6.40 \pm 0.05 \) and \( 7.38 \pm 0.08 \), respectively. Rats given free access to a non-purified diet have high cecal content weights and low cecal pH, as compared with rats fed a purified diet, and produce large amounts of volatile fatty acids in the cecum. Fasting has been reported to decrease total volatile fatty acids in the cecum in rats fed a non-purified diet [11]. We confirmed that cecal \( p \)-cresol and the pH of rats fed a non-purified diet increased with fasting (data not shown). Fasting also enhances \( p \)-cresol production in the cecum in rats fed a non-purified diet.

Total amounts of cecal ammonia decreased with fasting. Ammonia is produced by degradation of protein and amino acids, as is \( p \)-cresol. Hydrolysis of urea is also a major metabolic pathway for ammonia production [22]. In humans, approximately 20% of the urea produced by the liver is transferred via diffusion from the bloodstream to the intestinal tract and is utilized as a substrate for ammonia production [22]. Decreased blood urea nitrogen with fasting may influence ammonia production in the cecum.

Serum \( p \)-cresol in fasted rats was found to be high relative to the non-fasted animals. Generally, hemconcentration is observed in fasted rats [18]. In the present study, RBC, HGB and HCT did not differ between non-fasted and fasted rats because water was given freely during the fasting period. Thus, accumulation of serum \( p \)-cresol observed with fasting was caused by enhancement of that production in the intestinal tract but not by hemococoncentration.

One possible explanation of the enhancement of \( p \)-cresol production and increase in serum \( p \)-cresol in fasted rats is as follows. Restriction of indigestible dietary fiber such as cellulose powder would decrease the contents of the intestinal tract and increase the intestinal transit time. A decrease in volume of the intestinal contents would cause an increase in the ratio of endogenous

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**Table 2.** Biochemical and hematological analysis in blood of fasted rats

<table>
<thead>
<tr>
<th>Parameter (Unit)</th>
<th>Non-fasting( a) )</th>
<th>12-h fasting( b) )</th>
<th>Sig.( d) )</th>
<th>18-h fasting( c) )</th>
<th>Sig.( d) )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( p )-Cresol (nmol/ml)</td>
<td>( 1.02 \pm 0.50 )</td>
<td>( 3.44 \pm 2.15 ) *</td>
<td></td>
<td>( 5.40 \pm 2.20 ) ***</td>
<td></td>
</tr>
<tr>
<td>Ammonia (nmol/ml)</td>
<td>( 42.9 \pm 2.5 )</td>
<td>( 42.2 \pm 2.7 )</td>
<td></td>
<td>( 41.2 \pm 3.1 )</td>
<td></td>
</tr>
<tr>
<td>Total protein (g/dl)</td>
<td>( 5.21 \pm 0.24 )</td>
<td>( 5.11 \pm 0.13 )</td>
<td></td>
<td>( 5.25 \pm 0.20 )</td>
<td></td>
</tr>
<tr>
<td>Albumin (g/dl)</td>
<td>( 2.55 \pm 0.13 )</td>
<td>( 2.57 \pm 0.06 )</td>
<td></td>
<td>( 2.65 \pm 0.07 )</td>
<td></td>
</tr>
<tr>
<td>Urea nitrogen (mg/dl)</td>
<td>( 19.5 \pm 1.6 )</td>
<td>( 10.9 \pm 2.5 ) ***</td>
<td></td>
<td>( 11.4 \pm 2.4 ) ***</td>
<td></td>
</tr>
<tr>
<td>Glucose (mg/dl)</td>
<td>( 199 \pm 8 )</td>
<td></td>
<td>( 152 \pm 27 ) ***</td>
<td></td>
<td>( 95 \pm 14 ) ***</td>
</tr>
<tr>
<td>RBC (( 10^4 ) cells/( \mu l ))</td>
<td>( 702 \pm 29 )</td>
<td>( 673 \pm 23 )</td>
<td></td>
<td>( 700 \pm 35 )</td>
<td></td>
</tr>
<tr>
<td>HGB (g/l)</td>
<td>( 135 \pm 5 )</td>
<td>( 134 \pm 4 )</td>
<td></td>
<td>( 136 \pm 7 )</td>
<td></td>
</tr>
<tr>
<td>HCT (%)</td>
<td>( 39.8 \pm 1.6 )</td>
<td></td>
<td>( 38.7 \pm 1.5 )</td>
<td></td>
<td>( 39.8 \pm 1.9 )</td>
</tr>
</tbody>
</table>

Values are means \( \pm \) SD. \( a) \) Food was given freely (n=7). \( b) \) Rats fasted from 21:00 to 09:00 (n=8). \( c) \) Rats fasted from 15:00 to 09:00 (n=8). \( d) \) Significantly different from the non-fasting group (*, ***, *** correspond to \( P<0.05 \) and 0.001, respectively, Dunnett’s test).
protein derived from mucin and dead intestinal epithelial cells. Thus, some bacteria in the intestinal microflora specifically might overgrow and/or activate nitrogen metabolism under these protein-rich conditions and produce p-cresol, using protein in the intestine as a substrate. The high amount of p-cresol produced would be absorbed into the body and serum p-cresol would, therefore, accumulate with fasting.

p-Cresol is frequently measured in feces, blood and urine of experimental animals and humans as indicators of intestinal putrefactive fermentation. Rats have been used as experimental animals to evaluate the suppressive effects on intestinal putrefactive fermentation by probiotic bacteria [19] and functional foods [10]. In animal experiments, fasting and restriction of the diet are often conducted. In the present study, fasting markedly changed the intestinal environmental conditions through modification of the intestinal microflora or metabolism in the intestine. Therefore, when intestinal metabolism such as p-cresol production is investigated in experimental animals, the influence of fasting or restriction of food intake on the intestinal environment merits consideration.

References

