Identification of a Toxic Mechanism of the Plasticizers, Phtahlic Acid Esters, which are Putative Endocrine Disrupters: Time-dependent Increase in Quinolinic Acid and Its Metabolites in Rats Fed di(2-ethylhexyl)phthalate

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Received May 7, 2002; Accepted August 13, 2002

We have reported that the administration of di(2-ethylhexyl)phthalate (DEHP) increased the formations of quinolinic acid (QA) and its lower metabolites on the tryptophan-niacin pathway. To discover the mechanism involved in disruption of the tryptophan-niacin pathway by DEHP, we assessed the daily urinary excretion of QA and its lower metabolites, and enzyme activities on the tryptophan-niacin pathway. Rats were fed with a niacin-free, 20% casein diet or the same diet supplemented with 0.1% DEHP or 0.043% phthalic acid and 0.067% 2-ethylhexanol added for 21 days. Feeding of DEHP increased the urinary excretions of QA and its lower metabolites in a time-dependent manner, and the increase of these excretions reached a peak at 11 days, but feeding of phthalic acid and 2-ethylhexanol had no effect. Feeding of DEHP, however, did not affect any enzyme activity including $\alpha$-amino-$\beta$-carboxymuconate-$\varepsilon$-semialdehyde decarboxylase (ACMSD), affecting the formation of QA, on the tryptophan-niacin pathway.

Key words: phthalate ester; di(2-ethylhexyl)phthalate; quinolinic acid; endocrine disrupters; tryptophan metabolism

Phthalate esters (PhE), such as di(2-ethylhexyl) phthalate (DEHP) and its monoester, mono(2-ethylhexyl)phthalate (MEHP), are low-level environment contaminants that can adversely affect rodent testis only at high doses,1 and chronic feeding of a high concentration of DEHP (1.2%) but not a low concentration (0.6%) to rodents resulted in an increased incidence of liver tumors.2 DEHP has subacute-chronic toxicity mediated by peroxisome proliferator-activated receptors $\alpha$ (PPAR$\alpha$) in liver, kidney, and testis, but DEHP can also act through PPAR $\alpha$-independent pathways in mediating renal and testicular toxicity.3 We have found that the growth of weaning rats fed a niacin-deficient diet was promoted by an administration of di-n-butylphthalate (DBP).4 Furthermore, we showed that the conversion of tryptophan to niacin was significantly promoted by feeding a diet containing DBP5 or DEHP6 (even at a low concentration (0.1%). Both plasticizers increased the formation of quinolinic acid (QA) and its lower metabolites on the tryptophan-niacin pathway, suggesting the inhibition of $\alpha$-amino-$\beta$-carboxymuconate-$\varepsilon$-semialdehyde decarboxylase (ACMSD) by the PhE or their metabolites. To discover the mechanism of disruption of the tryptophan-niacin pathway by DEHP, we assessed the daily urinary excretions of QA and its lower metabolites, and enzyme activities on the tryptophan-niacin pathway.

Vitamin-free milk casein, sucrose, L-methionine, and Nam were purchased from Wako Pure Chemical Industries (Osaka, Japan). $N^4$-methyl-2-pyridone-5-carboxamide (2-Py) and $N^4$-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized by the methods of Pullman and Colowick7 and of Shibata et al.,8 respectively. The mineral and vitamin mixtures were obtained from Oriental Yeast Kogyo (Tokyo, Japan), all other chemicals used being of the highest purity available from commercial sources.

The animal room was kept at a temperature of around 22°C and at about 60% humidity with a 12-hr light/12-hr dark cycle. Body weight and food intake were measured daily at around 10:00 a.m., and food and water were renewed daily. 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 (6 weeks old) were obtained from Clea Japan (Tokyo, Japan) and immediately placed...
Table 1. Composition of the Diets

<table>
<thead>
<tr>
<th></th>
<th>Control 0.1% DEHP</th>
<th>0.043% PhA*1</th>
<th>0.067% 2-EH*2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>%</td>
<td>%</td>
<td>%</td>
</tr>
<tr>
<td>Casein</td>
<td>20</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>1-Methionine</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Gelatinized cornstarch</td>
<td>45.9</td>
<td>45.9</td>
<td>45.9</td>
</tr>
<tr>
<td>Sucrose</td>
<td>22.9</td>
<td>22.8</td>
<td>22.7</td>
</tr>
<tr>
<td>Corn oil</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Mineral mixture*3</td>
<td>5</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>(NIA-free)*4</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Vitamin mixture</td>
<td>DEHP</td>
<td>0</td>
<td>0.1</td>
</tr>
<tr>
<td>Phthalic acid</td>
<td>0</td>
<td>0</td>
<td>0.043</td>
</tr>
<tr>
<td>2-Ethylhexanol</td>
<td>0</td>
<td>0</td>
<td>0.067</td>
</tr>
</tbody>
</table>

*1 PhA = phthalic acid.
*2 2-EH = 2-ethylhexanol.

Table 2. Effects of Dietary DEHP on the Body Weight and Food Intake in Rats

<table>
<thead>
<tr>
<th></th>
<th>Control 0.1% DEHP</th>
<th>0.043% PhA</th>
<th>0.067% 2-EH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(g)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Initial body weight</td>
<td>144.4 ± 2.1</td>
<td>143.2 ± 1.2</td>
<td>144.2 ± 0.7</td>
</tr>
<tr>
<td>Final body weight</td>
<td>280.5 ± 3.5</td>
<td>279.4 ± 5.1</td>
<td>283.3 ± 5.9</td>
</tr>
<tr>
<td>Body weight change</td>
<td>136.0 ± 2.2</td>
<td>136.2 ± 4.8</td>
<td>139.1 ± 6.2</td>
</tr>
<tr>
<td>Food intake (g/21 days)</td>
<td>394.5 ± 3.0</td>
<td>399.2 ± 6.2</td>
<td>402.8 ± 7.8</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>12.4 ± 0.7a</td>
<td>15.6 ± 0.9b</td>
<td>12.2 ± 0.6c</td>
</tr>
</tbody>
</table>

Male rats of the Wistar strain (6 weeks old) 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 fed ad libitum for 21 days (Table 1). Urine samples (10:00 a.m.–10:00 a.m.; 24-hr urine) were collected in amber bottles with 1 ml of 1 M HCl on days 2, 3, 5, 9, 11, 15, 18, and 21, and stored at −25°C until needed. The rats were killed by decapitation at around 10:00 on the last day of the experiment. The liver of each animal was dissected, and approximately 1 g of the liver was treated as described in the literature[9] to measure the enzyme activities involved in the metabolism of tryptophan to niacin.

The contents of QA, nicotinamide (Nam), N'-methyl-N'-adenosine (MNA), 2-Py, 4-Py, kynurenic acid (KA), xanthurenic acid (XA), 3-hydroxyanthranilic acid (3-HA) and anthranilic acid (AnA) in the urine were measured by the HPLC methods. Tryptophan oxygenase (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), ACMSD (EC 4.1.1.45), and quinolinate phosphoribosyltransferase (QPRT) (EC 2.4.2.19) were measured as described in the literature.

The body weight gain and food intake were not different in the three groups (Table 2). The liver weights were significantly higher in the rats given 0.1% DEHP diet than in the rats on the other two diets in agreement with the previous report (Table 2).[8]

We previously showed that QA and its lower metabolites on the tryptophan-niacin pathway were significantly increased by dietary concentration of DEHP above 0.01% for 21 days. In this experiment, daily changes of urinary outputs of QA and its lower metabolites such as Nam, MNA, 2-Py, and 4-Py were investigated when rats were fed the diet containing 0.1% DEHP. Under these experimental conditions, QA, Nam, MNA, 2-Py and 4-Py originated from dietary tryptophan, since the diets were niacin-free. Feeding of DEHP increased the urinary outputs of QA, Nam, MNA, 2-Py, and 4-Py in a time-dependent manner, and the increase of excretions reached a peak at 11 days (Figs. 1(A), 1(B), 1(C), 1(D), and 1(E)). The upper metabolites on the tryptophan-niacin pathway such as AnA, KA, XA, and 3-HA were not affected by the DEHP diet (data not shown), as reported previously.[6] The conversion ratio of tryptophan to niacin, (sum of Nam, MNA, 2-Py and 4-Py (nmol/day))/tryptophan intake during urine collection (nmol/day) × 100, also increased in a day-dependent manner corresponding with the results of the urinary outputs of QA, Nam, MNA, 2-Py and 4-Py (Fig. 1(G)). Feeding of phthalic acid (PhA) and 2-ethylhexanol (2-EH), hydrolysis products of DEHP, did not affect the urinary excretions and the conversion ratio of tryptophan to niacin (Fig. 1).

To discovery the action point of DEHP, we assessed the effects of DEHP on enzyme activities on the tryptophan-niacin pathway. Feeding of DEHP did not affect any enzyme activities such as tryptophan oxygenase, kynureninase, kynurenine aminotransferase, 3-hydroxyanthranilic acid oxygenase, ACMSD, and QPRT in the liver (Table 3). PhA and 2-EH also did not change enzyme activities on the tryptophan-niacin pathway (Table 3).

In this study, the urinary excretions of the lower metabolites from QA, but not the upper metabolites from 3-HA, on the tryptophan-niacin pathway were dramatically increased by feeding of DEHP, consistent with our previous finding.[6] 3-HA is catalyzed to α-aminobutyroxy-3-aminomuconate-ε-semialdehyde (ACMS) by 3-hydroxyanthranilic acid oxygenase, and ACMS is to α-aminomuconate-ε-semialdehyde.
Identification of Toxic Mechanism of Phthalic Acid Esters

Fig. 1. Time-dependent Increase in QA (A), Nam (B), MNA (C), 2-Py (D), 4-Py (E), and SUM (F), and the Conversion Ratio of Trp to Niacin (G).

Male rats of the Wistar strain (6 weeks old) 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 fed *ad libitum* for 21 days (Table 1). Urine samples (10:00 a.m.-10:00 a.m.; 24-hr urine) were collected in amber bottles with 1 ml of 1 M HCl. ○, Control; ●, DEHP; ▲, PhA + 2-EH. Each point is the mean ± SEM for five rats.
led to the glutarate pathway by ACMSD or to QA led to the NAD pathway by autcyclization. Therefore, these results suggest that feeding of DEHP have some inhibition effect on ACMSD. Shin et al. showed that ACMSD activity in the liver of rats fed diverse peroxisome-proliferators, including DEHP, contained in their diets was severely lowered, suggesting the association of PPARα with the regulation of ACMSD. However, we have been unable to observe inhibition of ACMSD activity in the liver of rats fed DEHP-containing diets. The precise reason for this discrepancy remains unclear, but it may result plausibly from differences in experimental design, that is the dietary concentration of DEHP, high (2%) or low (0.1%). Anyway, the PPARα pathway may be one of the candidate pathways involved in enhancement of the tryptophan-niacin metabolism by DEHP.

ACMSD activity is detected only in the liver and kidney, and its activity is much higher in the kidney than the liver. Low hepatic ACMSD activity induced by injection of d-galactosamine led to liver injury, and increased the conversion of tryptophan to niacin. Adenine-induced renal failure lowered the kidney ACMSD activity and the liver QPRT, a rate-limiting enzyme that led to the formation of niacin, activity and increased the liver ACMSD activity, and the levels of niacin and related compounds were decreased consistent with the enzyme activities in the liver but not kidney. Therefore, it is considered that the conversion of tryptophan to niacin is mainly due to the liver enzymes, and the role of the kidney would be extremely low. It is reported that increased QA levels in serum, brain, and urine in renal-insufficient rats are mainly due to the decreased ACMSD activity in the liver and kidney. In this study, we did not measure ACMSD activity in the kidney. Whether DEHP would affect ACMSD in the kidney remains to be seen.

These present data showed that PhA and 2-EH, hydrolysis products of DEHP, did not affect the metabolism of tryptophan to niacin at all, and that DEHP or its metabolite, presumably MEHP, affected the metabolism at the point of QA formation. Although DEHP led to rats soon accumulated in the liver, the hepatic concentration of DEHP was gradually lowered in time-dependent manner. On the other hand, the hepatic concentration of MEHP and PhA were elevated in a time- and dietary dose-dependent manner. If MEHP is a disrupter of tryptophan-niacin metabolism, the increase of excretion of QA and its lower metabolites, reaching a peak at 11 days, may be explained.

Acknowledgment

This investigation was partly supported by The University of Shiga Prefecture Grant for Keynote Research, and Suntory Institute for Bioorganic Research (SUNBOR) Grant.

References

6) Fukuwatari, T., Suzuki, Y., Sugimoto, E., and


