Effect of a Hypolipidemic Drug, Di (2-ethylhexyl) phthalate, on mRNA-Expression Associated Fatty Acid and Acetate Metabolism in Rat Tissues

Ai Itsuki-Yoneda, Masumi Kimoto, Hideaki Tsujii, Miki Hiemori, and Hiromi Yamashita†

Department of Nutritional Science, Faculty of Health and Welfare Science, Okayama Prefectural University, 111 Kuboki, Soja-shi, Okayama 719-1197, Japan

Received September 4, 2006; Accepted December 9, 2006; Online Publication, February 7, 2007

Di (2-ethylhexyl) phthalate (DEHP) is a peroxisome proliferator and a drug having a hypolipidemic effect. The body-weight change of rats treated with DEHP was lower than that of rats in an untreated control group. Expressions of long-chain acyl-CoA dehydrogenase and 3-ketoacyl-CoA thiolase, which are involved in fatty acid oxidation and acetate formation in mitochondria, showed an increase in the liver and testes of rats treated with DEHP. The expression of acetyl-CoA synthetase 1 was significantly decreased in the testes and relatively decreased in the liver, while the expression of acetyl-CoA synthetase 2 was significantly increased in the heart. Furthermore, the expressions of acetyl-CoA carboxylase in heart and testes showed a tendency to decrease. From these results, it is suggested that DEHP-treatment increased fatty acid oxidation and acetate formation in liver and testes, and that acetate utilization was increased in peripheral tissues such as the heart.

Key words: peroxisome proliferator; fatty acid metabolism; β-oxidation; acetate metabolism; di (2-ethylhexyl) phthalate

The hypolipidemic drug di (2-ethylhexyl) phthalate (DEHP), known as a peroxisome proliferator, markedly increases both peroxisomal and mitochondrial enzymes involved in β-oxidation of fatty acid and stimulates fatty acid catabolism in the liver.1–4) Peroxisome proliferators such as DEHP, clofibrate acid, nafenopin, WY-14,643, and some unsaturated fatty acids activate members of the steroid/nuclear receptor superfamily known as the peroxisome proliferator-activated receptor (PPAR).5–12) Peroxisome proliferator-activated receptor α (PPARα) is a member of PPAR, and it modulates expression of genes encoding several mitochondrial and peroxisomal fatty acid-catabolizing enzymes.13–17) DEHP is metabolized to mono (2-ethylhexyl) phthalate (MEHP) and 2-ethylhexanol, and the major metabolite, MEHP, activates PPARα.10,18,19) Transcription of the genes for many fatty-acid oxidation-related enzymes and fatty-acid transport proteins in the liver is up-regulated by peroxisome proliferator in a PPARα-dependent manner.16

Peroxisome proliferator has also found to cause an induction of hepatic ketogenic capacity and increases in plasma ketones by 2-fold in clofibrate-treated animals.20) In our previous observation, perfused liver of rats treated with DEHP accelerated β-oxidation of fatty acid, and in addition, acetate generation was enhanced as well as ketogenesis.21) We examined the acetate generation system. Its formation in the liver was derived from acetyl-CoA hydrolase activity, which appeared to be a side reaction of 3-ketoacyl-CoA thiolase that has been reported as an inducible enzyme by DEHP.3,22) We hypothesized that DEHP treatment induces the transcription of several fatty acid-catabolizing and acetate-metabolizing enzymes in tissues as well as in the liver. The effect of DEHP on expression of fatty acid oxidizing enzymes in the liver have been reported, but the effects on those expressions in other tissues or on the expression of metabolic enzymes of acetate, a final metabolite of fatty acid oxidation, remain to be investigated. In order to study the effect of DEHP on fatty acid metabolism in tissues, the expression of mRNA involving fatty-acid and acetate metabolism was analyzed.

Materials and Methods

Experimental animals and treatment. Six-week-old male Sprague-Dawley rats weighing 260–280 g were housed individually and maintained in an air-condi-
tioned room at approximately 25 °C with alternating 12-h periods of light and dark (lighting, 7:30–19:30). They were supplied with a standard laboratory animal diet (CE-2, Nihon Clea, Tokyo, Japan) for 1 week to stabilize the metabolic conditions. Then the rats were divided randomly into two groups: a control group and a DEHP group (n = 3 in each group). The DEHP group was fed the CE-2 diet with an added 2% (by mass) DEHP for 3 weeks for treatment with di (2-ethylhexyl) phthalate (DEHP). All animals were allowed free access to water and food. At 10 weeks of age, the rats were anesthetized, and the liver, heart, lungs, kidneys, brain, brown adipose tissue (BAT), testes, and abdominal muscle were dissected out. All of the tissues removed were immediately frozen in liquid nitrogen and stored at −80 °C until they were used for RNA extraction. The care and use of the animals in this study followed the guidelines of Okayama Prefectural University and the laws and notifications of the Japanese Government.

Materials. DEHP was obtained from Sigma (Tokyo, Japan). All other chemicals were of analytical grade, unless otherwise stated.

Preparation of cRNA probe for Northern blot. The cDNA fragments for rat long-chain acyl-CoA dehydrogenase (LCACD), 3-ketoacyl-CoA thiolase/acyetyl-CoA hydroxylase (3-KACT/ACh), cytosolic type acetyl-CoA synthetase (AceCS1), mitochondrial type acetyl-CoA synthetase (AceCS2), and acetyl-CoA carboxylase (ACC) were obtained by polymerase chain reaction from first-strand cDNA using rat heart cDNA library (Takara Shuzo, Shiga, Japan). The PCR primers used were as follows: LCACD: 5′-GTTTTTCTGGTGAT-3′, and 3′-primer, 5′-GTTTTTCTGGTGAT-3′; 3-KACT/ACh: 5′-primer, 5′-AGAAGACTGGCACTAGGACCC-3′, and 3′-primer, 5′-TATTTTCACTTCGACGCTTA-3′; AceCS1: 5′-primer, 5′-TTTACTGGAAACACTGCAATCCC-3′, and 3′-primer, 5′-ACTCAGGCTCACACTCGTCTCC-3′; AceCS2: 5′-primer, 5′-ACTGTATGCGCCATTGACGCAC-3′, and 3′-primer, 5′-CAGCGATGACACTGATGATG-3′; ACC: 5′-primer, 5′-GTGGGCTCTTACCGTGTC-3′, and 3′-primer, 5′-GTGGGCTCTTACCGTGTC-3′. Polymerase chain reaction was performed with a Taq DNA polymerase (Takara Shuzo). Forty-one cycles of amplification were done according to the following program: 94 °C, 1 min; 66 °C, 1 min; and 72 °C, 2 min. The amplified products were subcloned into pGEM-T Easy vector (Promega, Madison, WI). These plasmid DNAs were used for DIG-RNA labeling with the DIG labeling system (Roche, Mannheim, Germany). These cRNA probes were used as probes for Northern blotting.

RNA extraction and Northern analysis. Total RNA was extracted from each tissue with Isogen (Nippon Gene, Toyama, Japan). The RNA was dissolved in diethylpyrocarbonate-treated water and quantitated by absorbance at 260 nm. Total RNA extracted was denatured with formaldehyde, and electrophoresed in 1% agarose gels containing formaldehyde, and the amounts of loaded RNAs were checked by staining of 28 and 18 S rRNA bands with ethidium bromide. After capillary transfer onto nylon membranes (Roche) and UV crosslinking, membranes were hybridized overnight at 68 °C with DIG-cRNA probes recognizing LCACD, 3-KACT/ACh, AceCS1, AceCS2, and ACC. The filter was washed twice with 2 × SSC, 0.1% SDS at room temperature, washed twice again with 0.1 × SSC, 0.1% SDS at 68 °C for 30 min, incubated with blocking solution and anti-DIG antibody conjugated alkaline phosphatase for 30 min each at room temperature, and then detected using CDP-Star (Roche) to expose to X-ray film. The amount of each transcript was quantitated with an image analyzer (Genomic Solutions, Tokyo).

Membranes were reprobed with β-actin to ensure that the changes observed did not reflect unequal loading of samples. The levels of β-actin mRNA in all tissues examined did not change due to treatment with DEHP.

Statistical analysis. The data are presented as means ± SE. The control and treated group were compared by unpaired Student’s t test. Statistical significance was defined as p < 0.05–0.001.

Results

The food intake and weight gain of rats fed the 2% (W/W) DEHP-treated diet were determined (Fig. 1A). A lower gain in body weight was observed in rats treated with DEHP, lower by 25% on average than that for untreated rats at three weeks after DEHP-administration, while food intake did not change significantly (Fig. 1B). The expression of long-chain acyl-CoA dehydrogenase (LCACD) mRNA in tissues is shown in Fig. 2. LCACD mRNA was distributed in tissues, and it was especially highly expressed in the liver, BAT, and heart. DEHP-treatment increased expression significantly in the testes, by 2.8-fold, and somewhat increased it in the liver, by 1.8-fold. Figure 3 shows 3-KACT/ACh expression in the tissues and the effect of DEHP on its expression in those tissues. 3-KACT/ACh mRNA was highly expressed in the liver, BAT, and heart, as was seen in the expression pattern of LCACD. Expression significantly increased in the liver, by 4.1-fold, and increased somewhat in the testes, by 1.6-fold.

Acceleration of fatty acid β-oxidation increases acetate generation in the liver. The cellular metabolism of acetate is absolutely dependent on the activity of acetyl-CoA synthetase, which catalyzes the initial step of catabolism as well as anabolism of the compound. The effects of DEHP on Acetyl-CoA synthetase 1 (AceCS1), which is localized in cytosol, and Acetyl-CoA synthetase 2 (AceCS2), which is localized in mitochondria, were examined. Northern blotting of
RNA from various rat tissues revealed hybridization of AceCS1 transcript expressed in a wide range of tissues of BAT, kidney, heart, and testes (Fig. 4). DEHP-treated rats show a lower level of AceCS1 mRNA in the liver, by 50%, and a significantly reduced level in the testes, by 60%, while AceCS2 mRNA was expressed at the highest level in the heart and at relatively high levels in the kidney, lungs, and abdominal muscles (Fig. 5). No AceCS2 transcript was detected in the liver, as was seen also in the mouse liver.23) AceCS2 transcript increased by 1.8-fold in the heart after treatment with DEHP, as compared with that of untreated controls.

The mRNA level of lipogenic genes such as ACC was also measured by Northern blotting (Fig. 6). ACC is responsible for fatty acid de novo synthesis. After treatment with DEHP, the heart and testes showed decrease in ACC mRNA, by 30% and 60% respectively, but expression in the liver did not change (data not shown).

**Discussion**

Previously, we showed that acetate was formed concomitantly with the production of ketone bodies under the ketogenic condition. \(\beta\)-oxidation of fatty acid and acetate formation were stimulated in the liver of rats fed a DEHP-contained diet.21) Miyazawa et al., have reported that weight gain for rats fed on a 2% (w/w) DEHP diet appeared to be low, and that the activities of enzymes of peroxisomal and mitochondrial \(\beta\)-oxidation markedly increased under DEHP treatment. Lower weight gain might have been due to enhancement of
the fatty-acid oxidation system in peroxisome and mitochondria. In order to investigate the effect of DEHP treatment on fatty-acid metabolism, the expression of mRNAs involved in fatty-acid and acetate metabolism was analyzed in the rats fed on the 2% DEHP.

The body-weight gain of rats fed the DEHP-treated diet was less than that of those fed the untreated diet. When treated with DEHP, expression of the LCACD and 3-KACT/ACH genes increased in the liver and testes, indicating that DEHP might increase mitochondrial fatty-acid oxidation in the liver and testes (Fig. 7). A lower gain in body weight might be caused by activation of fatty-acid oxidation in those tissues under treatment with DEHP. The liver is the principal organ responsible for the catabolism and anabolism of fatty acids, depending on the animal’s physiological condition. Among extrahepatic tissues, the BAT, heart, and muscles, organs that actively oxidize fatty acids similarly to the liver, LCACD and 3-KACT/ACH mRNA are expressed at higher levels, but they were not stimulated to those expression levels by DEHP-treatment as was seen in the liver. While, the testes are a lipogenic organ, the expressions of LCACD and 3-KACT/ACH mRNA in the testes was stimulated by DEHP-treatment. Aoyama et al. reported that expression of the LCACD gene was induced by peroxisome proliferator and that induction was mediated by PPARγ, a member of the nuclear receptor superfamily. DEHP is a peroxisome proliferator, and its stimulating effect on the expression of LCACD and 3-KACT/ACH mRNAs in the liver and testes might also be mediated by PPARγ. In a previous study, we found that acetate formation results from mitochondrial acetyl-CoA hydrolase activity, that the enzyme was identical to 3-ketoacyl-CoA thiolase, which catalyzes the final reaction of β-oxidation, producing acetyl-CoA from 3-ketoacyl-CoA, and that the acetyl-CoA hydrolase activ-
Fig. 4. Effect of DEHP Treatment on Expression of the Cytosolic Acetyl-CoA Synthetase (AceCS1) Gene in Tissues of Rats. Assay conditions were the same as in Fig. 2.

Fig. 5. Effect of DEHP Treatment on Expression of the Mitochondrial Acetyl-CoA Synthetase (AceCS2) Gene in Tissues of Rats. Assay conditions were the same as in Fig. 2.

Fig. 6. Effect of DEHP Treatment on Expression of the Acetyl-CoA Carboxylase (ACC) Gene in Tissues of Rats. Assay conditions were the same as in Fig. 2.
ity was a side reaction of 3-ketoacyl-CoA thiolase. Free acetate might be formed in other tissues the same manner, as was found in a study that acetate was final product of β-oxidation in brown adipose tissue.25)

Free acetate formed and excreted from the liver or BAT might be utilized mainly as a fuel in tissues through the activation to acetyl-CoA, a metabolic intermediate. The metabolism of acetate is absolutely dependent on the activity of acetyl-CoA synthetase. Two isoforms of acetyl-CoA synthetase were reported to be AceCS1 and AceCS2.23,26) The major function of AceCS2 was to produce acetyl-CoA for oxidation through the TCA cycle in the mitochondrial matrix,23) while AceCS1 was a cytosolic enzyme that provided acetyl-CoA for the synthesis of fatty acids and cholesterol.26) In order to examine the capacity for acetate utilization as a fuel or fatty acid synthesis in tissues, the transcripts of AceCS2 and AceCS1 were analyzed. The AceCS2 gene was expressed in the heart at the highest level, and in the muscles, lungs, and brain at a somewhat higher level. However, it is apparently absent from the liver, suggesting that the acetate formed was utilized in extrahepatic tissues, especially in the heart or muscle. The transcript significantly increased, by 1.7-fold, in the heart under DEHP treatment, implying that the capacity for acetate oxidation is enhanced in that organ, while the transcript of AceCS1 in the liver was decreased, and that in the testes significantly decreased under DEHP treatment, suggesting that the acetate formed in the liver and testes was utilized for fuel in peripheral tissues rather than for fatty acid synthesis in the liver and testes. These data obtained here suggest that DEHP treatment enhanced fatty acid oxidation and acetate generation in the liver and testes, while it stimulated acetate utilization in the heart, an organ responsible for higher energy metabolism. ACC is a key enzyme of fatty acid synthesis, and it is widely distributed among tissues. ACC transcript did not change in the liver (data not shown), but it showed a decrease in the heart and testes of 35% and 70% respectively under DEHP treatment, suggesting that the synthesis of fatty acid is decreased in these tissues of DEHP treatment. The suppression mechanism of ACC transcript is unknown at this point. Whether the other genes involved in fatty-acid synthesis were affected by DEHP-treatment is under investigation. It has been reported that mono (2-ethylhexyl) phthalate (MEHP), a major metabolite of DEHP, activates mouse liver PPARα.10) PPARα regulates expression of the genes that cause peroxisome proliferation and of genes encoding enzymes that are responsible for fatty-acid metabolism.13–17) But the action of MEHP and PPAR in the testes and heart is unclear. Further studies are necessary to elucidate the precise function of this transcription factor on fatty-acid and acetate metabolism in the testes and heart.

In summary, peroxisome proliferator, DEHP, stimulated the expression of genes involved β-oxidation and acetate formation in the liver and testes. Acetate utilization for fuel might be stimulated in the heart, while fatty-acid and cholesterol synthesis from acetate declined in the liver and testes under DEHP treatment, indicating that fatty acid-oxidizing activity was enhanced and acetate metabolic activity was increased by treatment with DEHP.

Acknowledgments

This work was supported in part by a Grant-in-Aid for Scientific Research from the Ministry Education, Culture, Sports, Science and Technology of Japan.
References


