Di(2-ethylhexyl)phthalate Induces Hepatic Tumorigenesis through a Peroxisome Proliferator-activated Receptor α-independent Pathway

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Abstract: Di(2-ethylhexyl)phthalate (DEHP), a commonly used industrial plasticizer, causes liver tumorigenesis presumably via activation of peroxisome proliferator-activated receptor alpha (PPARα). The mechanism of DEHP tumorigenesis has not been fully elucidated, and to clarify whether DEHP tumorigenesis is induced via PPARα, we compared DEHP-induced tumorigenesis in wild-type and Pparα-null mice. Mice of each genotype were divided into three groups, and treated for 22 months with diets containing 0, 0.01 or 0.05% DEHP. Surprisingly, the incidence of liver tumors was higher in Pparα-null mice exposed to 0.05% DEHP (25.8%) than in similarly exposed wild-type mice (10.0%). These results suggest the existence of pathways for DEHP-induced hepatic tumorigenesis that are independent of PPARα. The levels of 8-OHdG increased dose-dependently in mice of both genotypes, but the degree of increase was higher in Pparα-null mice exposed to 0.05% DEHP (25.8%) than in similarly exposed wild-type mice (10.0%). These results suggest the existence of pathways for DEHP-induced hepatic tumorigenesis that are independent of PPARα. The levels of 8-OHdG increased dose-dependently in mice of both genotypes, but the degree of increase was higher in Pparα-null mice than in wild-type mice. NFκB levels also significantly increased in a dose-dependent manner in Pparα-null mice. The protooncogene c-jun-mRNA was induced, and c-fos-mRNA tended to be induced only in Pparα-null mice fed a 0.05% DEHP-containing diet. These results suggest that increases in oxidative stress induced by DEHP exposure may lead to the induction of inflammation and/or the expression of protooncogenes, resulting in a high incidence of tumorigenesis in Pparα-null mice. (J Occup Health 2007; 49: 172–182)

Key words: Di(2-ethylhexyl)phthalate, Pparα-null mouse, Tumorigenesis, NFκB, 8-OHdG, c-jun, Inflammation

Di(2-ethylhexyl)phthalate (DEHP) is a plasticizer used in polyvinyl chloride products that have become ubiquitous in our daily living. These include building materials such as wallpaper and flooring, wire covering, vinyl sheeting for agriculture, food packages, and medical devices such as intravenous and hemodialysis tubing and blood bags. DEHP has potentially adverse effects on the liver, kidney, lung, heart, reproductive organs and endocrine systems1, 2). Peroxisome proliferator-activated receptor alpha (PPARα) belongs to the nuclear receptor superfamily and has various functions such as lipid transport and catabolism, and activation of PPARα can result in potential anti-inflammatory effects3, 4). Mono- and dicarboxylic acid metabolites of DEHP act as ligands for PPARα, similar to hypolipidemic fibrate drugs5, 6). As for DEHP, there are species differences in the metabolism, especially in lipase activity, which is highest in mice.
followed by rats and marmosets in that order, suggesting the existence of species differences in the production of ligands of PPARα also exist⁹.

Ppara-null mice, developed in the mid-1990s, have been found to be a very useful tool for investigating the function of PPARα¹⁰. Using this mouse line, Ward et al. studied the receptor- and nonreceptor-mediated organ-specific toxicity of DEHP. They found that exposure to 12,000 ppm DEHP for six months induced peroxisomal enzymes, liver enlargement, histopathological increases in eosinophil counts and peroxisomes in the cytoplasm of wild-type mice, while there were no toxic liver lesions in Ppara-null mice⁷. From these results, it was concluded that DEHP-induced tumorigenesis occurred via PPARα.

Although the degrees of peroxisome proliferation in rat livers exposed to DEHP are comparable to that by Wy-14,643, the latter induces liver tumors with shorter exposure periods than DEHP and the tumor incidence is higher in the former than in the latter¹¹. Thus, the differences between the rates and onsets of hepatic tumors induced by Wy-14,643 and DEHP cannot be explained merely as a phenomenon of peroxisome proliferation. These findings suggest that other mechanisms may also be involved in DEHP tumorigenesis¹². In addition, some studies have supported the idea of a non-peroxisome proliferation pathway. Tumorigenesis of hepatocellular adenomas induced by DEHP in rasH2 mice is not related to overexpression of PPARα¹³; peroxisome proliferators (PPs)-induced tumorigenesis is related to the genes involved in cellular proliferations of, for example, p38 mitogen-activated protein kinase which is not involved in peroxisome proliferations.⁴ These PPARα-independent mechanisms, however, have still not been determined. Recently, it was reported that activated PPARα exerts anti-inflammation effects by repressing nuclear factor kappa B (NFκB), which regulates inflammation⁶,¹⁵,¹⁶ and the inflammation-associated model of liver cancer¹⁷,¹⁸. These results suggest that Ppara-null mice may be more vulnerable to tumorigenesis induced by exposure to environmental carcinogens.

The Lowest-Observed-Effect-Level and No-Observed-Effect-Level (NOEL) for DEHP-induced hepatic tumors have been reported to be 0.3% and 0.1% in male mice, respectively, exposed to DEHP for 103 wk³,¹⁹. In contrast, male mice exposed to 0.05% DEHP for 78 wk exhibited a significant increase in the tumor incidence rate compared with controls⁸-²⁰. Therefore, two DEHP dosages were selected in the present study, with high exposure groups set as 0.05% which is half that of the NOEL (0.1%)³-¹⁹, or the LOAEL (Lowest-Observed-Adverse-Effect-Level) reported by David et al.²⁰, and low exposure groups of 0.01% which is one fifth that of the LOAEL (0.05%). Using these dosages for wild-type and Ppara-null mice, the tumorigenesis of DEHP and the mechanism were examined.

Materials and Methods

Animals and DEHP treatment

This study was conducted according to the Guidelines for Animal Experiments of the Shinshu University Animal Center. Two mouse genotypes, Ppara-null male mice on a Sv/129 genetic background produced as described elsewhere²⁰ and wild-type male Sv/129 mice, were used to identify PPARα-dependent or -independent carcinogenesis of DEHP in this experiment. All mice were housed in a temperature- and light-controlled environment (25°C, 12-h light/dark cycle), and maintained on stock rodent chow and tap water ad libitum. DEHP-containing diets (0.01 and 0.05%) were prepared with the rodent chow every two weeks, according to the method of Lamb et al.²¹. Both mouse genotypes (from three weeks to 22 months of age) were exposed to 0, 0.01% or 0.05% DEHP diets throughout the experiment. The animals were sacrificed by decapitation at about 23 months of age, and livers and plasma were collected to investigate DEHP-mediated pathological and biochemical changes, respectively. Livers were removed and small portions were fixed in 10% neutral buffered formalin, while the remainder was stored at –80°C until use; plasma was collected from trunk blood after centrifuging at 3,500 g for 10 min, and stored at –80°C until use. All analyses except histopathology were conducted using macroscopic non-tumorous parts of livers. There was no difference in the internal exposure levels of DEHP between the two mouse genotypes. The plasma concentrations of a metabolite, mono (2-ethylhexyl) phthalate (MEHP), were 0.047 ± 0.014, 0.370 ± 0.097, 1.404 ± 0.371 µg/mL in wild-type mice exposed to diets containing 0% (control), 0.01% and 0.05% DEHP diets throughout the experiment. The animals were sacrificed by decapitation at about 23 months of age, and livers and plasma were collected to investigate DEHP-mediated pathological and biochemical changes, respectively. Liver homogenates for all proteins except for Histopathological evaluation of livers

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Liver homogenates for all proteins except for
cyclooxygenase-2 (Cox-2) adjusted to 10 µg protein were subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. For Cox-2, the supernatant after centrifuging at 105,000 g was used by adjusting to 10 µg protein. The primary polyclonal antibodies to p50, p65, c-Jun, c-Myc, c-Fos, Bax, Bcl-2, IκBα and Cox-2 were purchased from Santa Cruz Biotechnology, Inc. (CA, USA). After blocking with 3% skim milk, membranes were incubated with the primary antibodies to p65, c-Jun, Bax, Bcl-2, Cox-2 respectively, followed by incubation with alkaline phosphatase-conjugated goat anti-rabbit IgG or donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA). The other membranes were incubated with the primary antibodies to p50, c-Myc, c-Fos and IκBα followed by incubation with biotin-conjugated goat anti-rabbit IgG or donkey anti-goat IgG (Jackson ImmunoResearch, West Grove, PA), and alkaline phosphatase-conjugated streptavidine (Jackson ImmunoResearch, West Grove, PA). 1-Step™ NBT/BCIP (Pierce Biotechnology, Rockford, IL) was used as the substrate of alkaline phosphatase. Each band was quantified by densitometry, using the Lane & Spot Analyzer version 5.0 (ATTO corporation, Tokyo, Japan).

RNA extraction and real-time quantitative PCR

The mRNA levels were monitored on the ABI PRISM 7000 Sequence Detector system (Applied Biosystems, Foster City, CA). Total RNA was isolated using RNAeasy Protect Mini Kit (QIAGEN, Tokyo, Japan). Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using Oligo(dT)12 primer. RNA quantity and quality were checked by GeneQuant II RNA/DNA Calculator (Pharmacia Biotech, Framingham, MA). Primers were designed using Primer Express software (Applied Biosystems) based on the sequence of GI numbers shown in Table 1.

Table 1. List of primers used for real-time quantitative PCR

<table>
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<tr>
<th>GI number</th>
<th>Forward</th>
<th>Reverse</th>
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<td>c-myc</td>
<td>GCCTGCCAGCATCCCTGAA</td>
<td>AGGATGTAAGCGGTTGGCCTT</td>
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<tr>
<td>c-fos</td>
<td>TAGCGCCATGCTCGCAGA</td>
<td>GCTCCAGATCTGCTGACATAGA</td>
</tr>
<tr>
<td>c-jun</td>
<td>GTCCAGCAATTGGGGAATC</td>
<td>TGCTCGTGCGTCAGTCTCTT</td>
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<td>ACCGGCTTGGAGTTGCTTGA</td>
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<td>Bax</td>
<td>388191</td>
<td>CAGGATGCTGCCACCAAGA</td>
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<tr>
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<tr>
<td>GAPDH</td>
<td>66396585</td>
<td>AGAACATCATCCCTGCAATC</td>
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</table>

DNA isolation and 8-hydroxydeoxyguanosine (8-OHdG) measurement

As an index of oxidative stress, the level of 8-OHdG was measured. DNA was extracted from the mouse liver using a DNA Extractor Kit (Wako Pure Chemical Industries, Ltd., Osaka, Japan), according to the manufacturer’s protocol. Each 10 µg of extracted DNA (final vol. 100 µl) was digested with 12 U of nuclease P1 (MP Biomedicals, Irvine, CA, USA) and 1.3 U of Escherichia coli alkaline phosphatase (Sigma, St. Louis, MO, USA) at 37°C for 1 h each. An ELISA kit (8-OHdG Check, Japan Institute for the Control of Aging, Fukuroi, Japan) was used to analyze the levels of 8-OHdG. An aliquot (50 µl) of the primary antibody and 50 µl of the digested DNA were added to a microplate that had been precoated with 8-OHdG. After the plate was incubated at 37°C for 1 h, it was washed thoroughly with 250 µl of phosphate buffered saline. Then, 100 µl of horseradish peroxidase-conjugated secondary antibody was added to each well and the plate was incubated at 37°C for 1 h, and
then washed with 250 \( \mu l \) of washing solution, followed by the addition of 100 \( \mu l \) of 3,3',5,5'-tetramethylbenzidine as a color development reagent. After the plate was incubated at room temperature for 15 min, the reaction was stopped by adding 100 \( \mu l \) of reaction terminating solution, 1 M phosphoric acid. After 3 min, the color development was measured at 450 nm using a Benchmark Microplate Reader (Bio-Rad Laboratories, CA, USA). These processes were performed in the dark. A concentration of 8-OHdG was calculated from a standard curve.

Alanine aminotransferase (ALT) measurement

Plasma ALT was measured using kits purchased from Wako (Osaka, Japan).

Statistical analyses

Comparisons were made using a two-way analysis of variance, followed by the Tukey-Kramer HSD post hoc test. Values of \( p<0.05 \) were considered statistically significant. A square-root transformation was applied to Bax and c-fos-mRNA before Tukey-Kramer analysis or Student’s \( t \)-test, respectively. Student’s \( t \)-test was used for comparisons of p65-mRNA and ALT levels between the \( Ppar \alpha \)-null mice with hepatic tumors and those without tumors. The Cochran-Armitage test was used to evaluate the significance of the histopathological changes.

Results

Body and liver weights, and liver macroscopic findings

There were no significant differences in the body and liver weights associated with DEHP exposure in either wild-type or \( Ppar \alpha \)-null mice (Table 2). DEHP treatment tended to increase the liver weights only in \( Ppar \alpha \)-null mice in the highest-exposure group due to small protruding lesions, which might have been caused by the vacuolar degeneration of hepatocytes (data not shown), and by the tumors observed in the livers of some mice. These macroscopic changes in the lesions were rare in the livers of the wild-type mice. Thus, the above macroscopic changes were seen profoundly in the livers of 0.05% DEHP-treated \( Ppar \alpha \)-null mice.

Histopathological changes

Hepatocellular carcinomas were seen in only two mice: one was a \( Ppar \alpha \)-null mouse in the control group and the other was a 0.05% DEHP-treated \( Ppar \alpha \)-null mouse (Fig. 1-A). No hepatocellular adenomas were seen in the liver from control group mice of either genotype, whereas they were seen in the livers from the 0.01% and 0.05% DEHP-treated groups in both the wild-type and \( Ppar \alpha \)-null type mice (Fig. 1-B). Total neoplastic changes including hepatocellular carcinomas, hepatocellular adenomas, and cholangiocellular carcinoma increased only in the livers of \( Ppar \alpha \)-null mice exposed to 0.05% DEHP (Table 2). Inflammatory cell infiltration was often observed in the portal vein area of the livers from \( Ppar \alpha \)-null mice exposed to 0.05% DEHP. The filtration was more prominent in the livers with tumors than in those without tumor (Fig. 1-C).

Oxidative damage

DEHP treatment dose-dependently increased 8-OHdG levels in the livers of \( Ppar \alpha \)-null mice (Fig. 2). DEHP also increased the levels in the wild-type mice, and the interaction between the DEHP dose and the genotypes was significant, indicating that the degree of increase was smaller than that in \( Ppar \alpha \)-null mice. When the 8-OHdG levels between the control groups of both genotypes were compared, the levels were significantly higher in \( Ppar \alpha \)-null mice.
null mice than in the wild-type mice. The 8-OHdG levels were also compared between livers from 16-week-old and 22-month-old mice of both genotypes, and the levels were found to be significantly higher in the older rather than the younger Ppara-null mice. No such difference was seen in the wild-type mice (data not shown). These results suggest that DEHP increases oxidative stress in the livers of mice, and that PPARα suppresses oxidative stress in the liver.

Western blotting

DEHP exposure clearly induced protein levels of the NFκB subunit, p65, and a protooncogene c-jun, only in the livers of Ppara-null mice (Fig. 3). However, induction of p50 protein was not seen, even in Ppara-null mice, nor were c-fos and c-myc identified by the antibodies used. DEHP did not influence the expression of Cox-2, Bax, Bcl-2 and IkBα in the livers of either mouse genotype (data not shown).

Real-time quantitative PCR of signaling molecules and protooncogenes

Trifunctional protein α subunit (long chain-specific hydratase + long chain-specific 3-hydroxyacyl-CoA dehydrogenase) (TPα) is one of the liver mitochondrial PPARα-related genes. DEHP treatment induced TPα mRNA only in wild-type mice, suggesting that 0.05% DEHP activated the signaling pathways via PPARα (Fig. 4-A). Overall, DEHP exposures tended to increase the mRNA levels of several signaling molecules or protooncogenes in the Ppara-null mice. DEHP exposure at a dose of 0.05% significantly induced hepatic expressions of p50 mRNA levels only in Ppara-null mice. However, the DEHP treatments did not induce p65- or p52-mRNA, which are involved in other NFκB signaling pathways, in both mouse genotypes. Interestingly, p65 mRNA levels in the macroscopic non-tumorous parts of the livers of only Ppara-null mice with hepatic tumors
were significantly higher (2.5-times) than in those without them ($p<0.05$) (Fig. 4-B).

DEHP at a dose of 0.05% also induced mRNA levels of the protooncogene c-jun, and tended to induce c-fos mRNA levels in \textit{Ppara-null} mice. In particular, the mean-induced levels of the c-fos mRNA were about 40-fold in mice exposed to 0.05% DEHP, though there were large individual differences. On the other hand, the induction of c-jun mRNA was dose-dependently increased, although the levels were not as high as those of c-fos mRNA. The mRNA levels of c-jun and c-fos were also significantly higher in wild-type mice exposed to 0.01% DEHP than wild-type controls, but the inductions were not dose-dependent, and the induced levels were only marginal compared to those in \textit{Ppara-null} mice exposed to 0.05% DEHP.

In contrast, DEHP treatment decreased Bax-mRNA, an apoptosis-associated gene, in the \textit{Ppara-null} mice, but not in the wild-type mice. DEHP did not influence Bcl-2 mRNA levels in both mouse genotypes (data not shown). Exposure to DEHP tended to increase Cox-2 mRNA levels in \textit{Ppara-null} mice but not to a statistically significant degree because of major individual differences. DEHP treatment did not influence the expression of 8-oxoguanine DNA-glycosylase 1 (ogg1), 8-OHdG repair enzymes, mRNA in both genotypes of mice. A significant positive correlation between hepatic 8-OHdG and ogg1 mRNA levels was observed in the wild-type mice without hepatic tumors ($p<0.05$), but not in the \textit{Ppara-null} mice, suggesting that 8-OHdG produced by oxidative stresses were accumulated more in \textit{Ppara-null} mice than in wild-type mice.

DEHP treatment did not influence the expression levels of TNF$\alpha$, IκB$\alpha$, IκB$\beta$ and IL-6 mRNA expression in both...
Fig. 4.
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Alt levels in plasma
DEHP treatment (0.05%) tended to increase the ALT levels in Ppara-null mice, but not to a statistically significant degree because of the great individual variations. However, the treatment did not increase the levels in the wild-type mice (Fig. 5-A). On the other hand, only the ALT levels in plasma from the Ppara-null mice with hepatic tumors were significantly higher only than in Ppara-null mice without them (Fig. 5-B) (p<0.05), a result mirroring the histopathological changes as well as the p65-mRNA levels as described above.

Discussion
DEHP has long been thought to induce hepatic tumor in rodents via PPARα-dependent pathways4, 7, 24, 25), although the mechanism has remained unknown. The present study clearly shows that DEHP induces such tumors in Ppara-null mice, suggesting the possibility of PPARα-independent tumorigenesis pathways in DEHP-induced hepatic tumors.

Prior to this study, there have been few long-term studies on DEHP carcinogenesis using Ppara-null mice. Such studies would be useful for determining the possibility of PPARα-dependent or -independent tumorigenesis of DEHP. One study reported that wild-type and Ppara-null mice exposed to 12,000 ppm DEHP for 24 wk had no tumors in mice of either genotype, although hepatic peroxisome proliferation was only observed in wild-type mice7). Therefore, it was speculated that DEHP tumorigenesis might be related to the presence of PPARα. In contrast, Wy-14,64326) or bezafibrate27) treatment induced hepatic tumors in wild-type, but not in Ppara-null mice during a one-year exposure, suggesting a critical role for PPARα in hepatocarcinogenesis of PPs. However, exposure to DEHP for only one year may not be sufficient to induce hepatic tumors, as suggested by Marsmanet al. who reported that DEHP tumorigenesis required longer exposure periods than that of Wy-14,64311). In addition, PPs stimulated growth regulatory pathways such as immediate early genes (c-jun, c-fos, junB, egr-1), mitogen-activated protein kinase kinase, extracellular...
signal-regulated kinase and phosphorylation of p38, which were dissociated from PPARα activation in rat primary cultures28). These findings also support the view that PPs, including DEHP, may have the potential for tumorigenesis via non-PPARα signal pathways.

The question may now be raised as to why hepatic tumors were induced more in Pparα-null than in wild-type mice. The 8-OHdG level is an index of oxidative stress, which was clearly induced to a greater degree in the livers of Pparα-null mice than in those of wild-type mice by DEHP treatment. Nevertheless, ogg1-mRNA levels were not changed by the DEHP treatment in both genotypes of mice, suggesting that 8-OHdG is accumulated more in Pparα-null mice. In addition, NfκB (p65 and/or p50) levels were induced in the livers of Pparα-null mice by DEHP treatment, and the protooncogenes c-jun and c-fos were also induced in the livers of the Pparα-null mice, but not in wild-type mice. It is interesting to note that NfκB (p65)-mRNA and the protein levels in macroscopic non-tumorous parts of livers from Pparα-null mice having hepatic tumors were greater than in those having no tumors. NfκB induces inflammation as shown by histopathological findings as well as the plasma ALT level, which is related to tumorigenesis17,18. In addition, c-jun and c-fos form the heterodimer called AP-1, which plays an important role in cell proliferation, growth, differentiation and tumorigenesis29). Thus, in Pparα-null mice, DEHP overproduces oxidative stress, which increases the level of inflammation, or may overexpress the AP-1 transcription factor. This signaling is associated with a higher incidence of hepatic tumors in Pparα-null mice. Indeed, there have been several reports supporting the relationships between tumorigenesis and the 8-OHdG levels, inflammation or protooncogene expression17,18,30-32). Exposure to nickel compounds significantly increased 8-OHdG levels as well as inflammation intensity30). The increases in 8-OHdG levels in patients with chronic cholecystitis were related to their duration and intensity, which may in turn be related to the incidence of gallbladder carcinogenesis31,32). Others have suggested that NfκB is essential for promoting inflammation-associated cancer, and is therefore a potential target for cancer prevention in chronic inflammatory disease17,18). On the other hand, PPARα inhibits the transfer of the heterodimer, consisting of p65 and p50, into nuclei, and exhibits anti-inflammatory effects33). A relatively low dose of DEHP (0.05%) weakly yet significantly activated PPARα in terms of TPα-mRNA levels; thus, PPARα expression itself has anti-inflammatory effects33). In the present study, signals inducing inflammation and tumorigenesis were not seen in the wild-type mice, which may have resulted in the lower incidence of tumorigenesis at exposures to the relatively low dose of DEHP. Interestingly, DEHP also induced glomerulonephritis more often in Pparα-null mice because of the absence of PPARα-dependent anti-inflammatory effects of antagonizing the oxidative stresses and NfκB signaling pathway34).

DEHP is known not to induce 8-OHdG levels dose-dependently in rodents41. In the present experiment, DEHP strongly induced 8-OHdG levels in the livers of Pparα-null mice, but only slightly in wild-type mice, indicating that PPARα is a major factor suppressing the induction of 8-OHdG adducts by DEHP. It is noteworthy that while a significant difference in the 8-OHdG levels was found between livers from the 23-month-old untreated wild-type and Pparα-null mice, no differences were observed between the two genotypes of control mice at 16 wk of age (data not shown), suggesting that PPARα itself suppresses the production of 8-OHdG levels due to aging, and also inhibits DEHP-induced ones. 8-OHdG levels were positively correlated with ALT levels in
Pparα-null mice without hepatic tumors exposed to 0.05% DEHP. This result is also supportive of a relationship between 8-OHdG levels and hepatocellular inflammation.

Since relatively low doses (0.01 and 0.05%) of DEHP were used in this study, the incidences of DEHP-induced hepatic tumors were low, compared to findings reported elsewhere. Thus, additional experiments using higher doses of DEHP, i.e. higher than LOAEL for the carcinogenesis, may be required.

In conclusion, the results of this study suggest the possibility of DEHP tumorigenesis via a non-PPARα pathway, the hypothetical route of which is shown in Fig. 6. Re-evaluation of the risk of DEHP may well be warranted, given that previous evaluations of its toxicity were based on PPARα-dependent mechanisms.

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