**INTRODUCTION**

Diheptyl phthalate (DHP), a type of phthalate ester (PAE), has been used as plasticizer in polyvinyl chloride, medical devices and industrial applications (Petrovic et al., 2001). PAEs are well-known to cause hepatic and testicular toxicities in rodents through the activation of peroxisome proliferator-activated receptors (PPARs) (Bhattacharya et al., 2005; David et al., 2000). Peroxisomes are membrane-bound organelles that contain enzymes responsible for the β-oxidation of fatty acids, cholesterol biosynthesis, and other biochemical pathways (Mannaerts et al., 1993). PPARs are members of the steroid hormone receptor superfamily, and three related isotypes, specifically PPARα, PPARβ and PPARγ, have been identified (Desvergne and Wahli, 1999). Based on target gene expression patterns, PPARα appears to have critical roles in the regulation of fatty acid metabolism, including fatty acid β-oxidation, apolipoproteins, and fatty acid transport proteins (Lee et al., 1995; Auwerx et al., 1996; Aoyama et al., 1998; Peters et al., 1997; Ren et al., 1997; Martin et al., 1997). Additionally, chronic exposure to numerous PPARα agonists has been reported to increase the incidence of hepatocellular tumors in rodents. 

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**Twenty-six-week oral toxicity of diheptyl phthalate with special emphasis on its induction of liver proliferative lesions in male F344 rats**

Fumiyuki Nakane¹, Masaki Kunieda¹, Shigekazu Shimizu¹, Yoshihiko Kobayashi¹, Hirotoshi Akane¹, Yasuki Akie¹, Akemi Saito¹, Masayoshi Noguchi¹, Toshihito Kadota¹ and Kunitoshi Mitsumori²

¹CMIC BIORESEARCH CENTER CO., LTD., 10221 Kobuchisawa-cho, Hokuto, Yamanashi 408-0088, Japan
²Laboratory of Veterinary Pathology, Tokyo University of Agriculture and Technology, 3-5-8 Saiwai-cho, Fuchu, Tokyo 183-8509, Japan

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**ABSTRACT** — The 26-week oral toxicity of diheptyl phthalate (DHP), a peroxisome proliferator-activated receptor α (PPARα) agonist, with special emphasis on the potential induction of hepatocellular proliferative lesions was investigated in this study. DHP was administered to male F344 rats via gavage at 0 (control), 1,000 or 2,000 mg/kg/day for 26 weeks. Body weight gain was significantly lower, whereas food and water consumption was significantly higher in DHP-treated rats compared with controls. DHP-treated rats exhibited decreases in blood triglyceride, total cholesterol, phospholipid and glucose levels, which were likely related to biological effects of the PPARα agonist. Absolute and relative organ weights of the livers with pale brown discoloration and dark brown spots significantly increased in DHP-treated rats. Histopathological examinations revealed remarkable diffuse hypertrophy of hepatocytes with ground-glass appearance, intracytoplasmic inclusion bodies and/or vacuolation in the DHP-treated groups. These findings were associated with increases in serum aspartate aminotransferase, alanine aminotransferase, alkaline phosphatase, and γ-glutamyltranspeptidase. The number and area of glutathione S-transferase placentiform positive foci, a marker of hepatocellular preneoplastic lesions in rats, significantly increased in DHP-treated groups. Additionally, proliferating cell nuclear antigen positive liver cell counts in DHP-treated groups were significantly higher than those of the controls. Testicular alterations were not detected histopathologically, whereas absolute and relative prostate weights significantly decreased at both doses. These results indicate that DHP induces liver pre-neoplastic foci, and suggest the possibility that DHP is a possible genotoxic carcinogen in the liver of rats.

**Key words:** 26-week toxicity, Diheptyl phthalate, Phthalate esters, Preneoplastic foci, PPAR agonist
(Rao and Reddy, 1996). However, because these chemicals do not show any mutagenic potential in genotoxicity tests, they are regarded as non-genotoxic hepatocarcinogens. On the contrary, it has been speculated that PPARα agonists markedly induce H₂O₂-generating enzymes, such as acyl-CoA oxidase and cytochrome p450 4A, resulting in increased levels of hydrogen peroxide, a reactive oxygen species (ROS), leading to lipid peroxidation and oxidative DNA damage (Yeldandi et al., 2000; Seo et al., 2004). Such an oxidative stress induced by PPARα agonists is considered to be an indirect mechanism involved in hepatocarcinogenesis (Reddy and Rao, 1989; Klaunig and Kamendulis, 2004; Seo et al., 2004).

Although DHP did not have any in vitro mutagenic effects, as determined by the Ames and chromosomal aberration tests (Yoshikawa et al., 1983), there are no available data on the in vivo genotoxic effects of DHP (e.g. micronucleus test). In a 28-day oral toxicity study, Jin et al. (2009) previously found that F344 rats given 5,000 mg/kg of DHP had altered hepatocellular foci, which were associated with an increased number and area of glutathione S-transferase placental form (GST-P) positive foci. Furthermore, cell migration, a marker of DNA damage as assessed by the Comet assay, was significantly induced in the livers of rats treated orally with DHP for 14 days (Jin et al., 2009). This observation paralleled the significant increase in the number and area of GST-P positive foci, as determined by an in vivo liver initiation assay (Jin et al., 2009). Therefore, DHP may be a genotoxic carcinogen in the livers of rats (Jin et al., 2009).

The objectives of the present study were to determine whether a prolonged treatment with DHP over 26 weeks increases the number and size of hepatocellular altered foci and examine the chronic effects of DHP on other organs.

**MATERIALS AND METHODS**

**Chemicals**

DHP (CAS No.046-28522; purity, > 98%) was purchased from Wako Pure Chemical Industries, Ltd (Osaka, Japan). All other chemicals were of analytical grade and obtained commercially.

**Animals**

Five-week old, male F344/N rats were purchased from Japan SLC Inc. (Shizuoka, Japan). Rats were housed individually in stainless steel cages and allowed ad libitum access to tap water and a commercial pellet diet (CRF-1; Oriental Yeast Industries Co. Ltd, Tokyo, Japan). All animals were handled under standard conditions (room temperature of 22 ± 3°C, relative humidity of 50 ± 20%, and 12 hr light and dark cycle), and acclimatized for 1 week prior to experimentation. Rats were randomly stratified into three groups (i.e. n = 12/group) according to body weight prior to dosing. This study was conducted in the CMIC BIORESEARCH CENTER CO., LTD., which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) International. The protocol was approved by the institutional animal care and use in the committee of our facility.

**Study design**

**Dosages**

There were three treatment groups (n = 12/group), specifically the control group, which received olive oil, and the two DHP-treatment groups, which received either 1,000 or 2,000 mg/kg/day of DHP via gavage for 26 weeks. Rats were administered olive oil or DHP five times per week. In a 28-day oral toxicity study, Jin et al. (2009) previously found that F344 rats given 5,000 mg/kg of DHP had altered hepatocellular foci, which were associated with an increased number and area of glutathione S-transferase placental form (GST-P) positive foci. In order to examine a progression of these hepatocellular changes and to confirm new other toxicological findings induced in longer dosing duration of DHP, the highest dose of DHP was set at 2,000 mg/kg/day and half of the highest dose was designated as the lower dose in the present study.

**Dosing duration**

The duration of 26 weeks was selected because there are lots of background data as a long term toxicity studies.

**Mortality and clinical observations**

Rats were observed daily for mortality, toxic signs, and changes in general health and behavior. Body weight and food consumption were measured once a week from study week 1 to 13, and once a month after study week 13. Water consumption was measured once a month. Ophthalmologic examinations were performed at study week 26 using a slit lamp and fundus camera after ocular instillation of a mydriatic (Mydrin® P, Santen Pharmaceutical Co., Ltd., Osaka, Japan).

**Clinical pathology**

Urine was collected using metabolic cages. Fresh urine was used for qualitative analyses of pH, protein, glucose, ketone bodies, urobilinogen, bilirubin, occult blood,
sediiments and color. Twenty-four-hour stock urine was
used for measurements of volume, specific gravity, sodi-
um, potassium and chloride. Blood samples were collect-
ed prior to necropsy at study week 26 for hematological
and blood chemical analyses. Hematological parameters
assessed included erythrocyte count (RBC), reticulocytes
(Reti), hemoglobin (Hb), hematocrit (Ht), mean corpus-
cular volume (MCV), mean corpuscular hemoglobin
(MCH), mean corpuscular hemoglobin concentration (MCHC), plate-
clet count, leukocyte and differential count (XT-2000iV,
Sysmex Corporation, Hyogo, Japan), prothrombin time
(PT), and activated partial thromboplastin time (APTT)
(CA-1500, Sysmex Corporation). Blood chemical
parameters assessed included glucose (Glu), urea nitro-
gen (BUN), creatinine (Crea), total protein (TP), albu-
min (Alb), albumin/globulin ratio (A/G), aspartate ami-
notransferase (AST), alanine aminotransferase (ALT),
alkaline phosphatase (ALP), γ-glutamyl transpeptidase
(γ-GTP), sodium (Na), potassium (K), chloride (Cl), cal-
cium (Ca), phosphorus (P), triglycerides (TG), choles-
terol (Cho), and total bilirubin (T.Bil) (Synchron CX-7,
Beckman Coulter, Inc., Brea, CA, USA).

Anatomical pathology

The rats were necropsied and weights of the follow-
ing organs were recorded: brain, pituitary, thyroid, sub-
mandibular glands, heart, thymus, spleen, lungs, liv-
er, kidneys, adrenals, testes, epididymides, prostate, and
seminal vesicles.

Histopathology

Eyes from all rats were fixed in Davidson’s fixative.
The remaining tissues were fixed in 10% neutral buffered
formalin. Sections of the following tissues were prepared,
stained with hematoxylin and eosin (HE), and examined
microscopically: cerebrum, cerebellum, spinal cord, sci-
catic nerve, optic nerve, pituitary, parathyroid, adrenals,
submandibular glands, sublingual glands, heart, thymus,
spleen, mesenteric lymph nodes, submandibular lymph
nodes, trachea, bronchus, lungs, aorta, liver, pancreas,
tongue, esophagus, stomach, duodenum, jejunum, ile-
um, cecum, colon, rectum, kidneys, urinary bladder, tes-
tes, epididymides, prostate, seminal vesicles, mammary
glands, skin, sternum, femur, bone marrow, femoral mus-
cle, eyes, and Harder’s glands.

Histopathology of the liver

Three serial sections of the liver were used for HE
staining and immunohistochemical staining for GST-P,
a marker of cell proliferation. Immunohistochemical stain-
ing was performed using the following procedure: depar-
affinized sections were treated with 3% H2O2 in phos-
phate-buffered saline for 10 min, then rabbit polyclonal
anti-GST-P antibody (1:1,000 dilution; Medical and
Biological Laboratories Co., Ltd., Aichi, Japan) for 90
min or mouse monoclonal anti-PCNA antibody (1:1,000
dilution; DAKO Japan, Kyoto, Japan) for 60 min at room
temperature. An avidin-biotin-peroxidase complex meth-
od (DAKO Japan) was employed with 3,3’-diaminoben-
zidine as a chromogen, followed by light counterstain-
ing with hematoxylin. With a computer-assisted image
analyzer (Win Roof; Mitani Corp., Japan), the number and
area of GST-P positive foci in at least five cells were
measured, and then the mean number and total area of
liver sections were calculated. PCNA-positive cells were
counted from a total of ten fields of view (approximately
600-800 hepatocytes in each fields) in each animal, and
the total number per area was calculated.

Statistical analysis

The mean and standard deviation were calculated
for the data of body weight, food and water consump-
tion, hematology, blood chemistry, urinalysis, organ
weight, number and/or area of GST-P-positive liver foci
and PCNA-positive cells. These data were analyzed for
homogeneity using Bartlett’s test. For homogeneous data,
significant differences were determined using Dunnett’s
multiple comparison test (Dunnett, 1955). For heteroge-
neous data, significant differences between the control
and DHP-treated groups were determined using Dunnett’s
rank test. The level of significance was set at a $P$-value
less than 0.05.

RESULTS

Clinical observations, body weight, food and
water consumptions

No abnormal clinical signs were observed in any DHP-
treated rats. There was a dose-dependent decrease in
body weights from study day 8 in DHP-treated groups.
Furthermore, DHP-treated rats had significantly lower
body weights compared with controls from study
days 29-182 at 2,000 mg/kg and study days 43-182 at
1,000 mg/kg (Fig. 1). At both doses, DHP-treated rats
demonstrated significantly higher food consumption from
study weeks 3-26 (Fig. 2), and water consumption from
study weeks 6-26 (data not shown) compared with con-
trols. The increase in food consumption was considered to
be related to the PPARα agonistic effects of DHP, which
induced lower Glu, TG, and Cho values. The increase in
water consumption was thought to be secondary to the increase in food consumption.

**Ophthalmology**

There were no DHP-related ophthalmologic changes.

**Urinalysis**

There were decreases in pH and specific gravity, and increases in protein and urine volume following treatment with DHP at 1,000 and 2,000 mg/kg. There were also increases in total Na, K and Cl excretion; however, urinary concentration of those electrolytes in DHP-treat-
ed groups was comparable with those in the controls (data not shown).

Hematology

Significant prolongation of PT was evident at both doses of DHP (data not shown), and may be attributed to decreased liver function resulting from DHP-induced hepatotoxic lesions. There were no apparent changes in the other hematological parameters.

Blood chemistry

There were significant decreases in TG, Glu, Cho, and PL, and increases in AST, ALT, ALP, and γ-GTP following DHP treatment at 1,000 and 2,000 mg/kg (Table 1). These decreases in TG, Glu, Cho, and PL may be due to DHP’s PPARα agonistic effects, and the slight increases in AST, ALT, ALP and γ-GTP is probably related to the hepatocellular hypertrophy associated with increased peroxisome proliferation. Additionally, there were significant increases in K, P and Ca at both doses (data not shown).

Necropsy findings

Pale brown discoloration and dark brown spots were evident in livers of rats treated with 1,000 and 2,000 mg/kg of DHP (Fig. 3). There were no changes attributable to DHP treatment observed in the other organs assessed.

Organ weights

Mean absolute and relative organ weights are shown in Table 2. Mean absolute and relative liver weights were significantly higher and drug-related in the DHP-treated compared with untreated rats. Mean absolute and rel-

### Table 1. Blood chemistry in male F344 rats treated orally with DHP for 26 weeks

<table>
<thead>
<tr>
<th></th>
<th>AST IU/l</th>
<th>ALT IU/l</th>
<th>γ-GTP IU/l</th>
<th>Glu mg/dl</th>
<th>T.Cho. mg/dl</th>
<th>TG mg/dl</th>
<th>PL mg/dl</th>
<th>TP g/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Mean</td>
<td>142.1</td>
<td>85.4</td>
<td>1.96</td>
<td>195</td>
<td>64</td>
<td>108</td>
<td>119</td>
</tr>
<tr>
<td></td>
<td>S.D.</td>
<td>47.1</td>
<td>24.3</td>
<td>0.47</td>
<td>37</td>
<td>4</td>
<td>40</td>
<td>8</td>
</tr>
<tr>
<td>1,000 mg/kg</td>
<td>Mean</td>
<td>229.6**</td>
<td>148.9##</td>
<td>3.17##</td>
<td>137##</td>
<td>32##</td>
<td>13##</td>
<td>81##</td>
</tr>
<tr>
<td></td>
<td>S.D.</td>
<td>65.0</td>
<td>55.6</td>
<td>1.07</td>
<td>15</td>
<td>3</td>
<td>3</td>
<td>6</td>
</tr>
<tr>
<td>2,000 mg/kg</td>
<td>Mean</td>
<td>198.7*</td>
<td>130.4#</td>
<td>3.22##</td>
<td>135##</td>
<td>31##</td>
<td>17##</td>
<td>82##</td>
</tr>
<tr>
<td></td>
<td>S.D.</td>
<td>45.8</td>
<td>36.1</td>
<td>0.79</td>
<td>13</td>
<td>2</td>
<td>3</td>
<td>5</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01: significantly different from control, as determined by Dunnett’s test.

#p < 0.05, ##p < 0.01: significantly different from control, as determined by Dunnett’s rank test.

S.D.: standard deviation (n = 12 per group).

Fig. 3. Macro photographs of livers from male F344 rats treated orally with DHP at 0 (1), 1,000 (2) or 2,000 (3) mg/kg for 26 weeks. Pale brown discoloration and diffuse dark brown spots are observed in both DHP-treated groups.
Table 2. Absolute and relative (per 100 g body weight) organ weights of male F344 rats treated orally with DHP for 26 weeks

<table>
<thead>
<tr>
<th>Body weight</th>
<th>Liver</th>
<th>Testes</th>
<th>Prostate</th>
<th>Thymus</th>
<th>Adrenals</th>
<th>Kidneys</th>
</tr>
</thead>
<tbody>
<tr>
<td>(g)</td>
<td>(g)</td>
<td>(g%)</td>
<td>(mg)</td>
<td>(mg%)</td>
<td>(mg)</td>
<td>(mg%)</td>
</tr>
<tr>
<td>Control</td>
<td>Mean</td>
<td>330</td>
<td>8.91</td>
<td>2.70</td>
<td>3.01</td>
<td>0.91</td>
</tr>
<tr>
<td></td>
<td>S.D.</td>
<td>13</td>
<td>0.48</td>
<td>0.10</td>
<td>0.29</td>
<td>0.09</td>
</tr>
<tr>
<td>1,000 mg/kg</td>
<td>Mean</td>
<td>295**</td>
<td>11.38**</td>
<td>3.86**</td>
<td>2.93</td>
<td>1.00**</td>
</tr>
<tr>
<td></td>
<td>S.D.</td>
<td>9</td>
<td>0.46</td>
<td>0.08</td>
<td>0.07</td>
<td>0.04</td>
</tr>
<tr>
<td>2,000 mg/kg</td>
<td>Mean</td>
<td>294**</td>
<td>11.57**</td>
<td>3.94**</td>
<td>2.80**</td>
<td>0.95</td>
</tr>
<tr>
<td></td>
<td>S.D.</td>
<td>14</td>
<td>0.60</td>
<td>0.10</td>
<td>0.16</td>
<td>0.06</td>
</tr>
</tbody>
</table>

*p < 0.05, **p < 0.01: significantly different from control, as determined by Dunnett’s test.

#p < 0.05, ##p < 0.01: significantly different from control, as determined by a Dunnett’s rank test.

S.D.: standard deviation (n = 12 per group).

a): g/100 g body weight.
b): mg/100 g body weight.

Table 3. Histopathological findings in male F344 rats treated orally with DHP for 26 weeks

<table>
<thead>
<tr>
<th>Sex</th>
<th>Male</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (olive oil)</td>
<td>DHP</td>
</tr>
<tr>
<td>Dose (mg/kg)</td>
<td>0</td>
</tr>
<tr>
<td>No. of animals</td>
<td>12</td>
</tr>
<tr>
<td>Grade</td>
<td>0</td>
</tr>
</tbody>
</table>

Liver

H.E. staining

- Hypertrophy*, hepatocyte, diffuse: 12 0 0 0 0 0 0 12 0 0 1 11
- Foci of cellular alterations: 12 0 0 0 0 4 8 0 0 3 9 0
- Anisokaryosis: 12 0 0 0 0 12 0 0 0 12 0 0
- Focal necrosis: 12 0 0 0 12 0 0 0 11 1 0 0
- Infiltration, Mononuclear cell, focal: 12 0 0 0 12 0 0 0 11 0 1 0
- Infiltration, Neutrophil, focal: 12 0 0 0 12 0 0 0 11 1 0 0

GST-P staining

- Foci of cellular alterations, positive for GST-P stain: 12 0 0 0 0 3 7 2 0 0 12 0

Numerals represent the number of animals.
Grade 0: no change, 1: slight, 2: moderate, and 3: marked.

*: with a ground-glass appearance, inclusion body and/or vacuolation.

Significant decreases in the mean absolute weights of the spleen, thyroid, submandibular glands and heart at 1,000 and/or 2,000 mg/kg of DHP (data not shown). These changes were considered to be secondary to decreases in body weights, as there were no histopathological findings related to DHP treatment.
DHP-related histopathological findings were limited to the liver. Marked diffuse hypertrophy of hepatocytes was found to be associated with a ground-glass appearance, slight anisokaryosis, intracytoplasmic inclusion bodies and/or vacuolation in all DHP-treated rats (Table 3). Additionally, altered hepatocellular foci were observed in all DHP-treated rats, whereas these foci were not detected in untreated rats (Fig. 4). Most of the DHP-induced altered hepatocellular foci were eosinophilic; however, some were basophilic and a few could not be distinguished as either eosinophilic or basophilic with HE.
staining. The demarcation line of eosinophilic foci was not as clear as that of the basophilic foci. The number and area of GST-P positive foci markedly increased in DHP-treated rats (Table 4). With respect to the regional distribution of the altered foci detected with HE-staining and GST-P positive foci detected with immunohistochemically-staining, it was found that GST-P negative and positive foci were generally basophilic and eosinophilic, respectively, whereas a few GST-P positive foci were found in areas where altered hepatocellular foci were not detected in HE-stained sections (Fig. 4). Additionally, PCNA positive liver cell counts in DHP-treated rats were markedly higher than those of the controls (Table 5). There were no clear differences in the incidence and severity of these altered hepatocellular foci between 1,000 and 2,000 mg/kg of DHP. Lastly, there were no DHP-related histopathological changes in the prostate, thymus and adrenal glands demonstrating decreases in absolute and relative organ weights, kidneys showing increases in absolute and relative organ weights, nor testicular alterations.

**DISCUSSION**

GST-P is considered the most sensitive and reliable marker for the identification of pre-neoplastic and neoplastic hepatic lesions in rats. However, it has been pointed out that GST-P is not an ideal marker for identifying peroxisome proliferator-induced hepatic lesions in rats, because more than 95% of neoplastic nodules and hepatocellular carcinomas induced by these chemicals were negative for this marker (Rao et al., 1986). Similar findings were reported with PPARα agonists: di(2-ethyl hexyl)
phthalate (DEHP), a type of PAEs, did not increase the incidence of GST-P positive foci in livers of rats in a long-term carcinogenicity test (Sano et al., 1999); DEHP or clofibrate increased the number of GST-P negative foci in a dose-dependent manner in two-stage hepatocarcinogenesis rat models (Sano et al., 1999; Hasegawa et al., 1994); and fenofibrate (FF), a PPARα agonist, increased the number of GST-P negative foci in a diethyl nitrosamine (DEN)-initiated two-stage liver carcinogenesis rat model (Nishimura et al., 2008). Conversely, in the present study, we demonstrated that the incidence of hepatocellular foci, and the number and area of GST-P positive foci markedly increased in rats treated orally with DHP at 1,000 and 2,000 mg/kg/day over a 26-week period. DHP-induced altered hepatocellular foci are composed of eosinophilic or basophilic hepatocytes, and may be characteristic of DHP treatment, since oral administration of common PPARα agonists induced altered foci with eosinophilic cytoplasm in rodents (Moody and Reddy, 1978; Nishimura et al., 2007). Therefore, altered foci with eosinophilic cytoplasm may be GST-P negative foci, but there was no clear relationship between GST-P positive and eosinophilic/basophilic altered foci in the present study. In addition, there was a significant increase in PCNA-positive cells among DHP-treated rats. It was reported that a 28-day DHP treatment increased the number and area of GST-P positive foci in the livers of rats (Jin et al., 2009). When comparing the severity of GST-positive foci observed at a dose of 2,000 mg/kg in the 26-week study with that of the 2,500 mg/kg dose in the 28-day study, the number of foci was 5.5-6.6-fold higher in the 26-week study (0.44-0.53/mm² of liver lobe for the 26-week study vs. 0.08/mm² of liver lobe for the 28-day study), and their area was 1.6-3.6-fold higher in the 26-week study (7.0-16.4 mm² of foci/cm² of liver lobe for the 26-week study vs. 4.5 mm² of foci/cm² of liver lobe for the 28-day study). These findings strongly suggest that DHP-induced GST-P positive foci are rarely induced by common PPARα agonists, and that long-term treatment with DHP results in an increase in the number and size of altered foci.

Suzuki et al. (2010) reported that a 14-day repeated administration of Wy14643, a PPARα agonist and non-genotoxic hepatocarcinogen, induces ROS generation and some DNA damage in the livers of rats. However, this DNA damage was repaired by increasing the activity of certain DNA repair genes without inducing mutations. Tawfeeq et al. (2010) also reported positive results with the in vivo comet assay 3 hr after a 2-week treatment with FF, a PPARα agonist and non-genotoxic hepatocarcinogen. However, negative results were obtained 24 hr after the last treatment of FF. Furthermore, DNA repair genes, such as Apex1, Mlh1, Ogg1 and Gadd45a, which repair DNA damage due to ROS generation, significantly increased in rats 3 hr after the last administration of FF, as determined by quantitative real-time reverse transcription-polymerase chain reaction (RT-PCR). Therefore, it was speculated that FF may cause DNA damage in liver by oxidative stress initially following treatment, and that this DNA damage is repaired via a simultaneous transcriptional upregulation of DNA repair genes. Thus, it appears that most PPARα agonists do not induce DNA mutations in livers of rats, despite the ROS-induced DNA damage. Conversely, it was reported that DHP treatment significantly induced DNA migration after 14 repeated oral doses, as determined by an in vivo comet assay (Jin et al., 2009). Furthermore, DHP-treated rats exhibited an increased number and area of GST-P positive foci after 14 times of the oral dose, as determined by an in vivo liver initiation assay (Jin et al., 2009). However, a single or three times of the oral dose of DHP demonstrated negative results in the two in vivo tests mentioned above. As a result, Jin et al. (2009) speculated that more time is necessary for an induction of DNA mutations following ROS-induced DNA damage associated with chemicals, such as DHP. Considering these findings, the mechanism by which DHP induces DNA damage appears to be different from other PPARα agonists, and thus DHP might be categorized as a possible genotoxic substance.

With regard to the molecular mechanisms by which DHP induces hepatocellular altered foci of DHP, Jin et al. (2009) reported that cell cycle/proliferation-related genes, such as Spp1, Mmp12, ATF3, Cyclin B1, Cyclin A2 and Cdc2a, and anti-apoptosis-related genes, such as Ccl2, Prnp and Timp1, were overexpressed in the livers of rats treated with DHP for 28 days. However, it was found that oxidative stress-related genes, such as Cyp2c, Cyp2c7 and Cyp1a2, were down-regulated in these rats, despite the fact that ROS generation was found to be upregulated with thiobarbituric acid-reactive substances (TBARS). On the contrary, microarray and quantitative RT-PCR analyses in male F344/N rats fed a diet containing 6,000 or 3,000 ppm of FF for 13 weeks following DEN initiation demonstrated a significant upregulation in genes related to lipid metabolism, such as Aco and Cyp4a1, metabolic oxidative stress, such as Gpx2, Yc2, Cat, Cyp2b15, and Ugt1a6, DNA repair, such as Apex1, Mgmt, Xrc5, Nbn, and Gadd45a, and cell cycle regulation, such as Ccnd1 (Nishimura et al., 2008). Thus, such an upregulation of genes related to lipid metabolism and metabolic oxidative stress suggest that a large amount of ROS was produced in FF-treated rats (Nishimura et al., 2008). The results of these molecular analyses suggest that the oxidative stress
resulting from both DHP- and FF-induced ROS generation may be involved in the development of pre-neoplastic hepatocellular foci. However, the major source of ROS appears to be different between DHP and FF. That is, the major source of ROS production following treatment with FF is in the endoplasmic reticulum and peroxisome, whereas the major source of ROS following treatment with DHP is in the peroxisome and other organelles, such as mitochondria, as Cyp4a1 was not overexpressed by DHP (Jin et al., 2009). Therefore, it appears that DHP has a distinct mechanism of action for ROS generation from the other PPARα agonist, such as FF. Qu et al. (2010) recently reported that the activation of cell cycle control and DNA repair-related genes in non-parenchymal cells is critical in PPARα-induced carcinogenesis, as demonstrated in transgenic mice expressing a constitutively active PPARα in hepatocytes (VP16PPARα). However, it is not apparent whether DHP induces an upregulation of DNA repair-related-genes in the liver of rats. Therefore, DHP may have a different mechanism for hepatocellular proliferating activity from other PPARα agonists.

It has been previously reported that PAEs elicit adverse effects in the testes of rodents, causing testicular atrophy (Bhattacharya et al., 2005; Corton and Lapinskas, 2005). It is known that DHP causes testicular atrophy in rats treated orally with doses of 1, 2.5 or 5 g/kg for 28 days (Matsushima et al., 1992; Jin et al., 2009). In the present study, however, no apparent morphological changes were detected in the testes at both doses. The testicular toxicity of phthalate esters depends in part on the length of the alcohol moiety (side chain) of the ester molecule (Heindel and Powell, 1992). In general, phthalates with medium- (e.g. dibutyl phthalates) or branched long-side chains (e.g. DEHP) induce degenerative testicular lesions, while those with short- (e.g. dibutyl phthalates) or linear long-side chains (e.g. di-n-octyl phthalate) are inactive. Since DHP had linear long-side chains in the aspect of chemical structure, it is speculated that DHP may have low capability to induce testicular toxicity and the dose, 2 g/kg of DHP, is not enough to induce degeneration of the testis.

In conclusion, we demonstrated that oral administration of DHP at 1,000 and 2,000 mg/kg/day to rats for 26 weeks induced hypolipidemic and toxic effects that are characteristic of PPARα agonists, with the exception of testicular toxicity. Furthermore, DHP treatment increased the number and area of GST-P positive foci that are not generally induced by PPARα agonists in the liver. Therefore, DHP is considered to be a possible genotoxic substance and have the potential of hepatocarcinogenicity in rats, although this notion needs to be confirmed by long-term carcinogenicity studies.

REFERENCES


