It has been reported that the two phthalates, DEHP and di-butylphthalate, possess anti-androgenic effects in vivo, but the effects of these phthalates on the male reproductive system is different from the directly acting androgen receptor antagonist, flutamid (Gray et al., 1999; Mylchreest et al., 1999). The androgen receptor immunoreactivity may be affected through indirect mechanisms during this 12 h study, and therefore we applied an immunohistochemical staining of the androgen receptor. One androgen receptor response element is identified within the first intron and one cAMP response element is found in the first exon of the *TRPM*-2 gene and both androgens and cAMP repress the expression of this gene (Rosemblit and Chen, 1994). In the testis the *TRPM*-2 gene product is identical to sulfated glycoprotein-2/clusterin, a major secretion product of normal Sertoli cells (Gaemers et al., 1998). In the prostate an increase in *TRPM*-2 expression is associated with apoptosis induced by castration, androgen depletion and ischemia (Bettuzzi et al., 1992; Furuya and Isaacs, 1993; Wong et al., 1993). If the androgen receptor or cAMP is influenced by MEHP, a change in *TRMP*-2 expression would be expected. Therefore, we investigated the relationship between MEHP induced testis toxicity and gene expression of the testosterone-repressed-prostatic-message-2-gene (*TRPM*-2) in testis and ventral prostate.

Programmed cell death (apoptosis) is a continual normal physiological process regulating the number of germ cells to the relative number of supportive Sertoli cells in the seminiferous tubules during spermatogenesis (Blanco-Rodriguez and Martinez-Garcia, 1998). Increased germ cell apoptosis is associated with abnormal spermatogensis in man (Lin et al., 1997). In rodents heat and irradiation as well as xenohormones and testis toxicants like MEHP, 2,5-hexandione, nitrobenzene, deltamethrin, and hydroxyurea are known inducers of germ cell apoptosis (Lee et al., 1997; Shinoda et al., 1998; El-Gohary et al., 1999; Shin et al., 1999). According to Billig et al. (1995) and our own preliminary studies, the level of germ cell apoptosis in male rats peaks during the first spermatogenetic cycle from postnatal day 16 to 32.

DEHP is a known Sertoli cell toxicant associated with detachment of germ cells and vimentin positive conglomerates of desquamated cells in the lumen of degenerating tubules (Dalgaard et al., 2000). Additionally, Richburg and Boekelheide (1996) studied the testis toxicity of a single very high oral dose of 2000 mg/kg bw MEHP in 28-day-old male Fisher rats. They found histopathological changes, changes in the cytoplasmatic distribution of vimentin in Sertoli cells, and a decrease in numbers of apoptotic germ cells 3 h after dosing followed by an increase after 6–12 h.

The present study is a multidisciplinary approach to investigate the effects of MEPH induced on testis in 28-day-old Wistar rats by a five-fold lower dose level than previous reported.

2. Materials and methods

2.1. Animals, exposure and tissue preparation

Forty-eight 28-day-old male Wistar rats (Bomholtgaard and Møllegaard breeding Centre, Denmark), weighing approximately 60 g, were randomised into four groups of 12 rats. During the acclimating period of 1 week the animals were housed two per cage, with a 12 h day:night cycle. The room temperature was 22 ± 1°C and the relative humidity 55 ± 5%. The rats were given a standard diet (Altromin, rat no. 1324, Brogården, Denmark). Three groups were dosed with 400 mg/kg bw MEHP (TCI, Japan, CAS nr. 4376-20-9) in corn oil by gavage, and sacrificed by decapitation in deep CO_2/O_2 narcosis 3, 6 or 12 h after dosing. The control rats, received a similar volume of corn oil, and were sacrificed 2 h after dosing. Due to the limited number of animals it was necessary to distribute the available testes between the applied methods. Therefore, each method used one testis sample from eight out of 12 rats per group. Testis was removed and fixed in Bouin's fixative for 24 h and embedded in paraffin. One cross section was stained with haematoxylin and eosin (HE) and used for the histopathological investigation, two sections were immunostained and the vimentin filament as well as androgen receptor distribution were deter-

mined, and one section was TUNEL-stained. For the caspase-3, LM-PCR and gene expression analysis, testis was cut in three pieces. The samples for caspase-3 activity and LM-PCR analyses were immediately snap frozen in liquid nitrogen and stored at −80°C until processing. The third piece and the ventral prostate were stored in RNAlater (Ambion) and kept at −20°C until total RNA purification was performed.

2.2. Immunohistochemical staining of vimentin

Testis vimentin was investigated with a method by Zhu et al., (1997) and modified in order to use Bouin's fixed paraffin sections (Dalgaard et al., 2000). Briefly, paraffin sections (5 μm) were deparaffinised, and heat induced epitope retrieval in a microwave oven was performed for 2 × 5 min in 0.01 M citrate buffer, pH 6.0. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide. Non-specific background staining was eliminated with 10% non-immune rabbit serum. The tissue sections were incubated overnight at 4°C with mouse–*anti*-vimentin monoclonal antibody (DAKO). This was followed by incubation with secondary biotinylated rabbit–*anti*-mouse antibody (DAKO). Sections were incubated with the avidin biotinylated horseradish peroxidase complex and visualised by the chromogenic substrate 3-amino-9-ethylcarbazole (Sigma). Tissue sections were counter-stained with haematoxylin, mounted with Aquamount (Gurr®) and examined by light microscopy. For validation of the immunostaining, three control slides were stained simultaneously: on one testis slide the primary antibody was omitted, on another testis slide the primary antibody was replaced with normal mouse serum, and on a third slide a tonsil, known to express vimentin, was examined.

2.3. Immunohistochemical staining of the androgen receptor

The staining protocol was kindly provided by K.J. Turner, MRC Reproductive Biology Unit, Centre for Reproductive Biology, Edinburgh, UK. Deparaffination, epitope retrieval and blockage of endogenous peroxidase activity were similar to the procedure used for the vimentin staining. Non-specific background staining was eliminated with 10% normal swine serum. The tissue sections were incubated overnight at 4°C with the primary rabbit polyclonal antibody (N20:sc-816, Santa Cruz Biotechnology). This was followed by incubation with secondary biotinylated swine–*anti*-rabbit antibody (DAKO). The steps of detection and visualisation were analogous to the procedure used for the vimentin staining mentioned above. For validation of the immunostaining, three control testis slides were stained simultaneously: on one slide the primary antibody was omitted, on another slide the primary antibody was replaced with normal rabbit serum, and on a third slide an adult rat testis, known to express the androgen receptor, was examined.

2.4. Expression of TRPM-2 gene in testis and ventral prostata

The testis and ventral prostate samples were collected, stored in RNAlater (Ambion) to minimise RNAase activity, and homogenised by an Ultra-Turrax. Subsequent extraction of total RNA was performed by the commercial available kit, RNeasy Midi-kit (QIAGEN), following the manufacturer's instructions. cDNA was produced from 0.5 to 1.0 μg of total RNA by using the display-THERMO-RT kit and the manufacturer's instructions (Display Systems Biotech, Kem-en-tec). Real-time reverse transcriptase–polymerase chain reaction (RT–PCR) with online detection of the PCR product based on fluorescence monitoring (LightCycler, Roche) was employed. Primers and hybridisation probes (TIB MolBiol) were designed and the hybridisation probes were employed to monitor the amount of specific target sequence produced. Quantitative results were obtained by the cycle threshold value, where a signal rose above background levels. Expression of the gene coding for *TRPM*-2 was compared to the steady expression of endogenous gene, β-actin. Optimal reactions were achieved with 4 mM $MgCl_2$, 5000 pg cDNA per reaction, 0.1 μM primers and probes for β-actin, and 0.7 μM of primers and 0.3 μM of hybridisation probes for

TRPM-2 as shown in Appendix A. The PCR program followed the manufacturer's instructions (LightCycler-DNA Master Hybridisation Probes, Roche), except that a *Taq* start antibody (0.16 per 20 μl reaction) (Clontech) was employed to prevent enzyme activity prior to the PCR amplifications, and the denaturation step therefore increased to 2 min. Gene expressions were analysed at least three times for each animal.

2.5. Measurements of caspase-3 activity

Testis samples frozen in liquid nitrogen and stored at −80°C were placed at room temperature. To the still frozen samples, 1250 μl ice-cold cell lysis buffer containing a proteinase inhibitor cocktail (Calbiochem Cat. No. 539134) were added and mixed in the relative volumes 98.75 and 1.25, respectively. Samples were homogenised with an Ultra-Turrax using an ice-jacket. One homogenate aliquot was taken for protein analysis, another was centrifuged for 10 min at 30 000 *g* at 4°C. The supernatant was decanted and used for caspase-3 activity measurements. The caspase-3 activity was measured at 37°C after incubation for 60 min. The method applied was essentially as described by Thornberry (1994), Nicholson et al. (1995). Briefly, samples were incubated with or without a specific caspase-3 inhibitor (Ac-DEVD-CHO, Calbiochem Cat. No. 235420) applying a colourimetric substrate (Ac-DEVD-pNA, Calbiochem, Cat. No. 235400) from which 4-nitroaniline (4-NA) is liberated due to caspase activity. The values are expressed in pmol 4-NA liberated per mg homogenate protein during 60 min. The composition of the cell lysis buffer was: 50 mM HEPES, 1 mM dithiotreitol, 0.1 mM Na_2EDTA, 10% glycerol, 0.1% CHAPS, pH 7.4. Protein was measured in the homogenate by use of a commercial available BicinChoninic Acid method kit (Pierce Cat. No. 23220/23225).

2.6. Detection of nucleosomal ladders in the testis

DNA from the testis was isolated according to Ausubel et al. (1994). Detection of nucleosomal ladders was based on ligation-mediated PCR (LM-PCR) (Staley et al., 1997) using the ApoAlert™ LM-PCR Ladder Assay Kit #K 905-1 from Clontech. In short, the first step is the ligation of dephosphorylated adaptors (composed of a 12-mer and a 24-mer) to the ends of the DNA fragments generated during apoptosis. In mammalian cells, such fragments generally have 5′-phosphorylated blunt ends. Thus, only the 24-mer is ligated to the DNA fragments. When the mixture of ligated DNA fragments is heated, the 12-mer is released. Next, the 5′ protruding ends of the molecules are filled in by a thermostable DNA polymerase. The 24-mer then serves as a primer in a PCR in which the fragments with adaptors on both ends are exponentially amplified. The resulting nucleosomal ladder can be visualised on an agarose/ethidium bromide gel. As a positive control, calf thymus DNA, provided by Clontech was used.

2.7. The TUNEL-assay

The occurrence of apoptosis is based on the in situ labelling of the free 3′ OH termini of single stranded or double stranded DNA breaks, cleaved in the characteristic nucleosomes consisting of 185 base pairs or multiplies of that size (Brinkworth et al., 1995; Hikim et al., 1995). A procedure previously described in Dalgaard (2000) was used. In brief, after deparaffination and rehydration, tissue sections (5 μm) were incubated with Proteinase-K (Sigma) (10 μl/ml) for 10 min, washed and treated with 2% hydrogen peroxide to block endogenous peroxidase activity. Sections were then incubated with a reaction mixture containing terminal reaction buffer, digoxigenin labelled nucleotide, and terminal deoxynucleotidyl transferase (TdT) following the suppliers' guidelines (ApopTag® peroxidase in situ apoptosis detection kit, Intergen Company). Subsequently, the sections were treated with an *anti*-digoxigenin-peroxidase antibody followed by the chromogenic substrate 3-amino-9-ethylcarbazole (Sigma). Finally, sections were counterstained with haematoxylin. A regressing mammary gland from a female rat 4 days after weaning, and testis tissue treated with DNAse were used as positive controls. In the negative control the TdT enzyme was substituted with distilled water. In eight rats per group (seven

rats in the group sacrificed after 3 h) all TUNEL positive and negative germ cells fulfilling morphological criteria for apoptosis: cell shrinkage, nuclear fragmentation and condensation were counted in 100 randomly selected tubules in one cross section per animal. In order to determine the number of apoptotic germ cells at each time point a modified version of the method by Richburg and Boekelheide (1996) was used. The percentage of tubules containing 0, 1–3, or >3 apoptotic germ cells per tubule was compared.

2.8. Statistical analysis

For the statistical analyses of gene expression, caspase-3 activity, and the quantitative analysis of germ cell apoptosis, a one-way analysis of variance (ANOVA) (Procedure GLM in SAS institute version 6.12) was used. If necessary, the data were log transformed in order to obtain variance homogeneity. When significant ($P < 0.05$) treatment related effects were detected in the ANOVA, two-tailed *t*-tests (Dunnett's method) were used to compare the control group with MEHP-exposed groups.

3. Results

3.1. Clinical effects, histopathology and immunohistopathology

No signs of general toxicity were observed during the study. In the testis no histopathological changes were observed in the control group and in the MEHP exposed group sacrificed after 3 h. Slightly disorganised tubules and detachment and sloughing of germ cells into the lumen of the seminiferous tubules were observed after 6 h, and this effect was enhanced after 12 h since only Sertoli cells and spermatogonia remained attached to the basal lamina (Fig. 1). The sloughing of germ cells was apparently most pronounced in stage IX–XIV tubules. Three to 12 h after administration of a single oral dose of MEHP, the vimentin filaments in the Sertoli cell cytoplasm collapsed, the apical extensions towards the lumen of the tubules vanished, and a perinuclear condensation of vimentin was observed (Fig. 1).

The intensity of the androgen receptor immunostaining was most prominent in the peritubular myoid cells, and the Leydig cells, but to a lesser extent in the Sertoli cells (Fig. 2). In the Sertoli cells the intensity of the reaction varied in a stage specific manner; in stage IV–VIII tubules, the androgen receptor immunostaining was more intense than in later stage tubules (IX–XIV). This pattern did not change between the control group and the dosed groups.

3.2. TRPM-2 gene expression

In testis the relative expression of the *TRPM*-2 was statistically significantly increased 3 h after MEHP administration, but returned to normal levels after 6 and 12 h (Fig. 3). In the ventral prostate no treatment related differences in the expression of *TRPM*-2 gene were observed (data not shown).

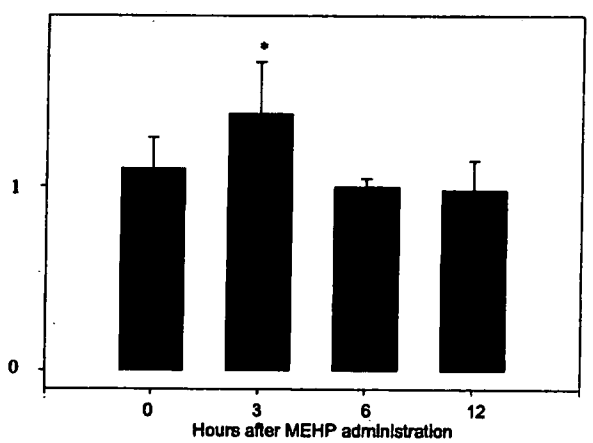

Fig. 3. Expression of *TRPM*-2 relative to the expression of β-actin (mean ± SEM) in testis of 28-days-old male rat exposed to 0 or 400 mg/kg bw of MEHP and sacrificed after 3, 6 or 12 h. The *TRPM*-2/β-actin value is significantly increased after 3 h of MEHP exposure compared to the control rats. Six and 12 h after exposure the expression returned to normal levels. * denotes a statistically significant difference (Dunnett's test) from the control rats ($P < 0.05$) (*y*-axis represents expression of *TRPM*-2).

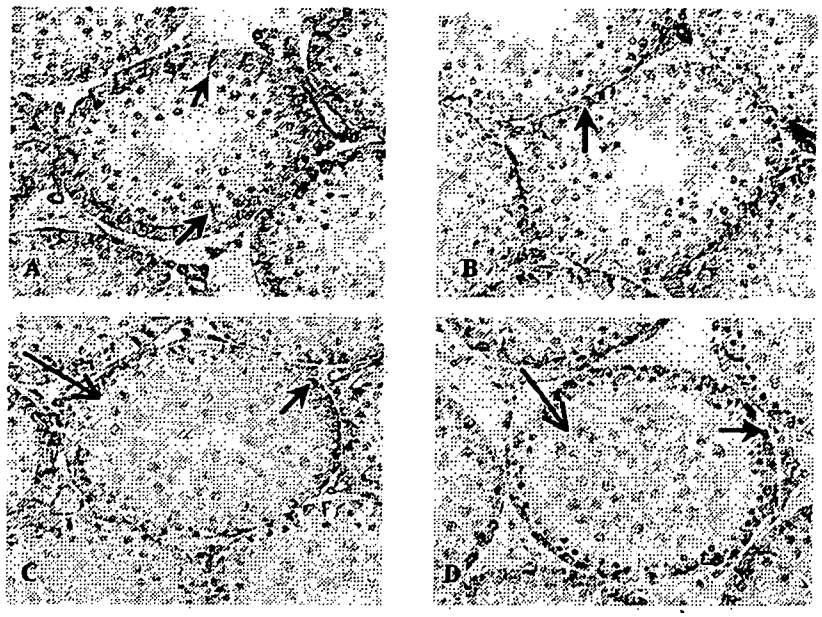

Fig. 1

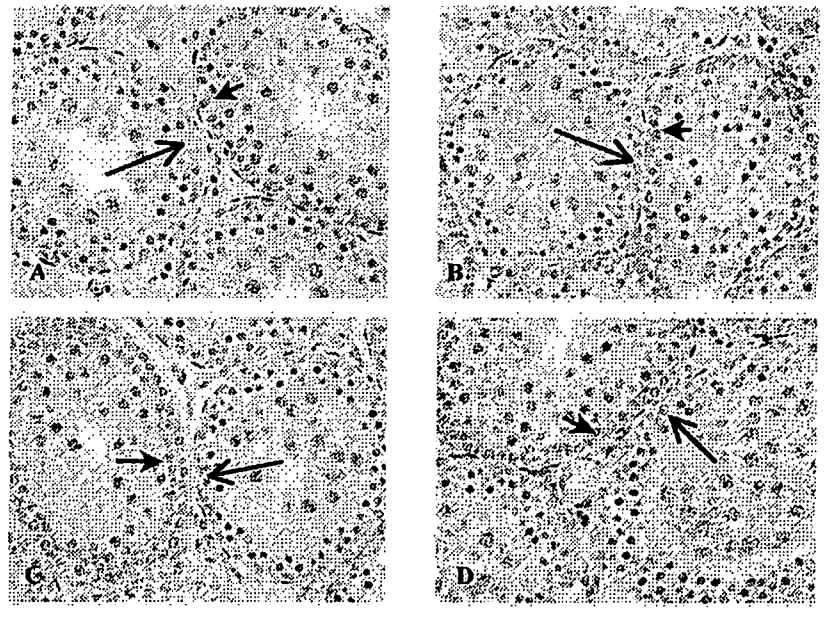

Fig. 2

Fig. 1. Vimentin staining of Sertoli cell cytoplasm of 28-days-old male rats exposed to 0 (A) or 400 mg/kg of MEHP and sacrificed after 3 (B), 6 (C), or 12 (D) h. In the Sertoli cells vimentin filaments (short arrows) in control animals are surrounding the nucleus and extending towards the apical part of the cell. Three, 6, and 12 h after dosing vimentin filments collapsed and perinuclear condensation of vimentin is observed in the Sertoli cell cytoplasm (B, C, D). The apical extentions of filaments towards the lumen of the seminiferous tubules have disappeared. Note the sloughing of germ cells into the lumen of the seminiferous tubules (long arrows) 6 and 12 h after treatment (C and D). Magnification × 400.

Table 1
The effect on testis caspace-3 activity in 28-days-old male rats after administration of 400 mg/kg bw MEHP by gavage [a]

	Caspase-3 activity[b]
Control	281 ± 133
3 h. after dosing	559 ± 264[c]
6 h after dosing	442 ± 261
12 h after dosing	677 ± 354[c]

[a] The animals were sacrificed after 3, 6, or 12 h. Caspase-3 activity is expressed in pmol4-nitroaniline liberated per mg homogenate protein during 60 min at 37°C.
[b] The values are means ± SD, $n = 8$.
[c] Statistical significant from control by Dunnett's test. $P < 0.05$.

3.3. Apoptosis

There was a significant spontaneous caspase-3 activity and a true inter-animal variation in the testis of control rats (Table 1), as demonstrated by the low intra-sample and day-to-day variation, which were both less than 5% (data not shown). MEHP treatment statistically significantly increased the activity of caspase-3, 3 and 12 h after administration. A tendency towards an increase in caspase-3 activity was observed 6 h after dosing, although this was not statistically significant (Table 1).

One small rat (31 g) with a substantial number of TUNEL-positive cells was considered an outlier and excluded from the quantitative analysis with the TUNEL staining. The level of apoptosis was similar in the control and MEHP exposed groups, when the number of apoptotic cells were counted in 100 randomly selected tubules per animal (Fig. 4). The results were confirmed by LM-PCR, where the characteristic 'ladder' pattern was detected in all investigated animals in all groups (Fig. 5). Some of the lanes were fainter compared to others, due to the minute individual variations in DNA quality and quantity among the samples. Therefore, quantitative comparison

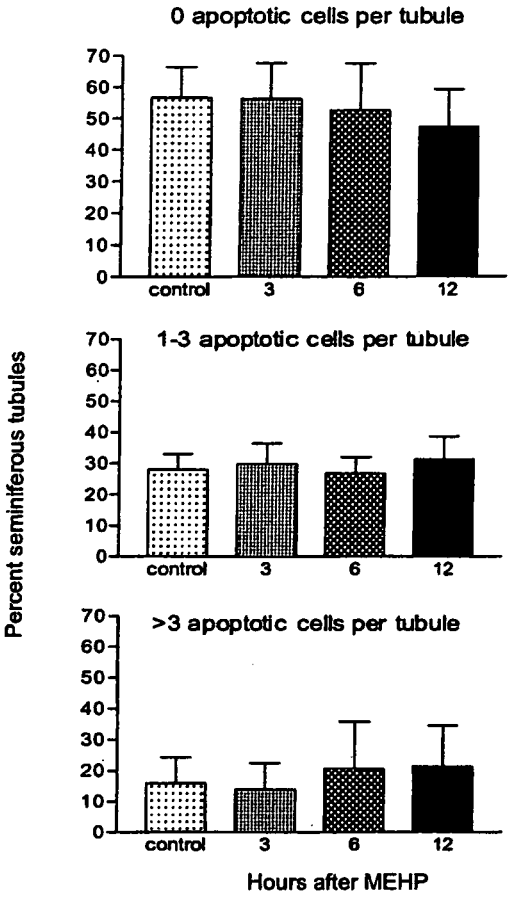

Fig. 4. The incidence of apoptotic germ cells in the testes 3, 6 or 12 h after treatment with MEHP, 400 mg/kg bw. Positive germ cells and/or cells fulfilling morphological criteria for apoptosis (cell shrinkage, nuclear fragmentation and condensation) were counted in 100 randomly selected tubules in one slide per animal. Bars represent the percentage of total tubules (mean ± SD) containing 0, 1–3, or > 3 apoptotic germ cells at each time point. $n = 8$ per group except in the group sacrificed after 6 h, $n = 7$.

Fig. 2. Immunostaining of the androgen receptor in 28-days-old male rat exposed to 0 (A) or 400 mg/kg bw of MEHP and sacrificed after 3 (B), 6 (C) or 12 (D) h. The control group was sacrificed after 2 h. The immunoreactivity of the androgen receptor was most pronounced in the peritubular myoid cells, the Leydig cells and to a lesser extent in the Sertoli cells. In the Sertoli cells the androgen receptor immunoreactivity altered with the cycle of the seminiferous epithelium. The staining of the androgen receptor in the Sertoli cells was, more intense in stage IV–VIII tubules (short arrows), compared to the weaker staining in stage IX–XIV tubules (long arrows). There was no change in the androgen receptor expression between controls rats and MEHP exposed rats. Magnification × 400.

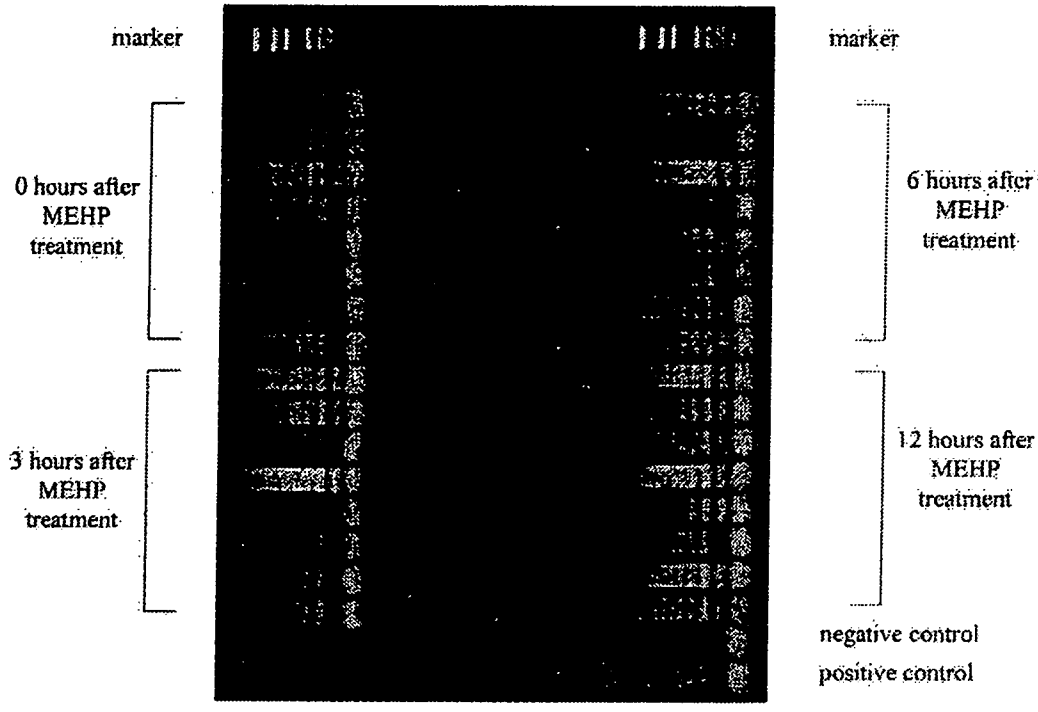

Fig. 5. Apoptotic banding pattern in testis of rats in 28-days-old male rat exposed to 0 or 400 mg/kg bw of MEHP and sacrificed after 3, 6 or 12 h. DNA by LM-PCR (20 cycles), run on 1.2% agarose electrophoresis. No differences in banding pattern between groups were observed.

between control and treated animals with respect to DNA-laddering as well as between DNA-laddering pattern and caspase-3 activity was not possible.

4. Discussion

Detachment and sloughing of germ cells into the lumen of the seminiferous tubules were observed 6 h after MEHP exposure, and this effect was enhanced after 12 h, as only Sertoli cells and spermatogonia remained attached to the basal lamina. Vimentin filaments in the Sertoli cells cytoplasm collapsed after 3 h and a perinuclear condensation remained throughout the study. Hence, the immunohistochemical investigation of vimentin filaments in the Sertoli cells is an early parameter of MEHP testis toxicity, as shown even at a dose five-fold lower than previously reported (Richburg and Boekelheide, 1996). The dose level was chosen in order to avoid general toxicity in our 28-day-old Wistar rats, as observed at higher doses in a preliminary study.

The increased expression of *TRPM*-2 in the testis 3 h after dosing probably reflects a temporary effect on Sertoli cells, since the expression of *TRPM*-2 returns to normal levels 6 and 12 h after MEHP treatment. MEHP can inhibit FSH-stimulated cAMP accumulation in primary Sertoli cell cultures (Heindel and Chapin, 1989). If cAMP represses *TRPM*-2 expression a decrease in cAMP accumulation in Sertoli cells may be responsible for the temporary increase in *TRPM*-2 expression. In this study there was apparently no immunohistochemical difference in the androgen receptor distribution between control animals and MEHP dosed animals. Besides, no effect on the *TRPM*-2 level in the ventral prostate was observed indicating that the function of androgen receptor was intact. This is supported by a recent publication by Parks et al., (2000), which reveals

that the anti-androgenic mechanism of DEHP during foetal sexual differentiation is disturbed by decreasing levels of testosterone production, while DEHP and MEHP do not have affinity for the human androgen receptor in vitro.

In the testis of control rats marked true spontaneous caspase-3 activity along with significant inter-animal variation were demonstrated. The caspase-3 activity most likely represents the normal physiological germ cell apoptosis as also reflected when applying DNA-laddering and TUNEL-staining (Table 1, Figs. 4 and 5). Thus, apoptosis is difficult to investigate unless the level of apoptosis is induced substantially above the spontaneous level. MEHP treatment statistically significantly increased the activity of caspase-3, 3 and 12 h after treatment. This is in accordance with the literature reporting that part of the mechanism of MEHP induced germ cells apoptosis is mediated through the Fas-signaling system (Richburg et al., 1999). Data on the time duration between Fas-stimulation and caspase-3 activation are sparse, but one study reports, that an agonistic Fas antibody can induce caspase-3 activation 2.5 h after exposure in vitro (Enari et al., 1996), indicating a short interval. Kim et al., (2000) has reported an overlap in time between caspase-3 and TUNEL-staining. They demonstrated that double staining with immunohistochemical investigation of caspase-3 and concomitant TUNEL-staining is overlapping in Leydig cells exposed to the cytotoxin ethane dimethanesulfonate. Likewise, it has been reported, that activation of effector caspases, DNA fragmentation by activated endonucleases, and morphological signs of apoptosis in the cells are closely related (Huppertz et al., 1999). However, during the present study, we observed an increase in caspase-3 activity after MEHP exposure without an increase in germ cell apoptosis measured by LM-PCR and the TUNEL assay. This could be explained in the variability in levels of apoptosis among individuals, as well as the limitations in the quantitative sensitivity of the methods. For example, it is generally believed that the TUNEL-assay has insufficiencies concerning sensitivity and specificity. However, an advantage of the TUNEL staining is that it identifies cell apoptosis in situ, whereas caspase-3 activity and semiquantitative LM-PCR are measured in testis homogenates. Despite the discrepancy in apoptotic results, we recommend employing several apoptotic endpoints, when investigating apoptosis for example a cytoplasmatic endpoint like the key protease, caspase-3, in combination with nuclear endpoints like DNA-laddering and TUNEL-staining (Ladefoged et al., 2000), in order to add further knowledge to the apoptotic process in vivo. In conclusion, MEHP testis toxicity does apparently not involve the androgen receptor either directly or indirectly. The increase in apoptosis in testis determined by increased caspase-3 activity after MEHP exposure could not be confirmed by the later events in the apoptotic pathway: DNA-fragmentation investigated by LM-PCR and TUNEL-staining. Immunostaining of vimentin in the Sertoli cells, and levels of caspase-3 appear to be appropriate, sensitive, and early markers of MEHP testis toxicity.

Acknowledgements

Karen Roswall, Ulla El-Baroudy, Vibeke Stenberg and Annette Landin are thanked for their excellent technical work. Part of this work was supported by The Danish Medical Research Council grant no. 9700832.

Appendix A

Sequences of the sense and antisense primers for *TRPM*-2 and β-actin, respectively

Gene	Sense primer, 5′–3′	Anti-sense primer, 5′–3′
TRPM-2	CTGACCCA-GCAGTACA-ACGA	TGACACGA-GAGGGGACT-TCT
β-Actin	ACCCACACT-GTGCCCATC-TA	GCCACAG-GATTCCATA-CCCA

Appendix B

Sequences of the two fluorescently labelled hybridisation probes for *TRPM*-2 and β-actin, respectively

Gene	Hybridisation probes, FL530; 5′–3′	Hybridisation probes, LC 640; 5′–3′
TRPM-2	TAACCTCA-CACAGGGC-GATGACCA	ACCTTCGG-GTCTCCAC-AGTGACAAC
β-Actin	GCCACGCT-CGGTCAG-GATCTTCAT	AGGTAGTCT-GTCAGGTCC-CGGCCA

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TOXICOLOGICAL SCIENCES 58, 377–385 (2000)

Chronic Toxicity of Di(2-ethylhexyl)phthalate in Mice

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Received May 23, 2000; accepted August 18, 2000

B6C3F1 mice were treated with 0, 100, 500, 1500, or 6000 ppm di(2-ethylhexyl)phthalate (DEHP) in the diet for up to 104 weeks. Blood and urine were analyzed at Weeks 26, 52, 78, and 104 from 10 animals per sex per group. Body weights and food consumption were measured weekly for the first 16 weeks, then monthly thereafter. Survival was reduced for mice receiving 6000 ppm DEHP. Overall weight gains were significantly lower for the 6000-ppm male group, but there was no difference among female groups. Food consumption was not affected by exposure. No biologically significant changes in clinical chemistry, hematology, or urinalysis were observed. After 104 weeks of exposure, kidney weights for the 500- and 1500-ppm male, and 6000-ppm male/female groups were significantly lower than for the controls. Significantly higher liver weight was seen for the 500-, 1500-, and 6000-ppm male groups and the 6000-ppm female group of mice. Testis weights for the 500-, 1500-, and 6000-ppm males were significantly lower than for the controls. Uterine weights for the 6000-ppm group were significantly lower than for the controls. All organs were examined for histopathology. The incidence of hepatocellular lesions has been reported separately (R. M. David *et al.*, 1999. *Toxicol. Sci.* 50, 195–205). Tumors were observed at ≥ 500-ppm dosages, where peroxisome proliferation was significantly increased. A NOEL for both tumors and peroxisome proliferation was 100 ppm. In the study presented here, bilateral hypospermia in the testes of male mice, hepatocyte pigmentation and cytoplasmic eosinophilia in the liver, and chronic progressive nephropathy of male and female mice were observed at 6000 ppm. Hypospermia and chronic progressive nephropathy were also observed at 1500 ppm, where peroxisome proliferation was 2.7–6.8-fold higher than controls. Many lesions observed in rats were not seen in mice. A dose level of 500 ppm (98.5–116.8 mg/kg/day) was identified as a no-observed-adverse-effect level (NOAEL) for noncarcinogenic effects.

Key Words: **DEHP; di(2-ethylhexyl)phthalate; chronic toxicity; liver; kidneys; testes.**

Di(2-ethylhexyl)phthalate (DEHP) was found by the National Toxicology Program (NTP) to be a hepatocarcinogen in F344 rats and B6C3F1 mice (Kluwe *et al.*, 1982); however, the noncarcinogenic effects of DEHP have not been investigated since the 1950s and few studies involved mice. There is a need to determine the noncarcinogenic effects based on the widespread use of DEHP as a plasticizer in vinyl products (ATSDR, 1993). Recently, risk assessments have been undertaken by a variety of U.S. and European agencies (European Union: risk assessment of DEHP; the National Toxicology Program [NTP] Center for the Evaluation of Risk to Human Reproduction, and the U.S. Food and Drug Administration). Exposure to DEHP is thought to be from many sources; however the ATSDR identifies some foods as main sources of exposure to DEHP, although DEHP is not used in food-wrap products. The ATSDR has estimated the total exposure to the general population from all sources to be 0.25 mg/person/day. Doull *et al.* (1999) has estimated this to result in an exposure of 3–30 μg/kg/day. This does not include exposure from medical devices such as vinyl tubing and blood bags, which could raise this estimate at least one order of magnitude higher (Huber *et al.*, 1996), but is intermittent. In addition, the route of exposure influences the extent of hydrolysis and metabolism (Huber *et al.*, 1996), which may affect the toxicity.

Since dietary administration represents the most widespread exposure, an assessment of the potential toxicity from dietary sources is appropriate. Data for rats have been published (Carpenter *et al.*, 1953; David *et al.* 2000; Harris *et al.* 1956), but relatively few long-term studies of DEHP have been performed using mice. The NTP conducted a chronic oncogenicity study in the 1980s, demonstrating liver tumors after 2 years of exposure to 3000 and 6000 ppm (Kluwe *et al.*, 1982), but few other effects were observed. There were no "toxic" lesions in the liver. Ward *et al.* (1986) reported that male B6C3F1 mice exposed to 3000, 6000, or 12,000 ppm DEHP for 18 months had substantially lower body weight at 6000 ppm (20%) and 3000 ppm (10%). Cytoplasmic eosinophilia of the liver and hepatomegaly were reported at all dose levels. Renal tubular degeneration, necrosis and cystic hyperplasia were seen in the kidneys, and some animals at 6000 ppm had degeneration of the seminiferous tubules. Other organs were not evaluated, and there was no assessment of clinical chemistry or hematology. The value of having data for mice is that it provides information on distinguishing species-specific effects from test substance-specific effects.

Long-term studies of the chronic effects of DEHP in mice and rats were recently undertaken to help evaluate the corre-

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lation of peroxisome proliferation, cell proliferation, and hepatocarcinogenicity in rodents (David *et al.*, 1999). Dose levels of 2500 and 12,500 ppm resulted in an increased incidence of hepatocellular neoplasm, hepatomegaly, and palmitoyl CoA oxidation in rats. Palmitoyl CoA oxidation was increased 1.7–1.9 times control for the 2500-ppm groups, and 3.6–5.1 times control for the 12,500-ppm dose groups. Dose levels of 500, 1500, and 6000 ppm resulted in an increased incidence of hepatocellular neoplasm, hepatomegaly, and palmitoyl CoA oxidation in mice. Palmitoyl CoA oxidation was increased 2.2–4 times control for the 500-ppm groups, 2.7–6.8 times control for the 1500-ppm groups, and 7.6–15.0 times control for the 6000-ppm dose groups. As part of those studies, the noncarcinogenic chronic effects of exposure were also evaluated. We previously reported that the noncarcinogenic effects of long-term oral exposure to DEHP were limited to minor alterations in clinical chemistry and hematology, increased incidence of liver lesions (pigment in Kupffer cells, spongiosis hepatis), pancreatic acinar cell adenoma, aspermatogenesis, and castration cells in the pituitary gland for rats (David *et al.*, 2000). DEHP exposure exacerbated age-, species- or strain-related lesions such as mineralization of the renal papilla and chronic progressive nephropathy in male rats. Those effects and the effect levels were compared with dose levels at which peroxisome proliferation occurred. Lesions such as pancreatic acinar-cell hyperplasia and spongiosis hepatis have been associated with exposure to peroxisome proliferators such as WY-14,643 (Obourn *et al.*, 1997) or weak hepatocarcinogens (Bannasch *et al.*, 1981) in rat. Ward *et al.* (1998) suggested that testicular and renal lesions were unrelated to peroxisome proliferation, based on a study in which PPAR-knockout mice given 20,000 ppm DEHP in the diet developed testicular and renal lesions similar to those found in the wild type. Other effects may also be unrelated.

Bilateral aspermatogenesis in the testes, castration cells in the pituitary gland, spongiosis hepatis, and pancreatic acinar cell adenoma were observed for 12,500 ppm male rats. Aspermatogenesis and spongiosis hepatis were observed for 2500 ppm-dosed male rats, and aspermatogenesis was seen in 500-ppm male rats. Kupffer cell pigmentation and renal tubule pigmentation were seen only in male and female high-dose rats (12,500 ppm), although peroxisome proliferation was also apparent at the next lower dose level (2500 ppm). The increased incidence of spongiosis hepatis correlated with increased palmitoyl CoA oxidase activity, but the incidence of pancreatic acinar-cell adenoma was increased only at the highest dose level. Thus, spongiosis hepatis, Kupffer cell pigmentation, renal tubule pigmentation, and pancreatic acinar-cell adenomas may be associated with high levels of peroxisome proliferation. The data for mice are presented here for comparison.

Animals were evaluated for evidence of progressive alterations in clinical chemistry, hematology, and urine parameters. Tissues were examined after 78 and 104 weeks for histopathology. These results are useful for assessing the risk from oral exposure and may provide some insight into the association of noncarcinogenic effects with peroxisome proliferation.

MATERIALS AND METHODS

Chemicals. Di-(2-ethylhexyl)phthalate (CAS No. 117–82–7), 99.7% pure, was supplied by Eastman Chemical Company (Kingsport, TN). The structure and purity were confirmed prior to use and the purity was reconfirmed at the termination of the study.

Animals. Four-week old male and female B6C3F1 mice (B6C3F1/CrlBR), obtained from Charles River Laboratories, Inc. (Portage, MI), were used for the study following a 2-week acclimation period. Animals were maintained on tap water and powdered PMI #5002 chow (Purina Mills, Inc., Richmond, VA). Animal husbandry conformed to standards outlined in the Guide for the Care and Use of Animals (National Research Council, 1996) using temperatures at 72 ± 6°F with a relative humidity of 50 ± 20%. A 12-h light/12-h dark cycle and 10 or greater air changes/h were maintained in the room housing the animals.

Treatment levels for bioassay. The animals were assigned to study using a computerized weight-randomization program, which first eliminated the animals with extreme body weights, then selected the random assignment that produced homogeneity of variance and means by Bartlett's test and 1-way analysis of variance (ANOVA). At randomization, the weight variation of the animals selected did not exceed 2 standard deviations of the mean body weight for each sex, and the mean body weight for each group of each sex was not statistically different. Mice were divided into 5 groups of 70, 60, 65, 65, and 70 for Groups 1–5, respectively. Dosage levels were 0, 100, 500, 1500, or 6000 ppm DEHP, given in the diet (Groups 1–5, respectively), based on the results of a 13-week study. These groups were treated continuously for up to 104 weeks. A set of 10–15 animals per group was terminated during Week 79. The remaining animals were terminated during Week 105. Diets containing DEHP were mixed weekly during the study, and the concentration of DEHP in the diet was verified periodically by high-performance liquid chromatography (HPLC) analysis.

Mortality and clinical observations. The mice were observed for mortality and moribundity twice daily. A thorough physical examination was conducted at each weighing interval. A careful cage-side observation for obvious indications of toxic effects was performed once daily.

Body weights, organ weight, and food consumption. Body weights and food consumption were measured weekly for Weeks 1–17, and once every 4 weeks thereafter. At necropsy, the terminal (fasted) body weight and the weights of the brain (with stem), lungs, liver, spleen, kidneys (paired), testes (paired with epididymides), and uterus were measured.

Clinical pathology. Blood was collected under anesthesia from the retro-orbital sinus of 10 fasted animals per sex per group during Weeks 26, 52, 78, and 104 for clinical chemistry and hematology analyses. Whole blood samples were analyzed for counting red blood cells (RBC), total and differential white blood cells (WBC), reticulocytes, and platelets; as well as hemoglobin (Hgb) and hematocrit (Hct), counts. If possible, blood was collected from the same animals at each respective interval. Serum samples were analyzed for albumin, protein, calcium, phosphorous, urea nitrogen (BUN), creatinine, glucose, sodium, potassium, chloride, bilirubin, gamma-glutamyltransferase (GGT), aspartate aminotransferase (AST), and alanine aminotransferase (ALT). Urine samples from the same animals were collected overnight for evaluation of appearance (color and turbidity); bilirubin, glucose, and ketone levels; and microscopic examination of sediment, occult blood, pH, protein, specific gravity, and urobilinogen. Hematology analyses (RBC, total WBC, Hgb, and Hct) were performed using a Coulter Counter Model S-Plus IV (Coulter Co., Hialeah, FL). Differential leukocyte, reticulocyte, and platelet counts were determined from blood smears. Serum and urine chemistry analyses were performed using a BMD/Hitachi 704/737 Chemistry Analyzer (Boehringer Mannheim Diagnostics, Indianapolis, IN). Semiquantitative urinalysis deter-

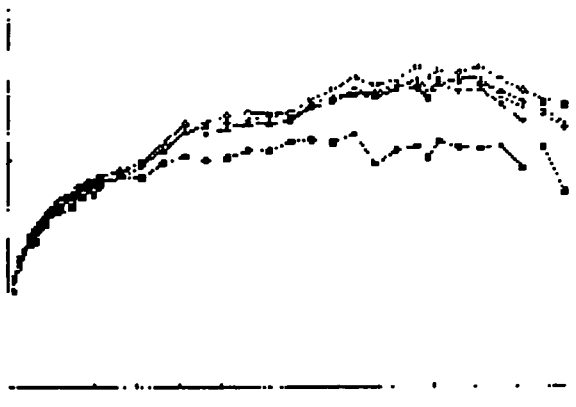

FIG. 1. Mean body weight for male mice exposed to 0 (plus sign), 100 (circle), 500 (triangle), 1500 (diamond), or 6000 ppm (square) DEHP in the diet for 104 weeks.

minations were performed using Ames Multistix (Miles, Inc., Diagnostic Division, Elkhart, IN) or equivalent. Specific gravity was determined with a refractometer.

Histopathology. A set of 10–15 animals per sex from all groups was sacrificed after Week 78 for histopathology studies. All surviving animals were terminated after 104 weeks of treatment. Animals were food-fasted overnight, weighed, given an intraperitoneal injection of sodium pentobarbital, and exsanguinated. All tissues listed in the U.S. EPA Health Effects Testing Guideline for Combined Chronic Toxicity/Oncogenicity Study (40 CFR 798.3320) from the high-dose and control groups were evaluated microscopically. Target tissues from other groups were also examined. The tissues were embedded in paraffin, sectioned at 5 μm, and stained with hematoxylin and eosin for microscopic examination.

Statistics. Body weights, feed consumption, clinical chemistry, hematology, and organ weights were analyzed by ANOVA, followed by a Dunnett's *t*-test. Tumor incidence was compared by Fisher's exact test. A probability of 0.05 was used to determine significance.

RESULTS

In-Life Data

Data for survival have been reported elsewhere (David *et al.*, 1999). Survival for mice was significantly decreased for the 6000-ppm males compared to concurrent controls. Adjusted survival rates at Week 104 in Groups 1–5 were 76, 80, 71, 72, and 31% for males and 63, 66, 73, 72, and 61% for the females, respectively. The most common cause of death was hepatocellular neoplasia and was most frequent in mice of Groups 4 and 5 (10/30 and 15/56, respectively).

Mean body weights for 6000-ppm male mice were significantly decreased compared with the control group for most of the study (Fig. 1). For female mice, significantly lower body weight means were noted occasionally for Group 5 (Fig. 2). Mean body weight changes for the groups were 10.5 ± 2.7, 11.4 ± 2.3, 11.7 ± 1.9, 10.8 ± 2.7, and 5.8 ± 2.5 g for males in Groups 1–5, respectively, and 11.8 ± 1.9, 12.8 ± 2.0, 13.0 ± 1.9, 12.5 ± 2.4, and 10.5 ± 2.4 g for females in Groups 1–5, respectively. Weight gain for Group-5 males (6000 ppm) was significantly lower than for the controls.

Significantly lower food consumption values were occasionally noted for the male mice from the 100-, 500-, 1500-, and 6000-ppm groups. For the female mice, significantly decreased food consumption mean values were occasionally noted for the 500- and 6000-ppm groups. There were numerous instances of significantly increased food consumption values when compared to control values; however, no treatment-related patterns were noted. Total food consumption, which was comparable among groups, was 1532 ± 63.5, 1521 ± 51.2, 1536 ± 79.8, 1543 ± 91.1, and 1460 ± 129.0 g for Groups 1–5 male mice, respectively, and 1625 ± 72.0, 1667 ± 61.6, 1582 ± 100.9, 1603 ± 56.5, and 1605 ± 76.5 g for Groups 1–5 male female mice, respectively. Based on the average daily feed consumption, the dose of DEHP was 19.2, 98.5, 292.2, and 1266.1 mg/kg for males, and 23.8, 116.8, 354.2, and 1458.2 mg/kg for females, respectively.

Clinical Chemistry

There were no significant differences in clinical chemistry or hematology at the interim sampling periods (data not shown). At termination, no toxicologically significant changes in clinical chemistry parameters were observed for mice exposed to DEHP. The serum potassium level for the male 6000-ppm group was significantly lower when compared with controls (Table 1). In addition, the concentration of potassium in the urine demonstrated a trend to lower concentrations at higher-dose levels (data not shown). However, the volumes of urine for the higher-dose-level groups were also higher, such that there was no apparent difference in urinary excretion of potassium. Therefore, the decrease in serum potassium was not considered to be toxicologically meaningful. Mean ALT value for the 1500-ppm group was substantially higher than values

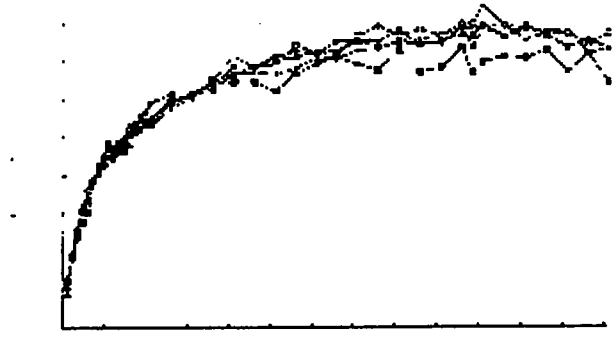

FIG. 2. Mean body weight for female mice exposed to 0 (plus sign), 100 (circle), 500 (triangle), 1500 (diamond), or 6000 ppm (square) DEHP in the diet for 104 weeks.

TABLE 1
Mean Serum Chemistry Values for Male Mice Exposed to DEHP for 104 Weeks

Parameter	Dose level (ppm)				
	0	100	500	1500	6000
Glucose (mg/dL)	124	137	138	142	111
	10.4	1.3	23.1	19.3	23.7
	4	4	6	5	4
BUN (mg/dl)	24	26	32	30	38
	4.9	4.5	20.9	10.3	19.8
	9	9	10	10	9
Creatinine (mg/dl)	0.3	0.4	0.4	0.4	0.4
	0.07	0.05	0.08	0.11	0.08
	9	9	10	10	9
ALT (IU/l)	96	93	104	289	108
	144.4	86.0	175.2	603.1	69.8
	10	10	10	10	10
AST (IU/l)	221	107	139	477	263
	422.1	40.3	100.7	1109.7	143.1
	10	10	10	10	10
Calcium (mg/dl)	10.6	10.4	10.5	10.8	11.3
	0.94	0.93	0.56	1.66	1.03
	8	9	9	8	9
Total bilirubin (mg/dl)	0.2	0.2	0.29	0.2	0.2
	0.08	0.05	0.15	0.24	0.07
	9	9	10	10	9
Phosphorous (mg/dl)	12.5	11.6	11.6	12.6	11.1
	0.98	0.67	1.21	0.9	0.9
	4	4	5	4	3
GGT (IU/l)	0	1	1	1	0
	0.7	1.3	1.4	1.3	0.7
	10	10	10	10	10
Sodium (meq/dl)	167	165	165	173	168
	6.3	4.2	4.1	11.7	4.6
	6	7	8	5	5
Potassium (meq/dl)	8.1	8.0	7.9	7.8	6.8*
	1.1	0.31	0.97	0.59	0.85
	9	9	9	9	9
Chloride (meq/dl)	137	129	131	134	135
	12.9	4.7	9.0	6.7	10.1
	9	9	9	9	9

Note. Values given are mean, standard deviation, and number of animals.
*Significantly different from control, $p \leq 0.05$.

for controls, but these were not statistically significant because of the large standard deviation for the groups. Similar results were seen for female mice. Because of limitations in the volume of blood collected, total protein, albumin, and globulin values are insufficient for good comparison among groups, although the mean values do not suggest any effect in these parameters.

For hematology, no changes in RBC, hemoglobin, or hematocrit values were seen for mice exposed to DEHP, as had been observed for rats (Table 2). There were significantly lower mean corpuscular hemoglobin values for all treated male groups of mice, but not for female mice. In addition, mean corpuscular hemoglobin content (MCHC) for the 6000-ppm group was significantly lower than for controls. No other significant differences were noted for hematology. No significant differences were observed in urinalysis (data not shown).

Organ Weights

Terminal body weights for the 6000-ppm groups were significantly lower than for the control group (Tables 3 and 4). Absolute kidney weights for the 1500-ppm male, and 6000-ppm male and female groups were significantly lower than for the control group. Kidney-to-body weight ratios for the 500-, 1500-, and 6000-ppm male groups were also significantly lower than for the control group. There was no change in relative kidney weight for the female groups. Significantly higher absolute liver weight was seen for the 500-, 1500-, and 6000-ppm male groups and the 6000-ppm female group of mice; significantly higher liver-to-body weight ratios were seen for the male and female 1500- and 6000-ppm groups compared with the control group. Mean relative lung-to-body weight ratios for the 6000-ppm male group was significantly higher than for the controls, but absolute values were not affected. Mean absolute brain weights for the 1500- and 6000-ppm male groups were significantly lower than for the controls; relative to body weight, brain weight for the 6000-ppm male group was significantly higher than for the control group. A significantly lower brain-to-body weight ratio was seen for the 100-ppm male group; the toxicological significance of this finding is questionable. Absolute testes weight for the 1500- and 6000-ppm males, and testes-to-body weight ratios for the 500-, 1500-, and 6000-ppm males were significantly lower than for the control group. Absolute and relative uterine weights for the 6000-ppm group were significantly lower than for the controls.

Histopathology

There was no difference in the types of lesions observed at Weeks 78 and 104, but there were differences in the incidence or severity per group. After 78 weeks of exposure, treatment-related effects were observed nearly exclusively for the 6000-ppm group. Hepatocyte pigmentation, cytoplasmic eosinophilia, and chronic inflammation were observed in the liver of all 6000-ppm animals; no other treatment-related liver lesions were observed (Table 5). Chronic progressive nephropathy (CPN) was observed in nearly all male mice including controls, and in most treated female mice. The incidence of CPN was significantly higher for the 1500-and 6000-ppm female groups compared with the controls. In addition, the mean severity was higher for the 6000-ppm groups than for other groups. All male 6000-ppm animals had immature or abnormal epididymal sperm and bilateral hypospermia of the testes. These lesions were not observed at other dose levels. No treatment-related lesions were observed in the uterus.

After 104 weeks of exposure, increased incidence of hepatocyte pigmentation and cytoplasmic eosinophilia in the liver were observed for the 6000-ppm groups, compared with the

TABLE 2
Mean Hematology Values for Mice Exposed to DEHP for 104 Weeks

Dose level (ppm)	Sex	RBC ($10^6/\mu L$)	Hgb (mg/dL)	Hct (%)	MCV (fL)	MCH (pg)	MCHC (g/dL)	Reticulocytes (%RBC)	Platelets ($10^3/\mu L$)	WBC ($10^3/\mu L$)	M/E ratio[a]
		8.82	14.5	41.5	47.8	16.7	34.9	2.6	1463	8.3	2.56
	Male	1.666	1.89	5.48	4.29	1.53	0.43	3.69	304	8.63	0.633
0		10	10	10	10	10	10	10	9	10	8
		9.82	15.7	44.7	45.5	16.0	35.2	1.5	848	3.5	2.09
	Female	1.472	2.26	6.69	0.86	0.32	0.41	1.03	170.4	1.97	0.563
		10	10	10	10	10	10	10	9	10	10
		9.53	14.9	43.0	45.1	15.7[b]	34.7	1.4	1224	5.9	2.43
	Male	0.262	0.38	0.97	0.85	0.27	0.47	0.38	127.7	2.4	0.711
100		9	9	9	9	9	9	9	9	9	10
		9.12	14.8	41.7	45.8	16.2	35.4	1.7	732	5.6	2.48
	Female	0.407	0.56	1.31	0.88	0.38	0.62	1.53	165.6	6.03	0.535
		10	10	10	10	10	10	10	9	10	10
		9.32	14.6	42.5	45.6	15.7[b]	34.3	1.4	1305	3.9	3.54
	Male	0.468	0.71	1.89	0.7	0.31	0.52	0.86	142.5	0.81	1.523
500		10	10	10	10	10	10	10	9	10	8
		9.31	14.8	42.0	45.3	16.0	35.3	2.0	851	4.0	2.96
	Female	0.968	1.25	3.57	2.64	1.15	0.89	1.93	163.6	1.8	1.829
		10	10	10	10	10	10	10	10	10	9
		9.52	14.9	42.9	45.0	15.6[b]	34.6	1.8	1092	6.3	3.27
	Male	0.527	0.88	2.58	1.05	0.26	0.52	0.97	378.1	6.75	1.252
1500		10	10	10	10	10	10	10	9	10	9
		9.4	14.9	42.9	45.7	15.9	34.9	1.6	854	2.6	2.55
	Female	0.688	0.79	2.33	1.38	0.5	0.37	0.56	169.4	0.92	0.547
		10	10	10	10	10	10	10	10	10	10
		10.21	15.3	45.3	44.6	15.1[b]	33.9[b]	1.8	1156	5.1	3.01
	Male	1.758	1.88	6.61	2.37	0.88	0.69	1.2	332.2	2.35	1.101
6000		8	8	8	8	8	8	8	8	8	5
		9.2	14.5	42.5	46.8	16.0	34.1[b]	2.7	737	5.0	2.69
	Female	1.478	1.49	4.39	4.01	1.36	0.63	2.71	191.3	3.68	0.971
		10	10	10	10	10	10	10	10	10	9

Note. Values given are mean, standard deviation, and number of animals.
[a]Myeloid/erythroid ratio.
[b]Significantly different from control, $p \leq 0.05$.

controls (Table 6). The incidence of chronic inflammation in the liver for male 6000-ppm mice was significantly higher than for controls. Incidences and mean severity were comparable among other groups. The only other treatment-related lesion observed in the liver was hepatocellular enlargement which was observed in nearly all 6000-ppm mice (67/70 males, 68/70 females) but not in the controls (0/70 males, 1/70 females) or other groups (data not shown). Spongiosis hepatis, a lesion observed in rats exposed to DEHP (David *et al.*, 2000), was not observed in any animals of this study. In the kidney, the incidences of CPN for the 1500- and 6000-ppm female groups were significantly higher than for the controls; the mean severity of CPN was higher for both male and female 6000-ppm groups compared with other groups. Renal tubule pigmentation, a lesion observed in rats exposed to DEHP (David *et al.*, 2000), or an increase in renal cysts were not observed in this study. Male mice in the 1500- and 6000-ppm groups had a significantly higher incidence of bilateral hypospermia of the testes, and the 6000-ppm group had a higher incidence of hypospermia of the epididymis. A higher incidence of immature or abnormal sperm cells in the epididymis were observed for the 1500- and 6000-ppm groups compared with the controls. No treatment-related lesions were observed in the uterus, ovaries, pituitary gland, thyroid, or pancreas.

DISCUSSION

We have previously shown that long-term exposure of mice to DEHP results in peroxisome proliferation at the same dose levels as hepatocarcinogenesis (David *et al.*, 1999). Dose levels of 500, 1500, and 6000 ppm resulted in an increased palmitoyl CoA oxidation and increased incidence of hepatocellular neoplasm. In the results presented here, we expand those findings to include the noncarcinogenic effects of DEHP. Exposure of mice to 6000 ppm of DEHP ($\geq$ 1200 mg/kg/day) resulted in hepatocyte pigmentation, increased cytoplasmic

TABLE 3
Mean Absolute Organ Weights for Mice at Termination of the Study

Dose level (ppm)	Sample size	Terminal body weight	Kidneys	Liver	Lungs	Testes/uterus	Brain	Spleen
Males								
0	42	28.6 ± 3.0	0.72 ± 0.07	1.51 ± 0.24	0.22 ± 0.03	0.35 ± 0.05	0.49 ± 0.02	0.08 ± 0.03
100	43	29.9 ± 3.1	0.71 ± 0.07	1.60 ± 0.30	0.23 ± 0.04	0.34 ± 0.05	0.48 ± 0.02	0.09 ± 0.04
500	39	29.3 ± 1.8	0.68 ± 0.05	1.73 ± 0.69[a]	0.23 ± 0.03	0.34 ± 0.04	0.49 ± 0.03	0.07 ± 0.02
1500	41	28.2 ± 3.3	0.61 ± 0.08[a]	1.92 ± 0.54[a]	0.25 ± 0.08	0.30 ± 0.07[a]	0.47 ± 0.02[a]	0.11 ± 0.11
6000	19	25.8 ± 3.1[a]	0.49 ± 0.05[a]	2.37 ± 0.59[a]	0.23 ± 0.05	0.15 ± 0.02[a]	0.47 ± 0.02[a]	0.08 ± 0.04
Females								
0	36	26.5 ± 2.8	0.52 ± 0.05	1.63 ± 0.55	0.25 ± 0.03	0.51 ± 0.21	0.49 ± 0.02	0.19 ± 0.14
100	37	26.7 ± 2.8	0.53 ± 0.05	1.74 ± 0.97	0.25 ± 0.04	0.49 ± 0.17	0.50 ± 0.02	0.18 ± 0.11
500	40	26.2 ± 2.1	0.53 ± 0.05	1.61 ± 0.48	0.25 ± 0.05	0.55 ± 0.28	0.49 ± 0.02	0.22 ± 0.28
1500	39	26.4 ± 2.9	0.51 ± 0.05	1.73 ± 0.32	0.25 ± 0.06	0.59 ± 0.46	0.49 ± 0.02	0.20 ± 0.22
6000	35	24.9 ± 2.7[a]	0.47 ± 0.05[a]	2.58 ± 0.84[a]	0.24 ± 0.03	0.30 ± 0.17[a]	0.48 ± 0.02	0.22 ± 0.21

Note. Values given are mean and standard deviation in grams.
[a]Significantly different from control; $p \leq 0.05$.

eosinophilia in the liver, and hypospermia where peroxisome proliferation was 7–15 times control. Only hypospermia was seen at a dose level of 1500 ppm, where peroxisome proliferation was 2.7 times control. Exposure to 1500- and 6000-ppm DEHP also resulted in exacerbation of chronic inflammation and chronic progressive nephropathy: naturally occurring lesions in the liver and kidneys. These lesions do not appear to be correlated with increased levels of peroxisome proliferation since a no-observed-effect level of 500 ppm was determined for noncarcinogenic effects, whereas peroxisome proliferation and hepatocellular neoplasia were seen at this dose level. These results contrast with those observed for rats. Mice failed to show effects on hematology (RBC parameters or mononuclear cell leukemia), spongiosis hepatis in the liver, lesions in the pituitary gland, or lesions in the pancreas that were observed in rats (David *et al.*, 2000), even though mice received higher dose levels.

Although few long-term studies of DEHP have been performed using mice, some of the effects observed in the study presented here were described by Ward *et al.* (1986). In that study, male B6C3F1 mice exposed to 3000, 6000, or 12,000 ppm DEHP for 18 months had substantially lower body weights at 6000 ppm (20%) and 3000 ppm (10%). Cytoplasmic eosinophilia of the liver and hepatomegaly were reported at the 12,000-ppm dose level. Renal tubular degeneration, necrosis, and cystic hyperplasia (lesions resembling chronic progressive nephropathy) were seen in the kidneys of all treated groups, and some animals at 6000 ppm had degeneration of the seminiferous tubules. Those results are consistent with the lesions observed in the study presented here. How closely linked these lesions are to cellular events associated with peroxisome proliferation is not clear. Pigmentation and eosinophilia may represent deposition of lipofuscin in the hepatocyte known to occur with peroxisome proliferators. Lake *et al.* (1987) ob-

TABLE 4
Mean Relative Organ Weights for Mice at Termination of the Study

Dose level (ppm)	Sample size	Kidneys	Liver	Lungs	Testes/uterus	Brain	Spleen
Males							
0	42	2.561 ± 0.236	5.334 ± 0.986	0.802 ± 0.109	1.241 ± 0.143	1.762 ± 0.156	0.292 ± 0.118
100	43	2.438 ± 0.189	5.380 ± 1.035	0.805 ± 0.162	1.172 ± 0.150	1.650 ± 0.142[a]	0.318 ± 0.176
500	39	2.345 ± 0.163[a]	5.967 ± 2.754	0.804 ± 0.116	1.156 ± 0.129[a]	1.638 ± 0.123	0.254 ± 0.088
1500	41	2.242 ± 0.336[a]	6.961 ± 2.491[a]	0.905 ± 0.296	1.091 ± 0.196[a]	1.717 ± 0.167	0.395 ± 0.394
6000	19	1.992 ± 0.199[a]	9.234 ± 2.522[a]	0.946 ± 0.222[a]	0.623 ± 0.080[a]	1.901 ± 0.118[a]	0.340 ± 0.177
Females							
0	36	2.016 ± 0.198	6.150 ± 1.891	0.963 ± 0.118	1.995 ± 0.814	1.922 ± 0.167	0.740 ± 0.445
100	37	2.039 ± 0.117	6.437 ± 3.293	0.955 ± 0.155	1.909 ± 0.738	1.914 ± 0.168	0.679 ± 0.366
500	40	2.084 ± 0.177	6.102 ± 1.608	0.966 ± 0.171	2.147 ± 1.043	1.927 ± 0.120	0.846 ± 0.917
1500	39	1.999 ± 0.160	6.566 ± 0.944[a]	0.986 ± 0.254	2.310 ± 1.691	1.935 ± 0.128	0.770 ± 0.771
6000	35	1.945 ± 0.225	10.279 ± 2.621[a]	0.982 ± 0.122	1.214 ± 0.649[a]	1.991 ± 0.151	0.901 ± 0.822

Note. Values given are mean and standard deviation in g/100 g body weight.
[a]Significantly different from control, $p \leq 0.05$.

TABLE 5
Incidence of Histopathologic Lesions in Mice Exposed to DEHP for 78 Weeks

Lesion	Incidence per dose level[a]				
	0 ppm	100 ppm	500 ppm	1500 ppm	6000 ppm
Hepatocyte pigmentation					
Males	0/15 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15/15 (100%)[c]
Females	0/15 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15/15 (100%)[c]
Increased cytoplasmic eosinophilia					
Males	0/15 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15/15 (100%)[c]
Females	0/15 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15/15 (100%)[c]
Chronic inflammation					
Males	0/15 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15/15 (100%)[c]
Females	0/15 (0%)	0/10 (0%)	0/10 (0%)	0/10 (0%)	15/15 (100%)[c]
Chronic progressive nephropathy					
Males	10/10 (100%)	10/10 (100%)	9/10 (90%)	9/10 (90%)	10/10 (100%)
Severity grade[b]	1.2	1.0	1.0	0.9	1.8
Females	4/10 (40%)	7/10 (70%)	6/10 (60%)	8/10 (80%)[c]	10/10 (100%)[c]
Severity grade[b]	0.4	0.7	0.6	0.8	2.2
Immature/abnormal epididymal sperm	0/10	0/10	0/10	0/10	10/10[c]
Bilateral hypospermia of the testes	0/10	0/10	0/10	0/10	10/10[c]

[a]Number of animals exhibiting lesion out of total examined. Percentage of animals exhibiting lesion in parentheses.
[b]Mean severity grade based on scores of 1–4 for minimal to severe.
[c]Significantly different from concurrent control, $p \leq 0.05$.

served a "golden-brown" pigment in hepatocytes stained with hematoxylin and eosin, which was identified, using a different stain, as lipofuscin. Ward *et al.* (1998) observed an absence of hepatocyte pigmentation and cytoplasmic eosinophilia in PPARα-knockout mice treated with 12,000 ppm DEHP for 24 weeks whereas these liver lesions were observed in Sv/129 wild-type mice following up to 16 weeks of exposure. There was also an absence of peroxisome proliferation in the PPAR-knockout mice whereas peroxisome proliferation was observed in the wild-type mice. Those data suggest that pigmentation and eosinophilia are associated with peroxisome proliferation. However, pigmentation in hepatocytes and cytoplasmic eosinophilia of hepatocytes were not observed in the study presented here at all dose levels where peroxisome proliferation was significantly increased. This information implies that there is a threshold in mice for peroxisome proliferator-induced noncarcinogenic liver effects.

The relationship of increased incidence of hypospermia to peroxisome proliferation is also not clear. Ward *et al.* (1998) evaluated whether peroxisome proliferation was a causative factor by studying the testicular effects in PPAR-knockout mice treated for up to 24 weeks with 12,000 ppm DEHP. Their data suggest that peroxisome proliferation plays some role in the onset of testicular toxicity, but not in the severity or extent of cellular damage. Focal tubular degenerative lesions of the testes were seen in wild-type mice and spermatogenesis was diminished by 8 weeks; whereas PPARα-knockout mice had normal testes at 8 weeks but lacked "normal indicators of spermatogenesis" only in the outer portion of the testis. Severe tubular degeneration was observed in the PPAR-knockout mice at 24 weeks (interim time periods were not investigated). The data from the study presented here support the hypothesis that testicular lesions and hypospermia are time-dose dependent relative to the increase in peroxisome proliferation. Male mice receiving 1500 ppm of DEHP for 78 weeks had no signs of hypospermia, whereas 30% of the animals treated for 104 weeks had hypospermia. All male mice receiving 6000 ppm of DEHP for 78 weeks had hypospermia. Peroxisome proliferation in these animals was 2.7 times control for the 1500-ppm groups and 7.6 times control for the 6000-ppm group. At 500 ppm, where the testes appeared normal, peroxisome proliferation was only 2.2 times control, although there was a slight decrease in relative testis weight. The significance of the decrease in testes weights is unclear since there was no histopathology at this dose level. By contrast, Ward *et al.* (1998) observed at least minimal hypospermia in PPAR-knockout mice given 12,000 ppm DEHP that exhibited a decrease in testes weights.

For the kidney, Ward *et al.* (1986) imply that lesions were dose- and time-dependent. However, the data from the study presented here indicate that the relationship must occur prior to Week 78. Based on the incidence of chronic progressive nephropathy among control animals at Week 78, this lesion is considered to be naturally occurring. It is also clear that exposure to high levels of DEHP (≥300 mg/kg) exacerbated the severity, but the mechanism is not clear. Ward *et al.* (1990) described increased severity of focal nephropathy in B6C3F1 mice exposed to 6000 ppm DEHP for 78 weeks. Ward *et al.* (1998) also evaluated the association of nephropathy with peroxisome proliferation using PPAR-knockout and wild-type

TABLE 6
Total Incidence of Histopathologic Lesions in Mice Exposed to DEHP

Lesion	Incidence per dose level[a]				
	0 ppm	100 ppm	500 ppm	1500 ppm	6000 ppm
Hepatocyte pigmentation					
Males	1/70 (0%)	0/60 (0%)	0/65 (0%)	1/65 (0%)	67/70 (96%)[c]
Females	0/70 (0%)	0/60 (0%)	0/65 (0%)	0/65 (0%)	55/70 (79%)[c]
Increased cytoplasmic eosinophilia					
Males	0/70 (0%)	0/60 (0%)	0/65 (0%)	0/65(0%)	69/70 (99%)[c]
Females	1/70 (1%)	0/60 (0%)	0/65(0%)	0/65(0%)	70/70 (100%)[c]
Chronic inflammation					
Males	34/70 (49%)	28/60 (47%)	27/65(42%)	35/65(54%)	51/70 (73%)[c]
Severity grade[b]	0.5	0.5	0.4	0.6	0.9
Females	50/70 (71%)	34/60 (57%)	53/65(82%)	49/65(75%)	58/70 (83%)
Severity grade[b]	0.8	0.6	0.9	0.8	1.1
Chronic progressive nephropathy					
Males	54/60 (90%)	56/60 (93%)	52/60 (87%)	54/60 (90%)	59/61(97%)
Severity grade[b]	1.1	1.0	0.9	1.2	2.3
Females	35/60 (58%)	32/60 (53%)	37/60 (62%)	51/60 (85%)[c]	60/60 (100%)[c]
Severity grade[b]	0.7	0.6	0.6	1.0	2.5
Bilateral hypospermia of the testes	2/60 (3%)	2/60 (3%)	1/60 (2%)	18/60 (30%)[c]	57/60 (95%)[c]
Hypospermia of the epididymis	3/60 (5%)	0/60 (0%)	1/60 (2%)	3/60 (5%)	36/60 (60%)[c]
Immature/abnormal epididymal sperm	10/60 (17%)	14/60 (23%)	11/60 (18%)	29/60 (48%)[c]	48/60 (80%)[c]

[a]Number of animals exhibiting lesion out of total examined. Percentage of animals exhibiting lesion are provided in parentheses.
[b]Mean severity grade based on scores of 1–4 for minimal to severe.
[c]Significantly different from control, $p \leq 0.05$.

mice. In that study, Ward and coworkers described nephropathy and severe cystic renal tubules in Sv/129 wild-type mice exposed to 12,000 ppm for 16 weeks. However, PPARα-knockout mice had minimal focal renal tubular lesions at 4 and 8 weeks, and diffuse and mildly cystic kidneys at 24 weeks. Thus, the nephropathy observed in mice is influenced by PPARα or sequelae of peroxisome proliferation, but are not entirely responsible for that effect. Peroxisome proliferation has been observed in the kidneys of rats following exposure to 20,000 ppm DEHP for 4 weeks (Ohno *et al.*, 1982), to 1 g/kg for 21 days (Cimini *et al.*, 1994), and 10000 ppm for 18 months (Price *et al.*, 1987). Thus, in the absence of associated kidney lesions, the most likely hypothesis for increased kidney weights is that they reflect peroxisome proliferation, which was observed in the liver at this dose level (David *et al.*, 1999).

Comparing the study presented here for mice to data generated for rats, it appears that there are clear differences in the responses of mice and rats to DEHP exposure. Absent in mice were several rat-specific lesions with questionable relevance to humans. For example, hematologic changes (decreased erythrocyte count, hemoglobin, and hematocrit), mononuclear cell leukemia, spongiosis hepatis, mineralization of the renal tubules, and pancreatic acinar-cell adenoma were not observed in mice but were observed in rats. Many of these lesions (leukemia, spongiosis hepatis, mineralization of the renal tubules, and pancreatic acinar-cell adenoma) appear to be associated with rat-specific mechanisms such as α2u-globulin nephropathy (David *et al.*, 2000). The lack of effect in mice given even higher dose levels of DEHP reinforces the hypothesis of species-specificity for these lesions.

In conclusion, exposure of mice to high levels of DEHP resulted in minimal signs of toxicity: histopathologic changes in the liver and testes similar to those observed for rats. Also observed was exacerbation of naturally occurring lesions in the liver and kidneys such as those observed for rats. All lesions occurred at dose levels where peroxisome proliferation was $\geq$ 2.7 times control. This is in contrast to the effects in rats, where testicular lesions occurred at a dose level of minimal peroxisomal enzyme activity. A dose level of 500 ppm (98.5–116.8 mg/kg/day) was identified as a no-observed-adverse-effect level for noncarcinogenic effects. By comparison, nonmedical lifetime human exposure levels are estimated to be 3–30 μg/kg/day (Doull *et al.*, 1999) using a total non-occupational exposure of 0.25 mg/person/day (ATSDR, 1993).

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THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 282, NO. 26, pp. 19152–19166, June 29, 2007

The Endocrine Disruptor Monoethyl-hexyl-phthalate Is a Selective Peroxisome Proliferator-activated Receptor γ Modulator That Promotes Adipogenesis*

Received for publication, March 30, 2007, and in revised form, April 25, 2007 Published, JBC Papers in Press, April 27, 2007, DOI 10.1074/jbc.M702724200

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The ability of pollutants to affect human health is a major concern, justified by the wide demonstration that reproductive functions are altered by endocrine disrupting chemicals. The definition of endocrine disruption is today extended to broader endocrine regulations, and includes activation of metabolic sensors, such as the peroxisome proliferator-activated receptors (PPARs). Toxicology approaches have demonstrated that phthalate plasticizers can directly influence PPAR activity. What is now missing is a detailed molecular understanding of the fundamental basis of endocrine disrupting chemical interference with PPAR signaling. We thus performed structural and functional analyses that demonstrate how monoethyl-hexyl-phthalate (MEHP) directly activates PPARγ and promotes adipogenesis, albeit to a lower extent than the full agonist rosiglitazone. Importantly, we demonstrate that MEHP induces a selective activation of different PPARγ target genes. Chromatin immunoprecipitation and fluorescence microscopy in living cells reveal that this selective activity correlates with the recruitment of a specific subset of PPARγ coregulators that includes Med1 and PGC-1α, but not p300 and SRC-1. These results highlight some key mechanisms in metabolic disruption but are also instrumental in the context of selective PPAR modulation, a promising field for new therapeutic development based on PPAR modulation.

Exposure to endocrine disrupting chemicals can lead to detrimental effects in human and animal populations by promoting or inhibiting the synthesis, elimination, and action of hormones (1, 2). At the molecular level, these compounds act by activating or inhibiting enzymatic activities of hormone biosynthesis and by targeting nuclear receptors (NRs),[4] a class of transcription factors that regulate gene expression programs in response to lipophilic hormones and mediators. In the past, research on endocrine disruption has focused on reproductive defects caused by interference with steroid signaling, largely linked to modulation of steroid NR activity (3). However, NRs constitute a large family of receptors regulating diverse physiological functions, out of which most members share the capacity to regulate gene expression in response to ligand binding. The concept of endocrine disruption is now being broadened to other receptors implicated in different aspects of homeostatic regulation (4).

Given their central role in metabolic regulations (5, 6), peroxisome proliferator-activated receptors (PPARs) potentially constitute important targets for environmental factors. PPARs are lipid sensors that cooperate in different organs to adapt gene expression to a given metabolic status. PPARγ (NR1C3) controls fat storage in the adipose tissue by promoting the differentiation and the survival of adipocytes, but also plays major roles in the control of insulin sensitivity (7). At the molecular level, PPAR action relies on its constitutive association with the retinoid X receptor (NR2B) (8, 9), which allows binding of the heterodimer to specific response elements located in target gene promoters. The unliganded receptors are engaged in large complexes of corepressors and coactivators that can promote repression and activation according to the promoter context, respectively (9–11). To induce full transcriptional activation, ligand binding enhances the recruitment of coactivators acting

* This work was supported by grants from the National Research Project 50, the Swiss National Science Foundation, the Etat de Vaud and the National Center for competence in Research "Frontiers in Genetics" (to W. W. and B. D.), the Swiss National Science Foundation and Oncosuisse (to O. M.), CNRS and ARC (to R. M.), the Research Council of the Katholieke Universiteit Leuven (to C. T.), and project G.0584.06 of the Fund for Scientific Research Flanders (to Y. E.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[4] The abbreviations used are: NR, nuclear receptors; FCS, fluorescence correlation spectroscopy; ACSBG1, acyl-CoA synthetase Bubblegum 1; FRET, fluorescence resonance energy transfer; PPAR, peroxisome proliferator-activated receptor; SPPARγM, selective peroxisome proliferator-activated receptor γ modulators; DEHP, diethyl-hexyl-phthalate; MEHP, monoethyl-hexyl-phthalate; EYFP, enhanced yellow fluorescent protein; siRNA, small interfering RNA; GST, glutathione *S*-transferase; LBD, ligand binding domain; CFP, cyan fluorescent protein; YFP, yellow fluorescent protein; FABP, fatty acid-binding protein; WB, Washing Buffer.