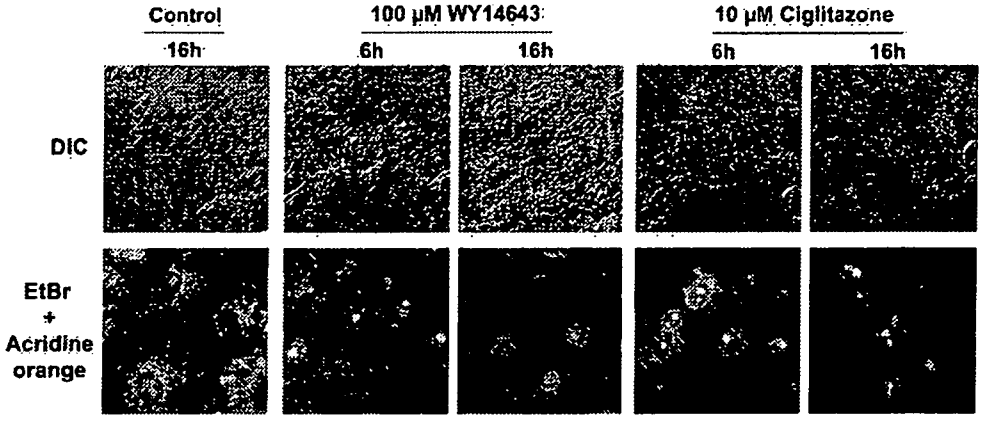

Fig. 5. Staining of LLC-PK1 cells with DNA intercalating dyes. Cells were incubated with 100 μM WY14643 or 10 μM ciglitazone for 6 and 16 h and then stained with ethidium bromide and acridine orange. Non-fixed cells were analyzed by DIC or fluorescence microscopy. Both treatments increase the number of cells that uptake ethidium bromide (stains red). Live cells show green as a consequence of acridine orange. No nuclear or cell morphology reminiscent of apoptotic cell can be appreciated.

No peripheral green staining (annexin V) on the cells could be found, thereby indicating that the phosphatidylserine had not been externalized. Similar results were observed at 6 h of treatment with the same concentration, but the number of cells stained with PI was dramatically reduced. At both incubation times, DIC optics also revealed a morphology pattern compatible with primary necrotic cell death. Treatment of the cells with 200 μM WY14643 simply increased the number of cells stained with PI at both times.

Similar results were obtained in LLC-PK1 cells treated with 5 or 20 μM ciglitazone, and for 6 or 12 h: All stainings revealed primary necrotic cell death.

3.7. Effect of WY14643 and ciglitazone on caspase-3 activity in LLC-PK1 cells

Caspase-3 activity was also assayed to check whether any apoptotic activity was present during WY14643 and ciglitazone treatments. LLC-PK1 cells were treated for 3 h with either 100 or 200 μM WY14643, and with 5 or 10 μM ciglitazone. Simultaneous analyses of LDH activity in assay medium and caspase-3 in cell lysate were performed (Fig. 7). While LDH activity increased in a concentration-dependent way, caspase 3 activity was not modified and maintained the basal levels of the control cells.

3.8. Effect of WY14643 and ciglitazone on DNA laddering in LLC-PK1 cells

As a final test in the characterization of WY14643 and ciglitazone cytotoxicities, the degradation of genomic DNA was tested in LLC-PK1 cells. The cells were incubated for 16 h with different concentrations of the drugs, as shown in Fig. 8. The genomic DNA was then extracted and separated by an agarose gel electrophoresis. After 16 h of treatment, WY14643 did not induce degradation of genomic DNA at 25 or 50 μM. However, at 100 and 200 μM, WY14643 degraded DNA in a necrotic, continuous pattern: no laddering of 200 bp or any multiples thereof were observed. Ciglitazone produced the same degradation of genomic DNA at either 10 or 20 μM, while no degradation was observed at 5 μM.

These results finally confirm that WY14643 and ciglitazone are cytotoxic to cells of proximal tubular lines and that the cells die from pure and classic necrosis.

4. Discussion

Peroxisome proliferator-activated receptors are nuclear receptors that, upon activation by specific ligands and in combination with RXR receptor, bind to PPAR response elements (PPRE) to initiate the expression of specific target genes. The receptors are involved in controlling a plethora of cell functions, including regulation of several metabolic pathways, such as lipid biosynthesis and glucose metabolism. PPARα and -γ are also the target for several environmental contaminants, as well as for drugs designed to correct metabolic disorders such as dyslipidemia or insulin resistance (for an extensive revision see Berger and Moller, 2002).

Many reports have described the existence of side effects after incubation with drugs initially designed to activate the PPARs (recently reviewed by Peraza et al., 2006). Some of them involve antiproliferative effects and even apoptosis in different cell types, such as the antineoplastic effects of PPARγ agonists (Grommes et al., 2004; Theocharis et al., 2004), apoptosis (Okuda et al., 2000; Redondo et al., 2005) or growth arrest (Bruemmer et al., 2003) induced by several TZDs and PPARα agonists (Roberts et al., 2002) in vascular smooth muscle cells; PPARγ mediated apoptosis of renal proximal tubular cells (Arici et al., 2003), mesangial cells (Tsuchiya et al., 2003) and interstitial fibroblasts (Parameswaran et al., 2003); etc. In this work

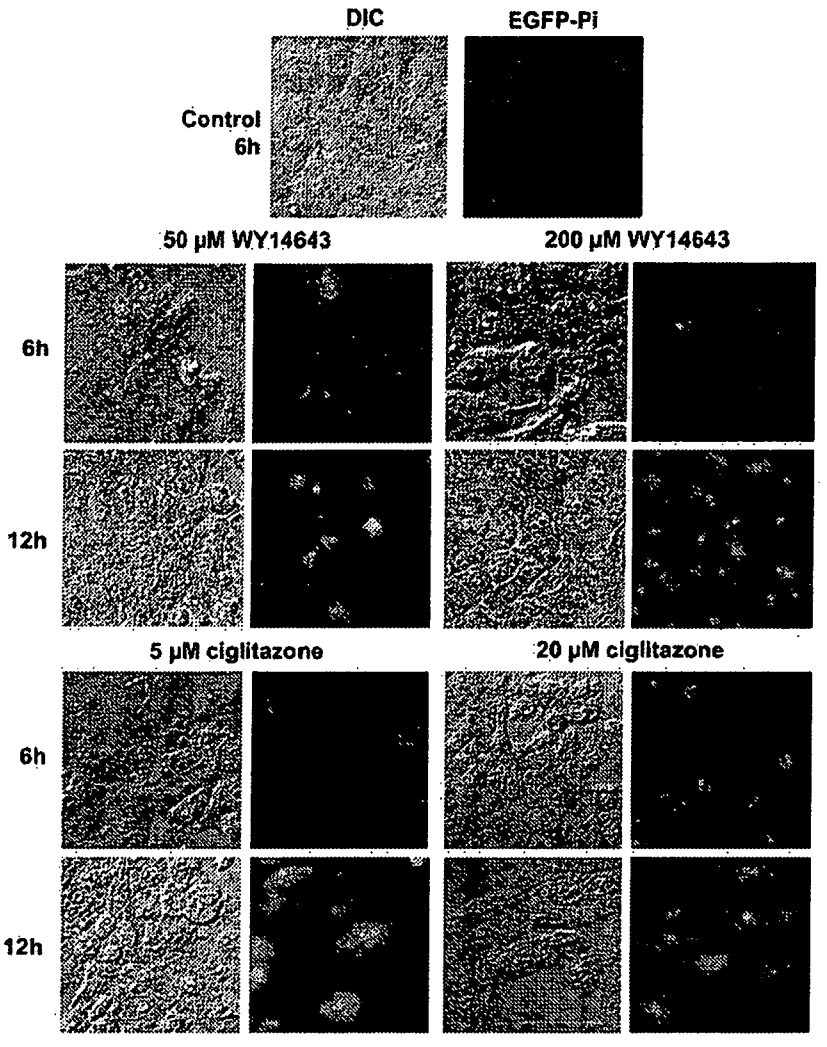

Fig. 6. Incubation of LLC-PK1 cells with EGFP-annexin V and propidium iodide. Cells were treated with WY14643 and ciglitazone at the indicated concentrations and incubation times. They were incubated with EGFP-tagged annexin V and propidium iodide and then fixed. The absence of green staining indicates that annexin V did not have access to phosphatidylserine in the outer layer of the plasma membrane. Propidium iodide stains for dead cells.

we show a direct necrotic effect of some PPAR agonists, namely WY14643 and ciglitazone using concentrations similar to the therapeutical plasma levels of the drugs (Berger and Moller, 2002; Brunton et al., 2005). We have shown that these effects are not universal for all PPAR activators, because clofibrate and pioglitazone (two alternative PPARα and -γ agonists, respectively) are non-cytotoxic in the same cells. In the case of PPARα agonists, WY-14643 has an EC_{50} of 0.63–5 μM (Willson et al., 2000), while we have determined that the LC_{50} is 92–124 μM in the three renal cell lines employed (Fig. 1). Likewise, clofibrate shows an EC_{50} of 50–55 μM (Willson et al., 2000), but it is not toxic even at 500 μM in any of the cell lines. With respect to PPARγ agonists, ciglitazone activates the receptor with 3 μM EC_{50} (Willson et al., 1996), but the LD_{50} is 8.6–14.8 μM, and pioglitazone does not show signs of toxicity at concentrations even 100 times higher than the EC_{50} (0.5 μM; Sakamoto et al., 2000). The absence of cytotoxic effects when the receptors are completely activated with clofibrate and pioglitazone suggests that the toxicities of WY-14643 and ciglitazone are mediated through PPAR-independent mechanisms. Similar findings have been published while reviewing our work (Soller et al., in press). The authors show that troglitazone induces apoptosis in Jurkat T cells, while ciglitazone induces necrosis. Both compounds inhibit the mitochondrial respiratory complex I, but ciglitazone also inhibits complex II and depletes the ATP cell levels.

We have also observed that the intensity of cytotoxity of PPAR agonists depends on the cell line assayed: mouse cortical tubular cells are the *in vitro* model with the narrowest threshold (high Hill coefficient in dose–response curves), while opossum kidney cells are the most sensitive cell line. These conclusions arise from the dose–response

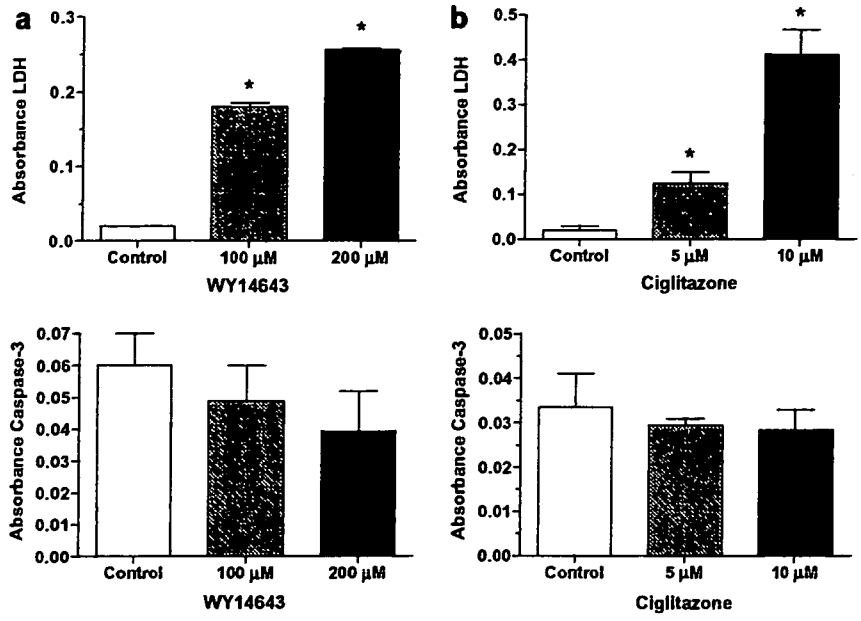

Fig. 7. Assay of caspase-3 activity in LLC-PK1 cells incubated for 3 h with the indicated PPAR agonists. As shown, LDH activity increases with the concentration of drugs (top panels). However, capase-3 activity remains unchanged.

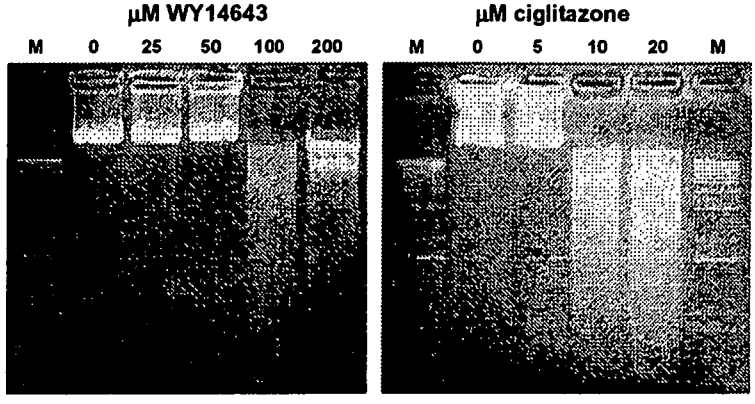

Fig. 8. Genomic DNA analysis. LLC-PK1 cells were incubated for 16 h with increasing concentrations of WY14643 and ciglitazone. Genomic DNA was extracted and run in horizontal electrophoresis as explained. Only 100 and 200 µM WY14643 and 10 and 20 µM ciglitazone produces degradation of genomic DNA. However, no laddering of 200 bp bands and onwards can be observed in the electrophoresis.

assays of LDH activity as a function of drug concentration (Fig. 1). At this point we do not know the toxic mechanisms that induce cell death in our experimental conditions, but one major conclusion is that these effects should not be related to PPAR activity, and therefore they should be independent of the affinity binding of the drugs. Moreover, the fact that the same drugs are toxic in a similar way to all three epithelial cell lines of proximal tubular origin suggests that a common toxic mechanism can take place in the kidney *in vivo*.

Since apoptosis does not follow a universal pattern of events and since many of the typical characteristics of apoptosis are not always expressed, we have characterized the cell death in different ways. Using either morphological and biochemical assays, as well as an analysis of genomic DNA, we have not found evidence of apoptotic cell death under any of our experimental conditions. However, LLC-PK1 cells have the ability to undergo apoptosis (Gennari et al., 2003; Liu and Baliga, 2005). In addition to previous reports, we have also checked this ability by treating the cells with 50 µM $CdCl_2$ for 16 h (see Section 2; data not shown). In conclusion, the cytotoxic effects of WY14643 and ciglitazone should consist of direct insults to the cells that overwhelm the homeostatic mechanisms and end in cell death. Therefore, further studies are needed to understand the likely mechanisms of cell death, and ongoing work should clarify this point. With respect to the induced apoptosis by PPAR agonists, several mechanisms have

been described, either through the PPAR receptors or through PPAR-independent mechanisms. The pathways include activation of the growth arrest and DNA damage-inducible gene 45 (GADD45) and p53 (Bruemmer et al., 2003; Okuda et al., 2000), upregulation of Bad and Bax (Zander et al., 2002) and p27 (Liu et al., 2004), or nuclear recruitment of phospho-Smad2 (Redondo et al., 2005). The effects of WY14643 are, however, contradictory. For example, this compound reduces both the apoptotic and necrotic cell death induced by cisplatin in mouse kidney, with a concomitant inhibition of proapoptotic renal endonuclease G (Li et al., 2004). In brain cortical cells, however, WY14643 can switch the cell death induced by cyanide from apoptosis to necrosis by increasing the expression of uncoupling protein-2 and mitochondrial dysfunction (Li et al., 2006).

Our results add new information to the possible toxic effect of the drugs. Renal side-effects were initially described for both fibrates and TZDs, and included dysuria, proteinuria and increases in plasma creatinine and urea (Lipscombe et al., 2001). These effects were eliminated with the design of new drugs, as in the case of ciglitazone that exhibited a significant liver toxicity and was substituted by pioglitazone. In agreement to our work, commercial drugs available today (eg. clofibrate, pioglitazone or rosiglitazone) show, fortunately, little or no renal toxicity. Our findings could reflect the side effects reported initially for these drugs, and highlight the importance of the characterization of side effects when designing pharmacological PPAR agonists.

In conclusion, fibrates and TZDs are drugs extensively used to control metabolic disorders, with important consequences to the kidneys, especially in patients with renal disease. The fact that some PPAR agonists are able to induce a direct necrotic cell death in epithelial cells of proximal tubular origin adds additional risk to the treatment of such disorders. Therefore, control of the dangerous side effects of PPAR agonists should be carefully determined.

Acknowledgements

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Phthalate Esters Enhance Quinolinate Production by Inhibiting α-Amino-β-Carboxymuconate-ε-Semialdehyde Decarboxylase (ACMSD), a Key Enzyme of the Tryptophan Pathway

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Tryptophan is metabolized to α-amino-β-carboxymuconate-ε-semialdehyde (ACMS) via 3-hydroxyanthranilate (3-HA). ACMS decarboxylase (ACMSD) directs ACMS to acetyl CoA; otherwise ACMS is non-enzymatically converted to quinolinate (QA), leading to the formation of NAD and its degradation products. Thus, ACMSD is a critical enzyme for tryptophan metabolism. Phthalate esters have been suspected of being environmental endocrine disrupters. Because of the structural similarity of phthalate esters with tryptophan metabolites, we examined the effects of phthalate esters on tryptophan metabolism. Phthalate esters containing diets were orally given to rats and the urinary excreted tryptophan metabolites were quantified. Of the phthalate esters with different side chains tested, di(2-ethylhexyl)phthalate (DEHP) and its metabolite, mono(2-ethylhexyl)phthalate (MEHP), most strongly enhanced the production of QA and degradation products of nicotinamide, while 3-HA was unchanged. This pattern of metabolic change led us to assume that these esters lowered ACMSD protein or its activity. Although DEHP could not be tested because of its low solubility, MEHP reversibly inhibited ACMSD from rat liver and mouse kidney, and also the recombinant human enzyme. Correlation between inhibition of ACMSD by phthalate esters with different side chains and urinary excretion of QA supports the notion that phthalate esters perturb tryptophan metabolism by inhibiting ACMSD. Quinolinate is a potential endogenous toxin and has been implicated in the pathogenesis of various disorders. Although toxicity of phthalate esters through accumulation of QA remains to be investigated, they may be detrimental by acting as metabolic disrupters when intake of a tryptophan-rich diet and exposure to phthalate esters occur coincidentally.

Key Words: phthalate ester; endocrine disrupter; tryptophan metabolism; quinolinate; metabolic disrupter.

INTRODUCTION

Phthalate esters are used as plasticizers in the manufacture of polyvinylchloride plastics, as solvents in certain industrial processes, and as vehicles for pesticides (Giam *et al.*, 1994). These esters are widely distributed in the ecosystem and have been suspected of being environmental endocrine disrupters. Of a variety of industrially important phthalate esters, di(2-ethylhexyl) phthalate (DEHP) has perhaps been most extensively used for the formation of plastics. A number of papers have reported that some phthalate esters are noxious to experimental animals (reviewed in Koizumi *et al.*, 2001; Shea *et al.*, 2003); administration of phthalate esters exhibits reproductive and developmental toxicity (David *et al.*, 2000; Davis *et al.*, 1994; Lamb *et al.*, 1987; Wine *et al.*, 1997). It is believed that phthalate esters taken orally are hydrolyzed in the intestine before absorption, and the resulting products, monoesters, are primarily responsible for the toxicity of phthalate esters (Lake *et al.*, 1977).

The tryptophan–NAD pathway consists of the kynurenine pathway and the NAD pathway. The kynurenine pathway is the main route of tryptophan metabolism (Fig. 1). This pathway is initiated by the oxidation of tryptophan by tryptophan oxygenase (TDO) in the liver or by indoleamine dioxygenase (IDO) in other tissues including the brain. The metabolite at a branching point in the tryptophan–NAD pathway is α-amino-β-carboxymuconate-ε-semialdehyde (ACMS), which is converted by ACMS decarboxylase (ACMSD, EC4.1.1.45) to α-aminomuconate-ε-semialdehyde (AMS). AMS eventually leads to acetyl-CoA through the glutarate pathway, or otherwise non-enzymatic cyclization of ACMS results in the formation of quinolinate (QA), from which NAD is synthesized through the NAD pathway. Thus, ACMSD activity plays a critical role in the tryptophan–NAD pathway. In mammals, NAD is also synthesized from niacin (nicotinate (NiA) and nicotinamide (Nam)) that can be obtained primarily from dietary sources.

Quinolinate is a potential endogenous toxin; QA is neurotoxic by acting as an agonist at the *N*-methyl-D-aspartate (NMDA)–sensitive glutamate receptors. Schwarcz *et al.* (1983) and more recently Pawlak *et al.* (2003) have shown that QA can be a

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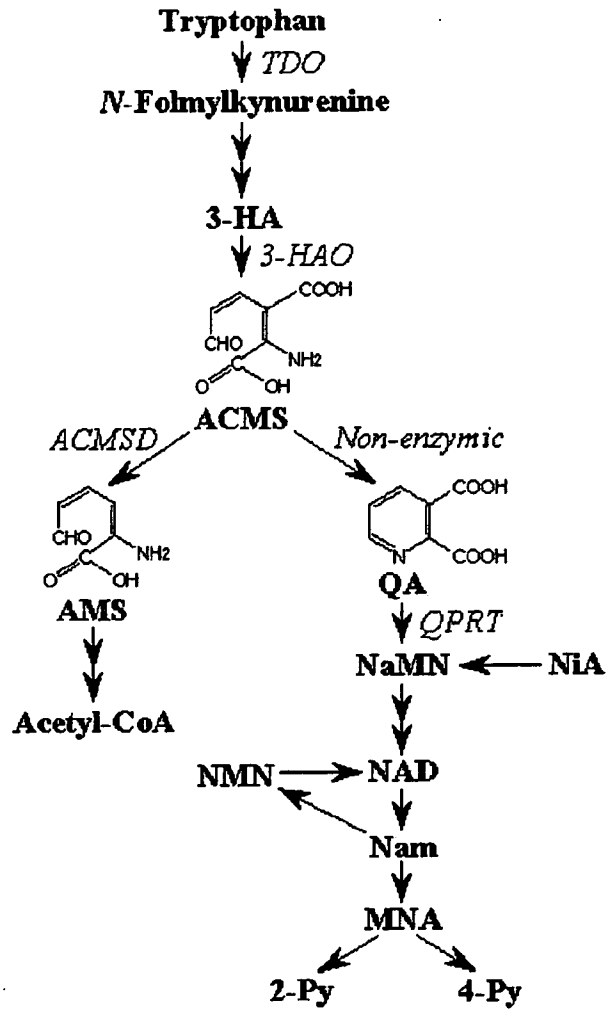

FIG. 1. Schematic diagram of the tryptophan–NAD pathway. Enzymes are underlined. 3-HA: 3-hydroxyanthranilate; ACMS: α-amino-β-carboxymuconate-ε-semialdehyde; AMS: α-aminomuconate-ε-semialdehyde; QA: quinolinate; NaMN: nicotinic acid mononucleotide; NiA: nicotinate; NMN: nicotinamide mononucleotide; Nam: nicotinamide; MNA: N^1-methylnicotinamide; 2-Py: N^1-methyl-2-pyridone-5-carboxamide; 4-Py: N^1-methyl-4-pyridone-3-carboxamide; TDO: tryptophan 2,3-dioxygenase; 3-HAO: 3-hydroxyanthranilic acid 2,3-dioxygenase; ACMSD: α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase; QPRT: quinolinate phosphoribosyltransferase.

uremic toxin responsible for anemia associated with renal failure by reducing production of erythropoietin, a glycoprotein that promotes erythrocyte formation. Elevation of QA concentration has been implicated in the pathogenesis of various diseases including cerebral ischemia, spinal cord injury, Huntington's disease, and multiple sclerosis (see review by Stone and Darlington, 2002).

The structural similarity of phthalates with tryptophan metabolites prompted us to examine the effects of phthalate esters on the pathway of tryptophan metabolism. NAD can be supplied from tryptophan in the dietary protein. Therefore, administration of a niacin-deficient diet containing phthalate esters to rats and measurement of the tryptophan metabolites excreted in the urine make it possible to estimate phthalate ester–induced changes in tryptophan metabolism (Fukuwatari *et al.*, 2002a, 2002b; Shibata *et al.*, 2001). We previously reported that di-*n*-butyly phthalate (DBP) (Shibata *et al.*, 2001) and DEHP (Fukuwatari *et al.*, 2002a, 2002b) stimulated conversion of tryptophan to NAD. In this article, we show that phthalate esters elevate QA and its downstream metabolites in the urine, whereas excretion of 3-hydroxyanthranilate (3-HA) remains unchanged. Of the phthalate esters tested, DEHP and its primary metabolite, mono(2-ethylhexyl)phthalate (MEHP), were the most potent disrupters of tryptophan metabolism. We also present results showing that direct inhibition of ACMSD by phthalate esters is primarily responsible for the phthalate ester–induced change in tryptophan metabolism.

MATERIALS AND METHODS

Chemicals. The materials used were obtained from the indicated sources: vitamin-free milk casein, sucrose, L-methionine, dimethyl phthalate (DMP), diethyl phthalate (DEP), DBP, di-*n*-octyl phthalate (DOP), DEHP, monoethyl phthalate (MEP), Nam, and QA (Wako Pure Chemical Industries, Osaka, Japan); mono-*n*-butyl phthalate (MBP), mono-*n*-hexyl phthalate (MHP), MEHP, and N^1-methylnicotinamide (MNA) chloride (Tokyo Chemical Industry, Tokyo); gelatinized cornstarch (Nichiden Kagaku, Tokyo); corn oil (Ajinomoto, Tokyo); mineral and vitamin mixtures (Oriental Yeast Kogyo, Tokyo). N^1-methyl-2-pyridone-5-carboxamide (2-Py) and N^1-methyl-4-pyridone-3-carboxamide (4-Py) were synthesized by the method of Shibata *et al.* (1988). All other chemicals used were of the highest purity available from commercial sources.

Animals and diets. The care and treatment of the experimental animals conformed to The University of Shiga Prefecture guidelines for the ethical treatment of laboratory animals. Rats and mice were obtained from Clea Japan (Tokyo), and housed in a room maintained at 22 ± 1°C with 60% humidity and a 12 h light/12 h dark cycle (light onset at 6:00 A.M.). Mice were used for preparation of ACMSD as described later. Body weight and food intake were measured daily at 10:00 A.M., and food and water were renewed daily. Male Wistar rats at 5 weeks old were placed in individual metabolic cages (CT-10; Clea Japan) and acclimated for 1 week. They were fed the control diet containing no phthalate esters. Experiments (five animals per group) were started by using rats at 6 weeks of age. The control diet consisted of 20% casein, 0.2% L-methionine, 45.9% gelatinized cornstarch, 22.9% sucrose, 5% corn oil, 5% mineral mixture (AIN-93 mineral mixture), and 1% vitamin mixture (niacin-free AIN-93 vitamin mixture). The phthalate esters tested were DMP, DEP, DBP, DOP, DEHP, MBP, MHP, or MEHP. Rats were fed with a diet containing 2.6 mmol phthalate ester/kg diet *ad libitum* for 21 days, and controls were fed without phthalate ester. The weight percent of individual phthalate esters in the diet ranges from 0.05% (500 ppm) of DMP to 0.1% (1000 ppm) of DEHP depending on their molecular weight values. Urine samples on the last day (10:00 A.M.–10:00 A.M.; 24-h urine) were collected in amber bottles containing 1 ml of 1 mol/l HCl, and stored at −25°C until use.

Determination of tryptophan metabolites in the urine. Tryptophan metabolites were determined by high-performance liquid chromatography (HPLC). To determine 3-HA (Shibata and Onodera, 1992), urine samples were filtered through a 0.45-μm microfilter, and 20 μl of the filtrates was injected into a STR ODS II column (4.6 × 250 mm I.D., particle size 7 μm) (Shinwa Chemical, Kyoto, Japan). The mobile phase was 50 mmol/l KH_2PO_4 (pH 3.0)-acetonitrile

(100:10 v/v) containing 3 mg/l ethylene diamine tetraacetic acid (EDTA)-2Na, the flow rate was 1 ml/min, the column temperature was maintained at 40°C, and 3-HA was detected at +500 mV electrochemical detection (ECD).

To determine QA (Mawatari *et al.*, 1995), urine samples were filtered through a 0.45-μm microfilter, and 20 μl of the filtrates was injected into a Unisil Q C18 column (4.6 × 250 mm I.D., particle size 5 μm) (GL Sciences, Tokyo). The mobile phase was 20 mmol/l KH_2Po_4, pH 3.8, containing 0.00045% tetramethylammonium hydroxide and 1.2% hydrogen peroxide, the flow rate was 0.6 ml/min, and the column temperature was maintained at 40°C. The fluorescence intensity at 380 nm was measured upon excitation at 326 nm.

Nam, 2-Py, and 4-Py in the urine samples were measured simultaneously (Shibata, 1987a). Briefly, 1 ml of urine samples was mixed with 10 μl of 1 mg/ml isonicotinamide as an internal standard, 1.2 g of potassium carbonate, and 10 ml of diethylether. The mixtures were shaken vigorously for 5 min, and centrifuged at 800 × g for 5 min. The organic layers were evaporated, and dissolved in 0.5 ml of water. Aliquots of each sample were filtered through a 0.45-μm microfilter, and 20 μl of the filtrates was injected into a CHEMCOSORB 7-ODS-L column (4.6 × 250 mm I.D., particle size 7 μm) (Chemco Scientific, Osaka, Japan). The mobile phase was 10 mmol/l KH_2Po_4 (pH 3.0)–acetonitrile (96:4 v/v), the flow rate was 1 ml/min, the column temperature was maintained at 40°C, and the detection wavelength was 260 nm.

To determine MNA (Shibata, 1987b), urine samples (0.1 ml each) were mixed with 0.7 ml of water, 0.2 ml of 1 mmol/l isonicotinamide, 0.5 ml of 0.1 mmol/l acetophenone, and 1 ml of 6 mol/l sodium hydroxide. After the mixtures were cooled on ice for 10 min, 0.5 ml of 99% formic acid was added, followed by boiling in a water bath for 5 min. The mixtures were cooled on ice, filtered through a 0.45-μm microfilter, and 20 μl of the filtrates was injected into a Tosoh 80Ts column (4.6 × 250 mm I.D., particle size 7 μm) (Tosoh, Tokyo). The mobile phase was a mixture of 20 mmol/l KH_2PO_4, pH 3.0-acetonitrile (97:3 v/v) containing 1 g/l sodium hepansulfonate and 1 mmol/l EDTA-2Na, the flow rate was 1 ml/min, and the column temperature was maintained at 40°C. The fluorescence intensity at 440 nm was measured upon excitation at 382 nm.

Enzymes and assays. Because the dietary protein has been shown to induce ACMSD in the rat liver (Fukuoka *et al.*, 1998), male Wistar rats (10 weeks old) were fed a high-protein diet (40% casein) for 4 weeks. Male ICR mice (9 weeks old) were fed the control diet (20% casein) for 1 week. Animals were sacrificed by decapitation, and the liver and kidneys were removed from rats and mice, respectively. The organs were immediately homogenized with a polytetrafluoroethylene (PTFE)-glass homogenizer in 5 volumes of cold 50 mmol/l potassium phosphate buffer, pH 7.0. The homogenate was centrifuged at 55,000 × g for 20 min, and the supernatant was used as an enzyme source. Four or five animals per group were used and the enzyme activities were assayed with the supernatant prepared from each organ.

Human ACMSD (Fukuoka *et al.*, 2002) or human quinolinate phosphoribosyltransferase (QPRT, EC 2. 4. 2. 19) (Fukuoka *et al.*, 1998) transiently expressed in COS-7 cells was prepared from cells cultured for 72 h after transfection. Cells were harvested and lysed with 50 mmol/l Tris-HCl buffer, pH 7.6, containing 137 mmol/l sodium chloride, 1% Triton X-100, 5 mmol/l EDTA, 100 μmol/l leupeptin, and 20 μg/ml FOY-305. The homogenates were centrifuged at 100,000 × g for 15 min, and the supernatants were used for assaying enzyme activity.

The activity of ACMSD was measured as described (Ichiyama *et al.*, 1965). The reaction mixture containing 10 μl of 3.3 mmol/l 3-HA (in 50 mmol/l Tris-acetate buffer, pH 8.0); 0.5 ml of 0.2 mol/l Tris-acetate buffer, pH 8.0; and 0.8 ml of water was incubated in a cuvette for 5 min at 25°C. ACMS was produced by the addition of an excess quantity of the purified 3-HA oxygenase (50 μl containing 0.4 mg protein). After the formation of ACMS was complete, as judged by its absorbance at 360 nm, 0.1 ml of the ACMSD preparation was added. The decrease in absorbance at 360 nm was followed for 5 min against a control incubation that contained all the ingredients except 3-HA. When the effects of phthalate monoesters were examined, 50 μl of the esters dissolved in ethanol was added before the addition of the enzyme. The control incubation contained 50 μl of ethanol. The effects of phthalate diesters could not be tested because of their low solubility in the enzyme assay mixture.

QPRT was assayed as described (Shibata *et al.*, 2000). The incubation medium contained 50 μl of 500 mmol/l potassium phosphate buffer, pH 7.0, 50 μl of 10 mmol/l QA, 50 μl of 10 mmol/l phosphoribosylpyrophosphate, 10 μl of 100 mmol/l $MgCl_2$, 20 μl of phthalate monoester dissolved in ethanol, 270 μl of water, and 50 μl of the enzyme preparation. The control incubation contained 20 μl of ethanol. The reaction was started by addition of the enzyme, and the incubation was carried out at 37°C for 1 h. The reaction tube was placed in a boiling water bath for 5 min to stop the reaction, cooled on ice for 5 min, and centrifuged at 10,000 x g for 5 min. The supernatant was filtered through a 0.45-μm microfilter, and 20 μl of the filtrate was injected into a HPLC column, Tosoh 80Ts (4.6 × 250 mm I.D., particle size 7 μm) (Tosoh, Tokyo). The mobile phase was 10 mmol/l potassium phosphate buffer, pH 7.8, containing 1.48 g/l tetra-*n*-butylammonium bromide-acetonitrile (90:10 v/v), the flow rate was 1.0 ml/min, and the column temperature was maintained at 40°C. The product was detected at 265 nm.

Statistical analysis. The values are expressed as the mean ± SEM. The statistical significance was determined by ANOVA followed by Tukey's multiple comparison test.

RESULTS

Body Weight, Food Intake, and Liver Weight

Body weight of rats (6 weeks of age) at starting point of experiments was 140 ± 3 g and their weight increased almost linearly with gains of 6.2 ± 0.4 g per day. There was no significant difference in growth between groups fed phthalate esters and the control group. Food intake (g/day) of rats was 10 ± 0.5 at 6 weeks of age and increased to 17 ± 0.5, 20 ± 0.7, and 22 ± 0.8 at 7, 8, and 9 weeks, respectively. Phthalate esters showed no significant effect on food intake. Food intake/kg body weight/day varies depending on age (66 g at 6 weeks and 77 g at 9 weeks). When we used an average value of food intake (70 g/kg body weight/day), intake of phthalate esters was calculated to be 0.182 mmol/kg body weight/day, and therefore the weight values of phthalate esters ingested ranged from 35 mg/kg body weight/day of DMP to 70 mg of DEHP, depending on their molecular weight values.

DEHP causes hepatomegaly in rodents by proliferating peroxisome (Elcombe and Mitchell, 1986; Ward *et al.*, 1986). However, the liver weights of phthalate ester–fed groups measured at the end point of experiments (9 weeks of age) did not differ from those of the control groups, indicating that DHEP at the dose level given in this experiment does not cause significant peroxisome proliferation.

Effects of Phthalate Diesters on the Urinary Excretion of the Tryptophan Metabolites

To assess the effects of various phthalate diesters on the tryptophan–NAD pathway, rats at 6 weeks of age were fed with a diet containing DMP, DEP, DBP, DOP, or DEHP for 21 days, and the urinary contents of tryptophan metabolites such as 3-HA, QA, Nam, MNA, 2-Py, and 4-Py were measured. The sum of Nam, MNA, 2-Py, and 4-Py was expressed as Nam metabolites. As shown in Figure 2A, the urinary excretion of 3-HA was not changed by any of the phthalate diesters used. In

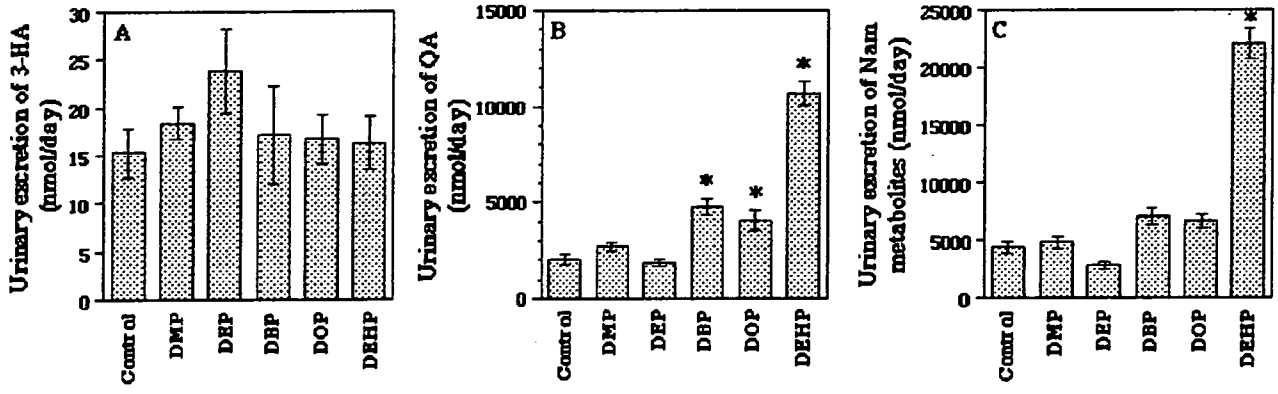

FIG. 2. Effects of phthalate diesters on the urinary excretion of 3-HA (A), QA (B), and Nam metabolites (C) in rats. Male Wistar rats at 6 weeks old were fed with a diet containing 2.6 mmol phthalate ester/kg diet *ad libitum* for 21 days. The phthalate esters used were DMP, DEP, DBP, DOP, or DEHP. Urine samples on the last day (10:00 A.M.–10:00 A.M.; 24-h urine) were collected in amber bottles containing 1 ml of 1 mol/L HCl. Values are means ± SEM; $n = 5$. *$P < 0.05$ versus the control.

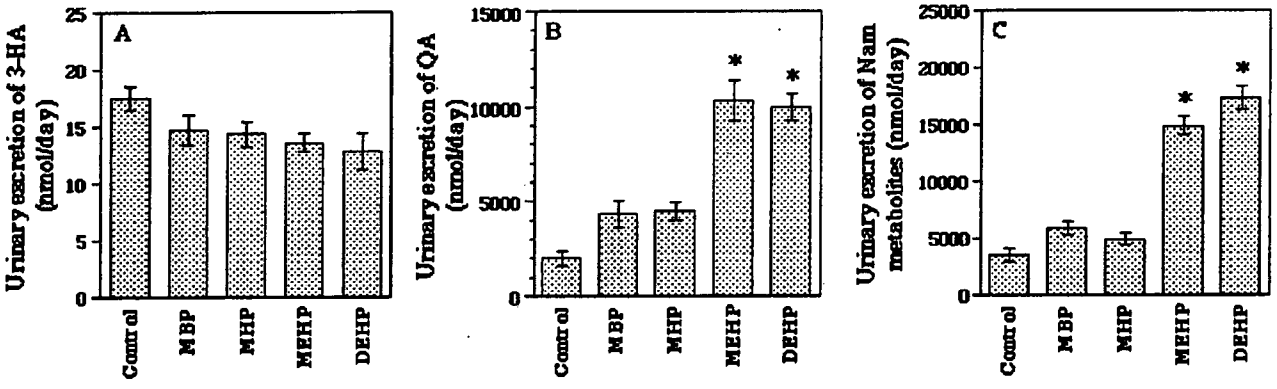

FIG. 3. Effects of phthalate monoesters on the urinary excretion of 3-HA (A), QA (B), and Nam metabolites (C) in rats. Male Wistar rats at 6 weeks old were fed with a diet containing 2.6 mmol phthalate ester/kg diet *ad libitum* for 21 days. The phthalate esters used were MBP, MHP, MEHP, or DEHP. Urine samples on the last day (10:00 A.M.–10:00 A.M.; 24-h urine) were collected in amber bottles containing 1 ml of 1 mol/L HCl. Values are means ± SEM, $n = 5$. *$P < 0.05$ versus the control.

contrast, QA (Fig. 2B) and its downstream metabolites (Nam metabolites in Fig. 2C) were markedly elevated by DEHP. Both DBP and DOP also increased the urinary excretion of QA but to a lesser extent; DMP and DEP, however, had no effect (Fig. 2B). DME, DEP, DBP, and DOP did not affect the excretion of Nam metabolites (Fig. 2C). Thus the length and structure of side chains in the esters appear to be crucial for the urinary excretion of tryptophan metabolites. DEHP that has long and branched side chains was the most powerful disruptor of tryptophan metabolism.

Effects of Phthalate Monoesters on the Urinary Excretion of the Tryptophan Metabolites

Because the phthalate diester–induced effects may be due to the monoesters that are produced in the digestive organs (Lake *et al.*, 1977), we also examined phthalate monoesters. Rats at 6 weeks of age were fed with a diet containing MBP, MHP, or MEHP for 21 days, and the urinary excretion of the tryptophan metabolites was assayed. The experiments with DEHP were performed again for comparison with MEHP. As shown in Figure 3, the results were very similar to those when the diesters were used. The urinary excretion of 3-HA was unchanged after administration of the monoesters (Fig. 3A). Large increases in QA and Nam metabolites were found when MEHP was given, and those increases were similar to the ones found with DEHP (Fig. 3B and 3C). When MBP and MHP were given, there was an increase in the mean values of urinary QA and Nam metabolites, but the increase was not statistically significant.

Effects of Phthalate Monoesters on ACMSD and QPRT

Feeding of MEHP or DEHP strongly increased urinary excretion of QA and its downstream metabolites in the

TABLE 1
Effects of Phtalate Monoesters on the Activity of ACMSD or QPRT[a]

	Vehicle	MEP	MBP	MHP	MEHP
Rat ACMSD activity (μmol/h/g liver)	9.3 ± 0.3	10.0 ± 0.4	7.2 ± 0.6*	3.9 ± 0.2*	0.9 ± 0.3*
Mouse ACMSD activity (μmol/h/g kidney)	3.07 ± 0.13	2.88 ± 0.15	2.63 ± 0.30*	1.12 ± 0.34*	0.21 ± 0.16*
Human ACMSD activity (μmol/h/mg protein)	21.5 ± 1.1	22.6 ± 1.3	17.7 ± 1.3	3.5 ± 1.0*	1.6 ± 0.4*
Rat QPRT activity (μmol/h/g liver)	1.27 ± 0.03	1.30 ± 0.07	1.29 ± 0.04	1.29 ± 0.05	1.25 ± 0.04
Human QPRT activity (μmol/h/mg protein)	10.7 ± 0.4	11.5 ± 0.3	11.7 ± 0.4	11.1 ± 0.2	11.1 ± 0.5

[a]Enzyme activities were measured in the presence or absence of 3 mmol/L phthalate monoester. ACMSD (α-amino-β-carboxymuconate-ε-semialdehyde decarboxylase) was assayed with extracts from rat liver, mouse kidney, and COS-7 expressing recombinant human enzyme, and QPRT (quinolinate phosphoribosyltransferase) was assayed with extracts from rat liver and COS-7 expressing human enzyme. Extracts prepared from five animals and five cultures of COS-7 cells were assayed; values are means ± SEM (n = 5). MEP: monoethyl phthalate; MBP: mono-n-butyl phthalate.
*P < 0.05 versus vehicle.

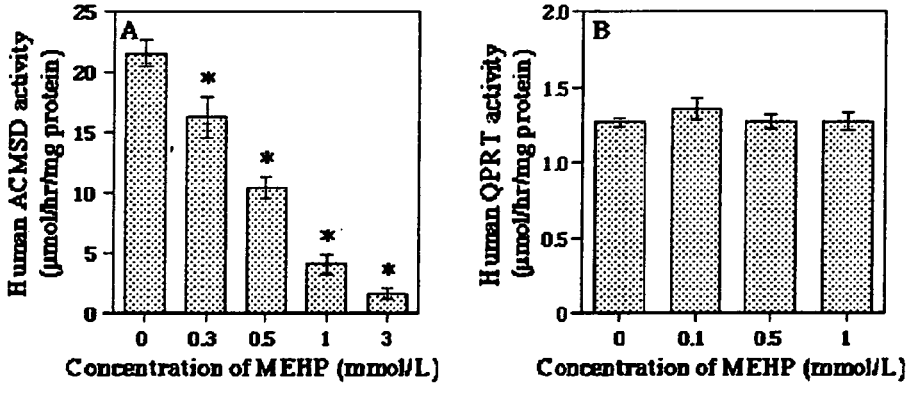

FIG. 4. MEHP inhibits human ACMSD activity in a dose-dependent manner. ACMSD activity is shown as the amount of enzyme that generates μmoles of AMS per hour per milligram of protein. QPRT activity is shown as the amount of enzyme that generates μmoles of nicotinic acid mononucleotide per hour per milligram of protein. Values are means ± SEM, n = 5. *P < 0.05 versus the control.

tryptophan–NAD pathway but did not change the excretion of 3-HA, suggesting involvement of ACMSD in this phthalate ester-induced change of the tryptophan metabolism; the cellular concentration of ACMSD protein may be decreased or ACMSD activity may be directly inhibited by these esters. To test the latter possibility, rat, mouse, and human ACMSD activities were measured *in vitro* in the presence and absence of phthalate monoesters such as MEP, MBP, MHP, and MEHP. Extracts from rat livers, mouse kidneys, and COS-7 cells that express human recombinant ACMSD were used as enzyme sources. To show the specificity of the inhibition, QPRT from rat livers and COS-7 cells producing human enzyme were also assayed. Assays with the corresponding diesters could not be performed because the addition of the diesters caused turbidity in the mixture for the enzyme assay. Table 1 shows the results when ACMSD was assayed with and without 3 mmol/l phthalate ester. The high activity of human ACMSD compared with that from rats and mice, is probably due to the high expression of this recombinant human enzyme in COS cells. The same would be true for QPRT. MEP, the monoester with the shortest chain, was not inhibitory whereas low inhibition was found with MBP. The inhibition became more potent as the length of the side chains in esters became longer; MHP and MEHP severely blocked the ACMSD activity from all sources, and MEHP was the most potent inhibitor. In contrast, neither human nor rat QPRT was affected by any of the phthalate monoesters. Figure 4A shows a dose-dependent inhibition of human ACMSD by MEHP; the presence of MEHP at 0.3 mmol/l caused a significant inhibition, and at 3 mmol/l the activity was inhibited by more than 90%. In agreement with Table 1, MEHP was not inhibitory to human QPRT at any of the concentrations tested (Fig. 4B).

To investigate whether the inhibition of ACMSD by MEHP was reversible or whether MEHP irreversibly inactivated the enzyme, human ACMSD was incubated for 10 min at 25°C with 3 mmol/l MEHP, at which the ACMSD activity would be inhibited by more than 90% (see Fig. 4). The enzyme was then diluted fivefold with 50 mmol/l Tris-HCl buffer, pH 7.6, and the diluted enzyme preparation was subjected to the enzyme assay. The final concentration of MEHP in the assay mixture was 0.06 mmol/l at which the inhibition of the enzyme should be negligible if the inhibition is reversible. The activity of the

diluted ACMSD was found to be comparable to that of the control enzyme diluted without treatment with MEHP (data not shown), indicating that the inhibition is reversible. A similar result was obtained using rat ACMSD.

DISCUSSION

This study was undertaken to investigate effects of phthalate esters orally given to rats on the tryptophan metabolism. DEHP, which has been most widely used in the formation of plastics, and its primary metabolite, MEHP, caused the most dramatic change in tryptophan metabolism. They markedly enhanced the urinary excretion of QA and its downstream metabolites, while the excretion of 3-HA was unchanged, raising the possibility that ACMSD, a critical enzyme acting at the branching point of tryptophan metabolism, is inhibited by these phthalate esters. In fact, MEHP reversibly inhibited ACMSD from human, mouse, and rat sources in a dose-dependent manner. Some of phthalate esters with different side chains promoted urinary excretion and inhibited ACMSD but their potency was far less than that of DEHP and MEHP. Thus it is very likely that the inhibition of ACMSD by phthalate esters blocks conversion of tryptophan to acetyl-CoA and directs tryptophan metabolism to the NAD pathway (see Fig. 1), and thus cellular QA and Nam metabolites are accumulated, resulting in their increased excretion into the urine.

Isenberg *et al.* (2000) explored the metabolism of DEHP given orally to rats. MEHP was the most prominent hepatic metabolite of DEHP, and elevation of the hepatic MEHP concentration was time-dependent and dose-dependent, whereas the levels of DEHP and phthalate were minimal and did not correlate with the dose of DEHP or the time after its administration (Isenberg *et al.*, 2000). Oral administration of phthalate and 2-ethylhexanol, hydrolysis products of DEHP, did not affect the conversion rate of tryptophan to NAD (Fukuwatari *et al.*, 2002b). In agreement with the proposal that phthalate monoesters are produced from the diesters in the intestine before absorption (Lake *et al.*, 1977), these results indicate that MEHP is mainly responsible for the perturbation of tryptophan metabolism.

According to Isenberg *et al.* (2000), hepatic MEHP concentration in rats fed 1000 ppm DEHP for 2 weeks, conditions similar to those used in the present experiments, was 9 μmol/g tissue. *In vitro* inhibition of ACMSD by MEHP was apparent (33%) at 0.3 mmol/l and greater than 90% at 3 mmol/l. These results suggest that the liver in rats fed 1000 ppm DEHP accumulates MEHP at the concentration sufficient to exhibit its inhibitory effect on ACMSD, although all of the MEHP molecules in the liver may not necessarily be available for this inhibition.

Previously we showed that ACMSD activity in the liver extracts from rats fed DEHP was similar to that of control animals (Fukuwatari *et al.*, 2002b). This result is not contradictory to our present finding that ACMSD is inhibited *in vitro* by MEHP. The inhibition is reversible, and therefore, even if MEHP is accumulated in the liver of rats fed DEHP at concentrations sufficient to block ACMSD, MEHP would be washed out during the preparation of the enzyme; the resulting enzyme preparations would contain MEHP at levels that show little inhibition of ACMSD. Taken together, we conclude that phthalate esters perturb tryptophan metabolism through direct inhibition of ACMSD but not by reducing the ACMSD protein level. The mechanism by which ACMSD is inhibited remains to be examined. The very labile nature of ACMS, the substrate of ACMSD, hampers kinetic studies of this enzyme.

Quinolinate is a potential endogenous toxin; it is neurotoxic and has been suspected of being involved in the development of a number of brain diseases (see review by Stone and Darlington, 2002). Although it is believed that the liver is a major site of tryptophan metabolism, expression of ACMSD, as well as its mRNA in the brain and kidney (Fukuoka *et al.*, 2002), suggests that the phthalate ester–induced metabolic alteration occurs in these organs. However, to date, there are no reports that show neurotoxicity of phthalate esters. When DEHP (0–200 mg/kg/day) was given to rats via oral gavage, few adverse effects on neurobehavioral evaluations were found (Moser *et al.*, 2003). DEHP given to mice through the diet to provide levels of 0.01–0.09% did not show detrimental effects on neurobehavioral parameters (Tanaka, 2002). Examination of tissue distribution by the use of the radioactive DEHP did not show significant accumulation of the radioactivity in the brain of rats (Tanaka *et al.*, 1975) and mice during the pre-weaning period (Eriksson and Darnerud, 1985). Quinolinate also can be a uremic toxin responsible for anemia with renal failure by reducing the renal production of erythropoietin, a growth factor essential for erythrocyte formation (Pawlak *et al.*, 2003). When DEHP at a high dose (12,500 ppm in the diet) was given to rats, the erythrocyte count, hemoglobin, and hematocrit values were significantly lower than controls, but these effects were not found with a lower dose (2500 ppm; David *et al.*, 2000). Although no data indicating that phthalate esters exhibit adverse effects through accumulation of QA are available, effects of the concurrent intake of a tryptophan-rich diet and phthalate esters are worthy of further investigation. Such a series of misfortunes may contribute to triggering and/or exacerbating various diseases.

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Phthalate Ester Effects on Rat Sertoli Cell Function *in Vitro*: Effects of Phthalate Side Chain and Age of Animal

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Mono(2-ethylhexyl) phthalate (MEHP), the active metabolite of the testicular toxicant di(2-ethylhexyl) phthalate, inhibits FSH-stimulated rat Sertoli cell cAMP accumulation, stimulates basal lactate production, and decreases intracellular ATP levels *in vitro*. Dibutyl phthalate and dipentyl phthalate but not diethyl-dimethyl or dipropyl are also age-dependent testicular toxicants *in vivo*. We therefore examined the effect of animal age and phthalate monoester on the Sertoli cell FSH-stimulated cAMP accumulation, lactate secretion, and ATP levels in order to determine if these effects are part of the mechanism of action of phthalate esters *in vivo*. MEHP, monobutyl and monopentyl phthalates but not the monoethyl, monomethyl, or monopropyl phthalates inhibited FSH-stimulated cAMP accumulation, a segregation which matches the *in vivo* toxicity potential of these agents. MEHP and monopentyl, but not monobutyl phthalates, also stimulated Sertoli cell lactate secretion. The effect of the active phthalates on FSH-stimulated cAMP accumulation and lactate secretion is not dependent on age of animal over a range of 13–80 days, suggesting that the age-related toxicity *in vivo* may be related to differences in metabolism and disposition rather than tissue sensitivity. Since the ED_{50} of MEHP inhibition of cAMP accumulation and lactate secretion is similar, these two effects may be related to a common initial effect of the active phthalates. Inhibition of intracellular ATP levels is specific for MEHP and is lost with age (>28 days of age) and thus is not likely to be an essential part of the *in vivo* mechanism of action of phthalate diesters.

Esters of *o*-phthalic acid are used extensively in consumer products and medical devices. While in general, acute toxicity of the most commonly encountered phthalates is low, some phthalates have been shown to be carcinogenic, to result in liver toxicity, and to cause reproductive toxicity at high doses (see Thomas and Thomas, 1984; Albro, 1986; and Woodward, 1988, for reviews). Specifically, several phthalate esters including di(2-ethylhexyl) phthalate (DEHP), dibutyl phthalate (DBP), dipentyl phthalate (DPP), and dihexyl phthalate (DHP) are male reproductive toxicants in rats and mice. The short chain diethyl, dimethyl, dipropyl, and the long chain dioctyl (DOP) derivatives, on the other hand, are not toxic to the male reproductive system of rats and mice (Gray *et al.*, 1977; Oishi and Hiraga, 1980b; Creasy *et al.*, 1983; Lamb *et al.*, 1987; Woodward, 1988; and Heindel *et al.*, 1989). The monoester derivatives of DBP, DOP, and DEHP have been shown to produce the same effect (or lack thereof) *in vivo* as the diesters. Metabolic studies indicate that orally administered phthalate diesters are rapidly hydrolyzed to the corresponding monoesters by nonspecific esterases in the gut and other tissues (Albro *et al.*, 1973; Lake *et al.*, 1977; and Oishi and Hiraga, 1980a,b). Since the hydrolysis of the second ester linkage yielding phthalic acid occurs much more slowly, the monoesters are the major metabolite and the proximal toxicant.

The testicular toxicity of DEHP can be mimicked *in vivo* by MEHP (mono-(2-ethylhexyl) phthalate) but not by MEHP-derived metabolites (Sjoberg *et al.*, 1986a). While similar detailed studies have not been completed for all the phthalate diesters toxic to the male reproductive system, the available data suggest that they cause a similar testicular lesion and that the toxicity is due to the mono-substituted derivative (Cater *et al.*, 1977; Oishi and Hiraga, 1980b; and Fukuota *et al.*, 1989).

The testicular lesion produced by DBP, DPP, DHP, and DEHP in immature rats is characterized by early sloughing of spermatids and spermatocytes and severe vacuolation of Sertoli cell cytoplasm (Cater *et al.*, 1977; Foster *et al.*, 1982; Fukuota *et al.*, 1989). Further studies have shown that with DPP (and presumably for the other active phthalates), the Sertoli cell is the initial and primary target (Foster *et al.*, 1982; Creasy *et al.*, 1983; Creasy *et al.*, 1987), with the result being a disturbance in Sertoli cell–germ cell interaction (Fukuota *et al.*, 1989).

In vitro studies using Sertoli cell–germ cell cocultures have shown that, in general, the monoesters of the active phthalates *in vivo* increase germ cell detachment *in vitro*, thereby

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mimicking their *in vivo* action (Gray and Beamand, 1984; Gray and Gangolli, 1986). The testicular lesion in DPP-treated adult rats was restricted to tubules in the successive stages XI–XIV, I, and II of the spermatogenic cycle, the stages with the highest FSH responsiveness (Parvinen, 1982). This prompted several investigators to examine an effect of phthalates, specifically MEHP, on FSH stimulation of cAMP accumulation in Sertoli cell cultures. Results indicated that MEHP specifically inhibits FSH-stimulated cAMP accumulation in cultured Sertoli cells (Lloyd and Foster, 1988; Heindel and Chapin, 1989). This effect appears to be due to a decreased affinity of FSH for its receptor (Grasso *et al.*, 1991). Other effects shown *in vitro* using Sertoli cell cultures include MEHP, monopentyl, monohexyl, and monooctyl stimulation of Sertoli cell lactate secretion (Chapin *et al.*, 1988; Moss *et al.*, 1988; Williams and Foster, 1989), MEHP decreased Sertoli cell pyruvate secretion (Chapin *et al.*, 1988; Williams and Foster, 1989) decreased ATP levels (MEHP) (Chapin *et al.*, 1988) and decreased mitochondrial succinate dehydrogenase activity (MEHP) (Chapin *et al.*, 1988). These data indicate that the mechanisms of action of either MEHP or phthalates in general on Sertoli cells are incomplete.

Immature animals are more sensitive to phthalate-induced testicular toxicity than adults (Gray and Butterworth, 1980; Creasy *et al.*, 1983; Sjoberg *et al.*, 1986b; Oishi, 1986). While there is not adequate data for all the phthalates, it has been shown that little or no DEHP reaches the systemic circulation after oral dosing due to activation to MEHP by stomach and intestinal lipases. Furthermore, the extent of absorption and hence the total systemic exposure to MEHP is higher in young rats (Sjoberg *et al.*, 1985). Thus the age-related differences in sensitivity are thought to be due to differences in pharmacokinetics and not tissue sensitivity (Sjoberg *et al.*, 1985). When DEHP is given iv, the age-related change in sensitivity is lost (Sjoberg *et al.*, 1986b). However, no one has examined the age-related *in vitro* sensitivity of phthalates on Sertoli cell function.

The purpose of this study is to determine whether the effects reported for MEHP in Sertoli cells cultured from immature rats are observed for other phthalates reported to produce similar testicular toxicity *in vivo*. *In vivo* phthalate testicular toxicity is both age dependent and phthalate side-chain-specific. Thus the effect of various phthalate monoesters on basal lactate secretion and FSH-stimulated cAMP accumulation were measured in Sertoli cell cultures derived from animals of various ages to determine if the *in vitro* results were related to the *in vivo* testicular toxicity.

METHODS

MEHP was synthesized by the method of Albro *et al.* (1973). Monomethyl, ethyl, propyl, butyl, and pentyl phthalates were generously supplied by Dr. Tim Gray, Sterling Research Group, England. Purity of all the monosubstituted phthalates (analyzed by HPLC and IR) ranged from 97.1–99.5%. For *in vitro* studies the phthalates were dissolved in dimethyl sulfoxide and added at 2 μl stock per ml culture medium. Controls received 2 μl/ml of DMSO. Eagle's culture media with Earle's salts (MEM) and Hanks' balanced salt solutions were obtained from GIBCO (Long Island, NY). Collagenase, trypsin, lactate, 3-acetylpyridine-adenine dinucleotide, and 1-methyl-3-isobutylxanthine (MIX) were purchased from Sigma Chemical Co. (St. Louis, MO). The RIA antibody to cAMP was prepared in collaboration with Biotek Research, Inc. (Lenexa, KS). All chemicals for the luminometric determination of ATP were obtained from LKB (Gaithersburg, MD). Iodinated cAMP tyrosine methyl ester was purchased from Biomedical Technologies, Inc. (Stoughton, MA). Ovine follicle-stimulating hormone (FSH: NIH-017) was generously supplied by the National Hormone and Pituitary Distribution Program, NIH.

Cultures. Litters of rats were reared by F344 parents in the NIEHS breeding colony under conditions described previously (Chapin *et al.*, 1987). Males at various ages (13–80 days as indicated in specific experiments) were terminated by asphyxiation with carbon dioxide, the testes removed, and Sertoli cell monolayer cultures prepared as described previously (Chapin *et al.*, 1987). Briefly, decapsulated testes were minced and sequentially incubated with trypsin and then collagenase. The dissociated peritubular cells, interstitial cells, and most germ cells were separated from clusters of Sertoli cells by consecutive washes with MEM, each wash being followed by centrifugation at 60*g* for 2 min. The general procedure for preparation of Sertoli cell monolayer cultures was similar for animals of all ages except that clusters of Sertoli cells from the older animals (≥36 days) required more washes for a shorter time (1 min) to separate them from the contaminating individual germ cells. All cultures contained a significant number of germ cells attached to the Sertoli cells. In general, the older the animals from which the cultures were prepared the more germ cells were attached to the Sertoli cells at the time of culture. However, by Days 3–5 of culture and after several media changes, the germ cell contamination (mostly spermatogonia) was in general similar in cultures from variously aged animals. Sertoli cells were cultured in stationary 24-well culture plates (16-mm Costar multiwell, Costar, Cambridge, MA) with 1 ml MEM at 34°C in 5% CO_2/95% air without antibiotics or additives. Medium was changed 24 hr before experimentation, on Day 3 or 4 of culture. Sertoli cells from each animal, regardless of age, were sufficient to provide a monolayer of cells (~100 μg protein/well) in approximately one costar plate of 24 wells. Sertoli cells from a given preparation (10–30 animals) were pooled and used to examine the effects of various phthalates.

Preincubation procedure. Unless noted in figure legends, phthalates were added at the time of medium change, 24 hr prior to hormone challenge for cAMP measurements, media removal for lactate measurement, or extraction of intracellular ATP.

Assays. Media lactate was assayed by the method of Maurer and Poppendiek (1974) using 3-acetylpyridine adenine dinucleotide (APAD) as cofactor. The method was modified to use 33% less APAD after first determining that this did not change the rate or endpoint of the reaction. At the times indicated under Results, media was removed from the culture wells and centrifuged to remove any cells, and the cell-free supernatant was frozen in dry ice and stored at −80°C until assayed (less than 7 days after collection). The data are expressed as μg lactate/mg protein/24 hr.

For determination of cellular ATP, the media were removed and the cells were overlayed with 1 ml of 5% trichloroacetic acid containing 2 mM ethylenediaminetetraacetic acid (EDTA) to inactivate phosphatases and extract the ATP. An aliquot was diluted 1:20 with 0.1 M Tris–acetate, pH 7.75, containing 2 mM EDTA, and either assayed fresh or frozen on dry-ice and stored at −80°C for <5 days. ATP was assayed luminometrically on an LKB 1251 luminometer. The assay was linear from 10^{-7} to 10^{-12} M ATP; standards were made up in Tris–acetate as indicated in the LKB luminometer manual. Each sample was corrected for internal quenching. The ATP data are expressed as nmol ATP/mg protein.

cAMP measurement. Cultures were rinsed with MEM and incubated for 30 min in 500 μl MEM containing varying concentrations of FSH as

indicated in the figures. MEHP was not present during the incubation period. Incubation was terminated by aspiration of the media and addition of 1 ml of 95% ethanol.

Cyclic nucleotide extraction and assay. Cyclic cAMP was released from the cells by disruption with a 3-sec burst from a Branson Sonifier equipped with a microtip and an output setting of 2. Sonicates were centrifuged at 1000*g* for 30 min at 4°C, and aliquots of the supernatants were dried for cAMP radioimmunoassay as previously described (Heindel *et al.*, 1975). Levels of intracellular cAMP are expressed as pmol cAMP/mg protein.

Protein determination. Cell monolayers were dissolved in 1 ml 0.1 N NaOH and protein was measured by the method of Schacterle and Pollack (1973).

Statistics. Differences between groups were identified by analysis of variance, and the significance of the difference between group means was assessed by Student's *t* test. All statistical comparisons were verses control values only. Table 1 and Figs. 1 and 4 were calculated based on fitting the data to the logistic model

$$A + B\frac{1}{1 + e^{-C(\ln[MEHP]-D)}},$$

where A is the control value, B is the amount added or subtracted at infinite dose, C is the parameter controlling steepness, and D is the log dose at which half the potential change has occurred. Results were considered statistically significant at $p < 0.05$.

RESULTS

The concentration dependence of MEHP inhibition of FSH-induced cAMP accumulation in Sertoli cells cultured from 18- or 42-day-old rats is evident in Fig. 1. MEHP inhibits FSH-induced cAMP accumulation approximately 60% in Sertoli cells from rats at either age. Also the concentration of MEHP which results in a half maximal inhibition of cAMP accumulation, 4 μM, is the same at both ages. This lack of effect of age on the ability of MEHP to inhibit FSH-induced cAMP accumulation is confirmed in Fig. 2. The effect of 100 μm MEHP is to inhibit this FSH effect by approximately 50–60% regardless of the age of the animals from which the Sertoli cells were cultured from 13 to 80 days of age.

MEHP has also been shown to stimulate basal lactate secretion. This action of MEHP is evident in Sertoli cells cultured from animals 13 to 60 days of age (Fig. 3). In addition, as shown in Fig. 4, the concentration of MEHP which results in a half maximal lactate stimulation (4–6 μM) does not vary with age of the cultured Sertoli cells. The age independence of the effect of MEHP on cAMP and lactate production is summarized in Table 1. The ID_{50} of MEHP on FSH-stimulated cAMP accumulation is similar to the ED_{50} for its effect on lactate production.

To determine whether the effects of MEHP on Sertoli cell cAMP accumulation and lactate production *in vitro* would be shared by other phthalate esters, the monoesters of other phthalates were examined. As can be seen from Tables 2 and 3, phthalate monoesters with side chains of one, two, or three carbons had no effect on the ability of FSH to stimulate either cAMP accumulation or lactate production. However, at least in Sertoli cells from the older animals, both monobutyl and monopentyl phthalate (100 μM) inhibited FSH-stimulated cAMP accumulation 40–60%.

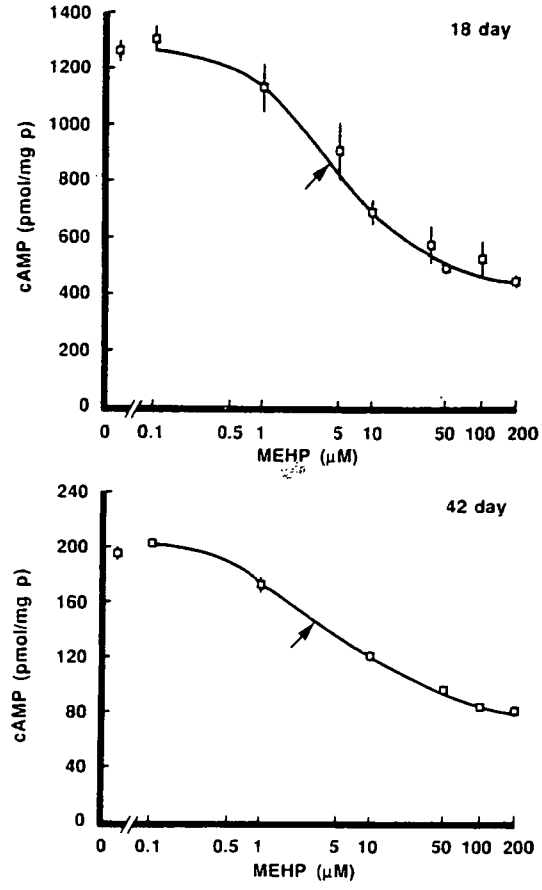

FIG. 1. Concentration dependence of MEHP inhibition of FSH-stimulated cAMP accumulation in Sertoli cells cultured from 18- and 42-day-old rats. Sertoli cells cultured from 18- or 42-day-old rats were incubated with MEHP at the concentrations indicated for 24 hr, the media was changed, and the cells were incubated with 1 μg/ml FSH plus 1 mM MIX for 30 min. Intracellular cAMP was measured by RIA. Each point is the average of quadruplicate determinations ± SE from a representative experiment. The arrows indicate the ED_{50} of 4 μM for both age groups.

Only monopentyl phthalate stimulated lactate production, doing so in Sertoli cells from animals of 13 through 60 days of age with no effect in cells from 80-day-old animals.

MEHP has also been shown to reduce intracellular levels of ATP. The data in Table 4 confirm this effect and also show that this effect is lost with age of animal from which the Sertoli cells are prepared. Also no other phthalate monoester had an effect on cellular ATP levels at any age of culture.

DISCUSSION

The fact that younger animals are more sensitive to the testicular toxicity of phthalates coupled with the spermato-

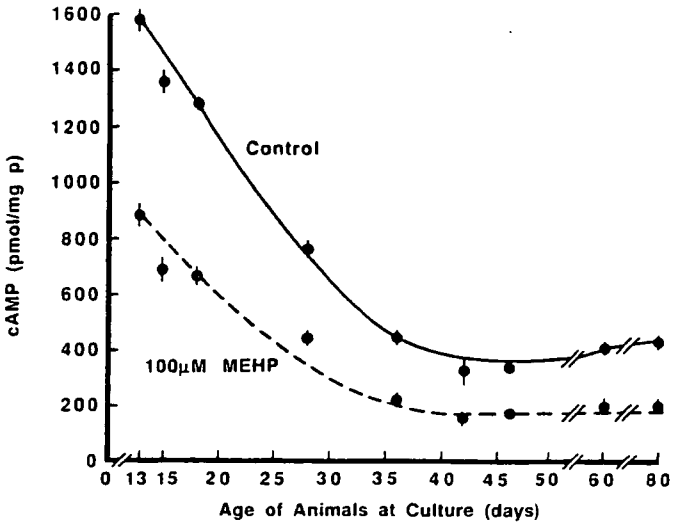

FIG. 2. Age dependence of MEHP-induced inhibition of FSH-stimulated cAMP accumulation in cultured Sertoli cells. Sertoli cells cultured from rats 13–80 days of age were preincubated with 100 μM MEHP or control (2 μl DMSO/ml) for 24 hr, the media were changed, and the cells were incubated with 1 μg/ml FSH plus 1 mM MIX for 30 min. Intracellular cAMP was measured by RIA. Each point is the average ± SE of quadruplicate determinations from a separate experiment. All MEHP values are significantly decreased from their corresponding control values.

genic stage specificity of the DPP testicular toxicity strongly suggests that there may be a hormonal component to the effects of phthalates on Sertoli cells. Indeed Sertoli cells from younger animals are more responsive to the FSH stimulation of cAMP accumulation than those from adults, and the FSH

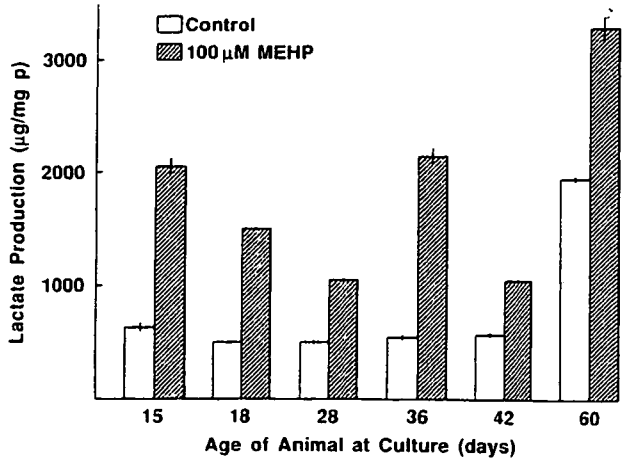

FIG. 3. Age independence of MEHP stimulation of lactate production by cultured Sertoli cells. Sertoli cells cultured from rats at the ages indicated were incubated for 24 hr in the presence of 100 μM MEHP or control media. Media were then assayed for lactate content as described under Methods. Each bar is the average of quadruplicate determinations from three separate experiments ± SE. All MEHP values are significantly increased over those of the corresponding controls.

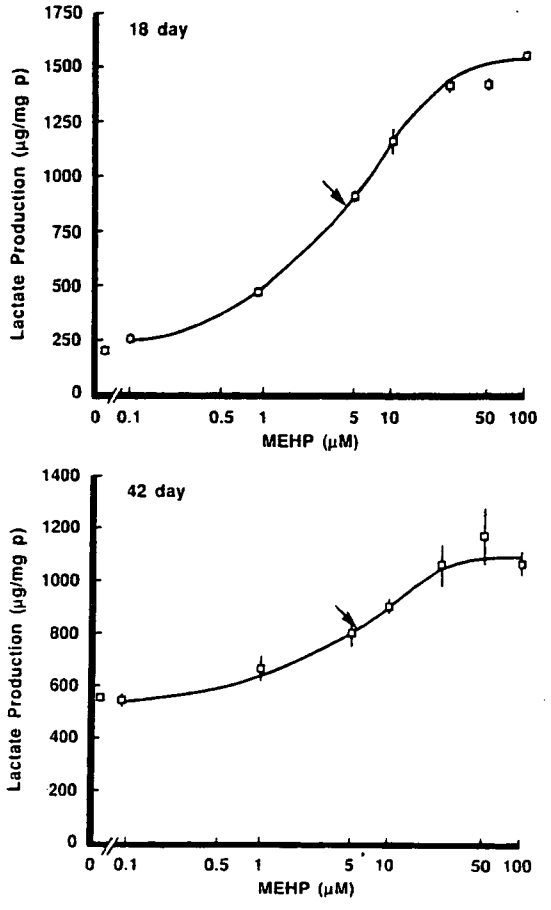

FIG. 4. Concentration dependence of MEHP stimulation of basal lactate production from Sertoli cells cultured from 18- and 42-day-old rats. Sertoli cells from 18- and 42-day-old rats were incubated with MEHP at the concentrations indicated for 24 hr and the media were removed and the lactate concentration was measured as described under Methods. Each point is the average ± SE of quadruplicate determinations from a representative experiment. The arrows indicate the ED_{50} concentration of 4 and 6 μM, respectively, for cells from 18- or 42-day-old rats.

response of Sertoli cells in adults is spermatogenic-stage specific. The stage specificity of Sertoli cell responsiveness to FSH matches that of their sensitivity to DPP. Thus the data of Lloyd and Foster (1988), Heindel and Chapin (1989), and Grasso *et al.* (1991) that showed an effect of MEHP *in vitro* to inhibit FSH-stimulated cAMP accumulation in cultured Sertoli cells pointed to a mechanism which could account for the phthalate Sertoli cell toxicity. The present data confirm this possibility in that not only did MEHP decrease FSH-stimulated cAMP accumulation but also the monobutyl and monopentyl phthalates. The fact that the order of potency of MEHP > MPP > MBP is slightly different from the *in vivo* toxicity which for the rat is DPP > DEHP > DBP (Foster *et al.*, 1980), may be related to differences in metabolism and disposition *in vivo*. Perhaps more importantly, the nontoxic phthalates monoethyl, monomethyl, and mono-

TABLE 1
Age Independence of MEHP Alteration of Sertoli Cell FSH-Stimulated cAMP Accumulation and Basal Lactate Production

	cAMP accumulation		Lactate production	
Age (days)	MEHP $ID_{50}(\mu M)$	Maximal inhibition (%)	MEHP $ED_{50}(\mu M)$	Maximal stimulation (fold)
13	7 (5,9)	66	6 (5,8)	1.9
15	5 (2,4,8)	67	5 (3,6,7)	3.1
18	4 (3,5,5,4)	68	4 (2,4,6.5)	3.1
28	9 (5,13)	65	5 (3,7)	2.2
36	6 (4,6,7)	60	6 (5,8,4)	3.2
42	4 (5,3)	60	6 (3,9)	2.0
60	6 (6,6)	54	12 (9,15)	1.9
82	2 (2,1,4)	66	4 (2,5,6)	2.0

Note. ID_{50} values were computed from 6-point dose–response curves (0.1–200 μM) (see under Methods). Maximal inhibition is that which occurred after a 24-hr preincubation with 100 μM MEHP. Values for both parameters are the averages from two to four separate experiments at each age (i.e., n = 2–4).

propyl had no effect on FSH-stimulated cAMP accumulation, indicating that this *in vitro* action has the proper specificity for an important *in vivo* mechanism.

The ability of MEHP to inhibit Sertoli cell cAMP accumulation *in vitro* is age independent, a phenomenon which is different from the *in vivo* data. This suggests the age-related differences in toxicity may be due to pharmacokinetic differences (i.e., absorption) (Sjoberg *et al.*, 1985) and not to tissue sensitivity. It must be noted, however, that while the actual MEHP inhibition is not age dependent, the effect of FSH to stimulate cAMP is age dependent. Therefore, the absolute decrease in Sertoli cell cAMP due to MEHP is greater in the cells from the younger animals which could also contribute to the age-related differences in sensitivity to DEHP *in vivo*. Thus we would conclude that the effect of phthalate monoesters to inhibit FSH-stimulated cAMP accumulation is likely to be at least a part of the mechanism responsible for their testicular toxicity.

Stimulation of lactate secretion has also been proposed as part of the action of phthalate monoesters on Sertoli cell–germ cell function. In this regard, we have confirmed the results of Chapin *et al.* (1988), Moss *et al.* (1988), and Williams and Foster (1989) which showed that MEHP increases Sertoli cell lactate production. We have extended these results to show that this stimulation of lactate production is evident in Sertoli cells cultured from animals aged 13–82 days. Indeed the ID_{50} for inhibition of cAMP accumulation and the ED_{50} for stimulation of lactate secretion are similar for all age groups, suggesting the possibility that these two effects may be somehow related. The stimulation of lactate production is also apparent and unrelated to age with monopentyl phthalate but not with the nontoxic methyl, ethyl, and propyl side-chain phthalates. We could not, however, show an effect of monobutyl phthalate on lactate production at any age which agrees with the data of Moss *et al.* (1988) who used Sertoli cells from 28-day-old rats. Whether this implies that stimulation of lactate production is not part of the general mechanism of action of phthalate esters or that this lack of stimulation is because monobutyl phthalate is the least toxic phthalate, as far as Sertoli cell toxicity is concerned, is not clear.

In the liver, MEHP and MBP have been shown to inhibit mitochondrial respiration by altering the permeability properties of the inner membrane and by inhibiting succinate

TABLE 2
Effect of Age of Animal on the Ability of Phthalate Monoesters to Inhibit FSH-Stimulated cAMP Accumulation in Cultured Sertoli Cells

Age of animal (days)	Phthalate monoester side chain (100 μM)					
	Control	Methyl	Ethyl	Propyl	Butyl	Pentyl
	cAMP (pmol/mgp)					
13	1157 ± 67	1328 ± 113	1180 ± 59	1248 ± 31	1190 ± 56	1046 ± 76
15	1191 ± 25	1315 ± 167	1362 ± 147	1081 ± 56	1176 ± 60	1163 ± 73
18	1224 ± 54	1428 ± 78	1368 ± 132	1260 ± 69	1415 ± 93	1284 ± 41
28	916 ± 34	1027 ± 53	837 ± 30	948 ± 56	940 ± 62	568 ± 45*
36	579 ± 22	543 ± 18	596 ± 26	569 ± 19	558 ± 30	454 ± 38*
46	330 ± 8	331 ± 6	320 ± 7	333 ± 14	266 ± 8*	223 ± 7*
60	466 ± 35	480 ± 20	405 ± 13	409 ± 9	243 ± 33*	268 ± 7*

Note. Sertoli cells cultured from animals at the ages indicated were preincubated with the phthalate monoesters for 24 hr and then incubated with FSH (1 μg/ml) for 30 min in the presence of 1 mM MIX. Values are the averages of quadruplicate determinations from a representative experiment. Each experiment was repeated two to four times with similar results.

* Denotes statistically different from the corresponding control value at $p < 0.05$.

TABLE 3
Effect of Age of Animal on the Ability of Phthalate Monoesters to Stimulate Basal Lactate Production in Cultured Sertoli Cells

Age of animal (days)	Phthalate monoester side chain (100 μM)					
	Control	Methyl	Ethyl	Propyl	Butyl	Pentyl
	Lactate production (μg/mg p/24 hr)					
13	362 ± 24	420 ± 10	435 ± 20	502 ± 23	550 ± 35	670 ± 22*
15	782 ± 46	718 ± 46	795 ± 178	790 ± 78	800 ± 73	1420 ± 105*
18	497 ± 46	568 ± 20	534 ± 32	561 ± 26	560 ± 57	671 ± 56*
28	492 ± 16	458 ± 9	434 ± 21	447 ± 20	453 ± 32	737 ± 19*
36	607 ± 47	754 ± 120	631 ± 45	612 ± 94	649 ± 54	1086 ± 105*
42	636 ± 54	641 ± 77	587 ± 29	666 ± 58	688 ± 55	1039 ± 105*
60	679 ± 44	674 ± 79	668 ± 27	743 ± 26	718 ± 16	1218 ± 26*
80	197 ± 7	292 ± 18*	191 ± 16	166 ± 10	164 ± 8	226 ± 26

Note. Sertoli cells cultured from animals at the ages indicated were incubated with phthalate monoesters for 24 hr. Media were analyzed for lactate. Values are the averages ± SE of quadruplicate determinations from a representative experiment. Each experiment was repeated two to four times with similar results.

* Denotes statistically different from the corresponding control values at $p < 0.05$.

dehydrogenase activity in a noncompetitive manner. DPP was not tested (Melnick and Schiller, 1982). Thus, there is precedence for an effect of phthalate monoesters to alter energy metabolism. *In vivo,* DPP was shown histochemically to reduce succinate dehydrogenase activity in mitochondria of Sertoli cells (Foster *et al.,* 1982; Gangolli, 1982). Chapin *et al.* (1988) reported that MEHP inhibited Sertoli cell succinate dehydrogenase activity in a mixed manner. They were, however, unable to detect changes in mitochondrial membrane potential as was shown for liver mitochondria (Inouye *et al.,* 1978; Melnick and Schiller, 1985). Additionally, hepatic mitochondria swell when exposed to specific phthalate esters (Lake *et al.,* 1975; Ohyama, 1977; Melnick and Schiller, 1985). The literature on Sertoli cell mitochondria are conflicting, with some reports showing mitochondrial condensation after *in vivo* exposure to phthalates (Foster *et al.,* 1982) while others show mitochondrial hypertrophy after phthalate exposure *in vivo* or *in vitro* (Creasy *et al.,* 1983, 1987).

Our data show that only MEHP results in reduced ATP levels, an effect which only occurred in Sertoli cells from immature animals. Thus, while the data suggest that at least some of the phthalate esters (MEHP, DPP) may alter energy metabolism, the effects are not as impressive as those reported for liver. More information is needed, perhaps with more sensitive probes of mitochondrial function, to ascertain the

TABLE 4
Effect of Age of Animal on the Ability of Phthalate Monoesters to Decrease Cellular ATP Levels in Cultured Sertoli Cells

Age of animal (days)	Phthalate monoester side chain (100 μM)						
	Control	Methyl	Ethyl	Propyl	Butyl	Pentyl	Ethylhexyl
	ATP ($\times 10^8$ mol/mgp)						
13	2.34 ± 0.14	2.22 ± 0.22	2.30 ± 0.09	2.10 ± 0.08	2.18 ± 0.07	2.05 ± 0.07	1.98 ± 0.05*
15	4.81 ± 0.26	4.31 ± 0.23	4.48 ± 0.24	4.30 ± 0.27	4.31 ± 0.21	4.44 ± 0.81	3.47 ± 0.12*
18	6.46 ± 0.18	6.70 ± 0.38	7.16 ± 0.38	7.18 ± 0.20	7.19 ± 0.27	6.60 ± 0.36	4.64 ± 0.08*
28	5.45 ± 0.31	4.81 ± 0.16	4.97 ± 0.31	5.26 ± 0.16	4.98 ± 0.19	5.40 ± 0.22	3.66 ± 0.09*
36	6.29 ± 0.62	6.04 ± 0.36	7.05 ± 0.33	6.69 ± 0.80	6.80 ± 1.0	7.00 ± 0.30	6.33 ± 0.40
46	4.39 ± 0.29	4.42 ± 0.12	3.89 ± 0.07	3.99 ± 0.18	3.97 ± 0.15	3.68 ± 0.09	4.48 ± 1.04
66	4.43 ± 0.01	4.45 ± 0.01	4.44 ± 0.02	4.47 ± 0.01	4.41 ± 0.02	4.40 ± 0.02	4.39 ± 0.01

Note. Sertoli cells cultured from animals at the ages indicated were incubated with phthalate monoesters for 24 hr. Cells were analyzed for intracellular levels of ATP. Values are the average ± SE of quadruplicate determinations from a representative experiment. Each experiment was repeated two to four times with similar results.

* Denotes statistically different from corresponding control at $p < 0.05$.

importance of mitochondria as a site of action of the active phthalate esters.

In summary, our studies *in vitro* with various phthalates indicate that the effect on FSH-induced cAMP accumulation is a part of the phthalate-induced Sertoli cell toxicity. Furthermore, evaluation of age differences of Sertoli cells *in vitro* to this effect suggest that the age-related sensitivity seen *in vivo* is likely due to differences in absorption, metabolism, and disposition and also to the differential sensitivity of Sertoli cells from different age animals to FSH. Lactate secretion is stimulated in Sertoli cells by phthalates from all ages and, at least for MEHP, occurs at the same concentrations as the inhibition of FSH-stimulated AMP accumulation. This effect on lactate secretion is specific for the most toxic phthalates, and thus may also be part of the mechanism of action of phthalates on Sertoli cells. However, it is not clear if the effects of MEHP on FSH-stimulated cAMP accumulation, lactate production, and ATP levels are interrelated or separate effects of MEHP. Also, it is not possible from the data obtained thus far to determine if these effects of MEHP are "the" cause of Sertoli cell toxicity and therefore gonadal toxicity seen *in vivo* or if they are the consequence of an as yet unidentified action(s) of the phthalate esters on Sertoli cells.

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