Alterations of Activities of Cytosolic Phospholipase A2 and Arachidonic Acid-Metabolizing Enzymes in Di-(2-Ethylhexyl)Phthalate-Induced Testicular Atrophy

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ABSTRACT. Di-(2-ethylhexyl) phthalate (DEHP), a peroxisome proliferator-activated receptor α (PPARα) ligand, alters the lipid composition of rat testis, yet the mechanism is unclear. In this study, we investigated the effect of DEHP on the synthesis and metabolism of arachidonic acid (AA), a precursor of eicosanoids, in the testis of prepubertal rats. DEHP (100 and 1,000 mg/kg, 5 days) administration caused a significant reduction in activity of cytosolic phospholipase A2 (cPLA2), the rate-limiting enzyme in the AA and eicosanoid synthesis pathways. DEHP increased the expression of 12-lipoxygenase (12-LOX) in rat testis, whereas cyclooxygenase-2 (COX-2) expression was not altered. Cytochrome P450 4A1 (CYP4A1), a product of a PPARα-regulated gene, was markedly increased in the testis by DEHP administration. Taken together, DEHP suppresses cPLA2 activity and induces the AA metabolizing enzymes such as 12-LOX and CYP4A1, resulting in the reduction of AA level. These data suggest that altered AA metabolic cascades may be related to the decrease of testosterone concentration in DEHP-induced testicular atrophy.

KEY WORDS: arachidonic acid, cytosolic phospholipase A2, di-(2-ethylhexyl) phthalate, 12-lipoxygenase, testis.


The exposure of animals to phthalate esters can result in a significant perturbation of normal lipid metabolism in liver, heart, testis, adrenal gland and brain [4]. The plasticizer, di-(2-ethylhexyl) phthalate (DEHP), is a widespread environmental chemical due to its common use in the production of plastic medical devices and food packaging. On the exposure to phthalates for the human, certain populations may be exposed to much higher levels. The range of DEHP concentrations in medical devises are 3.1–650 mg/L [49]. Patients undergoing hemodialysis or blood transfusion received as much as 0.5–360 mg or 14–600 mg of DEHP via plastic medical devices, respectively [17, 23]. Ingestion of DEHP, a peroxisome proliferator-activated receptor α (PPARα) ligand, by rats resulted in the increase of non-esterified fatty acids and phospholipids and changes in lipid composition in the testis [36]. Actually, altered lipid metabolism in the testis is frequently associated with testicular atrophy [11].

Peroxisomes are ubiquitous eukaryotic organelles that play a key role in regulating lipid homeostasis in mammals. A peroxisome proliferator induces a broad spectrum of responses such as cell proliferation, decreased apoptosis, altered estradiol levels, increased metabolism of fatty acids and eicosanoids and carcinogenesis in the rodent liver [12]. DEHP activates many genes involved in lipid metabolism including fatty acyl-CoA oxidase, 3-ketoacyl-CoA thiolase and cytochrome P450 4A1 (CYP4A1) through PPAR in the liver [3]. Although DEHP is a well-known reproductive toxicant, the mechanism of its toxicity on lipid metabolism in the testis is still unclear.

Phospholipase A2 represents a large superfamily of enzymes that catalyze the hydrolysis of phospholipids at the sn-2 position, liberating free fatty acids and lysophospholipids. Cytosolic phospholipase A2 (cPLA2, 85-kD) is responsible for the release of arachidonic acid (AA), a precursor for the synthesis of eicosanoids and bioactive lipid mediators, from cellular phospholipids in many cell types in response to a variety of physiological and pharmacological agonists. AA and its metabolites play an important role in a variety of biological processes, including signal transduction, chemotaxis, cell proliferation, differentiation, apoptosis and testicular steroidogenesis [42, 50]. AA may be involved in apoptosis in vascular smooth muscle [39] and also influence cellular processes at a molecular level, capable of modulating breast cancer cell growth [9]. Otherwise, free fatty acids (FFA) such as palmitic acid and stearic acid induced apoptosis of the Leydig cells by ceramide production and AA can partly prevent the apoptotic effect induced by FFA [31]. In addition, administration of exogenous AA induced a dose-dependent increase of testosterone production by rat Leydig cells [42]. The role of cPLA2 in AA metabolism has been highlighted by recent experiments using cPLA2 knockout mice [6]. Intracellular calcium levels, protein kinase C (PKC) and mitogen-activated protein kinase (MAPK) activities are involved in the regulation of cPLA2 activity [2, 8, 34]. AA, which is generated by cPLA2, is metabolized to thromboxane A2, 12-hydroxyeicosatetraenoic acid (12-HETE) and 20-HETE by cyclooxygenase-2 (COX-2), 12-lipoxygenase (12-LOX) and CYP4A1, respectively [20].

We have recently demonstrated that DEHP disrupts the metabolism of testosterone in prepubertal rat testis [24]. There are several factors, such as hormones, zinc concentration, reactive oxygen species and apoptosis, involved in the toxic mechanisms of DEHP-induced testicular atrophy [15,
22]. However, the molecular mechanisms of testicular atrophy remain unclear, and the effect of DEHP on the lipid metabolism pathway has not been reported. We hypothesized that reduced AA concentration might be related to the decrease of testosterone concentration in the DEHP-induced testicular atrophy. The present study was designed to evaluate effects of DEHP on synthesis and metabolism of AA in the testis of prepubertal rats.

MATERIALS AND METHODS

Animals and treatment: All experiments using animals were performed under the supervision and approval of the Institutional Animal Care and Use Committee of Hokkaido University. Male Wistar rats of 3 weeks old were supplied from Japan SLC Co. (Hamamatsu, Japan). They were housed, 3 per cage, under standard laboratory conditions (24 ± 1°C, 12-hr light: 12-hr dark cycle) with food and water available ad libitum and were used for experiments after 1 week of acclimatization. Four week-old rats were orally administered with 100 or 1,000 mg/kg of di-(2-ethylhexyl) phthalate (Kanto Chemical Co., Tokyo, Japan) or vehicle (corn oil, 2 ml/kg) for 5 consecutive days. Two weeks after last dose, rats were killed with carbon dioxide, and testes were removed and weighed. A part of each testis was placed in ice-cold PBS for preparation of the S-9 fraction. Cytosol and microsomes were isolated from S-9 fractions by centrifugation at 105,000 × g for 70 min, and samples were stored at –80°C until assayed. Protein concentration was determined by the method of Lowry [30] using bovine serum albumin (Sigma Chemical Co., St. Louis, MO, U.S.A.) as a standard.

Cytosolic phospholipase A2 activity: Cytosolic phospholipase A2 (cPLA2) activity was measured with a kit from Cayman Chemicals (Ann Arbor, MI, U.S.A.) according to manufacturer’s protocol. Briefly, the reaction solution was prepared by adding 10 µl sample to 5 µl buffer. The reaction was initiated by adding 200 µl substrate solution to the wells. The plate was gently shaken for 30 sec and incubated for 60 min at room temperature. The reaction medium (blank) was recorded, and the reaction was stopped by the addition of the phospholipase A2. The absorbance at 405 nm was measured using a plate reader (Multiskan MS-UV, Labsystems, Helsinki, Finland).

Immunoblot analysis: Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was carried out with 10% polyacrylamide gel. Following electrophoresis, proteins (25 µg) were transferred to a nitrocellulose membrane by semi-dry electroblotting. The membrane was blocked in TBST (10 mM Tris-HCl, pH 7.4, 0.1 M NaCl and 0.1% Tween-20) solution containing 5% skim milk for 1 hr and then incubated with anti-cPLA2 antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, U.S.A.), anti-12-LOX antiserum, anti-COX-2 antibody (Cayman Chemicals) and CYP4A1 antibody (Daiichi Pure Chemicals Co., Tokyo, Japan) at room temperature. The membrane was incubated with horseradish peroxidase-conjugated anti-rabbit or mouse secondary antibodies. The signal was detected by chemiluminescence using the ECL reagent (Amersham, Little Chalfont, UK).

Arachidonic acid measurement by reverse phase HPLC: Arachidonic acid (AA) was measured as described previously with some modifications [33]. The sample was acidified with 100 µl citric acid (10 mM, pH 3.0) and extracted with ethyl acetate and centrifuged at 2,000 rpm for 10 min. The organic phase was transferred to another glass tube and vortexed with 500 µl of water followed by a further centrifugation. Organic phase was aspirated again and samples were dried under liquid nitrogen. Standard (AA) was obtained from Cayman Chemicals. Samples were dissolved in mobile phase, consisting of acetonitrile/water (85:15), was pumped at a flow rate of 0.9 ml/min through the ODS column (5-µm, 4.5 × 150 mm). AA was assayed using a Shimadzu LC-6A liquid chromatograph with an SPD-6AV spectrophotometric detector at the wavelength of 200 nm.

Statistical analysis: Results are expressed as mean ± SD. Data were statistically analyzed by Dunnett’s test. Significance was defined at the level of p<0.05.

RESULTS

As reported in our recent study, there was no significant change in the body weight of prepubertal rats orally treated with DEHP (100 and 1,000 mg/kg) for 5 days. As shown in Fig. 1A, however, at the dose of 1,000 mg/kg DEHP, a marked decrease of serum testosterone level with testicular atrophy (62% reduction of testis weight) was observed (published data) [24]. In addition, AA level was measured using an HPLC system as described previously with some modifications [33]. DEHP treatment (100 and 1,000 mg/kg) decreased serum AA level to 82% and 76% of the vehicle control, respectively (Fig. 1B).

Table 1 shows the effect of DEHP on the cPLA2 activity, a rate-limiting enzyme in the formation of AA. The enzyme activity of testicular cPLA2 was significantly reduced to 62% of the control by 1,000 mg/kg DEHP administration. This result was confirmed by immunoblot analysis, which also showed a marked decrease of cPLA2 expression by DEHP administration (Fig. 2).

We next examined the effect of DEHP on AA-metabolizing enzymes, 12-LOX, COX-2 and CYP4A1. Because previous studies demonstrated that 12-LOX and COX-2 are dominantly expressed in the vas deferens and kidney, these tissues were used as positive controls, respectively [21, 27]. DEHP administration markedly enhanced the expression level of 12-LOX and CYP4A1, whereas no change in COX-2 expression was observed in the testis of prepubertal rats (Fig. 3).

DISCUSSION

Testis has a very high concentration of polyunsaturated fatty acids, and approximately 70% of the fatty acids located in rat testis are polyunsaturated, with a prevalence of AA [16, 35]. Essential fatty acids are necessary for the mainte-
nance of normal testicular functions in rats, and their deficiency was shown to result in impairment of testicular function leading to male sterility [26]. There are several references about the relation between AA and testosterone level. Exogenous AA induced a dose-dependent increase of testosterone secretion [42]. Dix et al. and Sullivan and Cooke indicated that AA lipoxygenase pathway is involved in HCG- and LHRH-induced testosterone production by rat Leydig cells [13, 46]. DEHP altered COX-2 expression levels and metabolic activity of AA in rat liver and leukocytes [28, 48]. Although the effects of DEHP on AA metabolizing enzymes varied from tissue to tissue, the effects in testis were still unclear. The plasticizer DEHP is a well-known testis toxicant. DEHP induces malformations such as reduced anogenital distance and testicular atrophy by decreasing fetal testosterone levels during sexual differentiation period in male rat [37]. Indeed, co-administration of DEHP with testosterone appeared to prevent testicular injury [38]. Thus, testosterone closely correlates with the testicular atrophy by DEHP. This study examined the effects of DEHP on the AA metabolic cascade, which related to testosterone level in the rat testis.

An important pathway in the release of AA from phospholipid pools and the eicosanoid signal transduction process involves cPLA$_2$-dependent hydrolysis of sn-2-acyl ester bonds. As shown in Table 1, testicular cPLA$_2$ activity was significantly reduced by 1,000 mg/kg DEHP administration. Recently, we have shown that this dose of DEHP caused a testicular atrophy with altered testosterone metabolism [24]. In previous studies, the PLA$_2$ inhibitors 4-bromophenacyl bromide and quinacrine blocked the LH- and Bt$_2$cAMP-stimulated testosterone production [32]. Thus,
the decrease of testosterone concentration after DEHP administration may be due to the reduced level of cPLA₂ activity. Although protein kinase C (PKC) activity is involved in the regulation of cPLA₂ activity [8], no alteration in PKC expression levels either in the cytosol and the microsomes of rat testis treated with DEHP was observed (data not shown).

On the other hand, cPLA₂ has been implicated in apoptosis in many different cell lines [25]. Indeed, DEHP induced marked apoptosis by up-regulating the Fas-signaling system in the testis [41]. A hypothesis that the cPLA₂ activity and its product AA might contribute to apoptosis has been proposed. However, our results indicated that DEHP reduced cPLA₂ activity. Our results are in agreement with those of Enari et al. [14] who showed that Fas-induced apoptosis is independent of cPLA₂ because cross-linking this receptor induces cell death equally well in both cPLA₂-deficient and -expressing L929 cells.

AA can be oxygenated enzymatically by three important pathways: the COX, LOX and CYP epoxygenase pathways [1]. A previous study reported that 12-LOX activity was detected in both cytosol and microsomes and that 12-HETE was the major metabolite in the testis [40]. COX-2 plays a major role in the synthesis of prostaglandins from AA and is rapidly induced by growth factors, inflammatory cytokines and oncogenes [29]. As shown in Fig. 3, 12-LOX expression was significantly induced by DEHP treatment, whereas there was no change in COX-2 expression level of the testis. It has been demonstrated that DEHP increased the generation of reactive oxygen species (ROS), with a concomitant decrease in the concentration of glutathione [22]. 12-LOX activation can regulate the oxidation process by generating superoxide [19, 43, 47]. These findings suggest that the 12-LOX expression activated by DEHP might contribute to ROS generation resulting in germ cell apoptosis.

We next sought to investigate the expression level of CYP4A1, mediated by PPARα. PPARα, a member of the nuclear hormone receptor superfamily, is most prominently expressed in tissues with high fatty acid turnover in its function as a regulator of lipid metabolism [7] and is activated by fatty acids [18]. Indeed, the activation of PPARα enhances lipid oxidation, alters the levels of an estrogen-metabolizing enzyme and induces Leydig cell tumors in certain rat strains [44, 45]. The regulation of gene expression via the PPAR is believed to be critical in the effects of peroxisome proliferators on lipid metabolism. The involvement of PPAR in the peroxisome proliferator-mediated induction of fatty acid metabolizing enzymes such as acyl-CoA oxidase, fatty acid-binding protein and CYP4A1 has been clearly demonstrated [5]. In this study, we demonstrated that the administration of DEHP caused a significant increase in CYP4A1 expression in the testis (Fig. 3).

DEHP induces malformations and testicular atrophy by decreasing testosterone levels in male rat, which was prevented by co-administration of DEHP with testosterone. We suggest that multiple factors may contribute to DEHP-induced testicular atrophy and testosterone closely correlated with testicular atrophy. Enzymatic activities and expression levels of testosterone hydroxylation (6β-hydroxylation and 16α-hydroxylation) were induced in rat testis exposed to DEHP [24]. In addition to previous studies, we observed DEHP suppresses cPLA₂ activity and induces the AA metabolizing enzymes such as 12-LOX and CYP4A1 (Fig. 4); it was suggested to be one of the factors which might reduce the level of testosterone, resulted in testicular atrophy. These data suggest that altered AA metabolic cascades may be related to the decrease of testosterone concentration, resulted in DEHP-induced testicular atrophy.

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