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ScienceDirect
Toxicology 240 (2007) 86–95
TOXICOLOGY
www.elsevier.com/locate/toxicol

# Differential expression of peroxiredoxin 6 in fetal rat testis following *in utero* exposure to di(*n*butyl) phthalate

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Received 15 May 2007; received in revised form 4 July 2007; accepted 24 July 2007
Available online 8 August 2007

**Abstract**

*Objectives:* To isolate and identify differentially expressed proteins in fetal rat testis following *in utero* exposure to di(*n*butyl) phthalate (DBP).
*Methods:* We used the technique of proteomic analysis to compare the testis protein patterns obtained by two-dimensional gel electrophoresis from fetal rats of gestation day 19.
*Results:* We found significant differences in protein spot intensities. Subsequently several of these variant protein spots were identified by mass spectrometry. Peroxiredoxin 6 (*Prdx6*) was one of them. *Prdx6*, which expressed a higher level in DBP-treated fetal rat testes compared with normal ones, is a member of the peroxiredoxins family (*Prdxs*). Recently, *Prdx6* had been shown to be important in protecting cells from oxidative injury. Further, immunohistochemical analyses of fetal rat testes sections were made to determine the cellular distribution of this protein, consequently a strong *Prdx6* staining was found out primarily in Leydig cells.
*Conclusions:* The present study had found several differentially regulated proteins and demonstrated the differential expression of *Prdx6* in fetal rat testis following *in utero* exposure to DBP, when compared with controls. Combining the cellular location of *Prdx6* and its function in other tissues, the results of this study could provide us with a possibility to interfere the reproductive toxicity of DBP for its specific antioxidant properties in testis tissues.


*Keywords:* Proteomic analysis; Peroxiredoxin 6; Di(*n*butyl) phthalate; Rat; Testis

## 1. Introduction

An array of male reproductive abnormalities such as testicular germ cell cancers, low sperm counts, cryptorchidism, and hypospadias are common and may be increasing in incidence (Toppari et al., 1996; Sharpe and Irvine, 2004; SEER, 2002). It has been hypothesized that they compose a "testicular dysgenesis syndrome" (TDS). Evidence suggests that this syndrome arises from abnormal development of Sertoli and Leydig cells either due to genetic defects or because of environmental factors (Boisen et al., 2001; Skakkebaek et al., 2001). Administration of the phthalate ester di(*n*butyl) phthalate (DBP) to female rats during pregnancy leads to a variety of male reproductive malformations, including underdeveloped or absent reproductive organs, malformation of the external genitalia, cryptorchidism, decreased anogenital distance, diminished sperm counts and Leydig cell

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0300-483X/$ – see front matter © 2007 Published by Elsevier Ireland Ltd.
doi:10.1016/j.tox.2007.07.021

adenomas (Mylchreest et al., 1998, 1999; Foster et al., 2001). These abnormalities correspond closely with the human conditions that compose TDS.

DBP is one of many phthalate esters (PAEs) which are found in a variety of environmental media. Structurally, phthalates are characterized by a diester structure consisting of a 1,2-benzenedicarboxylic acid head group linked to two ester side chains. The most common phthalates possess ester side chains ranging from $C_1$ to approximately $C_{13}$. Side chains may be linear, e.g. dibutyl phthalate (DBP), branched, e.g. diisobutyl phthalate (DIOP) or a combination of linear, branched and ringed structures, e.g. butyl benzyl phthalate (BBP). The structural characteristics of the ester side chains affect the physicochemical and toxicological properties of the phthalate. DBP initially attracted attention as a potential endocrine disruptor because it was found to be a weak estrogen receptor agonist in some cell-based assays (Joblin et al., 1995; Harris et al., 1997). DBP is used as a plasticizer for nitrocellulose, polyvinyl chloride, and polyvinyl acetate. It is also used in adhesives, plastic coatings, and cosmetic formulations. A variety of consumers' products contain DBP, including plastic food wrap, perfumes, skin emollients, hair spray, nail polish, and insect repellents. DBP is ubiquitous in the environment, and dietary intake appears to be the principal source of human exposure. The estimated total average daily human intake from air, drinking water, and food is 7.4 μg/kg/day (International Programme on Chemical Safety, 1997). Upon ingestion, DBP is rapidly absorbed through the gastrointestinal tract, mainly as the monosubstituted phthalate ester mono (*n*butyl) phthalate (MBP). In the rat, MBP has a half-life in blood of less than 24 h (National Toxicology Program, 1995). DBP is toxic to the Sertoli cells of the testis (Foster et al., 1982; Gray and Beamand, 1984) and acute or subacute high doses (greater than 1 g/kg/day) impair spermatogenesis in rats by inducing widespread exfoliation of the seminiferous epithelium in the rat (Cater et al., 1977). Neonatal and pubertal rats are more sensitive than sexually mature animals to the testicular toxicity of DBP and other PAEs (Foster et al., 1980; Dostal et al., 1988) which are mediated by the monosubstituted phthalate ester metabolite (Foster et al., 1981).

To understand the protein expression profile related to reproductive toxicity of DBP, we compared the protein patterns in DBP-exposed with that in control fetal rat testes by high-resolution two-dimensional gel electrophoresis (2-DE); and identified the differentially expressed proteins by tryptic in-gel digestion and subsequent mass spectrometry combining with computer analysis. Here we concentrated on one such protein—Peroxiredoxin 6 (*Prdx6*). Western blot analysis authenticated the result of mass spectrometry. Immunohistochemistry studies were performed to localize the expression of this protein in fetal rat testis.

## 2. Materials and methods

### 2.1. Reagents

Immobilized pH gradient strips (IPG strips), urea, immobilized pH gradient buffer (IPG buffer), Tris-(hydroxymethyl)-aminomethane (Tris), molecular weight markers, acrylamide, methylenebisacrylamide, sodium dodecyl sulfate (SDS), ammonium persulfate (APS), *N*,*N*,*N*′,*N*′-tetramethylethylenediamine (TEMED), and glycerol were from Amersham Pharmacia Biotech (Uppsala, Sweden). Di(*n*butyl) phthalate (DBP; AR, 1.04 g/ml), 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), thiourea, iodoacetamide, acetonitrile (ACN), ammonium bicarbonate ($NH_4HCO_3$), trifluoracetic acid (TFA), and formaldehyde were from Sigma Chemical (St. Louis, MO, USA). Dithiothreitol (DTT) was from Shenggong (Shanghai, China). Peptide calibration standards and the matrix material (a-cyano-4-hydroxycinammic acid, a-HCCA) were purchased from Bruker (Bruker Daltonik, Bremen, Germany).

### 2.2. Samples

Twenty-eight-week-old female Sprague–Dawley rats were kept in the laboratory animal center of Nanjing Medical University under controlled temperature (20–22 °C), light (12/12 h light dark cycle), and humidity (50–70%) and received food and water in accordance with the Guide for the Care and Use of Laboratory Animals. The day sperm was found in the vagina of the mated female, was considered gestation day 0 (GD0). Dams from GD14 to GD18 were daily treated by gavage with 1 ml/kg corn oil (vehicle control, Sigma Chemical Co.) or 750 mg/kg DBP (99.8%, Aldrich Chemical Co., Milwaukee, WI). The dosage level of DBP was based on previous studies which included the teratogenic range of DBP on rats (Ema et al., 1993, 1994, 2000). In our previous study, it was shown that this dosage had induced higher incidence of reproductive malformation without significant maternal toxicity (Jiang et al., 2007). The DBP exposure period was associated with the prenatal period of sexual differentiation in the male rats that was demonstrated to be the critical period for DBP-induced malformations of the male reproductive tract (Ema et al., 1994, 2000; Mylchreest et al., 2002). Body weights were recorded daily before dosing. Food consumption was monitored biweekly throughout the dosing period. Ten dams in each dose group were sacrificed by carbon dioxide asphyxiation at GD19 within 24 h of last dose, when there was a rapid increase to the peak of testosterone production (El-Gehani et al., 1998). Upon sacrifice, fetuses were immediately removed and sexed by measuring anogenital distance under a dissect-