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Toxicoproteomic Analysis of Differentially Expressed Proteins in Rat Liver by DEHP

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Abstract

The endocrine disrupting chemical, di (2-ethylhexyl) phthalate (DEHP) is a plasticizer used in polyvinyl chloride products ubiquitous in our daily lives. DEHP has potentially adverse effects on the liver, kidney, lung, heart, reproductive organs and endocrine systems. Many toxicological data on the DEHP toxicity have been stated, but complete protein profiles have not yet been reported. In this study, DEHP-induced oxidative DNA damage in rat lymphocyte was evaluated by Comet assay (single-cell gel electrophoresis) for the first time. Moreover, DEHP-induced protein profile alterations were examined in rat liver by using toxicoproteomic tools. 34 protein spots in the liver were identified to be significantly deregulated by DEHP on the 2-dimensional gel. Among them, 20 spots were up-regulated and 14 spots down-regulated by DEHP.

Keywords: DEHP, Rat liver, Toxicoproteomics

Phthalates, chemicals used as plasticizers, are economically significant due to industrial applications. Di-(2-ethylhexyl)phthalate (DEHP) has been commonly used as a phthalate plasticizer for manufacturing vinyl products such as plastic food wraps, children's toys, blood dialysis bag, and catheters[1,2]. There are accumulating evidences that DEHP is an endocrine disruptor, an environmental contaminant that perturbs the male reproductive process such as spermatogenesis[3]. More specifically, DEHP causes considerable damage to the somatic cells of the seminiferous tubules, Sertoli cells, and to the shedding of spermatocytes and spermatids to eventually decrease sperm production[4,5]. Moreover, the long-term exposure effects of DEHP on female rats revealed that the serum estradiol, Follicle-Stimulating Hormone (FSH), pituitary FSH and luteinizing hormone levels were significantly reduced[6].

DEHP has potentially adverse effects on the liver, kidney, lung, heart, reproductive organs and endocrine systems[7,8]. Treatment with DEHP promotes abnormal proliferation, leading to hepatocarcinogenesis and alteration in the expression of genes associated with testis development and steroid hormone synthesis in mice[9]. DEHP-fed rats show a deficiency in muscle glucose and lactate transport, a reduction of the flux through muscle hexokinase and hepatic glucokinase, and a reduction in glycogen synthesis[10,11]. A recent study suggested that DEHP induced hepatic tumorigenesis through a peroxisome proliferator-activated receptor α-independent pathway via increase in oxidative stress[12].

Toxicoproteomic approaches are becoming more acceptable in toxicology because proteomics are a promising tool for supporting classical and predictive toxicology[13] and for improving risk assessment by dissecting toxicity mechanism in greater detail prior to pathological observation[14,15]. Numerous studies have been focused on the toxicity of DEHP and their adverse effects on living organism. In contrast, data on protein expression changes due to DEHP exposure is very limited. To identify liver proteins in rats that were significantly changed by DEHP treatment, 2-DE analyses of rat liver proteins were performed.

Assessment of DNA Damage by DEHP Using Single Cell Gel Electrophoresis (Comet) Assay

To investigate whether DEHP could induce oxidative DNA damage in rat lymphocytes, we performed single cell gel electrophoresis (Comet) assay following the guideline recommended by IWGTP. Comet assay, also known as Single Cell Gel electrophoresis (SCG), is a microgel electrophoresis technique which detects DNA damage and repair in individual cells. The damage is represented by an increase in DNA fragments that have migrated out of the cell nucleus in a streak characteristically similar to the tail of a

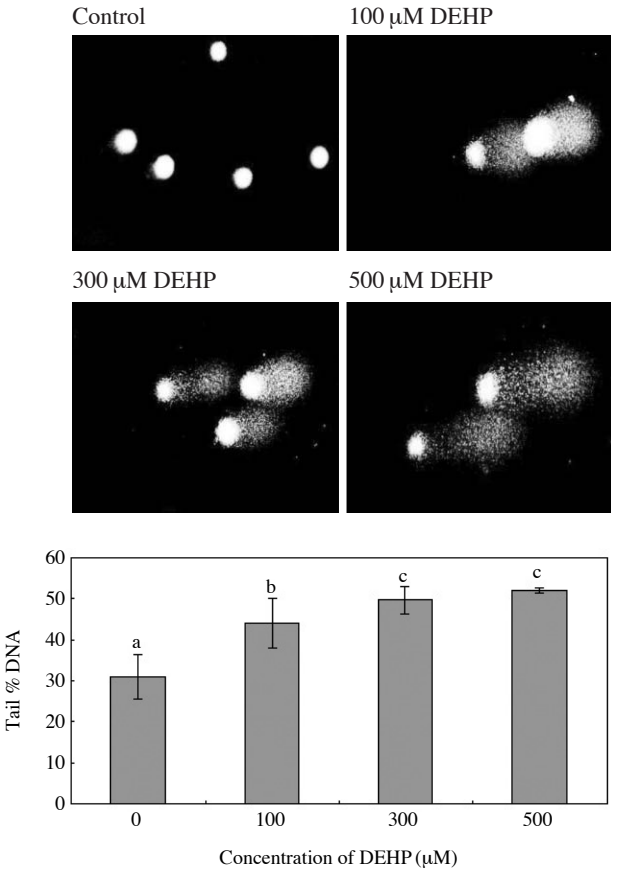

Figure 1. Photomicrographs showing comet images from rat blood lymphocytes following DEHP treatment.

comet. The DNA fragments are generated by DNA double strand breaks, single strand breaks and/or strand breaks induced by alkali-labile sites in the alkaline version of the assay. The length and fragment content of the tail is directly proportional to the amount of DNA damage. As shown in Figure 1, DEHP treatment for 1 hour caused a statistically significant increase in tail length (%) in rat lymphocytes, indicating oxidative damage in rat lymphocyte DNA by DEHP.

Differentially Expressed Proteins in Rat Liver by DEHP

We chose to focus our proteomics study on the protein alteration by DEHP toxicity in the liver, since the organ is an important site of various metabolisms and a known DEHP target organ[16]. Figure 2 shows the gel images of 2-DE maps showing the separation of rat liver proteins. More than 300 protein spots with *p*Is between 3 and 10 and with relative molecular masses between 6.5 and 205 kDa were detected on the 2-DE gels. The protein patterns from three DEHP -treated rats and untreated rats were compared by a student's t-test, and 34 protein spots in the DEHP-administered rat liver were significantly different in comparison to the control. The spot maps of the control and treated livers are shown in Figure 2A and Figure 2B, respectively.

34 spots were significantly deregulated on the 2-DE gel as indicated by the arrows. Among them, 14 spots were found to be down-regulated (Figure 3) and 20

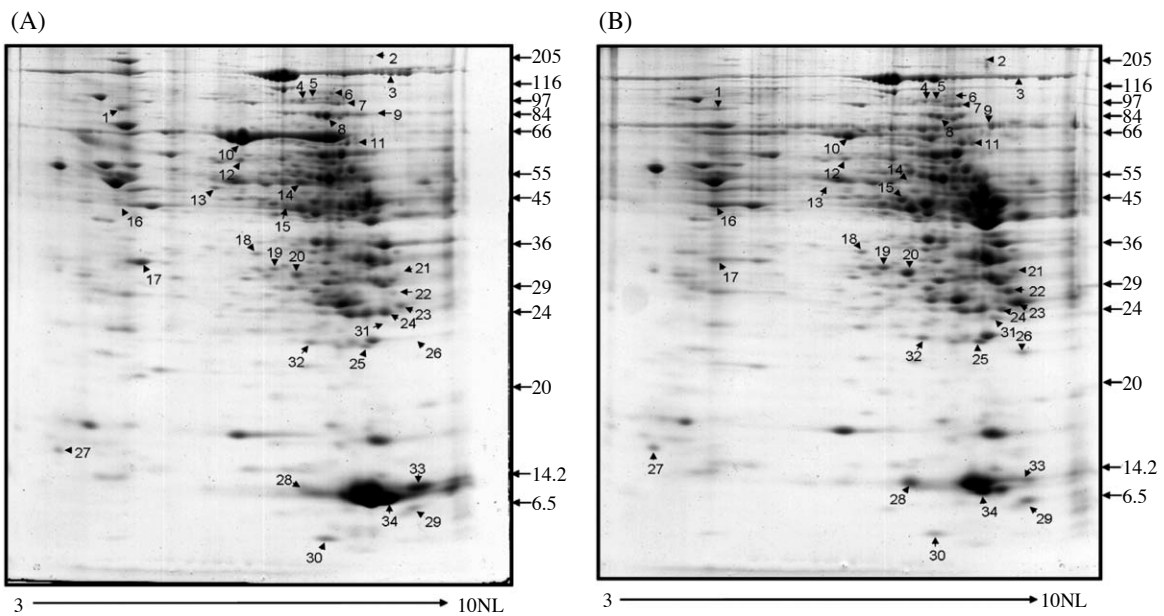

Figure 2. 2-Dimensional gel electrophoresis (DE) of proteins from rat liver tissue. Differentially expressed proteins on the 2-DE gel was indicated by the arrows. (A) Control (B) DEHP-treated liver.

Table 1. Differentially expressed proteins in the liver of rat with DEHP.

Spot no.	Identified protein	Accession no.	Cov. %	Matching peptide no.	Tr/M.W/ pI	Change
1	Heat shock protein	AAT99568	21	15	83.61/5.0	↓
2	3-hydroxy-3-methylglutaryl-Coenzyme A synthase 2	NP_775117	19	10	57.35/9.2	↑
3	Carbamoyl-phosphate synthetase 1, mitochondrial	NP_058768	26	32	165.76/6.3	↓
4	Carbamoyl-phosphate synthetase 1, mitochondrial	NP_058768	21	23	165.76/6.3	↓
5	Glutamate-ammonia ligase (glutamine synthase) domain containing 1	NP_852048	24	12	62.94/5.4	↑
6	Plasminogen precursor	Q01177	15	12	93.26/6.8	↓
7	Aconitase 1	NP_059017	32	25	98.79/6.7	↑
8	Liver regeneration related protein LRRG03	AAP97736	32	25	78.55/7.3	↓
9	Enoyl-Coenzyme A, hydratase/3-hydroxyacyl Coenzyme A dehydrogenase	NP_598290	40	24	79.21/9.5	↑
10	Albumin	NP_599153	31	19	70.70/6.1	↓
11	Acyl-Coenzyme A dehydrogenase, very long chain	NP_037023	29	17	71.07/9.2	↑
12	Protein disulfide-isomerase A3 precursor	P11598	37	18	57.06/5.9	↓
13	Phenylalanine hydroxylase	NP_036751	47	15	52.32/5.8	↓
14	Mitochondrial aldehyde dehydrogenase	AAS75815	32	20	56.10/7.8	↓
15	Cytosolic acyl-CoA thioesterase 1	NP_112605	34	14	46.15/7.2	↑
16	Actin, cytoplasmic 2 (Gamma-actin)	P63259	50	15	42.12/5.3	↑
17	Regucalcin	NP_113734	44	13	33.94/5.3	↓
18	Malate dehydrogenase 1, NAD (soluble)	AAH59124	24	6	36.64/5.9	↑
19	Glycerol-3-phosphate dehydrogenase 1 (soluble)	NP_071551	47	18	38.12/6.2	↑
20	Acyl sulfotransferase	2021280A	42	11	34.21/6.6	↑
21	Peroxisomal trans-2-enoyl-CoA reductase	NP_579833	35	9	32.70/9.2	↑
22	2, 4-dienoyl CoA reductase 1, mitochondrial	AAH59120	40	12	36.50/9.4	↑
23	Chain A, Crystal structure of rat mitochondrial 3, 2-enoyl-coa	1XX4_A	41	9	29.42/9.1	↑
24	Chain A, Glutahione transferase mutant Y115f	1MTC_A	55	12	25.92/8.6	↓
25	Superoxide dismutase 2	NP_058747	44	9	24.88/9.3	↑
26	Chain A, Crystal structure of peroxisomal Acyl-Coa oxidase-Ii from rat liver	1IS2_A	13	7	75.07/8.5	↑
27	Yif1b protein	AAH58153	38	5	25.31/9.0	↑
28	Fatty acid binding protein 1	NP_036688	54	6	14.31/7.8	↑
29	Chaperonin 10	AAB27570	44	5	10.75/8.9	↑
30	Ubiquitin	P62989	70	5	8.55/6.6	↓
31	Glutathione S-transferase, theta 3	NP_598755	22	8	27.73/7.9	↑
32	Similar to billverdin reductase B (flavin reductase (NADPH))	XP_214823	49	10	22.19/6.3	↑
33	Unnamed protein product	CAA34440	73	10	16.03/9.0	↓
34	Hemoglobin alpha 1 chain	NP_037228	58	7	15.48/7.9	↓

↓ : down-regulation ↑ : up-regulation

spots up-regulated (Figure 4). The identification results on the differentially expressed spots by MALDI -TOF mass spectrometry are summarized in Table 1.

Discussion

In DEHP-treated rat liver tissue, many proteins involved in stress defense were significantly deregulated after DEHP administration (Table 1). Increase in superoxide dismutase by DEHP, which is known to eliminate superoxide radicals, shows enhanced cellular stress by DEHP[13]. Heat Shock Protein (HSP), an important protein for facilitating correct protein assembly, was disturbed under chaperonin-10 enhancement by DEHP[17]. Decreased expression of HSP resulted in inhibition of apoptotic processes, consequently promoting uncontrolled cell growth as a secondary effect triggered by oxidative cell damage[18].

Defense processes related to cellular stress were accompanied by deregulations in anabolic and catabolic metabolism. Aldehyde dehydrogenase, malate dehydrogenase and glycerol-3-phosphate dehydrogenase were altered after DEHP administration.

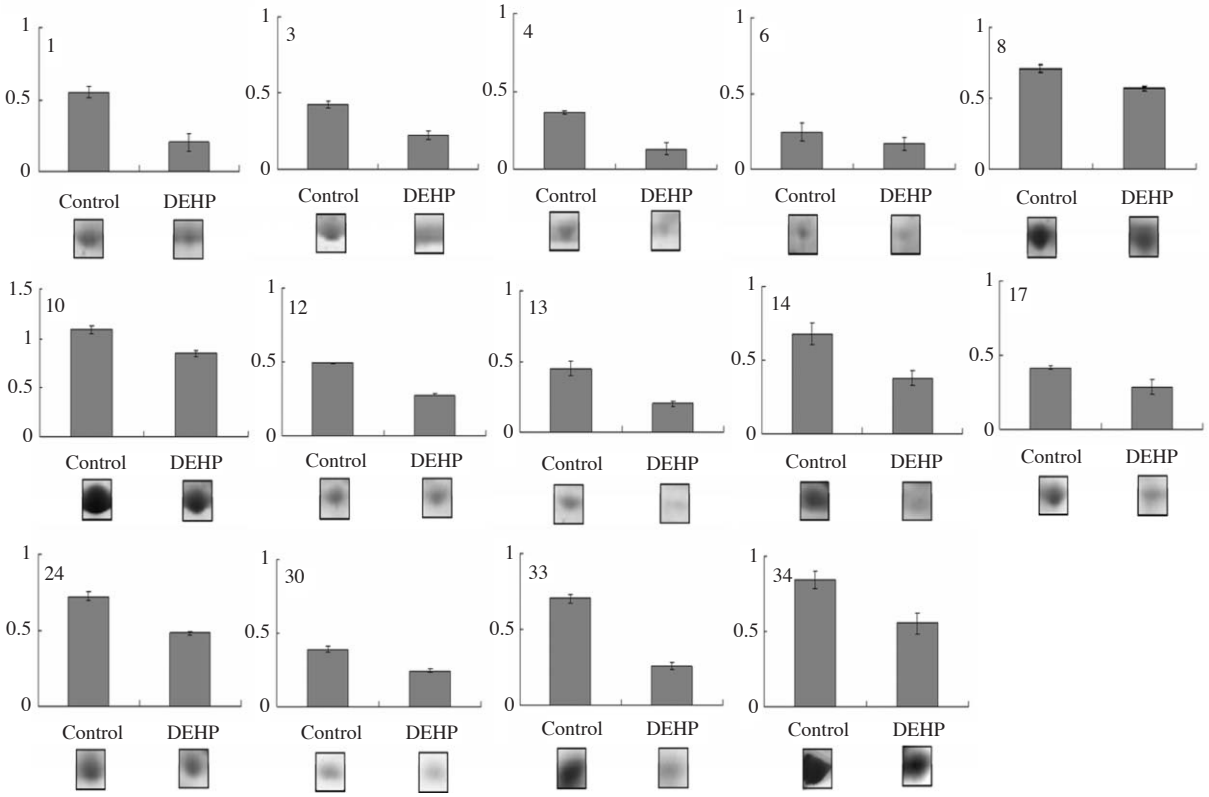

Figure 3. Down-regulated proteins from rat liver by DEHP. Protein expression levels were determined by relative intensity using image analysis. Normalized spot intensities of the DEHP versus normal control group were compared. Mean spot intensities on individual gels are shown. $P < 0.05$ by Student's t-test.

Aldehyde dehydrogenase (ALDH) was down-regulated in the DEHP-treated liver, enabling the elimination of toxic biogenic and xenobiotic aldehydes. The reduction of ALDH by DEHP might cause an increase of lipid peroxides because oxidative damage could result in lipid peroxidation and the production of highly reactive aldehydes.

Glutathione transferase (GST), capable of reacting with lipid hydroxyperoxides, was also up-regulated. When the enzymes become depleted, intracellular H_2O_2 should accumulate, resulting in more severe oxidative damage through Fenton and Haber-Weiss reactions. GST was also known to be notably expressed at the very early stages of chemical hepatocarcinogenesis[19]. Besides being capable of conjugating glutathione to electrophilic compounds derived from the biotransformation of xenobiotics, GST also shows a glutathione peroxidase activity to eliminate lipid peroxides and DNA hydroxyperoxides[20].

It was expected that serum components such as albumin could be detected in this study, because the sample preparation protocol does not involve liver perfusion. Serum albumin was reduced after DEHP treatment, potentially due to changes in blood flow through liver. The decrease in serum albumin seems more likely due to changes in tissue blood content rather than a change in serum albumin production by the liver cells.

Long chain acyl-CoA dehydrogenase was up-regulated by DEHP treatment. The up-regulation of this catabolic enzyme might enhance fatty acid β-oxidation. Besides enhancing ATP production, increased β-oxidation can result in cellular reducing power as one of the survival mechanisms against DEHP toxicity[21].

Carbamoylphosphate synthetase was reported to be the most abundant protein in rat liver tissue[15]. Several isoforms or fragments of this enzyme were detected on the 2-DE gel. Carbamoylphosphate synthetase 1 isoforms are known to be highly reactive and specific toward different toxic compounds; however, since more than 15 isoforms with unknown roles in toxicity related mechanism exist, its use as toxicity markers seems to be difficult.

DEHP was reported to have an indirect effect on

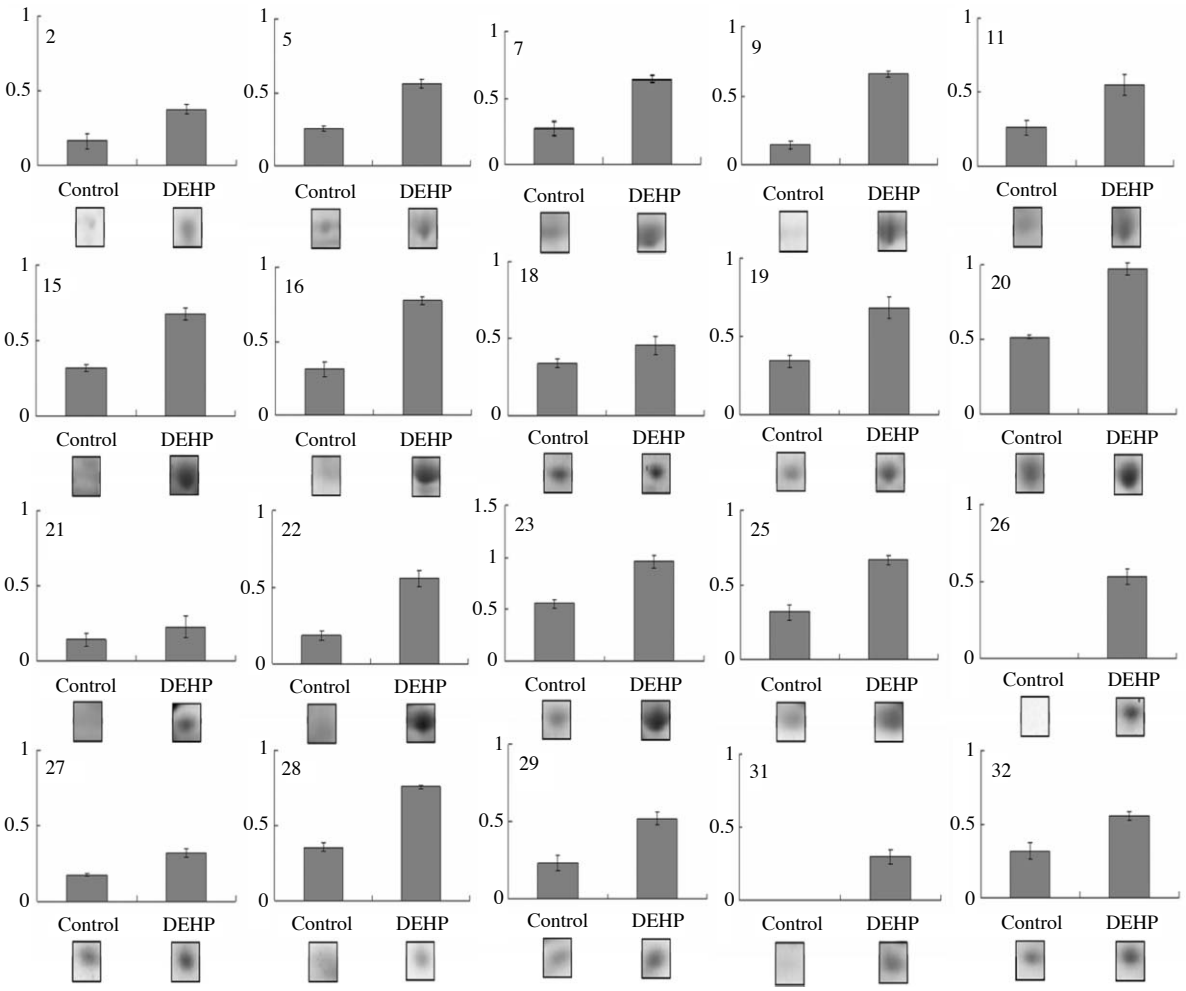

Figure 4. Up-regulated proteins from rat liver by DEHP. Protein expression levels were determined by relative intensity using image analysis. Normalized spot intensities of the DEHP versus normal control group were compared. Mean spot intensities on individual gels are shown. $P < 0.05$ by Student's t-test.

the endocrine system that is not mediated by the estrogen receptor through the analysis of an endocrine organ, the pituitary[6]. Proteome-based analyses of the pituitaries showed that actin, hemoglobin alpha, glutathione S-transferase, malate dehydrogenase, aconitase and 2-enoyl-coa hydratase have been deregulated by DEHP, as also detected in the liver in this study.

In summary, our approach revealed 34 differentially expressed proteins after three weeks of DEHP exposure. The identified protein signature may act as a candidate for the predictive biomarker set. This has to be proven in further experiments and verified by immunoblot analysis. This study explores the potential of proteomics to identify unexpected and novel targets for toxic chemicals in environmental toxicology.

Methods

Animals

Adult male Sprague-Dawley rats weighing about 100 g were acclimatized in a temperature and light-controlled environment (23 ± 2°C, 12-hr light/dark cycle) for 1 week before use. They were fed *ad libitum* with commercial rodent diet and tap water. At least three rats per group were treated with 150 mg/kg/day of DEHP in 0.5 mL corn oil for 15 days by gavage. Control group was given the same amount of corn oil. After 1 week of final treatment, rats were sacrificed by decapitation and their livers were removed after a midline abdominal incision, frozen, and stored in liquid nitrogen until homogenizing and analysis.

Isolation of Lymphocytes

Lymphocytes were isolated from the rat whole blood by the method of Sudheer[22] with slight modifications. A total of 6 mL of peripheral blood was collected from two healthy male Sprague-Dawley rats by cervical dislocation for each experiment. Every time blood was collected freshly in heparinised sterile glass vials on the same day of experiment. Whole blood (6.0 mL) was diluted with 6.0 mL of Phosphate Buffered Saline (PBS), pH 7.4, and carefully layered over 3.0 mL of Histopaque 1077. After centrifugation at 400 g for 30 min at room temperature, the upper layer was discarded and the opaque interface containing mononuclear cells was transferred into a clean centrifuge tube. After repeated washing of the lymphocytes with PBS, cells were recentrifuged at 250 g, the resulting pellet was resuspended in 0.5 mL of PBS and finally washed with RPMI-1640 media.

Comet Assay

The comet assay was performed according to the method of Singh, N. P. *et al*.[23] with some modifications. Clean frosted slides were precoated with normal melting point agarose (1%) and allowed to air dry for two days. Rat lymphocytes were exposed to 0-500 μM DEHP for 1 hr on the ice. At the end of the incubation with the DEHP, the cells were removed from the plates with trypsinisation and aliquots (1 mL) of the cell suspension were centrifuged at 2,000 g for 5 min. The cell suspension was diluted in 100 μL of low melting point agarose (1%). Aliquots (100 μL) of the resulting suspension were embedded on previously prepared gels on frosted slides and the addition of 100 μL of low melting point agarose (0.5%) followed. The slides were then immersed in lysis buffer (1% Triton X-100, 10% DMSO, 2.5 M NaOH, 100 mM Na_2 EDTA, 10 mM Tris HCl, pH 10.0) for 2 hrs at 4°C. Then the slides were placed in alkaline electrophoresis buffer (300 mM NaOH, 1 mM Na_2 EDTA, pH 13) for 30 min at 4°C to produce single stranded DNA and to express alkali labile sites. Electrophoresis was carried out at 4°C for 30 min at 25 V. Then the slides were washed twice in neutralizing buffer (0.4 M Tris HCl pH 7.5) for 5 min. The DNA was stained by ethidium bromide to each slide.

Comets were scored using a DMLB microscope from Leica and the kinetic imaging program Komet 5.5 (Kinetic Imaging Liverpool). Fifty cells were scored per each of two replicate slides and results were expressed as tail % DNA.

Liver Sample Preparation

For homogenization, about 100 mg of liver tissue was ground with a mortar and pestle under liquid nitrogen. The powdery homogenate was extracted with lysis buffer containing 50 mM Tris HCl (pH 7.5), 0.1% Triton X-100 and 1 mM PMSF protease inhibitor. After centrifugation the supernatant was collected, and protein concentration was determined using the Bradford assay kit. Protein was precipitated with 10% TCA in acetone. The protein pellet was washed with ice-cold acetone at least 5 times in order to remove contaminants.

2-Dimensional Electrophoresis (2-DE)

For IEF in the first dimension, dried protein samples were dissolved in 500 μL rehydration buffer (7 M urea, 2 M thiourea, 4% CHAPS, 0.5% ampholytes, 100 mM DTT and 0.01% bromophenol blue). The sample solution was applied on immobilized pH 3-10 nonlinear gradient dry strips using an IPG phor system (Amersham Pharmacia Biotech, Uppsala, Sweden). Focusing was performed using the following steps: rehydration for 12 hr, 250 V/100 Vhrs, 500 V/500 Vhrs, 1,000 V/1,000 Vhrs, 8,000 V/38,000 Vhrs. After IEF, the individual IPG strips were equilibrated in 6 M urea, 2% SDS, 50 mM Tris-HCl (pH 8.8), 2% DTT and 10% glycerol for 15 min and subsequently incubated in the same buffer for another 15 min after replacing DTT with 2.5% iodoacetamide. Gel was run at 10 mA/gel for 15 min for the initial migration and then 35 mA/gel for 8 hrs.

Image Analysis

The gels were visualized using CBB-G250 (Coomassie brilliant blue G-250) staining method. The stained gels were scanned using a UMAX scanner (UMAX Technologies, Plano, TX), and the data were analyzed using Image Master 2D Elite software (Amersham Pharmacia Biotech, Uppsala, Sweden).

Protein Digestion and Mass Spectrometric Analysis

Protein spots were excised from the gel. Gel pieces were washed twice with 25 mM ammonium bicarbonate, pH 8.2, 50% v/v acetonitrile (ACN) and then dehydrated by the addition of 100% ACN. 30 ng of trypsin in 25 mM ammonium bicarbonate was added to each gel piece and incubated at 30°C for 16 hr. The peptide solution was automatically desalted and concentrated using Zip-Tips from Millipore on the Xcise apparatus and spotted on to the Axima (Kratos, Manchester. UK) MALDI target plate. Peptide mass fingerprints of and Post-Source Decay (PSD) tryptic peptides were generated by Matrix Assisted Laser Desorption/Ionization-Time-Of-Flight-Mass Spectrometry (MALDI-TOF-MS) using Ettan MALDI-TOF. Tryptic peptides derived from protein spots were analyzed

and amino acid sequences were deduced using a de novo peptide-sequencing program, PepSeq (Gibbsland). To identify the proteins, sequences were searched against the NCBInr and EST databases using the PROFOUND search program (http://www.rockefeller.edu/labheads/chait/novel_tandem.php) and BLAST.

Statistical Analysis

The proteins from the DEHP-treated and untreated control rats were analyzed for the differences in expression levels using Student's t-test. Values of $P < 0.05$ were considered significant.

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