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### Proteomic Analysis of the Rat Ovary Following Chronic Low-Dose Exposure to 2,3,7,8-Tetrachlorodibenzo-p-Dioxin (TCDD)

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# Proteomic Analysis of the Rat Ovary Following Chronic Low-Dose Exposure to 2,3,7,8-Tetrachlorodibenzo-*p*-Dioxin (TCDD)

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**2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitously distributed endocrine-disrupting chemical and reproductive toxicant. In order to elucidate low-dose TCDD-mediated effects on reproductive or endocrine functions, female Sprague-Dawley rats were orally administered various concentrations (20, 50, or 125 ng/kg once weekly) TCDD for 29 wk. A proteomic analysis of the ovaries by two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization (MALDI) tandem mass spectrometry showed distinct changes in the levels of several proteins that are relevant markers of TCDD toxicity. Serum estradiol (E2) levels of TCDD-treated animals were markedly lower than control. There were no significant differences in bone mineral density (BMD) of femurs. The body weight of the 125-ng/kg TCDD group was significantly decreased relative to control and there was also a significant reduction in absolute and relative ovarian weights. Expressions of selenium binding protein 2, glutathione *S*-transferase mu type 3, Lrpap1 protein, NADPH, and peptidylprolyl isomerase D were upregulated, while prohibitin and *N*-ethylmaleimide-sensitive factor expression levels were downregulated. Data provide further insight into the mechanisms by which TCDD disrupts ovarian function by indicating which differential protein expressions following low-dose TCDD exposure.**

An endocrine-disrupting chemical (EDC) is defined as an exogenous substance/mixture that alters function(s) of the

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endocrine system and consequently produces adverse health effects in intact organism, or progeny, or (sub)populations (Roy et al., 1997; Choi et al., 2004). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is a ubiquitously distributed EDC and is recognized to be the most potent toxic compound among the polychlorinated dibenzo-*p*-dioxins (PCDD) and dibenzofurans (PCDF). In mammals, TCDD and other dioxin-like compounds exert effects on various biological systems, including the so-called “wasting syndrome,” hepatotoxicity, immunotoxicity, reproductive disorders, carcinogenicity, and disturbances in endocrine systems (Okey et al., 1994; Diehl-Jones & Bols, 2000). Dioxin initially binds and subsequently activates the aryl hydrocarbon receptor (AhR). The TCDD–AhR complex is subsequently translocated to the nucleus of the cell, forming a heterodimer with the AhR nuclear translocator (ARNT). The TCDD–AhR–ARNT complex adheres to dioxin-responsive elements (DRE), inducing the transcription of a number of genes (Tang et al., 2008), and alters the expression of a wide range of proteins (Kim et al., 2004).

Proteomic methods enable a systematic screening of the expressed protein profile (Conrad et al., 2008), and can detect a number of proteins whose levels are changed in response to a diverse spectrum of toxic agents including TCDD (Joo et al., 2003). In the thymus of marmosets treated with TCDD, the deregulated proteins are related to immune responses (Oberemm et al., 2005). Some investigators, using two-dimensional gel electrophoresis to determine placental proteins, showed that rat placentas exposed to TCDD were in a hypoxic state at the end of pregnancy (Ishimura et al., 2002). Analysis of the changes in the rat hepatoma cell proteome indicated a mechanism by which TCDD may affect cellular homeostasis and survival (Sarioglu et al., 2008).

Reproductive and endocrine disorders are considered as the most sensitive adverse effects correlated with TCDD exposure

(Mutoh et al., 2006). The ovary has been recognized as the main organism responsible not only for germ-cell storage but also for secretion of various steroidal hormones. Therefore, ovarian dysfunction may lead to adverse consequences, some of which are due to hormonal imbalance (Yoshida et al., 2005). AhR and ARNT were reported to localize in the ovary (Khorram et al., 2002). Bruggeman et al. (2006) showed the effects of TCDD on the ovary and liver in 1-d-old chickens as evidenced by alterations in proteins that could not be directly linked to drug on xenobiotic metabolism but appeared to be involved in oxidative stress, blood clotting, calcium regulation, and electron transport. Some studies found that the toxicity following chronic exposure was different from acute exposure (Franczak et al., 2006; Goldstein et al., 1982). Few studies have addressed the proteomic reactions of ovary tissue following chronic exposure to low doses of TCDD. In order to explore the chronic effects of low-dose treatment with TCDD, two-dimensional gel electrophoresis and matrix-assisted laser desorption/ionization (MALDI) tandem mass spectrometry (2-DE and MALDI-TOF/TOF) were used to examine the rat ovary proteome following TCDD exposure.

## MATERIALS AND METHODS

### Animals

All animal experiments were performed according to the guidelines for Animal Welfare of the National Ethics Committee. As the Sprague-Dawley rat is sensitive to the effects of TCDD (Kociba et al., 1978), in total 32 female Sprague-Dawley rats were obtained from the Chinese Academy of Military Medical Sciences (SCXK-2002-001). Upon receipt they were 6 to 6.5 wk old, weighing approximately 215 g. The rats were randomly assigned to control or one of three dose groups. Rats were housed 4–5 per cage in the specific-pathogen-free (SPF) barrier unit with a 12-h light/dark cycle at  $21 \pm 1^\circ\text{C}$  and  $50 \pm 10\%$  relative humidity. Rats had free access to distilled water and rodent food. They were held for 1 wk of acclimation prior to the start of the study.

Animals were administered TCDD by a gastric tube once per week (0, 140, 350, or 875 ng TCDD/kg/wk) and were weighed every week at the same time for 29 wk. Control animals received the vehicle only. The once-weekly TCDD administration schedule was chosen in order to reduce the irritation. These doses of TCDD were equivalent to concentrations of 0, 20, 50, or 125 ng TCDD/kg.

Vaginal cytology was conducted before sacrifice to determine the estrous cycle phase of rats. Only 3 rats were in the metaestrous phase, belonging to the 0-, 20-, and 125-ng/kg groups, respectively, and the rest were all diestrous.

Rats were sacrificed by  $\text{CO}_2$  asphyxiation. Serum samples were collected for hormonal assays, and ovary tissues were removed, weighed, and frozen in liquid nitrogen for subsequent analysis. Femurs were excised, and soft tissue was removed and stored at  $-20^\circ\text{C}$  until further study.

### Chemicals

TCDD (lot ER011005-01) was purchased from Cambridge Isotope Laboratories, Inc. (CIL, USA). Purity and identity were established by using a variety of chromatographic and spectroscopic methods, such as gas chromatography with flame ionization detector (GC/FID) and gas chromatography–mass spectrometry (GC/MS). Chemical purity was determined to be over 99%. The TCDD powder was dissolved and stored in analytic-grade acetone. Dose formulations for treatment were prepared weekly by mixing the TCDD solution in corn oil vehicle.

### Estradiol (E2) Measurements

Serum concentrations of estradiol (E2) were measured by enzyme-linked immunosorbent assay (ELISA) using kits provided by Adlitteram Diagnostic Laboratories (USA), according to the procedures recommended by the supplier. The concentrations were determined by using a microplate spectrophotometer and were read at an optical density of 450 nm.

### Bone Densitometry

The bones were scanned with Challenger osteodensitometers (Montpellier, France) using the dual energy x-ray absorptiometry (DEXA) principle. Each bone was scanned at the distal region.

### Ovary Protein Preparation

Frozen ovary samples were mashed into powder using a ceramic mortar and pestle chilled with liquid nitrogen. Then the ovary powders from eight animals in the same group were pooled together before further analysis.

The pooled powdered tissue (approximately 300 mg/sample) was subsequently dissolved in lysis buffer (8 M urea, 2 M thiourea, 4% CHAPS, 1% DTT, 0.8% ampholine, pH 3–10) followed by sonicating for 5 min and were centrifuged at  $25,000 \times g$  for 30 min at  $4^\circ\text{C}$ . The Bradford protein assay kit was used for measuring protein concentrations.

### Two-Dimensional Electrophoresis

The first dimension of gel separation was carried out with 18-cm pH 3–10 immobile pH gradient (IPG) strips following the manufacturer's protocol (Bio-Rad, Hercules, CA) with minor modifications. Samples containing up to 500  $\mu\text{g}$  protein were loaded and isoelectric focusing (IEF) was performed in the IPG-phor isoelectric focusing system (Pharmersham) by stepwise increase of the voltage as follows: 0–500 V for 2 h, 500 V for 5 h, 500–3500 V for 3 h, and finally maintained at 3500 V until the total volt-hours reached 45 kVh. The temperature was kept at  $16^\circ\text{C}$ .

Strips were equilibrated for 15 min in equilibration buffer (6 M urea, 2% sodium dodecyl sulfate [SDS], 0.05 M Tris-HCl, pH 6.8,

30% glycerol, and trace bromophenol blue containing 2% DTT), and then equilibrated again for 15 min in the same buffer containing 2.5% iodoacetamide instead of DTT. Equilibrated IPG strips were transferred onto 12% uniform polyacrylamide gels and run in a PROTEAN II Xi cell tank at 15 mA per gel for the first 15 min and then at a constant voltage of 250 V until the dye front reached the bottom of the gel. The gels were visualized using Coomassie brilliant blue R250 staining, after which two-dimensional (2D) gels were imaged using Powerlook 2100XL (UMAX, Fremont, CA).

### Image Analysis

Image analysis was performed by using PDQuest 7.3.0 software (Bio-Rad, Hercules, CA). After spot detection and background subtraction, gels were aligned and matched for the quantitative determination of the spots.

### Identification of Proteins with MALDI Tandem Mass Spectrometric Analysis and Database

For mass spectrometric analysis, the individual protein spots were excised from a polyacrylamide gel, washed with doubly deionized water, and destained after treating with ammonium bicarbonate and acetonitrile. Trypsin solution (0.005  $\mu\text{g}/\mu\text{l}$  in 25 mM ammonium bicarbonate) was added and samples were digested at 37°C overnight. The next day they were analyzed by the MALDI-TOF/TOF method. Proteins with the corresponding peptide mass were identified using the NCBI search engine (<http://www.ncbi.nlm.nih.gov/sites/entrez/>).

The mass spectrometric analysis was carried out using a MALDI-TOF/TOF instrument (4700 Proteomics analyzer, Applied Biosystems) with reflector positive ion mode. For MS analysis, 700–4000  $m/z$  mass range was used with 1000 shots per spectrum. A maximum of 5 precursors per spot with minimum signal/noise ratio of 50 were selected for data-dependent MS/MS analysis. A 1-keV collision energy was used for collision-induced dissociation (CID), and 1500 acquisitions were accumulated for each MS/MS spectrum. All the analyses were performed using default calibration, and the mass accuracy was calibrated to within 0.1 Da using calibration standards (Applied Biosystems) before each run.

### Western Blot Analysis

To verify the identification procedure by MALDI-TOF/TOF, one of the proteins detected by us for a Western blot analysis was selected. Prohibitin was chosen, because it is known that its function is related to E2. One hundred and twenty micrograms of the homogenate of each group of pooled ovaries was separated by SDS-PAGE and then blotted onto polyvinylidene fluoride (PVDF) membranes. The membranes were incubated with a blocking solution containing 1:200 dilution of anti-prohibitin (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and subsequently incubated with blocking solution containing 1:10,000 dilution of horseradish peroxidase-conjugated anti-rabbit

immunoglobulin (Ig) G secondary antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA). Detection was performed with BeyoECL Plus system (Beyotime Biotechnology, Jiangsu, China).

### Statistical Analysis

Data were represented as means  $\pm$  standard deviation (SD) ( $n = 8/\text{group}$ ), and the statistical evaluations of weight, E2, and BMD were performed by analysis of variance (ANOVA), with subsequent Student–Newman–Keuls test using SPSS (13.0) software. The partial correlation coefficients were used to identify the association between E2 and prohibitin expression. The criterion for significance was set at of  $p < .05$ .

## RESULTS

### Body and Ovary Weight

To examine the effect of TCDD on body weight of Sprague-Dawley rats, all animals were weighed every week during the treatment. As shown in Figure 1, the 125-ng/kg group had a significantly lower weight gain relative to control at age 32 wk, and the significant differences continued until sacrifice at wk 34. There was also a statistically significant reduction in absolute and relative ovary weight in all TCDD groups compared with the control (Table 1).

### Estradiol (E2)

The E2 levels in all three TCDD-treated groups were significantly lower than in the control group (Figure 2). Compared with the 20-ng/kg TCDD-treated group, the E2 levels in the 50- and 125-ng/kg groups were markedly lower.

### Bone Mineral Density (BMD)

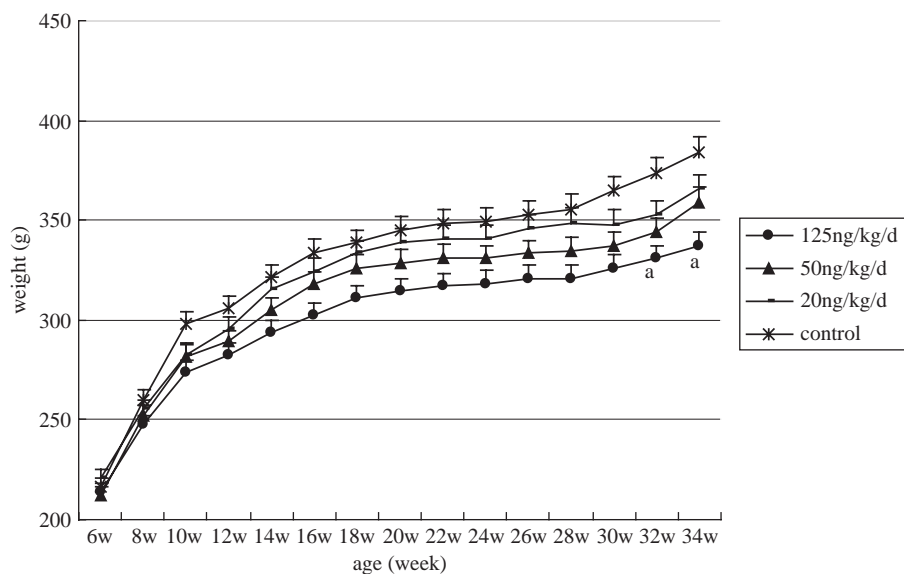
There was no significant difference of femur bone density between the control and the three TCDD groups (Figure 3).

### 2-DE and Image Acquisition

Following quantitative comparison of the gels obtained from the ovary tissues of each of the 4 groups, 610 spots were identified in the control group, 598 spots in the 20-ng/kg group, 590 spots in the 50-ng/kg group, and 599 spots in the 125-ng/kg group. These spots were matched, normalized, and quantified. Because differences cannot be detected visually, only the 2D gel images of the control group and the highest exposure group are shown in Figure 4. However, after PDQuest image analysis of the 2D images, seven spots, which showed a significant twofold change in the amount relative to control, were selected for further analysis.

### MALDI-TOF/TOF and Proteins Identification

The results of this identification procedure, such as the pI/Mw (kD), accession number, and sequence coverage, are shown in



**FIG. 1.** Effects of chronic treatment with various doses of TCDD on body weight gain. Animals were administered orally 0, 20, 50, or 125 ng/kg TCDD for 29 wk. Data are mean body weights of 8 animals per group. a, Significantly different from control ( $p < .05$ ).

**TABLE 1**  
Body Weight, Ovary Weight, and Relative Ovary Weight

Treatment	Body weight (g) at 6 wk old	Body weight (g) at sacrifice time	Ovary weight (g)	Relative ovary weight (g/kg)
Control	216.88 ± 13.87	384.00 ± 33.54	0.042 ± 0.005	0.109 ± 0.017
20 ng/kg/d	220.71 ± 24.23	365.43 ± 24.86	0.031 ± 0.006 <sup>a,b</sup>	0.090 ± 0.023 <sup>a,b</sup>
50 ng/kg/d	212.50 ± 12.54	359.13 ± 29.46	0.028 ± 0.006 <sup>a,b</sup>	0.078 ± 0.013 <sup>a</sup>
125 ng/kg/d	212.50 ± 10.34	337.44 ± 29.33 <sup>a</sup>	0.019 ± 0.003 <sup>a</sup>	0.060 ± 0.012 <sup>a</sup>

Note. Data represent mean ± standard deviation (SD) for 8 rats/group.

<sup>a</sup>Significantly different vs. control group.

<sup>b</sup>Significantly different vs. the 125ng/kg/d group.

Table 2. The following up-regulated proteins were identified: selenium binding protein 2 (SBP2), glutathione *S*-transferase mu type 3 (GSTM3), Lrpap 1 protein, NADPH, and peptidylprolyl isomerase D. The levels of these upregulated proteins, presented as relative intensity in the pooled gel spots, are shown in Figure 5 for each exposure dose. Because of the pooling prior to this identification, no standard deviations could be calculated. Two downregulated proteins were identified as prohibitin and *N*-ethylmaleimide-sensitive factor (NSF). Figure 5 shows the relative intensity of these proteins.

### Partial Correlation Analysis

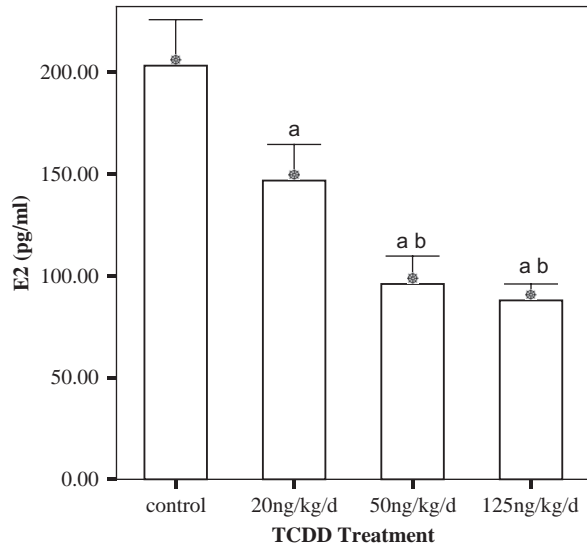
Because prohibitin was identified among the changed proteins, and because prohibitin is related to the serum E2 levels, a partial correlation analysis was performed where the TCDD dose is controlled. A positive correlation coefficient of 0.81 was noted between prohibitin and E2 levels (Figure 6).

### Detection of Prohibitin by Western Blot

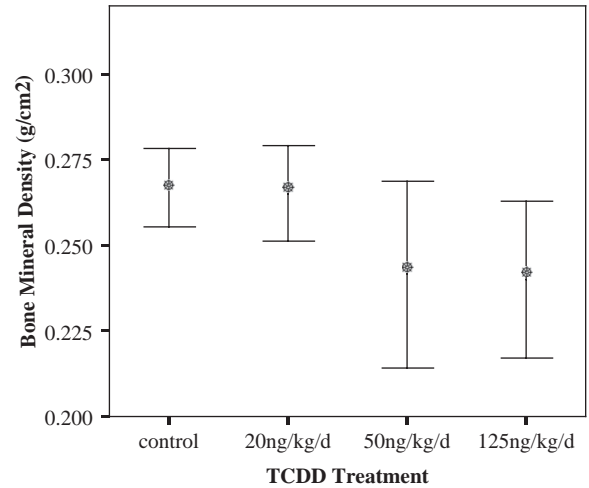
To verify the identity of one of the proteins identified by MALDI-TOF/TOF, the expression level of prohibitin was analyzed by Western blot. Prohibitin (molecular mass = 32.839 kD) was detected and confirmed to be correctly identified in the ovaries (Figure 7).

### DISCUSSION

Although the body weights of all rats increased with age, this was less pronounced in the TCDD treatment animals. The 125-ng/kg group had a significantly lower weight gain relative to control from age 32 wk until wk 34. The present findings are in agreement with a study on the effects of 0.0125, 0.05, or 0.2 µg/kg TCDD for 20 wk (Croutch et al., 2005). On the other hand, B6C3F1 mice treated with 0, 1.5, or 150 ng/kg TCDD showed no adverse effects in body weights or organ/body weight ratios (Diliberto et al., 2001). In agreement with other



**FIG. 2.** Effects of chronic treatment with varying TCDD doses on serum estradiol (E2). Data are presented as mean ± standard deviation of 8 animals per group. a, Significantly different from control ( $p < .05$ ). b, Significantly different from 20-ng/kg group ( $p < .05$ ).

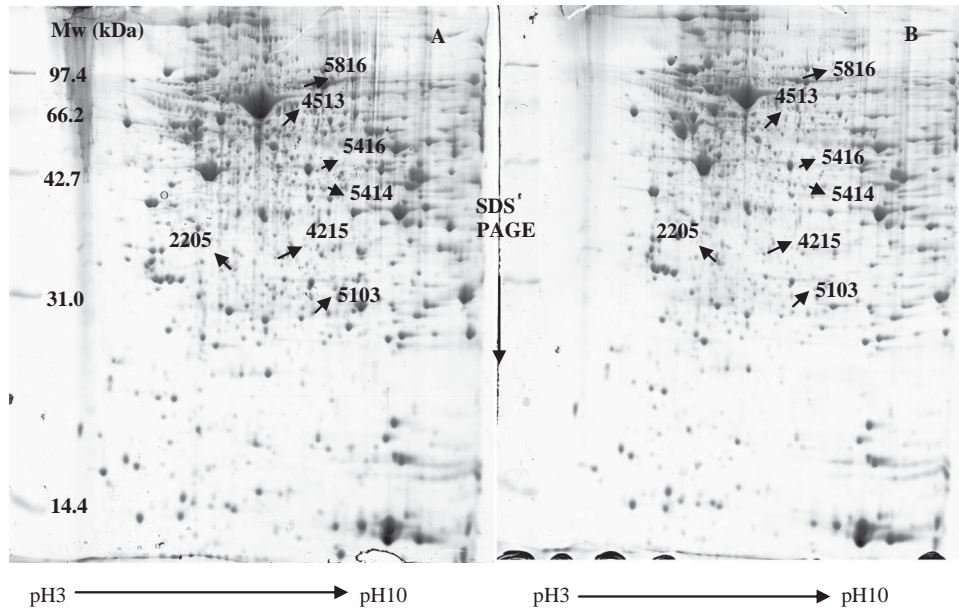


**FIG. 3.** Influence of chronic treatment with TCDD on femur bone mineral density (BMD). Data are presented as mean ± standard deviation of 8 rats per group.

studies in mice and rats, we noted a decreased ovarian weight (Johnson et al., 1997; Cummings et al., 1999), which is likely due to a direct influence of TCDD on the ovary (Heiden et al., 2006).

Lower E2 levels were found in the TCDD-treated groups. Estradiol, an important endocrine hormone, is essential for the

regulation of growth, differentiation, and function of target cells (Richards, 1980). Some investigators indicated that a decrease is partly because of enhanced estrogen metabolism (Pocar et al., 2003, 2005) and functional impairment of the ovary or hypothalamic–pituitary–gonadal (HPG) axis (Heiden et al., 2006). TCDD also inhibits the expression and activity of aromatase mRNA (Dasmahapatra et al., 2000; Tang et al., 2008), which is responsible for converting androgens to estrogens (Young et al., 1983).



**FIG. 4.** Coomassie blue-stained 2D gel images of pooled rat ovaries proteome: (A) control, (B) 125 ng/kg. Mw, Molecular mass marker; IEF, isoelectric focusing; SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis. The numbers on the map are the codes of specific proteins indicated by an arrow, which correspond to the numbers in Table 2 and Figure 5.

**TABLE 2**  
Identification of Differentially Expressed Proteins Among Four Groups

Code	Accession number in NCBI	Protein identification	Exp. pI/Mw (kD)	Theor. pI/Mw (kD)	Sequence coverage
Upregulated					
4513	EDL85767	Selenium binding protein 2	6.74/55.83	5.94/50.58	65%
6103	NP_112416	Glutathione S-transferase, mu type 3	7.75/27.80	6.84/25.68	74%
5416	AAH98947	Lrpap 1 protein	7.34/41.96	6.90/41.90	46%
4215	CAA59088	Carbonyl reductase (NADPH)	6.62/33.46	8.21/30.58	48%
5414	AAH19778	Peptidylprolyl isomerase D (cyclophilin D)	7.47/40.07	7.07/40.74	29%
Downregulated					
2205	AAH72518	Prohibitin	5.92/32.84	5.57/29.82	57%
5816	AAD39485	<i>N</i> -Ethylmaleimide sensitive factor	7.06/82.56	6.55/82.65	17%

*Note.* Exp. pI/Mw (kD), experimental molecular mass and pI. Theor. pI/Mw (kD), theoretical molecular mass and pI. Theoretical masses and pI were predicted by entering the sequence at ExPASy Molecular Biology Server ([http://cn.expasy.org/tools/pi\\_tool.html](http://cn.expasy.org/tools/pi_tool.html)).

Some studies suggest that TCDD retards sexual and skeletal maturation (Ohyama et al., 2007; Fukuzawa et al., 2004), with the latter including a decrease in BMD and a reduced bending breaking force and stiffness (Murtomaa et al., 2007). Bone changes are sensitive endpoints of dioxin exposure, but high variability limits their use as biomarkers (Murtomaa et al., 2007). TCDD exposure did not change BMD significantly in our experiment. Marked bone changes may need a higher TCDD dose in adult rats (Miettinen et al., 2005). In our study it is possible the starting age of treatment was too late to affect bone development.

Our study focused on the identification of the proteins whose levels were affected after TCDD treatment. The upregulated proteins identified were selenium binding protein 2, glutathione *S*-transferase mu type 3, Lrpap 1 protein, NADPH, and peptidylprolyl isomerase D. Prohibitin and *N*-ethylmaleimide-sensitive factor proteins were expressed in lower amounts compared with controls.

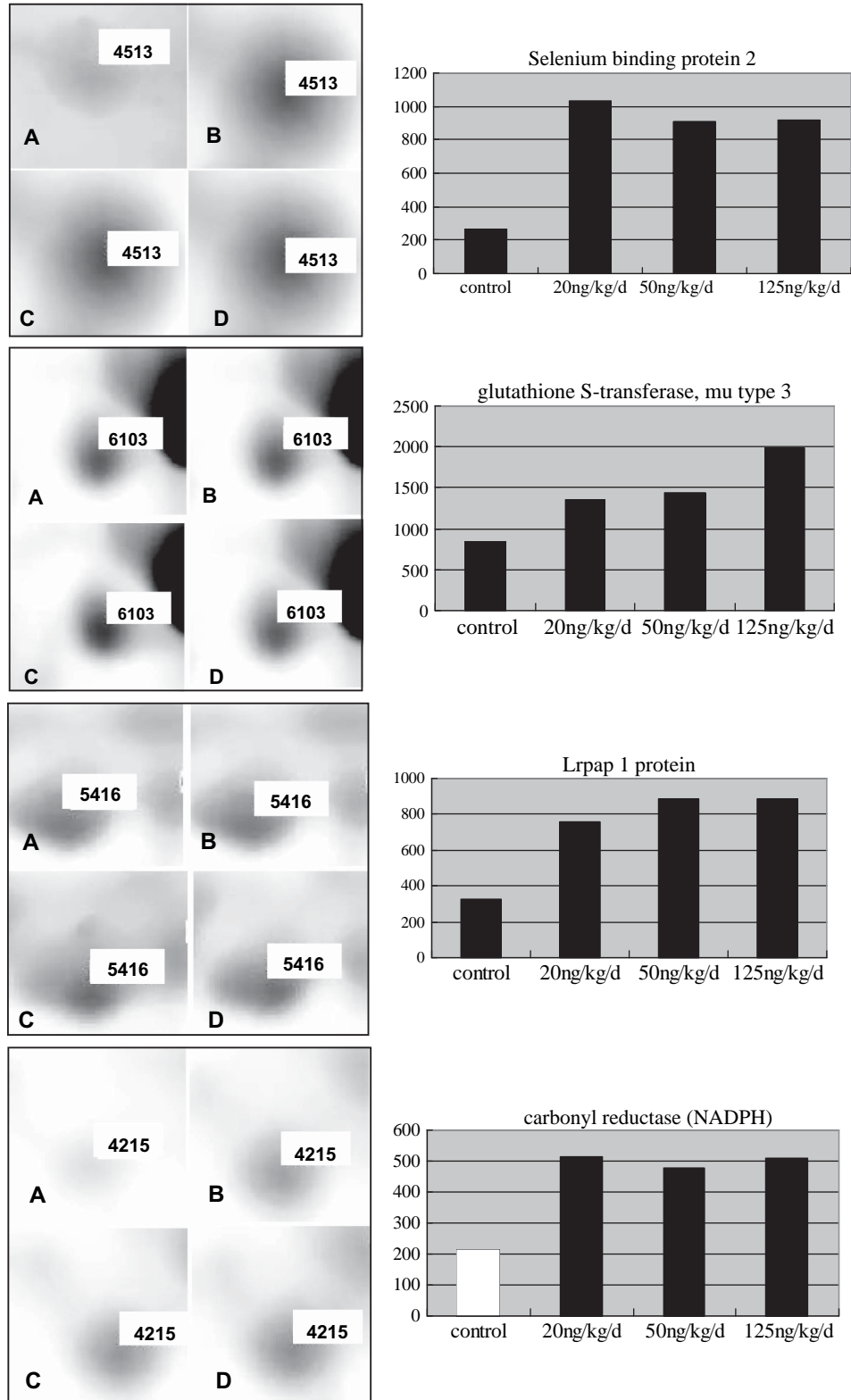
Selenium-binding protein 2 (SBP2), also known as 56-kD acetaminophen-binding protein (Mattow et al., 2006), has specific binding properties for selenium and APAP (*N*-acetyl-*p*-aminophenol) (Lanfear et al., 1993). Our findings show that SBP2 expression in the ovary was significantly higher in TCDD-treated female rats. However, the precise mechanism and function of SBP2 in the ovary are not known. Ishida et al. (2002) postulated that SBP2 is concomitantly induced by dioxin binding to AhR and by TCDD-induced oxidative stress. Transition to reproductive senescence involves changes in ovarian functions, and this process may be accelerated by TCDD (Franczak et al., 2006; Valdez & Petroff, 2004). Recent studies identified SBP2 as a crucial factor for accelerated senescence (Cho et al., 2003); thus, increased level of SBP2 in our study may be related to reproductive senescence produced by TCDD.

The prohibitins Phb1 and Phb2 are highly conserved proteins that are present in multiple cellular compartments (Kasashima et al., 2006). Craig et al. (2007) showed that Phb is expressed in all cells of the ovary. Initial investigations focused on the role of Phb1 as an inhibitor of cell proliferation, hence the original name prohibitin (Mishra et al., 2006). In animals and yeast, prohibitins were reported to play important roles in cell cycle regulation, senescence, and steroid hormone modulation (Mishra et al., 2006; Gamble et al., 2004; Kurtev et al., 2004; Montano et al., 1999; Park et al., 2005; Kasashima et al., 2006).

Phb functions as a transcriptional co-repressor for estrogen receptor  $\alpha$  (ER $\alpha$ ) in vitro and in vivo, and depletion of Phb enhanced the expression of estrogen receptor (ER) target genes (He et al., 2008). In our study, the expression of Phb was decreased in TCDD-treated groups. After controlling for the TCDD dose variable, the coefficient of correlation between estrogen and Phb levels was 0.81. It is postulated that this TCDD-induced downregulation of Phb might involve a compensatory or protective role via the reduction of ER target genes expression and decrease of E2 concentration observed.

*GSTM3*, one of the oxidative stress response genes, was upregulated in humans with chloracne (McHale et al., 2007), in waste incineration workers (Kim et al., 2004), and in animal models (Shertzer et al., 1998) following TCDD exposure. Our study identified the "glutathione *S*-transferase, mu type 3" as an upregulated protein spot, the intensity of which in the 125-ng/kg/day group was around 2.5-fold higher than for the control.

The intensity of Lrpap-1 (low-density lipoprotein-related receptor-associated protein-1) was increased in the TCDD treated groups. Lrpap-1 functions as a chaperone protein in the



**FIG. 5.** Magnified 2-DE map of spots and quantitative comparison of 7 altered protein expressions among 4 groups. The volume of the spots was normalized and quantified by PDQuest Software. Bar graphs represent the volume intensity of spots 4513, 6103, 5416, 4215, 5414, 220,5 and 5816. The upregulated proteins include selenium binding protein 2 (4513), glutathione S-transferase (6103), Lrpap1 protein (5416), NADPH (4215), peptidylprolyl isomerase D (5414). Down-regulated proteins are prohibitin (2205) and N-ethylmaleimide-sensitive factor (5816). (A) Control; (B) 20 ng/kg; (C) 50 ng/kg; (D) 125 ng/kg.



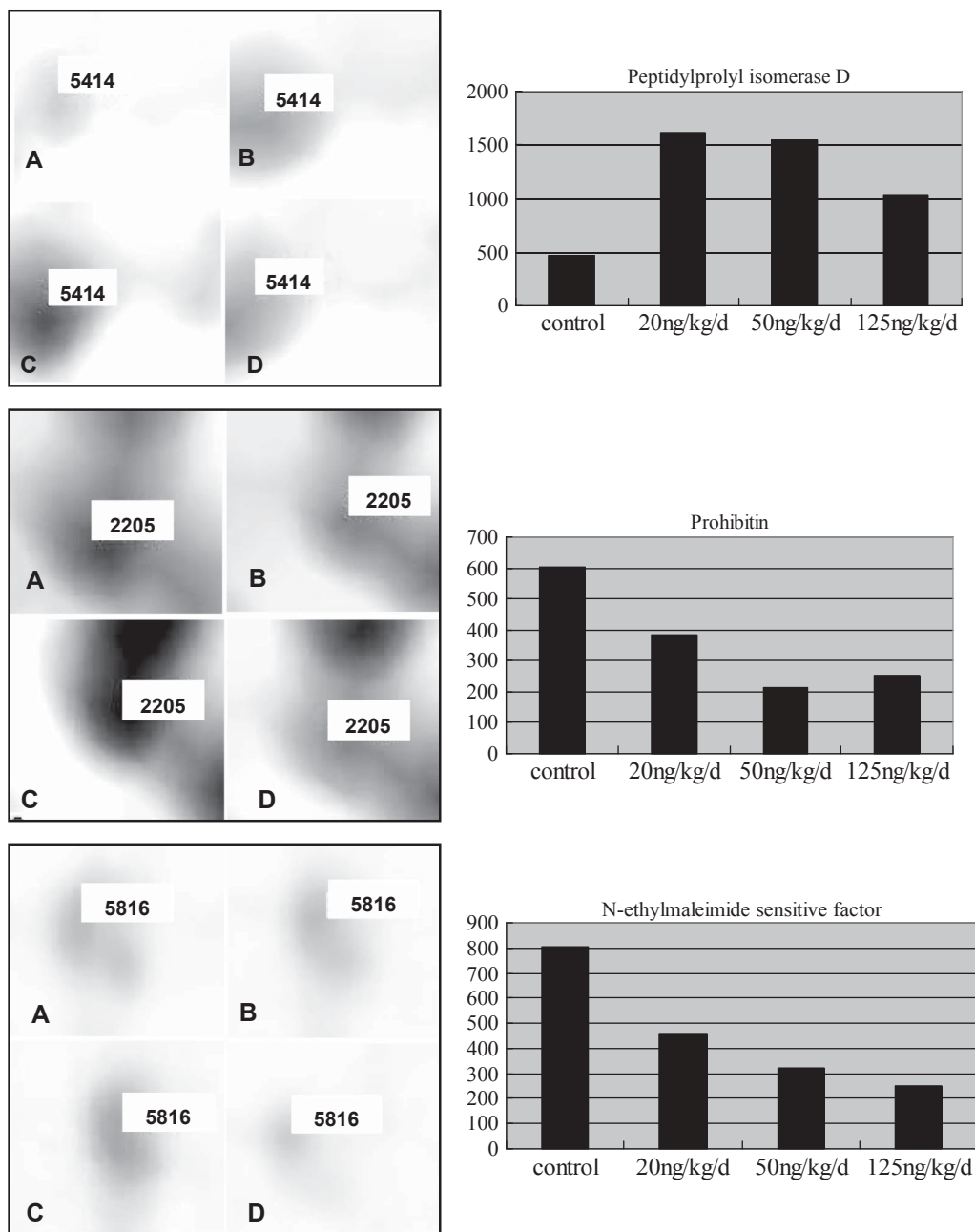
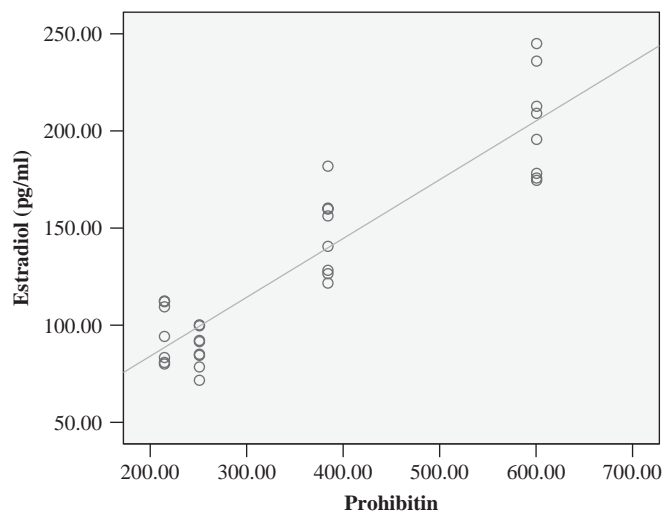


FIG. 5. (Continued).

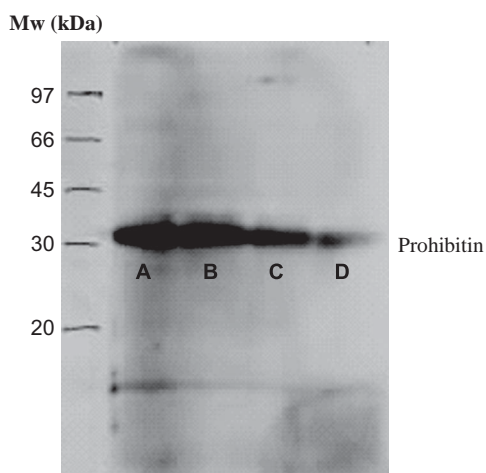
transport of low-density lipoprotein-related receptor (Gonzalez et al., 2002; Zhang et al., 2005). The Lrpap-1 gene is related to aging, and the changed expression may result from the role of TCDD in accelerating senescence.

*N*-Ethylmaleimide (NEM)-sensitive factor (NSF) is an important part of the exocytic machinery and plays a critical role in endothelial granule exocytosis (Yamakuchi et al., 2008). NSF was decreased with increasing TCDD dose, and in the 125-ng/kg group it was almost threefold lower than

the control. The xenobiotics TCDD, 2,3,4,7,8-pentachlorodibenzofuran (PeCDF), and polychlorinated biphenyl (PCB) 126 induce oxidative stress in rats after chronic treatment, and TCDD is the most potent among the congeners (Hassoun et al., 2002). TCDD elevates  $H_2O_2$  and other biomarkers of oxidative stress (Senft et al., 2002; Shen et al., 2005). The free radicals  $H_2O_2$  and nitrous oxide (NO) inhibit NSF expression, which may explain the downregulation of NSF in our study. Cyclophilin D is upregulated by oxidative



**FIG. 6.** Correlation between estradiol levels and prohibitin expression.



**FIG. 7.** Western blot analysis for ovarian prohibitin. The homogenate of each group of pooled ovaries was separated by SDS-PAGE, and then blotted onto PVDF membranes. The membranes were treated with anti-prohibitin and subsequently incubated with blocking solution containing horseradish peroxidase-conjugated anti-rabbit IgG secondary antibody. Detection was performed with BeyoECL Plus system. (A) Control; (B) 20 ng/kg; (C) 50 ng/kg; (D) 125 ng/kg. Mw, molecular mass marker.

stress (Juhaszova et al., 2008; Schneider, 2005) and was found to be upregulated in our study. Data indicate that TCDD-mediated oxidative stress may play a role in the ovaries of exposed rats.

In summary, our proteomic analysis of the rat ovary revealed altered protein level expression following chronic low-dose exposure to TCDD. Several proteins with higher up- or downregulation, such as SBP2, GSTM3, NADPH, Lrpap 1 protein, cyclophilin D, prohibitin, and NSF, may yield clues to the mechanisms underlying TCDD-mediated oxidative stress on reproductive and endocrine functions.

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