Proteome Analysis of the Effects of 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin on Murine Testicular Leydig and Sertoli Cells

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We treated testicular cell lines Leydig TM3 and Sertoli TM4 with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and used two-dimensional electrophoresis to investigate the resulting protein alterations. Cells were cultured in a medium containing 10^{-5} to 10 nM TCDD for 4 hr, under which condition viability was not affected. Protein expression was compared semi-quantitatively by silver staining, by autoradiography of [^{35}S] methionine-labeled proteins, and by anti-phosphotyrosine antibody. In TM3, 34 protein spots were altered by TCDD, 26 of which were increased and 8 of which were decreased; in TM4, the amount of total protein appeared to be reduced and 19 protein spots were altered by TCDD, 12 of which were increased and 7 of which were decreased. Four of these altered proteins were identified by N-terminal protein microsequencing and by a homology search against protein databases. Whereas a pyruvate dehydrogenase E1 beta subunit was decreased by TCDD exposure, ATP synthase beta chain, mitochondrial matrix protein P1 and 78 kDa glucose-regulated protein were relatively increased. The precise role of these proteins in TCDD toxicity remains to be determined, but the observed alterations suggest the proteins to be important in the effects of TCDD on testicular cells.

Key words —— 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (dioxin), testis, proteome, two-dimensional electrophoresis, Leydig cell, Sertoli cell

INTRODUCTION

Polychlorinated dibenzo-*p*-dioxins, which vary in terms of toxicity and persistence in the environment, are of major concern. These compounds are formed as trace contaminants in waste or as by-products of herbicides. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) is the most toxic congener and is a good candidate for investigation of the physiological and toxicological effects of this class of chemicals and their mechanisms.[1] At doses of acute and overt toxicity, TCDD can cause wasting syndrome,[2] thymic atrophy,[3] immunotoxicity,[4] teratogenicity,[5] and carcinogenesis,[6] which are commonly seen in many species.[1] TCDD also produces a variety of adverse effects on the endocrine system at small doses in laboratory animals. Its effect on the male reproductive system has been widely discussed, and many such effects are reported in rodents exposed to TCDD: reduced testis and sex accessory gland weight, impaired spermatogenesis, decreased ejaculation, and decreased plasma androgen concentrations.[7–9] Since maternal TCDD exposure in rodents could similarly affect the perinatal androgenic status in male offspring *in utero* and during lactation,[8,10–13] the effects on future generations are worrisome. The probability of developing any one of these disorders varies greatly with dose, length of exposure, and, most importantly, the species exposed,[1] making it difficult to determine the TCDD threshold for toxicity. A great deal of information is available on the histological and pathological effects of TCDD *in vivo*, and the molecular mechanism of its toxicity is gradually being clarified.

Several enzymes are known to be induced by TCDD.[14] It is well established that cytochrome P450 proteins are induced by the activation of an enhancer via an aryl hydrocarbon receptor (AhR) and AhR nuclear translocator (ARNT).[15] The expression of

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several genes has also been reported to be altered by TCDD.[16–18] Since it appears, however, that there is not a good correlation between mRNA and protein level in cells at a given time,[19] gene alteration may not necessarily correspond quantitatively to protein alteration. Thus, the effects of TCDD on both nucleic acids and proteins must be assessed.

We used two-dimensional electrophoresis (2-DE) to examine proteins in Leydig TM3 and Sertoli TM4 cells treated with TCDD to understand changes in protein expression caused by TCDD in the male reproductive system. These cell lines are derived from BALB/c mouse testis and are continuous, nontransformed cell lines that share morphological and hormonal properties with resident Leydig and Sertoli cells *in situ*.[20–22] 2-DE is a well known technique used to separate many proteins in tissues or cells and is one of the core methods in many pharmaceutical and toxicological studies.[23–25] We attempted to investigate the alteration of total proteins, newly synthesized proteins, and phosphorylated proteins induced by TCDD in murine testicular cells to better understand the mechanism of TCDD toxicity in cells.

MATERIALS AND METHODS

Cell Cultures —— Murine testis Leydig TM3 and Sertoli TM4 cell lines were obtained from the American Type Culture Collection (MD, U.S.A.). Cells were cultured in a 1 : 1 mixture of Dulbecco's modified Eagle's and Ham's F12 (DME/F12) medium (GIBCO BRL, NY, U.S.A.) supplemented with 5% horse serum (HS) (Nacalai, Kyoto, Japan) and 2.5% fetal bovine serum (FBS) (JRH Biosciences, KS, U.S.A.). Cells were cultured in a humidified 95% air/ 5% CO_2 atmosphere at 37°C.

Cells from exponentially growing stock cultures were removed from the plate with Ca^{2+}- and Mg^{2+}-free phosphate-buffered saline (PBS) (Nissui Pharmaceutical, Tokyo, Japan) containing 0.1% trypsin (DIFCO, NJ, U.S.A.) and 0.02% ethylenediamine-*N*,*N*,*N*′,*N*′-tetraacetic acid (EDTA). Cells were plated into tissue culture dishes at a density of 3×10^6 cells/ml.

Quantitation of Viable Cells —— Cells were seeded at a concentration of 8×10^4 cells/ml in microtiter plates in 100 μl of DME/F12 medium. After 42 hr, 100 μl of TCDD (GL Sciences, Tokyo, Japan) was added to wells at final concentrations of 10^{-5}, 10^{-4}, 10^{-3}, 10^{-2}, 10^{-1}, 1, and 10 nM. In control groups, cells were cultured in a medium containing the same concentrations of dimethyl sulfoxide, a TCDD solvent, as the TCDD-containing medium. After additional incubation for 4, 12, 24, and 72 hr, TM3 cells were stained with crystal violet, and viable cells were counted at 595–450 nm with a microplate reader (Model 550, Bio-Rad, CA, U.S.A.).[26] TM4 cells were counted with a WST-1 kit (Dojindo, Kumamoto, Japan) at 450 nm.

Cell Protein Preparation and Quantification —— Cells were washed three times with PBS and suspended in 10% trichloroacetic acid (TCA) in PBS at 0°C for 15 min. The following procedures were carried out at 0°C. Cells were collected by scraping and were centrifuged at 428 *g* for 3 min. The precipitate was washed twice with ice-cold PBS and centrifuged at 1711 *g* for 10 min. After removal, the supernatant was mixed with acetone containing 10% TCA, 0.07% 2-mercaptoethanol (2-ME), 1 mM phenylmethylsulphonyl fluoride, and 2 mM EDTA, then homogenized with a sonicator (Model UR-200P, Tommy Seiko, Tokyo, Japan). The mixture was allowed to stand at –20°C for 45 min, then centrifuged at 1711 *g* for 5 min. The precipitate was mixed with acetone containing 0.07% 2-ME, allow to settle at –20°C for 1 hr, and centrifuged at 1711 *g* for 5 min. After being washed with the same acetone solution, the precipitate was centrifuged at 1711 *g* for 5 min and dried at 4°C. The resultant powder was subjected to quantification of total protein and first dimensional isoelectric focusing (IEF).

The total protein concentration was quantified using a Protein Assay kit (Bio-Rad) against the ovalbumin standard.[27] Protein dissolved in the sample solution was subjected to 2-DE; 70 μg was used for [^{35}S] methionine-labeling or silver staining, 1 mg for the analysis of tyrosyl phosphorylated proteins, and 2 mg for *N*-terminal sequencing.

Two-Dimensional Polyacrylamide Gel Electrophoresis —— Protein powder was dissolved in a buffer (400 μl) consisting of 9 M urea, 2% 2-ME, 2% Pharmalyte (pH 3–10, Amersham Pharmacia Biotech, Uppsala, Sweden), 0.5% Triton X-100, and 0.003% Bromophenol Blue (BPB), and applied to an 18 cm Immobiline DryStrip (Amersham Pharmacia Biotech). An immobilized pH gradient (IPG) gel with a linear gradient of pH 3–10 was used for IEF, as described by Görg *et al.*,[28,29] on a horizontal electrophoresis system, Multiphor II (Amersham Pharmacia Biotech). Immediately prior to IEF, the protein solution was mixed with 2 μg of external standard marker, (SDS-PAGE Molecular

Weight Standards, Low Marker, Bio-Rad). The IPG gel was rehydrated with the protein solution overnight at room temperature according to the manufacturer's instructions. The run condition was 500 V for 1 hr, followed by a linear increase from 500 to 3500 V over 1 hr, and then 3500 V for 15 hr. After IEF, the IPG gel was pre-equilibrated at 25°C for 15 min in a buffer consisting of 50 mM Tris-HCl (pH 6.8), 6 M urea, 30% glycerol, 1% sodium dodecyl sulfate (SDS), 16 mM dithiothreitol, and 0.01% BPB.

Two-dimensional SDS-polyacrylamide gel electrophoresis (PAGE) on a 12.5% running gel (20 × 20 × 0.15 cm) was carried out without the stacking gel, essentially as described by Laemmli.[30] IPG gels were placed on top of polyacrylamide gels, and electrophoresis was conducted at 20 W/gel.

Silver Staining of 2-DE Gel —— Silver staining of the 2-DE gels was carried out with the 2D-silver stain II "Daiichi" kit (Daiichi, Tokyo, Japan) according to the manufacturer's instructions, but with minor modifications.[31]

[^{35}S] Methionine-Labeling of Cellular Proteins —— Testicular cells were cultured at a density of 1 × 10^6 cells/dish for 2 days and then washed with DME methionine-free medium. After incubation in the medium containing TCDD, proteins were biosynthetically labeled for 1 hr by the addition of 1.85 MBq of [^{35}S] methionine (Expre ^{35}S^{35}S[^{35}S] Protein Labeling Mix, > 37 TBq/mmol, NEN, DE, U.S.A.) to the medium. Cells were cultured at 37°C for 1 hr in a humidified 95% air/ 5% CO_2 atmosphere.

Immunoblotting of Phosphorylated Tyrosine in Testicular Cells —— After 2-DE, gels were shaken gently for 5 min in a transfer buffer consisting of 250 mM Tris, 200 mM glycine, and 20% methanol. The proteins were electrophoretically transferred onto a nitrocellulose membrane (Optitran BA-S 85 reinforced NC, Schleicher & Schuell, NH, U.S.A.) at a constant current (1 mA/cm^2) for 2 hr at room temperature. A semi-dry blotting system (Model NA-1512, Nihon-Eido, Tokyo, Japan) was used. The membrane was incubated in a solution of 5% bovine serum albumin in 1 M Tris-HCl buffer (pH 7.4) and 0.1% Tween 20 for 1 hr to block nonspecific protein-binding sites. Immunodetection was carried out by incubation of the membrane with anti-phosphotyrosine coupled with monoclonal IgG2b$_k$ (1 : 5000, Upstate Biotechnology, NY, U.S.A.), followed by reaction with a secondary antibody, anti-mouse IgG, and horseradish peroxidase complex for 1 hr. Protein spots were detected by the addition of 50 mM Tris-HCl (pH 7.4) containing 0.2% 3,3′-diaminobenzidine and 30% H_2O_2.

Protein Identification —— The proteins separated with 2-DE were electroblotted onto a polyvinylidene difluoride (PVDF) membrane (Fluorotrans, Pall Corporation, NY, U.S.A.) at 1 mA/cm^2 for 4 hr at room temperature. The transfer buffer consisted of 100 mM 3-(cyclohexylamino)-1-propanesulfonic acid and 10% methanol (pH 11.0).[32] The membrane was stained with 0.1% Coomassie Brilliant Blue R-250 (CBB) in 50% methanol for 5 min, destained with 10% acetic acid in 40% methanol for 5 min, then washed with water for 10 min.[33] Spots on the PVDF membrane were excised, washed with ethanol, and transferred into the cartridge of the Procise 494cLC sequencing system (PE Applied Biosystems, CA, U.S.A.). Sequencing was carried out according to the sequencer instruction manual, and phenylthiohydantoin-amino acids were identified with an on-line high performance liquid chromatography system consisting of a model 140D gradient pump and a model 785A detector (PE Applied Biosystems).[34] The amino acid sequence obtained from each protein spot was searched for sequence similarity against Protein Information Resource International Database release 63.01 (http://www-nbrf.georgetown.edu/)[35] and/or SWISS-PROT release 38 (http://www.expasy.ch/sprot/).[36] The theoretical isoelectric point and molecular weight were calculated with an ExPASy proteomics tool (http://www.expasy.ch/tools/pi_tool.html).[37,38]

Statistical Analysis —— Viable cell numbers are shown as the mean ± S.D. of four determinations. Statistical significance was determined by Student's *t*-test, and a *p*-value of less than 0.05 was considered significant.

RESULTS AND DISCUSSION

Viability of Cells Exposed to TCDD

The initial experiments were undertaken to determine the effect of TCDD on the viability of Leydig TM3 and Sertoli TM4 cells (Fig. 1). TCDD did not significantly affect the viable cell count at the concentration of 10 nM for 72 hr. This result was supported by the findings of another study in which no particular change in dead cell numbers measured by the release of L-lactate dehydrogenase was observed for 48 hr at concentrations up to 30 nM.[17] Accordingly, we decided to expose TM3 and TM4 cells to 10 nM TCDD for 4 hr to examine altered proteins

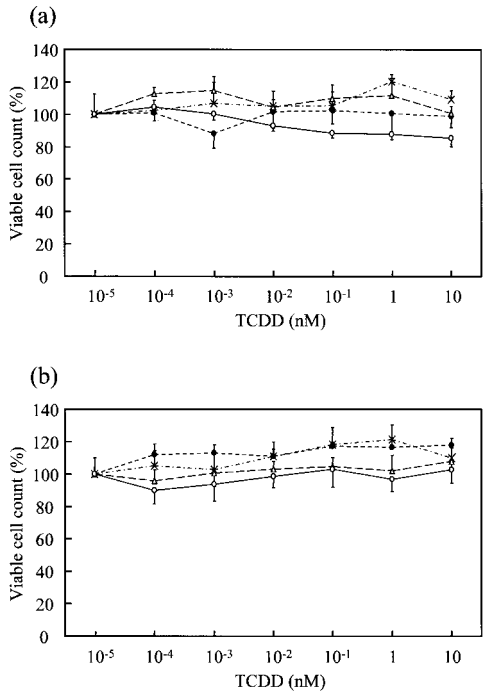

Fig. 1. Effects of TCDD Exposure on Testicular Cell Viability

Values are means ± S.D. for four determinants. (a) Leydig TM3 and (b) Sertoli TM4 cell lines. The number of viable cells was counted using crystal violet staining for TM3 and WST-1 for TM4 at 4 (●), 12 (×), 24 (△), and 72 hr (○).

over a short period.

Analysis of Proteins Altered by TCDD Exposure

The 2-DE profile spots altered by TCDD in Leydig TM3 and stained by silver nitrate are shown in Fig. 2. TCDD exposure led to an increase in 3 proteins and a decrease in 4 proteins in TM3 cells (Tables 1 and 2). Changes in Sertoli TM4 cells are also listed in Tables 1 and 2. These are summarized in Table 3. Several spots which were relatively increased by TCDD and detected with autoradiography were subjected to N-terminal sequencing, but all were unidentified (Table 4). There were 2 proteins observed at the same molecular weight and the same isoelectric point in both TM3 and TM4 cells.

The 2-DE profile of each staining differed markedly, with no spots in common between silver staining and autoradiography (Figs. 2 and 3). We considered this difference to be due to the composition of amino acids or affinity of each protein to the dye reagent. This illustrates the importance of using more than one detection method in the examination of proteins in 2-DE.

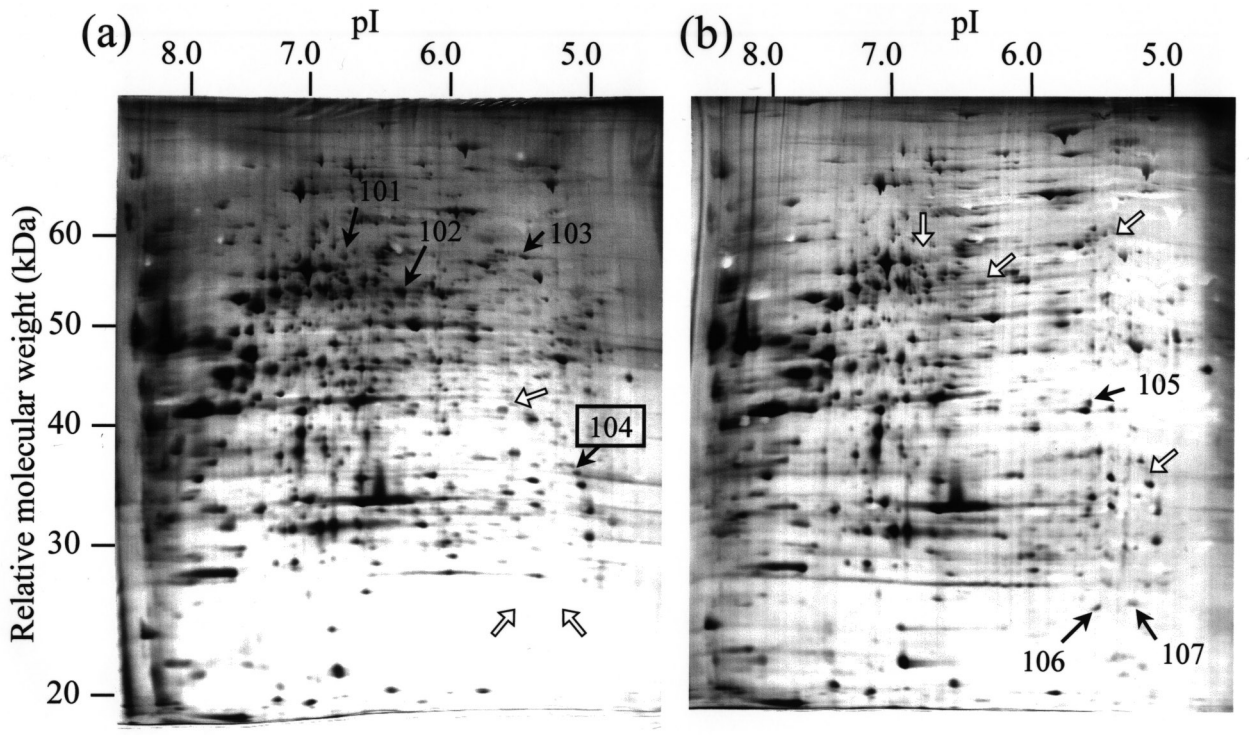

Fig. 2. Protein Changes Due to TCDD Exposure in Leydig TM3 Cell Line

The cells were grown (a) in DME/F12 medium and (b) in the medium with 10 nM TCDD for 4 hr. The numbers with arrows mark spots which semi-quantitatively varied on 2-DE. (The derived data are shown in Tables 1 and 2.) The number in the rectangle indicates the spot identified by N-terminal sequencing. Decreased spots after exposure to TCDD are marked by black arrows in (a), and increased spots are marked by black arrows in (b). White arrows indicate the spots corresponding to the black ones on the other gel. The first dimension used an immobilized pH gradient gel (pH 3–10), and the second dimension used a 12.5%T polyacrylamide gel. The gels were stained with silver nitrate.

Table 1. Semi-Quantitatively Varied and Identified Proteins Exposed to TCDD

Cell line	Quantity[a]	Protein name	N-terminal sequence[b]	Observed		Theoretical		Detection method[d]	Number of spots[e]
				MW[c] (kDa)	pI[c]	MW (kDa)	pI		
TM3	–	Pyruvate dehydrogenase (EC 1.2.4.1) E1 beta subunit, mitochondrial	VQLTVXEAINQ/	34.1	5.0	35.840	5.3	Ag	104
TM4	+	ATP synthase (EC 3.6.1.34) beta chain, mitochondrial	AAQAXAAPKA/	51.6	4.9	51.800	5.0	AR	—
	+	Mitochondrial matrix protein P1	AKDVKFGADAR/	54.3	5.4	57.926	5.4	AR	—
	+	78 kDa glucose-regulated protein	EEEDKKEDV/	62.4	5.0	70.464	5.1	AR	—

a) +, Relatively increased by TCDD exposure; −, decreased. *b*) N-terminal sequences are shown using the single-letter for amino acid residues. X indicates that no interpretation could be made. *c*) MW, molecular weight; pI, isoelectric point. *d*) Spots were stained with silver nitrate (Ag) or detected with autoradiography (AR). *e*) See Fig. 2.

Table 2. Semi-Quantitatively Varied and Unidentified Proteins Exposed to TCDD by Analysis of Silver Staining

Cell line	Quantity[a]	Observed		N-terminal sequence[c]	Number of spots[d]
		MW[b] (kDa)	pI[b]		
TM3	+	39.4	5.6	—	105
	+	24.8	5.2	—	107
	+	24.5	5.5	—	106
	–	58.2	7.0	Blocked	101
	–	57.4	5.2	—	103
	–	53.1	6.4	—	102
TM4	+	41.2	6.9	—	—
	+	18.4	6.5	Blocked	—
	–	23.0	5.1	—	—
	–	18.3	6.0	Blocked	—

a) +, Increased by TCDD exposure; −, decreased. *b*) MW, molecular weight; pI, isoelectric point. *c*) Minus signs stand for spots not large enough to be stained with Coomassie Brilliant Blue R-250. *d*) These numbers are derived from Fig. 2. Minus signs indicate the spots whose electrophoretic patterns are not shown.

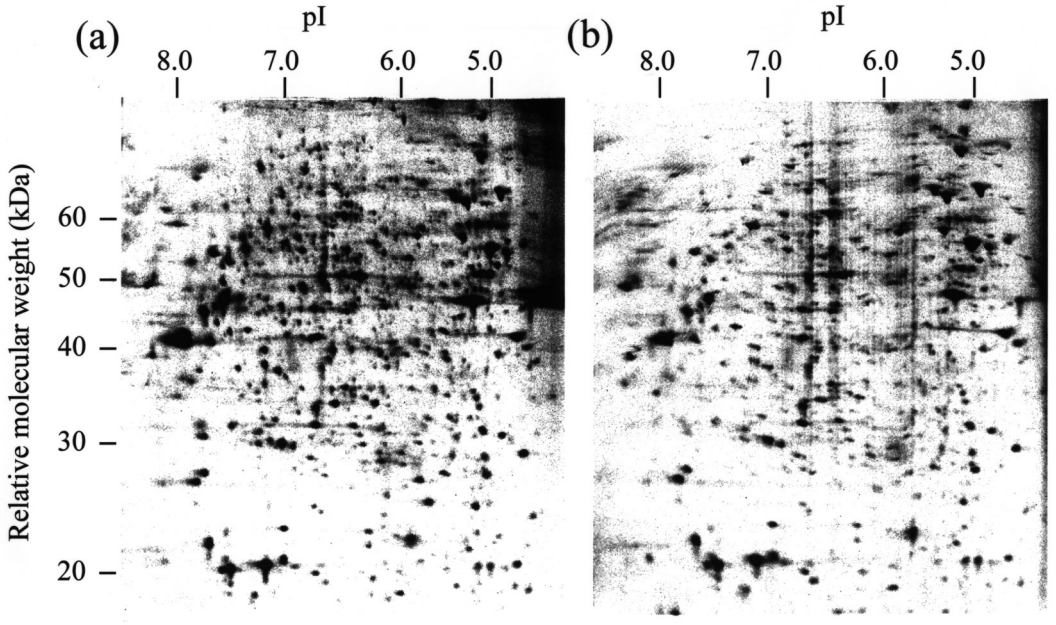

Fig. 3. Autoradiograms from 2-DE of Proteins from Leydig TM3 Exposed to TCDD

The cells were grown (a) in DME/F12 medium and (b) in a medium containing 10 nM TCDD for 4 hr, then labeled with [^{35}S] methionine for 1 hr. All other conditions are as described in MATERIALS AND METHODS.

Table 3. Number of Spots Affected by TCDD Exposure on Two-Dimensional Electrophoretic Patterns

	Quantity[a)]	Silver staining	Auto-radiography	Phosphorylation
TM3	+	3	13	10
	−	4	2	2
TM4	+	2	8	2
	−	2	1	4

a) +, Increased by TCDD exposure; −, decreased.

Table 4. Relatively Increased and Unidentified Proteins Detected with Autoradiography in TM3 and TM4 Cell Lines Exposed to TCDD

Cell line	Observed		N-terminal sequence[b)]
	MW[a)] (kDa)	pI[a)]	
TM3	63.7	5.6	—
	61.5	5.0	—
	60.1	5.3	Blocked
	54.7	5.3	Blocked
	51.6	4.9	—
	51.6	5.1	Blocked
	33.8	8.3	—
	32.6	8.0	—
	20.9	5.6	—
	19.4	8.1	—
	18.1	6.6	Blocked
TM4	60.1	5.3	Blocked
	52.0	5.0	—
	20.2	5.4	—
TM3, TM4	48.0	5.1	Blocked
	44.3	5.2	Blocked

a) MW, molecular weight; pI, isoelectric point. *b*) Minus signs indicate spots not large enough to be stained with Coomassie Brilliant Blue R-250.

Table 5. Spots Wherein Phosphorylation was Affected by TCDD Exposure

Cell line	Phosphorylation[a)]	Observed		N-terminal sequence[c)]
		MW[b)] (kDa)	pI[b)]	
TM3	+	54.9	6.7	—
	+	48.2	6.9	Blocked
	+	44.8	7.6	—
	+	41.5	6.9	Blocked
	+	41.2	6.7	Blocked
	+	39.8	7.1	Blocked
	+	39.2	8.1	—
	+	38.3	6.6	Blocked
	+	36.5	7.4	Blocked
	+	33.7	6.7	Blocked
	−	52.5	7.6	Blocked
	−	44.1	5.3	—
TM4	+	44.3	7.6	Blocked
	+	33.6	6.9	Blocked
	−	52.9	7.5	Blocked
	−	45.6	5.2	—
	−	42.3	5.2	—
	−	18.4	7.2	Blocked

a) +, Relatively increased; −, decreased. *b*) MW, molecular weight; pI, isoelectric point. *c*) Minus signs indicate spots not large enough to be stained with Coomassie Brilliant Blue R-250.

Analysis of Phosphorylated Proteins Affected by TCDD Exposure

The mechanism of TCDD toxicity via AhR/ARNT is well known, and protein kinases such as c-Src have been proposed as another signal transfer pathway.[39–43)] With this in mind, we investigated phosphorylated proteins with an anti-phosphotyrosine antibody (2-DE patterns not shown). Several spots were observed to be altered in phosphorylation (Tables 3 and 5). The fact that more proteins were phosphorylated in TM3 than in TM4 recalls the reported elevation in tyrosine kinase and protein kinase C activities in response to TCDD exposure.[42,43)] Since there were no identical spots (Tables 1, 2, 4, and 5), we surmised that TCDD exposure induced not the expression of phosphorylated proteins, but rather the actual phosphorylation of the proteins. N-terminal sequencing attempted to determine those characteristic proteins, but they were not identified because all were blocked at the N-termini (Table 5).

Identification of Proteins Semi-Quantitatively Affected by TCDD Exposure

The proteins affected by TCDD were examined by N-terminal amino acid sequencing. Spots with a common molecular weight and isoelectric point on different gels were considered to be the same proteins, since standard marker proteins were found at the same positions by the different gels. Due to the relatively low sensitivity of CBB staining, however, only 28 spots (15 for TM3 and 13 for TM4) were subjected to N-terminal sequencing. Four proteins that were identified by the homology of N-terminal sequences and the observed and theoretical molecular weight and isoelectric point are shown in Table 1. Each N-terminal sequence was identical to the amino acid sequence without the signal peptide predicted by similarity on the databases. We found that more than 70% of spots were blocked at the N-termini because stepwise degradation could not progress; also, the TM3 and TM4 cell lines had more N-ter-

Table 6. Spots Wherein N-Terminal Sequencing was Conducted

	Variation in quantity[a)]	Analyzed	Sequenced[b)]	N-Terminally blocked
Mouse Leidig TM3	34	15	1	14 (93.3%)
Mouse Sertoli TM4	19	13	3	10 (76.9%)
Mouse brain[47)]	—	301	124	180 (59.8%)
Arabidopsis thaliana[46)]	—	136	100	57 (41.9%)
Rice[45)]	—	282	127	157 (55.7%)
Fusarium sporotrichioides[44)]	—	106	58	48 (45.3%)

a) Sum of proteins altered by TCDD and detected with silver staining, autoradiography, and anti-phosphotyrosine antibody. *b*) Internally sequenced spots were also counted.

minal blockage than other species (Table 6).[44–47)]

Seventy-eight kDa glucose-regulated protein (shown in Table 1), which is identical to immunoglobulin heavy chain binding protein, is localized in the endoplasmic reticulum lumen[48)] and belongs to the hsp70 family, sharing several family structural and biochemical characteristics.[49)] It has two domains, a peptide-binding domain and an ATPase domain,[50)] and is involved in the molecular chaperone of secretory proteins.[51)] Mitochondrial matrix protein P1, or heat shock protein 60, functions in additional refolding of misfolded or denatured proteins in a mitochondrial matrix.[52)] It is localized in Leydig and Sertoli cells of rat testis and is expressed coincidentally with the stages of spermatogonia mitotic division.[53)] In the present study, these two heat shock proteins were found only in Sertoli TM4 cells, so they may play some role in the intrinsic function of Sertoli cells to protect against stresses brought on by TCDD. The other proteins shown in Table 1 are housekeeping proteins, but these have not been reported in relation to TCDD so far.

We consider further research necessary 1) to identify the other proteins that were changed in this study as a result of TCDD exposure, 2) to apply several other methods of detection for more in-depth examination of altered proteins, and 3) to clarify the relation of these proteins to the effects of TCDD on testicular cells.

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