Toxicology and Applied Pharmacology 231 (2008) 374–383

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Critical role of cyclooxygenase-2 activation in pathogenesis of hydronephrosis caused by lactational exposure of mice to dioxin

Noriko Nishimura [a,*], Fumio Matsumura [b], Christopher F.A. Vogel [b], Hisao Nishimura [c], Junzo Yonemoto [a], Wataru Yoshioka [d], Chiharu Tohyama [a,d,*]

[a] Research Center for Environmental Risk, National Institute for Environmental Studies, Tsukuba 305-8506, Japan
[b] Department of Environmental Toxicology and Center for Environmental Health Sciences, University of California, Davis, CA 95616, USA
[c] Department of Human Sciences, Aichi Mizuho University, Toyota 470-0394, Japan
[d] Laboratory of Environmental Health Sciences, Center for Disease Biology and Integrative Medicine, Graduate School of Medicine, The University of Tokyo, Tokyo 113-0033, Japan

ARTICLE INFO

Article history:
Received 18 February 2008
Revised 12 May 2008
Accepted 14 May 2008
Available online 24 May 2008

Keywords:
Cyclooxygenase-2
Inflammation
Hydronephrosis
Lactational exposure
2,3,7,8-tetrachlorodibenzo-p-dioxin

ABSTRACT

Congenital hydronephrosis is a serious disease occurring among infants and children. Besides the intrinsic genetic factors, *in utero* exposure to a xenobiotic, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD), has been suggested to induce hydronephrosis in rodents owing to anatomical obstruction in the ureter. Here, we report that hydronephrosis induced in mouse pups exposed lactationally to TCDD is not associated with anatomical obstruction, but with abnormal alterations in the subepithelial mesenchyma of the ureter. In the kidneys of these pups, the expressions of a battery of inflammatory cytokines including monocyte chemoattractant protein (MCP)-1, tumor necrosis factor α (TNFα) and interleukin (IL) -1β were up-regulated as early as postnatal day (PND) 7. The amounts of cyclooxygenase (COX) -2 mRNA and protein as well as prostaglandin E2 (PGE_2) were conspicuously up-regulated in an arylhydrocarbon-receptor-dependent manner in the TCDD-induced hydronephrotic kidney, with a subsequent down-regulation of the gene expressions of Na^+ and K^+ transporters, NKCC2 and ROMK. Daily administration of a COX-2 selective inhibitor to newborns until PND 7 completely abrogated the TCDD-induced PGE_2 synthesis and gene expressions of inflammatory cytokines and electrolyte transporters, and eventually prevented the onset of hydronephrosis. These findings suggest an essential role of COX-2 in mediating the TCDD action of inducing hydronephrosis through the functional impairment rather than the anatomical blockade of the ureter.

Introduction

Congenital obstructive nephropathy is the most frequent cause of renal failure in infants and children (Chevalier, 2004). Fetal hydronephrosis was found in 0.59% to 1.4% of fetuses, with at least 20% being clinically significant (Belarmino and Kogan, 2006; Grasso and Gitlin, 2006). Hydronephrosis is characterized by a marked dilatation of the pelvis and calyces and thinning of the renal parenchyma, and is caused mainly by anatomical obstruction, most commonly found at the ureteropelvic junction of the ureter, but in some cases, by the abnormal peristalsis of the ureter (Zeidel and Pirtskhalaishvili, 2004).

More than 40 types of mouse strain, mutated either spontaneously or by artificial techniques such as gene targeting or ethylnitrosourea exposure, exhibit hydronephrosis (as shown by the Mouse Genome Informatics Database of the Jackson Laboratory), were categorized into several groups on the basis of the coded proteins, such as electrolyte transporters, renin–angiotensin system related proteins, transcription factors necessary for development, and structural proteins. In particular, mice that were subjected to a homologous recombination to knock out a gene coding for the NaK2Cl cotransporter (NKCC2) (Takahashi et al., 2000) or the renal outer medullary potassium channel (ROMK) (Lorenz et al., 2002; Lu et al., 2002) were shown to have symptoms of polyuria and electrolyte imbalance, resulting in hydronephrosis of varying severities. The NKCC2 gene is expressed in the renal epithelial cells of the thick ascending limb of Henle's loop (TAL) and macula densa, and the ROMK gene is responsible for the transport of potassium ions at the apical potassium ion channel in the TAL, with a subsequent reabsorption of NaCl. The NKCC2 and ROMK genes are known to be responsible for Bartter's syndrome. In addition, mice whose arginine vasopressin receptor 2 (AVPR2) (Yun et al., 2000) or aquaporin 2 (AQP2) (McDill et al., 2006) were knocked out were reported to develop hydronephrosis. In humans, the urine volume that overwhelms the capacity of transfer of urine from the kidney to the bladder can contribute to hydronephrosis induction in the absence of anatomical obstruction (Zender et al., 1992; Uribarri and Kaskas, 1993). These findings suggest that the abnormal handling of electrolyte and water by these transporters may lead to hydronephrosis.

* Corresponding authors. N. Nishimura is to be contacted at Fax: +81 29 850 2561. C. Tohyama, Fax: +81 3 5841 1434.
E-mail addresses: nishimura.noriko@nies.go.jp (N. Nishimura), mtohyama@mail.ecc.u-tokyo.ac.jp (C. Tohyama).

0041-008X/$ – see front matter © 2008 Elsevier Inc. All rights reserved.
doi:10.1016/j.taap.2008.05.012

Besides these intrinsic genetic factors, *in utero* exposure to xenobiotic chemicals such as 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) has been shown to induce hydronephrosis in rodent fetuses (Birnbaum et al., 1985; Abbott et al., 1987; Thomae et al., 2006). In these studies, pregnant mice were treated with TCDD during gestation, and the fetuses were autopsied and examined for anomalies. Hydronephrosis and cleft palate were documented as the main typical teratogenic phenotypes, and the hydronephrosis was considered to be a more sensitive phenotype of TCDD toxicity than cleft palate (Theobald et al., 2003; Thomae et al., 2006). The lactational exposure of newborn mice to TCDD was also reported to induce hydronephrosis (Couture-Haws et al., 1991). This teratogenic phenotype was proven to be arylhydrocarbon receptor (AhR)-mediated, because TCDD-administered AhR-null mutant progeny showed no signs of hydronephrosis (Mimura et al., 1997; Peters et al., 1999). The TCDD-induced hydronephrosis of mouse fetuses was reported to be induced by anatomical osbtruction in the ureter, and this obstruction was suggested to be caused by hyperplasia of the ureteric epithelium mediated by epidermal growth factor (EGF) (Abbott and Birnbaum, 1990; Bryant et al., 2001). However, studies using EGFR-null mice or EGF-null mice suggested that the EGF signaling pathway is not essential for the TCDD-induced hydronephrosis. The molecular mechanism of the TCDD-induced hydronephrosis thus remained to be elucidated.

In the present study, we report that newborn mice lactationally exposed to TCDD showed no overt anatomical obstruction in the ureter, but exhibited abnormal morphology in the ureteral subepithelial mesenchyma, and that the enhancement of COX-2 activity by TCDD, which increased prostaglandin E2 (PGE_2) synthesis, plays an essential role in the disruption of electrolyte (Na^+ and K^+) transporters and the onset of hydronephrosis. We also suggest that TCDD-altered inflammation signaling, as well as AhR signaling, is responsible for the etiology of hydronephrosis, probably via the functional impairment of the ureter.

Methods

Animals and exposure to TCDD. Male and female C57Bl/6J mice were purchased from Clea Japan Inc (Tokyo, Japan), given laboratory chow (Clea Rodent Diet CE-2, CLEA Japan Inc.) and maintained at the National Institute for Environmental Studies and the University of Tokyo. AhR-null mice (Mimura et al., 1997), a kind gift from Prof. Yoshiaki Fujii-Kuriyama, University of Tsukuba, were backcrossed with C57Bl/6J for 10 generations. Heterozygous AhR mice (AhR+/−) were mated with each other to produce AhR-null (AhR−/−) mice. All the animals were kept in a controlled room at 23 ± 1°C with humidity at 50 ± 10% on a 12-h light–dark cycle, provided with food and distilled water *ad libitum*, and were handled humanely following the guidelines for animal experiments of both institutions.

TCDD (purity > 99.5%) was purchased from Cambridge Isotope Laboratory (Andover, MA). TCDD solution dissolved in nonane was diluted with corn oil so that the intended dose of TCDD would be delivered in a final volume of 2.5 mL/kg body weight. Ten-week-old C57Bl/6J female mice in proestrus were mated 1:1 with males overnight. After spontaneous delivery, the dams were administered TCDD at a single oral dose of 10 μg/kg body weight or an equivalent volume of corn oil on postnatal day PND 1. Pups were culled to six, if possible, and were exposed to TCDD via lactation. To minimize possible litter effects, we selected a pup from each dam to form TCDD-exposed and control groups. Male and female pups were used, but only the data of male pups are described in this paper in detail, unless otherwise specified.

We performed multiple sets of experiments under the identical protocol in terms of lactational TCDD exposure. We collected kidney, ureter and urine specimens form TCDD-exposed mice or control mice in a total of five experiments, and compiled the data for Table 2, and other sets of experiments were performed for histology of the ureter and ink injection. The reason for performing multiple sets of experiments is three-fold. First, the size of kidneys of mouse pups was not large enough to be used for all of the analyses performed in the present study. In reality, we used the dataset of identical mice for histochemistry, real-time RT-PCR and urinalyses, but not for TCDD determination. Second, mice often spontaneously urinated just before autopsy and the success rate to collect sufficient volume of urine from the bladder was approximately 30–40%. Third, we repeated experiments to confirm the reproducibility of our observations presented in this article. Thus, the total number of kidney specimens that were used to analyze the incidence of hydronephrosis varied among time-points, treatment groups or sex (Table 2).

Collection of kidney tissues and urine samples. In a series of experiments, kidneys and urine were collected on PNDs 7, 14, and 21, and subjected to analyses ($n = 6$ for each treatment group per each time point). In particular, when sufficient volume of urine was not collected from the bladder, we performed another set of experiment under the identical exposure condition to obtain complete sets of tissue and urine samples from the identical mouse to secure the number of animals for each treatment group per each time point to be six. Right kidneys were excised, fixed in Zamboni solution for 24 h at 4 °C, and processed for immunohistochemical and histological examinations. Left kidneys were snap-frozen in liquid nitrogen and stored at −80 °C until analysis for mRNA analyses. In another series of experiments, right kidneys ($n = 3$) were collected for TCDD analysis.

AhR knock-out mouse experiments. Heterozygous AhR female and male mice were mated to obtain wild-type and homozygous knock-out mice. The essentially same protocol as used for C57Bl/6J mice in the present study was also used. Wild-type and homozygous AhR knock-out pups were autopsied on PND 7 ($n = 6$ per each treatment group) for histology and mRNA determinations.

Incidence of hydronephrosis. To evaluate the incidence of hydronephrosis in male and female pups of C57'BL/6J mice, we compiled all the data of pups from PNDs 7, 14, 21 and 56 from five independent experiments (Table 2).

Measurement of TCDD concentration. TCDD in the kidney of mice was determined using a high-resolution gas chromatograph equipped with a high-resolution mass spectrometer, as previously described (Nishimura et al., 2002, 2003). In brief, the right kidney were collected from three TCDD-exposed pups from a different dam on PNDs 7, 14 and 21 ($n = 3$ per each time point). Because kidneys from 21-day-old control mice did not contain of TCDD (the limit of detection, 0.1pg/g tissue), TCDD was determined in the kidney from 21-day-old control mice only. Quantified values were calculated by the internal standard method.

Histology of the ureter. To examine the anatomical obstruction in the ureter of TCDD-exposed mice, C57Bl/6J TCDD-exposed mice ($n = 4$ per each sex) and control mice ($n = 4$ per each sex), selected from different dams, were killed by decapitation on PNDs 7, 14 and 21. The lower trunk having the kidney, ureter and bladder was fixed in Zamboni solution for 24 h at 4 °C and embedded in paraffin. Serial sections (5 μm in thickness) were prepared, from the renal pelvis to bladder, and subjected to histological analysis by hematoxylin and eosin staining.

Ink injection experiments. To study the possible anatomical obstruction of the urinary tract, we injected ink solution into the pyelocaliceal space of the hydronephrotic kidney from 7-week-old mice that were exposed to TCDD via dams' milk and that of the normal kidney of 7-week-old male mice ($n = 4$). Ink infusion was made by using a 30-G needle connected to a tubing (approx. 60 cm long), the end of which was fixed to an ink container. Hydrostatic pressure was applied to push the ink from the pyelocaliceal space to the bladder,

and the height of the ink container was increased stepwise every 60 s until the ink was introduced into the ureter.

PGE_2 measurements. The PGE_2 concentrations in urine, and culture media were measured using an enzyme immunoassay kit according to the manufacturer's instructions (Cayman Chemical Co, Ann Arbor, MI, USA). Urine specimens were collected from the bladder of mice on PNDs 7 and 14.

Immunohistochemistry. Cytochrome P450 (CYP) 1A1, COX-2 and PGE_2 in the kidney sections were visualized by an indirect immunohistochemical technique (Nishimura et al., 2003). Briefly, for the first antibody, goat polyclonal antibody against CYP1A1 (G-18; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and rabbit polyclonal antibodies against murine COX-2 (Cayman) and PGE_2 (Assay Designs, Ann Arbor, MI, USA) were used. For the subsequent reactions, either biotinylated rabbit anti-goat IgG or goat anti-rabbit IgG and an avidin-biotinylated peroxidase complex were purchased from Vector Lab (Peterborough, UK). Immunoreactions were performed using hydrogen peroxide-activated 3-diaminobenzidine tetrahydrochloride (Sigma, St Louis, MO, USA). Sections were counterstained for 10s in Mayer's hematoxylin. Negative controls, in which the primary antibody was replaced with normal rabbit IgG, did not show nonspecific staining.

Real-time RT-PCR. Total RNA was isolated from the kidney of mice using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA synthesis was carried out using the Omniscript RT Kit (Qiagen). The quantitative detection of differentially expressed genes, including the internal standard gene glyceraldehyde-3-phospate dehydrogenase (GAPDH), was performed using the Light Cycler instrument (Roche Molecular Biochemicals, Indianapolis, IN, USA) and the QuantiTech SYBR Green PCR Kit (Qiagen). The primers for each gene were designed on the basis of the respective cDNA or mRNA sequences using the OLIGO primer analysis software. PCR

Table 1
Primer sequences for cDNA amplification

Gene		Primer sequences (5′ to 3′)
AQP2	Forward	GCT GTC AAT GCT CTC CAC AA
	Reverse	AGG CAA AGA TGC ACA GCA C
AQP3	Forward	TGC AAA GGC AAG GGA CCA A
	Reverse	GGC ACA CGC ATA CTT AGA AAC TC
AVPR	Forward	ATG GGA AGC AAC TGG GAC A
	Reverse	CAG AGG GAG GAA TGA CAG GA
COX-2	Forward	AGA AGG AAA TGG CTG CAG AA
	Reverse	GCT CGG CTT CCA GTA TTG AG
Cyclophilin	Forward	TCG GCA AAG TTC TAG AGG GC
	Reverse	TCT GTG GGG ATT GAC AGG
Cyp1a1	Forward	GGC CAC TTT GAC CCT TAC AA
	Reverse	CAG GTA ACG GAG GAC AGG AA
Cyp1a2	Forward	CAC TAA CGG CAA GAG CAT GA
	Reverse	TCT GAA GCT TGC TGA CGA GA
Cyp1b1	Forward	ATA GCC CTG GAT GAG GCT TT
	Reverse	CCA AAT GAG GTT CGG CTT TA
GAPDH	Forward	AAC TTT GGC ATT GTG GAA GG
	Reverse	ACA CAT TGG GGG TAG GAA CA
IL-1b	Forward	GCC CAT CCT CTG TGA CTC AT
	Reverse	AGG CCA CAG GTA TTT TGT CG
IL6	Forward	TTT GGG TTG CTT CTC TGT GTC
	Reverse	GTC GTC TTG CTT TCC TTC TCA
KC	Forward	CTT GAA GGT GTT GCC CTC AG
	Reverse	TGG GGA CAC CTT TTA GCA TC
MMP12	Forward	TTT CTT CCA TAT GGC CAA GC
	Reverse	GGT CAA AGA CAG CTG CAT CA
NFκB	Forward	AGG TCC ACT GTC TGC CTC TC
	Reverse	GGA AGG ATG TCT CCA CAC CA
NKCC2	Forward	CGT TTC CTT GCT CAG GTA GC
	Reverse	GTG GTC TCC CAT GCA AAC TT
ROMK	Forward	GGT AAG ACG GTG GAA GTG GA
	Reverse	GCA GGA TGC TTC TGA ACA CA
TNFα	Forward	CAC CAC CAT CAA GGA CTC AA
	Reverse	ACA GAG GCA ACC TGA CCA CT

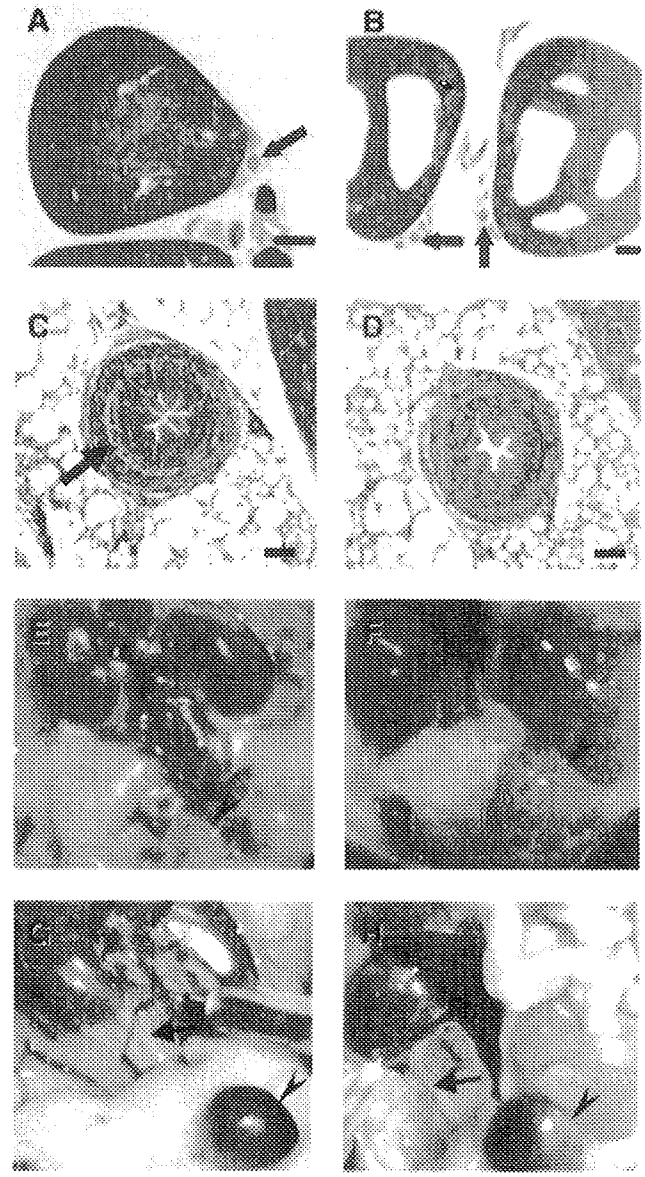

Fig. 1. TCDD-induced hydronephrosis in the absence of anatomical obstruction in the ureter of mice. Newborn mice were lactationally exposed to TCDD until PND 21 by being nursed by dams administered TCDD on PND 1. Control dams received corn oil (vehicle). To examine the anatomical obstruction in the ureter of TCDD-exposed mice on PNDs 7, 14 and 21, the lower trunk having the kidney, ureter and bladder was collected from TCDD-exposed mice (n=4 per each sex) and control mice (n=4 per each sex). Serial sections (5 μm in thickness) were prepared from the renal pelvis to bladder. Representative pictures of kidneys from control and TCDD-exposed 14-day-old male mice (n=6 for each group) are shown. Control pups showed normal kidney morphology without a sign of hydronephrosis (A), whereas TCDD-exposed pups showed hydronephrosis (B). Hematoxylin and eosin staining. Scale bar=500 μm. Magnified pictures (C, D) of the ureter from (A) and (B), respectively. Scale bar=50 μm. No sign of anatomical obstruction was observed in the ureter of either control pups (C) or TCDD-exposed pups (D). However, the subepithelial mesenchymal cells (C, arrow) visible in the ureter of a control mouse did not develop normally in the ureter of a TCDD-exposed mouse (D). Normal kidney of 7-week-old control mouse (E). Bilateral hydronephrotic kidneys of 7-week-old mouse offspring exposed lactationally to TCDD (F). Ink infused into the pyelocaliceal space of the right kidney of a 7-week-old control mouse (G) or a TCDD-exposed mouse (H) was introduced to the bladder with identical hydrostatic pressure (n=4 per group). Arrows (A, B, G, H) and arrowhead (E, F, G, H) indicate ureter and bladder, respectively.

Table 2
Incidence of hydronephrosis in mouse pups exposed to TCDD via dams' milk

Postnatal day	Male				Female			
	Control		TCDD		Control		TCDD	
	Incidence	%	Incidence	%	Incidence	%	Incidence	%
7	0/11	0	11/14	78.6	1/14	7.1	11/15	73.3
14	1/18	5.6	17/20	85.0	0/10	0	11/18	61.1
21	0/17	0	15/18	83.3	0/11	0	14/14	100
56	0/30	0	9/11	81.8	1/28	3.6	6/8	75

Values of incidence represent the number of pups having hydronephrosis over the number of pups examined. All the incidence values of TCDD-exposed male and female mice were significantly higher than the corresponding control group ($P<0.001$).

amplification was carried out in a total volume of 20 μl, containing 2 μl of cDNA, 10 μl of 2× QuantiTect SYBR Green PCR Master Mix, and 0.2 μM of each primer. The PCR cycling conditions were 95 °C for 15 s followed by 30 to 40 cycles of 94 °C for 15 s, 60 °C for 20 s and 72 °C for 10 s. Detection of the fluorescent product was performed at the end of the 72°C extension period. Negative controls were run concomitantly to confirm that the samples were not cross-contaminated. To confirm the amplification specificity, we performed melting curve analyses for PCR products. The intra-assay variability was < 7%. For quantification, data were analyzed with the Light Cycler analysis software (Vogel et al., 2007). The primer sequences are shown in Table 1.

COX-2 selective inhibitor experiment. Indomethacin *N*-octylamide (1-(*p*-chlorobenzoyl)-5-methoxy-2-methyl-1H-indole-3-acetic acid, 4-methoxyphenyl ester; Calbiochem-Merck Co, Tokyo, Japan), a selective COX-2 inhibitor, was injected subcutaneously to newborn mice ($n = 6$ for each group per each time point) at a daily dose of 5 μg/capita from PND 1 to PND 7. On PND 1, the inhibitor was injected 1 h before dams were administered TCDD by gavage.

Cell culture. Mouse macula-densa-derived 1(MMDD1) cells, a kind gift from Dr J. B. Schnermann (NIDDK and NHLBI of NIH), were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and antibiotics. The cells were plated onto six-well culture dishes and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. The medium was changed every 2 days, and once the cells reached confluence (typically in 3–4 days), the cells were transferred to serum-free medium, followed by the addition of chemicals 24 h later. At selected times, the cells were lysed using the RNeasy Mini Kit.

Data analysis. StatView for Windows (version 5.0, SAS Institute, Cary, NC, USA) was used for statistical analysis. Data are expressed as X ± SE. Differences in means among the groups between three time points were analyzed by one-way analysis of variance, followed by a post-hoc Scheffe's test. Differences in means between TCDD-exposed group and the corresponding control group at a specific time point were analyzed by Student's *t*-test. In these tests, it was confirmed that there is no difference in variance among groups. Differences in incidence of hydronephrosis between TCDD-exposed group and the corresponding control group were analyzed by *z*-test. *P* values less than 0.05 were considered statistically significant, unless otherwise stated.

Results

Incidence of TCDD-induced hydronephrosis in mouse pups and absence of anatomical obstruction in ureter

No significant difference in body weight gain was observed between TCDD-exposed and control pups. Histological examination revealed that lactational exposure to TCDD induced visually recognizable kidney lesions characteristic of hydronephrosis, such as marked pelvic dilatation and decreased thickness of the renal parenchyma no later than PND 7 (Fig. 1B) when compared with those of control mouse kidney (Fig. 1A). This disease state was persistent throughout the experimental period of 8 weeks, suggesting its irreversibility. Table 2 summarizes the incidence of hydronephrosis on PNDs 7, 14 and 21 and 8-week post-delivery for male and female mice. The overall incidences were 82.2 and 77.3% for TCDD-exposed male and female mice, respectively, whereas control male and female mice had incidences of 1.4 and 2.7%, respectively. These results were consistent with those of a previous study on mice lactationally exposed to TCDD (Couture-Haws et al., 1991). Because previous studies showed the presence of anatomical obstruction in the ureter of TCDD-exposed fetal mice owing to epithelial

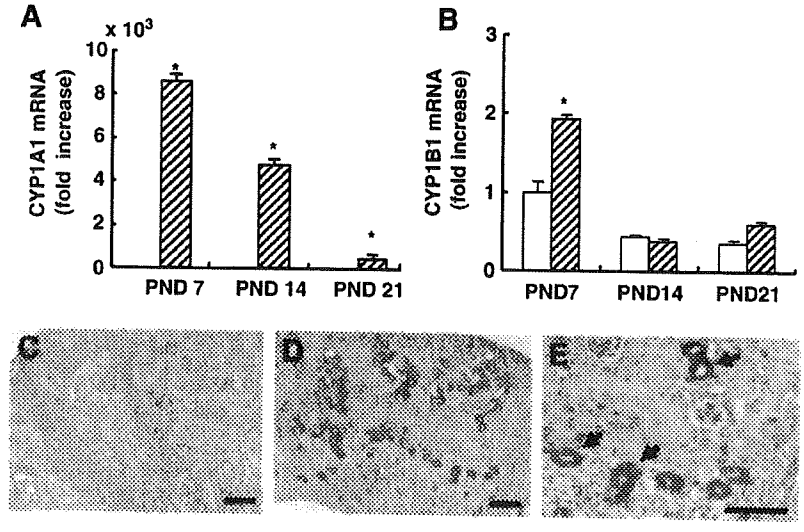

Fig. 2. CYP1A1 and CYP1B1 mRNA expression levels and immunohistochemical localization of CYP1A1 in control and TCDD-exposed mouse kidney on PND 7. CYP1A1 mRNA (A) and 1B1 mRNA (B) of kidney specimens from TCDD-exposed (hatched) and control (open) pups were quantified by real-time RT-PCR. The mRNA expression level was normalized to control values on PND 7. Each bar represents the X±SE for 6 mice. * $P<0.05$ versus control. (C–E) Note the presence of intense CYP1A1 immunostaining in the distal tubular epithelial cells (arrows in E) of the TCDD-exposed hydronephrotic kidney (D) and at higher magnification (E), compared with almost no immunostaining in the control kidney (C). All the kidney specimens showed similar CYP1A1 localization ($n=6$). Counterstained with hematoxylin. Scale bars: 100 μm (C–E).

hyperplasia (Abbott et al., 1987), we examined microscopically the ureters of TCDD-exposed mice (Fig. 1D) and control mice (Fig. 1C) of both sexes on PNDs 7, 14 and 21 (n = 8 for each time point, 4 males and 4 females). Serial sections of the ureter were examined from the pelvis to the bladder, with particular attention to the ureteropelvic junction because an abnormality above this junction may lead to hydronephrosis (Zeidel and Pirtskhalaishvili, 2004), and indeed, we did not find a case of hydroureter in this study. Surprisingly, TCDD-exposed hydronephrotic male and female mice on PNDs 7, 14, and 21 (n = 8 for each time point; 4 males and 4 females each) did not present with an anatomical obstruction in the ureter, i.e. the openings of the ureter was not morphologically different from those of the control mice. In the ureter, smooth muscle cells are differentiated fully to become a thick layer by embryonic day 18.5 in the mouse (Airik et al., 2006), and the ureters of control mice showed a normal histology of the smooth muscle cell layer, mesenchymal cells and urothelium (Fig. 1C). On the other hand, the ureters of TCDD-induced hydronephrotic male and female mice were apparently devoid of the normal mesenchymal cell populations (Fig. 1D).

To study the possible obstruction of the ureter further, we infused ink into the pyelocaliceal space of normal and TCDD-induced hydronephrotic kidneys. Representative pictures before (Figs. 1E, F) and after (Figs. 1G, H) the ink infusion are shown. Hydrostatic pressure below 30cm H_2O was not sufficient to allow ink to be introduced into the bladder of both normal mice and TCDD-induced mice. When the ink container was raised to maintain a hydrostatic pressure of 30cm H_2O for at least 45s, ink was successfully introduced from the pelvis to the bladder in the normal mice (n = 6, Fig. 1G) and the TCDD-induced mice having hydronephrosis (n = 4, Fig. 1H). Taken together with the histological observations (Figs. 1C, D), these findings support the notion that TCDD-induced hydronephrosis is not associated with anatomical obstruction.

TCDD concentration and induction of CYP1A1 and CYP1B1 expression in mouse kidney by lactational exposure to TCDD

Kidneys were harvested from mouse pups exposed to TCDD via dams' milk or those given milk by vehicle-administered dams. TCDD concentrations (X ± SE for three animals) in the kidney reached a maximum of 811 ± 99 pg/g wet weight by PND 7, with a decrease to 303 ± 14 pg/g wet weight by PND 14, and then remained constant at 339 ± 14 pg/g wet weight on PND 21. TCDD was not detected in the kidney from control pups on PND 21 (below 0.1pg/g tissue).

CYP1A1 and 1B1 enzymes are induced by TCDD through the activation of the AhR in various types of tissue, and have been used as markers of AhR activation by its ligands. As expected, CYP1A1 mRNA expression in the TCDD-exposed mouse kidney was markedly induced with a maximum level on PND 7, with a progressive decline but still a significantly elevated level compared with that of the control on PND 21 (Fig. 2A). In the kidney, CYP1B1 mRNA expression was also induced significantly on PND 7 with a decrease to the control level by PND 14

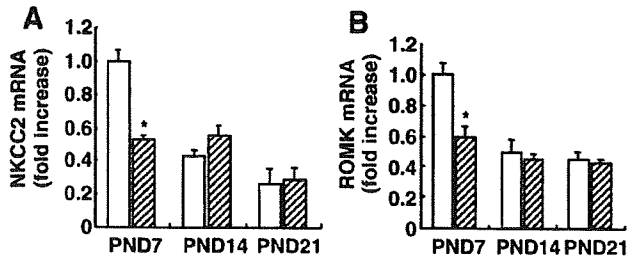

Fig. 3. NKCC2 (A) and ROMK (B) mRNA expression levels in control (open) and TCDD-exposed (hatched) kidneys of newborn mice. The mRNA expression level was normalized to control values on PND 7. Each bar represents the X±SE for 6 mice.* $P<0.05$ versus control.

Table 3
Effects of lactational exposure to TCDD on the gene expression of inflammatory cytokines and water transporters in the kidney of mouse pups on PND 7[a]

Gene	Control	TCDD
MCP1	1.00±0.06[b]	2.47±0.28*
IL-6	1.00±0.09	1.30±0.13*
TNFα	1.00±0.07	2.64±0.62*
mF4/80	1.00±0.01	2.27±0.21*
AQP2	1.00±0.08	1.03±0.08
AQP3	1.00±0.04	0.93±0.10
AVPR	1.00±0.03	1.06±0.12

[a] Male pups born to dams that were administered either TCDD or corn oil on PND 1 were sacrificed on PND 7 for the harvest of kidney samples. The expression of mRNA of each gene was determined by real-time RT-PCR.
[b] Values represent X±SE of five pups.
* $P<0.05$ versus control.

(Fig. 2B). Although almost no immunostaining for CYP1A1 was observed in the kidney of pups nursed by vehicle-administered dams (Fig. 2C), intense immunostaining for CYP1A1 was found in the kidney of pups lactationally exposed to TCDD, with a prominent staining in the distal

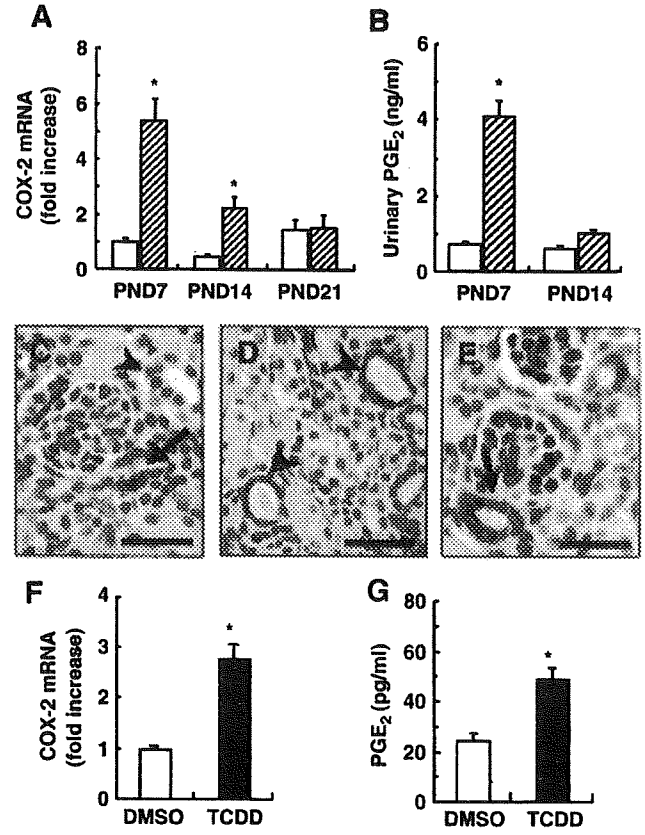

Fig. 4. TCDD-induced COX-2 mRNA (A) and COX-2 immunoreactivity in the kidney (C–E) and PGE_2 concentration in the urine (B) of TCDD-exposed newborn mice and COX-2 mRNA and PGE_2 levels in macula densa cells *in vitro* (F, G). (A) The COX-2 mRNA expression levels in the kidney of control (open) and TCDD-exposed (hatched) mice were determined by quantitative RT-PCR. The mRNA expression level was normalized to control values on PND 7, and each bar represents the X±SE for 6 mice. * $P<0.05$ versus control. Immunohistochemical staining for COX-2 in control mouse kidney (C) and TCDD-exposed mouse kidney (D, E) on PND 7. Arrows and arrowheads indicate macula densa cells and distal epithelial cells, respectively. Counterstained with hematoxylin. Scale bar = 100 μm. Elevated induction of COX-2 mRNA expression in MMDD1 cells (F) and increased PGE_2 release into culture medium (G) in response to 10 nM TCDD. The mRNA expression level was normalized to a DMSO value. Each bar represents the X±SE for 3 culture dishes. * $P<0.05$ versus DMSO control.

tubules of the TCDD-induced hydronephrotic kidney (Figs. 2D, E). An elevated level of TCDD-induced CYP1A1 mRNA expression in the kidney seemed to be comparable to the increased degree of immunostaining for CYP1A1 in the kidney.

Effects of TCDD on expression of genes of salt and water transporters in the developing kidney

We next tested our hypothesis that lactational exposure to TCDD may disrupt the harmonious regulation of Na^+ and K^+ ions and/or water and the cortico-medullary osmotic gradient, because mice lacking some of these proteins, such as NKCC2, ROMK, AQP2 and AVPR2, were reported to develop hydronephrosis (Takahashi et al., 2000; Yun et al., 2000; Lorenz et al., 2002; Lu et al., 2002; McDill et al., 2006). We found that the NKCC2 mRNA level in the kidney of control mice was highly elevated on PND 7, followed by a significant decrease until PND 21. Interestingly, the NKCC2 gene expression in the TCDD-exposed mice was down-regulated to nearly half of the control level ($P < 0.05$; Fig. 3A), but such a difference was diminished by PND 14. The time-course of the gene expression of another important transporter, ROMK, showed a pattern very similar to that of NKCC2 in control animals. The constitutive expression of ROMK mRNA was highly elevated on PND 7, with a 50% decrease by PND 14. The ROMK gene expression in the TCDD-exposed animals was down-regulated to approximately 60% of the corresponding expression in the control on PND 7 (Fig. 3B).

Because polyuria, an important causative factor for the onset of hydronephrosis, develops as a result of the abnormality of water transporters, we determined mRNA levels of AVPR2 and AQPs 2 and 3 in the kidney. However, no significant difference in the expressions of these genes was observed between TCDD-exposed hydronephrotic kidney and control kidney on PND 7 (Table 3).

Increased COX-2 activity and PGE_2 synthesis by TCDD exposure in the kidney and cultured macula densa cells

Next, we studied the possible increase in COX-2 mRNA and protein amounts in the TCDD-exposed kidney because of the following two reasons. First, COX-2 is induced in an AhR-dependent manner *in vivo* and *in vitro* (Puga et al., 1997; Vogel et al., 1998; Degner et al., 2007).

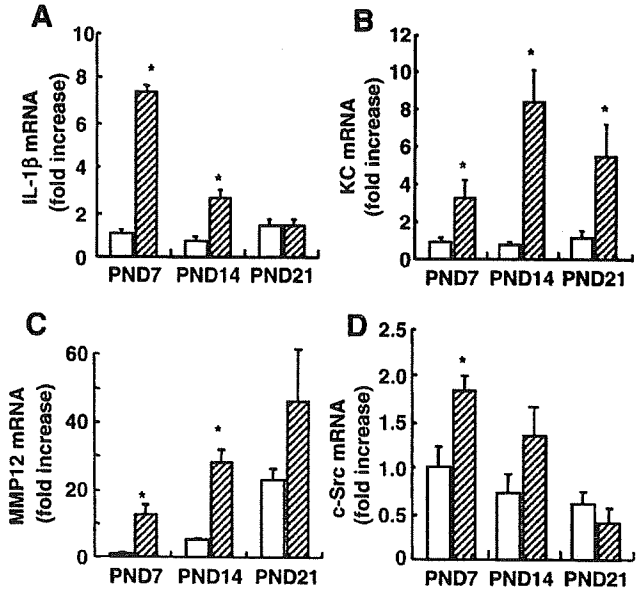

Fig. 5. mRNA expression levels of inflammation-related genes (IL-1β, KC, MMP12) and c-Src in control kidney (open) and TCDD-exposed kidney (hatched) of newborn mice. The mRNA expression level was normalized to control values on PND 7. Each bar represents the X±SE for 6 mice.* $P<0.05$ versus control.

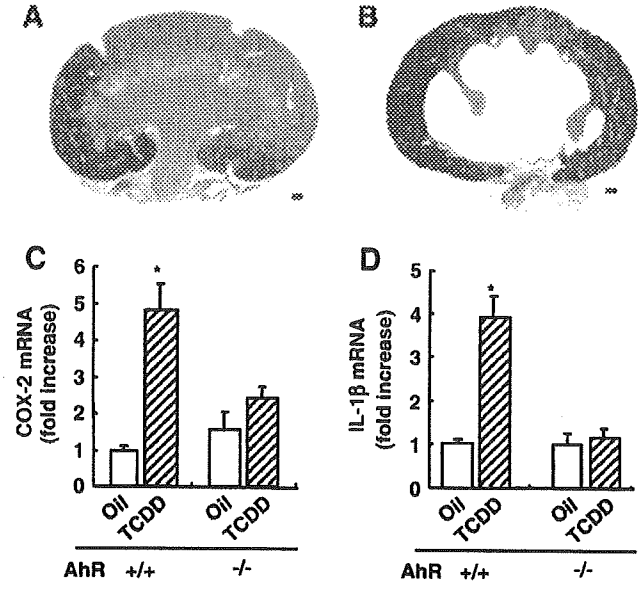

Fig. 6. AhR-dependent effects of lactational exposure to TCDD on onset of hydronephrosis in newborn mice and COX-2 and IL-1β mRNA expression levels. Dams received TCDD at an oral dose of 10 μg/kg b.w on the first day of delivery, and newborn mice were lactationally exposed to TCDD from PND 1. Representative pictures of histological sections of the entire kidney of TCDD-exposed AhR−/− (A) and AhR+/+ (B) mice on PND 7. Hematoxylin and eosin staining. An increase in the COX-2 (C) and IL-1β (D) mRNA levels was observed in the kidney of AhR+/+ mice, but not in that of AhR−/− mice. The mRNA expression level was normalized to control values on PND 7. * $P<0.05$ versus control.

Second, PGE_2 could down-regulate NKCC2 and ROMK transporter activities (Kaji et al., 1996; Jin et al., 2007). We found that TCDD administration markedly induced the COX-2 mRNA expression by PND 7 (Fig. 4A), with a subsequent decrease to the control level by PND 21. On the other hand, the COX-2 mRNA levels in control pups nursed by vehicle-administered dams remained relatively constant during the entire experimental period (Fig. 4A). Consistent with the quantitative RT-PCR data of COX-2 mRNA, intense immunostaining for COX-2 protein was clearly detected in the distal tubular cells of the TCDD-exposed kidney on PND 7 (Fig. 4D) in comparison with those of the control kidney (Fig. 4C). In particular, macula densa cells, which function as chemoreceptors that sense the sodium or chloride load in the distal tubule (Madsen and Tisher, 2004), showed stronger immunostaining for COX-2 protein in the kidney of TCDD-exposed pups (Fig. 4E) than in the kidney of control pups (Fig. 4C).

The above-mentioned increases in COX-2 mRNA and protein amounts in the kidney of TCDD-exposed mouse pups were consistent with a finding on the significant increase in PGE_2 excretion into the urine on PND 7 (Fig. 4B). In addition, the PGE_2 concentration in the urine decreased to the control level by PND 14. The PGE_2 concentrations in the urine of the control pups were found to be unchanged on PNDs 7 and 14 (Fig. 4B).

As a further study, mouse macula-densa-derived 1 (MMDD1) cells were cultured in a medium containing 10 nM TCDD and were found to have 2.5- and 2-fold increases in COX2 mRNA expression and PGE_2 release, respectively, into the culture medium in comparison with those of DMSO-treated control cells (Figs. 4F, G).

Effects of TCDD on mRNA expressions of inflammation-related and c-Src genes in the developing kidney

We next determined the mRNA levels of inflammatory cytokines and c-Src in the TCDD-exposed mouse kidney, because inflammatory cytokine and/or c-Src signaling have been known to regulate COX-2 activities (Vogel et al., 2000). The most noticeable alteration in gene

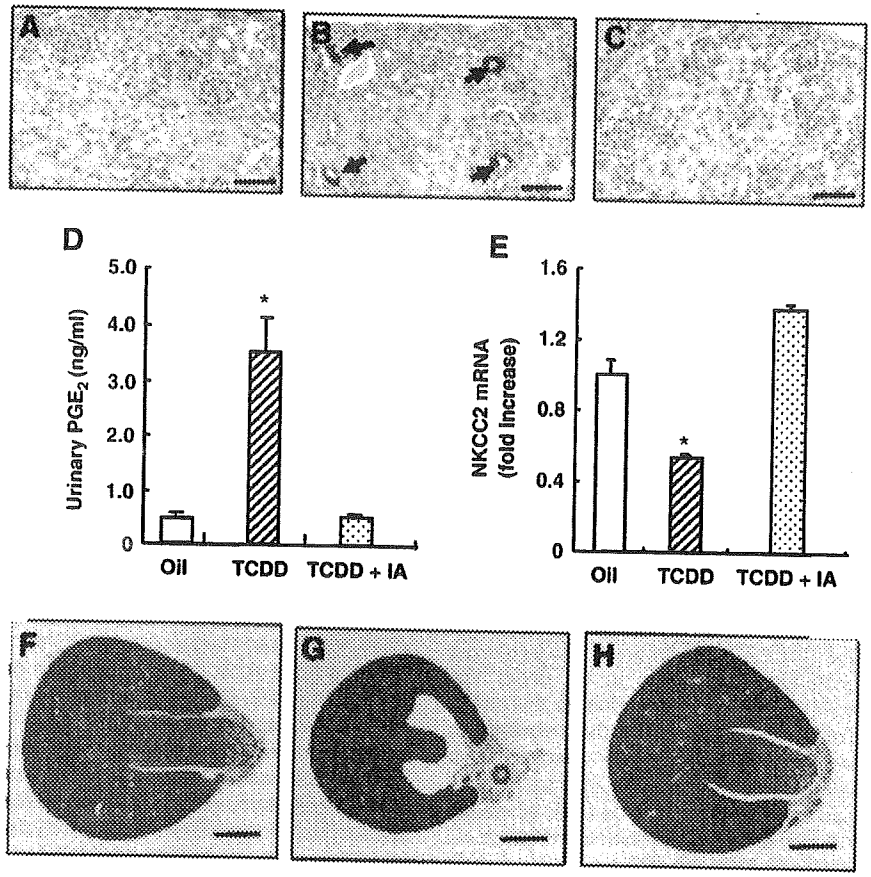

Fig. 7. COX-2 selective inhibitor suppressed the TCDD-induced elevation of PGE_2 levels in the kidney and urine, reduction of NKCC2 mRNA expression in the kidney and hydronephrosis development. (A–C) TCDD administration increased PGE_2 synthesis in some of the distal tubular cells (arrow) (B), in comparison with those of the control kidney (A). In contrast, daily administration of indomethacin N-octylamide (IA) from PNDs 1–7 suppressed PGE_2 synthesis (C), and the histological findings were similar to those of the control kidney (A). Representative pictures of kidneys from each group (n=6 mice) are shown. (F, G) TCDD-exposed pups showed hydronephrosis on PND 7 (G), but IA administration suppressed the onset of this disease (H), the result of which was indistinguishable from that of normal kidney of pups nursed by vehicle-administered dams (F). Hematoxylin and eosin staining. Bar = 50 μm (A–C); 500 μm (F–H). (D, E) Both the increased excretion of PGE_2 in the urine and the decreased expression of NKCC2 mRNA in the kidney in TCDD-exposed pups on PND 7 were abrogated by IA administration from PNDs 1–7. The NKCC2 mRNA expression level was normalized to an oil value. * $P<0.05$ versus vehicle-control.

expression was that the expressions of a battery of genes related to inflammation were significantly up-regulated as early as PND 7. Among them, IL-1β mRNA expression was markedly induced in the TCDD-exposed kidney on PND 7 and decreased to control level by PND 21, whereas its expression remained constant in control pups (Fig. 5A). The levels of TNFα, MCP1, mF4/80 and IL-6 mRNAs were significantly up-regulated in the TCDD-exposed kidney by PND 7 compared with those in the control kidney (Table 3). On the other hand, TCDD up-regulated the KC (equivalent of human IL-8) mRNA level significantly throughout the experimental period with the maximum level on PND 14, whereas its level remained constant in the control kidney (Fig. 5B). The matrix metalloproteinase (MMP) 12 mRNA level increased with kidney development in the control pups, and further increased significantly following TCDD exposure (Fig. 5C).Thus, these data suggest that lactational exposure to TCDD from PNDs 1 to 7 triggered a signal transduction pathway that may lead to inflammation. Similarly, c-Src mRNA expression significantly increased on PND 7 with a decrease to the control level by PND 21 (Fig. 5D).

Role of AhR in TCDD-induced hydronephrosis

Using AhR-null mice, we next studied whether TCDD-activated AhR signaling is required to induce COX-2 activity because most of the TCDD-induced toxicity phenotypes, including hydronephrosis in the fetus, depend on the AhR-mediated mechanism (Mimura et al., 1997; Peters et al., 1999; Gasiewicz and Park, 2003). We first confirmed that lactational exposure to TCDD induced hydronephrosis in male and female AhR+/+ (wild-type) pups on PND 7 (Fig. 6B) and thereafter, but did not induce such anomaly in AhR−/− pups (Fig. 6A). We also found that lactational exposure to TCDD induced COX-2 mRNA expression in the kidney only in TCDD-exposed wild-type pups but not in the AhR(−/−) mice (Fig. 6C). Because IL-1β mRNA expression was found to be markedly induced in the kidney exposed to TCDD in the early postnatal period, we investigated whether this response was also AhR-mediated. As shown in Fig. 6D, lactational exposure to TCDD led to a 4-fold increase in the IL-1β mRNA level in the kidney from wild-type mice on PND7, but not in AhR(−/−) mice.

Suppression of TCDD-enhanced COX-2 activity and onset of hydronephrosis by a COX-2 selective inhibitor

On the basis of our findings obtained so far, we hypothesized that TCDD-enhanced COX-2 activity plays an essential role in the onset of hydronephrosis in the developing rodent kidney, which led us to anticipate that cotreatment of a COX-2 selective inhibitor together with TCDD exposure may suppress the development of TCDD-induced hydronephrosis. Indomethacin N-octylamide, a COX-2 selective inhibitor, was injected daily to TCDD-exposed pups from PNDs 1 to 7. In accordance with our hypothesis, the stimulatory effect of TCDD on PGE_2 synthesis in the kidney (Fig. 7B) was completely suppressed by the COX-2 selective inhibitor (Fig. 7C), the result being

indistinguishable from that of the control kidney (Fig. 7A). The quantification of PGE_2 in urine supports these immunohistochemical data: The elevated excretions of PGE_2 in urine of TCDD-exposed pups were completely suppressed by the COX-2 selective inhibitor to a level similar to that of the control (Fig. 7D). The COX-2 selective inhibitor also reversed the decreased expression of NKCC2 mRNA to a level similar to that of the control (Fig. 7E), supporting the hypothesis that the downstream target of PGs was actually affected by this treatment. Finally, we found that TCDD exposure induces hydronephrosis in the newborn mice on PND 7 (Fig. 7G), and that the morphology of the kidney rescued by the inhibitor (Fig. 7H) is visually indistinguishable from that of the normal kidney (Fig. 7F). The reproducibility of this data was confirmed by using 6 animals per group.

Discussion

Hydronephrosis is a serious disease that has been regarded to be mainly due to anatomical obstruction in the ureter, but in some cases due to abnormal peristaltic movement of the ureter (Zeidel and Pirtskhalaishvili, 2004). Studies on the pathogenesis of hydronephrosis at the molecular level have just begun to address the more intricate causes of this disease. In this regard, the present study is the first to show that TCDD-enhanced COX-2 activity is essential for the onset of hydronephrosis and that the TCDD-induced hydronephrosis is not associated with anatomical obstruction in the ureter but perhaps with the functional impairment of urination. In this study, we used a dosing protocol of lactational exposure rather than *in utero* exposure because of the following two reasons. First, previous studies already used the *in utero* exposure protocol and reported the possible toxicity mechanism for hydronephrosis. Second, newborns take up greater amounts of dioxins than fetuses (Nishimura et al., 2005), and such exposure protocol is relevant to exposure situations in humans. Here, we describe the characteristic features of the TCDD-induced hydronephrosis from the following four perspectives. First, our observation of the absence of anatomical obstruction in the ureter of TCDD-induced hydronephrotic mice may challenge the generally accepted view concerning the anatomical obstruction that is caused by TCDD-induced hyperplasia in the ureter (Abbott et al., 1987; Abbott and Birnbaum, 1990; Theobald et al., 2003). A possible reason for this inconsistency may be partly attributable to a difference in the exposure period (*in utero* exposure vs. lactational exposure) in terms of the developmental stages of the kidney and ureter. Another possible reason for the inconsistency may be due attributable to a difference in the experimental approach used in the previous study (Abbott et al., 1987). In this study, C57Bl/6 pregnant mice were injected with TCDD on gestation day (GD) 10, and the fetuses were examined for abnormality on GDs 14, 15, 16 (a.m.), 16 (p.m.) and 17. A dye solution was infused into the bladder to examine its flow to the kidney, in a direction opposite to that used in our present study. The authors showed that the percentages of fetuses with dye remaining in the bladder were 37% and 22% in the control and TCDD-exposed fetuses, respectively, on GD 16 (a.m.), and 59% and 38% in the control and TCDD-exposed fetuses, respectively, on GD 16 (p.m.) when severe hydronephrosis significantly developed. Since statistical difference was not shown in these data, it was difficult to conclude that there is any difference in the presence of the anatomical obstruction in the ureter between control mice and TCDD-exposed mice from the ink infusion experiment. Thus, these data suggest that there might be causes more directly related to hydronephrosis than anatomical obstruction.

Besides anatomical obstruction, functional impairments of the initiation and propagation of peristaltic contractions, which transport urine from the renal pelvis to the bladder, have been documented as causes of the onset of hydroureter and/or hydronephrosis (Zeidel and Pirtskhalaishvili, 2004). The developmental mechanisms underlying the molecular and cellular events of the peristaltic contractions of smooth muscle cells are beginning to be clarified. In particular, transgenic mice whose T-box 18 transcription factor gene or discs-large homolog 1 gene was targeted were found to develop hydronephrosis at the fetal stage. The onset of hydronephrosis was shown to be ascribed mainly to reduced differentiation of the smooth muscle layer of the ureter in the T-box 18-null mice (Airik et al., 2006) or abnormal misalignment of the circular smooth muscle cells in discs-large homolog 1-null mice (Mahoney et al., 2006). Interestingly, in the latter transgenic mouse strain, the absence of the subepithelial mesenchymal cells in the ureter is suggested to be associated with this abnormal development of the smooth muscle layer. In contrast to the findings of these studies, mutant mice having a single base change in codon 256 of the AQP2 gene had normal smooth muscle cells and nerves along the ureter, but developed progressive hydronephrosis owing to the incomplete transfer of urine because the production of a large quantity of hypotonic urine overwhelms the capacity of peristalsis (McDill et al., 2006). It can be speculated that either of these abnormalities or both in combination is an underlying cause of TCDD-induced hydronephrosis. In addition, it has been reported that the release of endogenous prostaglandins as well as sensory nerves plays an essential role in the maintenance of peristalsis although effects of prostaglandins can have either an excitatory or inhibitory action on the smooth muscle contractility of the upper urinary tract, depending on the type and concentrations of the prostanoid, and the tissue and animal species involved (Lang et al., 2002). It is worth studying how an increased activity of COX-2 affects the contractility of the ureter in the TCDD-exposed newborn mice.

Second, TCDD-exposed mouse pups showed an increased production of PGE_2 and a decrease in the levels of NKCC2 and ROMK mRNAs on PND 7. This observation seems to be similar to the signs of antenatal Bartter's syndrome, known to have a mutation in one of at least four genes encoding membrane proteins of the nephron segment, including NKCC2 and ROMK (Bonnardeaux and Bichet, 2004). As for the appropriate precedence, NKCC2-null mice (Takahashi et al., 2000) and ROMK-null mice (Lorenz et al., 2002; Lu et al., 2002) were shown to have severely impaired salt absorption in the TAL and polyuria without a sign of anatomical obstruction in the ureter, and developed hydronephrosis that is presumably induced by a very severe degree of polyuria that overwhelmed the capacity of peristalsis. Fernandez-Llama et al. (1999) reported that administration of COX inhibitors to rats markedly increases the expression of the NKCC2 cotransporter in the TAL and concluded that cyclooxygenase inhibitors enhance urinary concentrating ability in part through effects to increase NKCC2 cotransporter expression in the TAL. An important question is how TCDD suppresses NKCC2 and ROMK expressions. A previous *in vitro* study showed that PGE_2 (<1 nM) inhibited K^+ influx by down-regulating the numbers of functioning NKCC2 in the medullary TAL (Kaji et al., 1996). Recently, an *in vivo* study using PGE_2 receptor KO mouse strains has revealed that the PGE_2 receptors, EP1, EP3 and EP4, all contribute to the enhanced PGE_2-mediated salt and water excretions via NKCC2 (Nusing et al., 2005). The increased production of PGE_2 was also shown to inhibit the ROMK-like small-conductance K^+ channels and the Ca^{2+}-activated big-conductance K^+ channels in the cortical collecting duct via the activation of EP1 receptors and MAPK (Jin et al., 2007). Taken together, although we cannot exclude the possibility that the expression of these Na and K transporters is affected after the onset of hydronephrosis, the above-mentioned lines of experimental evidence suggest that the imbalance in electrolyte and water caused by the lactational exposure to TCDD could contribute to hydronephrosis development without inducing anatomical obstruction. Furthermore, these animal models may serve as a disease model, which is useful for uncovering the molecular basis of kidney diseases, such as Bartter's syndrome.

Third, the TCDD-induced COX-2 activation in the kidney occurred not only in an AhR dependent manner but also in temporal- and tissue-specific manners. We demonstrate that TCDD exposure induced COX-2 activity in distal tubules and macula densa cells of

the kidney and exacerbate the renal teratology in the developing kidney. In a previous study (Vogel et al., 1998), TCDD administration to adult female mice was reported to induce COX-2 mRNA expression in the lung and thymus, but not in the liver, kidney and other organs. Using antibody specific to CYP1A1, we found that lactational exposure of newborn rats to TCDD induced CYP1A1 expression in a specific region of the developing kidney, but that TCDD administration to adult rats did not induce CYP1A1 expression in the kidney (Nishimura et al., 2006). Thus, it is reasonable to speculate that the induction of COX-2 mRNA expression by TCDD is not only cell- or tissue-specific, but also temporal-specific.

Fourth, the TCDD-induced COX-2 activation has been proved to be AhR-dependent by our present experiment using the AhR-null mouse and a previous *in vitro* study using mouse hepatoma cells (Puga et al., 1997). In addition, in the present study, inflammatory cytokine and c-Src signaling pathways were suggested to mediate the COX-2 activation in the TCDD-induced hydronephrotic kidney. How COX-2 is activated by TCDD exposure through either of these signaling pathways or through cross-talk manners is an intriguing question. Recent studies on the human COX-2 promoter have demonstrated that COX-2 expression is critically governed by several major binding sites for transcription factors in its promoter region, including CRE, which is bound by both CREB and AP-1 proteins,, C/EBP protein binding site, and nuclear factor (NF)-κB response element in a highly cell type-specific and stimulus-specific manner (Nie et al., 2003). Inflammatory stimuli by lipopolysaccharide were reported to transmit signals via CD14/Toll-like receptor 4, perhaps followed by mitogen-activated protein kinases (MAPKs)/NF-κB to activate COX-2 to produce PGE_2 for the homeostatic regulation in the inner medulla of the kidney (Yang, 2003). In the previous studies, IL-1β has been shown to act as a potent inducer of COX-2 in various kinds of cells (Diaz et al., 1998; Pang et al., 1998; Walch and Morris, 2002; Ohama et al., 2007). In the present study, we found that gene expressions of both COX-2 and IL-1β are significantly elevated by TCDD in AhR+/+ wild type newborn mice, but not in AhR−/− mice (Figs. 6C, D). These results suggest a specific association between IL-1β and COX-2 and the pivotal role of IL-1β in COX-2 up-regulation, at least in part, under the pathological situation. A more recent study showed that COX-2 activation is mediated via NF-κB activation in murine macrophages (Kang et al., 2006). Because lactational exposure to TCDD increased the mRNA levels of inflammatory cytokines (TNFα, IL1β, KC and MMP12) and a macrophage marker (mF4/80), on PND 7, and because NF-κB is also known to be activated by TNFα, it is conceivable that COX-2 activated by TCDD is mediated via proinflammatory factors during kidney development. Recently, it has been reported that TCDD injection induces the expressions of MCP-1 and KC mRNAs on day 1 in various organs, including the kidney, followed by an increased expression of mF4/80 mRNA in adult mice, suggesting the infiltration of macrophages in these organs (Vogel et al., 2007).

The c-Src tyrosine kinase pathway is a plausible candidate for another possible mechanism of TCDD-enhanced COX-2 activity because Src has been shown to be initially involved in the triggering event of toxic signaling of TCDD in many other types of cells (Matsumura, 2003), and is known as a regulator of COX-2 gene expression (Herschman, 1996). It was also reported that the C/EBP binding site of the COX-2 gene is an important regulatory *cis*-acting factor for the *trans*-activation of this gene by TCDD and that *in vitro* experiments with c-Src inhibitors and *in vivo* studies with c-Src-deficient mice indicated that the up-regulation of COX-2 gene by TCDD is mediated via a c-Src-dependent pathway (Vogel et al., 2000). Because lactational exposure to TCDD induced c-Src mRNA expression by PND 7 (Fig. 5D), it is plausible that the c-Src signaling pathway is actively involved in the induction of COX-2 activity. Taken together, the analysis of the sequential steps of the inflammatory signaling pathway that proceeds and regulates COX-2 activity, as well as the possible interaction of the inflammatory cytokine signaling with those of AhR and c-Src, may provide yet unidentified signaling pathways.

In conclusion, the present study demonstrates that the mice lactationally exposed to TCDD develop hydronephrosis in the developing kidney, without anatomical obstruction in the ureter. The AhR-dependent TCDD-activated COX-2 activity is essential for the disruption of NKCC2 and ROMK transporters and for the onset of hydronephrosis. In addition, the enhanced inflammation signaling pathway is highly involved in the development of hydronephrosis, presumably via the functional impairment of peristalsis due to the abnormal development of the ureter. A toxicological approach focusing on a specific receptor of a given xenobiotic and on its toxicity phenotype, as applied in this study, may reveal the pathogenesis of the disease state, and that this is a typical example of a study that links toxicology and clinical medicine.

Acknowledgments

We thank K. Taki, F. Saito, Y. Takeuchi, C. Yokoi, C. Miyata (NIES) and T. Shibata (Univ. of Tokyo) for their technical assistance, K. Hayashi (NIES) for the animal care, Dr. J. Schnermann (NIDDK and NHLBI of NIH) for kindly providing the MMDD1cells, and Dr. Y. Fujii-Kuriyama (University of Tsukuba) for kindly providing the AhR-null mice. This work was supported in part by a Grant-in-Aid from the Japan Society for the Promotion of Science (to N.N. and C.T.), and Research Grant Nos. ES05233 and ES05707 from NIEHS (to F. M.).

References

Abbott, B.D., Birnbaum, L.S., 1990. Effects of TCDD on embryonic ureteric epithelial EGF receptor expression and cell proliferation. Teratology 41, 71–84.
Abbott, B.D., Birnbaum, L.S., Pratt, R.M., 1987. TCDD-induced hyperplasia of the ureteral epithelium produces hydronephrosis in murine fetuses. Teratology 35, 329–334.
Airik, R., Bussen, M., Singh, M.K., Petry, M., Kispert, A., 2006. Tbx18 regulates the development of the ureteral mesenchyme. J. Clin. Invest. 116, 663–674.
Belarmino, J.M., Kogan, B.A., 2006. Management of neonatal hydronephrosis. Early Hum. Dev. 82, 9–14.
Birnbaum, L.S., Weber, H., Harris, M.W., Lamb, J.C.T., McKinney, J.D., 1985. Toxic interaction of specific polychlorinated biphenyls and 2,3,7,8-tetrachlorodibenzo-*p*-dioxin: increased incidence of cleft palate in mice. Toxicol. Appl. Pharmacol. 77, 292–302.
Bonnardeaux, A., Bichet, D.G., 2004. Inherited disorders of the renal tubule. In: Brenner, B.M. (Ed.), Brenner and Rector's The Kidney. Saunders, Philadelphia, pp. 1697–1741.
Bryant, P.L., Schmid, J.E., Fenton, S.E., Buckalew, A.R., Abbott, B.D., 2001. Teratogenicity of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the expression of EGF and/or TGF-alpha. Toxicol. Sci. 62, 103–114.
Chevalier, R.L., 2004. Biomarkers of congenital obstructive nephropathy: past, present and future. J. Urol. 172, 852–857.
Couture-Haws, L., Harris, M.W., McDonald, M.M., Lockhart, A.C., Birnbaum, L.S., 1991. Hydronephrosis in mice exposed to TCDD-contaminated breast milk: identification of the peak period of sensitivity and assessment of potential recovery. Toxicol. Appl. Pharmacol. 107, 413–428.
Degner, S.C., Kemp, M.Q., Hockings, J.K., Romagnolo, D.F., 2007. Cyclooxygenase-2 promoter activation by the aromatic hydrocarbon receptor in breast cancer mcf-7 cells: repressive effects of conjugated linoleic acid. Nutr. Cancer 59, 248–257.
Diaz, A., Chepenik, K.P., Korn, J.H., Reginato, A.M., Jimenez, S.A., 1998. Differential regulation of cyclooxygenases 1 and 2 by interleukin-1 beta, tumor necrosis factor-alpha, and transforming growth factor-beta 1 in human lung fibroblasts. Exp. Cell Res. 241, 222–229.
Fernandez-Llama, P., Ecelbarger, C.A., Ware, J.A., Andrews, P., Lee, A.J., Turner, R., Nielsen, S., Knepper, M.A., 1999. Cyclooxygenase inhibitors increase Na-K-2Cl cotransporter abundance in thick ascending limb of Henle's loop. Am. J. Physiol. 277, F219–226.
Gasiewicz, T.A., Park, S., 2003. Ah receptor: involvement in toxic responses. In: Schecter, A., Gasiewicz, T.A. (Eds.), Dioxins and Health. John Wiley & Sons, Hoboken, N.J. USA, pp. 491–532.
Grasso, M., Gitlin, J.S., 2006. Ureteropelvic junction obstruction. eMedicine. <http://www.emedicine.com/med/topic3074.htm>.
Herschman, H.R., 1996. Prostaglandin synthase 2. Biochim. Biophys. Acta 1299, 125–140.
Jin, Y., Wang, Z., Zhang, Y., Yang, B., Wang, W.H., 2007. PGE2 inhibits apical K channels in the CCD through activation of the MAPK pathway. Am. J. Physiol., Renal Physiol. 293, F1299–1307.
Kaji, D.M., Chase Jr., H.S., Eng, J.P., Diaz, J., 1996. Prostaglandin E2 inhibits Na-K-2Cl cotransport in medullary thick ascending limb cells. Am. J. Physiol. 271, C354–361.
Kang, Y.J., Wingerd, B.A., Arakawa, T., Smith, W.L., 2006. Cyclooxygenase-2 gene transcription in a macrophage model of inflammation. J. Immunol. 177, 8111–8122.
Lang, R.J., Davidson, M.E., Exintaris, B., 2002. Pyeloureteral motility and ureteral peristalsis: essential role of sensory nerves and endogenous prostaglandins. Exp. Physiol. 87, 129–146.
Lorenz, J.N., Baird, N.R., Judd, L.M., Noonan, W.T., Andringa, A., Doetschman, T., Manning, P.A., Liu, L.H., Miller, M.L., Shull, G.E., 2002. Impaired renal NaCl absorption in mice

lacking the ROMK potassium channel, a model for type II Bartter's syndrome. J. Biol. Chem. 277, 37871–37880.
Lu, M., Wang, T., Yan, Q., Yang, X., Dong, K., Knepper, M.A., Wang, W., Giebisch, G., Shull, G.E., Hebert, S.C., 2002. Absence of small conductance K+ channel (SK) activity in apical membranes of thick ascending limb and cortical collecting duct in ROMK (Bartter's) knockout mice. J. Biol. Chem. 277, 37881–37887.
Madsen, K.M., Tisher, C.C., 2004. Anatomy of the kidney. In: Brenner, B.M. (Ed.), Brenner and Rector's The Kidney. Saunders, Philadelphia, pp. 17–19.
Mahoney, Z.X., Sammut, B., Xavier, R.J., Cunningham, J., Go, G., Brim, K.L., Stappenbeck, T.S., Miner, J.H., Swat, W., 2006. Discs-large homolog 1 regulates smooth muscle orientation in the mouse ureter. Proc. Natl. Acad. Sci. U. S. A. 103, 19872–19877.
Matsumura, F., 2003. On the significance of the role of cellular stress response reactions in the toxic actions of dioxin. Biochem. Pharmacol. 66, 527–540.
McDill, B.W., Li, S.Z., Kovach, P.A., Ding, L., Chen, F., 2006. Congenital progressive hydronephrosis (cph) is caused by an S256L mutation in aquaporin-2 that affects its phosphorylation and apical membrane accumulation. Proc. Natl. Acad. Sci. U. S. A. 103, 6952–6957.
Mimura, J., Yamashita, K., Nakamura, K., Morita, M., Takagi, T.N., Nakao, K., Ema, M., Sogawa, K., Yasuda, M., Katsuki, M., Fujii-Kuriyama, Y., 1997. Loss of teratogenic response to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice lacking the Ah (dioxin) receptor. Genes Cells 2, 645–654.
Nie, M., Pang, L., Inoue, H., Knox, A.J., 2003. Transcriptional regulation of cyclooxygenase 2 by bradykinin and interleukin-1beta in human airway smooth muscle cells: involvement of different promoter elements, transcription factors, and histone h4 acetylation. Mol. Cell. Biol. 23, 9233–9244.
Nishimura, N., Miyabara, Y., Sato, M., Yonemoto, J., Tohyama, C., 2002. Immunohistochemical localization of thyroid stimulating hormone induced by a low oral dose of 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in female Sprague–Dawley rats. Toxicology 171, 73–82.
Nishimura, N., Yonemoto, J., Miyabara, Y., Sato, M., Tohyama, C., 2003. Rat thyroid hyperplasia induced by gestational and lactational exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Endocrinology 144, 2075–2083.
Nishimura, N., Yonemoto, J., Nishimura, H., Ikushiro, S., Tohyama, C., 2005. Disruption of thyroid hormone homeostasis at weaning of Holtzman rats by lactational but not in utero exposure to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicol. Sci. 85, 607–614.
Nishimura, N., Yonemoto, J., Nishimura, H., Tohyama, C., 2006. Localization of cytochrome P450 1A1 in a specific region of hydronephrotic kidney of rat neonates lactationally exposed to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin. Toxicology 227, 117–126.
Nusing, R.M., Treude, A., Weissenberger, C., Jensen, B., Bek, M., Wagner, C., Narumiya, S., Seyberth, H.W., 2005. Dominant role of prostaglandin E2 EP4 receptor in furosemide-induced salt-losing tubulopathy: a model for hyperprostaglandin E syndrome/antenatal Bartter syndrome. J. Am. Soc. Nephrol. 16, 2354–2362.
Ohama, T., Hori, M., Momotani, E., Elorza, M., Gerthoffer, W.T., Ozaki, H., 2007. IL-1beta inhibits intestinal smooth muscle proliferation in an organ culture system: involvement of COX-2 and iNOS induction in muscularis resident macrophages. Am. J. Physiol.: Gasterointest. Liver Physiol. 292, G1315–1322.
Pang, L., Holland, E., Knox, A.J., 1998. Role of cyclo-oxygenase-2 induction in interleukin-1beta induced attenuation of cultured human airway smooth muscle cell cyclic AMP generation in response to isoprenaline. Br. J. Pharmacol. 125, 1320–1328.
Peters, J.M., Narotsky, M.G., Elizondo, G., Fernandez-Salguero, P.M., Gonzalez, F.J., Abbott, B.D., 1999. Amelioration of TCDD-induced teratogenesis in aryl hydrocarbon receptor (AhR)-null mice. Toxicol. Sci. 47, 86–92.
Puga, A., Hoffer, A., Zhou, S., Bohm, J.M., Leikauf, G.D., Shertzer, H.G., 1997. Sustained increase in intracellular free calcium and activation of cyclooxygenase-2 expression in mouse hepatoma cells treated with dioxin. Biochem. Pharmacol. 54, 1287–1296.
Takahashi, N., Chernavvsky, D.R., Gomez, R.A., Igarashi, P., Gitelman, H.J., Smithies, O., 2000. Uncompensated polyuria in a mouse model of Bartter's syndrome. Proc. Natl. Acad. Sci. U. S. A. 97, 5434–5439.
Theobald, H.M., Kimmel, G.L., Peterson, R.E., 2003. Developmental and reproductive toxicity of dioxins and related chemicals. In: Schecter, A., Gasiewicz, T.A. (Eds.), Dioxins and Health. Wily-Interscience, Hoboken, N.J., USA, pp. 329–432.
Thomae, T.L., Stevens, E.A., Liss, A.L., Drinkwater, N.R., Bradfield, C.A., 2006. The teratogenic sensitivity to 2,3,7,8-tetrachlorodibenzo-*p*-dioxin is modified by a locus on mouse chromosome 3. Mol. Pharmacol. 69, 770–775.
Uribarri, J., Kaskas, M., 1993. Hereditary nephrogenic diabetes insipidus and bilateral nonobstructive hydronephrosis. Nephron 65, 346–349.
Vogel, C., Boerboom, A.M., Baechle, C., El-Bahay, C., Kahl, R., Degen, G.H., Abel, J., 2000. Regulation of prostaglandin endoperoxide H synthase-2 induction by dioxin in rat hepatocytes: possible c-Src-mediated pathway. Carcinogenesis 21, 2267–2274.
Vogel, C., Schuhmacher, U.S., Degen, G.H., Bolt, H.M., Pineau, T., Abel, J., 1998. Modulation of prostaglandin H synthase-2 mRNA expression by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin in mice. Arch. Biochem. Biophys. 351, 265–271.
Vogel, C.F., Nishimura, N., Sciullo, E., Wong, P., Li, W., Matsumura, F., 2007. Modulation of the chemokines KC and MCP-1 by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) in mice. Arch. Biochem. Biophys. 461, 169–175.
Walch, L., Morris, P.L., 2002. Cyclooxygenase 2 pathway mediates IL-1beta regulation of IL-1alpha, -1beta, and IL-6 mRNA levels in Leydig cell progenitors. Endocrinology 143, 3276–3283.
Yang, T., 2003. Regulation of cyclooxygenase-2 in renal medulla. Acta Physiol. Scand. 177, 417–421.
Yun, J., Schoneberg, T., Liu, J., Schulz, A., Ecelbarger, C.A., Promeneur, D., Nielsen, S., Sheng, H., Grinberg, A., Deng, C., Wess, J., 2000. Generation and phenotype of mice harboring a nonsense mutation in the V2 vasopressin receptor gene. J. Clin. Invest. 106, 1361–1371.
Zeidel, M.L., Pirtskhalaishvili, G., 2004. Urinary tract obstruction. In: Brenner, B.M. (Ed.), Brenner and Rector's The Kidney. Saunders, Philadelphia, pp. 1867–1894.
Zender, H.O., Ruedin, P., Moser, F., Bolle, J.F., Leski, M., 1992. Traumatic rupture of the urinary tract in a patient presenting nephrogenic diabetes insipidus associated with hydronephrosis and chronic renal failure: case report and review of the literature. Clin. Nephrol. 38, 196–202.

A GENOME INFORMATICS AND EPIDEMIOLOGICAL STUDY IDENTIFIES ALLELES IN ARNT2 ASSOCIATED WITH RISK OF HYPOSPADIAS AND MICROPENIS

Hideko Sone and Junzo Yonemoto

Research Center for Environmental Risk, National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, 305-8506 Japan

Introduction

At the early developmental stages (embryonic, foetal and infant), humans are highly vulnerable to environmental hazards. Several epidemiological studies have suggested the association of perinatal exposure to diethylstilbestrol, allylestrenol, and environmental chemicals, such as organochlorine pesticides and PCBs, with male genital organ abnormalities[1-4]. These chemicals exert their effects via binding to nuclear receptors and activating various genes, including steroid- or drug-metabolizing enzymes. The expression of some of these effects may be promoted by predisposing genetic traits. Ogata et al. recently identified the association of male genital abnormalities with homozygosity for the specific ESR1 (estrogen receptor α) "AGATA" haplotype[5]. In the present study, we systematically selected SNPs in the nuclear receptor genes interacting with ESR1 using informatics and examined the association of certain SNPs with male genital organ abnormalities.

Materials and Methods

Subjects

A total of 234 genome DNA samples were obtained from Dr. Ogata (National Research Institute for Child Health and Development, Tokyo, Japan); they consisted of 134 samples from patients aged 1-13 yrs (62 cryptorchidism (CO), 30 hypospadias (HS), 42 micropenis (MP)) and 100 control samples from 34 boys and 66 adult men.

This study was approved by the Institutional Review Board Committees at the National Institute for Environmental Studies and National Center for Child Health and Development, and informed consent was obtained from each subject and parent(s).

Target gene selection by informatics

As homozygosity for the specific ESR1 (estrogen receptor α) "AGATA" haplotype associated with male genital organ abnormalities, we searched genes, proteins and low-molecular-weight chemicals which interact with ESR1 using KeyMolnet, a comprehensive bioinformatics platform (IMMD, Tokyo, Japan). Metabolism maps containing responsive genes to nuclear receptors that interact with ESR1 were obtained from KEGG (Kyoto Encyclopedia of Genes and Genomes: http://www.genome.jp/kegg/). We then searched for genes that are responsive to environmental chemicals via nuclear receptors that are involved in the biosynthesis pathway from cholesterol to steroid hormones.

Genotyping

SNP genotyping was performed on 221 samples with the Golden Gate Assay (Illumina, CA). The genotyping success rate was 95.2%, and the locus success rate was 98.43%.

SNP selection

The precise human allele information of locus genes selected using informatics was obtained from HUGO (The Human Genome Organization (http://www.hugo-international.org/)). Information on SNPs and tagSNPs was obtained from the dbSNP database (National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/)) and the HapMap Project (http://www.hapmap.prg). The selected SNPs were 20kb upstream to 10kb downstream from the coding region of each gene and more than 60kb from each other; tagSNPs were given priority. Table 1 shows the list of selected genes and the number of SNPs examined in this study.

Statistical analysis

The tests of Hardy-Weinberg equilibrium and correlation between the ESR1 and ARNT2 haplotype block were carried out with GeneSpring GT (ver2.0). Case-control incidences were compared by the χ^2 test. Analysis of the haplotype block was carried out using Haploview (http://www.broad.mit.edu/mpg/haploview/). Other statistical analysis was performed using SAS (SAS Analysis Pro, SAS Institute, Inc., Cary, NC).

Results and Discussion

A total of 378 SNPs of 210 samples (91 controls and 119 cases: 62 cryptorchidism; 23 hypospadias; 34 micropenis) were genotyped. In the case-control analysis, 7 SNPs from three genes were significantly different from the control for all phenotypes combined. Twenty-five SNPs from 5 genes, i.e., CYP17A1, ARNT2, CYP1A2, NR1I2, and AHR, were significantly different from the control in any single phenotype. For these 25 SNPs, the odds ratio for the most frequent allele of each SNP was calculated. Alleles from CYP17A1, ARNT2, and CYP1A2 showed odds ratios of more than 2.0. Five SNPs from CYP17A1 and CYP1A2 showed odds ratios of more than 3.0 (95% CI > 1.0). As many SNPs from CYP17A1, ARNT2, and CYP1A2, such as rs206951 and rs4778607, had no variant, the odds ratio could not be calculated. Instead, trend analysis was performed. Table 2 shows SNPs from CYP17A1, ARNT2, and CYP1A2 with significantly different frequency of allele variants by trend analysis.

As Ogata et al. had shown 4 SNPs in an ESR1 haplotype block related to male genital organ abnormality, the correlation between the 4 SNPs and 31 SNPs which revealed significant differences by case-control analysis (χ^2 test) from 5 genes (AHR, ARNT2, CYP17A1, CYP1A2, NR1I2 (PXR)) was tested. ESRI and ARNT2 were highly correlated. The correlation coefficient for rs3020371 (ESR1) and rs4778607 (ARNT2) was 1.0.

Case-control analysis revealed differences in 5 genes (AHR, ARNT2, CYP17A1, CYP1A2, NR1I2 (PXR)). As these genes are related to dioxin binding (AHR, ARNT2), dioxin induction (CYP1A2), estrogen synthesis (CYP17A1), and bisphenol A induction (NR1I2 (PXR)), the relevance of genetic susceptibility to a dioxin and estrogenic endocrine disruptor in the development of male genital organ abnormality was suggested.

Acknowledgment

We thank Dr. Tsutomu Ogata, National Research Institute for Child Health and Development, for supplying case and control samples.

References

1. Gill, W. B., Schumacher, G. F., Bibbo, M., Straus, F. H., 2nd & Schoenberg, H. W. (1979) *J Urol* **122,** 36-9.
2. Czeizel, A. & Huiskes, N. (1988) *Clin Ther* **10,** 725-39.
3. Longnecker, M. P., Klebanoff, M. A., Brock, J. W., Zhou, H., Gray, K. A., Needham, L. L. & Wilcox, A. J. (2002) *Am J Epidemiol* **155,** 313-22.
4. Fujita, H., Kosaki, R., Yoshihashi, H., Ogata, T., Tomita, M., Hasegawa, T., Takahashi, T., Matsuo, N. & Kosaki, K. (2002) *Teratology* **65,** 10-8.
5. Watanabe, M., Yoshida, R., Ueoka, K., Aoki, K., Sasagawa, I., Hasegawa, T., Sueoka, K., Kamatani, N., Yoshimura, Y. & Ogata, T. (2007) *Hum Reprod* **22,** 1279-84.

Table 2. *p* values for trend analysis for *CYP17A1, CYP1A2* and *ARNT2* SNPs

	Analysis Type	All diseases	Cryptorchidism	Hypospadias	Micropenis
CYP17A1					
rs10786712	Fisher's exact	0.3970	0.2471	0.2101	0.6837
	Cochran-Armitage	0.2477	0.1667	**0.0712**	0.5260
Number		119	62	23	34
rs743572	Fisher's exact	0.4215	0.2711	0.2188	0.5436
	Cochran-Armitage	0.3329	0.2151	**0.0864**	0.4615
Number		119	62	23	34
CYP1A2					
rs2069521	Fisher's exact	**0.0634**	**0.0102**	0.1389	0.2573
	Cochran-Armitage	**0.0888**	**0.0551**	0.7488	0.1237
Number		119	62	23	34
rs2069522	Fisher's exact	0.1038	**0.0287**	0.1803	0.2111
	Cochran-Armitage	**0.0803**	0.1862	0.2858	0.1174
Number		119	62	23	34
rs2069526	Fisher's exact	0.1273	**0.0287**	0.1803	0.1717
	Cochran-Armitage	0.1496	0.1862	0.2858	0.1765
Number		119	62	23	34
ARNT2					
rs4778607	Fisher's exact	0.1644	0.1038	1.0000	0.1795
	Cochran-Armitage	**0.0333**	**0.0392**	1.0000	**0.0536**
Number		119	62	23	34
rs8024819	Fisher's exact	0.9561	0.2505	0.7440	0.3782
	Cochran-Armitage	1.0000	**0.0994**	0.8034	0.1030
Number		119	62	23	34

Number of control samples is 91. Bold figure indicates statistically significant at $p < 0.01$.

Table 1. Selected genes and number of SNPs examined in this study.

HUGO approved gene symbol	Approved Gene Name	Location	Sequence	Previous symbol	Aliases	SNP[a]	tgSNP[b]
AHR	aryl hydrocarbon receptor	7p15	L19872, NM_001621			17	10
AHRR	aryl-hydrocarbon receptor repressor	5p15.33	AB033060, NM_020731	AHH, AHHR	KIAA1234	29	14
ARNT	aryl hydrocarbon receptor nuclear translocator	1q21	AF001307		HIF-1beta	31	6
ARNT2	aryl-hydrocarbon receptor nuclear translocator 2	15q24	AB002305		KIAA0307	69	32
CYP17A1	cytochrome P450, family 17, subfamily A, polypeptide 1	10q24.3	M19489, NM_000102	CYP17	P450C17, CPT7, S17AH	18	4
CYP19A1	cytochrome P450, family 19, subfamily A, polypeptide 1	15q21	D14473	CYP19	ARO, P-450AROM, CPV1, ARO1, CYAR	40	21
CYP1A1	cytochrome P450, family 1, subfamily A, polypeptide 1	15q24.1	BC023019, NM_000499	CYP1	P450DX, P1-450, P450-C, CP11	9	2
CYP1A2	cytochrome P450, family 1, subfamily A, polypeptide 2	15q24.1	AF182274, NM_000761		P3-450, CP12	10	2
CYP1B1	cytochrome P450, family 1, subfamily B, polypeptide 1	2p22.2	U56438, NM_000104	GLC3A	CP1B	20	6
CYP2B6	cytochrome P450, family 2, subfamily B, polypeptide 6	19q13.2	AF182277, NM_000767		CPB6, CYPIIB6, CYP2B	13	9
CYP3A4	cytochrome P450, family 3, subfamily A, polypeptide 4	7q21.1	AF280107	CYP3A3		27	1
NR1I2	nuclear receptor subfamily 1, group I, member 2	3q12-q13.3	AF061056		ONR1, PXR, BXR, SXR, PAR2	21	14
RXRA	retinoid X receptor, alpha	9q34	X52773		NR2B1	46	19
RXRB	retinoid X receptor, beta	6p21.3	M84820		NR2B2, H-2RIIBP, RCoR-1	0	0
RXRG	retinoid X receptor, gamma	1q22-q23	U38480, NM_006917		NR2B3	34	19

CONCENTRATION OF PCBS AND OH-PCBS IN PRESERVED UMBILICAL CORDS

Junzo Yonemoto[1], Junko Kawahara[1], Tatsuya Hattori[2], Tohru Matsumura[2], Seiichi Sugama[3]

[1]National Institute for Environmental Studies, 16-2 Onogawa, Tsukuba, 305-8506 Japan, [2]Idea Consultants Inc., 1334-5 Riemon, Ohigawa, 421-0212 Japan, [3]National Center for Child Health and Development, 2-10-1, Okura, Setagaya, 157-8535 Japan

Introduction

In recent years, the prevalence of certain neurodevelopmental disorders such as learning disabilities, autism, attention deficit and hyperactivity disorders might be increasing[1]. Evidence has been accumulating over several decades that industrial chemicals can cause neurodevelopmental damage and that subclinical stages of these disorders might be common[2]. Much attention has been directed to fetal exposure to chemicals with possible neurodevelopmental effects as the developing human brain is much more susceptible to these chemicals than is the brain of adults[3]. PCB is one of these chemicals of concern because PCB and its metabolite are neurotoxic[2] and are still found in humans, especially in fish eating population such as Japanese at comparatively high concentrations.

In the present study, the concentration of PCB and its hydroxylated metabolites were measured in preserved umbilical cords from subjects of Seiiku Birth Cohort (National Center for Child Health and Development, Tokyo, Japan) to examine the usefulness of preserved umbilical cords as a surrogate for monitoring fetal exposure to PCBs.

Materials and Methods

Subjects and sample collection

Subjects were participants of ongoing Seiiku Birth Cohort (2003-; National Center for Child Health and Development, Tokyo, Japan). As a pilot study, 17 preserved umbilical cords of babies (6 males and 11 females; mean birth weight: 2955 ± 422g) were obtained from mothers (median age: 34.0) with informed consent. This study was approved by the Institutional Review Board Committees at the National Institute for Environmental Studies and National Center for Child Health and Development.

Analysis

Umbilical cord sample was ground into a fine powder with a multibeads shocker (Yasui Kikai, Tokyo, Japan) and was spiked with internal standards. PCBs and OH-PCBs were extracted with 25% ethanol/hexane, washed with distilled water and dehydrated with unhydrous sodium sulfate and then

concentrated by rotary evaporator. OH-PCBs were methylated by reaction with tetramethylsilyldiazomethane. The derivatized solution was concentrated and passed through activated silica-gel packed in a glass column. PCBs were eluted with hexane (Fraction 1) and then CH_3O-PCBs were eluted with 25% dichloromethane/hexane (Fraction 2) and concentrated. Identification and quantification of PCBs and CH_3O-PCBs were performed using GC (Agilent HP6890)- HRMS (Micromass AutoSpec-Ultima). PCB and OH-PCB congeners in samples were quantified using isotope dilution method to $^{13}C_{12}$-internal standards.

Results and Discussion

The weight of the samples obtained was so small (mean 0.27g dry weight) that the detection limit for OH-PCBs in preserved umbilical cords was 10pg/g dry weight in the present study. The concentrations of OH-PCBs and PCBs in preserved umbilical cords were shown in Fig. 1. The median levels of total TeCBs, PeCBs, HxCBs, HpCBs and OcCBs in the preserved umbilical cords were 330, 180, 510, 370 and 60.5 pg/g dry weight, respectively. Hydroxylated PCBs (OH-PCBs) were detected in all the preserved umbilical cord samples examined. The median level of total OH-PCBs was 451 pg/g dry weight (range 194- 879 pg/g). The predominant OH-PCBs detected were 4-OH-2,2',3,4',5,5'-hexachlorobiphenyl (4M146), 3'-OH-2,2',3,4,4',5'-hexachlorobiphenyl (3PM138) and 4-OH-2,2',3,4',5,5',6-heptachlorobiphenyl (4M187) followed by 4-OH-2,3,3',4,5'-pentachlorobiphenyl (4M107). These four OH-PCBs were also detected in human blood as predominant OH-PCBs[4]. The relative abundance of the predominant OH-PCBs in the preserved umbilical cords varies between individuals.

A retrospective case-control study to investigate the association between fetal OH-PCBs exposure and neurodevelopmental disorders is now underway as it was reported that OH-PCBs disturbed thyroid hormone homeostasis in experimental animals[5] and human newborns[6].

References

1. Rutter, M. (2005) *Acta Paediatr* **94,** 2-15.
2. Grandjean, P. & Landrigan, P. J. (2006) *Lancet* **368,** 2167-78.
3. Rice, D. & Barone, S., Jr. (2000) *Environ Health Perspect* **108 Suppl 3,** 511-33.
4. Sjodin, A., Hagmar, L., Klasson-Wehler, E., Bjork, J. & Bergman, A. (2000) *Environ Health Perspect* **108,** 1035-41.
5. Kato, Y., Ikushiro, S., Haraguchi, K., Yamazaki, T., Ito, Y., Suzuki, H., Kimura, R., Yamada, S., Inoue, T. & Degawa, M. (2004) *Toxicol Sci* **81,** 309-15.
6. Otake, T., Yoshinaga, J., Enomoto, T., Matsuda, M., Wakimoto, T., Ikegami, M., Suzuki, E., Naruse, H., Yamanaka, T., Shibuya, N., Yasumizu, T. & Kato, N. (2007) *Environ Res* **105,** 240-6.

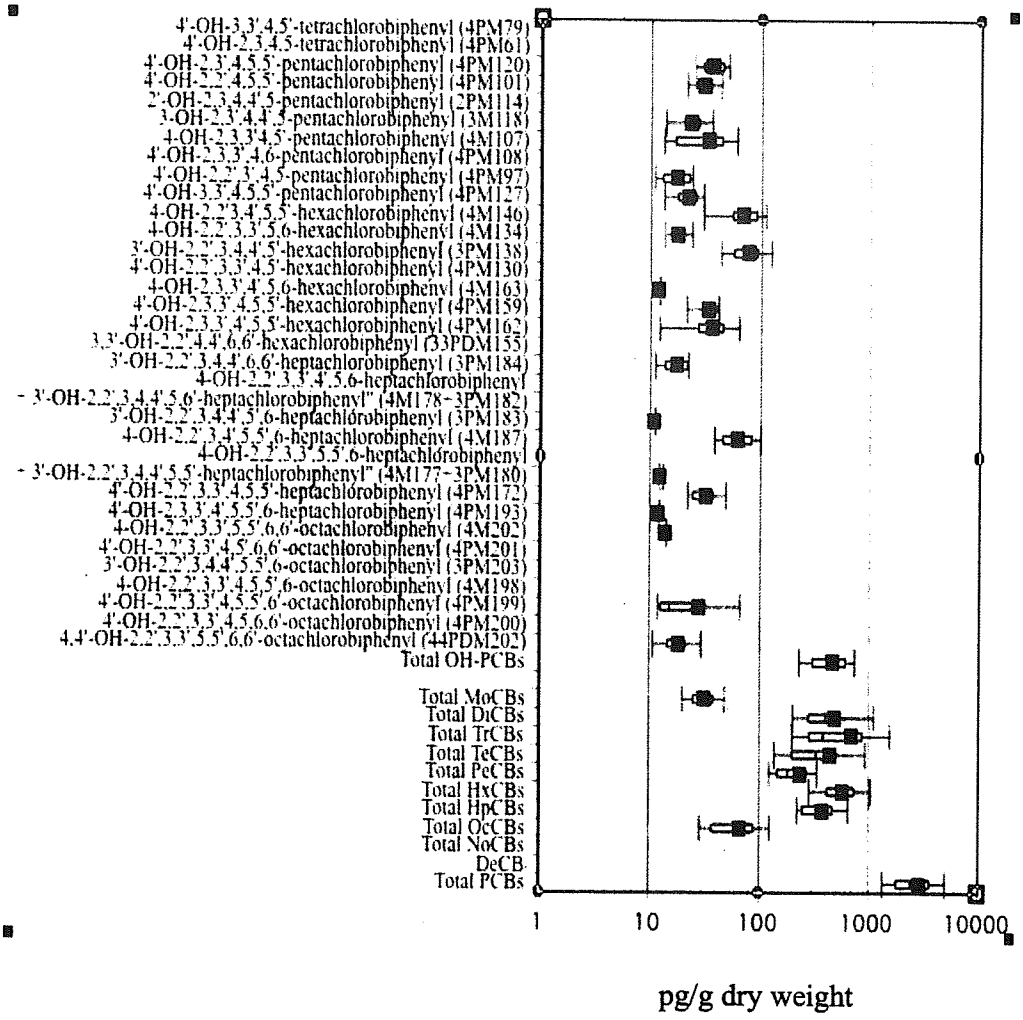

Fig 1. Concentration of PCBs and OH-PCBs in preserved umbilical cords

保存臍帯中の OH-PCB 、PCB 濃度 ―軽度発達障害児における予備的検討―

米元純三[1]、河原純子[1]、服部達也[2]、松村徹[2]、洲鎌盛一[3]
[1]国立環境研究所環境リスク研究センター、[2]（株）いであ環境創造研究所、[3]国立成育医療センター小児期診療部

【はじめに】胎児期の化学物質のばく露がヒトの脳神経発達や行動に与える影響について関心が持たれている。本研究は、国立成育医療センターにおいて 2003 年 11 月から 2005 年 12 月までに出生した児及びその母親を調査対象候補者とし、胎児期における水酸化 PCB および PCB へのばく露と生後の軽度発達障害の発症との関連性を明らかにすることを目的とし、後ろ向き症例対照研究を行った。【方法】国立成育医療センターで出産した母親のうち、研究協力に対する同意が得られたものを対象者とし、そのうち生後の診断によって軽度発達障害と判定されたものを症例、発達障害と判定されなかったものを対照として選定し、胎児期の水酸化 PCB および PCB へのばく露と軽度発達障害の発症との関連性について解析を行った。児の軽度発達障害の検査・診断は、発達心理士および医師により遠城寺式発達検査、デンバー式発達検査法等を用いて行った。ばく露の評価には、保存臍帯を用い、これに含まれるダイオキシン類ばく露の指標となる PCB 類、水酸化 PCB を GC/MS にて分析した。本研究計画は機関における医学研究倫理審査委員会の承認を受けた。【結果及び考察】310 名に本研究への参加を呼びかけ、45 名（症例 8 名、対照 37 名）から保存臍帯の提供を受けた。男女の割合は、症例で 7：1、対照で 19：18 であり、症例では男児が圧倒的に多かった。水酸化 PCB のプロフィールは個人差が大きかったがすべてのサンプルで水酸化 PCB が検出された。総水酸化 PCB の中央値は 451.0 pg/g dry weight (範囲 141−819) であった。検出された主要な水酸化 PCB は、4-OH-2,2',3,4',5,5'-hexachlorobiphenyl (4M146)、3'-OH-2,2',3,4,4',5'-hexachlorobiphenyl (3PM138)、 4-OH-2,2',3,4',5,5',6-heptachlorobiphenyl (4M187) であり、4-OH-2,3,3',4,5'-pentachlorobiphenyl (4M107) がこれに続いた。水酸化 PCB では、総水酸化 PCB、4'-OH-2,3',4,5,5'-pentachlorobiphenyl (4PM120)、4-OH-2,3,3'4,5'-pentachlorobiphenyl (4M107)、4-OH-2,2'3,4',5,5'-hexachlorobiphenyl (4M146)、4-OH-2,2',3,3',5,6-hexachlorobiphenyl (4M134)、3'-OH-2,2',3,4,4',5'-hexachlorobiphenyl (3PM138)、4'-OH-2,3,3',4,5,5'-hexachlorobiphenyl (4PM159)、4-OH-2,2',3,4',5,5',6-heptachlorobiphenyl (4M187)、4'-OH-2,2',3,3',4,5,5'-heptachlorobiphenyl (4PM172)において、PCB では、総 HxCBs, 総 HpCBs, 総 OcCBs において、症例の方が対照に比べ有意に高かった。水酸化 PCB は *in vitro* の系で神経細胞への影響、実験動物、ヒトでの研究において脳神経系の発達に深く関わっている甲状腺ホルモンのかく乱が報告されており、例数は少ないながら水酸化 PCB が軽度発達障害の発症に関わる環境因子として寄与している可能性も考えられる。今後、例数を増やして検討を加える予定である。【謝辞】本研究の一部は環境省の補助を受けた。

Concentration of OH-PCBs and PCBs in preserved umbilical cords -preliminary study of case (neurodevelopmental disorders) and control comparison-

Junzo Yonemoto[1], Junko Kawahara[1], Tatsuya Hattori[2], Tohru Matsumura[2], Seiichi Sugama[3]
[1]National Institute for Environmental Studies, [2]Idea Consultants, Inc., [3]National Center for Child Health and Development

As a pilot study to investigate the association between fetal OH-PCBs exposure and neurodevelopmental disorders, 45 preserved umbilical cords of babies (8 cases (7 males and 1 female), 37 controls (19 males and 18 females were analyzed for hydroxylated PCBs (OH-PCBs) and PCBs by HRGC/HRMS. Hydroxylated PCBs (OH-PCBs) were detected in all the preserved umbilical cord samples examined. The median level of total OH-PCBs was 451 pg/g dry weight (range 141-819 pg/g). The predominant OH-PCBs detected were 4-OH-2,2',3,4',5,5'-hexachlorobiphenyl (4M146), 3'-OH-2,2',3,4,4',5'-hexachlorobiphenyl (3PM138) and 4-OH-2,2',3,4',5,5',6-heptachlorobiphenyl (4M187) followed by 4-OH-2,3,3',4,5'-pentachlorobiphenyl (4M107). In the case-control comparison, total OH-PCBs, 4'-OH-2,3',4,5,5'-pentachlorobiphenyl (4PM120), 4-OH-2,3,3'4,5'-pentachlorobiphenyl (4M107), 4-OH-2,2'3,4',5,5'-hexachlorobiphenyl (4M146), 4-OH-2,2',3,3',5,6- hexachlorobiphenyl (4M134), 3'-OH-2,2',3,4,4',5'-hexachlorobiphenyl (3PM138), 4'-OH-2,3,3',4,5,5'- hexachlorobiphenyl (4PM159), 4-OH-2,2',3,4',5,5',6-heptachlorobiphenyl (4M187), 4'-OH-2,2',3,3',4,5,5'-heptachlorobiphenyl (4PM172), total HxCBs, total HpCBs, and total OcCBs were significantly higher in the cases than controls. Further study is needed to confirm these differences.

PE-6

ヒト血管内皮細胞とマウスES細胞を用いたペルメトリンの血管毒性の評価

今西　哲、米元純三、曽根秀子
（独）国立環境研究所　環境リスク研究センター

ガンや心臓・脳血管障害の治療を目的に血管新生調節剤が注目され、サリドマイドなど様々な化学物質に血管新生を調節する作用があることが示されている。これらの物質は多様な骨格を持っており、共通する特徴は見られない。このような観点から考えると、環境中の化学物質にも、血管形成や血管新生に影響を及ぼす物質が存在するのではないかという懸念が浮上してくる。そこで、広く用いられている殺虫剤ペルメトリンについて、血管形成への影響をマウスES細胞を用いて、また血管新生への影響をヒト正常脳毛細血管内皮細胞を用いて調べた。

マウスES細胞を、DMSO（溶媒）、サリドマイドまたはペルメトリンを 10^{-7}M、10^{-6}M または 10^{-5}M の濃度で含む培地中で浮遊培養して作成した胚様体をI型コラーゲンのゲル中に包埋して、伸長してくるPECAM1陽性の血管様構造の出芽率を調べた。DMSO添加群では約60%の胚様体から出芽がみとめられた。しかし、ペルメトリン添加群では約30%、サリドマイド添加群では約10%にまで減少しており、ペルメトリンには弱い血管形成阻害作用があると考えられた。ペルメトリン添加群とサリドマイド添加群では、約35%の胚様体から中胚葉様の細胞が遊走している様子が見られた。

ヒト正常脳毛細血管内皮細胞を用いた管腔形成試験では、ペルメトリン添加によって分岐間距離の短縮と、盲管状の管腔の増加がみとめられた。これらの結果はサリドマイド添加群でもみとめられ、ペルメトリンはサリドマイドと同様に血管新生を阻害する作用があると考えられた。

以上の結果から、ペルメトリンは *in vitro* で血管形成、血管新生の双方を阻害する作用を持つことが明らかになった。

Evaluation of Vascular Toxicity of Permethrin in Human Endothelial Cells and Mouse ES Cells

Satoshi Imanishi, Junzo Yonemoto, Hideko Sone
Research Center for Environmental Risk, National Institute for Environmental Studies. Japan.

We evaluated the effects on angiogenesis of permethrin, a widely used insecticide with tubular formation assay and differentiation systems of mouse ES cells.

In the tubular formation assay using human brain endothelial cells, treatment with permethrin reduced the length between branches and increased not-connected branches. Same changes were detected in the endothelial cells treated with thalidomide. The resuls suggested that permethrin acted as an angiogenesis inhibitor.

To evaluate effects of permethrin on differentiation of endothelial cells, embryoid bodies (EBs) were formed from mouse ES cells in medium added permethrin or thalidomide. Vascular-like sprouts were observed in about 60% of DMSO treated EBs in type I collagen gel. However, 30% and 10% of permethrin-treated and thalidomide-treated EBs, respectively were sprouted. Mesoderm like cells were migrated from 35-40% of EBs treated with permethrin or thalidomide.

In conclusion, permethrin is an inhibitor of endothelial differentiation and angiogenesis.