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Predominant Role of Cytosolic Phospholipase A₂ α in Dioxin-induced Neonatal Hydronephrosis in Mice

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Hydronephrosis is a common disease characterized by dilation of the renal pelvis and calices, resulting in loss of kidney function in the most severe cases. 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD) induces nonobstructive hydronephrosis in mouse neonates through upregulation of prostaglandin E₂ (PGE₂) synthesis pathway consisting of cyclooxygenase-2 (COX-2) and microsomal prostaglandin E synthase-1 (mPGES-1) by a yet unknown mechanism. We here studied possible involvement of cytosolic phospholipase A₂ α (cPLA₂ α) in this mechanism. To this end, we used a cPLA₂ α -null mouse model and found that cPLA₂ α has a significant role in the upregulation of the PGE₂ synthesis pathway through a noncanonical pathway of aryl hydrocarbon receptor. This study is the first to demonstrate the predominant role of cPLA₂ α in hydronephrosis. Elucidation of the pathway leading to the onset of hydronephrosis using the TCDD-exposed mouse model will deepen our understanding of the molecular basis of nonobstructive hydronephrosis in humans.

Hydronephrosis, defined as dilation of the renal pelvis and calices, is a common disease, found in 1.5–3.3% of autopsies¹. In the most severe cases, kidney function is lost as a result of complete destruction of the renal parenchyma. Obstruction of urine flow at any point along the urinary tract causes retention of urine and increased hydrostatic pressure in the renal pelvis and calices, which is one of the known causes of hydronephrosis. The prevalence of antenatal hydronephrosis was reported to be in the range of 1–5% of pregnancies diagnosed by the use of fetal ultrasonography². While the underlying etiologies of antenatal hydronephrosis are considered to be diverse, most cases lack obstruction^{3,4}. Nonobstructive hydronephrosis is clinically defined by the absence of urinary tract obstruction and vesicoureteral reflux. However, its molecular pathophysiology is unclear.

Certain types of chemical agents induce hydronephrosis. Administration of lithium chloride results in hydronephrosis in mouse neonates⁵. Drugs such as mitomycin⁷ and adriamycin⁸ are also known to produce hydronephrosis. Dioxins, a group of halogenated aromatic hydrocarbons, induce hydronephrosis in rodent fetuses and neonates⁶. They are produced during combustion processes and are ubiquitously present in the environment. They bioaccumulate in humans and wild animals⁹ and have a variety of toxic effects, including teratogenicity, reproductive toxicity, neurobehavioral disorders, immune dysfunction, and carcinogenicity⁶. The most potent congener among dioxins is 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD administration induces hydronephrosis in rodents when they are exposed to it during gestation or via lactation^{10–12}. A pathological characteristic of TCDD-induced hydronephrosis in mouse neonates is the absence of ureteral morphological obstruction¹³. Therefore, TCDD-induced hydronephrosis is a potential model of nonobstructive hydronephrosis in mammals. Another important characteristic is that the critical exposure period leading to the development of TCDD-induced hydronephrosis is confined to the perinatal period^{14,15}, which makes TCDD-induced hydronephrosis a potential model of the perinatal hydronephrosis that is frequently found in human fetuses^{3,4}.

The toxic effects of dioxins, including hydronephrosis, are mediated by aryl hydrocarbon receptor (AhR), a ligand-activated transcription factor. AhR is essential for dioxin toxicity as is demonstrated by the absence of toxicity in AhR-null mice^{13,16–18}. Several genes in addition to that for AhR have been shown to be involved in

dioxin toxicity¹⁹. Among them, genes encoding enzymes for the production of prostaglandins are responsible for the onset of TCDD-induced neonatal hydronephrosis in mice^{13,20}. AhR-dependent upregulation of COX-2 plays a critical role in the onset of hydronephrosis induced by lactational exposure to TCDD in mice. COX-2 upregulation by TCDD is AhR-dependent *in vivo* and the requirement for COX-2 was confirmed by the abrogation of TCDD-induced hydronephrosis by a COX-2-selective inhibitor¹³. COX-2 is an inducible form of cyclooxygenase, which converts arachidonic acid to prostaglandin H₂ (PGH₂). Microsomal prostaglandin E synthase-1 (mPGES-1) transforms PGH₂ to prostaglandin E₂ (PGE₂) and is required for TCDD-induced hydronephrosis, as demonstrated by the lack of hydronephrosis in mPGES-1-null mice²⁰. These studies indicate that PGE₂ production, mediated by COX-2 and mPGES-1, plays a key role in the onset of TCDD-induced hydronephrosis.

In the present study, we hypothesized that there is at least one upstream mediator that is necessary for the TCDD-induced upregulation of COX-2 and/or mPGES-1. Such a mediator is thought to affect the pathogenesis of TCDD-induced hydronephrosis via an increase in the production of PGE₂. Arachidonic acid, the substrate of COX-2, is one of the main precursors of eicosanoids, including leukotrienes, thromboxanes, and prostaglandins. Phospholipase A₂ (PLA₂) enzymes catalyze the hydrolysis of membrane phospholipids to release arachidonic acid²¹. Of more than 30 enzymes with PLA₂ activity, cytosolic phospholipase A₂α (cPLA₂α) is transcriptionally upregulated^{22,23} or enzymatically activated²⁴ in response to TCDD

exposure. Upregulation and/or activation of cPLA₂α can modulate a number of biological processes through arachidonic acid-derived eicosanoids, including PGE₂. Therefore, cPLA₂α has the potential to mediate the toxic effects in response to TCDD exposure. The present study examined the possible role(s) of cPLA₂α in the onset of TCDD-induced hydronephrosis and in the upregulation of COX-2 and mPGES-1.

Results

Role of cPLA₂α in TCDD-induced hydronephrosis. To investigate the potential role of cPLA₂α in the onset of TCDD-induced hydronephrosis in mouse neonates, cPLA₂α KO mice were produced by mating heterozygous cPLA₂α KO mice with each other. After delivery, dams were administered TCDD (20 μg/kg) or vehicle on PND 1, and consequently WT [cPLA₂α (+/+)] and KO [cPLA₂α (-/-)] pups were exposed to TCDD through lactation. The cPLA₂α KO pups did not show any overt abnormality until PND 14 when they were sacrificed for analyses. The body weight of the TCDD-exposed KO pups and that of the WT pups were similar to each other on PND 14 (6.25 ± 0.41 g in female WT pups vs 6.30 ± 0.50 g in female KO pups, and 6.39 ± 0.27 g in male WT pups vs 6.83 ± 0.25 g in male KO pups), which indicates that the KO pups were fed normally and suggests that these two groups of mice were exposed to a similar dose of TCDD via milk. This supposition was confirmed by the nearly identical expression levels of the AhR target genes, CYP1A1²⁵, AhRR²⁶, and IGFBP-1²⁷, upon TCDD exposure

Figure 1 | cPLA₂α-dependent and -independent increases in gene expression in 7-day-old female pups lactationally exposed to TCDD.

CYP1A1 (A), AhRR (B), IGFBP-1 (C) and cPLA₂α (D) mRNA abundances in the kidney after normalizing to the cyclophilin B mRNA level. Results are presented as mean ± SEM for 4–6 dams/group. Histograms with different letters indicate a statistically significant difference at $P < 0.05$ by one-way ANOVA with the post hoc test. No statistical comparison was performed for cPLA₂α KO pups expressing truncated cPLA₂α³¹.

Figure 2 | Representative pictures of cPLA₂α-dependent hydronephrosis on PND 14 induced by lactational exposure to TCDD in male pups. Dams were administered TCDD (20 μg/kg) on PND 1. Kidney of vehicle-exposed cPLA₂α (+/+) (A) and cPLA₂α (-/-) (B) mice and TCDD-exposed cPLA₂α (+/+) (C) and cPLA₂α (-/-) (D) mice on PND 14. The degree of hydronephrosis in (B) and (D) was diagnosed as 4 and 2, respectively, using the scale, reported previously³⁴. Scale bars = 500 μm. Refer to Table 1 and Supplementary Table 1 for information on all pups examined on PND 14.

between the groups of KO pups and WT pups (Fig. 1A–C and Supplementary Fig. 1A–C).

In histological examination, compared with the normal morphology of kidneys from vehicle-treated WT pups (Fig. 2A) and that of KO pups (Fig. 2C), the most severe degree of hydronephrosis was found in TCDD-exposed WT pups (Fig. 2B), while only modest (Fig. 2D) or no effects were observed in TCDD-exposed KO pups. Incidence and severity of hydronephrosis were examined in TCDD-exposed WT and KO pups and control pups on PND 14. All TCDD-exposed female (Table 1) and male (Supplementary Table 1) WT pups developed hydronephrosis by PND 14 and most mice of both sexes showed the most severe degree of hydronephrosis (Table 1 and Supplementary Table 1). By contrast, the incidence of hydronephrosis in TCDD-exposed female and male cPLA₂α KO pups on PND 14 was 33% and 36%, respectively, and none showed the most severe degree of hydronephrosis (Table 1 and Supplementary Table 1). No hydronephrosis was observed in female and male control pups (Table 1 and Supplementary Table 1). These results demonstrate that a lack of cPLA₂α markedly reduces the incidence and severity of TCDD-induced neonatal hydronephrosis.

Role of cPLA₂α in upregulation of the PGE₂ production pathway.

The possible role of cPLA₂α in the TCDD-induced increase in PGE₂ synthesis was examined on PND 7. This time point was chosen because TCDD-induced increase in urinary PGE₂, and upregulation of enzymes responsible for the PGE₂ production, COX-2 and mPGES-1, are prominent on PND 7^{13,20}. In female TCDD-exposed WT pups, COX-2 mRNA abundance, mPGES-1 mRNA abundance, and urinary PGE₂ concentration were significantly increased 5.5, 6.1, and 13.3 fold, respectively, compared with those in the control pups (Fig. 3A–C). In female TCDD-exposed KO pups, COX-2 mRNA abundance, mPGES-1 mRNA abundance, and urinary PGE₂ concentration were 1.9, 1.6, and 2.2 fold, respectively, compared with those in the vehicle-treated KO pups, and none of these increase was significant (Fig. 3A–C). The TCDD-dependent fold induction of COX-2

Table 1 | Severity and incidence of hydronephrosis in female pups

PND ^a	Dose (μg/kg)	cPLA ₂ α genotype	N ^b (pup/dam)	Severity ^c					Incidence ^d (%)
				0	1	2	3	4	
14	0	+/+	6/5	4	2	0	0	0	0
	0	-/-	5/4	4	1	0	0	0	0
	20	+/+	9/6	0	0	1	0	8	100
	20	-/-	8/6	6	0	1	1	0	33*

Note:

^aDams were treated once by gavage on PND 1 and the pups were analyzed for hydronephrosis on PND 14.

^bNumber of pups and dams in each group. Among two groups of mice with different genotypes subjected to the same treatment, dams are counted redundantly.

^cNumber of pups with each severity score. Possible litter effects were not normalized.

^dIncidence in each group, with normalization by litter to minimize possible litter effects.

**P* < 0.05 vs WT. See Experimental Procedures for details.

mRNA, mPGES-1 mRNA, and urinary PGE₂ concentration in female cPLA₂α KO pups was significantly reduced by 74%, 65%, and 84%, respectively, compared with those in female WT mice. The degree of such reductions by the genetic ablation of cPLA₂α was comparable to the degree of suppression in the incidence of TCDD-induced hydronephrosis (i.e., 67%). Essentially the same results were observed in TCDD-exposed male pups. TCDD exposure considerably increased abundance of COX-2 and mPGES-1 mRNAs and urinary PGE₂ concentration in WT pups, and such increases were suppressed to nearly the control level by genetic ablation of cPLA₂α (Supplementary Fig. 2). These results demonstrate that cPLA₂α plays a predominant role in TCDD-dependent increases in COX-2 and mPGES-1 mRNAs and urinary PGE₂ excretion in WT C57BL/6J mouse pups.

Changes in gene expression in the kidney associated with TCDD-induced hydronephrosis.

We analyzed expression of several genes the deficiency of which have been reported to induce hydronephrosis^{28–30} and that are downregulated in the kidney of mouse pups exposed to TCDD^{13,20}. In female pups on PND 7, expression of mRNAs of electrolyte transporters, Na-K-Cl cotransporter 2 (NKCC2) and renal outer medullary K channel (ROMK), were significantly downregulated in the TCDD-exposed WT pups (46% and 32%, respectively) compared with those in the vehicle-treated WT pups (Figs. 4A and B). Expression of mRNA for a water channel, aquaporin 2 (AQP2), in the TCDD-exposed WT pups was also significantly reduced to 50% compared with the vehicle-control WT pups (Fig. 4C). In particular, no such decreases in NKCC2, ROMK, and AQP2 mRNAs were observed in cPLA₂α KO pups (Fig. 4). In male WT and cPLA₂α KO pups (Supplementary Fig. 3), lactational exposure to TCDD led to results essentially identical to those observed in the above-described female pups (Fig. 4).

CYP1A1, AhRR and IGF1P-1 mRNAs were quantified to monitor AhR transactivation activity and found to be significantly increased by TCDD exposure not only in female WT pups but also in female cPLA₂α KO pups (Fig. 1A–C). The expression level of these AhR dependent genes were used as surrogate marker of TCDD exposure as already described above. Essentially, the same result was observed in male pups (Supplementary Fig. 1), although the difference in IGF1P-1 mRNA abundance was not statistically significant probably due to a low induction level by TCDD. The abundance of cPLA₂α mRNA was increased by TCDD exposure in female and male WT pups (Fig. 1D and Supplementary Fig. 1D, respectively), but was barely detected in female and male KO pups, possibly reflecting the expression of a truncated cPLA₂α lacking enzymatic activity³¹.

Discussion

This study was performed to elucidate the possible roles of cPLA₂α, a member of PLA₂ enzyme family, in the pathogenesis of hydronephrosis.

Figure 3 | cPLA₂α-dependent increase in urinary PGE₂ production in 7-day-old female pups lactationally exposed to TCDD. COX-2 (A) and mPGES-1 (B) mRNA abundances in the kidney on PND 7 are shown after normalizing to the cyclophilin B mRNA level. (C) PGE₂ concentration in urine. Results are presented as mean ± SEM for 4–6 dams/group. Histograms with different letters indicate a statistically significant difference at $P < 0.05$ by one-way ANOVA with a post hoc test.

phrosis in mouse neonates caused by lactational exposure to TCDD. Heterozygous female and male cPLA₂α KO mice were mated, and the resulting dams were given TCDD on PND 1 at a dose sufficient to induce hydronephrosis in all WT pups by PND 14. In contrast to WT pups, only 33% of female and 36% of male cPLA₂α KO pups showed signs of hydronephrosis (Table 1 and Supplementary Table 1). This marked reduction in the incidence of hydronephrosis by genetic ablation of cPLA₂α, as well as the concurrent reduction in PGE₂ excretion in the urine, i.e., 84% in KO females and 72% in KO males, compared with the WT pups, demonstrates a significant role of cPLA₂α in the onset of TCDD-induced neonatal hydronephrosis, and suggests that cPLA₂ is the major player among many other PLA₂ enzymes in this role.

Approximately one-third of female and male cPLA₂α KO pups developed hydronephrosis upon TCDD exposure, although the degree of hydronephrosis was much less severe than those in female and male WT pups (Table 1 and Supplementary Table 1). The mechanism by which TCDD-exposure developed hydronephrosis in cPLA₂α KO neonates is thought to be dependent on PGE₂ because of the following two reasons. (1) Genetic ablation of mPGES-1, a PGE₂ synthase, completely blocks the onset of hydronephrosis in TCDD-exposed mouse neonates²⁰, which demonstrates PGE₂ is essential for the pathogenesis. (2) Synthesis of PGE₂ is not completely blocked in the cPLA₂α KO pups (Fig. 3C and Supplementary Fig. 2C), and the minor increase in PGE₂ could elicit onset of the less severe hydronephrosis.

Another important finding of this study is that cPLA₂α is necessary for the TCDD-induced upregulation of the expression of COX-2 and mPGES-1. The indispensable roles of these two enzymes in the

TCDD-induced neonatal hydronephrosis were previously demonstrated by pharmacological inhibition of COX-2¹³ and by genetic ablation of mPGES-1²⁰; each of these treatments completely abolished TCDD-induced hydronephrosis. These studies also revealed that TCDD exposure upregulates the mRNA expression of both COX-2 and mPGES-1 *in vivo*, but did not clarify the component that mediates upregulation of the two genes. The present study showed that cPLA₂α has a role to mediate TCDD-induced upregulation of COX-2 and mPGES-1 expression, because the TCDD-induced upregulation of COX-2 and mPGES-1 mRNAs was markedly reduced in the absence of cPLA₂α (Fig. 3 and Supplementary Fig. 2). It has been established that the expression of CYP1A1²⁵, AhRR²⁶ and IGFBP-1²⁷ is induced by a canonical AhR signaling pathway (also referred to as a genomic action pathway of AhR) because they require the transactivation activity of AhR for their expression in response to TCDD. TCDD-induced increase in mRNA abundance of CYP1A1, AhRR and IGFBP-1 was not influenced by the presence or absence of cPLA₂α (Fig. 1 and Supplementary Fig. 1), while that of COX-2 and mPGES-1 was dependent on cPLA₂α, indicating that the upregulation mechanism of these three genes is distinct from that of COX-2 and mPGES-1.

Two distinct cPLA₂α pathways in AhR signaling can mediate responses to TCDD. The first pathway, referred to as the genomic action pathway²⁴, is well established and is the basis of a dogma to explain TCDD toxicity. This response requires translocation of the TCDD-AhR complex into the nucleus to form a heterodimer with the AhR nuclear translocator (ARNT)²⁵, which upregulates a number of genes, including CYP1A1²⁵, AhRR²⁶ and IGFBP-1²⁷. The cPLA₂α gene is also expected to be a target of the genomic action pathway of

Figure 4 | cPLA₂α-dependent alterations in gene expression in 7-day-old female pups lactationally exposed to TCDD. NKCC2 (A), ROMK (B), and AQP2 (C) mRNA abundances in the kidney (after normalizing to the cyclophilin B mRNA level) are shown. Results are presented as mean ± SEM for 4–6 dams/group. Histograms with different letters indicate a statistically significant difference at $P < 0.05$ by one-way ANOVA with the post hoc test.

AhR for the following reasons: the gene has an AhR binding site in its intron²³, and the TCDD-induced increase in cPLA₂α mRNA is dependent on AhR in hepatoma-derived cell lines^{22,23}. The second pathway of AhR, on the other hand, does not require dimerization of AhR with ARNT or binding of the AhR-ARNT-ligand complex to the promoter region of its target genes; it is named the nongenomic AhR pathway²⁴. This response requires phosphorylation of cPLA₂α

through activation of protein kinases upon Ca²⁺ influx, as has been demonstrated in MMDD1 cells³² and several other cell lines²⁴. As a logical consequence, a question arises about the dominance of either the genomic or the nongenomic AhR pathway in the pathogenesis of TCDD-induced hydronephrosis. However, the question has not been explored yet because an appropriate experimental model to differentiate the two mechanisms on TCDD toxicity via cPLA₂α is unavailable. The present study demonstrated for the first time that TCDD-induced neonatal hydronephrosis is a suitable model to explore the question because this toxicity phenotype was revealed to be dependent on cPLA₂α, which mediates both genomic and nongenomic actions of AhR.

Three enzymes, cPLA₂α, COX-2, and mPGES-1, are the main components of the PGE₂ synthesis pathway. In this pathway, cPLA₂α catalyzes the hydrolysis of membrane phospholipids to release arachidonic acid, which is then converted to PGH₂ by COX-2. PGH₂ is converted to PGE₂ by mPGES-1. TCDD activates this pathway. These three enzymes were upregulated in the kidneys of mouse pups exposed to TCDD, and the PGE₂ concentration in the urine was increased (Figs. 1 and 3, and Supplementary Figs. 1 and 2). The indispensable roles of COX-2¹³ and mPGES-1²⁰ and the crucial role of cPLA₂α (demonstrated in the present study) clearly indicate that PGE₂ elicits TCDD-induced neonatal hydronephrosis. The next step in elucidating the pathogenesis pathway of TCDD-induced neonatal hydronephrosis will be to identify a receptor for PGE₂ responsible for the onset of hydronephrosis, and to characterize the biological components downstream of the PGE₂ receptor. Electrolyte transporters NKCC2 and ROMK and a water channel AQP2 are presumably the candidate components, since the lack of them can result in polyuria leading to the onset of hydronephrosis^{28–30}.

Table 2 | Sequences of primers used for qPCR

Target Gene		Primer Sequences (5' to 3')
AhRR	Forward	CAG GGC AGA CAT TGT GGT TA
	Reverse	CTC CAT TGC TCT TTC CTG CT
AQP2	Forward	GCT GTC AAT GCT CTC CAC AA
	Reverse	AGG CAA AGA TGC ACA GCA C
COX-2	Forward	TGT GAA CAA TCA AAC AAA ATG ATG
	Reverse	GCG TAA ATT CCA ACA GCC TAA GT
cPLA ₂ α	Forward	AGC ATT CAA AAG GCT TCA CG
	Reverse	GGG AAA CAG AGC AAC GAG AT
Cyclophilin-B	Forward	GAC TTC ACC AGG GGA GAT GG
	Reverse	TGT GAG CCA TTG GTG TCT TTG
CYP1A1	Forward	GGC ACC TCT GTT CAC CCT A
	Reverse	GAA TCT CTC CCT CTG TTC TTG
IGFBP-1	Forward	GGA ACG CCA TCA GCA CCT AT
	Reverse	GCA GCT AAT CTC TCT AGC ACT
mPGES-1	Forward	CTC AAG CCC TGC TAC CAC A
	Reverse	GGC CTC AGA CAA GAG ACC AT
NKCC2	Forward	GCT TTA GAC AAC GCT CTG GAA
	Reverse	CGC CTG TTA AGA CAA TGC AC
ROMK	Forward	GCC AAG CCC ATT CCT TAG AC
	Reverse	TCT TCA GCC TCT TCA CCA ACA

A variety of biological phenomena, such as reproduction, cancer, immune responses, and atherosclerosis, are influenced by cPLA₂α through the production of eicosanoids, such as prostaglandins, thromboxanes, and leukotrienes, which are metabolites of arachidonic acid²¹. Using hydronephrosis as a model, we demonstrate for the first time that cPLA₂α is involved in the pathogenesis of TCDD toxicity. Given that the expression of cPLA₂α is deregulated by TCDD (Fig. 1D and Supplementary Fig. 1), other cPLA₂α-related phenomena could be disrupted through changes in eicosanoid levels. TCDD exposure was recently reported to alter the levels of many eicosanoids in several organs³³, although the impact of the alterations on a variety of the endpoints of TCDD toxicity remains to be identified.

In conclusion, we have shown that cPLA₂α predominantly affect the onset of TCDD-induced neonatal hydronephrosis, and is responsible at least partly for the TCDD-induced increase in mRNAs of COX-2 and mPGES-1. The identification and characterization of downstream components of the cPLA₂α/COX-2/mPGES-1 pathway will clarify the molecular mechanisms underlying the pathogenesis not only of TCDD-induced neonatal hydronephrosis, but also human nonobstructive hydronephrosis induced by yet uncharacterized mechanisms.

Methods

Animals and treatment. A TCDD solution (50 µg/mL in n-nonane) was purchased from Cambridge Isotope Laboratory (Andover, MA, USA) and diluted in corn oil (Wako Pure Chemicals, Osaka, Japan). Corn oil containing 2% n-nonane was used as vehicle.

C57BL/6J strain mice were purchased from CLEA Japan (Tokyo, Japan). cPLA₂α-null mice were generated as described previously³¹, and backcrossed more than 12 times in the laboratory of T. Shimizu and more than 4 times in the laboratory of C. Tohyama. Mice were housed at 23 ± 1°C and 50 ± 10% humidity on a 12/12 h light-dark cycle. Laboratory rodent chow (Labo MR Stock; Nosan, Yokohama, Japan) and distilled water were provided *ad libitum*. The study protocols were approved by the Animal Care and Use Committee of the Graduate School of Medicine, The University of Tokyo.

Heterozygous cPLA₂α knockout (KO) mice on the C57BL/6J background were mated to produce WT [cPLA₂α (+/+)] and homozygous KO [cPLA₂α (−/−)] mice. Parturition was checked twice daily, and the day of birth was designated postnatal day 0 (PND 0). Dams were orally administered either a single dose of TCDD (20 µg/kg, 20 mL/kg body weight) or an equivalent volume of vehicle on PND 1. Consequently, pups were exposed to TCDD via lactation, and were sacrificed on PND 7 or PND 14 to collect the kidneys and urine from the bladder. The numbers of dams and pups used for gene expression analysis on PND 7 were 6 and 4–6, respectively, for the vehicle groups, and 5 and 4–5, respectively, for the TCDD groups. The pups used for histological analysis on PND 14 are described in Tables 1 and 2. Data derived from individual pups of the same sex, age, genotype, and TCDD dose were averaged within a litter and then averaged across litters.

The genotypes of the mice (WT or cPLA₂α KO) were determined using PCR to amplify genomic DNA collected from tail snips using the primer sequences CTCTGGTGTGATGAAGGCACTCTATGAGTC and CCTACCTACAATGTTCCACCAAACTAGC for WT [cPLA₂α (+/+)], and CCGTGCTTTACGGTATCGCGCTCCCGATT and CCTACCTACAATGTTCCACCAAACTAGC for KO [cPLA₂α (−/−)] mice.

Histology of the kidney. Kidney specimens were fixed in 10% neutral-buffered formalin, cryoprotected in 20% sucrose solution, embedded in O.C.T. compound (Sakura Finetek Japan, Tokyo, Japan), and snap-frozen in liquid nitrogen. Tissues were sectioned (5-µm thick sections) and stained with hematoxylin and eosin.

Hydronephrosis severity scores were assigned using a previously described scoring system, ranging from ‘0 = no hydronephrosis’ to ‘4 = the most severe hydronephrotic kidney’³⁴. For determining the severity score, blind scoring was performed for all samples (vehicle/TCDD and WT/KO). Incidence of hydronephrosis in the pups was calculated as the percentage of pups with a severity score ≥2 on either the left or right kidney, in accordance with previous reports^{34,35}.

Quantitative RT-PCR. Total RNA was isolated from the kidneys using the RNeasy Mini Kit (Qiagen, Tokyo, Japan). cDNA synthesis of a given mRNA was performed using an oligo-dT₂₀ primer and SuperScript III (Invitrogen, Carlsbad, CA, USA).

Gene expression levels were quantitatively determined using a LightCycler System (Roche Molecular Biochemicals, Indianapolis, IN, USA) with the Thunderbird SYBR qPCR Mix (Toyobo, Osaka, Japan). Primers for the genes (Table 2) were designed from the respective cDNA or mRNA sequences using Primer3 software³⁶. No-template controls were analyzed in every PCR to monitor cross-contamination. To verify amplification specificity, melting curve analyses of the products were performed for

every PCR. The mRNA expression levels were calculated using the ΔΔC_t method and normalized to cyclophilin B expression.

PGE₂ measurement. Urine was collected from the bladders of 7-day-old mice using a syringe. Urinary PGE₂ concentrations were measured using a Prostaglandin E2 EIA kit (Cayman Chemical, Ann Arbor, MI, USA).

Data analysis. To normalize possible litter effects, the data for individual pups of the same sex, age, genotype, and TCDD dose were averaged within a litter and then averaged across litters. All data are expressed as the mean ± SEM for the indicated numbers of litters. Differences in means were analyzed by one-way analysis of variance (ANOVA) followed by Tukey-Kramer post hoc analysis. The statistical significance level was set at <0.05.

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Author contributions

C.T. raised the initial idea of this study. W.Y. and C.T. designed experiments. W.Y., T.K., N.F. and K.A.Y. performed experiments. W.Y., C.T. and F.M. wrote and edited the paper. T.S. provided cPLA $_2\alpha$ -null mice. All the authors discussed the results and provided comments on the manuscript.

Additional information

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