INTRODUCTION

The kidney of mammals is not completely developed at birth in terms of the insufficient ability of concentrating urine (Liu et al., 2001), low glomerular filtration rate (Horster, 1977), and the incomplete structure of the renal pelvis that is required to efficiently remove urine from the renal parenchyma (Miyazaki and Ichikawa, 2001). During the late gestation period of mammals, the placenta plays a pivotal role in discharging the waste from the fetus, and this function is taken over by the kidney after birth. Thus, the neonates are faced with a higher risk of exposure to hazardous factors, such as a certain kinds of drugs, physical agents, and environmental chemicals (Solhaug et al., 2004) because of the vulnerability of the immaturely developed kidney.

The vulnerability of the kidney to environmental chemicals during the early postnatal period has been clearly shown by lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a member of polychlorinated polycyclic aromatic hydrocarbons and a ubiquitous environmental contaminant, in rodents (Couture et al., 1990; Couture-Haws et al., 1991). As a hallmark of toxicities of TCDD, the rodent pups develop hydronephrosis in as early as the first week after birth. Although arylhydrocarbon receptor (AhR) has been known to have an extremely high binding capacity for TCDD (Poland et al., 1976), the molecular mechanism to induce a wide variety of toxicities besides the onset of hydronephrosis is nearly unknown (Kerzee et al., 2003). Our search for a molecular target of TCDD toxicity has recently revealed that cyclooxygenase (COX)-2, an inducible isoform of the rate limiting enzyme for prostanoid synthesis, plays an essential role in the onset of hydronephrosis (Nishimura et al., 2008). The essentiality of COX-2 in the pathogenesis of hydronephrosis was shown in the experiment in

Original Article

Severe toxicity and cyclooxygenase (COX)-2 mRNA increase by lithium in the neonatal mouse kidney

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ABSTRACT — Functions of the kidney of mammals are immature during the neonatal period, and the neonatal kidney could be susceptible to chemicals, including drugs and environmental toxicants. Among these chemicals, cyclooxygenase (COX)-inducing chemicals should be given attentions as the potential kidney toxicants during the period, and we hypothesized that lithium chloride (LiCl) has such toxicity. Neonatal mice of C57BL/J strain were intraperitoneally injected with LiCl (2 mmol/kg body weight) daily until 21 days of age, and examined on 7 days and 21 days of age. Neonatal treatment of LiCl caused a significant increase in COX-2 mRNA and a decrease in mRNAs of aquaporins on day 7 of age. Osmolarity of urine from LiCl-treated neonates was significantly lower than that of control neonate. Most of the LiCl-treated neonates died during the second week of age. Histological examination revealed renal cysts on day 7 and hydronephrosis on day 21 in the surviving neonates. The present results showed that the kidney of mouse neonates is vulnerable to lithium, and suggested the possibility that COX-2 upregulation is responsible for the severe renal toxicity including hydronephrosis.

Key words: COX-2, Dioxin, Hydronephrosis, Lithium, Mouse

INTRODUCTION

The kidney of mammals is not completely developed at birth in terms of the insufficient ability of concentrating urine (Liu et al., 2001), low glomerular filtration rate (Horster, 1977), and the incomplete structure of the renal pelvis that is required to efficiently remove urine from the renal parenchyma (Miyazaki and Ichikawa, 2001). During the late gestation period of mammals, the placenta plays a pivotal role in discharging the waste from the fetus, and this function is taken over by the kidney after birth. Thus, the neonates are faced with a higher risk of exposure to hazardous factors, such as a certain kinds of drugs, physical agents, and environmental chemicals (Solhaug et al., 2004) because of the vulnerability of the immaturely developed kidney.

The vulnerability of the kidney to environmental chemicals during the early postnatal period has been clearly shown by lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), a member of polychlorinated polycyclic aromatic hydrocarbons and a ubiquitous environmental contaminant, in rodents (Couture et al., 1990; Couture-Haws et al., 1991). As a hallmark of toxicities of TCDD, the rodent pups develop hydronephrosis in as early as the first week after birth. Although arylhydrocarbon receptor (AhR) has been known to have an extremely high binding capacity for TCDD (Poland et al., 1976), the molecular mechanism to induce a wide variety of toxicities besides the onset of hydronephrosis is nearly unknown (Kerzee et al., 2003). Our search for a molecular target of TCDD toxicity has recently revealed that cyclooxygenase (COX)-2, an inducible isoform of the rate limiting enzyme for prostanoid synthesis, plays an essential role in the onset of hydronephrosis (Nishimura et al., 2008). The essentiality of COX-2 in the pathogenesis of hydronephrosis was shown in the experiment in

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that a selective COX-2 inhibitor can suppress not only the overproduction of prostaglandin E₂ (PGE₂) by TCDD but also the thinning of the renal parenchyma and pelvis, a typical characteristic features of hydronephrosis.

PGE₂, a member of prostaglandins synthesized by PGE synthase from prostaglandin H₂ (PGH₂), is converted by both constitutive and inducible forms of the cyclooxygenase, COX-1 and COX-2, respectively, from arachidonic acid liberated from the cellular membrane. The prostaglandins exert complex and diverse functions within the kidney (Hao and Breyer, 2008) and play a prominent role in physiological and pathological conditions in neonates (Seyberth and Kühl, 1988; Chemtob et al., 1996). In particular, in the developing kidney, the targeted disruption of the COX-2 gene but not that of the COX-1 gene results in renal dysgenesis (Dinchuk et al., 1995), and COX-2 is a key regulator of neonatal kidney development. The COX-2 activity is required during only the latter phase of nephrogenesis in humans, corresponding to the postnatal period of nephrogenesis in rodents (8). On the other hand, excessive activation of COX-2 in the developing kidney by TCDD was a prototypical example to show that the activation of COX-2 activity is essential to be responsible for the onset of hydronephrosis. However, it was not clear whether the activation of COX-2 is sufficient to explain the etiology of the TCDD-induced hydronephrosis in the neonates. To address such an important question, we formulated the hypothesis that the excessive activation of COX-2 in the neonatal period in rodents results in adverse effects including hydronephrosis. To this end, we selected lithium chloride (LiCl) to neonatal mice and found that the kidney of mouse neonates has an increased level of COX-2 and a very severe degree of hydronephrosis.

**MATERIALS AND METHODS**

**Experimental animals and treatment**

Male and female C57Bl/6J mice were purchased from Clea Japan Inc. (Tokyo, Japan), given laboratory chow (Lab Stock MR, Nosan Corporation, Kanagawa, Japan) and maintained at the University of Tokyo. All the animals were kept in a controlled room at 23 ± 1°C on a 12-hr light-dark cycle, provided with diet and water ad libitum, and were handled humanely following the guidelines for animal experiments of the University of Tokyo. Female mice were mated 1:1 with male, and parturition was checked twice a day and the day of birth was designated post-natal day (PND) 0. Male pups from each dam were assigned to form control- and LiCl- groups to minimize possible litter bias. From PND 1, pups of LiCl-group were carefully administered LiCl (2 mmol/kg body weight, ip) daily for 21 days.

**Analysis of urine**

Urine was collected from bladder using an injection syringe. Measurement of urine osmolarity was carried out by freezing-point depression method (Osmometer 210; Fiske, Norwood, MA, USA). Urine concentrations of lithium, sodium, and potassium were determined by ICP-MS using Agilent 7500ce (Agilent, Santa Clara, CA, USA).

**Histology of the kidney**

Mice from the LiCl- and control- groups were killed at 7 and 21 days in age, and left kidneys were fixed in 4% paraformaldehyde, embedded in paraffin and cut to make 5 μm-sections. The sections were stained with hematoxilin and eosin.

**Real-time RT-PCR**

Total RNA was isolated from the right kidneys of mice using the RNeasy Mini Kit (Qiagen, Valencia, CA, USA). cDNA synthesis was carried out using Super Script III (Invitrogen, Carlsbad, CA, USA). The quantitative detection of differentially expressed genes, including the internal standard gene cyclophilin B, was performed using a Light Cycler instrument (Roche Molecular Biochemicals, Indianapolis, IN, USA) and QuantiTech SYBR Green PCR Kit (Qiagen). Primers for each gene were designed on the basis of respective cDNA or mRNA sequences using primer3 analysis program. PCR 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 1 s, 60°C for 20 s and 72°C for 10 s. Fluores-
cent product was subjected to detection at the end of the 72°C extension period. Negative controls were run con-
comitantly to confirm that the samples were not cross-
contaminated. To confirm the amplification specificity,
we performed melting curve analyses for PCR products.
For quantification, data were analyzed with Light Cycler
analysis software. The primer sequences are summarized
in Table 1.

Data analysis
Data are expressed as mean ± S.E.M. Differences in
means between control group and LiCl group at a specific
time point were analyzed by Student's t-test. In this test,
it was confirmed that there is no difference in variance
among groups. P values less than 0.05 were considered
statistically significant, unless otherwise stated.

RESULTS

Body weight and survival of mice after
administration of LiCl
To study effects of lithium on neonatal mice, newborn
male mice were daily administered LiCl (2 or 4 mmol/
kg body weight) from PND 1. A higher dose of LiCl (4
mmol/kg body weight) was found to be extremely toxic,
and all the pups died within 3 days, and we terminated
the experiment under this dosing condition. In mice given
a lower dose (2 mmol/kg body weight) of Li, body weight
of LiCl-treated mice became significantly lower than that
of control (3.7 ± 0.1 g for control mice vs 3.1 ± 0.2 g
for treated mice, P < 0.05) at 7 days of age, and contin-
ued to be lower thereafter (Fig. 1). At the end of the first
week of age, all the pups of both group was survived. By
the end of the second week of age, approximately 73% of
the LiCl-injected mice (8 out of 11) died (Fig. 1B). Pups
that gained nearly no body weight for a few days became
yellowish in skin color and died. However, the pups that
could survive until the end of the 2nd week of age did not
die at least until 21 day of age, the day of the end of this
experiment.

Effects of LiCl on developing kidney
Determination of lithium amounts in urine by ICP-
mass spectrometry confirmed a significantly elevat-
ed excretion of lithium in urine of lithium-treated pups
on PND 7 compared to the urine from the control mice
(Table 2). Urine osmolality was found to be significant-
ly lower in lithium-treated pups compared with control
pups. Although we were not able to measure the 24-hr
urinary excretion for the pups in the nursing period, the
low osmolality of urine was considered to reflect the diu-
retic effect of lithium. Sodium concentration in urine sig-
ificantly increased in lithium-treated pups, 4 times high-
er compared to control pups (Table 2). This could be
associated with the effect of lithium to enhance the sodi-
um excretion (Nielsen et al., 2008). Potassium concentra-
tion was not significantly affected by lithium administra-
tion (Table 2).

Table 1. Primer sequences for cDNA amplification

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer sequences (5' to 3')</th>
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<tbody>
<tr>
<td>AQP2</td>
<td>Forward: GCT GTC AAT GCT CTC CAC AA</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGG CAA AGA TGC ACA GCA C</td>
</tr>
<tr>
<td>AQP3</td>
<td>Forward: TGC AAA GGC AAG GGA CCA A</td>
</tr>
<tr>
<td></td>
<td>Reverse: GGC ACA CGC ATA CTT AGA AAC TC</td>
</tr>
<tr>
<td>COX-2</td>
<td>Forward: AGA AGG AAA TGG CTC CAG AA</td>
</tr>
<tr>
<td></td>
<td>Reverse: GCT CGG CTT CCA GTA TTG AG</td>
</tr>
<tr>
<td>Cyclophilin B</td>
<td>Forward: TCG GCA AAG TTC TAG AGG GC</td>
</tr>
<tr>
<td></td>
<td>Reverse: TCT GTG GGG ATT GAC AGG</td>
</tr>
<tr>
<td>NKCC2</td>
<td>Forward: CGT TTC CTT GCT CAG GTA GC</td>
</tr>
<tr>
<td></td>
<td>Reverse: GTG GTC TCC CAT GCA AAC TT</td>
</tr>
<tr>
<td>TNFa</td>
<td>Forward: CAC CAC CAT CAA GGA CTC AA</td>
</tr>
<tr>
<td></td>
<td>Reverse: ACA GAG GCA ACC TGA CCA CT</td>
</tr>
<tr>
<td>IL-1β</td>
<td>Forward: GCC CAT CTT CTG TGA CTC AT</td>
</tr>
<tr>
<td></td>
<td>Reverse: AGG CCA CAG GTA TTT TGT CG</td>
</tr>
</tbody>
</table>
It has been reported that lithium administration affects kidney functions including diuresis in a COX-2-mediated manner (Rao et al., 2005), and that COX-2 negatively regulates gene expression of water channels such as AQP2 and 3, and transporters such as Na-K-2Cl cotransporter type 2 (NKCC2), in the kidney (Kim et al., 2008; Norregaard et al., 2007). In this context, we quantified mRNA of COX-2, AQP2, AQP3, and NKCC2 in the kidney from LiCl-injected mice and control mice on PND 7. As expected, COX-2 was increased approximately 5-fold by LiCl injection (Fig. 2A), and AQP2 and AQP3 were downregulated to be 0.21- and 0.44-fold of the control level, respectively (Figs. 2B and C), but no alterations in mRNA levels of NKCC2 was observed (Fig. 2D).

Previously we found that TCDD-induced upregulation of COX-2 activity was accompanied with the increase in mRNA of several inflammatory cytokines including TNFα and IL-1β in the kidney of mouse neonates (Nishimura et al., 2008). Since COX-2 mRNA was also increased in the kidney of mouse pups during lactational period by LiCl (Fig. 3).

Lithium induced severe abnormality of kidney

Histological examination of kidneys at 7 days of age revealed that LiCl injection did not induce a gross abnormality (Figs. 4A and B) but induced small cysts located in the cortex (Figs. 4C and D) and tubular dilations in the medulla (Figs. 4E and F). Although only 3 mice survived at 21 days of age, they have visually recognizable kidney lesions, such as marked pelvic dilatation and decreased thickness of the renal parenchyma, and diagnosed to be afflicted with a severe degree of hydronephrosis (Fig. 4H). Another series of independent experiments confirmed the occurrence of hydronephrosis on PND21 by LiCl (data not shown). On the other hand, all the control mice were alive during this experimental period, and had no histological alterations in the kidney (Fig. 4G).

**Table 2.** Osmolality and electrolyte concentrations in urine of lithium-treated mouse pups

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Lithium</th>
</tr>
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<tbody>
<tr>
<td>Osmolality, mOsm/kgH₂O</td>
<td>470 ± 33</td>
<td>352 ± 34*</td>
</tr>
<tr>
<td>Li⁺, mEq/l</td>
<td>0.41 ± 0.08</td>
<td>18.7 ± 4.9 **</td>
</tr>
<tr>
<td>Na⁺, mEq/l</td>
<td>5.1 ± 2.9</td>
<td>24.2 ± 5.2 **</td>
</tr>
<tr>
<td>K⁺, mEq/l</td>
<td>77.9 ± 10.2</td>
<td>51.6 ± 21.9</td>
</tr>
</tbody>
</table>

Urine was analyzed on PND 7. Urine osmolality was measured by freezing-point depression method. Urine concentrations of lithium, sodium and potassium were determined by ICP-MS. The lower limits of detection of lithium, sodium and potassium were 0.019 mEq/l, 0.404 mEq/l and 0.197 mEq/l, respectively. n.d. indicates not detected below the detection limit.

**DISCUSSION**

Lithium compound has been widely used as an effective drug to treat bipolar disorders and Alzheimer’s disease, but the possible toxicities on the developing kidney as side effects have been less studied. In the present study, we tested the hypothesis that the neonatal kidney of mammals is vulnerable to a lithium compound and the lithium-exposed kidney is prone to develop kidney abnormalities, such as hydronephrosis. This hypothesis was formulated by two lines of already-published experimental evidence. First, lithium administration increased COX-2 protein in the kidney of adult mice, which results in polyuria (Rao
et al., 2005). Second, induction of COX-2 by lactational exposure to TCDD, a polychlorinated polycyclic aromatic hydrocarbon, was shown to induce hydronephrosis in the neonate of rodents, but the administration of a selective inhibitor to COX-2 abolished such a pathogenesis (Nishimura et al., 2008). Our present study showed that LiCl injection induces an increase in COX-2 mRNA and an associated decrease in AQP2 mRNA (Norregaard et al., 2007; Kim et al., 2008) in the neonatal kidney (Fig. 2) and resulted in severe histopathological alterations leading to hydronephrosis. This observation strongly supported the above-mentioned hypothesis. Furthermore, the present study showed a reduction in body weight gain starting in the first week and lethality in the subsequent week (Fig. 1), and such severe effects by lithium was not observed even by a higher dose in adult mice of the same strain (Rao et al., 2005), indicating that the developing kidney is highly vulnerable to lithium toxicity.

Toxicities elicited by LiCl and TCDD in the neonatal kidney share a common characteristics in that these chemicals cause upregulation of COX-2 and TNFα, and that the resultant histopathological alterations such as

![Fig. 2. Expression levels of mRNA of COX-2 (A), AQP2 (B), AQP3 (C), NKCC2 (D) in the kidney of lithium-treated and control mice on PND 7. The mRNA amounts were quantified by real-time RT-PCR, were normalized by that of cyclophilin B and designated as the fold increase to control value. *p < 0.05, **p < 0.01.](image1)

![Fig. 3. Expression levels of mRNA of TNFα (A) and IL-1β (B) in kidney of lithium-treated and control mice on PND 7. The mRNA amounts were quantified by real-time RT-PCR and were normalized by that of cyclophilin B and designated as the fold increase to control value. *p < 0.05.](image2)
marked pelvic dilatation and decreased thickness of the renal parenchyma. The precise molecular mechanism has yet to be proven, but integration of various lines of fragmentary experimental evidence could propose the plausible mechanism of the pathogenesis of hydronephrosis by lithium and TCDD administration in neonatal rodents. Exposure of rodent neonates to either lithium or TCDD induces levels of prostaglandins, particularly PGE_2 owing to the upregulation of COX-2. The COX-2 upregulation in the kidney is presumably regulated by inhibition of glycogen synthase kinase-3β activity (Rao et al., 2004, 2005) or possibly other kinases (Li and Matsumura, 2008; Dong and Matsumura, 2008). The elevation of PGE_2 will suppress the expression of AQP2 (Norregaard et al., 2005) and the trafficking of AQP2 to be inserted into the plasma membrane of the collecting duct epithelium (Nejsum et al., 2005), which subsequently suppresses the water permeability and leads to polyuria (Rao et al., 2005; Kim et al., 2008).

There were some differences between the effects of LiCl and TCDD. The main differences in toxicity phenotype were the presence of renal cyst and a late onset of hydropnephrotic state by LiCl. These differences could be attributable, in part, to a difference in kinetics of LiCl and TCDD. The elimination half-life of LiCl and TCDD are about 4 hr (Smithberg and Dixit, 1982) and 10-24 d (Birnbaum, 1986), respectively. We administered LiCl daily to keep its effects, which should have resulted in the oscillation of serum lithium level with a high level immediately after injection and a low level before the next injection. This oscillation could produce the above-described differences.

In addition, we observed essentially the same changes related to COX-2, such as decrease in urinary osmolarity and increase in COX-2 mRNA, in the neonatal kidney as those reported for adults (Rao et al., 2005), although the changes were more severe in the neonates. The differences of toxic effects between the neonates and adults might depend on temporally specific inducibility of COX-2 in the neonatal mice, or maybe other factors specifically induced by LiCl in this sensitive period.

In conclusion, lithium is contraindicated for use in the pregnant and lactating women because teratogenicity, such as fused ribs and defective vertebra, was reported in mouse (Smithberg and Dixit, 1982) and rat (Marathe and Thomas, 1986), and is suspected in humans from retrospective studies (Yacobi and Ornoy, 2008). However, the prospective studies of lithium treated women in pregnancy did not show significant risk of teratogenicity (Yacobi and Ornoy, 2008). These studies were focused on in utero exposure to lithium. Thus, the results of our study may suggest that more attentions should be paid to adverse effect of lithium on kidneys in the neonatal period.

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