Cadmium causes delayed effects on renal function in the offspring of cadmium-contaminated pregnant female rats

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Cadmium is one of the most common and toxic heavy metals present in our environment. The major sources of exposure to cadmium are contaminated food and water, tobacco, and industrial fumes and dusts (13). Cadmium accumulates in the body, and chronic exposure causes severe nephrotoxicity in humans (12) and animals (3, 8). Furthermore, its biological half-life is 30 yr (11, 13). Renal dysfunction induced by cadmium may be due to proximal tubular damage affecting the passive paracellular pathway (12, 32) and a decrease in active transcellular ion transport (35). In fact, it has been established that the proximal tubule is the main site of cadmium reabsorption, since more than 90% of the filtrated cadmium is reabsorbed along this segment (6). Recently, our group (18) showed that, in adult rats, chronic cadmium exposure causes renal failure with a decrease in glomerular filtration rate (GFR) and an increase of fractional electrolyte excretion with a Fanconi-like pattern (18). In pregnant rats, it has been shown that cadmium absorption from the gut is increased, leading to more accumulation of cadmium in target tissues like kidney and liver (22). Moreover, previous studies have demonstrated that maternal smoking, which is one of the main sources of human exposure to cadmium, is correlated with lower average birth weight and an increase in congenital malformations (1, 5). However, the effect of an in utero cadmium exposure on newborn renal function is unknown. Therefore, we thought this topic would be of interest to study, whether contamination of pregnant women with cadmium could have a deleterious effect on the renal function in the offspring. Thus, in the present study, we investigated the consequences of cadmium intoxication in pregnant rats in their offspring at different stages of postnatal development. For this purpose, we performed renal clearance experiments of offspring from birth to adulthood (postnatal day 2 to postnatal day 60) from cadmium-contaminated pregnant rats, and we also examined the expression of the tight-junction proteins claudin-2, -3, and -5, (CLDN2, CLDN3, and CLDN5), which are located at the proximal tubule, the distal tubule, and the glomerular endothelial cells, respectively.

MATERIALS AND METHODS

The use of animals was in accordance with the ILAR Guide for Care and Use of Laboratory Animals. All animal experimentation was conducted in accord with French government animal welfare policies represented by the Comité Régional d’Ethique pour l’Expérimentation Animal (CREAA).

Animals. After 1 wk of adaptation in a room with controlled temperature (24 ± 2°C) and lighting (12:12-h light-dark), female Wistar rats (200–220 g body wt) were mated with male rats of the same strain and divided into two groups: the 0.5 mg/kg cadmium group, CdCl2 was dissolved in tap water to a concentration of 0.25 mg/ml, and then the appropriate volume (2 μl for 1 g of body wt) of CdCl2 solution was administered to the animals every day by intragastric injection using an oral cannula during 21 days of pregnancy. Daily cadmium exposure for the pregnant rats was stepped at delivery. The control group received only tap water via the same way. Pregnant rats were housed individually in cages under standard laboratory condi-
tions and were given free access to food and drink. After delivery, the two groups of pups were kept with their dams for breastfeeding until PND21.

Measurement of GFR and fractional ion excretion. Clearance experiments were performed to analyze the whole kidney function of PND2, PND6, PND14, PND21, PND45, and PND60 rats intoxicated in utero. Anesthesia was induced by intraperitoneal injection of pentobarbital sodium (Nembutal; 5 mg/100 g body wt) and maintained by additional doses when necessary for PND14, PND21, PND45, and PND60 rats and by chloroform inhalation for PND2 and PND6 rats. The animals were placed on a heated table to maintain their body temperature between 37 and 39°C. Polyethylene catheters were inserted into the jugular vein for each group and in the femoral artery for PND21, PND45, and PND60 rats. For PND45 and PND60 rats, urine was collected with a catheter introduced in the ureter; however, for PND2 to PND21 rats, urine was collected with a catheter inserted into the bladder. Clearance experiments were carried out in rats intravenously infused with a 0.9% NaCl solution at a rate of 0.5 ml/min. FITC-inulin was used to measure the GFR. Urine samples were collected serially every 20 min, and blood samples were obtained halfway through each urine collection for rats from PND21 to PND60. Between blood collections, the arterial catheter was connected to a pressure transducer (Harvard Apparatus) for arterial blood pressure measurement. For rats from PND2 to PND14, one collection of urine of 1 h had been done, and blood was directly punctured in the heart at the end of the period. In all experiments, a loading dose of FITC-inulin (0.02 mg/g body wt) was given intravenously, followed by a continuous infusion as mentioned above for the duration of the experiment. In all of the experiments, urine collection began 1 h after the administration of the FITC-inulin priming dose.

Cadmium measurement in tissues. The technique used to measure cadmium was described earlier by Playle et al. (27). Liver, kidney, and stomach content (for PND2 to PND14 rats) samples were collected, weighted, and digested in a solution of nitric acid (1 N, TraceMetal grade HNO3; Fisher Scientific) at a temperature of 80°C for 4 h. At the end of this time, the supernatant was taken and its volume measured to calculate cadmium concentration in livers, kidneys, and maternal milk.

Rat frozen kidney tissue sections. Kidneys of experimental rats were removed at the end of each experiment and washed with PBS. Cubes (0.5 cm/side) were cut and immediately immersed for 2 min in 2-methylbutane (263-1; Aldrich), which was previously cooled in liquid nitrogen. The cubes were then transferred for 10 min to liquid nitrogen. Eight-micrometer sections were cut in an IEC microtome cryostat (International Equipment). For immunofluorescence, the sections were fixed for 20 min in 70% ethanol at 4°C, washed with PBS, and quenched for unspecific staining with 0.2% IgG-free albumin (1331-A; Research Organics) in PBS for 20 min at 4°C. The immunofluorescence protocol used is described below.

Immunofluorescence. The frozen sections were incubated overnight with one of the following rabbit polyclonal antibodies: CLDN2 (51-6100, dilution 1 g/ml; Zymed), CLDN3 (34-1700, dilution 5 g/ml; Zymed), or CLDN5 (34-1600, dilution 20 g/ml; Zymed). The sections were washed three times with PBS and incubated for 1 h with

Table 1. Stomach cadmium content of PND2, PND6, and PND14 offspring from contaminated dams

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<thead>
<tr>
<th>Experimental Rat Group</th>
<th>Stomach Cadmium Content, μg of cadmium/g of stomach content</th>
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<tr>
<td>PND2 (n=7)</td>
<td>14.48±3.61</td>
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<tr>
<td>PND6 (n=6)</td>
<td>2.67±0.73</td>
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<tr>
<td>PND14 (n=7)</td>
<td>1.30±0.23</td>
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Values are means ± SE, where n = no. of samples. PND, postnatal day.
an FITC-conjugated goat anti-rabbit IgG (65-6111, 6 μg/ml; Zymed).

After three more washes with PBS, the sections were transferred to glass coverslips and mounted with the antifade reagent Fluorogard (170-3140; Bio-Rad). The fluorescence of the sections was examined with a confocal microscope (Leica SP2, with krypton-argon laser). The images collected had an optical thickness of 1 μm. The images shown represent a projection of the sections made for each slide.

**Analytical procedure.** Na⁺, K⁺, Mg²⁺, Ca²⁺, and cadmium concentrations were determined by atomic absorption spectrometry with the use of a Zeeman furnace system (Solaar 969, Thermo Optek), and FITC-inulin was measured by spectrophotometer (SAFAS Monaco). PO₄³⁻ concentration was determined by colorimetry using a microplate reader (Bio-Tek Instruments).

**Statistical analyses.** Unpaired t-test was used to compare quantitative variables between control and experimental groups at the same developmental time points, as well as between each developmental time point. The data are expressed as means ± SE. Differences were considered significant at \( P < 0.05 \).

**RESULTS**

**Body weight.** Figure 1A shows the change in total body weight of offspring from cadmium-contaminated and control female rats throughout postnatal development. Results show that cadmium-intoxicated pups have a lower body weight during early development up to 6 days than that shown in control pups. Thereafter, at later postdevelopmental time points, no difference in body weight between the two groups of rats was observed. Concerning kidney and liver weights, no

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**Fig. 2.** Cadmium content in the kidney (A) and liver (C) in rats from contaminated dams, at different stages of development. Cadmium content normalized to organ weight (cadmium content per gram wet wt) in kidney (B) and in liver (D). E: total amount of cadmium in the rats. For gestational day 20 (GD20), \( n = 8 \); for PND2, \( n = 4 \); for PND6, \( n = 3 \); for PND14, \( n = 7 \); for PND21, \( n = 3 \); for PND45, \( n = 4 \); for PND60, \( n = 4 \). Values are means ± SE. * \( P < 0.05 \), ** \( P < 0.01 \), and *** \( P < 0.001 \), significant vs. the previous cadmium amount.
differences were observed between control and cadmium-contaminated offspring (Fig. 1, B and C).

**Cadmium presence in maternal milk.** To determine whether cadmium is present in the milk of cadmium-intoxicated pregnant dams, we measured the cadmium stomach content in PND2 to PND14 offspring (Table 1). In that period of postnatal development, pups obtain food only by breastfeeding. Results show a high level of cadmium at an early age (PND2), followed by a decrease at late postdevelopment time points. These results are compatible with the presence of cadmium in the milk of contaminated dams.

**Cadmium levels in the kidney and liver.** Figure 2 illustrates cadmium accumulation in the kidney and liver throughout postnatal development in the contaminated offspring. First, cadmium is present at significant levels at a late gestational developmental time point (gestational day 20) in both the kidney and liver, suggesting the transfer of cadmium from mother to embryos through the placental barrier. After birth, in the kidney (Fig. 2A), the amount of cadmium increases steadily throughout postnatal development until day 60. In the liver (Fig. 2B), cadmium increases, reaching a peak at day 14, followed by a drastic decrease. The combined liver and kidney cadmium content (Fig. 2E), which represents the total amount of cadmium in the body of intoxicated rats, shows that the cadmium level increases up to PND14 and thereafter stays at a steady-state level for later developmental time points. Cadmium content normalized per gram of organ weight is shown in Fig. 2, B and D. After delivery, in kidney (Fig. 2B), results show that cadmium content per gram of kidney decreases until PND14 and then increases at PND21 followed by a steady-state level until PND60. In contrast, in liver, after delivery, cadmium content per gram of liver decreases per gram decreases throughout all developmental days with a drastic decrease between PND14 and PND21.

**Arterial blood pressure.** Arterial pressure measurements of PND21, PND45, and PND60 rats demonstrated that offspring from contaminated dams have a higher arterial pressure than offspring from control females (Table 2).

**Renal function.** The effects of in utero exposure to a daily dose of 500 μg cadmium/kg during gestation (21 days) in renal function of offspring (PND2 to PND60) are shown in Fig. 3. Results showed that U/P inulin and GFR increase with age of development (P < 0.001), which is compatible with maturation of the kidney and in renal development. Before PND60, there is no difference in GFR between control and contaminated rats. However, at PND60, rats born from cadmium-treated females had a significant GFR decrease, from 1.16 ± 0.04 μl/min-1.g-1 to 0.52 ± 0.04 ml/min-1.g-1 (n = 12).

Fractional excretion and plasma concentrations of Na⁺, K⁺, Ca²⁺, Mg²⁺, and PO₄²⁻ for each experimental group are expressed in μl/min-1.g-1 of kidney and in ml/min-1.g-1 of kidney. Results for urinary flow rate and GFR of 2-, 6-, 14-, and 21-day-old pups were normalized for one day (P < 0.001), represented by a change from birth to adulthood consecutive to the normal maturation in renal function. At PND60 rats from contaminated dams had a significant increase in fractional excretion of all ions compared with rats from control dams, compatible with a tubular dysfunction. Regarding phosphate handling, its fractional excretion did not change significantly during the time of development, except for later time points.

**Intercellular junctions.** Immunofluorescence of renal cryosections detected CLDN2 in the tight-junction complex at the cell borders. We analyzed the tubular distribution of CLDN2 in

<table>
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<th>Table 2. Arterial blood pressure of PND21, PND45, and PND60 offspring from control and contaminated dams</th>
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<td><strong>Experimental Rat Group</strong></td>
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<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Control</td>
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<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>PND21</td>
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<tr>
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<tr>
<td>(n = 11)</td>
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<tr>
<td>PND60</td>
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<td>(n = 11)</td>
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Values are means ± SE, where n = no. of periods. *P < 0.05, † P < 0.01, and ‡P < 0.001, significant compared with results of control values for the same time points.
Fig. 4. Plasma concentrations (mM) and fractional excretions (%) of Ca$^{2+}$, Mg$^{2+}$, Na$^{+}$, K$^+$, and PO$_4^{3-}$ during postnatal development in rats from control (open bars) and contaminated dams (closed bars). Values are means ± SE. *P < 0.05, **P < 0.01, and ***P < 0.001, significance vs. the control group at the same time of development.
kidney slices from control rats and cadmium-treated rats (Fig. 5). As shown previously, CLDN2 is only present in the proximal tubules (31). The expression pattern shows a clear “chicken fence” appearance at the cell borders. In offspring from intoxicated rats, no difference in CLDN2 expression was observed compared with control PND2, PND6, PND14, and PND21 animals. In contrast to the observations in PND2 to PND21 animals, sections from PND60 rats from cadmium-treated mothers (Fig. 5J) showed a severely altered pattern of CLDN2 expression compared with control animals: the defined linear pattern was changed to a dotted mark at cell borders of the proximal epithelium leading to a complete disappearance of fluorescence in the most serious cases (Fig. 5J). These results are confirmed in Table 3, where data show the percentage,
evaled by double-blind counting, of proximal tubules with an altered fluorescence of CLDN2 distribution and expression at different postnatal ages.

We studied the tubular, glomerular, and arterial distributions of CLDN3 and CLDN5 with the same immunofluorescence technique. The presence of CLDN5 has been shown mainly in the tuft of the glomerulus and in the endothelium of blood vessels and arteries (30). Until day 21, the fluorescence of CLDN5 demonstrated a classic pattern in both control (Fig. 6, A, C, E, G) and cadmium-treated rats (Fig. 6, B, D, F, H); linear in small blood vessels and punctuated in the glomerulus tuft. As observed with CLDN2, the deleterious effects of cadmium appeared in PND60 rats. Thus, in contrast to results in control rats (Fig. 6I), the labeling of CLDN5 is weak, ill-defined, and shows disruption in the glomerulus (Fig. 6J). It was noted that the glomerulus shows an abnormally enlarged space between the tuft and the Bowman’s capsule, suggesting severe capillary damage and retraction. Occasionally, we also observed the presence of CLDN5 in the tubular epithelium (Fig. 6, C, D, and I).

CLDN3 has also been demonstrated in the endothelium of small blood vessels and arteries and in the tubular epithelium of the distal tubules and collecting ducts (18, 31). Here, we have shown that CLDN3 is not present in the tuft of the glomerulus (Fig. 7, A and C–H). In contrast to CLDN2 and CLDN5, CLDN3 did not show changes or alterations of the distribution in blood vessels and tubular epithelium at any of the studied ages; even at day 60, when damage was more evident for CLDN2 and CLDN5, CLDN3 labeling was preserved in cadmium-treated rats (Fig. 7I), showing the classical “chicken fence pattern” at the cell border of the tubular epithelium. CLDN3 expression differences between control and intoxicated offspring are shown in Table 4.

**DISCUSSION**

Heavy metals are present in our environment, either in the form of industrial pollutants or naturally in soils, and can contaminate food and drinking water; cadmium is also found in tobacco. In recent years, a large number of studies have examined the renal effects of cadmium intoxication. However, the consequence of maternal contamination with cadmium during pregnancy to renal function of the offspring is unknown. Thus the aim of the present study was to investigate the impact of cadmium on newborn renal function after exposure in utero. We intoxicated female Wistar rats with 0.5 mg cadmium/kg added to the drinking water during the whole gestation. According to Liu et al. (23), Dudley et al. (10), and Goyer and Cherian (13), this dose corresponds to low-dose chronic cadmium intoxication.

First, we observed a lower birth weight in pups from cadmium-exposed rats than in those from controls. The decrease in birth weight could be due to a deficit in iron and/or zinc in cadmium-contaminated dams. It has been shown that cadmium induces maternal zinc retention, which is responsible for fetal zinc deficiency and impaired fetal growth (34). Cadmium has also been shown to be transported in competition with iron by the iron transporter DMT1 (6, 14), a protein overexpressed during pregnancy in the intestinal tubule (22). This may cause anemia and fetal growth retardation from a lack of iron. However, even if our results had shown cadmium in the gestational day 20 fetal kidney, it seems that the placental barrier is still a good filtration barrier, related to the low amount of cadmium measured at this stage. In contrast, after delivery, the primary source of cadmium intake, but this time not negligible, is via contaminated milk, as reported in results showing that cadmium is present in the stomach of PND2, PND6, and PND14 offspring from intoxicated dams, thus indicating that offspring continue to get contaminated via milk, before weaning. This significant amount of cadmium via breastfeeding, in addition to the cadmium gain during gestation, could be the reason that a low body weight was observed for young offspring.

During postnatal development, we have seen in offspring from intoxicated dams a decrease in the amount of cadmium in the liver and an increase of cadmium in the kidney, suggesting a process of decontamination that eliminates cadmium from the liver. Metallothionein (MT), an intracellular metal binding protein, is synthesized in the liver in response to cadmium exposure to protect against its toxic effect by binding cadmium in a cadmium-MT complex, the nontoxic form (19). At saturation, this complex is released into the circulation, crossing the glomerular filtration barrier, and is endocytosed by the apical megalin receptor in the proximal tubule cells (37).

Table 3. Percentage of proximal tubules showing an altered fluorescence of claudin-2 (distribution and expression) at different postnatal ages

<table>
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<tr>
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<th>Control, %</th>
<th>Cadmium, %</th>
<th>n</th>
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<tr>
<td>PND2</td>
<td>14.5±1.5</td>
<td>22.4±10.0</td>
<td>15</td>
<td>NS</td>
</tr>
<tr>
<td>PND7</td>
<td>17.4±2.2</td>
<td>19.9±2.2</td>
<td>18</td>
<td>NS</td>
</tr>
<tr>
<td>PND14</td>
<td>3.3±1.6</td>
<td>2.8±1.4</td>
<td>8</td>
<td>NS</td>
</tr>
<tr>
<td>PND21</td>
<td>9.9±3.9</td>
<td>14.1±3.3</td>
<td>9</td>
<td>NS</td>
</tr>
<tr>
<td>PND60</td>
<td>12.0±0.7</td>
<td>66.2±3.4</td>
<td>12</td>
<td>P &lt; 0.0001</td>
</tr>
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</table>

Values are means ± SE, where n is no. of kidney slices evaluated by double-blind counting. Level of significance was calculated with a Student’s t-test comparing control and cadmium groups from each age. NS, not significant.

Free cadmium, the toxic form, is then released from the endothelium and contributes to cadmium-induced renal damage. In a study of MT-null knockout mice, repeated administration of cadmium results in nephrotoxicity at one-tenth the dose that produces nephrotoxicity in control mice (24). Thus, in the case of a chronic intoxication, it appears that MT would prevent or delay cadmium-induced renal injuries. In the present study, the decrease in the amount of cadmium in the liver of PND21 to PND60 offspring is consistent with the saturation of this protection system and a redistribution of cadmium in the kidney. Furthermore, it is known that, at PND21, the number of nephrons in the kidney has reached its final level (33). Thus,
after PND21, the increase of kidney weight is principally due to increase of interstitial tissue as well as loop of Henle length. Therefore, as shown in Fig. 2B, cadmium quantity per nephron after PND21 is more and more elevated and then contributes with time to its renal toxic effect. It is well known that after birth the kidney is not fully mature. Its excretory and reabsorptive functions are still developing (7). The final stages of nephron maturation are characterized by extensive elongation of the tubular nephron, with development of the loop of Henle and the renal diluting capacity segment, the expression of specialized nephron segment-specific transport proteins, and the structural integrity of the epithelium, the tight junction (27, 28). All of these phenomena lead to the production and excretion of a small quantity of concentrated urine. The evolution of the renal parameters observed is consistent with the maturation of the kidney. In this way, U/P inulin and GFR increase during

Fig. 6. Claudin-5 (CLDN5) label in kidney sections from control (A, C, E, G, and I) and cadmium-treated rats (B, D, F, H, and J) at 2, 6, 14, 21, and 60 days postpartum. CLDN5 is one of the specific proteins from the endothelial tight junction in blood vessels. It is observed in that location at all ages. Occasionally, CLDN5 fluorescence was also observed at the cell border of the tubular epithelium. Asterisks indicate glomeruli, arrows indicate arteries, and arrowheads indicate labeled tubular epithelium. Until day 45, fluorescence of CLDN5 of the tuft of glomeruli, the small blood vessels, and the tubular epithelium from cadmium-treated and control rats showed no differences. At PND60, cadmium-treated rats (J) show an enlarged space between tuft and Bowman’s capsule and a disrupted localization of claudin-3 (CLDN3) in glomerulus compared with PND60 control rats (I).
this time, whereas the fractional excretion of ions decreases. The evolution of the fractional excretion of phosphate during development suggests that its reabsorption is not dependent, compared with the other ions, on the structural maturation of the nephron. Thus it has been shown that the renal Na\(^+\)-P\(_i\) transport capacity is already high at birth (17) and decreases after 21 days old, the date of weaning for the rat. This reabsorption of phosphate is necessary for the animal’s growth and skeletal ossification (15). Our results did confirm such handling of phosphate.

The effects of cadmium on renal function have been observed at 60 days, when the amount of cadmium in the kidney reaches its highest level, suggesting a critical dose and/or exposure time for delayed nephropathy, as previously reported (18) for the induction of a hazardous effect of cadmium. The diminution of the GFR and the augmentation of the fractional excretion of all ions suggest that, in PND60 rats from intoxicated dams, there is damage to both glomerular and proximal tubules.

The measurement of arterial blood pressure in PND21, PND45, and PND60 offspring from intoxicated dams and

<table>
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<th>CADMIUM</th>
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<tr>
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<td><img src="A" alt="Image" /></td>
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<td>21 DAYS</td>
<td><img src="G" alt="Image" /></td>
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<tr>
<td>60 DAYS</td>
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Fig. 7. Immunofluorescence of CLDN3 in kidney sections obtained from control (A, E, G, and I) and cadmium-treated rats (B, D, F, H, and J) at 2, 6, 14, 21, and 60 days postpartum. In these sections, the endothelia of arteries and blood vessels show a CLDN3 well-defined punctuated labeling (arrowheads). In the epithelium of the distal tubule and collecting ducts, CLDN3 depicts a “chicken fence pattern” (arrows) in these areas. The glomeruli do not show specific labeling of CLDN3. A weak autofluorescence allows the identification of their presence (asterisks). In contrast to CLDN2 and CLDN5, no difference is observed between the control and cadmium-treated rats, even at 60 days old (I and J).
control rats shows that cadmium-exposed offspring have a hypertensive phenotype. Cadmium induces hypertension, as previously reported (4, 21). The cadmium hypertensive effect has been shown to involve the renin-angiotensin system (21). It should also be mentioned that hypertension observed in these rats could be involved in kidney dysfunction.

Tubular transport occurs through two main routes: the transepithelial and the paracellular pathway. The proximal tubule might be associated with a defect in the tight-junction organization, in addition to the abnormal transepithelial transport. The tight junction constitutes the main barrier in the epithelia to the passive and paracellular movement of electrolytes and macromolecules and is also responsible for epithelial polarity (9). Claudins are constitutive junctional proteins in all epithelia. Reyes et al. (31) showed that, in the kidney, CLDN2 is predominantly located in the proximal tubule. In a recent study (18), our group showed that the expression and localization of CLDN2 were altered after chronic cadmium intoxication. It is noteworthy that, in the present study, expression of CLDN2 was also disorganized in the adult offspring from intoxicated mothers but was normally expressed in younger individuals also exposed to cadmium. This disruption of the CLDN2 expression in the proximal tubule suggests a link between this protein and the renal dysfunction caused by exposure to cadmium. Our results show a delayed nephrotoxic effect of cadmium after exposure in utero, which seems to depend on the developmental stage and function of the kidney of rats chronically exposed to Cd. I. Micropuncture study of pressures in proximal tubules and peritubular capillaries of the rat kidney and their relation to ureteral and renal venous pressures. Am J Physiol 205: F1459–F1468, 1988.

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