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Cadmium-induced teratogenicity: Association with ROS-mediated endoplasmic reticulum stress in placenta

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ABSTRACT

The placenta is essential for sustaining the growth of the fetus. An increased endoplasmic reticulum (ER) stress has been associated with the impaired placental and fetal development. Cadmium (Cd) is a potent teratogen that caused fetal malformation and growth restriction. The present study investigated the effects of maternal Cd exposure on placental and fetal development. The pregnant mice were intraperitoneally injected with $CdCl_2$ (4.5 mg/kg) on gestational day 9. As expected, maternal Cd exposure during early limb development significantly increased the incidences of forelimb ectrodactyly in fetuses. An obvious impairment in the labyrinth, a highly developed tissue of blood vessels, was observed in placenta of mice treated with $CdCl_2$. In addition, maternal Cd exposure markedly repressed cell proliferation and increased apoptosis in placenta. An additional experiment showed that maternal Cd exposure significantly upregulated the expression of GRP78, an ER chaperone. Moreover, maternal Cd exposure induced the phosphorylation of placental eIF2α, a downstream molecule of PERK signaling. In addition, maternal Cd exposure significantly increased the level of placental CHOP, another target of PERK signaling, indicating that the unfolded protein response (UPR) signaling was activated in placenta of mice treated with $CdCl_2$. Interestingly, alpha-phenyl-N-t-butylnitrone, a free radical spin-trapping agent, significantly alleviated Cd-induced placental ER stress and UPR. Taken together, these results suggest that reactive oxygen species (ROS)-mediated ER stress might be involved in Cd-induced impairment on placental and fetal development. Antioxidants may be used as pharmacological agents to protect against Cd-induced fetal malformation and growth restriction.

Introduction

Cadmium (Cd) is one of major occupational and environmental toxicants. Cd is frequently used in electroplating, pigments, paints, welding, and Ni–Cd batteries. Workers in these occupations are exposed to Cd at significantly higher levels than the general population (Beveridge et al., 2010). The general population is exposed to Cd via drinking water, food and cigarette smoking (Honda et al., 2010). Cd, at a high dose, is a potent teratogen in rodents (Barr, 1973; Thompson and Bannigan, 2008). When administered to mice during gestation, Cd induced malformations of the neural tube, craniofacial region, limbs, trunk, viscera, and axial skeleton in fetuses (Hovland et al., 1999; Paniagua-Castro et al., 2007; Robinson et al., 2009; Scott et al., 2005). In addition, maternal Cd exposure during pregnancy induced fetal growth restriction (Ahokas et al., 1980; Ji et al., 2011a). Nevertheless, the molecular mechanism for Cd-induced development toxicity remains obscure.

Increasing evidence demonstrated that placenta could deter most of Cd from passing from dams to fetuses. According to several earlier reports, only less than 0.1% of Cd was passed from dams to fetuses when pregnant mice were chronically exposed to tracer levels of ^{109}Cd in drinking water (Brako et al., 2003; Whelton et al., 1993). Our recent report showed that only traces of blood Cd were measured in fetuses whose mothers were exposed to Cd during the late pregnant period. Importantly, no significant elevation of blood Cd concentration was observed in fetuses whose mothers were exposed to Cd during the late pregnant period (Ji et al., 2011a). Thus, Cd-induced development toxicity cannot be completely attributed to its direct toxic effect on fetuses.

The placenta is essential for sustaining the growth of the fetus during gestation, and defects in its function result in fetal growth restriction or, if more severe, fetal death and birth defects (Watson and Cross, 2005). The endoplasmic reticulum (ER) is an important organelle required for cell survival and normal cellular function (Ferri and Kroemer, 2001; Rao

Abbreviations: ATF6, activating transcription factor 6; Cd, cadmium; CHOP, CCAAT/enhancer binding protein (C/EBP) homologous protein; ER, endoplasmic reticulum; GSH, Glutathione; HO-1, heme oxygenase 1; iNOS, inducible nitric oxide synthase; NT, nitrotyrosine; PBN, alpha-phenyl-N-t-butylnitrone; peIF2α, phosphorylates eukaryotic translation initiation factor 2α; pIRE 1α, phosphor-inositol requiring ER-to-nucleus signal kinase 1α; ROS, reactive oxygen species; TUNEL, terminal dUTP nick-end labeling; UPR, unfolded protein response.

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et al., 2004). In the ER, nascent proteins are folded with the assistance of ER chaperones. The ER is sensitive to alterations in cellular homeostasis. If the client protein load is excessive compared with the reserve of ER chaperones, ER stress occurs. Several recent studies demonstrated that an increased ER stress was associated with the impaired placental development and fetal growth restriction (Iwawaki et al., 2009; Lian et al., 2011; Yung et al., 2008). Thus, we hypothesize that ER stress-mediated placental damage is involved in Cd-induced developmental toxicity. Indeed, Cd could accumulate in placenta (Boadi et al., 1991; Ji et al., 2011a). A recent report showed that an increased concentration of Cd was observed in placentas of mothers delivering low birth weight neonates (Llanos and Ronco, 2009).

In the present study, we investigated the effects of maternal Cd exposure during pregnancy on placental and fetal development. We found that maternal Cd exposure during pregnancy significantly increased the incidence of external malformations in fetuses. Moreover, maternal Cd exposure during pregnancy induces ER stress in placenta. Alpha-phenyl-N-t-butylnitrone (PBN), a free radical spin-trapping agent and antioxidant, significantly alleviated Cd-induced placental ER stress.

Materials and methods

Chemicals and reagents

$CdCl_2$ and alpha-phenyl-N-t-butylnitrone (PBN) were from Sigma Chemical Co. (St. Louis, MO). GRP78, CCAAT/enhancer binding protein (C/EBP) homologous protein (CHOP) and phosphor-eukaryotic translation initiation factor (eIF)2α antibodies were from Cell Signaling Technology (Beverley, MA). PCNA, heme oxygenase (HO)-1, inducible nitric oxide synthase (iNOS), 3-nitrotyrosine (3-NT) and phosphor-JNK antibodies were from Santa Cruz Biotechnologies (Santa Cruz, CA). β-Actin antibody was from Boster Bio-Technology Co. LTD (Wuhan, China). Phosphor-inositol requiring ER-to-nucleus signal kinase (IRE) 1α and chemiluminescence (ECL) detection kit was from Pierce Biotechnology (Rockford, IL). TRI reagent was from Molecular Research Center, Inc (Cincinnati, Ohio). RNase-free DNase was from Promega Corporation (Madison, WI). All other reagents were purchased from Sigma Chemical Co. (St. Louis, MO) if not otherwise stated.

Animals and treatments

The ICR mice (8–10 week-old; male mice: 28–30 g; female mice: 24–26 g) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. The animals were allowed free access to food and water at all times and were maintained on a 12-h light/dark cycle in a controlled temperature (20–25 °C) and humidity (50 ± 5%) environment for a period of 1 week before use. For mating purposes, four females were housed overnight with two males starting at 21:00 h. Females were checked by 7:00 h the next morning, and the presence of a vaginal plug was designated as gestational day (gd) 0. The present study consisted of five independent experiments.

Experiment 1. To investigate Cd-induced teratogenesis in mice, twenty pregnant mice were divided randomly into two groups. In Cd group, the pregnant mice received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) between 08:00 and 09:00 h on gd 9. The saline-treated pregnant mice served as controls. The doses of $CdCl_2$ used in the present study referred to others (Chen et al., 2008; Liao et al., 2007; Lutz and Beck, 2000; Paniagua-Castro et al., 2007; Robinson et al., 2009, 2011; Zhao et al., 2008). All animals were inspected daily for clinical signs and determined whether a pregnancy loss had occurred according to clinical signs and maternal weight. The dams were sacrificed on gd 18. The uterine horns were exposed and weighed. Live, dead and resorbed fetuses were counted. Live fetuses were sexed, weighed, and examined for external morphological malformations.

Experiment 2. To investigate the effects of maternal Cd exposure during pregnancy on cell growth in placenta, twenty-four pregnant mice were divided randomly into two groups. In Cd group, the pregnant mice received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) between 08:00 and 09:00 h on gd 9. The saline-treated pregnant mice served as controls. The pregnant mice were sacrificed 24 h after Cd injection. Placentas were collected for PCNA staining using immunohistochemistry.

Experiment 3. To investigate the effects of maternal Cd exposure during pregnancy on placental histopathology and apoptosis, twelve pregnant mice were divided randomly into two groups. In Cd group, the pregnant mice received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) between 08:00 and 09:00 h on gd 9. The saline-treated pregnant mice served as controls. All pregnant mice were sacrificed on gd 18. Placentas were weighed and then collected for measurement of placental histopathology and apoptosis.

Experiment 4. To investigate Cd-induced placental oxidative stress and ER stress, thirty-six pregnant mice were divided randomly into two groups. In Cd group, the pregnant mice received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) between 08:00 and 09:00 h on gd 9. The saline-treated pregnant mice served as controls. The pregnant mice were sacrificed at different times (2, 8, and 24 h) after $CdCl_2$ injection. Placentas were collected for measurement of oxidative stress and ER stress.

Experiment 5. To investigate the effects of PBN on Cd-induced placental ER stress, forty-eight pregnant mice were divided randomly into four groups. In Cd + NS group, the pregnant mice received two doses of NS, one injected 30 min before $CdCl_2$ (4.5 mg/kg) injection, and the second injected 4 h after $CdCl_2$ injection. In PBN + Cd group, the pregnant mice received two doses of PBN, one (100 mg/kg) injected 30 min before $CdCl_2$ (4.5 mg/kg) injection, and the second (100 mg/kg) injected 4 h after $CdCl_2$ injection. In PBN + NS group, the pregnant mice received two doses of PBN, one (100 mg/kg) injected 30 min before NS injection, and the second (100 mg/kg) injected 4 h after NS injection. In NS + NS group (Control), the pregnant mice received three doses of NS as above schedule. The doses of PBN used in the present study referred to others (Liao et al., 2007; Zhao et al., 2008). All pregnant mice were sacrificed at 8 h after $CdCl_2$ injection. Placentas were collected for measurement of oxidative stress and ER stress.

All procedures on animals followed the guidelines for humane treatment set by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University.

Histology in labyrinth

Freshly collected placentas were fixed in 4% paraformaldehyde and embedded in paraffin. Paraffin-embedded placentas were serially sectioned. Hematoxylin and eosin (H&E) stained placental sections were analyzed for vascular space quantification according to the previous study (Neres et al., 2008). In each section, 5 fields were randomly selected in the labyrinthine region at magnification ×400. We performed an image analysis using the public domain NIH Image J Program. Briefly, the images were given a color threshold to cover the internal space of maternal and fetal blood vessels in the labyrinth layer after noise removal. The blood sinusoids area in the labyrinthine region was estimated from the analysis of two nonconsecutive sections in each placenta. The coverage percentage was calculated as the ratio between the number of pixels covered by the area defined by the threshold and the overall number of pixels in the image. The reported

results in the present study represent the average results for six placentas in each group.

Terminal dUTP nick-end labeling (TUNEL) staining

For the detection of apoptosis, paraffin-embedded sections were stained with the TUNEL technique using an in situ apoptosis detection kit (Promega) according to the manufacturer's protocols. Sections were counterstained with hematoxylin. TUNEL-positive cells were counted in twelve randomly selected fields from each slide at a magnification of ×400. Three cross sections were chosen from each placenta. The litter was considered the unit for statistical comparison among different groups. The percentage of TUNEL-positive cells was analyzed in six placentas from six pregnant mice.

Isolation of total RNA and real-time RT-PCR

Total RNA was extracted using TRI reagent. RNase-free DNase-treated total RNA (1.0 μg) was reverse-transcribed with AMV (Pregmega). Real-time RT-PCR was performed with a LightCycler® 480 SYBR Green I kit (Roche Diagnostics GmbH) using gene-specific primers as listed in Table 1. The amplification reactions were carried out on a LightCycler® 480 Instrument (Roche Diagnostics GmbH) with an initial hold step (95 °C for 5 min) and 50 cycles of a three-step PCR (95 °C for 15 s, 60 °C for 15 s, 72 °C for 30 s). For analysis of *xbp-1* mRNA splicing, 2.5 μl of cDNA was amplified with specific primers described by others (Iwakoshi et al., 2003): forward, 5′-CCA TGG GAA GAT GTT CTG GG-3′; reverse, 5′-ACA AGC TTG GGA ATG GAC AC-3′. PCR products were separated by electrophoresis on 2.5% agarose gels and visualized by ethidium bromide staining. The level of *sXBP-1* mRNA was normalized to *uXBP-1* mRNA level in the same samples. *sXBP-1* mRNA level of the control was assigned as 1.

Immunoblotting

Total lysate from placenta was prepared by homogenizing 50 mg placenta tissue in 300 μl lysis buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% sodium deoxycholate, 0.1% sodium dodecylsylphate, 1 mM phenylmethylsulfonyl fluoride) supplemented with a cocktail of protease inhibitors (Roche). Total lysate was separated electrophoretically by SDS-PAGE and transferred to a polyvinylidene fluoride membrane. The membranes were incubated for 2 h with the following antibodies: GRP78, phosphor-eIF2α, phosphor-IRE1α, CHOP, iNOS, HO-1, 3-NT and phosphor-JNK. β-Actin was used as a loading control for total proteins. After washes in DPBS containing 0.05% Tween-20 four times for 10 min each, the membranes were incubated with goat anti-rabbit IgG or goat anti-mouse antibody for 2 h. The membranes were then washed for four times in DPBS containing 0.05% Tween-20 for 10 min each, followed by signal development using an ECL detection kit.

Table 1
Primers for real-time RT-PCR.

Name	Sequence	Size (bp)
GAPDH	Forward: 5′-ACCCCAGCAAGGACACTGAGCAAG-3′ Reverse: 5′-GGCCCCTCCTGTTATTATGGGGGT-3′	109
GRP78	Forward: 5′-CTGGCCGAGACAACACTGACCT-3′ Reverse: 5′-GCGACGACGGTTCTGGTCTCAC-3′	68
GRP94	Forward: 5′-GGCACAAGCATACCAGACGGGC-3′ Reverse: 5′-TCCTGACCGAAGTGTTGCCGT-3′	190
ATF4	Forward: 5′-GACCGCGGCTGGTCGTCAAC-3′ Reverse: 5′-TACCGCAGCGCCACCAAACC-3′	155
EDEM	Forward: 5′-CCCACCGCTCTACGTCAACGT-3′ Reverse: 5′-GCTCAGGGAGGGCCCCGTAC-3′	176
iNOS	Forward: 5′-GCTCGCTTTGCCACGGACGA-3′ Reverse: 5′-AAGGCAGCGGGCACATGCAA-3′	146
HO-1	Forward: 5′-CGTCACTTCGTCAGAGGCCTGC-3′ Reverse: 5′-TCTGGGGTTTCCCTCGGGGTG-3′	75
CHOP	Forward: 5′-GTTGAAGATGAGCGGGTGGCAGC-3′ Reverse: 5′-GCACGTGGACCAGGTTCTGCTT-3′	125

Glutathione measurement

Glutathione (GSH) was determined according to the previous method (Griffith, 1980). Proteins of 0.4 ml placenta homogenates were precipitated by the addition of 0.4 ml of a metaphosphoric acid solution. After 40 min, the protein precipitate was separated from the remaining solution by centrifugation at 5000 rpm at 4 °C for 5 min. 400 μl of the supernatant was combined with 0.4 ml of 300 mM Na_2HPO4, and the absorbance at 412 nm was read against a blank consisting of 0.4 ml supernatant plus 0.4 ml H2O. Then, 100 μl DTNB (0.02%, w/v; 20 mg DTNB in 100 ml of 1% sodium citrate) was added to the blank and sample, and absorbance of the sample was read against the blank at 412 nm. The GSH content was determined using a calibration curve prepared with an authentic sample. GSH values were expressed as nmol mg^{-1} protein.

Immunohistochemistry

Two cross sections from each placenta were embedded in paraffin. Paraffin-embedded placental tissues were serially sectioned. 5 μm of sections was mounted onto coated slides, dewaxed, and rehydrated. Antigen retrieval was performed by pressure cooking slides for 5 min in 0.01 M citrate buffer (pH 6.0). Slides were incubated for 30 min in 3% (vol/vol) hydrogen peroxide in methanol to block endogenous peroxidase activity and then washed in Tris–buffered saline [TBS; 0.05 M Tris, 0.85% (wt/vol) NaCl (pH 7.4)]. Nonspecific binding sites were blocked with an appropriate normal serum diluted 1:5 in TBS containing 5% (wt/vol) BSA before the addition of polyclonal antibody PCNA, 3-NT or HO-1 and overnight incubation at 4 °C. After washing in TBS, slides were incubated for 30 min with the appropriate secondary antibody conjugated to biotin goat anti-rabbit or goat anti-mouse (Santa Cruz, USA), diluted 1:500 in the blocking mixture. This was followed by 30 min incubation with horseradish peroxidase-labeled avidin–biotin complex (Dako). Immunostaining was developed by application of diaminobenzidine (liquid DAB^+; Dako), and slides were counterstained with hematoxylin, dehydrated, and mounted using Pertex mounting medium (Cell Path, Hemel Hempstead, UK). PCNA-positive cells were counted in twelve randomly selected fields from each slide at a magnification of ×400. Three cross sections were chosen from each placenta. The litter was considered the unit for statistical comparison among different groups. The percentage of PCNA-positive cells was analyzed in six placentas from six pregnant mice.

Statistical analysis

The litter was considered the unit for statistical comparison among different groups. Fetal malformation was calculated per litter and then averaged per group. For fetal weight and crown–rump length, the means were calculated per litter and then averaged per group. Each band of immunoblotting was scanned and its intensity analyzed by Image J software (http://rsb.info.nih.gov/ij/). GRP78, phosphor-eIF2α, phosphor-IRE1α, CHOP, HO-1, iNOS, 3-NT and phosphor-JNK were normalized to β-actin level in the same samples. The level of the control was assigned as 1. All quantified data were expressed as means ± SEM at each point. ANOVA and the Student–Newmann–Keuls post hoc test were used to determine differences among different groups. Differences were considered to be significant only for $P<0.05$.

Table 2
The effects of maternal Cd exposure on pregnancy and fetal outcomes.

	Control	Cd
Number of litters (n)	10	10
Litters of pregnancy loss (n)	0	0
Litters of successfully pregnancy (n)	10	10
Body weight gain of pregnant mice (g)[a]	21.9 ± 1.0	19.7 ± 1.7
Average placental weight (g)	0.107 ± 0.005	0.084 ± 0.003**
Resorptions per litter (n)	0.4 ± 0.2	0.9 ± 0.3
Dead fetuses per litter (n)	0.3 ± 0.2	0.7 ± 0.3
Live fetuses per litter (n)	11.4 ± 1.1	10.7 ± 0.7
Incidence of external malformations (%)	0.0 ± 0.0	22.8 ± 5.7
Fetal weight (g)	1.32 ± 0.04	1.16 ± 0.03**
Crown–rump length (cm)	2.44 ± 0.04	2.25 ± 0.04**

[a] Indicates the body weight gain of pregnant mice from gestational day (gd) 9 to gd18. All data were expressed as means ± SEM.
** $P<0.01$ as compared with the controls.

Results

Cd-induced teratogenicity

No pregnant mice were dead after the pregnant mice were injected with $CdCl_2$. In addition, a single dose of Cd had no effect on weight gain of the pregnant mice (Table 2). No abortion and preterm delivery were observed in mice injected with 4.5 mg/kg $CdCl_2$. All pregnant mice in Cd-treated group completed the pregnancy (Table 2). The effects of maternal Cd exposure on fetal outcomes were analyzed. As shown in Table 2, no significant difference on the number of implantation sites, resorptions per litter, live fetuses per litter and dead fetuses per litter was observed between Cd-treated mice and controls. As expected, maternal Cd exposure during pregnancy significantly increased the incidence of external malformations in fetuses. Among mice treated with $CdCl_2$, 80% (8/10) of litters were affected. Forelimb ectrodactyly and tail deformity were two of the most common malformations (Figs. 1A–D). Among dams exposed to Cd during pregnancy, 22.8% of fetuses were either forelimb ectrodactyly or tail deformity (Table 2). The effects of maternal Cd exposure on fetal weight and crown-rump length were evaluated. As shown in Table 2, average fetal weight in Cd-treated group was significantly decreased as compared with the control (1.16 ± 0.03 *versus* 1.32 ± 0.04, $P<0.01$). In addition, maternal Cd exposure significantly reduced crown-rump length (Table 2, 2.25 ± 0.04 *versus* 2.44 ± 0.04, $P<0.01$).

Effects of maternal Cd exposure on placental development

The effects of maternal Cd exposure during pregnancy on placental weight were analyzed. As expected, placental weight was significantly decreased in Cd-exposed mice as compared with controls (Table 2, 0.084 ± 0.003 *versus* 0.107 ± 0.005, $P<0.01$). To investigate whether maternal Cd exposure during pregnancy induces placental vascular space impairment, we used a computerized morphometry method to analyze cross-sectional areas of blood sinusoids in placental labyrinthine region. The results showed that the average blood

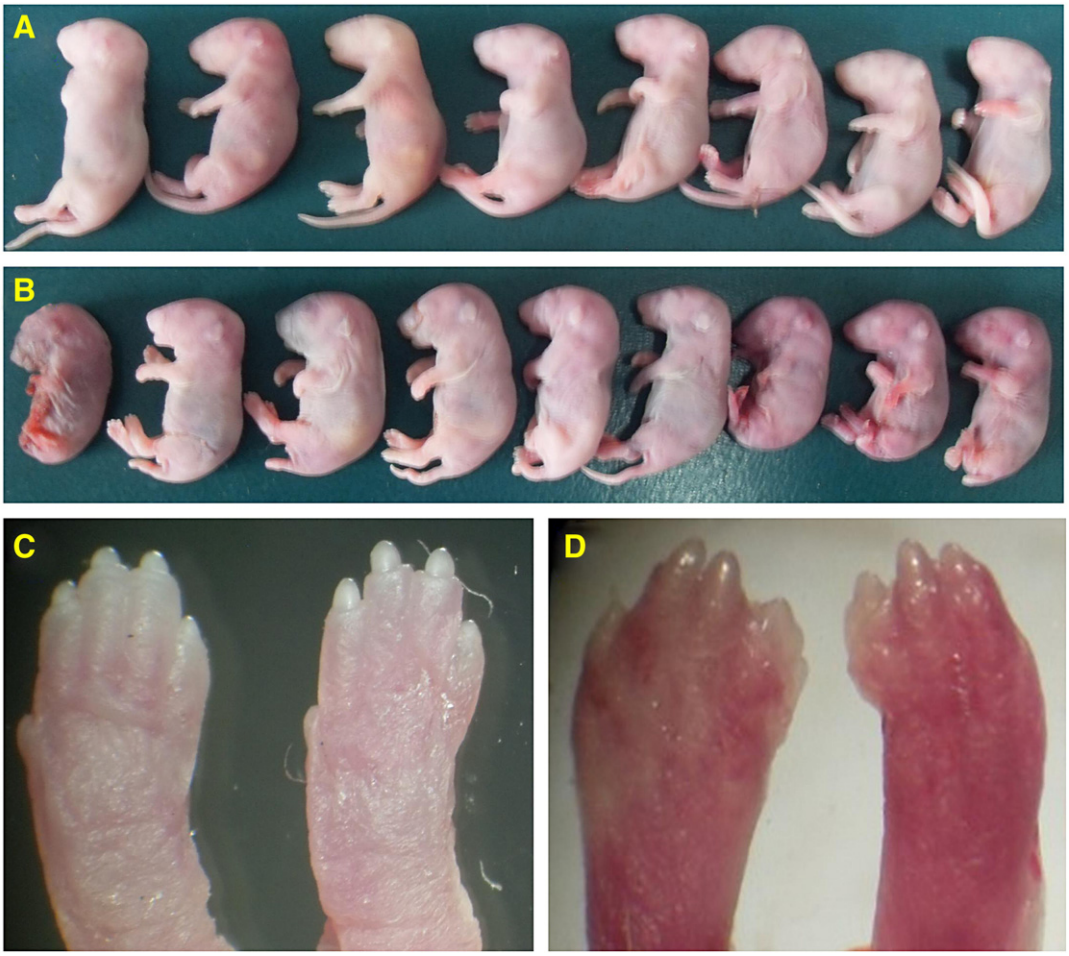

Fig. 1. Cd-induced external malformations. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. Fetal external malformations were examined on gestational day (gd) 18. (A) Fetuses from the control group. (B) Cd-induced tail deformation. From left to right, (1, 7 and 8) no tail; (2, 3, 4, 5 and 9) short tail; (4, 5 and 6) kink tail. (C) Normal forepaws. (D) Cd induced syndactyly (left) and ectrodactyly (right).

sinusoid area in the labyrinth layer was markedly decreased in placenta of mice treated with $CdCl_2$ (Fig. 2, 28.3 ± 3.3 *versus* 39.2 ± 2.4, $P<0.05$). Cell proliferation and apoptosis were then analyzed in placental labyrinthine region. Immunohistochemistry showed that the percentage of PCNA-positive cells in labyrinthine region was significantly decreased in placenta of mice treated with $CdCl_2$ (Figs. 3A, B and E). By contrast, the percentage of TUNEL-positive cells in labyrinthine region was significantly increased in placenta of mice treated with $CdCl_2$ (Figs. 3C, D and F).

Maternal Cd exposure induces placental ER stress

To examine whether maternal Cd exposure during pregnancy induces placental ER stress, the expression of placental ER chaperones was analyzed. As expected, mRNA level of GRP78, an ER chaperone and ATF6 target, was slightly upregulated in placenta of mice treated with a single dose of $CdCl_2$ (Fig. 4A). Correspondingly, placental GRP78 protein wan significantly increased in Cd-treated mice (Fig. 4B). However, a single dose of had no effect on the expression of GRP94, another ER chaperone in placenta (Fig. 4C). Next, the effects of maternal Cd exposure during pregnancy on PERK pathway were analyzed. As shown in Fig. 5A, the level of phosphorylated eIF2α, a downstream target of the PERK pathway, was significantly increased in placenta of mice treated with a single dose of $CdCl_2$. CHOP is another downstream target of the PERK pathway. As shown in Fig. 5B, the level of CHOP protein was significantly increased in placenta of mice treated with $CdCl_2$. In addition, ATF4, another downstream target of the PERK pathway, was upregulated in placenta of Cd-treated mice (Fig. 5C). To investigate the effects of maternal Cd exposure on the IRE1 branch of the UPR pathway, the level of phosphorylated IRE1α was measured in placenta. As shown in Fig. 6A, no significant difference on the level of phosphorylated IRE1α in placenta was observed between Cd-treated mice and controls. The effects of maternal Cd exposure during pregnancy on mRNA level of sliced X box-binding protein (sXBP)-1 in placenta were analyzed. As shown in Fig. 6B, there was no significant difference on mRNA level of placental sXBP-1 between Cd-treated mice and controls. JNK, a downstream target of the IRE1 pathway, was then analyzed. As shown in Fig. 6C, maternal Cd exposure had no effect on the level of phosphorylated JNK in placenta. In addition, no significant difference on the expression of placental EDEM, a downstream target of XBP-1, was observed between Cd-treated mice and controls (Fig. 6D).

Maternal Cd exposure induces oxidative stress and upregulates HO-1 in placenta

The expression of HO-1, a marker of oxidative stress, was analyzed in placenta of mice treated with $CdCl_2$. As shown in Fig. 7A, the level of HO-1 protein was significantly increased in placenta of mice administered with a single dose of $CdCl_2$. 3-NT is a specific marker for protein nitration. To investigate whether maternal Cd exposure during pregnancy induces protein nitration in placenta, 3-NT residue was measured. As shown in Fig. 7B, 3-NT intensity was significantly enhanced in placenta of mice treated with a single dose of $CdCl_2$. The effects of maternal Cd exposure during pregnancy on the expression of placental iNOS were analyzed. As shown in Fig. 8A, maternal Cd exposure during pregnancy significantly downregulated the expression of iNOS mRNA in placenta. Correspondingly, maternal Cd

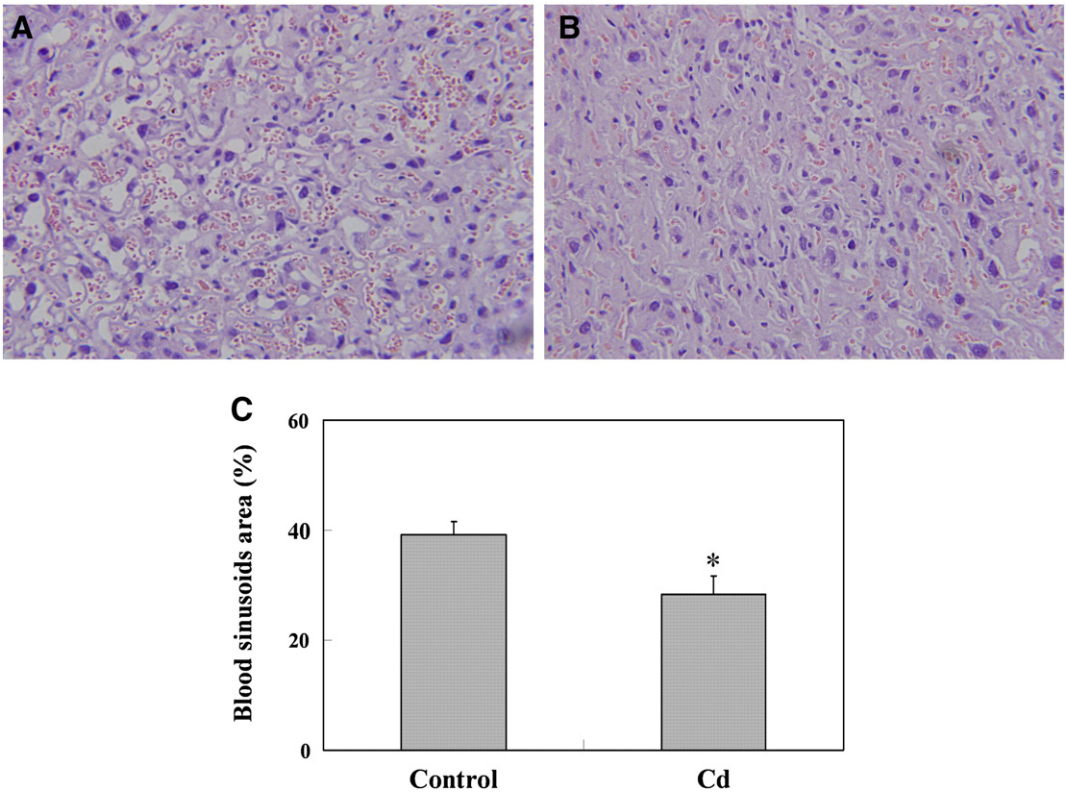

Fig. 2. Cd-induced placental vascular space impairment. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. Placentas were collected on gestational day 18. Placental cross sections were stained with H&E. Original magnification: 400×. (A) Placenta from the control group. (B) Placenta from Cd-treated group. (C) Vascular area in the labyrinthine region was estimated from two nonconsecutive sections in each placenta using the public domain NIH Image J Program. The blood sinusoid area (%) was calculated as the ratio between the number of pixels covered by the area defined by the threshold and the overall number of pixels in the image. All data were expressed as means ± SEM of six placentas from six pregnant mice. *$P<0.05$ *vs* the control.

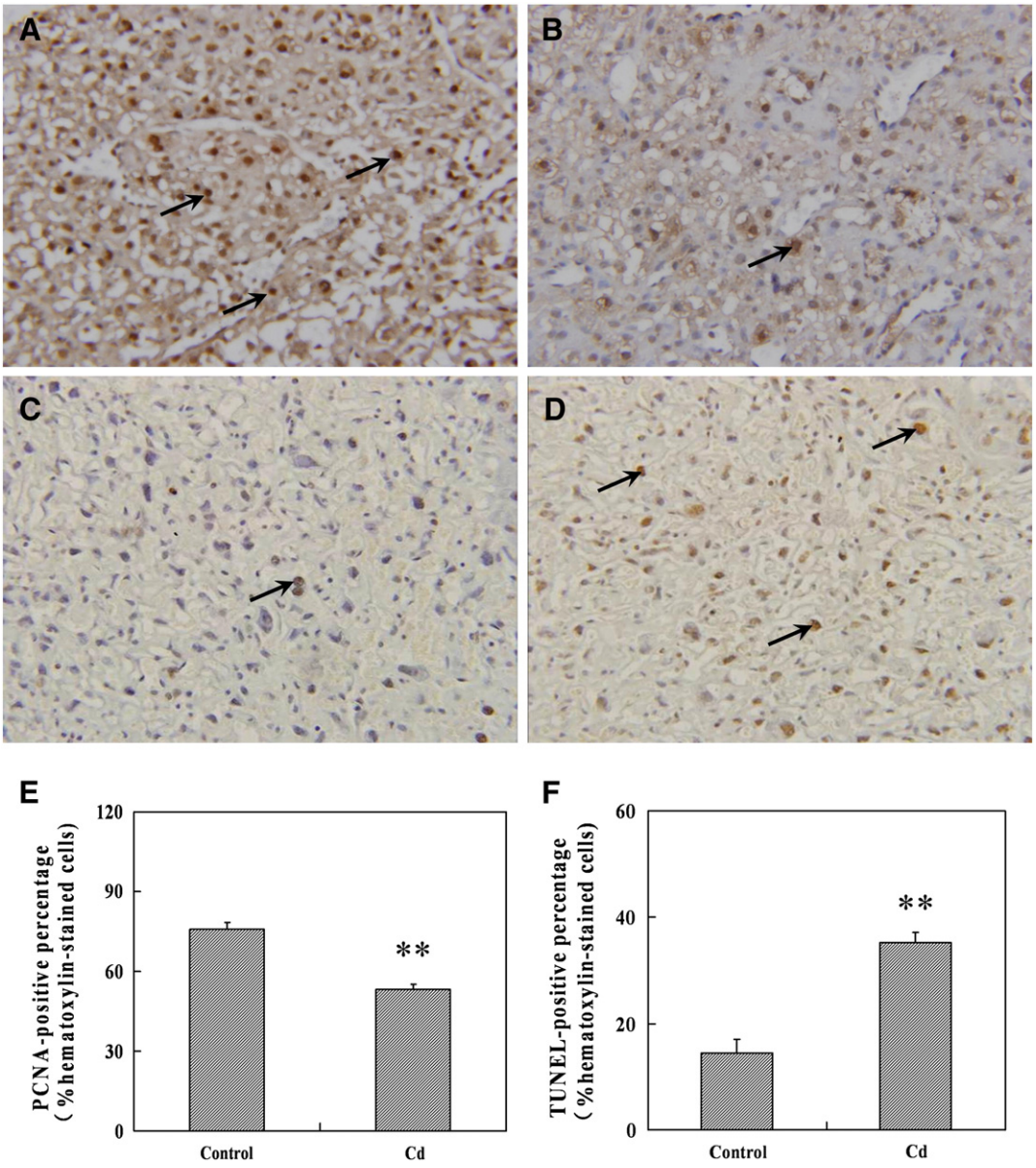

Fig. 3. The effects of maternal Cd exposure during pregnancy on placental cellular proliferation and apoptosis. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. (A and B) Placentas were collected at 24 h after Cd injection. PCNA immunohistochemically stained placental sections. Sections were counterstained with hematoxylin. Arrows indicate PCNA-positive cells. Original magnification: 400×. (C and D) Placentas were collected on gestational day 18. TUNEL assays placental sections. Sections were counterstained with hematoxylin. Arrows indicate TUNEL-positive cells. Original magnification: 400×. (A and C) Placental cross sections were from control mice. (B and D) Placental cross sections were from Cd-treated pregnant mice. (E) The percentage of PCNA-positive cells. (F) The percentage of TUNEL-positive cells. All data were expressed as means ± SEM of six placentas from six pregnant mice. $^{**}P<0.01$ *vs* the control.

exposure during pregnancy significantly reduced the level of iNOS protein in placenta (Fig. 8B).

PBN attenuates Cd-induced placental oxidative stress

The effects of maternal Cd exposure during pregnancy on placental GSH were analyzed. As shown in Fig. 9A, the level of placental GSH was significantly decreased 24 h after the pregnant mice were injected with a single dose of $CdCl_2$. PBN, a free radical spin-trapping agent, significantly alleviated Cd-induced GSH depletion in placenta. To investigate the role of ROS in Cd-evoked placental protein nitration, the effects of PBN on Cd-evoked placental 3-NT residue were analyzed. As shown in Fig. 9B, PBN pretreatment almost completely inhibited Cd-evoked 3-NT residue in placenta. Further analysis showed that intense 3-NT staining was mainly observed in trophoblast giant cells of the junctional region. Interestingly, pretreatment with PBN almost completely counteracted Cd-evoked 3-NT residue in mouse placenta trophoblast giant cells of the junctional region (Fig. 9C).

PBN attenuates Cd-induced placental HO-1 upregulation

To investigate the role of ROS in Cd-induced placental HO-1 upregulation, the effects of PBN on the expression of placental HO-1 were analyzed. As expected, the level of placental HO-1 mRNA was significantly increased 8 h after Cd injection. Interestingly, PBN pretreatment significantly attenuated Cd-induced upregulation of HO-1 mRNA in placenta (Fig. 10A). In addition, pretreatment with PBN significantly alleviated Cd-induced elevation of placental HO-1 protein in placenta (Fig. 10B). Immunohistochemistry revealed that an increase in HO-1 staining was mainly observed in trophoblast giant cells of the junctional region. PBN pretreatment significantly attenuated Cd-induced elevation of

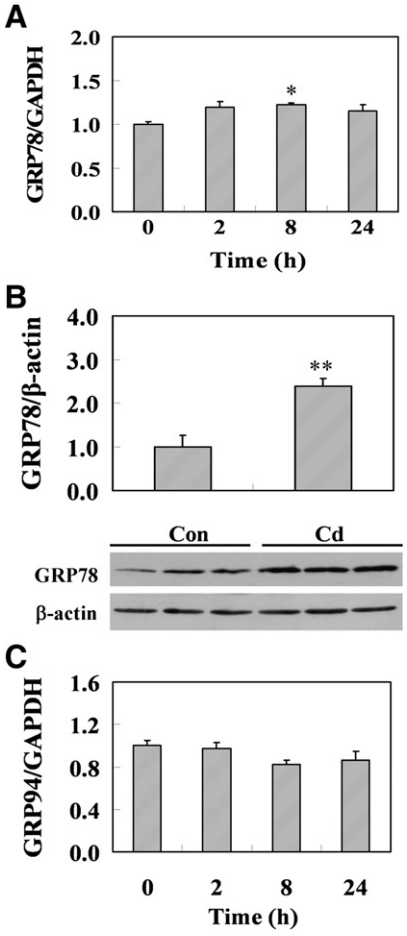

Fig. 4. The effects of maternal Cd exposure during pregnancy on the expression of placental ER chaperones. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. Placentas were collected at different times (2, 8 or 24 h) after Cd injection. (A) Placental GRP78 mRNA was determined using real-time RT-PCR. (B) Placentas were collected at 24 h after Cd injection. Placental GRP78 protein was measured using immunoblotting. A representative gel for GRP78 (upper panel) and β-actin (lower panel) was shown. (C) Placental GRP94 mRNA was determined using real-time RT-PCR. All data were expressed as means ± SEM of six samples from six pregnant mice. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control.

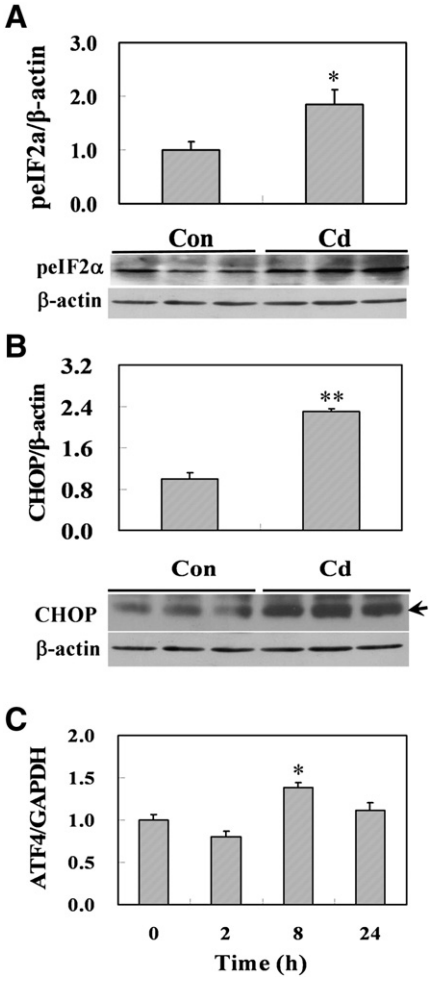

Fig. 5. The effects of maternal Cd exposure during pregnancy on placental PERK pathway. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. Placentas were collected at 24 h after Cd injection. (A) Placental peIF2α was measured using immunoblotting. A representative gel for peIF2α (upper panel) and β-actin (lower panel) was shown. (B) Placental CHOP protein was measured using immunoblotting. A representative gel for CHOP (upper panel) and β-actin (lower panel) was shown. (C) Placentas were collected at different times (2, 8 or 24 h) after Cd injection. Placental ATF4 mRNA was determined using real-time RT-PCR. All data were expressed as means ± SEM of six samples from six pregnant mice. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control.

HO-1 staining in trophoblast giant cells of the junctional region (Fig. 10C).

PBN attenuates Cd-induced placental ER stress

The effects of PBN on Cd-induced placental ER stress were analyzed. As shown in Fig. 11A, PBN pretreatment significantly alleviated Cd-induced elevation of GRP78 in placenta. The effects of PBN on Cd-induced eIF2α phosphorylation were then analyzed. As shown in Fig. 11B, PBN pretreatment significantly attenuated Cd-induced placental eIF2α phosphorylation. The effects of PBN on Cd-induced placental CHOP expression are presented in Figs. 11C and D. As expected, PBN pretreatment significantly attenuated Cd-induced upregulation of CHOP mRNA in placenta (Fig. 11C). Correspondingly, pretreatment with PBN significantly attenuated Cd-induced elevation of CHOP protein in placenta (Fig. 11D).

Discussion

Increasing evidence demonstrates that maternal Cd exposure causes a relatively specific forelimb ectrodactyly in the C57BL/6J mouse strain when administered during early limb development (Lutz and Beck, 2000; Robinson et al., 2009; Robinson et al., 2011). In the present study, we showed that the incidences of external malformations were significantly increased in fetuses when CD-1 pregnant mice were exposed to Cd during early limb development. Among mice treated with $CdCl_2$ on gd 9, 80% (8/10) of litters were affected. Forelimb ectrodactyly and tail deformity were two of the most common malformations. Among dams exposed to Cd on gd 9, 22.8% of fetuses were with either forelimb ectrodactyly or tail deformity. Interestingly, crown-rump length was significantly decreased in fetuses whose mothers were injected with $CdCl_2$ on gd9. In addition, administration of a single dose Cd on gd 9 significantly reduced fetal weight. These results suggest that maternal Cd exposure induces forelimb ectrodactyly and fetal growth restriction in CD-1 mouse strain when administered during early limb development.

Several reports showed that placenta could deter most of Cd from passing from dams to fetuses (Ji et al., 2011a). Thus, Cd-induced forelimb ectrodactyly and fetal growth restriction cannot be completely attributed to its direct toxic effect on fetuses. Increasing evidence demonstrates that the placenta is essential for sustaining the growth of the fetus during gestation (Cetin and Alvino, 2009; Scifres and Nelson, 2009; Yung et al., 2008). In the present study, we investigated the effects of maternal Cd exposure on placental development. We

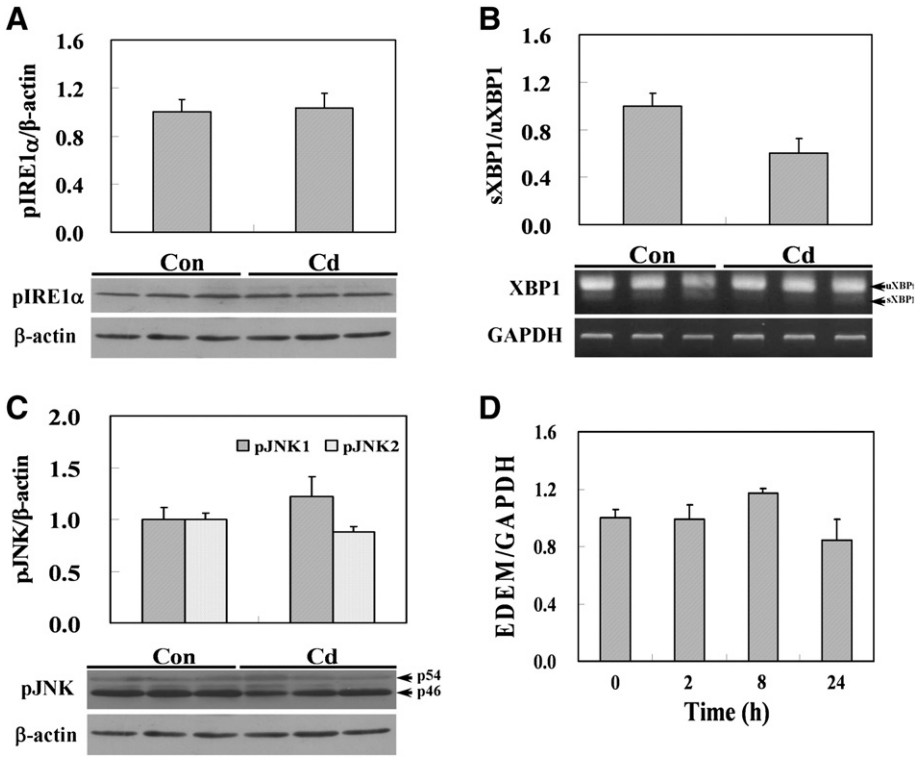

Fig. 6. The effects of maternal Cd exposure during pregnancy on placental IRE1 pathway. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. (A) Placentas were collected at 24 h after Cd injection. Placental pIRE1α was measured using immunoblotting. A representative gel for pIRE1α (upper panel) and β-actin (lower panel) was shown. (B) Placentas were collected at 24 h after Cd injection. Placental uXBP-1 and sXBP-1 mRNA were determined using RT-PCR. A representative gel for XBP-1 (upper panel) and GAPDH (lower panel) was shown. (C) Placentas were collected at 24 h after Cd injection. Placental pJNK was measured using immunoblotting. A representative gel for pJNK (upper panel) and β-actin (lower panel) was shown. (D) Placentas were collected at different times (2, 8 or 24 h) after Cd injection. Placental EDEM mRNA was determined using real-time RT-PCR. All data were expressed as means ± SEM of six samples from six pregnant mice.

showed that placental weight was significantly decreased in Cd-treated mice. Moreover, the percentage of PCNA-positive cells in labyrinthine region was significantly decreased in placenta of mice treated with $CdCl_2$. By contrast, the percentage of TUNEL-positive cells in labyrinthine region was significantly increased in placenta of mice treated with $CdCl_2$. Indeed, the labyrinth is the site of oxygen and nutrient exchange between the mother and the fetus. According to a recent report, the content of essential fatty acids, an important index of placental transport capacity, was significantly reduced in fetuses whose blood sinusoid area in the labyrinth layer was markedly decreased in placenta (Iwawaki et al., 2009). The present study showed that the internal space of maternal and fetal blood vessels in the labyrinth layer was markedly decreased in placenta of mice treated with $CdCl_2$. Thus, Cd-induced fetal malformations might be, at least partially, attributed to the impairments in placental development and the reduction in placental transport capacity.

Several studies have demonstrated that ER stress is associated with the impaired placental development and fetal growth restriction (Lian et al., 2011; Yung et al., 2008). Indeed, Cd could induce ER stress in renal epithelial cells (Biagioli et al., 2008; Komoike et al., 2011; Liu et al., 2006; Yokouchi et al., 2007). Recently, we showed that a single dose of Cd evoked ER stress and UPR in testes (Ji et al., 2011b, 2011c). To investigate whether maternal Cd exposure during pregnancy could induce placental ER stress, the expression of GRP78, an ER chaperone,

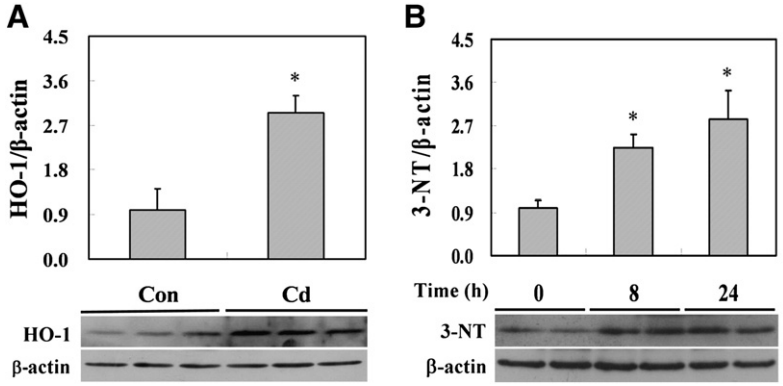

Fig. 7. Maternal Cd exposure during pregnancy induces oxidative stress and upregulates HO-1 in placenta. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. (A) Placentas were collected at 24 h after Cd injection. Placental HO-1 protein was measured using immunoblotting. A representative gel for HO-1 (upper panel) and β-actin (lower panel) was shown. (B) Placentas were collected at different times (8 h or 24 h) after Cd injection. Placental 3-NT was measured using immunoblotting. A representative gel for 3-NT (upper panel) and β-actin (lower panel) was shown. All data were expressed as means ± SEM of six samples from six pregnant mice. *$P<0.05$ *vs* the control.

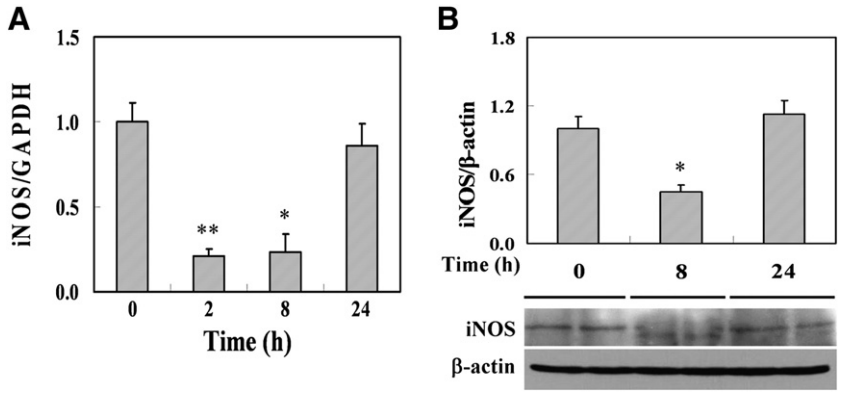

Fig. 8. The effects of maternal Cd exposure during pregnancy on placental iNOS expression. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. Placentas were collected at different times (2, 8 or 24 h) after Cd injection. (A) Placental iNOS mRNA was determined using real-time RT-PCR. (B) Placental iNOS was measured using immunoblotting. A representative gel for iNOS (upper panel) and β-actin (lower panel) was shown. All data were expressed as means ± SEM of six samples from six pregnant mice. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control.

was measured. As expected, placental grp78 mRNA was slightly upregulated in mice treated with $CdCl_2$. Importantly, a single dose of $CdCl_2$ significantly increased the level of GRP78 protein in placenta. These results suggest that maternal Cd exposure during pregnancy could induce placental ER stress.

Under ER stress, a signal response termed the unfolded protein response (UPR) is activated (Wek and Cavener, 2007; Wu and Kaufman, 2006). The UPR is mediated by three transmembrane ER proteins: inositol requiring ER-to-nucleus signal kinase (IRE)1, activating transcription factor (ATF)6 and double-stranded RNA-activated kinase (PKR)-like ER kinase (PERK) (Kohno, 2007). IRE1 is an ER-located transmembrane protein with a kinase domain and an RNase domain in the cytosolic region (Tirasophon et al., 1998). Under ER stress, IRE1 induces splicing of XBP-1 mRNA through activation of its RNase domain. The spliced XBP-1 mRNA is then translated into a functional transcription factor (Calfon et al., 2002; Yoshida et al., 2001). On the other hand, IRE1 can also activate JNK signaling through activation of its kinase domain (Urano et al., 2000). A recent report demonstrated that IRE1α was essential for placental development and embryonic viability (Iwawaki et al., 2009). In the present study, we investigated whether maternal Cd exposure could activate placental IRE1 signaling branch. Unexpectedly, a single dose of $CdCl_2$ had no effect on the level of phosphorylated IRE1α in placenta. Moreover, no significant difference on placental sXBP-1 mRNA level was observed between Cd-treated mice and controls. In addition, a single dose of $CdCl_2$ did not affect the level of phosphorylated JNK, a downstream target of IRE1 pathway. These results suggest that maternal Cd exposure during pregnancy cannot activate IRE1 signaling branch.

PERK is an ER-resident transmembrane protein ubiquitously expressed, but highly enriched in secretory cells. Under ER stress, activated PERK phosphorylates eukaryotic translation initiation factor

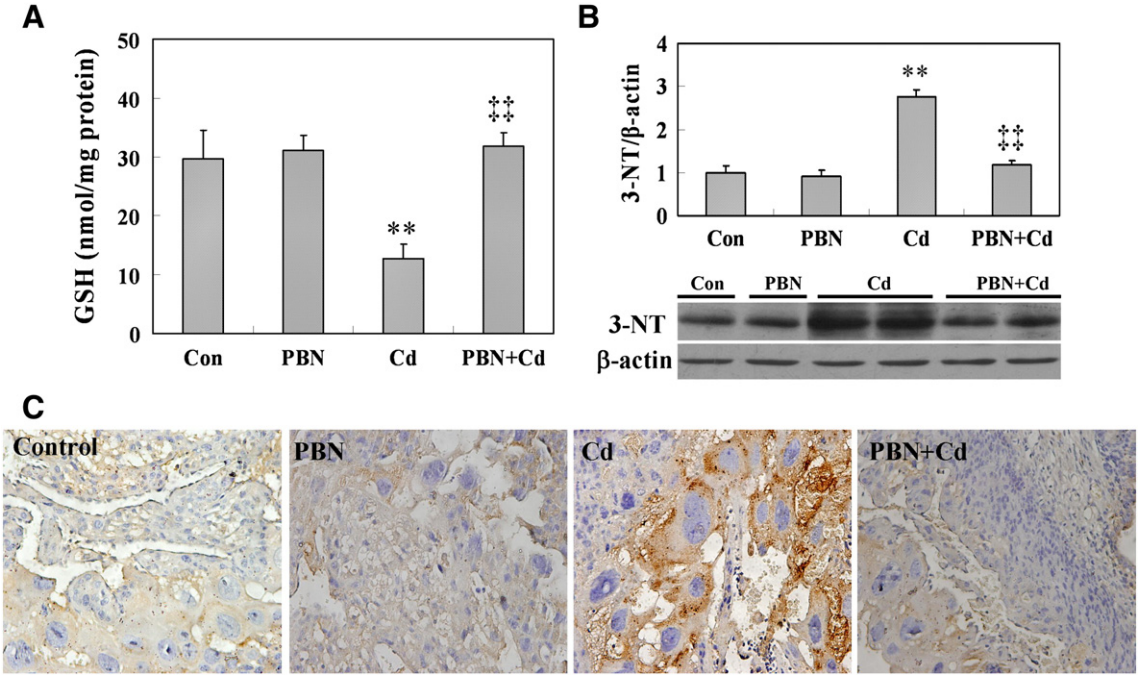

Fig. 9. The effects of PBN on Cd-induced placental oxidative stress. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. In PBN + Cd group, the pregnant mice were injected with PBN as described in Materials and methods. Placentas were collected at 24 h after Cd injection. (A) Placental GSH was measured. (B) Placental 3-NT was measured using immunoblotting. A representative gel for 3-NT (upper panel) and β-actin (lower panel) was shown. (C) Placental 3-NT was measured using immunohistochemistry. Original magnification: 200×. All data were expressed as means ± SEM of six samples from six pregnant mice. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control.

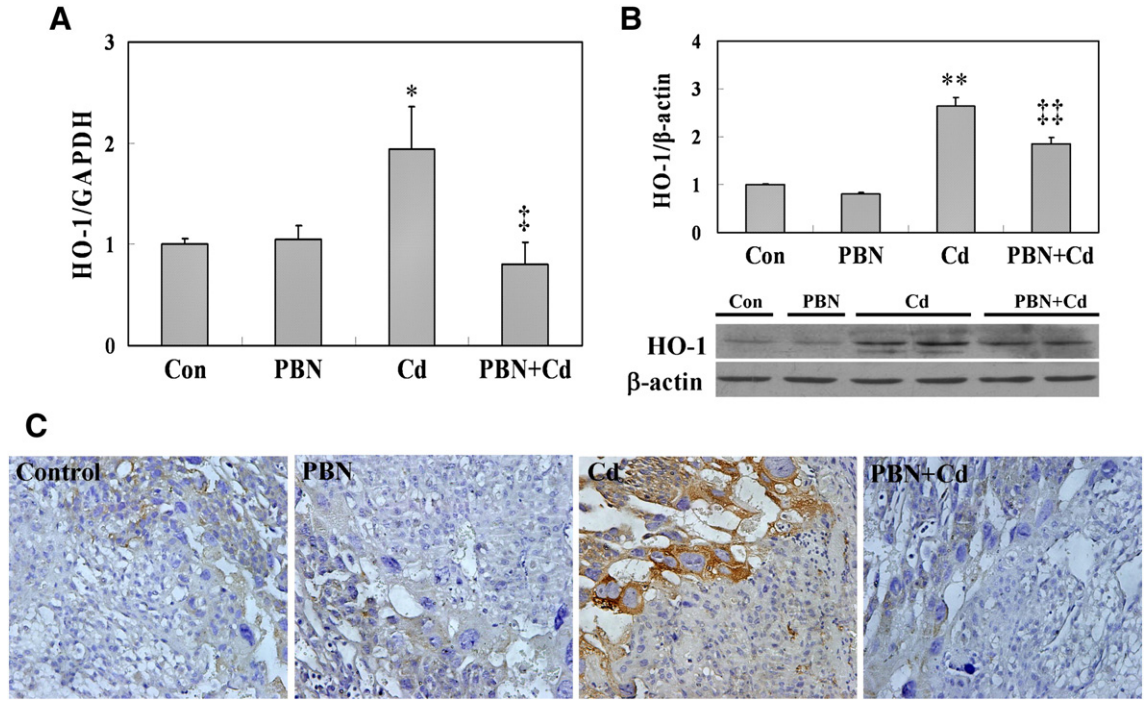

Fig. 10. The effects of PBN on Cd-induced upregulation of HO-1 in placenta. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. In PBN + Cd group, the pregnant mice were injected with PBN as described in Materials and methods. Placentas were collected at 8 h after Cd injection. (A) Placental HO-1 mRNA was determined using real-time RT-PCR. Data were expressed as means ± SEM of six samples from six pregnant mice. (B) Placental HO-1 protein was measured using immunoblotting. A representative gel for HO-1 (upper panel) and β-actin (lower panel) was shown. (C) Placental HO-1 protein was measured using immunohistochemistry. Original magnification: 200×. All experiments were repeated for three times. Data were expressed as means ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control. $^{‡}P<0.05$, $^{‡‡}P<0.01$ *vs* Cd group.

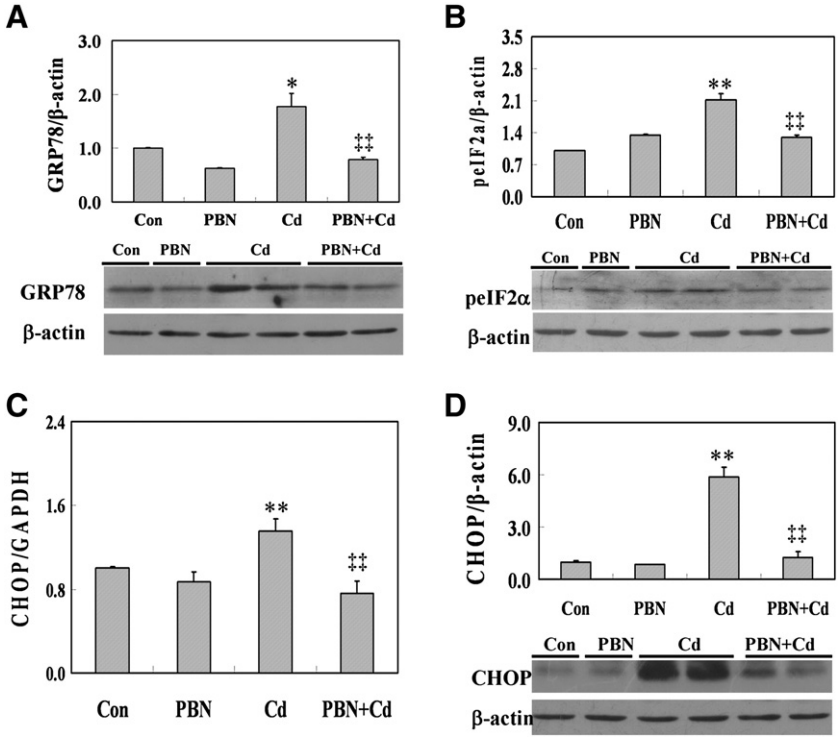

Fig. 11. The effects of PBN on Cd-induced ER stress in placenta. The pregnant mice except controls received an intraperitoneal injection of $CdCl_2$ (4.5 mg/kg) on gestational day 9. In PBN + Cd group, the pregnant mice were injected with PBN as described in Materials and methods. Placentas were collected at 8 h after Cd injection. (A) Placental GRP78 protein was measured using immunoblotting. A representative gel for GRP78 (upper panel) and β-actin (lower panel) was shown. (B) Placental peIF2α was measured using immunoblotting. A representative gel for peIF2α (upper panel) and β-actin (lower panel) was shown. All experiments were repeated for three times. Data were expressed as means ± SEM. (C) Placental CHOP mRNA was determined using real-time RT-PCR. Data were expressed as means ± SEM of six samples from six pregnant mice. (D) Placental CHOP was measured using immunoblotting. A representative gel for CHOP (upper panel) and β-actin (lower panel) was shown. All experiments were repeated for three times. Data were expressed as means ± SEM. $^{*}P<0.05$, $^{**}P<0.01$ *vs* the control. $^{‡‡}P<0.01$ *vs* Cd group.

2α (eIF2α), thereby inhibiting translation of messenger RNA into protein (Harding et al., 1999). A recent report demonstrated that an increased PERK-peIF2α signaling was associated with decreased cellular proliferation in placenta and the impaired placental development in mothers delivering low birth weight neonates (Lian et al., 2011). The present study investigated the effects of maternal Cd exposure during pregnancy on placental PERK signaling branch. We showed that the level of phosphorylated eIF2α, a downstream target of the PERK pathway, was significantly increased in placenta of mice treated with $CdCl_2$. Moreover, maternal Cd exposure significantly increased the level of placental CHOP, another target of PERK signaling branch. In addition, the expression of ATF4, a downstream target of the PERK pathway, was upregulated in placenta of mice treated with $CdCl_2$. These results indicate that maternal Cd exposure during pregnancy can activate placental PERK signaling branch.

The ER is especially sensitive to alterations in homeostasis of redox (Malhotra and Kaufman, 2007). When excess ROS produce, proteins formed in the ER fail to attain correct conformation (Dickhout et al., 2005; Gorlach et al., 2006). Accumulation of unfolded and misfolded proteins that aggregate in the ER lumen causes activation of the UPR signaling (Lai et al., 2007). According to a recent report, Cd caused ER stress in LLC-PK1 cells via generation of excess ROS (Yokouchi et al., 2008). Indeed, Cd could induce lipid peroxidation in placenta (Enli et al., 2010). The present study showed that a single dose of $CdCl_2$ significantly reduced the level of GSH in placenta. Moreover, HO-1, a marker of oxidative stress, was obviously upregulated in placenta of mice treated with $CdCl_2$. In addition, a single dose of $CdCl_2$ significantly increased the intensity of 3-NT, a marker of protein nitration, in placenta. Further analysis revealed that an increase in 3-NT and HO-1 staining was mainly observed in trophoblast giant cells of the junctional region. To investigate the role of ROS on Cd-evoked placental ER stress, the pregnant mice were pretreated with PBN before Cd injection. As expected, pretreatment with PBN, a free radical spin-trapping agent and antioxidant, significantly alleviated Cd-induced placental GSH depletion. Moreover, PBN significantly attenuated Cd-induced increase in HO-1 and 3-NT staining in trophoblast giant cells of the junctional region. Importantly, PBN significantly attenuated Cd-induced upregulation of GRP78 in placenta. In addition, PBN pretreatment significantly alleviated Cd-evoked eIF2α phosphorylation and CHOP expression in placenta. These results suggest that antioxidant protects against Cd-induced placental ER stress and UPR. ROS contribute, at least partially, to Cd-induced ER stress and UPR in placenta.

The protection of PBN against Cd-induced placental ER stress may have therapeutic implications. According to several studies, PBN, as a free radical spin-trapping agent and an antioxidant, could protect against teratogenicity and fetal growth restriction caused by endotoxin (Xu et al., 2006; Zhao et al., 2008), which has been demonstrated to induce ER stress and UPR in placenta (Wang et al., 2011). A recent report showed that melatonin, another antioxidant, significantly alleviated endotoxin-evoked placental ER stress (Wang et al., 2011). Correspondingly, melatonin could protect mice from endotoxin-induced fetal death and growth restriction (Chen et al., 2006). Indeed, ROS-mediated ER stress in decidual cells is associated with the development of early pregnancy loss (Liu et al., 2011). Thus, antioxidants may be used as pharmacological agents to protect against not only Cd-evoked placental ER stress but also Cd-induced fetal defects and growth restriction.

In summary, the present study indicates that maternal Cd exposure during early limb development results in fetal growth restriction and the incidence of a relatively specific forelimb ectrodactyly in fetuses. Cd-induced fetal malformations might be associated with the impairments in placental development and the reduction in placental transport capacity. Maternal Cd exposure during pregnancy induces placental ER stress and UPR, which may partially contribute to Cd-induced impairment on placental and fetal development. ROS, possibly sourced from trophoblast giant cells, mediate Cd-evoked placental ER stress and UPR. Thus, antioxidants may be used as pharmacological agents to protect against Cd-induced placental impairment, fetal defects and growth restriction.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

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