N-acetylcysteine alleviates cadmium-induced placental endoplasmic reticulum stress and fetal growth restriction in mice

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Abstract

Cadmium (Cd) is a developmental toxicant that induces fetal growth restriction (FGR). Placental endoplasmic reticulum (ER) stress is associated with FGR. This study investigated the effects of N-acetylcysteine (NAC) on Cd-induced placental ER stress and FGR. Pregnant mice were intraperitoneally injected with CdCl₂ daily from gestational day (GD)13 to GD17. As expected, Cd reduced fetal weight and crown-rump length. Cd decreased the internal space of blood vessels in the placental labyrinth layer and inhibited placental cell proliferation. Several genes of growth factors, such as Vegf-a, placental growth factor, Igf1 and Igf1r, and several genes of nutrient transport pumps, such as Glut1, Fatp1 and Snat2, were down-regulated in placenta of Cd-treated mice. Moreover, Cd evoked placental ER stress. Of interest, NAC alleviated Cd-induced FGR. Additional experiment showed that NAC inhibited Cd-induced impairment of placental development and placental ER stress. Therefore, NAC may be exploited for prevention of Cd-induced placental insufficiency and FGR.

Instruction

Cadmium (Cd) is a kind of toxic heavy metals. Cd contamination is pervasive in soil and food, which has been widely concerned as one of major environmental problem. Increasing cohort studies demonstrated that high Cd level during pregnancy is associated with increased risks of preterm delivery, low birth weight and fetal growth restriction [1–4]. Several animal experiments also found that Cd exposure at middle gestational stage resulted in neural tube defects in mice [5–9]. Moreover, Cd exposure at late gestational stage resulted in fetal growth restriction in mice [9–12]. Nevertheless, the mechanism for Cd induced developmental toxicity remains unclear.

The placenta is important for sustaining the growth of the fetuses. Increasing evidence has demonstrated that the defects in placental development result in fetal death, birth defects and fetal growth restriction [13]. The endoplasmic reticulum (ER) is an important organelle that is
required for cell survival and normal cellular function [14]. The ER is sensitive to alterations in cellular homeostasis. Several recent studies demonstrated that Cd was an inducer of ER stress [15,16]. On the other hand, an increased ER stress was associated with the impaired placental development and fetal growth restriction [17–22]. Our recent report showed that reactive oxygen species (ROS)-mediated placental ER stress was associated with Cd-induced teratogenicity in mice [8].

N-acetylcysteine (NAC) is a direct antioxidant and a GSH precursor [23]. Previous study showed that NAC prevented Cd-induced oxidative stress [24]. The aim of the present study was to investigate whether NAC protects against Cd-induced placental ER stress and fetal growth restriction in mice. Our results found that NAC attenuated Cd-induced placental ER stress. Importantly, NAC alleviated Cd-induced placental development and fetal growth restriction.

Materials and methods

Chemicals and reagents

CdCl$_2$ and NAC were from Sigma Chemical Co. (St. Louis, MO). Antibody against CD34 was from Abcam (Cambridge, MA). Antibodies against phosphor-eukaryotic translation initiation factor 2α (eIF2α), eIF2α and GRP78 were from Cell Signaling Technology (Beverley, MA). Antibody against β-actin was from Santa Cruz Biotechnologies (Santa Cruz, CA). Antibodies against phosphor-inositol requiring ER-to-nucleus signal kinase (IRE) 1α and IRE1α were from Pierce Biotechnology (Rockford, IL). 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 CD-1 mice (8~10 week-old) were purchased from Beijing Vital River whose foundation colonies were all introduced from Charles River Laboratories, Inc. Mice take food and water freely and were maintained on a 12-h dark/light cycle in a ambient temperature 23±3°C and humidity 50±5%. Females and males were mated at a ratio of 2: 1. Females were checked in the following morning, and the presence of a vaginal plug was considered as gestational day (GD) 0. This study consisted of two experiments. Experiment 1. To investigate the effects of NAC on Cd-induced ER stress and FGR in mice, pregnant mice were divided randomly into four groups. In Cd group, pregnant mice were i.p. injected with CdCl$_2$ (1.0 mg/kg) daily from GD13 to GD17. In NAC+Cd group, pregnant mice were i.p. injected with NAC (1.0 g/kg) daily from GD12 to GD17, one dose (300 mg/kg) at 8 h before CdCl$_2$ injection, one (400 mg/kg) at 30 min before CdCl$_2$ injection, another (300 mg/kg) at 8 h after CdCl$_2$ injection. The saline-treated or NAC-treated pregnant mice served as controls. The doses of CdCl$_2$ used in this study referred to others [6,7]. The doses of NAC used in this study referred to others [25]. All dams were anesthetized with pentobarbital (75 mg/kg, i.p.) treatments and sacrificed on GD18. The uterine horns were exposed and weighed. Live, dead and resorbed fetuses were counted. Live fetuses were sexed and weighed. The rate of FGR was calculated referred to others [26]. Placentas were weighed and then collected for measurement of histopathology, immunohistochemistry and ER stress. Experiment 2. To investigate the effects of NAC on Cd-induced down-regulation of growth factors and nutrient transport pumps in mouse placenta, twenty-four pregnant mice were divided randomly into four groups. In Cd group, pregnant mice were i.p. injected with CdCl$_2$ (1.0 mg/kg) daily from GD13 to GD15. In NAC+Cd group,
pregnant mice were injected with NAC (1.0 g/kg, i.g.) daily from GD12 to GD15, one dose (300 mg/kg) at 8 h before CdCl$_2$ injection, one (400 mg/kg) at 30 min before CdCl$_2$ injection, another (300 mg/kg) at 8 h after CdCl$_2$ injection. The saline-treated or NAC-treated pregnant mice served as controls. All dams were anesthetized with pentobarbital (75 mg/kg, i.p.) treatments and sacrificed on GD15. Placentas were collected for real-time RT-PCR. This work was approved by the Association of Laboratory Animal Sciences and the Center for Laboratory Animal Sciences at Anhui Medical University (Permit Number: 15–0013). All procedures on mice 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.

**Isolation of total RNA and real-time RT-PCR**

Total RNA was extracted using TRI reagent and reverse-transcribed with AMV (Promega). 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 levels of mRNA were normalized using 18S. The comparative $C_T$ method was used to determine the amount of target, normalized to an internal reference (18S) and relative to a calibrator ($2^{-\Delta\Delta C_T}$) using the Lightcycler480 software (Roche, version 1.5.0).

**Immunoblots**

Fifty milligram placenta tissue was homogenized in 300 µl lysis buffer. Total lysate was separated electrophoretically by SDS-PAGE and transferred to a polyvinylidene fluoride

| Genes | Forward | Reverse | Sizes (bp) |
|-------|---------|---------|------------|
| 18S   | 5' - GTAACCGGTGAGGCCATT-3' | 5' - CACATCAAATGTTAGGCG-3' | 151 |
| Vegf-a| 5' - TATTCAGGCTACCAAGG-3' | 5' - ACCAACCTCGTCGGGCT-3' | 156 |
| Pgf   | 5' - AACTTGGAAACACAAGAGCT-3' | 5' - CGACCCCAACTGGTGGAA-3' | 131 |
| Igf1  | 5' - AAGGCAATTACCAGGCT-3' | 5' - GCCCGGATTGAACACAAAC-3' | 125 |
| Igf2  | 5' - CTTTACGAGGGCTTCCATTCA-3' | 5' - TTGGTACCAAAAGGCGAAG-3' | 105 |
| Igf1r | 5' - CCAAGCTAACGGTACACTACT-3' | 5' - GAAAGTTTCAGGCAGG-3' | 110 |
| Glut1 | 5' - ACCATCTGGAAGCTTGTCG-3' | 5' - GCCCTTCTGAAAGATGCTCG-3' | 131 |
| Fatp1 | 5' - CGCCGATGTTCTATGCTACT-3' | 5' - ACACAGTCTCCCGAGACGG-3' | 138 |
| Fatp4 | 5' - GGCTCAGGGCCTAAACTC-3' | 5' - TCCCAAGGCTAAGGGCGAAG-3' | 102 |
| Snat2 | 5' - ACCCTACCTGTCGTCAAGA-3' | 5' - TGGTTCTACGGGACTTCC-3' | 117 |
| Snat4 | 5' - GGGCAGGAATTAAGCTGTG-3' | 5' - ACCCTCCTTCGTGCTTGC-3' | 156 |
| Pcft  | 5' - CTACCCTACCCTCCGCAGCT-3' | 5' - GCAAACCGCAAAGCACCACAT-3' | 119 |
| Rfc-1 | 5' - TGGGTGTGTTAGCTGGTG-3' | 5' - CAATCCACCTGGGCAACACCC-3' | 114 |

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membrane. The membranes were incubated for 2 h with the following antibodies: GRP78, p-eIF2α, eIF2α, pIRE1α and IRE1α. β-actin was used as a loading control. After washes in DPBS, the membranes were incubated for 2 h with goat anti-rabbit or goat anti-mouse IgG. The membranes were then washed for four times in DPBS, followed by signal development using an ECL detection kit.

**Immunohistochemistry**

Placental sections were dewaxed and rehydrated. Antigen retrieval was performed by pressure cooking slides in 0.01 M citrate buffer (pH 6.0). After nonspecific binding sites were blocked with 5% normal bovine serum, slides were incubated for 12 h with polyclonal antibody Ki67 or CD34 at 4˚C. After washing in TBS, slides were incubated with the biotin conjugated goat anti-rabbit IgG for 30 min, and then horseradish peroxidase-labeled avidin-biotin complex for 30 min. Immunostaining was developed by application of diaminobenzidine. Ki67-positive cells were counted in twelve randomly selected fields from each slide at a magnification of ×400.

**Placental glutathione content**

Placental glutathione (GSH) content was determined by the method of Griffith [27].

**Statistical analysis**

The litter was considered the unit for statistical comparison among different groups. Rates of fetal growth restriction were 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. All quantified data were expressed as means ± SEM. ANOVA and the Student-Newmann-Keuls post hoc test were used to determine differences among different groups. P < 0.05 was considered statistically significant.

**Results**

**NAC attenuates Cd-induced fetal growth restriction**

No pregnant mice were dead and no preterm delivery was observed in pregnant mice intraperitoneally injected with CdCl₂. The effects of maternal Cd exposure on fetal outcomes are presented in Table 2. There was no significant difference on the number of resorptions per litter,

| Table 2. Fetal outcomes among different groups. |
|-----------------------------------------------|
| Control | NAC | Cd | Cd+NAC |
| Number of litters(n) | 19 | 14 | 17 | 20 |
| Resorptions of per litter (n) | 0.4±0.2 | 0.3±0.2 | 0.5±0.2 | 0.5±0.2 |
| Death fetuses per litter (n) | 0.3±0.1 | 0.4±0.2 | 0.5±0.2 | 0.5±0.2 |
| Live fetuses per litter (n) | 12.7±0.5 | 13.0±0.2 | 13.1±0.6 | 12.4±0.4 |
| Fetal weight (g) | 1.397±0.023 | 1.371±0.026 | 1.247±0.017** | 1.309±0.012* |
| Crown-rump length (mm) | 24.46±0.16 | 24.35±0.24 | 22.92±0.17** | 23.56±0.16* |
| FGR per litter (%) | 10.3±2.7 | 14.6±4.5 | 51.9±8.2** | 23.0±5.0* |
| Placenta weight(g) | 0.100±0.003 | 0.095±0.003 | 0.091±0.003* | 0.095±0.003 |

* P<0.05
** P<0.01 as compared with the control
* P<0.05 as compared with Cd group.
live fetuses per litter and dead fetuses per litter among different groups. As expected, maternal Cd exposure at late gestational stage did not induce external malformations in fetuses. The effects of maternal Cd exposure at late gestational stage on fetal weight and crown-rump length are shown in Table 2. The fetal weight in the Cd group was lower as compared with controls. Correspondingly, crown-rump length was obviously reduced in the Cd group (Table 2). Further analysis showed that the rate of FGR per litter was markedly elevated in the Cd group (Table 2). The effects of NAC on Cd-induced fetal growth restriction were then analyzed. As shown in Table 2, NAC significantly attenuated Cd-induced reduction of fetal weight and crown-rump length. Correspondingly, NAC significantly attenuated Cd-induced elevation of FGR per litter.

**NAC alleviates Cd–induced impairment of placental development**

The effects of maternal Cd exposure at late gestational stage on placenta weight and histopathology were analyzed. As shown in Table 2, placenta weight was lower in Cd-exposed mice as compared with controls. Histopathology showed that the internal space of maternal and fetal blood vessels in the labyrinth layer was markedly reduced in the placenta of mice exposed to Cd (Fig 1A and 1B). Further analysis observed that the number of fetal blood vessels, as determined by CD34 immunostaining, was significantly reduced in the placenta of mice exposed to Cd (Fig 1C and 1D). The effects of NAC on Cd-induced impairment of placental vascular space were then analyzed. Of interest, NAC inhibited Cd-induced impairment of placental vascular space in the labyrinth layer (Fig 1). The effects of maternal Cd exposure at late gestational stage on cell proliferation in the placenta are presented in Fig 2. As expected, Ki67-positive cells in the trophoblast layer were reduced in the placenta of mice treated with Cd (Fig 2A and 2B). In addition,
Ki67-positive cells in the labyrinthine layer were reduced in the placenta of mice treated with Cd (Fig 2C and 2D). The effects of NAC on Cd-induced inhibition of placental cell proliferation were then analyzed. Of interest, NAC obviously attenuated Cd-induced inhibition of cell proliferation in the placenta (Fig 2).

NAC alleviates Cd–induced down-regulation of growth factors in mouse placenta

The effects of maternal Cd exposure at late gestational stage on growth factors in mouse placenta are presented in Fig 3. As shown in Fig 3A, placental Vegfα mRNA level was reduced in Cd-exposed mice. In addition, Pgf expression was down-regulated in the placenta of mice exposed to Cd (Fig 3B). Although maternal Cd exposure at late gestational stage had little effect on placental Igf2 expression (Fig 3E), placental Igf1 and Igf1r mRNAs were reduced in Cd-exposed mice (Fig 3C and 3D). The effects of NAC on Cd-induced down-regulation of growth factors in placenta were analyzed. Interestingly, NAC significantly attenuated Cd-induced down-regulation of growth factors in mouse placenta (Fig 3A–3D).

NAC alleviates Cd–induced down-regulation of placental nutrient transporters

The effects of maternal Cd exposure at late gestational stage on nutrient transport pumps in placenta are presented in Fig 4. As shown in Fig 4A, mRNA level of Glut1, a gene of a glucose transport pump, was significantly reduced in the placenta of mice exposed to Cd. Although maternal Cd exposure at late gestational stage had little effect on placental Fatp4, a fatty acid transport pump, mRNA levels of Fatp1, another fatty acid transport pump, was reduced in the placenta of Cd-exposed mice (Fig 4B and 4C). Maternal Cd exposure at late gestational stage reduced mRNA levels of S nat2, a gene of amino acid transport pump (Fig 4D). Maternal Cd exposure...
exposure at late gestational stage had little effect on placental Snat4, another gene of amino acid transport pump, and Pcft, a gene of folic acid transport pump (Fig 4E and 4F). The effects of NAC on Cd-induced down-regulation of nutrient transport pumps in placenta were analyzed. Interestingly, NAC significantly attenuated Cd-induced down-regulation of nutrient transport pumps in mouse placenta (Fig 4A, 4B and 4D).

NAC alleviates Cd-induced placental GSH depletion

The effects of NAC on placental GSH depletion were analyzed in placentas of mice exposed to Cd. As shown in Fig 5, placental GSH content was significantly reduced in placentas of mice exposed to Cd. NAC almost completely inhibited Cd-evoked placental GSH depletion (Fig 5).
NAC attenuates Cd-induced placental ER stress

The effects of maternal Cd exposure at late gestational stage on placental ER stress were analyzed. As expected, placental GRP78 protein was up-regulated in Cd-exposed mice (Fig 6A and 6B). Moreover, the level of phosphorylated IRE1α in placenta was elevated in Cd-exposed mice (Fig 6C and 6D). The level of phosphorylated eIF2α, a downstream target of the PERK pathway, was increased in placenta of mice exposed to Cd. (Fig 6E and 6F). Interestingly, NAC attenuated Cd-induced up-regulation of placental GRP78 (Fig 6A and 6B). In addition, NAC obviously inhibited Cd-induced phosphorylation of IRE1α and eIF2α in mouse placenta (Fig 6C–6F).

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Discussion

Our previous study mainly investigated the effects of maternal Cd exposure at the second trimester on fetal teratogenesis [8]. The previous results showed that maternal exposure to cadmium (4.5 mg/kg, i.p.) on gestational day 9 induced forelimb ectrodactyly in fetal mice, caused oxidative stress and endoplasmic reticulum stress in mouse placenta [8]. In the current study, we further explored the effects of maternal Cd exposure at the third trimester on fetal growth and development in mice. We found that maternal exposure to cadmium (1 mg/kg, i.p.) daily from gestation day 13 to 17 resulted in fetal growth restriction, induced oxidative stress and endoplasmic reticulum stress in mouse placenta. Further results showed that N-acetylcysteine (NAC) alleviates cadmium-induced placental endoplasmic reticulum stress and fetal growth restriction in mice.

An early study found that NAC prevented Cd-induced nephrotoxicity in rats [28]. A recent report showed that NAC protected mice from Cd-induced germ cell apoptosis in mouse testis [29]. In addition, NAC inhibited Cd-induced mitophagy in mouse brain [30]. In the present study, we investigated the effects of NAC on Cd-induced fetal growth restriction in mice. As expected, fetal weight and crown-rump length were significantly reduced in Cd-exposed mice. By contrast, FGR rate per litter was elevated in Cd-exposed mice. Of interest, NAC alleviated Cd-induced reduction of fetal weight and crown-rump length. These results suggest that NAC protects mice from Cd-induced fetal growth restriction.

The placenta is important for sustaining fetal growth and intrauterine development. The labyrinth is the site of oxygen and nutrient exchange between the mother and the fetus [31].
Increasing evidence demonstrates that placental insufficiency is major reason of fetal growth restriction [32–34]. Indeed, the present study showed that the weight of placenta in Cd-exposed mice was lower than in controls. Moreover, the percentage of Ki67-positive cells, a marker of cell proliferation, was decreased in the placenta of mice exposed to Cd. In addition, the internal space of maternal and fetal blood vessels in the labyrinth layer was reduced in the placenta of mice exposed to Cd, indicating that maternal Cd exposure induces placental insufficiency. To investigate whether NAC alleviates Cd-induced placental insufficiency, the present study analyzed the effects of NAC on cell proliferation and the internal space of vessels in mouse placenta. Of interest, NAC obviously alleviated Cd-induced impairment of maternal and fetal blood vessels in the labyrinth layer. In addition, NAC counteracted Cd-induced suppression of cell proliferation in the labyrinth layer. These results suggest that NAC might alleviate fetal growth through inhibiting Cd-induced placental insufficiency.

Increasing evidence demonstrated that ER stress was associated with the impaired placental development and fetal growth restriction [18–21]. A report from our laboratory showed that maternal Cd exposure on GD9 evoked placental ER stress [8]. Moreover, Cd-induced placental ER stress was associated with an increased incidence of forelimb ectrodactyly in fetuses [8]. The present study investigated the effects of maternal Cd exposure during late gestational stage on placental ER stress. We showed that placental GRP78 was up-regulated in mice exposed to Cd daily from GD13 to GD17. Moreover, the level of placental pIRE1α was elevated in mice exposed to Cd. In addition, the level of peIF2α, a downstream molecule of PERK signaling, was increased in the placenta of Cd-exposed mice. Of interest, NAC attenuated Cd-induced
placental GRP78 up-regulation. In addition, NAC inhibited Cd-induced placental IRE1α and eIF2α phosphorylation. These results suggest that NAC might protect against Cd-induced fetal growth restriction, at least partially, through inhibiting ER stress in mouse placenta.

The mechanism through which ER stress mediates Cd-induced placental insufficiency and fetal growth restriction remains obscure. A recent study from our laboratory indicated that disturbance of placental folate transport contributed, at least partially, to Cd-induced neural tube defects and fetal growth restriction [9]. On the other hand, a recent study showed that mRNA levels of placental glucose transporter 1 (Glut1), a major transporter for glucose, placental growth factor (Pgf) and vascular endothelial growth factor receptor-1 (Vegfr-1), two vascular-related genes, were down-regulated under tunicamycin-evoked ER stress [21]. Further analysis found that placental ER stress negatively regulates transcription of placental growth factor via ATF4 and ATF6β [22]. In the present study, we investigated the effects of maternal Cd exposure on growth factors and nutrient transport pumps in mouse placenta. Our results showed that mRNA levels of placental Vegfa, Pgf, Igfl and Igf1r, several key genes of growth factors, were reduced in mice exposed to Cd daily from GD13 to GD17. In addition, placental Glut1, Fatp1 and Snat2, several genes of nutrient transport pumps, were down-regulated in Cd-exposed mice. Of interest, NAC significantly attenuated Cd-induced down-regulation of growth factors and nutrient transport pumps in mouse placenta. These results suggest that maternal Cd exposure at late gestational stage induces placental insufficiency partially through ROS-mediated ER stress. Therefore, we guess that NAC alleviates Cd-induced fetal growth restriction, at least partially, through attenuating ER stress-mediated placental insufficiency. Additional experiments need to determine the protein expression of the placental growth factors and nutrient transporters in future studies.

The inhibitive effect of NAC on Cd-induced placental ER stress has preventive and therapeutic implications. An early study found that NAC reduced Cd-induced mesangial cell autophagy through inhibiting ROS-activated GSK-3β signaling [35]. Two recent studies showed that NAC protected against Cd-induced neuronal cell death through abrogating ROS-dependent activation of mTOR signaling [36, 37]. According to a recent report, NAC mediated Cd-induced mitophagy in mouse brain through blocking ROS-mediated activation of PINK1/Parkin pathway [30]. Indeed, a report from our laboratory showed that NAC protected against Cd-induced germ cell apoptosis by inhibiting testicular ER stress [29]. The present study showed that NAC alleviated Cd-induced fetal growth restriction, at least partially, through inhibiting ER stress in mouse placenta. Our results suggest that NAC may be exploited for prevention and treatment of Cd-induced fetal growth restriction.

In summary, the present study investigated the effects of NAC on Cd-induced placental ER stress and fetal growth restriction in mice. Our results showed that NAC attenuated Cd-induced placental ER stress. We found that NAC prevented Cd-induced impairment of placental development. Importantly, NAC alleviated Cd-induced fetal growth restriction. We demonstrate that NAC may be exploited for prevention and treatment of Cd-induced placental insufficiency and fetal growth restriction.

Supporting information

S1 Fig. The original blot of GRP78 from Fig 6A. The order of samples are Control1, Control2, NAC1, NAC2, Cd1, Cd2, Cd+NAC1 and Cd+NAC2, respectively. (TIF)

S2 Fig. The original blot of beta-actin from Fig 6A. The order of samples are Control1, Control2, NAC1, NAC2, Cd1, Cd2, Cd+NAC1 and Cd+NAC2, respectively. (TIF)
Author Contributions

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