Cadmium-induced immune abnormality is a key pathogenic event in human and rat models of preeclampsia

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Abstract
With increased industrial development, cadmium is an increasingly important environmental pollutant. Studies have identified various adverse effects of cadmium on human beings. However, the relationships between cadmium pollution and the pathogenesis of preeclampsia remain elusive. The objective of this study is to explore the effects of cadmium on immune system among preeclamptic patients and rats. The results showed that the cadmium levels in the peripheral blood of preeclamptic patients were significantly higher than those observed in normal pregnancy. Based on it, a novel rat model of preeclampsia was established by the intraperitoneal administration of cadmium chloride (CdCl2) (0.125 mg of Cd/kg body weight) on gestational days 9–14. Key features of preeclampsia, including hypertension, proteinuria, placental abnormalities and small foetal size, appeared in pregnant rats after the administration of low-dose of CdCl2. Cadmium increased immunoglobulin production, mainly angiotensin II type 1-receptor-agonistic autoantibodies (AT1-AA), by increasing the expression of activation-induced cytosine deaminase (AID) in B cells. AID is critical for the maturation of antibody and autoantibody responses. In addition, angiotensin II type 1-receptor-agonistic autoantibodies, which emerged recently as a potential pathogenic contributor to PE, was responsible for the deposition of complement component 5 (C5) in kidneys of pregnant rats via angiotensin II type 1 receptor (AT1R) activation. C5a is a fragment of C5 that is released during C5 activation. Selectively interfering with C5a signalling by a complement C5a receptor-specific antagonist significantly attenuated hypertension and proteinuria in Cd-injected pregnant rats. Our results suggest that cadmium induces immune abnormalities that may be a key pathogenic contributor to preeclampsia and provide new insights into treatment strategies of preeclampsia.

1. Introduction

Preeclampsia (PE) is a pregnancy-specific disorder that is characterized by hypertension, proteinuria and vascular abnormalities and often by intrauterine growth retardation (Roberts and Cooper, 2001) after the 20th week of gestation. It affects 2%–8% of pregnant women worldwide (Steegers et al., 2010), resulting in increased maternal and foetal morbidity and mortality. Numerous recent studies have shown that women with preeclampsia possess angiotensin II type 1-receptor-agonistic autoantibodies (AT1-AAs) that bind to and activate AT1 angiotensin receptors (AT1R) (Yang et al., 2015; Xia and Kellems, 2013; LaMarca et al., 2012; Sahay et al.,...
The introduction of these autoantibodies into pregnant rats results in hypertension, proteinuria and a variety of other features of preeclampsia (LaMarca et al., 2009). These findings raise the intriguing possibility that preeclampsia may be a pregnancy-induced autoimmune condition that is characterized by the presence of disease-causing angiotensin-receptor-activating autoantibodies. However, the factors that contribute to the increased levels of AT1-AAs in preeclampsia and how these auto-antibodies result in preeclampsia are still unclear.

With increased industrial development, cadmium (Cd) is an increasingly important environmental pollutant to both humans and animals (Thevenod and Lee, 2013; Kah et al., 2012). The population is exposed to Cd daily via the consumption of food and water polluted with this metal (Schlecht and Saumel, 2015; Cai et al., 2009), as well as by smoking (Al and Omu, 1999). Pregnant women are more vulnerable to Cd because of the greatly increased absorption and retention of Cd caused by nutritional deficiencies during pregnancy (Nishijo et al., 2004). Cadmium is a known endocrine disruptor (Kniazick et al., 2015) that affects the synthesis and/or regulation of several hormones. Studies have shown that cadmium has potent oestrogen-like activity in vivo (Nasiadek et al., 2011; Silva et al., 2013). According to several clinical and epidemiological studies, females can have stronger and more rapid immune responses than males upon antigen encounter (Kovats, 2015). This may occur because oestrogen can enhance immunoglobulin production by upregulation of the expression of activation-induced cytosine deaminase (AID) (Asaba et al., 2015), which is critical for the maturation of antibody and autoantibody responses. These results favour the hypothesis that Cd plays an important role in the immune system as well as oestrogen and it increases the production of autoantibodies that may induce pregnancy-specific hypertension.

A well regulated complement system is a prerequisite for a healthy pregnancy (Chow et al., 2009). The complement system can be initiated through the classical, lectin or alternative pathways. The activation of complement component 5 (C5) is a point of convergence for all three of the major complement activation pathways. The classical complement pathway is mainly initiated by antibody-dependent. Complement is activated by immune complexes of AT1-AA plus AT1R leading to generation of activation products C5a. C5a is a 74-amino acid fragment of C5 that is released during C5 activation, mediates the hypertension and functions as an anaphylatoxin that provokes a strong inflammatory response by activation of complement C5a receptors (C5aR) on multiple target cells, including inflammatory cells, endothelial cells, vascular smooth muscle cells, and epithelial cells (Rafail et al., 2015; Li et al., 2015; Shagarsuren et al., 2010). Besides, a case report was published, where treatment with the C5 inhibitor eculizumab prolonged PE pregnancy by 17 days (Burwick and Feinberg, 2012). We found it reasonable to hypothesize that increased AT1-AA may contribute to C5 activation and an imbalance between C5a activation and regulation could be involved in PE.

Studies on the relationship of Cd and preeclampsia may provide new insights into the pathogenesis of preeclampsia and shed some light on getting new treatment strategies of preeclampsia.

2. Materials and methods

2.1. Patients

Patients at 28–40 weeks of gestation admitted to the First Affiliated Hospital of Wenzhou Medical University were identified by the obstetrics faculty. Severe PE was defined as either severe hypertension (systolic blood pressure ≥ 160 mmHg or diastolic blood pressure ≥ 110 mm Hg) or severe proteinuria (urinary protein excretion ≥ 5.0 g per 24 h). The blood pressure of all patients returned to normal levels and symptoms of proteinuria disappeared by 6 weeks postpartum. Patients with chronic hypertension, renal disease, collagen vascular disease, premature rupture of membrane and other complications of pregnancy were excluded from this study. Pregnant women with uncomplicated pregnancies were randomly selected to serve as controls. Preeclamptic patients diagnosed with severe disease (n = 20) and women with normotensive pregnancies (n = 20) were included in this study. The research protocol was approved by the Institutional Committee for the Protection of Human Subjects. Detailed information on the human subjects is presented in supplemental material.

2.2. Plasma analyses

Plasma samples from a cohort of preeclampsia patients (n = 20) and from women with normal pregnancies (n = 20) were used for biochemical assays. Plasma was prepared by centrifugation for 20 min at 4000 rpm at 4 °C. Cd in maternal blood and umbilical cord blood was measured by inductively coupled plasma mass spectrometry (ICP-MS; Agilent 7500 with a Cd program). AT1-AA and C5a levels in plasma were measured with ELISA kits (YAJI Biological Technology Co. Ltd, Shanghai, China and USCN Business Co. Ltd, Wuhan, China) according to the manufacturer’s instructions.

2.3. Animals

All animal studies were performed in Wistar rats purchased from Beijing Vital River Laboratory Animal Technology Co. Ltd (Beijing, China). The animals were housed in a temperature-controlled room (23 °C) with a 12:12 light:dark cycle. All experimental procedures used in this study were approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University.

2.3.1. Protocol 1 to confirm suitable timing and dosage of cadmium chloride to establish the PE model

Twenty-five pregnant rats were divided into five groups (normal pregnant control, D7 group, D9 group, D11 group and D13 group; n = 5 animals per group) according to a random number table. The rats were intraperitoneally injected with cadmium chloride (CdCl2; Sinopharm Chemical Reagent Co. Ltd, Mainland, China) at concentrations of 0.25 mg of Cd/kg body weight (b.w.) daily for six days from gestational day (GD) 7–12 for the D7 group, from GD 9 to 14 for the D9 group, from GD 11 to 16 for the D11 group and from GD 13 to 18 for the D13 group. The other animals served as normal pregnant controls. The blood pressure of the rats in all groups was measured and compared to determine suitable injection times for the establishment of the preeclampsia model.

Another 20 pregnant rats were divided into four groups: a normal pregnancy group (n = 5), 0.0625 mg/kg Cd group (n = 5), 0.125 mg/kg Cd group (n = 5) and 0.25 mg/kg Cd group. The animals were intraperitoneally injected with sterile saline or CdCl2 at concentrations of 0.0625, 0.125 or 0.25 mg of Cd/kg b.w. at the suitable time as determined in the first step. Blood pressure, maternal body weight and foetal body weight were compared among the groups to determine the lowest effective dose.

2.3.2. Protocol 2 cadmium chloride administration to pregnant rats to induce a preeclampsia-like syndrome

Finally, 25 rats were randomly divided into a normal pregnant + Cd (NP + Cd) group (n = 5), a normal pregnant + normal saline (NP + NS) group (n = 5), a normal pregnant (NP) group (n = 5), a non-pregnant + (Non-p + Cd) group (n = 5) and a non-

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pregnant (Non-P) group (n = 5). Experimental PE was induced in rats of the (NP + Cd) group by intraperitoneal administration of CdCl₂ (0.125 mg of Cd/kg b.w.) on GD 9–14. The administration time and dose were determined according to the procedures described above. The five non-pregnant rats in the (Non-p + Cd) group received intraperitoneal injections of CdCl₂ (0.125 mg of Cd/kg b.w.). The (NP + NS) group received only sterile saline, and the NP and Non-P groups received no injections. For the neutralization experiments, losartan, an AT-1 receptor blocker, (Merck, Whitehouse Station, NJ, USA), was administered by gavage at a dose of 10 mg/kg of body weight per day from GD 14–19 (Wang et al., 2014) after CdCl₂ administration (n = 5). The CsAR antagonist (PMX53), ACF- [OP(2-Cha)W] (AcetylPhe; Orn-Pro-o-cyclohexylalanine-Trp-Arg), (Shanghai GL Biochemicals, Shanghai, China) (Li et al., 2014; Zhang et al., 2014) (0.5 mg/kg of body weight) was administered by intraperitoneal injection to rats daily, starting from 1 day before CdCl₂ infusion. Blood pressure, urinary protein levels, the characteristics of the foetuses and pathological changes were compared among the groups.

To confirm that the effect of cadmium on pregnant rats was not due to its toxicity, two groups of rats (n = 5 each) received CdCl₂ (1 mg of Cd/kg b.w.) (Demenesku et al., 2014) for one day or CdCl₂ (0.125 mg of Cd/kg b.w.) for six days by intraperitoneal injection. At 48 h post-exposure, the rats were euthanized and selected tissues were collected for use in experiments.

2.4. Measurement of systolic blood pressure

Systolic blood pressure (SBP) was measured in conscious, restrained pregnant rats each morning before and during pregnancy until 8 days after delivery. An automated system with a photometric sensor linked to a dual-channel recorder (BP-98A, Softron, Japan), tail cuff and sphygmomanometer were used to obtain blood pressure measurements. For the non-pregnant animals, comparable time periods were utilized.

2.5. Urine protein quantitation

The 24-h urine protein in each group on the 3rd and 19th day of pregnancy was, respectively, determined with a BCA protein assay kit (Thermo, Rockford, USA).

2.6. Specimen collection

On day 21 of pregnancy, maternal blood was collected by cardiac puncture after the rats were anaesthetized with 4% chloral hydrate. Plasma was prepared by centrifugation for 20 min at 4000 rpm at 4 °C. The foetuses, placentas, decidua and kidneys were removed and weighed. Three placentas, decidua and one kidney were randomly selected from each rat and fixed with 4% paraformaldehyde for histological evaluation. The plasma samples and the remaining placentas, decidua and kidney were stored at –80 °C until analysis.

2.7. Histology assay

Placentas, kidneys, liver, decidual and aortal specimens in 4-μm paraffin sections were stained with haematoxylin and eosin for conventional morphological evaluation under a light microscope (Olympus BX51, Tokyo, Japan). To compare the level of expression of C5a in kidney samples, kidney samples in 4-μm paraffin sections were stained with anti-rat C5a (Boster, Wuhan, China) at a dilution of 1:400 in a humidified chamber at 4 °C overnight. Following the primary antibody incubation, an anti-rabbit/mouse Autostainer Immunostaining System (DAKO, Carpinteria, CA, USA) was used to detect C5 staining. Sections 4 μm in thickness were obtained from the placenta samples and stained with anti-rat CD34 (Abcam, Cambridge, UK) at a dilution of 1:100 in a humidified chamber at 4 °C overnight. Following the primary antibody incubation, Cy-3 anti-rabbit IgG (Goodbio Technology Co. Ltd, Wuhan, China) was used to detect immunostaining for CD34.

2.8. Cell culture and treatment

2.8.1. Protocol 1 determination of a suitable dose of cadmium chloride for the treatment of B cells

Single-cell suspensions were prepared from the spleens of pregnant rats. After removal of red blood cells, splenic B cells were isolated using a rat B cell-positive isolation kit (Miltenyi Biotec, Bergisch Gladbach, Germany) (Wen et al., 2014) according to the manufacturer’s instructions. The cells were cultured in phenol red-free RPMI medium supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (FBS-RPMI) at 37 °C in the presence of 5% CO₂. For exposure of the cells to CdCl₂, the cells were cultured at 5 × 10⁸ cell/ml in FBS-RPMI containing CdCl₂ at the indicated doses or 17β-oestradiol (0.01 μM) (Sun et al., 2007) in 12-well plates and stimulated with either rIL-4 (3 ng/ml) (PeproTech, NJ, USA) and LPS (5 μg/ml) from E. coli (Sigma Aldrich, Saint Louis, MO, USA) or with no addition for class switch DNA recombination (CSR) to IgG3 (Mai et al., 2010). The amounts of total IgG in the supernatants were measured by ELISA; the AID level of B cells was measured by qPCR and western blot.

2.8.2. Protocol 2 effect of inhibitors of the oestrogen receptor and NF-κB on AID expression

To investigate the mechanism of increased AID expression by cadmium, the effects of ICI182780 (an oestrogen receptor blocker, Tocris Bioscience, Bristol, UK), G15 (a GPR30 antagonist, Cayman Chemical, Michigan, USA), and MG–132 (a selective inhibitor of NF-κB, Selleckchem, Houston, TX, USA) on AID levels in the cells were examined. After pre-incubation of the cells for 30 min with ICI182780 (10 mM), G15 (100 μM) or MG–132 (0.3 μM), CdCl₂ was administered for 48 h. The expression of AICDA mRNA and AID protein by the B cells was measured.

2.9. B cell proliferation and viability

Cell proliferation was analysed by flow cytometry. In vitro proliferation was analysed using the CellTrace™ CFSE Cell Proliferation Kit (Molecular Probes). The viability of the cells was assessed by trypan blue exclusion.

2.10. Quantitative real-time RT-PCR (qRT-PCR)

B cells were lysed with TRizol reagent (Invitrogen, Carlsbad, CA), and the total RNA was extracted according to the manufacturer’s instructions. The total RNA (2 μg) was used for reverse transcription using ReverTrna Ace (TOYOBO, Osaka, Japan) in a volume of 20 μl. Next, 1.5 μl cDNA was amplified with Thunderbird SYBR qPCR Mix (TOYOBO, Osaka, Japan) in duplicate. The resulting data were analysed with the comparative cycle threshold method for relative gene expression quantification against GAPDH. The primers used in our study were: AICDA forward (GGA CAG CCT CTT GAT GAA GC), AICDA reverse (GCC AAA GTC CAC TGA GAA GG); CsAR forward (ATG CCT GCA CAT GCC GTC TA), CsAR reverse (CAG AAA CCA AAT GCC GTT CAC); GAPDH forward (GCC ACA GTC AAG GCT GAC AAT C), GAPDH reverse (ATG GTG GTG AAG ACC CGA CTA).

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2.11. Protein isolation and western blot

Protein was extracted from B cells in lysis buffer. Proteins (40 μg) were separated on a 10% gel and transferred to nitrocellulose membranes. Immunodetection was performed using standard procedures. After blocking in 5% non-fat milk in TBST for 1 h, the membranes were incubated with primary rabbit anti-AID (1:200) (Santa Cruz Biotechnology Inc., CA, USA) overnight at 4 °C. The membranes were then washed three times in TBST and incubated with HRP-conjugated anti-rabbit secondary antibody (1:1000), followed by washing in TBST. The signals were developed with an ECL kit (Millipore, Bedford, MA, USA). GAPDH served as an internal control. Bound antibody was detected by enhanced chemiluminescence on X-ray film. We analysed the relative densities of the bands with Image J 1.47.

2.12. Statistical analysis

Data are presented as the mean ± SEM from at least three independent experiments. Differences between two means were determined using Student’s t-test. The means of the different groups were compared by a one-way ANOVA followed by a Tukey multiple comparison test. P-values of less than 0.05 were considered significant.

3. Results

3.1. Cadmium concentrations and C5a concentrations in the blood of preeclamptic patients

The Cd level in the peripheral blood of preeclamptic patients (37.65 ± 1.84 μg/L) was significantly higher than that in the blood of patients with normal pregnancies (18.65 ± 1.22 μg/L) (p < 0.001) (Fig. 1A). Interestingly, there was no difference in the Cd level in umbilical cord blood between the groups (1.149 ± 0.126 μg/L vs 1.266 ± 0.0845 μg/L, PE vs normal pregnancy) (Fig. 1B). In PE patients, the mean C5a level was higher than in NP patients (Fig. 1C).

3.2. Administration of cadmium chloride to rats in early pregnancy induces a preeclampsia-like syndrome

3.2.1. Timing and dosage of cadmium chloride required to establish the preeclampsia model

There were no significant differences in systolic blood pressure (SBP) among the five groups of rats (D7, D9, D11, D13 and normal pregnancy group) prior to CdCl2 administration. After CdCl2 administration, the SBP of the D9 group increased steadily; and the SBP of the D7, D11 and D13 groups increased slightly. SBP for pregnant rats exposed to CdCl2 of D9 group was significantly higher than the blood pressure of rats in the control group (p < 0.01) and the other groups (p < 0.01) (Fig. 2A). Therefore, GD 9–14 was chosen as the suitable injection time period for the establishment of the preeclampsia model. Compared with the normal pregnancy group treated with saline, the 0.0625 mg/kg Cd group, the 0.125 mg/kg Cd group and the 0.25 mg/kg Cd group all had higher SBP (113.50 ± 1.19 mmHg, 134.00 ± 2.27 mmHg, and 131.80 ± 1.10 mmHg, respectively, vs 101.00 ± 2.04 mmHg for the normal pregnancy control), lower maternal body weight (336.50 ± 1.66 g, 312.00 ± 3.19 g, and 300.80 ± 3.95 g, respectively, vs 346.30 ± 3.07 g for the normal pregnancy control) and lower foetal body weight, especially the 0.125 mg/kg Cd group and the 0.25 mg/kg Cd group (p < 0.01). Based on these results, 0.125 mg of Cd/kg b.w. was chosen as the dose for the establishment of the preeclampsia model (Fig. 2B–D).
By injecting pregnant rats with CdCl2 at a dose of 0.125 mg of Cd/kg body weight daily from GD 9 to 14, a novel rat model of preeclampsia was established. This model exhibited significant and characteristic hypertension, proteinuria and adverse pregnancy outcomes compared with the pregnant control.

3.2.2. Cadmium chloride administration to pregnant rats induced a preeclampsia-like syndrome

Hypertension is a dominant feature of preeclampsia. Fig. 2E shows that exposure of pregnant rats to CdCl2 (0.125 mg of Cd/kg b.w.) resulted in a significant pregnancy-dependent increase in SBP.
compared with (Non-p + Cd) animals at GD 19 (p < 0.01). The SBPs of pregnant rats exposed to CdCl2 (0.125 mg of Cd/kg b.w.) were significantly higher than those of rats in the NP and saline-treated pregnant groups (p < 0.01). Interestingly, there was no difference in SBP between the (Non-p + Cd) group and the Non-p group. The SBP of the animals in the model group recovered to baseline six days after delivery, and no changes in blood pressure were found in the other groups.

Proteinuria, another defining feature of preeclampsia, was measured in rats. As shown by the results of 24-hr urine protein quantitation, the urinary protein levels of the rats in the five groups were similar prior to CdCl2 administration. A pregnancy-dependent increase in urinary protein occurred in animals that received injections of CdCl2 (0.125 mg of Cd/kg b.w.) (p < 0.001); the urinary protein level in the Cd-treated pregnant rats was also significantly higher than that of the animals in the normal pregnancy group and of those in the saline-treated pregnant group (p < 0.001) (Fig. 2F). There was no difference in the level of protein excretion in the urine between the (non-pregnant + Cd) group and the non-pregnant group.

The pups of rats in the 0.125 mg Cd/kg group were significantly smaller than those of the animals in the normal pregnancy group (Fig. 2G, Table 1).

3.2.3. Cadmium chloride administration to rats in early pregnancy induces renal/decidual/placental/aortal damage

As shown in Fig. 3A, the kidneys of animals in the Cd-treated group displayed swelling of endothelial cells that appeared to reduce the capillary space (glomerular endotheliosis), thickening of the media of renal vessel walls via smooth muscle cell proliferation and protein casts in the renal tubules (Fig. 3A1, A2). A thickening uterine spiral artery wall in the decidua was found in the 0.125 mg Cd/kg group (p < 0.001); the renal protein level in the Cd-treated pregnant rats was also significantly higher than that of the animals in the normal pregnancy group and of those in the saline-treated pregnant group (p < 0.001) (Fig. 2F). There was no difference in the level of protein excretion in the urine between the (non-pregnant + Cd) group and the non-pregnant group.

3.3. Induction of preeclampsia-like syndrome by cadmium chloride does not occur via its toxicity

To confirm that the histological damage observed in pregnant rats treated with low doses of CdCl2 was not due to its toxicity, we administered various doses of CdCl2 to pregnant animals. Throughout the dosing period, maternal daily weight gain was significantly lower in the 1 mg/kg Cd group than in the 0.125 mg/kg Cd group (p < 0.05). As shown in Fig. 3B, administration of 1 mg of Cd/kg b.w. resulted in reduced capillary space in the kidney at 48 h post-injection (Fig. 3B1). Fatty degeneration of hepatocytes was found in the 1 mg/kg Cd group (Fig. 3B2). This group of animals also showed neutrophil infiltration into the placenta at 48 h post-treatment (Fig. 3B3). These phenomena were not observed in the 0.125 mg/kg Cd group at 48 h after exposure. These results provide evidence that the effects of hypertension induced by low doses of CdCl2 were probably not mediated via its toxicity.

3.4. Cadmium enhances LPS- and IL-4-mediated AID expression in B cells via the oestrogen receptor

To determine the effects of cadmium on activation-induced cytotoxic deaminase (AID) induction, we measured the expression of AID in rats spleen. We found that AICDA mRNA and AID protein expression was significantly higher in rats of the (NP + Cd group) than in NP rats (Fig. 4A, B). To further address the role of cadmium in AID induction in B lymphocytes, we used LPS and recombinant rat IL-4 (or no addition as a control) to induce rat B cells to undergo CSR in the presence of 1 μM, 5 μM, 10 μM or 50 μM Cd for 48 h. In order to confirm that cadmium has potent oestrogen-like activity, 17β-oestrogen was used as a positive control for E2 in parallel experiments. Using qRT-PCR, we were able to determine that CdCl2, as well as 17β-oestradiol (Mai et al., 2010) alone, slightly increased AICDA mRNA expression in isolated splenic B cells and that this powerfully enhanced AICDA mRNA expression in B cells stimulated by LPS and rIL-4. The enhancement of AICDA mRNA expression by CdCl2 occurred in a dose-dependent manner and was almost abolished at the highest CdCl2 dose (50 μM) (Fig. 4C, D). Fig. 4E and F show the proliferation and viability of the B lymphocytes after these treatments. CdCl2 did not alter B cell proliferation or viability until the concentration reached 50 μM. Based on these results, 10 μM CdCl2 was chosen as a suitable dose for B cells.

To determine how cadmium enhances LPS/rIL-4-mediated AID induction in B cells, ICI182780, G15 or MG-132 was added to the cells 30 min prior to administration of the indicated amount of CdCl2. The CdCl2-mediated up-regulation of AID induction was abrogated by ICI182780, an oestrogen receptor antagonist, but not by the other compounds (Fig. 4G, H). It was concluded that cadmium, like oestrogen, can induce AID and that these signalling events are mediated by possible interaction with ERs but not by interactions with membrane oestrogen receptor or NF-κB.

3.5. Cadmium up-regulates AT1-AA and induces hypertension via AT1R activation

3.5.1. Stimulatory effect of cadmium on immunoglobulin production in B lymphocytes and AT1-AA levels in pregnant rats

To determine whether cadmium causes increased IgG production and the role of AID in this process, the effect of CdCl2 at 10 μM concentration on spontaneous immunoglobulin production by rat B cells was examined. CdCl2 significantly enhanced IgG production (29.48 ± 2.36 μg/ml) compared with control cells (5.93 ± 2.63 μg/ml) (p < 0.05) (Fig. 5A). After co-culture with ICI182780, immunoglobulin production was detected, and the stimulatory effect of Cd was reduced.

AT1-AA levels increased from 71.09 ± 1.76 ng/L to 80.96 ± 2.62 ng/L after intraperitoneal injection of CdCl2 into normal pregnant rats (p < 0.05, Fig. 5B).

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>Placental weight (g)</th>
<th>Implanted embryos (n)</th>
<th>Resorbed and stillborn foetuses (n)</th>
<th>Live foetuses (n)</th>
<th>Female foetal weight (g)</th>
<th>Male foetal weight (g)</th>
<th>Crown-rump length (cm)</th>
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<td>NP group</td>
<td>0.55 ± 0.09</td>
<td>14.75 ± 0.86</td>
<td>0.25 ± 0.25</td>
<td>14.50 ± 0.65</td>
<td>5.51 ± 0.10</td>
<td>6.22 ± 0.11</td>
<td>3.82 ± 0.10</td>
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<tr>
<td>NP + NSgroup</td>
<td>0.54 ± 0.07</td>
<td>15.00 ± 0.91</td>
<td>0.13 ± 0.13</td>
<td>14.88 ± 0.83</td>
<td>5.40 ± 0.10</td>
<td>6.18 ± 0.13</td>
<td>3.79 ± 0.26</td>
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<tr>
<td>Cpd group</td>
<td>0.55 ± 0.12</td>
<td>14.75 ± 0.25</td>
<td>0.25 ± 0.25</td>
<td>14.50 ± 0.29</td>
<td>4.71 ± 0.06*</td>
<td>5.26 ± 0.14*</td>
<td>3.12 ± 0.28*</td>
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</table>

Data are the mean ± SEM *p < 0.05 compared with the NP control group.
Fig. 3. Haematoxylin-eosin staining for pathological analysis of tissue specimens. (A) Kidney, decidua, placenta and aorta specimens were obtained from CdCl2-treated (0.125 mg of Cd/kg b.w.) rats and normal-pregnancy rats on day 21 of pregnancy. The photos in the upper panel every pair show tissues from the Cd-treated group and the lower panel photos are from the control group. Haematoxylin-eosin staining was used for pathological analysis. (A1, A2) Representative images of the kidney (original magnification 200×). The black arrow in (A1) indicates swelling of endothelial cells that appears to reduce the capillary space (glomerular endotheliosis) and the yellow arrow in (A1) indicates thickening of the media of renal vessel walls via smooth muscle cell proliferation; the arrows in (A2) indicate protein in renal tubules. (A3) Representative images of the decidua (original magnification 200×). The arrows in (A3) indicate thickening of the uterine spiral artery wall in decidua of the NP + Cd group. (A4, A5) Representative images of the placenta (original magnification 200×). Degeneration in the placental labyrinth and placental thickening in the media of vessel walls (black arrow) are shown in (A4) and (A5), respectively. (A6) Representative images of the aorta (original magnification ×200). The black arrows indicate rupture of the internal elastic membrane of the aorta. (B) Kidney, liver and placenta specimens were obtained from pregnant rats that received various doses of CdCl2. The photos in the upper panel every pair show tissues obtained 48 h after a single injection of 1 mg of Cd/kg; the photos in the lower panel show tissues obtained 48 h after injection of 0.125 mg of Cd/kg for six days. (B1) The black arrow indicates reduced capillary space in the kidney (original magnification 200×). (B2) The yellow arrows indicate fatty degeneration of hepatocytes (original magnification 400×). (B3) The red arrows indicate enhanced neutrophil infiltration into the placenta (original magnification 400×) in an animal that received a single injection of 1 mg of Cd/kg. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)
Fig. 4. Regulation of expression of AICDA by cadmium in the spleen and in B lymphocytes. Upregulation of AICDA was induced by Cd in rat spleen and B lymphocytes. (A) and (B) AICDA mRNA and protein level in rats spleen was analysed by RT-PCR and western blot. Cd enhanced expression of AICDA in the spleens of pregnant rats compared with controls. (C) Cd as well as oestrogen alone increased AICDA mRNA expression slightly. (D) Cd enhanced AICDA expression in a dose-dependent manner in B cells stimulated by LPS and IL-4. (E) and (F) Proliferation and viability of B lymphocytes treated with the indicated concentrations of Cd for 48 h. Cd did not alter B cell proliferation or viability until the concentration reached 50 μM. (G) and (H) The indicated amounts of DMSO, ICI182780, G15, and MG-132 were added to B cells 30 min prior to Cd administration, respectively. Cadmium-mediated up-regulation of AID induction was abrogated by ICI182780. The data shown are representative of three independent experiments.
3.5.2. Hypertension in response to cadmium is blocked by an AT1 receptor antagonist

Next, to determine whether CdCl2-induced hypertension occurs via AT1R activation, we co-injected CdCl2 with losartan, an AT1R blocker. We found that the increased blood pressure response to CdCl2 (134.00 ± 2.27 mmHg) was markedly decreased by losartan (102.30 ± 2.66 mmHg) (p < 0.001, Fig. 5C). This finding illustrates that the CdCl2-induced hypertension was mediated via AT1 receptors.

3.6. Cadmium-mediated complement C5a receptor activation contributes to the pathogenesis of preeclampsia

3.6.1. C5a concentrations in the blood of preeclamptic rats

The results with animals showed that the level of C5a in plasma was significantly higher in Cd-treated pregnant rats (177.70 ± 14.61 μg/ml) than in rats in the normal pregnancy group (117.00 ± 5.67 μg/ml) or the Cd-losartan group (103.00 ± 6.63 μg/ml) (p < 0.01, Fig. 6A). This finding indicates that CdCl2-induced hypertension was mediated via AT1 receptors.

3.6.2. C5 activation of pregnant rats occurs via AT1R activation

This preeclamptic animal model with cadmium administration is a valuable investigative tool to determine whether AT1-AA contributes to elevated C5. Injected rats were euthanized on GD21, and the C5 deposition in kidneys was examined. Immunohistochemical analysis revealed that C5 deposition in the renal tubular epithelial cells of pregnant rats injected with CdCl2 (Fig. 6B) was significantly enhanced compared with Cd5 deposition in the kidney tissues of animals in the normal pregnancy groups. Next, to determine whether CdCl2-induced C5 deposition is via AT1R activation, we co-injected CdCl2 with losartan. The result showed that losartan significantly reduced C5 deposition in the kidney of pregnant rats injected with CdCl2. These results showed that AT1-ΑΑ, which was up-regulated by CdCl2, stimulating C5 deposition in the kidneys of the pregnant rats via AT1R.

3.6.3. Blocking C5aR activation attenuates cadmium chloride-induced hypertension and proteinuria in pregnant rats

Although we observed an increase in C5aR mRNA in placentas from rats treated with CdCl2 compared to placentas from normal pregnant rats (Fig. 7A), it was essential to determine whether elevated C5 activation signalling via C5aR contributes to the pathophysiology of PE. We treated pregnant rats with PMX53, a C5aR antagonist, and then with CdCl2. We found that the increased systolic blood pressure seen in CdCl2-injected pregnant rats was notably attenuated by administration of PMX53 (p < 0.05, Fig. 7B). An injection of normal saline (NS), which is the solvent used for PMX53, had no effect on CdCl2-induced hypertension. There was no difference in SBP between the normal pregnancy groups treated with no injection or with PMX53. These findings demonstrate that CdCl2-induced hypertension via increased AT1-AA was significantly inhibited by administration of a C5aR antagonist.

The level of urinary protein was also observed to decrease in pregnant rats treated with Cd-PMX53 compared with CdCl2-treated pregnant animals. NS, the solvent for PMX53, had no effect on CdCl2-induced proteinuria. Animals in the normal pregnancy groups treated with no injection or with PMX53 showed no difference in urinary protein levels (Fig. 7C).
3.6.4. Renal and placental damage induced by cadmium chloride are prevented by treatment with a C5aR antagonist

Assessment of kidney tissue by haematoxylin-eosin staining indicated that CdCl2-injected pregnant rats had damaged kidneys; in these animals, swelling of endothelial cells appeared to reduce the capillary space (glomerular endotheliosis), and smooth muscle cell proliferation occurred. Blocking C5aR activation by PMX53 significantly attenuated renal damage (Fig. 7D).

Defective angiogenesis is important in the process of PE. In order to clarify the angiogenesis in placentas of PE, we quantitatively evaluated angiogenesis by CD34 immunostaining. CD34 staining in the placentas of CdCl2-injected pregnant rats was significantly decreased. PMX53 treatment significantly increased CD34 staining in the placentas of CdCl2-injected pregnant animals (Fig. 7E).

4. Discussion

In this study, we demonstrated that a higher level of cadmium is present in the peripheral blood of preeclamptic women than in that of women undergoing normal pregnancies. Hypothesising that cadmium is closely related to preeclampsia, we exposed pregnant rats to low doses of CdCl2 and assessed them for parameters linked to PE. We found that administration of CdCl2 at concentration of 0.125 mg Cd/kg body weight of to pregnant rats daily from GD 9 to 14 increased blood pressure and proteinuria. Pathological changes in the maternal placenta, kidney and blood vessels typical of those seen in preeclampsia were also observed in the pregnant rats after low-dose of CdCl2 treatment. Thus, a novel rat model of preeclampsia was established.

Growing evidence indicates that altered immune mechanisms may have an important role in the pathophysiology of preeclampsia. PE is associated with the presence of pathogenic AT1- AA, which activates the AT1R. However, it is unclear what factors contribute to the production of AT1- AA in PE. In this study, we demonstrated that administration of CdCl2 to pregnant rats significantly increased AT1- AA levels. Females may have stronger and more rapid immune responses than males upon antigen encounter (Kovats, 2015), and oestrogen has been shown to have a role in establishing a gender bias in autoimmunity (Li and McMurray, 2007; Nalbandian and Kovats, 2005). Cadmium is a known endocrine disruptor (Knapicka et al., 2015). Previous studies have shown that cadmium acts like steroidal oestrogens in breast cancer cells, shown by its ability to form a high-affinity complex with the hormone-binding domain of the oestrogen receptor (Stoica et al., 2000). Johnson et al. found that cadmium also has potent oestrogen-like activity in vivo. Exposure to cadmium increased uterine wet weight and induced hormone-regulated gene expression in ovariectomized animals (Johnson et al., 2003). Thus, it is vital to determine whether cadmium, as well as oestrogen, has an important role in the immune system.

During the normal immune response, activation-induced cytidine deaminase (AID) is expressed in a B cell differentiation stage-specific manner. AID is essential for immunoglobulin (Ig) gene CSR and somatic hypermutation (SHM) (Laffleur et al., 2014). AID is tightly controlled, and AID levels are critical in balancing efficient immunity with an autoimmune state. Whereas lack of AID results in primary immunodeficiency, up-regulation of AID expression is associated with autoantibody-mediated autoimmune diseases such as systemic lupus (Zan and Casali, 2013). Oestrogen upregulates the expression of AID. SiimPauklin (Pauklin et al., 2009) found that the oestrogen-oestrogen receptor complex binds to the AID promoter and enhances AID messenger RNA expression, leading to an increase in AID protein levels and alterations in SHM and CSR at the immunoglobulin locus. In this way, oestrogen can enhance immunoglobulin production by up-regulating the expression of AID (Asaba et al., 2015). Cadmium has the potential to mimic the endogenous steroid hormone E2. Accordingly, we hypothesised that cadmium, like oestrogen, might cause IgG production via the induction of AID expression (Strumylaite et al., 2014). In the present study, the metalloestrogen cadmium, as well as 17β-oestradiol alone, slightly increased AICDA mRNA expression in B cells but powerfully enhanced AICDA mRNA expression in B cells stimulated by LPS and rIL-4. This phenomenon suggests that cadmium potentiates AID expression only or mainly in the course of specific antibody responses such as those triggered by microbial pathogens. It has been reported that cadmium can activate NF-κB (Yuko et al., 2007), oestrogen receptor alpha (Pauklin et al., 2009), and the membrane oestrogen receptor GRP30 (Yu et al., 2010). Our study showed that pre-incubation with a nuclear oestrogen receptor antagonist but not with a NF-κB inhibitor or a membrane oestrogen receptor antagonist suppressed the enhancement of AID by CdCl2. It is concluded that cadmium increases immunoglobulin production mainly by increasing AID gene expression via nuclear oestrogen receptors. The results also support the idea that cadmium may act as an important stimulator of humoral immunity. We therefore suggest that Cd-induced autoimmunity may derive from AID-dependent DNA instability. The ability of an antiestrogen to block the effects of CdCl2 suggests that the effects of the metal are mediated by the oestrogen receptor.

Consistent with the idea that cadmium, like oestrogen, can disturb the immune system and increase the production of auto- antibodies, a marked increase in the concentration of AT1- AA was
Fig. 7. C5aR activation attenuates cadmium-induced hypertension, proteinuria and tissue damage in pregnant rats. (A) The level of C5aR mRNA in placentas from rats treated with CdCl2 increased compared with that in the placentas of animals undergoing normal pregnancy. (B) Pregnant rats were treated with PMX53 (a C5aR antagonist) and then infused with CdCl2. The increased systolic blood pressure seen in Cd-injected pregnant rats was notably attenuated by administration of PMX53 (p < 0.05). Normal saline (NS), the solvent for PMX53, had no effect on CdCl2-induced hypertension. There was no difference in SBP between the normal pregnancy groups treated with no injection and those treated with PMX53. Urine protein excretion is shown in (C). (D) Kidneys assessed by haematoxylin-eosin staining indicate that blocking C5aR activation by PMX53 significantly attenuated renal damage. (E) Rat placental angiogenesis was assessed by CD34 dual immunostaining. CD34 staining in the placentas of Cd–injected pregnant rats was significantly decreased. PMX53 treatment significantly improved CD34 staining in the placentas of Cd–injected pregnant rats. All data are expressed as the mean ± SEM.
observed in rats treated with CdCl₂. Moreover, we found that CdCl₂-induced hypertension in pregnant rats was attenuated by oral administration of the AT1 receptor antagonist losartan. These data indicate that increased levels of AT1-AA in response to low-dose of CdCl₂ play an important role in hypertension during pregnancy via AT1R activation.

A well regulated complement system is a prerequisite for a healthy pregnancy. We investigated the possible contributory role of increased complement activation by elevated AT1-AA levels to the pathogenesis of PE. Complement is reportedly increased in the circulation of preeclamptic women, but studies have mainly focused on C3a (Lynch et al., 2011). Only a few studies have demonstrated that C5a levels are increased in preeclamptic women; the exact cause of the increased C5a level remains unclear, and its pathogenic role is poorly understood. Here, using a novel Cd-induced model of PE in pregnant rats, we demonstrated that increased autoantibody-mediated AT1R activation induces C5 deposition in kidney and C5a production. The fact that the effect could be blocked by co-injection of the animals with losartan provided additional evidence for its specificity.

Eventually, the important anaphylatoxin C5a is produced by all three pathways in the terminal cascade (Ehrnthaller et al., 2011). Existing data suggest that C5a is a critical complement component that contributes to tissue injury by activating C5aRs on target cells (Li et al., 2012). C5a-induced C5aR activation has a deleterious role in SLE and in neural disorders, including pain and Alzheimer's disease (Mahajan et al., 2015; Landlinger et al., 2015; Liang et al., 2012). Inhibition of C5a or C5AR is beneficial for protection against these diseases in animal models. Here, we provide strong evidence that interfering with C5aR activation by a specific antagonist significantly attenuates almost all of the features of PE seen in the CdCl₂-induced preeclamptic model, including hypertension, proteinuria, and impaired placental angiogenesis. The present study highlights the role of C5a/C5AR signalling in CdCl₂-induced PE in pregnant rats.

5. Conclusions

The results of the in vivo and in vitro studies described in this work show that cadmium-mediated immune abnormalities, as well as oestrogen, are key mechanisms underlying the features of PE induced by low doses of CdCl₂ in pregnant rats. Our data demonstrate that cadmium increases the production of immunoglobulins, mainly AT1-AA, by increasing the expression of AID in B cells. In addition, AT1-AA-mediated AT1 receptor (AT1R) activation is responsible for C5 elevation. Elevated C5a functions via C5AR activation and contributes to key features of PE seen in the CdCl₂-injection model of PE (Fig. 8). These findings suggest novel therapeutic options for the management of this serious condition.

Competing interests

The authors declare that they have no competing interests.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.envpol.2016.07.073.

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