Prorenin Receptor and ERK Are Associated with Kidney Development in the Fetal Rat administered Prenatal Glucocorticoid

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Abstract

The hypothesis of the “development origins of health and diseases” addresses the risk of chronic kidney disease in adulthood. This study aimed to investigate whether prenatal glucocorticoid (GC) administration is associated with fetal kidney maturation. We investigated the effects of prenatal GC administration on the expression of the prorenin receptor (PRR) and extracellular signal-regulated kinase (ERK) required for development in the fetal rat.

Dexamethasone (DEX) was administered to pregnant rats for 2 days on days 17 and 18 or days 19 and 20 of gestation, and the kidney tissues of 19- and 21-day fetuses and 1-day-old neonates were analyzed by immunohistochemistry. The viability of human embryonic kidney (HEK)293 cells exposed to DEX for 24 h was determined by MTT assay, and mRNA and protein expressions of the PRR, ERK, and phospho(p)-ERK were analyzed using real-time PCR and Western blotting.

ERK-positive areas were observed in primitive perivascular mesenchymal cells and immature glomeruli of the fetal rats. ERK-positive areas were significantly increased in the kidneys of 21-day fetuses compared with those of 19-day fetuses. DEX tended to increase ERK- and p-ERK-positive areas in the kidney of 19-day fetuses, and their levels tended to reach the expression levels of 21-day fetuses. Although the number of PPR-positive areas did not change with DEX administration, they were localized in ureteric bud branches and collecting ducts. DEX also significantly increased the mRNA and protein levels of ERK, p-ERK, and PRR in HEK293 cells.

Taken together, these results indicate that prenatal DEX administration may contribute to kidney development through an increase in ERK in the immature fetal rat.

Key words
ERK, prorenin receptor, prenatal glucocorticoid administration, kidney, fetus

Introduction

Nephrogenesis is still continuing in infants born preterm, and the kidneys remain vulnerable. The hypothesis of the “development origins of health and diseases” proposes that preterm infants are at risk of developing chronic kidney disease in adulthood. Glomerular and tubular immaturity may therefore have adverse consequences for life-long renal health. Prenatal glucocorticoid (GC) therapy after preterm delivery has been shown to help the lungs mature and to prevent breathing difficulties such as respiratory dis-

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tress syndrome. In addition, several reports indicated that prenatal GC therapy increases organizational maturation\(^2\). However, the optimal prenatal GC dose and treatment regimens have not yet been established and vary among institutions.

Animal-based studies are one means through which key questions on prenatal GC dosing and safety may be resolved. Prenatal GC administration was shown to lead maturation of the lungs and heart in rat models\(^3\)\(^-\)\(^5\), whereas in the kidney, it was reported to increase the glomerular filtration rate and renal development\(^6\), however, exposure to GC resulted in impairment of renal function in infant rams\(^7\).

The renin-angiotensin system (RAS) plays an important role in development of the mammalian kidney. It is involved in extracellular volume and salt metabolism and in the regulation of blood pressure. Mice lacking a functional RAS develop renal failure\(^8\)\(^-\)\(^10\). The prorenin receptor (PRR) not only activates the RAS but also stimulates the intracellular signaling pathways of the receptor itself independent of the RAS. The existence of a receptor for renin in its inactive precursor prorenin form was cloned in 2002 and named ATPase 6 accessory protein 2 (ATP6AP2)/PRR\(^11\). The PRR triggers intracellular signaling involving ERK\(^12\)\(^13\), which accelerates cell proliferation and differentiation. ERK is highly expressed in the embryonic rat kidney, and its expression correlates with kidney development\(^14\)\(^15\). However, to our knowledge, the effects of prenatal GC administration on the expression of the PRR and ERK in fetal rats have not been investigated. We therefore attempted to determine whether prenatal GC administration affects the expression of ERK and the PRR in the kidneys of fetal rats.

**Materials and Methods**

**Animal experiments**

Pregnant Wistar rats at 8 weeks of gestation were purchased from CLEA (Tokyo, Japan). The animals were maintained under conditions of constant humidity (55 ± 5%) and temperature (23 ± 1°C) with an electrically controlled 12-h light–dark cycle. Dexamethasone (DEX) was obtained from Wako Pure Chemical Industries (Osaka, Japan) and dissolved in sesame oil. The pregant rats were randomly divided into 2 groups to receive either DEX or vehicle (sesame oil) as a control at a dose of 1.0 or 2.0 mg/kg administered subcutaneously on days 17 and 18 or days 19 and 20 of gestation. In humans, prenatal administration of GC is performed for threatened abortion on the 24th to 34th weeks of pregnancy. We determined that the schedule of the animal experiment should correspond to this period of human gestation. The fetuses were delivered by cesarean section on gestational days 19 and 21, respectively, and the 1-day-old neonates were euthanized under isoflurane anesthesia. The kidney tissues were immediately removed and frozen at –80°C until use.

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Institute of Experimental Animals, St. Marianna University School of Medicine. The protocol was approved by the Animal Research Committee, Institute for Animal Experimentation, St. Marianna University Graduate School of Medicine (permit number: 1802011). All efforts were made to minimize animal suffering.

**Immunohistochemistry**

Paraffin-embedded renal tissues were cut into slices 3-μm thick and then deparaffinized and rehydrated. Endogenous peroxidase activity in tissues was quenched with 0.3% H\(_2\)O\(_2\). Immunoreactivity in the sections was shown using the Envision System (Agilent Technologies, Santa Clara, CA, USA) according to the manufacturer’s instructions. Finally, tissues were counterstained with hematoxylin. Observations were carried out under an Olympus CX41 microscope (Tokyo, Japan).

The positively stained areas in each kidney were measured with a WinROOF image analyzer (Mitani Co., Tokyo, Japan) and expressed as the ratio of the relative to total area of the renal sections.

**Cell culture**

Human embryonic kidney cells (HEK293 cell line) were cultured in Dulbecco’s modified Eagle’s medium (Thermo Fisher Scientific, Tokyo, Japan) or supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin while maintained at a constant temperature of 37°C under an atmosphere of 5% CO\(_2\).

**MTT assay**

HEK cells (3×10\(^3\)) were cultured with DEX for 24 h on 96-well collagen type I-coated flat-bottomed plates (AGC Techno Glass, Corning, NY, USA) in 100 μL of the medium. Cell viability was evaluated with a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-
zolum bromide (MTT) assay kit (Sigma-Aldrich, St. Louis, MO, USA).

After incubation, 10 µL of the MTT substrate was added to each well, and the plates were incubated for a further 4 h. Absorbance at 570 nm was measured with a microplate reader (Viento nano, Bio-Tek Instruments, Inc., Winooski, VT, USA). Proliferation of the cells was expressed as the ratio of the optical density of the DEX-treated cells to that of the untreated cells.

**Real-time reverse transcription-polymerase chain reaction (RT-PCR)**

Total RNA in HEK293 cells was extracted using a Sepasol-RNA I Super G kit (Nacalai Tesque, Kyoto, Japan). cDNA was synthesized from 1 µg of total RNA using a Transcriptor First Strand cDNA Synthesis Kit (Roche Diagnostics, Basel, Switzerland). Quantitative analyses of cDNA were performed using the StepOnePlus System (Thermo Fisher Scientific, Waltham, MA, USA). The cycling protocol consisted of one cycle of 30 s at 95°C, followed by 45 cycles of denaturation for 15 s at 95°C, annealing for 30 s at 72°C, and extension for 40 s at 72°C. The primers were as follows: for ERK1, 5'-CGCTACACGCAGTTGCAGTACA-3' (sense) and 5'-AAGCGCAGCAGGATCTGGA-3' (antisense); for ERK2, 5'-TGTTCCCAAATGCTGACTCCAA-3' (sense) and 5'-TCGGGTCGTAATACTGCTCCAGATA-3' (antisense); for the renin receptor, 5'-CATTGTCCATGGGCTTCTCT-3' (sense) and 5'-GCATTCTCCAAAGGGTACGA-3' (antisense); and for glyceraldehyde 3-phosphate dehydrogenase (GAPDH), 5'-TGAACGGGAAGCTCACTGG-3' (sense) and 5'-TCCACCACCTGTTGCTGTA-3' (antisense). Standard curves were plotted (Ct versus log cDNA), and sample quantities were determined from these curves. Data on expression were analyzed using the multiplex comparative threshold method, in which the gene level was normalized to that of the housekeeping genes for GAPDH in each sample.

**Western blotting**

The cells were homogenized with CelLytic M solution (Sigma-Aldrich). Cell debris was removed by centrifuging the samples for 30 min at 15,000 rpm at 4°C. Each sample (20 µg) was resolved using 5–20% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (e-PAGEL, ATTO, Tokyo, Japan). The membrane was blocked overnight with 5% skim milk in Tris-buffered saline containing Tween (TTBS; NaCl 150 mmol, Tris-HCl 100 mmol/L, pH 7.5, Tween 0.5%) overnight at 4°C. The blots were probed with anti-ERK polyclonal IgG antibody (Ab), anti-phosphorylated p-ERK polyclonal Ab (Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-ATP6IP2 (prorenin) polyclonal Ab (Abcam, Tokyo, Japan), and anti-α-tubulin polyclonal antibody (Wako, Tokyo, Japan) for 2 h at room temperature. Reactive proteins were viewed under enhanced chemiluminescence (ImmuNoStar, Wako). The band density was detected using a C-Digit Blot scanner (LI-COR, Lincoln, NE, USA), and the intensity of the detected bands was analyzed using Image Studio Software version 4.0.21 (LI-COR).

**Statistical analysis**

All results are presented as mean ± SEM. Differences between groups were analyzed for statistical significance using Dunnett’s test. A *P* value of less than 0.05 was considered to represent a statistically significant difference. Analysis was performed using JMP software version 13.2.1 (Tokyo, Japan).

**Results**

**ERK and p-ERK expression in kidney tissues**

ERK was expressed in the undifferentiated mesenchyme in the nephrogenic zone and ureteric bud (UB) branches in the kidneys of the 19-day fetal controls (Fig. 1A, B). It was also expressed in mesenchymal cells, blastemas, vesicles, and UB branches in the 19-day DEX-treated groups. ERK-positive areas in the 19-day DEX-treated groups indicated ERK expression levels similar to those in the 21-day fetal controls (Fig. 1C). Although we found widespread expression of ERK in mesenchymal cells, blastemas, and vesicles in the 21-day fetal controls, DEX administration did not change the expression of ERK. ERK expression gradually decreased with the maturation of the kidneys and was localized in the distal tubules in the 1-day-old neonates and adult rats (Fig. 2A).

**p-ERK-positive areas were stained in almost the same places as ERK-positive areas (Fig. 2A, B). Similar to ERK, p-ERK-positive areas in the 19-day DEX-treated groups showed that they had nearly the same expression levels of ERK as seen in the 21-day fetal controls (Fig. 2C). Furthermore, p-ERK expression gradually decreased with the maturation of the kidneys and was localized in the distal tubules in the 1-day-old neonates and adult rats (Fig. 2A).**
PRLR expression in kidney tissues

The PRLR was widely expressed in the undifferentiated mesenchyme in the nephrogenic zone and in UB branches in the kidneys of the 19-day fetal controls (Fig. 3A, B). PRLR expression in the DEX-treated groups was localized in UB cells and tended to decrease in the 1-day-old neonates. PRLR was widely found in some glomeruli, the proximal tubules, and collecting ducts in the adult rats (Fig. 3A, B). The number of PRLR-positive areas did not change in the fetal kidneys after DEX administration (Fig. 3C).

Cell viability in DEX-treated HEK293 cells

To investigate the effects of DEX on fetal kidney cells, we next evaluated the viability of HEK293 cells after DEX administration in the in vitro MTT assay. Little change in HEK293 cell viability was seen after co-culture with DEX (0.03–10 μM). However, cell viability was significantly decreased in HEK293 cells after treatment with DEX at 30 μM and 100 μM (Fig. 4).

PRLR and ERK mRNA and protein expression in DEX-treated HEK293 cells

When the mRNA and protein expression of the PRLR and ERK in DEX-treated HEK293 cells was examined, PRLR mRNA levels did not change in HEK293 cells after co-culture with DEX. Protein levels of the PRLR also remained unchanged. The mRNA levels of ERK1 and ERK2 increased in the DEX-treated HEK293 cells, whereas the protein levels of ERK and p-ERK showed results similar to those of the mRNA levels (Fig. 5).

Discussion

There is evidence showing that individuals born prematurely exhibit an elevated risk for the development of hypertension and renal disease later in life, thus supporting the developmental origins of disease hypothesis. Nephrogenesis is ongoing in preterm infants, and they have fewer nephrons at birth in proportion to gestational age. Although the prenatal administration of steroids decreases the mortality and pulmonary morbidity of premature infants, the effects on renal structure and function remain to be clarified.
Sheen et al. reported that prenatal maternal DEX administration results in an imbalance between endothelium-derived hyperpolarizing and contractile factors that might lead to renal programming and hypertension, suggesting long-term effects on offspring health\(^1\). Adult offspring of rats that received DEX on days 15 and 16 of gestation were shown to have fewer nephrons and to develop hypertension\(^1\). In contrast, antenatal GC exposure was associated with an increase in the number of mature nephrons\(^1\).

The present study investigated whether prenatal GC administration exerts beneficial effects on the fetal kidney, focusing on the key effectors ERK and the PRR associated with kidney maturation\(^1\). Our results showed that prenatal DEX administration tended to increase ERK expression in the 19-day fetuses. ERK was expressed in the undifferentiated mesenchyme in the nephrogenic zone and UB branches in the 19-day fetal kidneys, whereas ERK was expressed in mesenchymal cells, blastemas, vesicles, and UB branches in 19-day fetal kidneys after prenatal DEX administration. Furthermore, ERK-positive cells were observed in glomerular mesangial cells in the DEX 2.0 mg/kg-treated groups. Thus, ERK expression changed with nephrogenesis. ERK-positive areas in the 19-day DEX-treated kidneys indicated similar ERK expression levels as in the 21-day fetal controls. p-ERK-positive areas were stained in almost the same places as ERK-positive areas.

**ERK expression was reduced in adult kidneys**

The present in vitro study showed that co-culture with DEX increased ERK and p-ERK levels in HEK293 cells. The ERK MAP-kinase pathway is required for UB morphogenesis\(^1\). Although low levels of ERK expression were seen in immature tubular segments, ERK and p-ERK were intensely detected in distal tubules and collecting ducts in the normal human fetal kidney from gestational ages 19 to 34 weeks\(^1\). Omori et al. reported that the localization of ERK and p-ERK generally correlates with the distribution of proliferating cells during kidney develop-
ERK plays a pivotal role in cellular proliferation and differentiation. Therefore, we studied whether DEX treatment increases ERK levels in HEK293 cells. Although co-culture with DEX at doses of 0.03 μM to 10 μM did not affect cell viability, DEX 30 μM and 100 μM significantly decreased the viability of HEK293 cells, indicating that the increases in ERK were independent of viability. Large-scale cell proliferation and cell death were observed to occur in the developing fetal kidney. Apoptotic cells were seen in immature glomeruli, collecting ducts, and the interstitium in fetal rats, and the apoptosis contributes to kidney development. The PRR was named after its biological characteristics, i.e., the binding of renin and of its inactive precursor prorenin, which trigger intracellular signaling involving ERK. We speculate that high expression levels of the PRR are required during fetal kidney development and that prenatal DEX administration upregulates PRR expression. PPR binding was shown to activate ERK directly via epidermal growth factor receptor-dependent signal transduction in rat vascular smooth muscle cells. Our results showed
that PRR-positive areas did not change in fetal kidneys after prenatal DEX administration. In addition, PRR expression in HEK293 cells co-cultured with DEX showed no tendency to increase in the in vitro study. PRR mRNA and protein were detected in the UB branches of early embryonic mouse kidneys\(^26\). The PRR present in the UB epithelia was found to play essential roles during the morphogenesis of UB branching and development of collecting ducts via gene expression of the Ret/Wnt11 pathway, UB cell survival, ERK activation, and terminal differentiation and function of collecting duct cells\(^{26(27)}\). Although the number of PRR-positive areas was not significantly increased after prenatal DEX administration, the localization of PRR immunoreactivity was most prominent in mature tubules, which resemble collecting ducts morphologically, suggesting that the PRR is required for kidney development.

The PRR-ERK pathway may promote the function of the RAS and contribute to postnatal kidney growth and maturation. However, increased PRR and ERK expression alone do not account for the fetal kidney development that occurs after prenatal DEX administration. Further studies are needed to elucidate the PRR-ERK signaling mechanisms for kidney development after prenatal DEX administration.

In conclusion, the aim of this study was to evaluate the effects of prenatal GC administration on nephrogenesis in preterm rats. The results suggested that prenatal GC administration increases the expression of ERK through the PRR and that ERK is involved in the process of kidney maturation.

Conflict of interest

The authors have no conflicts of interest to declare.

References

Fig. 5. mRNA and protein levels of the PRR and ERK in HEK293 cells co-cultured with DEX.
(A) Graphic representations of mRNA levels of PRR, ERK1, and ERK2. The respective mRNA levels relative to GAPDH mRNA are shown as mean ± SEM. Data are representative of 4 independent experiments. *P* < 0.05 vs. controls.
(B) Graphic representations and results of Western blotting of protein levels of PRR, ERK, and p-ERK in HEK293 cells co-cultured with DEX for 24 h. α-Tubulin was used as a control. Values are shown as mean ± SEM and are representative of 5 independent experiments. *P* < 0.05 vs. controls.

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