ORIGINAL

Expression of NADPH oxidase and production of reactive oxygen species contribute to ureteric bud branching and nephrogenesis

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Abstract : Ureteric bud branching and nephrogenesis are performed through large-scale proliferation and apoptosis events during renal development. Reactive oxygen species (ROS), produced by NADPH oxidase, may contribute to cell behaviors, including proliferation and apoptosis. We investigated the role of NADPH oxidase expression and ROS production in developing kidneys. Immunohistochemistry revealed that NADPH oxidase components were expressed on epithelial cells in ureteric bud branches, as well as on immature glomerular cells and epithelial cells in nephrogenic zones. ROS production, detected by dihydroethidium assay, was strongly observed in ureteric bud branches and nephrogenic zones, corresponding with NADPH oxidase localization. Organ culture of E14 kidneys revealed that the inhibition of NADPH oxidase significantly reduced the number of ureteric bud branches and tips, consistent with reduced ROS production. This was associated with reduced expression of phosphorylated ERK1/2 and increased expression of cleaved caspase-3. Organ culture of E18 kidneys showed that the inhibition of NADPH oxidase reduced nephrogenic zone size, accompanied by reduced ROS production, fewer proliferating cell nuclear antigen-positive cells, lower p-ERK1/2 expression, and increased expression of cleaved caspase-3. These results demonstrate that ROS produced by NADPH oxidase might play an important role in ureteric bud branching and nephrogenesis by regulating proliferation and apoptosis. J. Med. Invest. 66 : 93-98, February, 2019

Keywords : *developing kidney, NADPH oxidase, oxidative stress*

INTRODUCTION

Reactive oxygen species (ROS) have been implicated as potential mediators in the development and progression of chronic kidney diseases (CKD), including diabetic nephropathy ; therefore, they have been suggested as therapeutic targets for the prevention of CKD progression. There are several potential pathways for the enhanced generation of ROS in CKD (1). NADPH oxidases comprise a family of multi-subunit enzyme complexes with the primary function of superoxide and/or hydrogen peroxide production (2). The prototype NADPH oxidase (Nox2 [gp91phox]-containing NADPH oxidase), found in phagocytic cells, possesses cytosolic subunits (p47phox, p67phox, or homologues) and membrane-bound subunits (gp91phox and p22phox), which form a functional enzyme complex upon activation. Nox isoforms (Nox1, Nox2, Nox3, Nox4, and Nox5) are expressed in a tissue-specific manner. Notably, several types of NADPH oxidases have been identified as primary sources of ROS in renal cells, such as tubular epithelial cells and glomerular cells (3-5).

ROS are well-known to induce direct cell and tissue damage ; notably, they also serve as signaling molecules for the regulation of cellular behaviors such as proliferation, differentiation, and apoptosis, thereby contributing to the pathogenesis of many human diseases (2). Furthermore, recent studies have indicated that ROS

constitute physiologically essential molecules for the organogenesis of several types of organs (6-8). For example, by using mouse embryonic stem cells, Li *et al.* revealed that Nox4-derived ROS play an important role in cell differentiation during the early stages of cardiogenesis (8).

In a recent Hungarian Case Control Surveillance study, Nielsen *et al.* reported that the prevalences of renal malformations, such as renal agenesis and congenital abnormalities of the kidney and urinary tract (CAKUT), are strongly associated with pre-gestational maternal diabetes, suggesting that a hyperglycemic intrauterine environment may influence ureteric bud (UB) branching morphogenesis in the fetal human kidney (9). Additionally, Chen *et al.* used in vitro and ex vivo approaches to investigate the link between hyperglycemia and the expression of Pax-2, a kidney-specific master gene. They found that high glucose specifically induced Pax-2 gene expression in mouse embryonic mesenchymal epithelial (MK4) cells and kidney explants, accompanied by ROS generation and activation of the NF-κB pathway (10). Subsequently, they demonstrated that high glucose alters UB branching morphogenesis. This occurs, at least in part, via enhanced ROS generation derived from the activation of NADPH oxidase and mitochondrial oxidase, activation of Akt signaling, and upregulation of Pax-2 gene expression (11). However, the physiological roles of ROS production in the process of UB branching morphogenesis of the developing kidney are incompletely understood. The present study investigated the participation of ROS in both UB branching morphogenesis and nephrogenesis, focusing on the developmental expression of kidney NADPH oxidase components involved in ROS production.

MATERIALS AND METHODS

Experimental animals and sampling

All procedures and protocols used in this study were approved by the Institutional Animal Care and Use Committee of the Tokushima University Graduate School. Sprague-Dawley rats were obtained from Japan SLC Inc. (Shizuoka, Japan). Pregnancy was determined by the detection of a vaginal plug. Before removal of the embryos, pregnant rats were anesthetized by isoflurane with oxygen gas. Embryos were removed and decapitated on day 16 (E16), and day 18 (E18) of gestation. Embryonic kidneys from E16 and E18, as well as postnatal kidneys from rats at 1 (P1), 7 (P7), and 20 (P20) days after birth, were harvested. For histology and immunohistochemistry, parts of kidneys were fixed with 10% neutral buffered formalin (4, 10).

Antibodies (Abs)

Antibodies (Abs) against Nox1, Nox3, and Nox4 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Abs for Nox2, p47phox, and p67phox were kindly provided by Dr. Quinn at Montana State University (12). A mouse monoclonal anti-proliferating cell nuclear antigen (PCNA) antibody was purchased from Calbiochem Merck KGaA (Darmstadt, Germany). Polyclonal rabbit anti-total-ERK1/2 antibodies and a monoclonal mouse anti-phospho-ERK1/2 antibody were purchased from Santa Cruz Biotechnology for western blotting analysis. Polyclonal rabbit anti-cleaved caspase-3 was purchased from Cell Signaling Technology (Danvers, MA, USA).

Western blotting

Whole kidneys from E16, E18, P1, P7, and P20 were harvested and homogenized using fifteen strokes of a motor-driven Teflon pestle in a tightly fitted glass tube in cell lysis buffer containing protease inhibitors (Cell Signaling Technology). After incubation of samples on ice for 15 min, insoluble materials were removed by centrifugation ($10,500 \times g$, 10 min). The protein content in kidney lysates was measured by using a BCA protein assay kit (Pierce Biotechnology, Rockford, IL, USA). Protein samples (30 μg) were separated by 12.5% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes (Amersham Bioscience, Piscataway, NJ, USA). The membranes were probed with primary antibodies, then incubated with horse-radish peroxidase-conjugated secondary antibodies. Immunoreactive proteins were detected with an enhanced chemiluminescence detection system (Amercham Corp., Arlington Heights, IL, USA). Bands were quantified by using ImageJ 1.33u (National Institutes of Health) (13).

Histology and immunohistochemistry

To examine the expression and localization of NADPH oxidase, paraffin sections (3-μm-thick) were deparaffinized and rehydrated. Endogenous peroxidase activity was quenched by incubating sections in 2.0% H_2O_2/methanol for 30 min. To unmask antigens, slides were autoclaved at 121℃ for 10 min in 0.01 M citrate buffer (pH 6.0). The expressions of NADPH oxidase components were detected by an immunoperoxidase technique as previous described (12). Briefly, the sections were incubated with primary antibodies against NADPH oxidase (all antibodies diluted to 1 : 100) for 24 h at 4℃. After washing, the sections were incubated with biotinylated secondary antibodies, then with avidin-biotin-peroxidase (ABC Elite ; Vector Laboratories, Burlingame, CA, USA) ; immunoreaction products were developed by using 3, 3'-diaminobenzidine (Dojindo, Kumamoto, Japan). The sections were then counterstained with Mayer's hematoxylin (Wako, Tokyo, Japan), dehydrated, and coverslipped prior to analysis.

Organ Culture

Embryonic day 14 (E14) and E18 metanephroi were explanted onto 3-μm Nucleopore filters (Corning Costar Corporation, Cambridge, MA, USA) at the interface between air/5% CO_2 atmosphere and medium ; they were then cultured at 37℃ in control medium comprising RPMI, 5% fetal bovine serum, 2 mM glutamine, 100 U/ml penicillin, and 100 mg/ml streptomycin, in accordance with previously described conditions with some modification (14). To determine the role of ROS produced by NADPH oxidase in UB branching and nephrogenesis, we incubated the kidneys with NADPH oxidase inhibitors (diphenyleneiodonium ; DPI and apocynin) for 48 h. At E14 + 2 days, UB branching was counted by using FITC-labeled *Dolichos biflorus* agglutinin (DBA) lectin, which binds to UB/collecting duct epithelia (15). At E18 + 2 days, nephrogenesis was assessed on PAS-stained sections. In addition, we examined the number of PCNA-positive cells, as well as the expression of phosphorylated-ERK1/2 (p-ERK1/2) and cleaved caspase-3, to assess proliferation, migration, and apoptosis.

In situ superoxide production

In situ production of $O_2{}^-$ was determined by using whole E14 metanephroi cultured for 2 days, or 30-μm sections of frozen tissue of E18 metanephroi cultured for 2 days that were incubated with dihydroethidium (DHE ; 10 μmol/L) in PBS for 30 min at 37℃ in a humidified chamber that was shielded from light. Upon reaction with $O_2{}^-$, DHE is oxidized to ethidium bromide, which binds to DNA in the nucleus and fluoresces red. To detect ethidium bromide, we used a 543-nm He-Ne laser combined with a 560-nm long-pass filter. To identify $O_2{}^-$-producing cells, we performed immunofluorescent staining combining the DHE assay (red) with FITC-labeled (green) anti-DBA-lectin antibody (12, 13).

Statistical analysis

Values are expressed as means $\pm$ standard deviation (SD). Differences were evaluated with the StatMate 3 software package (ATMS Co., Ltd., Tokyo, Japan). Comparisons of variables among groups were performed by using one-way ANOVA and Dunnett's test (13). All experiments were repeated at least three times. Values of $P < 0.05$ and $P < 0.01$ were considered statistically significant.

RESULTS

Western blotting

Western blotting was performed to examine protein levels of each NADPH oxidase component during different stages of rat kidney development. The same amount of protein samples was applied and separated by electrophoresis to detect each component of NADPH oxidase. All components were expressed in embryonic and postnatal kidneys (Figure 1a). The protein levels of Nox1, Nox3, Nox4, and p67 were significantly increased at P7, P20, P1, and P1 respectively. The levels of Nox2 and p47phox were not significantly altered in whole kidneys (Figure 1b).

Immunohistochemistry

Immunohistochemistry revealed that Nox1, an NADPH oxidase component was primarily expressed in UB cells and condensed mesenchymal cells at E16 and E18 (Figure 2a). At E18 and P1, they were also detected in immature glomerular endothelial and mesangial cells, and in tubular epithelial cells in nephrogenic zones (Figure 2b). Expression of Nox1 was weak in the cortex of adult kidney (P20). In contrast, Nox1 was expressed in renal tubules in the medulla of adult kidney (P20) (Figure 2c, 2d). The localization and expression of other NADPH oxidase components (Nox2, Nox3,
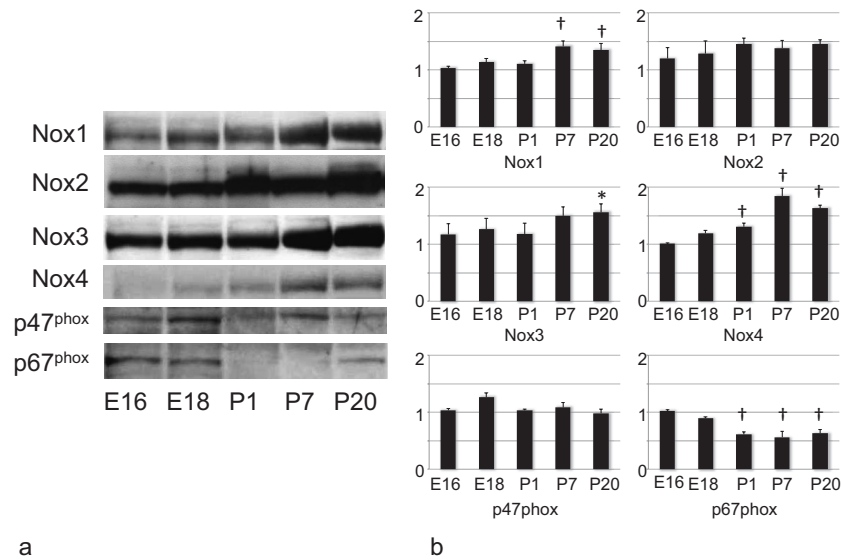
Figure 1.   Expression of NADPH oxidase components, Nox1, Nox2, Nox3, Nox4, p47phox, and p67phox during the development of rat kidneys
(a) Western blotting bands showed the expression level of NADPH oxidase components during renal development (E16, E18, P1, P7, and P20). (b)
Results are shown as means $\pm$ standard deviations of at least three independent experiments. Significant differences versus E16 : *$p < 0.05$; †$p < 0.01$.
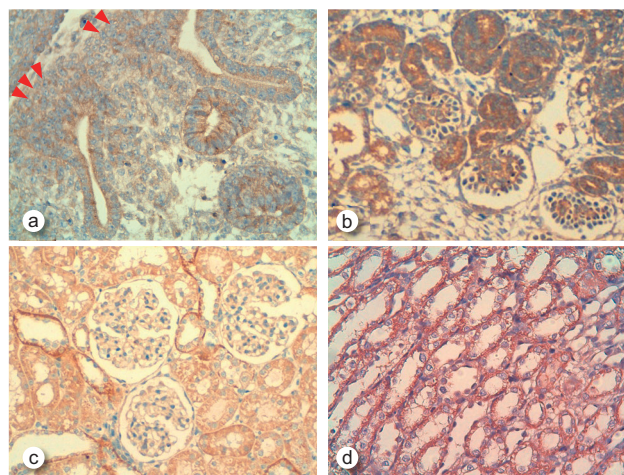

Figure 2.   Representative expression and localization of Nox1 in developing kidney
(a) In E16 kidneys, Nox1 expression was detected in ureteric bud and condensed mesenchymal cells, indicated by red arrows.
(b) In E18, Nox1 was expressed in immature mesangial and epithelial cells in the nephrogenic zone.
(c) In P20, Nox1 expression was reduced in mature glomeruli in the cortex of adult kidney.
(d) In P20, Nox1 was expressed in renal tubules in the medulla of adult kidney.
The localization and expression of other NADPH oxidase components (Nox2, Nox3, Nox4, p47phox, and p67phox) were comparable to those of Nox1.

Nox4, p47phox, and p67phox) were comparable to those of Nox1.
These results suggested that the expression of NADPH oxidase
components was spatiotemporally regulated during kidney devel-
opment.

Effect of NADPH oxidase inhibitor on UB branching

Double labeling with FITC-DBA lectin and the DHE assay
showed ROS production in UB epithelia. DPI treatment (1×10^{-6} M)
for 2 days suppressed ROS production and reduced the kidney size
and number of ureteric bud branches (71.4%, P< 0.05) and tips
(66.7%, P< 0.05) in E14 kidneys (Figure 3). Both ROS production

and NADPH oxidase localization were observed in UB branches
and nephrogenic zones. In addition, organ culture with apocynin
(1×10^{-3} M), another NADPH oxidase inhibitor, showed similar
results (data not shown).

Effect of NADPH oxidase inhibitor on nephrogenesis

At E18, inhibition of NADPH oxidase suppressed ROS produc-
tion in nephrogenic zones, as well as nephron formation ; it also
reduced the width of nephrogenic zones (P< 0.01), but did not
change overall kidney size. Notably, it reduced the number of
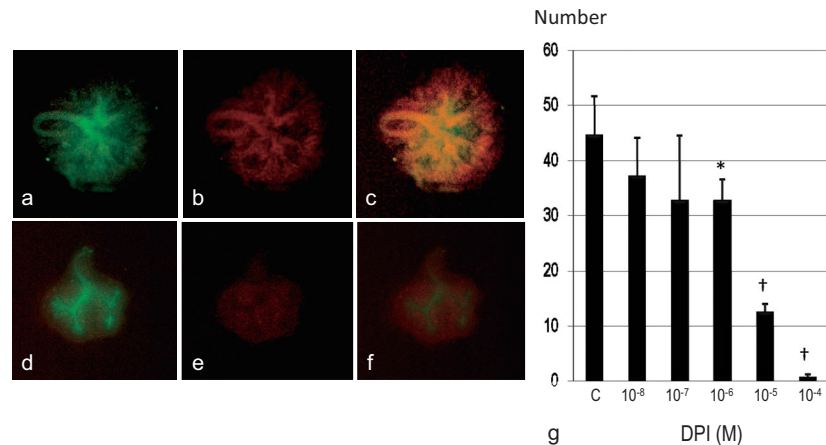PCNA-positive cells and levels of p-ERK1/2, and increased the

Figure 3.   The effect of Nox inhibitor (diphenyleneiodonium ; DPI) on ureteric bud (UB) branching
Whole kidneys were simultaneously stained with FITC-DBA lectin (green ; a and d) and by DHE assay (red ; b and e) after organ culture of E14 kidneys for 2 days. FITC-DBA lectin (green) and DHE assay (red) demonstrated UB branching and superoxide production, respectively. The merged images are shown (c and f). The representative organ culture experiments were performed by incubation for 2 days with 1×10^{-5} M of DPI, the Nox inhibitor. The representative images were shown as control (a,b,c) and the kidneys treated by 1×10^{-5} M of DPI (d,e,f).
Superoxide production was detected in UB and suppressed by DPI treatment. UB branching in E14 organ culture was reduced in a dose-dependent manner in the presence of DPI (g). Results are shown as means $\pm$ standard deviations of at least three independent experiments. Significant differences versus control (C) : $^{*}p < 0.05$; $^{\dagger}p < 0.01$. Kidney growth was also inhibited by the addition of apocynin.

level of cleaved caspase-3 (Figure 4). Western blotting demonstrated that inhibition of superoxide production by DPI reduced levels of p-ERK1/2 and increased levels of cleaved caspase-3 in organ cultures of both E14 and E18 kidney (P< 0.01) (Figure 5).

DISCUSSION

Metanephric kidney develops through reciprocal inductive interactions between the UB and metanephric mesenchyme (16). Induced by mesenchymal signals, the UB elongates and undergoes repetitive branching to form the renal collecting system (branching morphogenesis). In turn, UB tips stimulate the mesenchyme to differentiate into nephron epithelia ; the progression occurs from renal vesicles to comma-shaped bodies, then to S-shaped bodies, and finally to mature nephron consisting of the proximal tubule, loop of Henle, and distal tubule (nephrogenesis). The two representative morphogenetic events are tightly controlled

by multiple gene regulatory networks, as well as signaling pathways (16-18). Abnormal UB branching results in a spectrum of CAKUT, the major cause of end-stage kidney disease in children (16). Maternal malnutrition often induces the development of intrauterine growth restriction (IUGR) accompanied by low nephron number in the fetus, which is ultimately associated with an increased risk of chronic kidney disease in adulthood (19).

In this study, we showed the crucial role of ROS, generated by NADPH oxidase, in the process of branching morphogenesis, as well as nephrogenesis in the kidney. Notably, the expression of ROS-producing NADPH oxidase components is spatiotemporally altered according to the stages of kidney development. In general, these expression levels correspond with the level of ROS production within the kidney in a regional and cell-specific manner (20). NADPH oxidase-derived ROS seems to affect the cell proliferation, migration, and apoptosis involved in UB branching and nephrogenesis via activation of ERK1/2 and expression of caspase-3. Thus, NADPH oxidase expression appears to be high in developing
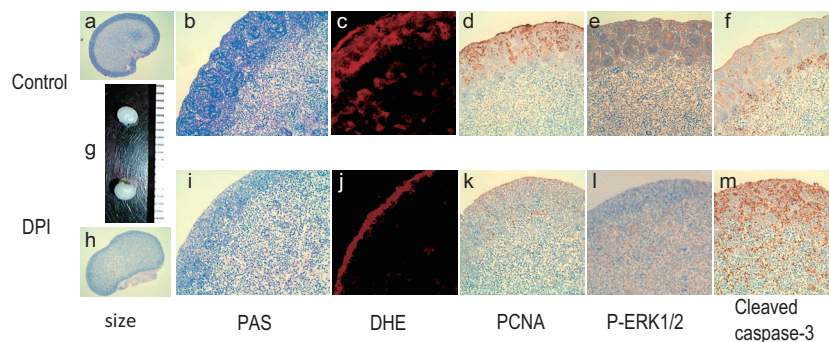

Figure 4.   The effect of Nox inhibitor (diphenyleneiodonium ; DPI) on nephrogenesis
Histochemical (PAS ; b and i) and immunohistochemical assessment (PCNA ; d and k, p-ERK ; e and l, and cleaved caspase-3 ; f and m) and the detection of superoxide production by DHE assay (c and j) were performed after organ culture of E18 kidneys for 2 days.
DPI treatment (1×10^{-5} M) impeded ROS production and reduced nephron formation in the nephrogenic zone, as well as the width of the nephrogenic zone. Differences in kidney size were not observed (a, g, h). DPI treatment also reduced the number of PCNA-positive cells and the levels of p-ERK1/2, while increasing the level of cleaved caspase-3.
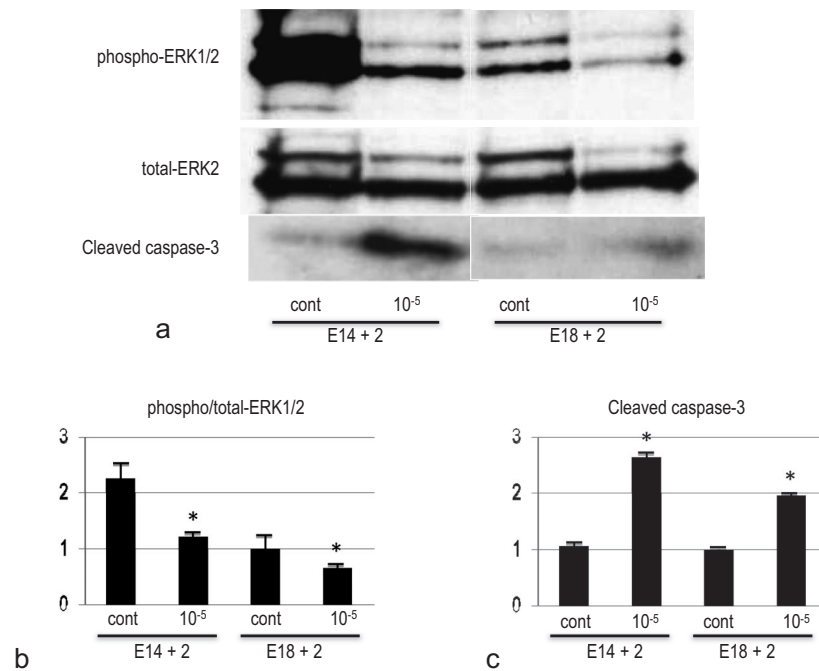
Figure 5.   The effect of Nox inhibitor (diphenyleneiodonium ; DPI) on ureteric bud (UB) branching and nephrogenesis by organ culture of developing kidneys

Western blotting demonstrated that inhibition of superoxide production by the treatment of DPI $(1 \times 10^{-5}$ M) reduced levels of p-ERK1/2, and increased levels of cleaved caspase-3 in developing kidneys after organ culture of both E14 and E18 kidneys for 2 days, respectively (a). Representative western blotting is shown (a). Bands indicating levels of p-ERK1/2 and cleaved caspase-3 were quantified by ImageJ 1.33u (National Institutes of Health) and are shown as b and c, respectively. The results are shown as means $\pm$ standard deviations of at least three independent experiments. Significant differences of addition of DPI (10^{-5}) versus control (cont) : *p < 0.01.

kidney while low in developed kidney.

Our observations in this study are partially in agreement with the work by Zhang *et al.*, which revealed that hyperglycemic intrauterine conditions increase UB branching morphogenesis by enhancing ROS generation through the activation of NADPH oxidase and mitochondrial oxidase (11). It was recently discovered that NADPH oxidase-dependent ROS are not limited to roles in cellular and tissue damage, and that they contribute to physiological processes of organ development in a carefully regulated manner (2). There is increasing evidence to indicate that adequate NADPH oxidase-mediated ROS production is required for neurogenesis and cardiogenesis, suggesting that physiological levels of NADPH oxidase-dependent ROS may be necessary for renal developmental, such as UB branching and nephrogenesis (7, 8).

Consideration should be given to the close relationship between activation of the renal renin angiotensin system (RAS) and kidney development during the fetal period (16). Genetic inactivation of RAS constituents such as AGT, ACE, prorenin receptor (PRR), combined AT1A/AT1BR, and AT2R in murine RAS induces abnormal renal phenotypes, such as CAKUT (e.g., hypodysplasia of the renal papilla, hydronephrosis, or renal tubular dysgenesis (RTD)), or immature glomerular filtration (16). Of note, the use of RAS inhibitors, such as angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs), is prohibited in pregnant women ; when these agents have mistakenly been used, the occurrence of renal dysplasia, including RTD (as a kidney phenotype of ACEI/ARB fetopathy), has been reported (21). In consideration of the extensive data that show Ang II can serve as a powerful inducer for UB branching, in combination with renal NADPH oxidase-derived ROS (22), the potential role of Ang II in kidney development may be partially mediated through the action of NADPH oxidase-derived ROS in the developing kidney.

Involvement of ROS-mediated signal pathways in kidney development remains unclear. The present study indicates that ERK1/2 phosphorylation, as well as cleaved caspase-3 expression, which are altered by ROS production within kidney, at least partly contribute to two key morphologic events in renal development. Large-scale proliferation and apoptosis events have been observed in these cells (18). Many reports have implicated MAP kinases, such as ERK1/2 and p38, in cell behaviors necessary for branching morphogenesis and nephrogenesis in kidney (23, 24). Additionally, high glucose-increased UB branching morphogenesis has been shown to occur, at least in part, via ROS generation, activation of Akt signaling, and upregulation of Pax-2 gene expression (11). Further investigations are needed to fully elucidate the roles of renal NADPH oxidase expression, ROS, and relevant signaling pathways in fine-tuned mechanisms of renal morphogenesis during the course of renal development.

In conclusion, the present study indicated that reduced ROS production impedes UB branching and nephrogenesis in the developing kidney. Considering the two critical findings of the involvement of RAS in renal development and Ang II-induced ROS production via NAPDH oxidases, appropriate modulation of ROS production for IUGR and premature babies might provide a therapeutic strategy to increase nephron number in these babies and prevent the development of CAKUT.

CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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