mPGES-1-derived PGE₂ contributes to adriamycin-induced podocyte injury

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PODOCYTES are terminally differentiated epithelial cells adhered to the outer layer of the glomerular basement membrane (GBM). Podocytes prevent urinary protein loss by maintaining foot processes and an interposed slit diaphragm. Chronic injury may cause podocyte morphology change, fusion, foot process effacement, apoptosis, and detachment from the GBM, which leads to albuminuria and glomerular disease. At the present time, there are no specific therapies targeting podocytes to prevent or attenuate podocyte damage. Therefore, exploration of pathogenic mechanisms of podocyte injury to define some novel therapeutic targets is becoming urgent and important. It has been well recognized that inflammation is the causative factor of podocyte injury in chronic kidney disease (CKD). PGE₂, an important inflammatory mediator, has been shown to be associated with multiple kidney diseases (13, 16).

PGE₂ is the product of arachidonic acid sequentially catalyzed by cyclooxygenase (COX)-1 and COX-2 and terminal PGE synthases (PGESs). COX-1 is constitutively expressed, whereas COX-2 serves as an inducible enzyme under many physiological and pathological conditions in multiple organs, including the kidney. Accumulating evidence has demonstrated that in vitro and in vivo experiments.

MATERIALS AND METHODS

Reagents and antibodies. Caelyx (liposomal ADR) was purchased from Merck (Whitehouse Station, NJ). The following antibodies were purchased from Cayman Chemicals (Ann Arbor, MI): anti-COX-2 (catalog no. 160106), anti-mPGES-1 (catalog no. 160140), anti-mPGES-2 (catalog no. 160145), and anti-cPGES (catalog no. 160150). The following antibodies were purchased from Abcam (Cambridge, MA): anti-IL-1β (catalog no. ab93650), anti-phospho-p65 (catalog no. 9242), anti-p65 (catalog no. 9246), anti-phospho-IκB-α (catalog no. 8242). The PGE2 enzyme immunoassay kit was from Cayman Chemicals. The IL-1β and TNF-α ELISA kit was from R&D Systems (Minneapolis, MN). Establishing of ADR-induced nephropathy in mice. Male adult (10–12 wk) BALB/C mice were treated with ADR (12 mg/kg) by a single tail vein injection. In another experiment, male adult mPGES-1 knockout (KO) mice and their wild-type (WT) littermates on a C57 genetic background were treated with ADR at a dose of 15 mg/kg by a tail vein injection. After 5 days, urine was collected using metabolic cages. All mice were maintained on a 12:12-h light-dark cycle in a temperature-controlled (19–21°C) room. Mice were fed standard rodent chow and were given free access to drinking water. All
procedures were in accordance with guidelines approved by the Institutional Animal Care and Use Committee of Nanjing Medical University (no. 20090053).

Cell culture. MPC5 conditionally immortalized mouse podocyte clonal cells [provided by Peter Mundel (Mount Sinai School of Medicine) and Dr. Jie Ding (Peking University)] were cultured and induced to differentiate as previously described (22). Cells were maintained in RPMI 1640 (HyClone) containing 10% heat-inactivated fetal calf serum (FCS) ( Gibco), 100 U/ml penicillin G, and 100 mg/ml streptomycin in the presence of 5% CO2. To sustain podocyte proliferation, 10 U/ml recombinant murine interferon-γ (Sigma) was added into the medium, and cells were maintained at 33°C. Podocytes were maintained without interferon-γ at 37°C for 10–14 days to induce differentiation before the experiments.

Knockdown of mPGES-1 using small interfering RNA. mPGES-1 small interfering (si)RNAs and the silencer negative control (scnRNA) were commercially available (GenePharma, Shanghai, China). Transfections with the siRNA oligomers were performed using the Lip2000 Kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions.

Analysis of podocyte apoptosis. For the apoptosis assay, podocytes were stained with FITC-annexin V and propidium iodide according to the manufacturer’s instructions. Stained cells were analyzed using a BD FACS Calibur flow cytometer (Bedford, MA), and data analysis was performed with FlowJo software.

Quantitative real-time PCR. Total RNA from cultured podocytes and the renal cortex were lysised in protein lysis buffer, and protein concentration was measured as described previously (9, 23). Immunoblot analysis was performed with primary antibodies against mPGES-1 (1:500), anti-COX-2 (1:500), anti-mPGES-2 (1:1,000), anti-cPGES (1:1,000), anti-podocin (1:1,000), anti-nephrin (1:500), and anti-GAPDH (1:1,000). Blots were visualized with the Amersham ECL Detection System (Amer sham, Buckinghamshire, UK). Densitometric analysis was performed using Quantity One software (Bio-Rad).

Enzyme immunoassay. Urine samples and cell culture medium were centrifuged for 5 min at 12,000 g. The concentration of PGE2 in the urine and medium was determined according to the manufacturer’s instructions.

Statistical analysis. All results are presented as means ± SE. Statistical analysis was performed using ANOVA followed by a Bonferroni’s test with SPSS 13 statistical software. P values of <0.05 were considered significant.

RESULTS

ADR treatment remarkably enhanced mPGES-1 expression in the kidney. To test whether ADR could induce mPGES-1 in kidney tissue, we treated BALB/C mice with ADR by a tail vein injection. As expected, ADR remarkably elevated mPGES-1 expression in animal kidneys. Meanwhile, protein levels of mPGES-2 and cPGES also increased after ADR administration (Fig. 1, A and B). We further validated mRNA expressions of mPGES-1 and COX-2 in ADR-treated mice via quantitative real-time PCR. As shown in Fig. 2, A and B, ADR caused a significant induction of COX-2 and mPGES-1 at the mRNA level. These data highly suggest a regulatory role of ADR in mPGES-1 expression in vivo.

ADR treatment significantly induced mPGES-1 expression in podocytes. To further study whether ADR could directly induce mPGES-1 expression in podocytes, we treated podocytes with ADR and found a remarkable induction of COX-2 and mPGES-1 protein expression in contrast with unaltered mPGES-2 and cPGES (Fig. 3, A and B). By quantitative real-time PCR, we observed a moderate but significant elevation of COX-2 mRNA expression (Fig. 4A). Meanwhile, the mRNA level of mPGES-1 displayed a significant upregulation

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Table 1. Sequences of primers for quantitative real-time PCR

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<tr>
<th>Gene</th>
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<td>Cyclooxygenase-2</td>
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<td>Caspase-3</td>
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<td>IL-1β</td>
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<td>GAPDH</td>
<td>5'-TGATGGAATCCATGAGAAGCG-3'</td>
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Fig. 1. Adriamycin (ADR) enhanced microsomal PGE synthase (mPGES)-1 protein expression in animal kidneys. A: Western blots of cyclooxygenase (COX)-2, mPGES-1, mPGES-2, and cytosolic PGE synthase (cPGES). B: densitometric analysis of Western blots. All values are means ± SE; n = 4 in each group. *P < 0.05 vs. the control (Ctrl) group; **P < 0.01 vs. the Ctrl group.
Fig. 2. ADR enhanced mPGES-1 mRNA expression in animal kidneys. A and B: quantitative real-time PCR analysis of COX-2 (A) and mPGES-1 (B). All values are means ± SE; n = 4 in each group. *P < 0.05 vs. the Ctrl group; **P < 0.01 vs. the Ctrl group.

Silence of mPGES-1 blocked ADR-induced PGE2 production. To examine the role of mPGES-1 in ADR-induced PGE2 production in podocytes, mPGES-1 siRNA was applied to podocytes (Fig. 5, A and B). Strikingly, mPGES-1 siRNA not only blocked the increase of mPGES-1 after ADR treatment but also remarkably blunted PGE2 production, as determined by quantitative real-time PCR and ELISA, respectively (Fig. 5, C and D). These data indicate that mPGES-1 was an important enzyme in mediating ADR-induced PGE2 production in podocytes.

Silence of mPGES-1 significantly blocked ADR-induced downregulation of podocin and nephrin and podocyte apoptosis. mPGES-1 siRNA was applied to podocytes to test the role of mPGES-1 in ADR-induced phenotypic alterations. Interestingly, the loss of podocyte marker proteins of podocin and nephrin was significantly attenuated by mPGES-1 silencing, as examined by Western blot analysis (Fig. 6, A and B). It is known that podocyte apoptosis is another pathogenic mechanism leading to podocyte loss and renal dysfunction. As shown in Fig. 7, A–C, ADR treatment caused a moderate but significant increase of podocyte apoptosis, which was entirely blocked by mPGES-1 silencing accompanied by a significant suppression of the caspase-3 mRNA level. These interesting data highly suggest that mPGES-1-derived PGE2 is of importance in mediating ADR-induced podocyte injury.

Silence of mPGES-1 ameliorated the ADR-induced inflammatory response in podocytes. PGE2 is one of the known mediators of inflammation and contributes to cellular injuries by promoting the inflammatory response. To explore the potential mechanism contributing to the detrimental effect of the mPGES-1/PGE2 cascade on podocyte injury induced by ADR, we detected the inflammatory cytokines of IL-1β, TNF-α, and ICAM-1 using quantitative real-time PCR. As expected, we observed a significant blockade of IL-1β and TNF-α and a trend toward a reduction of ICAM-1 in mPGES-1 siRNA-treated cells after the ADR challenge (Fig. 8, A–C). By ELISA, we further confirmed the protein regulation of IL-1β and TNF-α (Fig. 9, A and B). Due to the central role of the NF-κB pathway in mediating inflammation, we examined the phosphorylation of p65 and IkB. Consistent with the regulation of proinflammatory cytokines, ADR-induced phosphorylation of p65 and IkB was entirely abolished by mPGES-1 siRNA treatment (Fig. 9, C and D).

mPGES-1 deletion in the mouse markedly blunted ADR-induced albuminuria and urinary PGE2 excretion. To further study the in vivo role of mPGES-1 in ADR-induced podocyte injury, mPGES-1 WT and KO mice were subjected to ADR treatment via tail vein injection. Very strikingly, mPGES-1 deletion resulted in a marked attenuation of albuminuria in line with a remarkable blockade of urinary PGE2 excretion and urinary TNF-α output (Fig. 10, A–C). These important in vivo data further supported our conclusion that mPGES-1-derived PGE2 participated in ADR-induced podocyte injury possible via promoting inflammation.

DISCUSSION

Podocyte injury is a common feature in many glomerular diseases. Accumulating evidence has demonstrated that podocyte injury is not only an outcome of a number of pathological insults but also a pathogenic factor leading to the progression of kidney diseases (5, 14). Prevention of podocyte injury is of vital importance in dealing with glomerular diseases in the
clinic. In animal studies, ADR was used to induce podocyte damage and focal segmental glomerulosclerosis (4, 22). In the present study, we investigated the role of mPGES-1 in podocyte injury induced by ADR via in vitro and in vivo experiments.

Although previous reports have demonstrated that both COX-2 as an upstream enzyme to provide the substrate (PGH2) for PGESs and EP4 were attributable to the pathogenesis of podocytopathy (2, 3), no evidence was provided to illustrate the role of PGE2Ss in podocyte injury. Therefore, it is worthwhile to define the particular PGES participating in the pathogenesis of podocyte damage. Among the three PGESs, mPGES-1 is the best characterized. In the kidney, many insults, including aldosterone, cisplatin, and ANG II, can activate mPGES-1 to exert multiple functions in renal tubules (7, 8, 20). However, evidence related to actions of mPGES-1 in podocyte injury is still absent. In ADR-treated animals, mPGES-1 was remarkably induced in renal cortex tissues at mRNA and protein levels. In in vitro podocytes, ADR directly enhanced mPGES-1 expression. All these data indicated a direct stimulation of mPGES-1 in podocytes by ADR. To further define the role of mPGES-1 in ADR-induced podocyte injury, mPGES-1 siRNA was applied to the cells before ADR administration. As expected, mPGES-1 siRNA remarkably blocked the induction of mPGES-1 and PGE2. Meanwhile, a reduction of podocyte protein markers of podocin and nephrin was also significantly attenuated by mPES-1 silencing, indicating that mPGES-1 contributed to the alteration of the podocyte phenotype. Another known pathological phenomenon in ADR-induced podocyte injury is apoptosis (6). In agreement with the attenuation of the cell phenotypic transition, ADR-induced cell apoptosis was markedly blunted by mPGES-1 siRNA in parallel with a significant blockade of caspase-3 induction. These data provide strong in vitro evidence showing that mPGES-1-derived PGE2 contributed to ADR-induced podocyte injury.

To investigate the in vivo function of mPGES-1 in ADR-induced podocyte injury, systemic mPGES-1-deficient mice on a C57BL/6 genetic background were treated with ADR. Strikingly, albuminuria, a key parameter reflecting podocyte injury, was markedly blunted in mPGES-1 KO mice in line with a remarkable blockade of urinary PGE2 excretion in response to ADR.
to ADR injection for 5 days. Due to the resistance to ADR in mice on a C57BL/6 genetic background (19), proteinuria in these mice quickly recovered to a normal level after 10 days of ADR administration (data not shown). However, the striking attenuation of peaked albuminuria on day 5 strongly suggested that mPGES-1 could have an in vivo role in mediating ADR-induced podocyte injury. In the future, experiments performed in podocyte-specific mPGES-1 KO mice on an ADR-sensitive genetic background (BALB/c) are definitely needed to further clarify the in vivo role of mPGES-1 in podocyte injury.

To explore the potential mechanism leading to the detrimental effect of the mPGES-1/PGE2 cascade on ADR-induced podocyte damage, we examined the inflammatory markers of IL-1β, TNF-α, and ICAM-1 in podocytes. As expected, both IL-1β and TNF-α displayed a significant reduction after mPGES-1 silencing in line with a trend toward a decrease of ICAM-1. Meanwhile, the activation of the NF-κB signaling pathway induced by ADR was also abolished by mPGES-1 silencing, indicating that mPGES-1-derived PGE2 exerted a proinflammatory action possibly through activation of NF-κB. In mPGES-1 KO mice, urinary excretion of TNF-α was also significantly blunted compared with WT control mice. All these data highly suggest that mPGES-1-derived PGE2 could trigger an inflammatory response to promote podocyte injury in response to pathological insults. Taken together, this study not only provided the first evidence showing the pathogenic role of mPGES-1 in mediating podocyte injury but also offered us...
confidence for the clinical use of mPGES-1 inhibitors for the treatment of podocyte disease.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

mPGES-1 AND Podocyte Injury

Fig. 10. mPGES-1 deficiency in mice improved albuminuria induced by ADR. A: ELISA analysis of urinary PGE2 output. B: enzyme immunoassay of urinary PGE2 output. C: ELISA assay of urinary TNF-α excretion. KO, mPGES-1 knockout animals; WT, wild-type animals. All values are means ± SE; n = 6–10 in each group. * P < 0.05 vs. the Ctrl group; **P < 0.01 vs. the Ctrl group.

REFERENCES