mPGES-1-Derived PGE\textsubscript{2} Contributes to Indoxyl Sulfate-Induced Mesangial Cell Proliferation

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Key Words
Indoxyl sulfate • MPGES-1 • PGE\textsubscript{2} • Mesangial cells • Proliferation

Abstract

Background/Aims: We previously reported that indoxyl sulfate (IS) could cause mesangial cell (MC) proliferation via a cyclooxygenase (COX)-2-dependent mechanism. However, the specific prostaglandin contributing to COX-2 effect on IS-induced MC proliferation remained unknown. Thus, the present study was undertaken to examine the role of microsomal prostaglandin E synthase-1 (mPGES-1)-derived Prostaglandin E2 (PGE\textsubscript{2}) in IS-induced MC proliferation.

Methods: IS was administered to the MCs with or without mPGES-1 siRNA pretreatment to induce the MC proliferation which was determined by cell cycle analysis, DNA synthesis, and the expressions of cyclins. In another experimental setting, PGE\textsubscript{2} was applied to the MCs to examine its direct effect on MC proliferation, as well as the regulation of prostaglandin E receptors (EPs) by qRT-PCR.

Results: With the administration of IS, mPGES-1 (not mPGES-2 and cytosolic PGES) was significantly upregulated at both protein and mRNA levels in line with a promoted MC proliferation. Interestingly, silencing mPGES-1 reduced cell number in S and G2 phases and blocked the upregulation of cyclin A2 and cyclin D1 in parallel with blunted PGE\textsubscript{2} release after IS treatment, indicating that mPGES-1-derived PGE\textsubscript{2} could contribute to MC proliferation. Furthermore, we confirmed that exogenous PGE\textsubscript{2} could directly trigger the proliferative response in MCs. Lastly, we observed a selective upregulation of EP2 after PGE\textsubscript{2} treatment and enhanced phosphorylation of NF-κB following IS administration in MCs, suggesting the potential involvements of EP2 and NF-κB in this pathological process.

Conclusion: mPGES-1-derived PGE\textsubscript{2} contributed to IS-induced mesangial cell proliferation.
**Introduction**

In end stage renal disease (ESRD) patients, residual kidney function (RKF) is associated with the status of nutrition, inflammation, cardiac hypertrophy, and life quality [1-4]. Besides helping to maintain the fluid balance, RKF is also important in removing the plasma solutes that could not be effectively cleared by the dialysis. In general, the clearance of middle to large molecular weight uremic toxins such as indoxyl sulfate more relies on the renal metabolism and tubular secretion [4, 5]. Therefore, loss of RKF not only indicates a progressive reduction of glomerular filtration rate (GFR) and loss of functional nephrons, but also results in more serious uremia in ESRD patients.

IS is a product of indole metabolism in the liver and is mainly excreted from the kidney [6]. However, IS cannot be efficiently removed by conventional hemodialysis because of its high binding affinity to albumin [7]. Inadequate clearance of IS leads to its accumulation in circulation, which may promote the glomerular injury of residual nephrons, leading to a faster loss of RKF [8-11]. In agreement with this notion, IS was shown to trigger mesangial cell (MC) proliferation and extracellular matrix deposition [12, 13]. As a type of native glomerular cells, MCs are very sensitive to various insults [14-18]. In a number of primary and secondary glomerular diseases, MC proliferation and subsequent glomerular sclerosis are dominant pathological features [19, 20]. Therefore, it is worthwhile to better understand the IS effects on MC damage, as well as the underlying mechanisms.

As a major product of arachidonic acid (AA) metabolism, PGE\(_2\) has been shown to have multiple biological actions in modulating the glomerular filtration, renin release, and cell proliferation in kidney [21]. COX-1 and COX-2 chiefly regulate the production of five major prostaglandins (PGE\(_2\), PGI\(_2\), PGD\(_2\), PGF\(_2\alpha\), and TXA2) by supplying the substrate (PGH\(_2\)) to the prostaglandin synthetic enzymes including mPGES-1 [22]. Our previous study showed that IS significantly increased the COX-2 (not COX-1) expression to promote MC proliferation [13]. However, it still needs evidence to define the specific prostaglandin in mediating COX-2 effect on promoting MC proliferation. As a specific enzymatic source of PGE\(_2\), mPGES-1 has been reported to participate in the pathogenesis of multiple renal diseases [23, 24]. Therefore, we speculated that mPGES-1-derived PGE\(_2\) might be involved in the MC proliferation induced by IS.

**Materials and Methods**

**Materials**

Indoxyl Sulfate and PGE\(_2\) were purchased from Sigma (St. Louis, MO). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, and trypsin-EDTA solution were purchased from Gibco (Invitrogen, Grand Island, NY). Cyclin D1 mouse monoclonal antibody and cyclin A2 rabbit polyclonal antibody were purchased from Abcam. mPGES-1 mouse monoclonal antibody was purchased from Cayman Chemicals (Ann Arbor, MI). Anti-GAPDH (catalog no. ab9485) and anti-p-NF-\(\kappa\)B (catalog no. 3031S) were bought from Cell Signaling Technology (Danvers, MA). The PGE\(_2\) enzyme immunoassay kit was from Cayman Chemicals (Ann Arbor, MI).

**MC culture and siRNA transfection**

The mouse MC line (SV40 MES 13) was obtained from the China Center for Type Culture Collection (CCTCC Wuhan, China). Cells were maintained at 37°C in a humidified 5% CO\(_2\) atmosphere in DMEM which contained 5.6 mM glucose, 10% fetal bovine serum (FBS, GIBCO), 100 U/ml penicillin 100mg/ml streptomycin, 44mM NaHCO\(_3\) and 14mM 4-(2-hydroxy-ethyl)-1-piperazineethanesulfonic acid. After 2 or 3 days, cells were digested with 0.25% trypsin and 0.02% EDTA, and seeded at a density of 3×10^4/ml in 6-well plates for experiment. siRNAs for mPGES-1 and the silencer negative control were bought from Gene Pharma (Shanghai, China). mPGES-1 siRNA consisted of an RNA duplex containing a sense strand: 5’-GCACACUGCUGGUCAUCAATT-3’ and an antisense strand: 5’-UUGAUGACCAGCAGUGUCTT-3’. Cells were transfected with siRNA using the Lip2000 Kit (Invitrogen, Carlsbad, CA) 24 h before the experiments were initiated according to the manufacturer’s instructions.
Determination of DNA synthesis

The thymidine incorporation assay was used to detect mesangial cell proliferation. After the centrifugation, cell pellets were resuspended into DMEM with 1% FBS, and then placed in 96-well plates. Following the indicated treatments, cells were incubated with \( ^{3}H \)-thymidine (5 μCi/ml), and then were harvested and incubated at 4°C with trichloroacetic acid (5%) followed by solubilization in 0.1 N NaOH. Radioactivity was determined by scintillation counting.

Cell cycle analysis

Cell cycle analysis was performed by flow cytometry as previously described [25]. MCs were induced with the indicated agents for 24 h. Cells were washed with PBS before digested with 0.25% trypsin and fixed in 70% ethanol for at least 2 h at 4°C. Permeated cells were collected by centrifugation, and then treated with RNase, stained with propidium iodide by using cell cycle detection kit (KeyGEN, Shanghai, China). The number of cells in G1, S, and G2/M cell cycle phases was analyzed by flow cytometry using a BD FACS Calibur flow cytometer (Bedford, MA), and data analysis was performed with modifit3.0 software.

Quantitative real-time PCR (qRT-PCR)

Total RNA was extracted from cultured MGs by using a Trizol reagent (TaKaRa) according to the manufacturer’s protocol. cDNA was synthesized using a PrimeScript RT reagent Kit (TaKaRa) according to the manufacturer’s protocol. The reactions were incubated at 37°C for 15 min and then at 85°C for 5 sec. cDNA amplification was performed using the ABI 7500 Real-Time PCR Detection System (Foster City, CA) and SYBR Premix Ex Taq (TaKaRa). Oligonucleotides were designed using Primer5 software (available at http://frodo.wi.mit.edu/) and the sequences are shown in Table 1. The cycling program consisted of a preliminary denaturation (95°C for 10 min), followed by 40 cycles (95°C for 15 s and 60°C for 1 min). Relative gene expression of mRNA was normalized to GAPDH and calculated using the ΔΔCt method (where Ct is threshold cycle).

Western blotting analysis

After the treatment, MCs were rapidly washed with ice-cold PBS and lysed on ice using lysis buffer containing protease inhibitors. Protein concentration was determined using a Micro BCA protein assay kit (Pierce, Thermo). Then 60 μg protein was separated by SDS-PAGE and transferred onto PVDF membrane. The membranes were blocked by TBS-T (0.1% Tween 20 in TBS) containing 5% nonfat milk for 1h at room temperature, and then incubated with primary antibodies against cyclin D1 (1:1000), cyclin A2 (1:500), p-NF-κB, and mPGES-1 (1:500) overnight at 4°C, followed by the incubation of HRP-labeled secondary antibodies at room temperature for 1 h. GAPDH was used as an internal control. Membranes were visualized by chemiluminescence reaction. Band intensity was quantified using Image J software (NIH, Bethesda, MD, USA).

Enzyme immunoassay

Cell culture medium was centrifuged for 5 min at 12, 000 g and the supernatant was collected for PGE\(_2\) analysis. The concentration of PGE\(_2\), in the cell culture medium was determined by enzyme immunoassay kit (Cayman Chemical) according to the manufacturers’ instructions.

Data analysis

All Data are presented as means ± SD. Statistical analysis was performed using ANOVA analysis followed by a Bonferroni posttest. \( P < 0.05 \) was considered statistically significant.

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<th>Gene symbol</th>
<th>Primer Sequence</th>
<th>Accession Number</th>
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<td></td>
<td>5'-GCAGTCAGGGGAATGTTG-3'</td>
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<td>cyclin A2</td>
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<td>NM_0098282</td>
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<td>5'-TACTCTCACTGTCCTTGT-3'</td>
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Results

**IS treatment enhanced mPGES-1 expression in MCs**

In order to verify the hypothesis that PGE\(_2\) derived from mPGES-1 contributes to the mesangial cell proliferation induced by IS, we measured the expressions of mPGES-1, mPGES-2, and cytosolic PGES (cPGES) and found that IS enhanced mPGES-1 while decreased mPGES-2 at mRNA levels in dose- and time-dependent manners (Fig. 1A-D). However, cPGES only showed a trend reduction (Fig. 1E-F). We further detected the mPGES-1 protein expression using Western blotting. As shown in Fig. 1G&H, mPGES-1 protein was significantly upregulated following IS treatment (500 μM). These data suggested that IS could directly and selectively upregulate mPGES-1 expression in MCs.

**Silencing mPGES-1 ameliorated MC proliferation and PGE\(_2\) production induced by IS in MCs**

As an inducible enzyme for PGE\(_2\) production, mPGES-1 is of importance in the pathogenesis of a number of inflammatory diseases. To define its contribution in IS-induced MC proliferation, MCs were transfected with mPGES-1 siRNA prior to the IS treatment (Fig. 2A&B). Then we found that mPGES-1 upregulation was strikingly blocked in line with a significant blockade of PGE\(_2\) secretion in the medium following IS treatment (Fig. 2C-F). Notably, DNA synthesis analysis showed that mPGES-1 silencing significantly reduced the amount of \[^3\]H \) thymidine incorporated into the newly synthesized DNA after IS treatment (Fig. 2G), suggesting an amelioration of MC proliferation. Furthermore, the flow cytometry analysis demonstrated that the cell cycle progression was also blocked as shown by the

![Fig. 1.](image-url)
reduced percentage of cells in S and G2/S phases and increased cell population in G1/G0 phase after silencing mPGES-1 (Fig. 3A-H). These data demonstrated that mPGES-1 was important in promoting the cell cycle progression in MCs.

**Silencing mPGES-1 blocked IS-induced cyclin expression**

It is known that the progression of cell cycle is mainly regulated by cyclins in mammalian cells. Thus, we examined cyclin A2 and cyclin D1 levels in IS-treated MCs with or without mPGES-1 silencing. As shown in Fig. 4A-D, the upregulation of cyclin A2 and cyclin D1 induced by IS was significantly blunted by silencing mPGES-1 at both mRNA and protein levels. These data further confirmed that mPGES-1 is of importance in mediating IS-induced cell cycle progression in MCs.

![Figure 2](https://www.karger.com/doi/10.1159/000480369)

**Fig. 2.** Silencing mPGES-1 via a siRNA approach ameliorated IS-induced increments of MC number and PGE₂ production. To examine the role of mPGES-1 in IS-induced cell cycle progression, mPGES-1 siRNA was applied to MCs. (A) Protein levels of mPGES-1 in MCs with mPGES-1 siRNA or negative control treatment. (B) mRNA levels of mPGES-1 in MCs with mPGES-1 siRNA or negative control treatment. (C) Representative images of Western blots of mPGES-1 with or without mPGES-1 silencing in response to IS treatment. (D) Quantitative analysis of Western blots in C. (E) qRT-PCR analysis of mPGES-1. (F) EIA assay of PGE₂ in medium. (G) The assay of [³H] thymidine incorporated into the newly synthesized DNA after IS treatment with or without mPGES-1 silencing. All values are means ± SD; n = 6 in each group. * P<0.05 vs. control or IS group, ** P<0.01 vs. control or IS group.
**Fig. 3.** Silencing mPGES-1 blunted IS-induced cell cycle progression in MCs. (A-D) Representative images of cell cycle following mPGES-1 siRNA transfection with or without IS treatment. (E-H) Percentage of cells at G1/G0 (E), G2/M (F), S (G), and (S + G2)/M (H) phases following mPGES-1 siRNA transfection with or without IS treatment. All values are means ± SD; n = 6 in each group. * P<0.05 vs. control or IS group, ** P<0.01 vs. control or IS group.

**Fig. 4.** Silencing mPGES-1 blocked IS-induced upregulation of cyclins in MCs. The cells were transfected with mPGES-1 siRNA for 24 h before IS (500 μM) administration. (A & B) qRT-PCR analysis of cyclin A2 (A) and cyclin D1 (B). (C) Representative images of Western blots of cyclin A2 and cyclin D1. (D) Quantitative analysis of Western blots in C. All values are means ± SD; n = 6 in each group. * P<0.05 vs. control or IS group, ** P<0.01 vs. control or IS group.
PGE$_2$-induced MC proliferation

To evaluate the direct role of PGE$_2$ in modulating cell cycle progression, we treated the MCs with PGE$_2$ at different concentrations (5, 10, 100 nM) for 24 h before analyzing the cell cycle progression. (A-D) Representative images of cell cycle following PGE$_2$ treatment. (E-H) Percentage of cells at G1/G0 (E), G2/M (F), S (G), and (S + G2)/M (H) phases following PGE$_2$ treatment. All values are means ± SD; n = 6 in each group; * P<0.05 vs. control, ** P<0.01 vs. control.

PGE$_2$ treatment selectively upregulated EP2 expression

To identify the potentially involved EP(s) in mediating PGE$_2$ effect on MC proliferation, we examined the expressions of EPs by qRT-PCR. As shown in Fig. 7A-D, PGE$_2$ selectively enhanced EP2 expression.

IS treatment stimulated NF-κB phosphorylation

Activation of NF-κB signaling has a known role in many diseases including chronic kidney diseases (CKDs). Thus, we examined the phosphorylation of NF-κB and observed that IS stimulated NF-κB phosphorylation in MCs (Fig. 7E and F), suggesting an involvement of NF-κB-mediated inflammation in this pathological process.
Discussion

In the clinical practice, preserving RKF in CKD patients is one of the major goals of nephrologists [4, 26, 27]. RKF is particularly important for maintaining a better status of health even in the patients with dialysis therapy [28-30]. RKF not only benefits the urinary excretion of salt and water, but also helps to remove the large molecular uremic toxins which can't be effectively cleared by dialysis [2, 4, 5]. As one of the well-known protein-bound uremic toxins [31], IS is markedly accumulated in the circulation of patients with advanced CKDs [6, 32, 33]. In this study, we reported that IS stimulated MC proliferation through mPGES-1/PGE2 cascade, which may contribute to the progressive loss of RKF to some extent.

The cell cycle is a set of events responsible for the duplication of cells. Its fundamental task is to ensure the faithful DNA replication and identical chromosomal copy in daughter cells [34]. The progression of the cell cycle is controlled by cyclins which are synthesized at specific stages of the cell cycle. The synthesis of cyclin D is initiated during G1 and drives the G1/S phase transition [35], and cyclin A-associated kinase activity is required for the entry into S phase, completion of S phase, as well as the entry into M phase [36]. Thus, the expressions of cyclin D1 and cyclin A2 were generally used as the markers for cell cycle progression. Here we showed IS significantly induced cyclin A2 and cyclin D1 expression in MCs in parallel with increased cell percentage in S phase. These findings suggest that IS serves as a potent contributor of cell cycle progression and cell proliferation in MCs.
In our previous study, we reported that IS significantly promoted cell cycle progression via upregulating COX-2 expression in mesangial cells [13]. However, whether mPGES-1/PGE\(_2\) (a downstream cascade of COX-2) plays a role in mediating COX-2 effect on MC proliferation is still unknown. Here we examined the regulation of mPGES-1 in IS-treated MCs and found that mPGES-1 was significantly induced by IS, suggesting a potential involvement of mPGES-1 in mediating IS-induced MC proliferation. At the same time, we found that the expression of mPGES-2 was significantly downregulated by IS in line with a trend reduction of cPGES. To our knowledge, although the \textit{in vitro} evidence suggested that mPGES-2 and PGES could contribute to the baseline PGE\(_2\) production [37], genetic deletion of mPGES-2 or cPGES in mice did not reduce PGE\(_2\) levels in different organs [38-40], which strongly argues against the nature of these two enzymes as the PGE\(_2\) synthases.

Next, we explored the functional role of mPGES-1 in mesangial cell proliferation induced by IS using mPGES-1 siRNA. Similar as COX-2 inhibition [13], mPGES-1 silencing inhibited IS-induced MC proliferation as evidenced by the blocked cell cycle progression and reduced cell number. Meanwhile, the PGE\(_2\) production induced by IS was also blunted. These data suggested that mPGES-1-derived PGE\(_2\) contributed to the mesangial cell proliferation. To further verify this conclusion, we treated mesangial cells with exogenous PGE\(_2\) and observed a direct effect of PGE\(_2\) on inducing mesangial cell proliferation.

Furthermore, we examined the potentially involved PGE\(_2\) receptors mediating PGE\(_2\) effect on MC proliferation. Following PGE\(_2\) treatment, we found a selective upregulation of EP2 but not other EPs, which suggests that EP2 might be the potential receptor mediating the PGE\(_2\) effect on MC proliferation. Finally, we found that IS induced the phosphorylation of NF-κB, indicating that NF-κB-mediated inflammation might be also involved in this pathological process.

In summary, in the present study, we demonstrated a pathogenic role of mPGES-1-derived PGE\(_2\) in IS-induced MC proliferation possibly through EP2 receptor. Currently, mPGES-1 is a
hot target for the development of new anti-inflammatory drugs. The findings from current study suggest that inhibition of mPGES-1 might potentially serve as a therapeutic strategy in protecting against the uremic toxin-related glomerular damage and RKF loss in advanced CKDs.

Acknowledgements

This work was supported by Grants from the National Natural Science Foundation of China (nos. 81370802, 81300591, 81670647, 81600557, 81600352, 81600532 and 81570616), the National Key Research and Development Program (no. 2016YFC0906103), the National Science Foundation of Jiangsu Province (no. BK2012001, BK20160137, and BK20160136), Projects of Jiangsu Province Science and Education Qiang Wei (no. QNRC2016091 and ZDRCA2016074), and Project of Nanjing National Commission on Health and Family Planning (no. ZKX16059 and ZKX16057).

Disclosure Statement

There is no Disclosure Statement to disclose.

References

Li et al.: mPGES-1-Derived PGE2 and Is-Induced Mesangial Cell Proliferation


