mPGES-1-derived PGE₂ Contributes to Adriamycin-induced Podocyte Injury

Jing Yu¹,²,³, Wei Gong¹,²,³, Yimei Wu¹,²,³, Shuzhen Li¹,²,³, Yiyun Cui¹,²,³, Yifei Ma¹,²,³, Yue Zhang¹,²,³, Guangrui Yang¹,²,³, Songming Huang¹,²,³, Zhanjun Jia¹,²,³, and Aihua Zhang¹,²,³

1. Department of Nephrology, Nanjing Children’s Hospital, Affiliated with Nanjing Medical University, Nanjing 210008, China.
2. Jiangsu Key Laboratory of Pediatrics, Nanjing Medical University, Nanjing 210029, China.
3. Nanjing Key Laboratory of Pediatrics, Nanjing 210008, China.

Correspondence to: Aihua Zhang, Department of Nephrology, Nanjing Children’s Hospital, Affiliated to Nanjing Medical University, 72 Guangzhou Road, Nanjing 210029, Jiangsu Province, P. R. of China, Tel: 0086-25-8311-7309, Fax: 0086-25-8330-4239, Email: zhaihua@njmu.edu.cn.

Running title: mPGES-1 and podocyte injury

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Podocyte damage is a common pathological feature in many types of glomerular diseases and is involved in the occurrence and progression of kidney disease. However, the pathogenic mechanisms leading to podocyte injury is still uncertain. The present study was undertaken to investigate the role of microsomal prostaglandin E synthase-1 (mPGES-1) in adriamycin (ADR)-induced podocyte injury, as well as the underlying mechanism. In both mouse kidneys and in vitro podocytes, application of ADR remarkably enhanced mPGES-1 expression in line with a stimulation of COX-2. Interestingly, inhibition of mPGES-1 with siRNA approach significantly attenuated ADR-induced downregualtion of podocin and nephrin. Moreover, ADR-induced podocyte apoptosis was also markedly blocked in parallel with blunted caspase-3 induction. In agreement with the improvement of cell phenotypic alteration and apoptosis, the enhanced inflammatory markers of IL-1β and TNF-α were also significantly suppressed by mPGES-1 silencing. More importantly, in mPGES-1 deficient mice, albuminuria induced by ADR showed a remarkable attenuation in line with decreased urinary output of PGE₂ and TNF-α, highly suggesting an in vivo role of mPGES-1 in mediating podocyte injury. In summary, findings from current study offered first evidence demonstrating a pathogenic role of mPGES-1 in mediating ADR-induced podocyte injury possibly via triggering inflammatory response.

**Key words:** mPGES-1, PGE₂, podocyte, apoptosis
Podocytes are terminally differentiated epithelial cells adhered to the outer layer of glomerular basement membrane (GBM). Podocytes prevent urinary protein loss by maintaining foot-processes and 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. By now, there are no specific therapies by targeting podocytes to prevent or attenuate podocyte damage. Therefore, exploration of pathogenic mechanism 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). Prostaglandin (PG) E2, an important inflammatory mediator, has been shown the increment in multiple kidney diseases (13, 16). PGE$_2$ is the product of arachidonic acid sequentially catalyzed by cyclooxygenase (COX) -1 and -2 and terminal PGE synthases (PGESs). COX-1 is constitutively expressed while COX-2 serves as an inducible enzyme under many physiological and pathological conditions in multiple organs including kidney. Accumulating evidence demonstrated that COX-2, an upstream enzyme of mPGES-1, plays a detrimental role in mediating podocyte injury (1, 15). Meanwhile, PGE$_2$ receptors EP1 and EP4 were also reported to be attributable to the podocyte damage in diabetic and ADR nephropathy (12, 21), highly suggesting a pathogenic role of PGE$_2$ in podocyte injury. However, it is still uncertain for the specific PGE$_2$ synthase responsible for the PGE$_2$ production and podocyte injury under pathological conditions.

Currently, three PGESs including microsomal prostaglandin E synthase-1 (mPGES-1),
mPGES-2, and cytosolic PGES (cPGES) have been identified. Among three PGESs, mPGES-2 and cPGES are constitutively expressed and are thought to be contributed to the baseline PGE2 production, while mPGES-1 is identified as an inducible enzyme in response to pathological stimuli coupling with COX-2(17, 18). Recently, mPGES-1 was reported to be of importance in both cisplatin-induced acute kidney injury (AKI) and 5/6 nephrectomy-induced chronic renal failure(10, 11), and is becoming a promising target for the development of novel anti-inflammatory drugs. The major goal of the present study was to define the role of mPGES-1-derived PGE2 in podocyte injury via in vitro and in vivo studies.

**Materials and Methods**

**Reagents and antibodies**

Caelyx (liposomal ADR) was purchased from Merck (Whitehouse Station, NJ, USA). Antibodies of anti-COX-2 (Cat#: 160106), anti-mPGES-1 (Cat#: 160140), anti-mPGES-2 (Cat#: 160145), and anti-cPGES (Cat#: 160150) were bought from Cayman Chemicals. Antibodies of anti-nephrin (Cat#: ab58968), anti-podocin (Cat#: ab93650) and anti-GAPDH (Cat#: ab9485) were purchased from Abcam (Cambridge, MA). Antibodies of anti-phospho-IκBα (Cat#: 9246), anti-IκBα (Cat#: 9242), anti-phospho-p65 (Cat#: 3031) and anti-p65 (Cat#: 8242) were purchased from CST (Danvers, MA). PGE2 EIA kit was from Cayman Chemicals (Ann Arbor, MI). IL-1β, TNF-α ELISA kit was from R&D systems (Minneapolis, MN).
Establishment of ADR-induced nephropathy in mice

Male adult (10-12 week) BALB/C mice were treated with ADR (12mg/kg) by single tail-vein injection. In another experiment, male adult mPGES-1 KO and littermate WT mice with C57 genetic background were treated with ADR at a dose of 15mg/kg by tail-vein injection. After 5 days, urine was collected using metabolic cages. All mice were maintained on a 12-h light–dark cycle in a temperature-controlled (19-21°C) room. They were fed standard rodent chow and were free access to drinking water. All procedures were in accordance with the guidelines approved by the Institutional Animal Care and Use Committee at Nanjing Medical University (No. 20090053).

Cell culture

MPC5 conditionally immortalized mouse podocyte clonal cells (provided by Peter Mundel at Mount Sinai School of Medicine and Dr. Jie Ding at Peking University) were cultured and induced to differentiate as described previously(22). The cells were maintained in RPMI1640 medium (HyClone, USA) containing 10% heat-inactivated fetal calf serum (Gibco, USA), 100U/ml penicillin G, and 100mg/ml streptomycin in the presence of 5% CO₂. To sustain podocyte proliferation, 10U/ml recombinant murine interferon-γ (Sigma, USA) was added into the medium and the 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 siRNA

mPGES-1 siRNAs and the silencer negative control (sncRNA) were commercially available
(GenePharma, Shanghai, China). Transfections with the siRNA oligomers were performed using the Lip2000 Kit (Invitrogen, Carlsbad, USA) according to the manufacturer’s instructions.

**Analysis of podocyte apoptosis**

For apoptosis assay, podocytes were stained with FITC-Annexin V and PI according to the instruction of manufacturer. 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 (qRT-PCR)**

Total RNA from cultured podocytes and renal cortex were extracted by Trizol reagent (Invitrogen, Carlsbad, CA). Oligonucleotides were designed using the Primer3 software (available at http://frodo.wi.mit.edu/) and synthesized by Invitrogen Company. The primer sequences were shown in table 1. qRT-PCR was used to detect mtDNA copy number and the target gene expression. Reverse transcription was performed by using Transcriptor First Stand cDNA Synthesis kit (Roche, Germany) according to the manufacture’s instruction. Real-time PCR amplification was performed using the ABI 7500 Real-time PCR Detection System (Foster City, CA) with Fast Start Universal SYBR Green master mix (Roche, Germany). Cycling conditions were 95°C for 10 min followed by 40 repeats of 95°C for 15 s and 60°C for 1 min. The relative gene expression level was calculated through Delta-delta Ct method and GAPDH was used as the internal control.
Western blotting
Podocytes or renal cortex were lysed in protein lysis buffer and protein concentration was measured as previously (9, 23). Immunoblotting was performed with primary antibodies against mPGES-1 (1:500), anti-COX-2 (1:500), anti-mPGES-2 (1:1000), anti-cPGES (1:1000), anti-Podocin (1:1000), anti-Nephrin (1:500), and anti-GAPDH (1:1000). The blots were visualized with Amersham ECL Detection Systems (Amersham, 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 12,000g. Concentration of PGE2 in urine and medium was determined according to the manufacturers’ instructions.

Statistical analysis
All results were presented as means± SE. The statistical analysis was performed using ANOVA followed by Bonferroni’s test with SPSS 13 statistical software. \( p < 0.05 \) was considered significant.
Results

**ADR treatment remarkably enhanced mPGES-1 expression in kidney.**

To test whether ADR could induce mPGES-1 in kidney tissue, we treated BALB/C mice with ADR by tail-vein injection. As expected, ADR remarkably elevated mPGES-1 and COX-2 protein expression in kidney cortex (Fig. 1A & B). Meantime, the protein levels of mPGES-2 and cPGES also exhibited increment after ADR administration (Fig. 1A & B). We further validated mRNA expressions of mPGES-1 and COX-2 in ADR-treated mice via qRT-PCR. As shown in Fig. 2A & B, ADR caused a significant induction of COX-2 and mPGES-1 at mRNA level. These data highly suggested 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 contrasting to unaltered mPGES-2 and cPGES (Fig. 3A & B). Via qRT-PCR, we observed a moderate but significant elevation of COX-2 mRNA expression (Fig. 4A). Meantime, the mRNA level of mPGES-1 displayed a significant upregulation (Fig. 4B). These data demonstrated a direct effect of ADR on mPGES-1 upregulation.

**Silencing mPGES-1 blocked ADR-induced PGE\(_2\) production**

To examine the role of mPGES-1 in ADR-induced PGE\(_2\) production in podocytes, mPGES-1 siRNA was applied to the podocytes (Fig. 5A & B). Strikingly, mPGES-1 siRNA not only
blocked the increment of mPGES-1 after ADR treatment but also remarkably blunted PGE₂ production determined by qRT-PCR and ELISA, respectively (Fig. 5C & D). These data indicated that mPGES-1 was an important enzyme in mediating ADR-induced PGE₂ production in podocytes.

Silencing mPGES-1 significantly blocked ADR-induced downregulation of podocin and nephrin and podocyte apoptosis.

mPGES-1 siRNA was applied to the podocytes to test the role of mPGES-1 in ADR-induced phenotypic alteration. Interestingly, the loss of podocyte marker proteins of podocin and nephrin was significantly attenuated by mPGES-1 silencing as examined by Western blotting (Fig. 6A & B). It is known that podocyte apoptosis is another pathogenic mechanism leading to the podocyte loss and renal dysfunction. As shown in Fig. 7A-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 caspase-3 mRNA level. These interesting data highly suggested that mPGES-1-derived PGE₂ is of importance in mediating ADR-induced podocyte injury.

Silencing mPGES-1 ameliorated ADR-induced inflammatory response in podocytes.

PGE₂ is one of the known mediators of inflammation and contributes to cellular injuries via promoting inflammatory response. To explore the potential mechanism contributing to the detrimental effect of mPGES-1/PGE₂ cascade on podocyte injury induced by ADR, we detected inflammatory cytokines of IL-1β, TNF-α, and ICAM-1 using qRT-PCR. As expected,
we observed a significant blockade of IL-1β and TNF-α and a trend reduction of ICAM-1 in mPGES-1 siRNA-treated cells following ADR challenge (Fig. 8A-C). By ELISA, we further confirmed the protein regulation of IL-1β and TNF-α (Fig. 9A & B). Due to the central role of NF-κB pathway in mediating inflammation, we examined the phosphorylation of p65 and IκB. Consistent with the regulation of proinflammatory cytokines, ADR-induced phosphorylation of p-65 and IκB was entirely abolished by mPGES-1 siRNA treatment (Fig. 9C & D).

**mPGES-1 deletion in 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, ADR was subjected to mPGES-1 WT and KO mice by 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. 10A-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 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 clinic. In animal studies, ADR was used to induce podocyte damage and focal segmental glomerulosclerosis (FSGS) (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 demonstrated that both COX-2 being an upstream enzyme to provide the substrate (PGH$_2$) for PGESs and EP4 were attributable to the pathogenesis of podocytopathy (2, 3), no evidence was provided to illustrate the role of PGE$_2$ synthases in podocyte injury. Thereby it is worthwhile to define the particular PGES participating in the pathogenesis of podocyte damage. Among three PGESs, mPGES-1 is best characterized. In kidney, many insults including aldosterone, cisplatin, Ang II can activate mPGES-1 to exert multiple functions in renal tubules (7, 8, 20). However, the report related to mPGES-1 action 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 PGE$_2$. Meanwhile, the 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 podocyte phenotype. Another known pathological phenomenon in ADR-induced podocyte injury is apoptosis (6). In agreement with the attenuation of 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 provided 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 with 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 injection for 5 days. Due to the resistance to ADR in mice with C57BL/6 genetic background (19), proteinuria in these mice quickly recovered to the 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 knockout mice with 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 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 decrease of ICAM-1. Meantime, the activation of NF-κB signaling pathway induced by ADR was also abolished by mPGES-1 silencing, indicating that mPGES-1-derived PGE$_2$ exerted a proinflammatory action possibly through activating NF-κB. In mPGES-1 KO mice, urinary excretion of TNF-α was also significantly blunted as compared to WT controls. All these data highly suggested that mPGES-1-derived PGE$_2$ could trigger inflammatory response to promote podocyte injury in response to pathological insults. Taken together, this study not only provided 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.

Acknowledgments

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Conflict of interest statement

The authors have declared that no conflict of interest exists.


Figure legends

Fig.1. ADR enhanced mPGES-1 protein expression in animal kidneys. (A) Western blots of COX-2, mPGES-1, mPGES-2, and cPGES. (B) Densitometric analysis of Western blots. All values are means ± SE (n=4 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.

Fig.2. ADR enhanced mPGES-1 mRNA expression in animal kidneys. (A & B) qRT-PCR analysis of COX-2 (A) and mPGES-1(B). All values are means ± SE (n=4 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.

Fig.3. ADR stimulated mPGES-1 protein expression in podocytes. (A) Western blots of COX-2, mPGES-1, mPGES-2, and cPGES. (B) Densitometric analysis of Western blots. All values are means ± SE (n=3 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.

Fig.4. ADR stimulated mPGES-1 mRNA expression in podocytes. (A & B) qRT-PCR analysis of COX-2 (A) and mPGES-1(B). All values are means ± SE (n=4 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.

Fig.5. mPGES-1 silencing in podocytes blunted ADR-induced PGE2 production. (A) qRT-PCR analysis of mPGES-1 after mPGES-1 siRNA treatment in podocytes. (B) Western blotting analysis of mPGES-1 after mPGES-1 siRNA treatment in podocytes. (C) qRT-PCR
analysis of mPGES-1 in ADR-treated podocytes with or without mPGES-1 siRNA administration. (D) EIA assay of PGE₂ in medium. All values are means ± SE (n=3 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.

**Fig.6. mPGES-1 silencing attenuated ADR-induced downregulation of nephrin and podocin.** (A) Western blots of nephrin and podocin. (B) Densitometric analysis of Western blots. All values are means ± SE (n=3 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.

**Fig.7. mPGES-1 silencing blocked ADR-induced podocyte apoptosis.** (A) Representative images of flow cytometry analysis of podocyte apoptosis. (B) Quantitative analysis of podocyte apoptosis. (C) qRT-PCR analysis of caspase-3. All values are means ± SE (n=3 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.

**Fig.8. mPGES-1 silencing ameliorated ADR-induced inflammatory response in podocytes.** (A-C) qRT-PCR analysis of IL-1β (A), TNF-α (B), and ICAM-1 (C). All values are means ± SE (n=3 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.

**Fig.9. mPGES-1 silencing attenuated NF-κB activation.** (A & B) ELISA analysis of IL-1β (A) and TNF-α (B) in cell culture medium. (C) Western blots of p-IκBα, IκBα, p-p65, and p65. (D) Densitometric analysis of Western blots. All values are means ± SE (n=3 in each group). * p<0.05 vs. control group.
Fig.10. mPGES-1 deficiency in mice improved albuminuria induced by ADR. (A) ELISA analysis of urinary albumin output. (B) EIA assay of urinary PGE2 output. (C) ELISA assay of urinary TNF-α excretion. All values are means ± SE (n=6-10 in each group). * p<0.05 vs. control group. ** p<0.01 vs. control group.
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Fig. 1

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B

Individual protein/GAPDH

- **Cox-2**: p<0.01
- **mPGES-1**: p<0.05
- **mPGES-2**: p<0.05
- **cPGES**: p<0.01
Fig. 2

A

Cox-2 mRNA/GAPDH

p < 0.05

Ctrl

ADR

B

mPGES-1 mRNA/GAPDH

p < 0.05

Ctrl

ADR
Fig. 3

(A) Western blot analysis of COX-2, mPGES-1, mPGES-2, cPGES, and GAPDH in cells treated with 1 and 2 μmol/L of a compound compared to the control (Ctrl).

(B) Quantitative analysis showing the expression levels of Cox-2, mPGES-1, mPGES-2, and cPGES normalized to GAPDH. Statistical significance is indicated by p<0.05.
Fig. 5

A. Bar chart showing the comparison of mPGES-1 mRNA/GAPDH levels between Ctrl and simPGES-1 groups. The p-value is less than 0.05.

B. Western blot analysis showing mPGES-1 and GAPDH levels between Ctrl and simPGES-1 groups.

C. Bar chart showing the comparison of mPGES-1 mRNA/GAPDH levels between Ctrl, Ctrl+ADR, and simPGES-1+ADR groups. The p-values are less than 0.01.

D. Bar chart showing the comparison of PGE2 secretion between Ctrl, Ctrl+ADR, and simPGES-1+ADR groups. The p-values are less than 0.01.
Fig. 6

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B

![Bar chart showing the comparison of individual protein levels with GAPDH normalized for Ctrl, simPGES-1, Ctrl+ADR, and simPGES-1+ADR conditions.](image)

- Podocin: Ctrl group is significantly different from simPGES-1 group with p<0.05.
- Nephrin: Ctrl+ADR group is significantly different from simPGES-1+ADR group with p<0.05.
Fig. 8

A

\[ \text{IL-1} \beta \text{mRNA/GAPDH} \]

\[ \begin{array}{c|c|c|c|c|c}
\text{Grp} & \text{Ctrl} & \text{simPGES-1} & \text{Ctrl+ADR} & \text{simPGES-1+ADR} \\
\hline
\text{IL-1} \beta \text{mRNA/GAPDH} & 1.0 & 2.0 & 3.0 & 4.0 \\
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\end{array} \]

\[ p < 0.05 \quad p < 0.05 \]

B

\[ \text{TNF-\alpha mRNA/GAPDH} \]

\[ \begin{array}{c|c|c|c|c|c}
\text{Grp} & \text{Ctrl} & \text{simPGES-1} & \text{Ctrl+ADR} & \text{simPGES-1+ADR} \\
\hline
\text{TNF-\alpha mRNA/GAPDH} & 1.0 & 2.0 & 3.0 & 4.0 \\
\hline
\end{array} \]

\[ p < 0.01 \quad p < 0.05 \]

C

\[ \text{ICAM-1 mRNA/GAPDH} \]

\[ \begin{array}{c|c|c|c|c|c}
\text{Grp} & \text{Ctrl} & \text{simPGES-1} & \text{Ctrl+ADR} & \text{simPGES-1+ADR} \\
\hline
\text{ICAM-1 mRNA/GAPDH} & 1.0 & 2.0 & 3.0 & 4.0 \\
\hline
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\[ p < 0.01 \quad p = 0.1192 \]
Fig. 9

A

IL-1β secretion (pg/ml)

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B

TNF-α secretion (pg/ml)

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C

p-IκBα

D

Individual protein/GAPDH

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</tbody>
</table>
Fig. 10

A. Urinary albumin output (ug/24h) for WT and KO mice. Ctrl group is represented by a white bar, ADR group by a black bar. Significant differences are marked with p<0.01 and p<0.05.

B. Urinary PGE2 excretion (pg/24h) for WT and KO mice. Significant differences are marked with P<0.01.

C. Urinary TNF-α excretion (pg/24h) for WT and KO mice. Significant differences are marked with p<0.05.