Effect of selective inhibition or activation of PGE2 EP1 receptor on glomerulosclerosis

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Abstract. Prostaglandin E2 (PGE2) is involved in numerous physiological and pathological processes of the kidney via its four receptors. A previous study has suggested that a defect in the PGE2 receptor 1 (EP1) gene markedly suppressed the transforming growth factor-β1 (TGF-β1)-induced mesangial cell (MC) proliferation and extracellular matrix aggregation. Therefore, the present study aimed to adopt a pharmacological method of specifically suppressing or activating the EP1 receptor to further verify and demonstrate these results. The EP1 receptor antagonist SC-19220 and EP1 receptor agonist 17-phenyl-trinor-PGE2 ethyl amide (17-pt-PGE2) were selectively used to treat five-sixths nephrectomy renal fibrosis model mice and TGF-β1-stimulated MCs. An Alpha screen PGE2 assay kit, flow cytometry, western blotting and immunohistochemical techniques were adopted to perform in vivo and in vitro experiments. The present results suggested that compared with the control group, the selective EP1 receptor antagonist SC-19220 improved renal function, markedly reduced the plasma blood urea nitrogen and creatinine levels (P<0.05) and alleviated glomerulosclerosis (P<0.05). By contrast, the EP1 receptor agonist 17-pt-PGE2 aggravated renal dysfunction and glomerulosclerosis (P<0.05). To verify the renal protection mechanisms mediated by suppression of the EP1 receptor, the expression levels of endoplasmic reticulum stress (ERS)-related proteins, including chaperone glucose-regulated protein 78 (GRP78), transient receptor potential channel 1 (TRPC1) and protein kinase R-like endoplasmic reticulum kinase (PERK), were further evaluated histologically. The expression of GRP78, TRPC1 and PERK in the antagonist treatment group were markedly downregulated (P<0.05), whereas those in the agonist treatment group were upregulated (P<0.05). The present in vitro experiments demonstrated that, compared with the control group, the EP1 receptor antagonist suppressed the expression of GRP78, TRPC1 and PERK (P<0.05), reduced the production of PGE2 (P<0.05) and decreased the MC apoptosis rate (P<0.05), thus alleviating TGF-β1-stimulated MC injury. Consequently, consistent with previous results, selectively antagonizing the EP1 receptor improved renal function and mitigated glomerulosclerosis, and its potential mechanism might be associated with the suppression of ERS.

Introduction

Chronic kidney disease (CKD) is associated with a high incidence, poor prognosis and high medical costs, which has become a global public health issue (1). Renal fibrosis characterized by glomerulosclerosis and renal tubulointerstitial fibrosis is the common manifestation of multiple types of CKD, with a major pathological feature of activation of renal extracellular matrix (ECM)-producing cells; mesangial cell (MCs) are one of the major inherent cells for renal ECM production (2). Multiple cytokines have been known to participate in the pathological process of renal fibrosis; transforming growth factor-β1 (TGF-β1) is a well-recognized pro-fibrosis growth factor (3), which is an important regulatory factor of ECM deposition and renal fibrosis progression (4). Additionally, TGF-β1 stimulates MC expression of cyclo-oxygenase 2/membrane-bound prostaglandin E2 synthase 1, and the synthesis of its metabolic product prostaglandin E2 (PGE2) (5).

PGE2 is one of the major arachidonic acid metabolites in the kidney, which binds with four types of specific G protein-coupled receptors, EP1, EP2, EP3 and EP4, to extensively participate in multiple physiological and pathological processes in kidneys (6). It has been demonstrated that PGE2 activates these receptors to induce various intracellular signal transduction pathways, which may explain the multiple conflicting effects of PGE2 (7-11). EP1 receptor mainly mediates the recruitment of cytosolic Ca2+ via the Gq protein, and participates in regulating intracellular Ca2+ level (12,13). The endoplasmic reticulum (ER) is the major intracellular site

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of Ca\(^{2+}\) accumulation and protein processing; however, ER homeostasis is broken when Ca\(^{2+}\) is released, which results in the ER reaction (14). An exaggerated ER reaction response contributes to kidney disease due to glomerular and tubular damage (15). Glucose-regulated protein 78 (GRP78), transient receptor potential channel 1 (TRPC1) and protein kinase R-like endoplasmic reticulum kinase (PERK) are important ER marker proteins (16,17). It is currently known that the EP1 in situ hybridization signal occurs in the mesentery region, and the high glucose-induced MC proliferation can almost be completely suppressed by an EP1 antagonist (18). Moreover, previous studies have suggested that selective prostaglandin EP1 receptor antagonists effectively prevent the development of streptozotocin-induced diabetic nephropathy (DN) (19) and alleviate hypertension-induced renal injury (20) in rats. A previous study, which utilized in vitro experiments, demonstrated that the EP1 receptor gene defect inhibited TGF-β1-induced MC proliferation and ECM accumulation (5). Consequently, the PGE2-EP1 signaling axis appears to have a vital role in the genesis and development of renal injury.

The present study aimed to further examine and analyze the previous data demonstrated by Chen et al (5), which was obtained in EP1-/- mice. This study used a pharmacological method of specifically suppressing or activating the EP1 receptor. Consistent with these prior results, the results in the present study demonstrated that selectively antagonizing the EP1 receptor improved renal function, alleviated glomerulosclerosis and downregulated the expression of ER-related proteins GRP78, TRPC1 and PERK. Whereas, treatment with an EP1 receptor agonist was found to aggravate renal damage.

Materials and methods

Experimental animal groups and drug treatments. All animals were purchased from The Laboratory Animal Center of Nantong University. Animal experiments were approved by The Research Ethics Committee on Laboratory Animal Use of The Nantong University (Nantong, China) and all procedures in this study were conducted in accordance with the Guide for the Care and Use of Laboratory Animals. All mice were housed under standard conditions, as described previously (21) and were sacrificed using an intraperitoneal (i.p.) injection of 1% sodium thiopental at a dose of 100 mg/kg and death was confirmed by observing no breathing, pupil dilation and no heartbeat. In total, 45 C57/BL6 male mice aged 8-12 weeks and weighing 15-20 g were kept in nine cages, with five mice per cage, randomly divided into three groups appropriately (21) and were sacrificed using an intraperitoneal (i.p.) injection of an equal amount of saline at the same frequency as the treatment-group injections.

Cell culture. Kidneys from 8-12 week-old male wild-type (WT) mice were obtained from The Laboratory Animal Center, Nantong University (Nantong, China). The glomeruli were purified from the renal cortex and digested with 0.1% type I collagenase (Abcam) at 37°C for 40 min. Glomeruli were then collected through 40 and 70 μm stainless steel sieves. The digested samples were centrifuged at 1,000 x g for 5 min at room temperature, and the pellets were resuspended in DMEM (Gibco; Thermo Fisher Scientific, Inc.) growth medium containing 20% FBS (Gibco; Thermo Fisher Scientific, Inc.). Cells were cultured in a humidifying incubator containing 5% CO\(_2\) at 37°C. Cells at passages 8-10 were used. Then, the primary mice glomerular MCs were subjected to different treatments. EP1 agonist glomerular MCs were divided into four groups: i) WT group; ii) WT+TGF-β1 group; iii) 17-pter-PGE2 group (10 μmol/l); iv) and 17-pter-PGE2+TGF-β1 group. EP1 antagonist MCs were also divided into four groups: i) WT group; ii) WT+TGF-β1 group; iii) SC-19220 group (1.0 μmol/l); and iv) SC-19220+TGF-β1 group. The cells were serum starved for 24 h before treatment. 17-pter-PGE2 or SC-19220 was added to the cells at 37°C for 30 min. According to the results of our previous experiment, the optimal reaction time point and dose of TGF-β1 (PeproTech, Inc.) was 10 ng/ml at 37°C for 24 h (24). Each experiment was repeated at least three times with different cellular preparations.

PGE2 level detection. The PGE2 concentration in the cell culture supernatant was measured by an immunoassay. WT MCs were treated with 17-pter-PGE2 or SC-19220 and then 10 ng/l TGF-β1 for 24 h. Then, the cell supernatant was collected. The level of PGE2 in each supernatant sample was determined using an Alpha screen PGE2 assay kit (cat. no. E07966m; PerkinElmer, Inc.) according to the manufacturer’s instructions and an EnVision® Multilabel plate reader.

Western blot analysis. Immunoprecipitation cell lysis buffer (cat. no. ab152163; Abcam) was added to the wells and cells were incubated on ice for 30 min. Then, the cell lysate was transferred to 1.5 ml Eppendorf Tubes and centrifuged at 10,000 x g for 15 min at 4°C. Protein concentrations were determined using a Bca assay kit (Pierce; Thermo Fisher Scientific, Inc.). The samples were diluted (1:1) in loading buffer and boiled at 100°C for 5 min. Next, 130 μg protein from each sample was separated via SDS-PAGE on 10% gels and then wet transferred onto a PVDF membrane for 2 h. After transfer, the blots were blocked in 5% (w/v) skimmed milk at room temperature for 1 h. Then, the PVDF membrane was incubated at 4°C overnight with primary polyclonal antibodies: Anti-GRP78 (cat. no. ab21685; 1:1,000; Abcam), TRPC1 (cat. no. ab51255; 1:1,000; Abcam), PERK (cat. no. ab65142; 1:1,000; Abcam) and β-actin (cat. no. ab8227; 1:1,000; Abcam). The membrane was then washed with Tris-buffered saline with 0.05% Tween-20 three times for 5 min and then incubated with an anti-rabbit IgG secondary antibody (cat. no. ab190492; 1:5000; Abcam) at room temperature for 2 h. Protein bands were visualized using the Bio-Rad ChemiDoc XRS Imaging System (Bio-Rad Laboratories, Inc.). The results were semi-quantified using ImageJ software (version 1.8.0.112; National Institutes of Health).
Flow cytometry assay. The apoptotic rate was evaluated by flow cytometry (Beckman Coulter, Inc.). Each group of MCs was suspended in 500 µl binding buffer (BD Biosciences) and incubated with 5 µl Annexin V-EGFP (BD Biosciences) and 5 µl PI (BD Biosciences) at room temperature for 15 min. Phosphatidyl serine translocation to the cell surface was detected and used as an indicator of early apoptotic cells. The Annexin V-positive and PI-negative cells were defined as apoptotic cells. The apoptotic rate was quantified using Cell Quest software (version 3.3, FCM; BD Biosciences).

Removal of 5/6 of the kidney. Adult male WT C57BL/6 mice were subjected to 5/6 Nx or sham operations (9 mice in each group). Age-matched mice (8-12 weeks) underwent removal of 5/6 of the total renal volume under sodium thiopental-induced anesthesia. The operation was performed by resecting the right kidney, and then cauterizing the higher and lower poles of the left kidney. Once the left side was stitched, the dorsal incision was closed with stainless steel wound clips, and the mice entered recovery. The control mice underwent sham operations without the removal of any renal mass.

Sample collection. Prior to sacrifice, mice were anaesthetized with 1% pentobarbital (40 mg/kg) via intraperitoneal injection for serum collection. Blood samples (~0.8 ml) were collected from the postcava in heparinized tubes and centrifuged at 5,000 x g for 15 min at 4˚C to obtain serum for the measurement of serum creatinine and urea nitrogen. Serum was preserved at -80˚C. Subsequently, animals were sacrificed as aforementioned.

Serum creatinine and urea nitrogen concentrations were determined by performing a Creatinine assay (cat. no. 1012; Exocell, Inc.) and Urea Nitrogen Assay (cat. no. 1016; Exocell, Inc.), according to the manufacturer’s instructions.

Renal histological analysis. Renal histology was analyzed according to a previously described protocol (21,23). Mice were sacrificed with an overdose of sodium thiopental 12 weeks after the 5/6 Nx or sham surgery. The left ventricle of each mouse was perfused with PBS (0.1 M; pH 7.4), followed by a fixative solution of formaldehyde (4%). The kidney was removed, cleaned of connective tissue and embedded in paraffin. Subsequently, 3-µm sections were fixed and stained with periodic acid-Schiff and Masson’s stain, and the glomeruli were imaged for further analysis. The images were captured with a color video camera (VKC150; Hitachi, Ltd.) connected to a confocal microscope (Olympus Corporation) and analyzed using Leica Microsystems Imaging software (version 1A 2.0; Leica Microsystems GmbH) by a person blinded to the experimental groups.

Immunohistochemistry. Kidneys were fixed with 10% neutral buffered formalin at room temperature for 3 h and processed for immunostaining using standard techniques. Expression levels of collagen type 1 (Col1), GRP78, TRPC1 and PERK were measured by immunohistochemical staining methods. Paraffin-embedded sections (3-mm thick) were mounted on poly-L-lysine-coated glass slides overnight at 4˚C. Subsequently, sections were incubated at 37˚C for 1 h with the following primary antibodies: Anti-Col1 (cat. no. ab34710; 1:1,000; Abcam), anti-GRP78 (cat. no. ab21685; 1:1,000; Abcam), anti-TRPC1 (cat. no. ab51255; 1:1,000; Abcam), anti-PERK (cat. no. ab65142; 1:1,000; Abcam) and anti-β-actin (cat. no. ab8227; 1:1,000; Abcam). Sections were incubated with horseradish peroxidase-conjugated di-antibody (cat. no. HAF008; 1:2,000; R&D Systems). Subsequently, chromogen detection was performed using 3,3-DAB. For the control test, the binding antibody was omitted and PBS was used instead. Images were captured using a VKC150 color video camera (Hitachi, Ltd.) and an AX70 microscope (Olympus Corporation).

Statistical analysis. Data were analyzed using SPSS 19.0 statistical software (IBM Corp.) and are presented as the mean ± SEM. Statistical analysis was performed using one-way ANOVA followed by Tukey’s test. P<0.05 was considered to indicate a statistically significant difference. Each experiment was repeated at least three times.

Results

Effects of blocking or stimulating EP1 receptor on renal function. In 5/6 Nx mice, the EP1 receptor antagonist SC-19220 or EP1 receptor agonist 17-pt-PGE2 was used to selectively treat mice until 12 weeks after surgery. In the 5/6 Nx mice, plasma blood urea nitrogen (BUN) levels were significantly higher than those in controls (WT, 17.83±1.13 mmol/l vs. 10.78±0.63 mmol/l; 17-pt-PGE2, 21.55±1.67 mmol/l vs. 10.14±1.07 mmol/l; SC-19220, 13.75±0.87 mmol/l vs. 9.14±0.69 mmol/l; P<0.05; Fig. 1A). The plasma creatinine (Cr) concentration in 5/6 Nx mice was significantly higher than that in controls (WT, 28.25±1.86 µmol/l vs. 11.93±0.89 µmol/l; 17-pt-PGE2, 36.82±2.15 µmol/l vs. 12.26±0.78 µmol/l; SC-19220, 18.15±2.38 µmol/l vs. 10.75±0.91 µmol/l; P<0.05; Fig. 1B). At 12 weeks after surgery, there were significant decreases in BUN and Cr levels in the SC-19220-treated 5/6 Nx mice compared with the levels in the WT 5/6 Nx mice (BUN, 13.75±0.87 vs. 10.78±1.13; Cr, 18.15±2.38 vs. 28.25±1.86; both P<0.05). Furthermore, relative to the WT 5/6 Nx mice, the 17-pt-PGE2 treated 5/6 Nx mice exhibited significant increases in BUN and Cr levels (BUN, 21.55±1.67 vs. 17.83±1.13; Cr, 36.82±2.15 vs. 28.25±1.86; both P<0.05).

Effects of blocking or stimulating EP1 receptor on glomerulosclerosis. At 12 weeks after 5/6 Nx, compared with the 5/6 Nx WT controls, the ECM of the SC-19220-treated 5/6 Nx mice was notably reduced, whereas the ECM was markedly increased in 17-pt-PGE2-treated 5/6 Nx mice, as determined from periodic acid-Schiff staining. However, no significant difference in interstitial fibrosis was observed compared with the sham-operative kidney group (Fig. 2A). The results of Masson's staining demonstrated that less collagen deposition (blue area) around the glomeruli was observed in the SC-19220-treated 5/6 Nx mice compared with in the 17-pt-PGE2-treated 5/6 Nx mice, and compared with the SC-19220-treated 5/6 Nx mice, the WT 5/6 Nx mice showed a markedly larger area of collagen deposition. In addition, the area of collagen deposition was markedly increased in the 17-pt-PGE2-treated 5/6 Nx mice compared with the WT 5/6 Nx mice (Fig. 2B). Compared with the WT 5/6 Nx group, expression of Coll1 was reduced in the SC-19220 treated 5/6 Nx mice, whereas in 17-pt-PGE2-treated 5/6 Nx mice expression was upregulated (Fig. 2C and D).
Effects of blocking or stimulating EPI receptor on the endoplasmic reticulum stress (ERS)-related proteins. To identify the renal protection mechanism mediated by suppression of the EPI receptor, the expression of ERS-related proteins, GRP78, TRPC1 and PERK, was further assessed histologically. The effects of blocking or stimulating the EPI receptor on the expression of GRP78, TRPC1 and PERK were compared using immunohistochemical staining of the mouse glomeruli. The results suggested that compared with the WT 5/6 Nx group, expression of GRP78, TRPC1 and PERK was significantly reduced in the SC-19220-treated 5/6 Nx mice, whereas in 17-pt-PGE2-treated 5/6 Nx mice expression was significantly upregulated (Fig. 3A and B).

Meanwhile, in vitro experiments demonstrated that, compared with the control group (WT+TGF-β1 MCs), the protein expression levels of GRP78, TRPC1 and PERK were significantly downregulated in WT MCs treated with SC-19220+TGF-β1 (*P<0.05; Fig. 3C); whereas in MCs treated with 17-pt-PGE2+TGF-β1 expression levels were significantly upregulated (P<0.05; Fig. 3D).

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Figure 2. Effects of blocking or stimulating the prostaglandin E2 receptor 1 on the degree of renal fibrosis. Images were captured with a Hitachi VKC150 camera. (A) Periodic acid-Schiff staining was used to visualize the accumulation of extracellular matrix. (B) Masson's staining was used to visualize collagen deposition. WT, wild-type; 5/6 Nx, five-sixths nephrectomy; con, control; 17-pt-PGE2, 17-phenyl-trinor-prostaglandin E2 ethyl amide.
alpha screen PG E2 assay kit results suggested that PG E2 protein expression in the TGF-β1-treated MC groups significantly increased compared with groups that were not treated with TGF-β1. Compared with the WT+TGF-β1 group, PGE2 levels were significantly decreased in the SC-19220+TGF-β1 group and significantly increased in the 17-pt-PGE2+TGF-β1 group (P<0.05; Fig. 4A).

Flow cytometry results demonstrated that, compared with cells not treated with TGF-β1, the TGF-β1-treated cells had a higher rate of apoptosis. Furthermore, compared with the WT+TGF-β1 group, the SC-19220+TGF-β1 group had a smaller number of apoptotic cells and the 17-pt-PGE2+TGF-β1 group exhibited a larger number of apoptotic cells (P<0.05; Fig. 4B). In the control group, limited apoptosis was detected.

Discussion

In previous years, with the culture of various EP gene knockout mice, the roles and mechanisms of action of prostaglandin receptors in respiratory, circulatory and urinary system diseases have been extensively investigated (21,25-28). In our previous study, EP1-/- mice and WT mice MCs were subjected to primary culture in vitro, which verified that EP1 receptor gene deletion markedly suppressed the TGF-β1-induced MC proliferation and ECM accumulation (5), this suggested that the EP1 receptor may be closely associated with the occurrence of MC injury. The present study further demonstrated the effects of EP1 receptors on glomerulosclerosis using the pharmacological method of specific selective suppression or activation of EP1 receptors.

In the present study, it was demonstrated that the selective EP1 antagonist SC-19220 attenuated the progression of glomerulosclerosis in mice. Its beneficial effects mainly manifested as reducing excessive glomerular proliferation, suppressing mesentery dilation, decreasing BUN and Cr levels, and delaying renal failure progression. During the entire experimental period, no increased mortality or other obvious side and toxic effects were observed in mice with renal fibrosis treated with the inhibitor. These findings suggested that suppressing the PGE2-EP1 signaling axis delayed the progression of glomerulosclerosis. Previously, Makino et al (19) identified that activation of the PGE2-eP1 system also exerted an important role in the progression of DN, whereas the EP1 antagonist completely blocked excessive glomerular proliferation and proteinuria production in rats with DN. Moreover, previous literature has reported that the EP1 receptor mediates histological and functional changes in the renal injuries of stroke-susceptible rats with spontaneous hypertension (29). Consistent with these results, the present results demonstrated that selectively suppressing the expression of the EP1 receptor had a protective effect on renal injury. However, the present study was different from previous studies in that the EP1 receptor agonist 17-pt-PGE2 was also used to specifically activate EP1 receptors to further verify the results; distinctly different results were observed, namely, renal dysfunction and glomerulosclerosis were aggravated, thus supporting the hypothesis that EP1 might be one of the important regulatory factors mediating renal injury.

The EP1 receptor is a type of multiple-transmembrane G protein-coupled receptor, and its activation triggers the release of extracellular Ca2+ and increases inflow of extracellular Ca2+. A previous study demonstrated that intracellular Ca2+ overload is one of the key steps resulting in aggravated ERS (30). However, to the best of the authors’ knowledge, at present, no previous study has determined whether the EP1 receptor-mediated renal injury mechanism is directly associated with ERS. The immunohistochemical staining results from the in vivo experiments demonstrated that the expression levels of ERS marker proteins GRP78, TRPC1 and PERK, were markedly downregulated in the antagonist SC-19220 treatment group, whereas they were upregulated...
in the agonist 17-pt-PGE2 treatment group. To verify the experimental results in vivo, the TGF-β1-induced MCs were cultured in vitro, and compared with the control group, the EP1 receptor antagonist SC-19220 suppressed the expression of GRP78, TRPC1 and PERK, reduced PGE2 production, and reduced the MC apoptosis rate. Opposite results were observed in the agonist 17‑pt‑PGE2 treatment group. It is commonly known that abnormality in ERS is cytotoxic and results in cell apoptosis (14). Consequently, based on the present in vivo and in vitro experimental results, selectively suppressing the EP1 receptor decreased GRP78, TRPC1 and PERK expression, alleviated ECM deposition in renal tissues and inhibited ERS-induced cell apoptosis, thereby alleviating renal fibrosis and delaying renal dysfunction.

There were some limitations in this study; for example the present study did not include the effects of small interfering RNA targeting EP1 expression in vitro, which would be a more accurate method to further understand the underlying mechanism of the EP1 receptor in renal dysfunction.

In conclusion, the present study provided results consistent with previous studies, suggesting that selectively suppressing the EP1 receptor protects from glomerulosclerosis, whereas selectively activating the EP1 receptor aggravates glomerulosclerosis and renal dysfunction. Furthermore, the present study provided novel insight regarding the role of the EP1 receptor in the development of glomerulosclerosis, and suggested that blocking the EP1 receptor may represent a novel therapeutic strategy of glomerulosclerosis.
Figure 3. Continued. Effects of blocking or stimulating the prostaglandin E2 receptor 1 on endoplasmic reticulum stress-related proteins. Images were captured with a Hitachi VKC150 camera. Western blotting was performed to detect GRP78, TRPC1 and PERK expression in groups treated with (C) SC-19220 and (D) 17-pt-PGE2 24 h following treatment with 10 ng/ml TGF-β1. *P<0.05. Con, control; WT, wild-type; GRP78, glucose-regulated protein 78; TRPC1, transient receptor potential channel 1; PERK, protein kinase R-like endoplasmic reticulum kinase; TGF-β1, transforming growth factor-β1; 17-pt-PGE2, 17-phenyl-trinor-prostaglandin E2 ethyl amide.
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Availability of data and materials
The datasets used and/or analyzed during the present study are available from the corresponding author on reasonable request.

Figure 4. Effects of blocking or stimulating the prostaglandin E2 receptor 1 on the TGF-β1-induced upregulation of PGE2 and apoptosis of mouse MCs. (A) PEG2 concentration was detected in each group at 24 h after 10 ng/ml TGF-β1 treatment. (B) Apoptotic rates were measured in the MCs using fluorescence-activated cell sorting analysis after Annexin-V/Pl staining. *P<0.05, **P<0.01. TGF-β1, transforming growth factor-β1; PGE2, prostaglandin E2; MCs, mesangial cells; PI, propidium iodide; WT, wild-type; 17-pt-PGE2, 17-phenyl-trinor-prostaglandin E2 ethyl amide.
Authors' contributions

XC, JY, YXY, ZQ and JL performed the experiments and analyzed the data. XC and JY drafted and revised the manuscript. XLC was responsible for financial support of the present study, conceived and designed the present study, and provided final supervision. All authors read and approved the final manuscript.

Ethics approval and consent to participate

All procedures and handling of animals in the present study were performed according to local guidelines for the Care of Laboratory Animals of Animal Experimental Center and were approved by The Research Ethics Committee on Laboratory Animal Use of The Nantong University.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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