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All-trans Retinoic Acid Protects Renal Tubular Epithelial Cells Against Hypoxia Induced Injury In Vitro

X. Wan, X. Li, H. Bo, Y. Zhao, L. Liu, W. Chen, Z. Yin, and C. Cao

ABSTRACT

Background. It has been reported that the all-trans retinoic acid (atRA)-mediated protective effects in various cells are related to the inhibition of nuclear factor (NF)-κB activities. There exists some evidence that an increase in vascular endothelial growth factor (VEGF), which is expressed by proximal tubular epithelial cells and regulated by NFκB, may play a critical role in maintaining peritubular capillary endothelium in renal disease. By stimulating the production of VEGF, hypoxia is involved in tubulointerstitial fibrosis processes in various renal diseases.

Methods. NRK52E cells survival rate was proportional to absorbance in dimethyl-thiazol-diphenyltetrazoliumbromide tests. Quantitative real-time polymerase chain reaction and Western blot were performed to assay the expression of VEGF, p65, and Scpep1. The activation of NFκB was determined by electrophoretic mobility shift assay. Co-immunoprecipitation analysis demonstrates that whether the Scpep1 and NFκB protein interacted.

Results. We demonstrated that the hypoxia-mimicking agent $CoCl_2$ triggered hypoxia injury of rat proximal tubular epithelial cells and significantly reduced cell viability. Addition of atRA increased the cell survival rate. Under $CoCl_2$-mimicking hypoxic conditions, the expression of VEGF and p65 increased. The addition of atRA significantly attenuated the expression of VEGF and p65. There was a similar variation of NFκB/DNA binding activities. atRA not only activated distinct pathways to stimulate the expression of Scpep1, a retinoid-inducible gene, under normoxic conditions, but also acted as a $CoCl_2$-mimicking hypoxia.

Conclusion. The protective effects of atRA against hypoxia-induced injury might be involved in suppression of VEGF expression via stimulating Scpep1 distinct pathways and inhibiting the NFκB pathway.

HYPOXIA has recently been proposed as a common mechanism of tubulointerstitial fibrosis in the progression of various renal diseases, regardless of their underlying causes.[1] By simulating proximal tubules to increase production of proinflammatory cytokines and chemokines, hypoxia induces production of the extracellular matrix and decreases turnover in renal fibroblasts, which are key events leading tu tubulointerstitial fibrosis.[2] Although the underlying mechanisms are not completely elucidated, several observations suggest that vascular endothelial growth factor (VEGF) may be involved in the cellular response to hypoxia.[3–5] Currently, there is great interest in the pathogenetic role of VEGF in renal fibrotic, cystic tubular, and glomerular diseases, including diabetic nephropathy.[6,7] Nam et al[8] found that the nuclear factor (NF)-κB/HIF-1/VEGF pathway was activated under hypyoxic conditions.

All-trans retinoic acid (atRA) is a metabolite of vitamin A, which is well known to modulate immunologic and inflammatory responses, most probably by regulating cytokine production.[9,10] In recent years, much attention has been focused on the effects of atRA on maintaining the

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normal renal tissue structure and attenuating the development of renal pathological changes.[11] It has been reported that the atRA-mediated protective effects in various cells were related to the inhibition of NFκB activities.[12–14] Because the NFκB pathway is activated in hypoxia, we tested the hypothesis that atRA-mediated protective effects in cultured renal epithelial cells could be involved in suppression of VEGF expression via acting distinct pathways and inhibiting NFκB pathway.

MATERIALS AND METHODS

Epithelial Cell Culture

NRK52E renal tubular epithelial cells were purchased from American Type Culture Collection (Rockville, Md) and cultured in Dulbecco's modified Eagle's medium (Hyclone, Logan, Ut) containing 10% heat-inactivated fetal bovine serum, 2 mmol/L L-glutamine, and 100 IU/mL penicillin/streptomycin.

Cells were plated at a concentration of 1×10^5 cells/mL and used for experiments when they reached 80% confluence. Cultures were maintained in a humidified incubator with 5% CO_2 at 37°C. After reaching confluence, cells were treated with various concentrations of $CoCl_2$ in the presence or absence of atRA for the indicated time intervals. After incubation, cells were lysed by adding lysis buffer containing 10 mmol/L Tris HCl (pH 7.5), 1 mmol/L EGTA, 1 mmol/L $MgCl_2$, 1 mmol/L sodium orthovanadate, 1 mmol/L dithiothreitol, 0.1% mercaptoethanol, 0.5% Triton X-100, and the protease inhibitor cocktails (at final concentrations of 0.2 mmol/L PMSF, 0.1% aprotinin, and 50 μg/mL leupeptin). Cells adhering to the plates were scraped off using a rubber policeman and stored at −70°C for further measurements.

Proliferation and Viability Assay

MTT (dimethyl-thiazol-diphenyltetrazoliumbromide) assays were used to measure the number of viable cells and performed as previously described.[15] The absorbance at 490 nm was measured spectrophotometrically and subtracted from that of a blank consisting of lysis buffer alone. The absorbance was proportional to the survival rate of the cells. Each experiment was repeated at least three times.

Quantitative Real-Time Reverse Transcriptase Polymerase Chain Reaction

Total cellular RNA was extracted using TRIzol reagent (Invitrogen). The primer sequences used were as follows: p65: (sense) 5′-ACGATCTGTTTCCCCTCATC-3′ and (antisense) 5-′-TGCTTCTCTCCCCAGGAATA-3′; Scpep1: (sense) 5′-AAGGGCTTGTGGAGAGGAAT-3′ and (antisense) 5′-TGTGAGGTGAGTCAGTACCCC-3′; VEGF: (sense) 5′-CCTGGCTTTACTGC TGTACCT-3′ and (antisense) 5′-GCTGGTAGACGTCCATGAACT-3′; and β-actin: (sense) 5′-CTG GAACGGTGAAGGTGACA-3′ and (antisense) 5′-AAGGGACTTCCTGTAACAACGC A-3′. The target mRNAs were quantified by real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis using a one-step real-time RT-PCR kit (Toyobo Co., Ltd., Osaka, Japan) according to the manufacturer's instructions. Cycling conditions were at 90°C for 30 seconds, 61°C for 20 minutes, 95°C for 60 seconds, then 40 cycles at 95°C for 15 seconds, 60°C for 1 minute. The mRNA level of each sample was normalized to that of the β-actin mRNA and presented as unit values of 2^ [Ct (β-actin) − CT (p65)]. The amplification was monitored on an ABI prism 7500 realtime PCR apparatus (Applied Biosystems, Calif).

Western Blot Analysis

Cells were harvested from 6-well plates, and lysed with ice-cold lysis buffer (50 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 1 mmol/L $MgCl_2$, 100 μg/mL PMSF, and 1% Triton X-100) for 30 minutes on ice. Cell lysates were then collected after centrifugation at 12,000 rpm for 5 minutes at 4°C. Protein concentrations were measured using BCA Protein Assay Kit (Keygen, Nanjing, China).[16] Equal amounts (30 μg) of lysate proteins were separated on 15% (for detection of p65), 12% (for detection of Scpep1) or 10% (for detection of VEGF) SDS-PAGE gels, transblotted onto PVDF membrane, and blocking with 5% skim milk powder in TBST buffer (10 mmol/L Tris, pH 7.5, 150 mmol/L NaCl, and 0.05% Tween 20). Subsequently, the membranes were probed with rabbit anti-p65 (1:500; Santa Cruz Biotech, Calif) or rabbit anti-Scpep1 (1:500; Santa Cruz Biotech), respectively, followed by horseradish peroxidase-conjugated goat anti–β-action IgG (1:800; Santa Cruz Biotech). Protein bands were detected using ECL detection system (Pierce Chemical, Rockford, Ill).

Electrophoretic Mobility Shift Assay

Nuclear proteins of NRK52E cells were isolated using Nuclear and Cytoplasmic Protein Extraction Reagents (Keygen) according to the manufacturer's protocol. Protein concentrations were measured using BCA Protein Assay Kit (Keygen).[17] Double-stranded oligonucleotides encoding the NFκB consensus sequence were 5′-AGTTGAGGGGACTTTCCCAGGC-3′ and 3′-TCAACTCCCCTGAAAGGGTCCG-5′, which were end-labeled with biotin (Beyotime, Haimen, China). Nuclear extracts, (5 μg) were added in 20 μL of binding reactions and incubated for 20 minutes at room temperature. Electrophoretic Mobility Shift assay (EMSA) was performed using Light-Shift Chemiluminescent EMSA Kit (Pierce) according to the manufacturer's protocol. To establish the specificity of the reaction, negative controls without cell extracts and competition assays with a 200-fold excess of unlabeled oligonucleotides were performed. In competition assays, the corresponding unlabeled probe was added to the reaction mixture 10 minutes before the addition of the labeled probe.

Co-Immunoprecipitation

Total cellular proteins (500 μg) were first precleared with 25 μL of protein A/G agarose beads for 30 minutes (Santa Cruz Biotech) and then incubated overnight with 3 μL of polyclonal serum. Protein A/G agarose beads (25 μL) were added for 4 hours. All steps were done on a rocker at 4°C. Protein A/G agarose beads were collected by centrifugation, and washed 4 times with buffer. Bound proteins were eluted by boiling in SDS-PAGE sample buffer and subsequently analyzed by Western blot using the indicated antibodies (anti-p65or anti-Scpep1). Immunoprecipitation was performed with a rabbit polyclonal anti–R-Ras (Santa Cruz Biotech) and a nonimmune rabbit immunoglobulin (pre-immune serum) as a negative control.

Statistical Analysis

We used SPSS13.0 software (SPSS, Inc, Chicago, Ill). Each assay was performed ≥3 times. The data were expressed as mean ± SD; ANOVA and Student's *t* tests were used. $P < .05$ was considered significant. The data shown in some figures (eg, Western blots)

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