Original Paper

All-Trans Retinoic Acid Attenuates Hypoxia-Induced Injury in NRK52E Cells via Inhibiting NF- κ B/VEGF and TGF- β_2 /VEGF Pathway

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Key Words

All-trans retinoic acid • VEGF • TGF- β_2 • NF- κ B • Scep1

Abstract

Background/Aims: Hypoxia has recently been proposed as one of the most important factors in progressive renal injury. Hypoxia-induced vascular endothelial growth factor (VEGF) expression may play a critical role in maintaining peritubular capillary endothelium in renal disease. This study was designed to investigate the effect and underlying mechanism of all-trans retinoic acid (ATRA) on hypoxia-induced injury in NRK52E cells. **Methods:** For mimicking hypoxia, cells were treated with 100 μ M of cobalt chloride (CoCl₂). The cell viability, expression of VEGF, p65, transforming growth factor- β_2 (TGF- β_2) and serine carboxypeptidase 1 (Scep1), and nuclear factor of kappaB (NF- κ B) activities after ATRA treatment were determined by MTT, western blot and electrophoretic mobility shift assay. Co-immunoprecipitation analysis was performed to demonstrate whether Scep1 interacted with TGF- β_2 . **Results:** It was found that CoCl₂ triggered hypoxia injury and significantly reduced cell viability. ATRA pretreatment increased the cell survival rate. Under hypoxic conditions, the expression of VEGF, p65 and TGF- β_2 increased. Addition of ATRA significantly attenuated the expression of VEGF, p65 and TGF- β_2 . There was a corresponding variation of NF- κ B/DNA binding activities. In addition, ATRA stimulated Scep1 expression under normoxic and hypoxia condition. Furthermore, TGF- β_2 interacted with Scep1. **Conclusions:** This study indicated that ATRA may attenuate hypoxia-induced injury in NRK52E cells via inhibiting NF- κ B/VEGF and TGF- β_2 /VEGF pathway.

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Introduction

Hypoxia has recently been proposed as one of the most important factors in progressive renal injury [1]. By simulating proinflammatory cytokines, hypoxia increases extracellular matrix and decreases turnover in renal fibroblasts, which are key events leading to tubulointerstitial fibrosis and may further impair oxygen diffusion and aggravate regional hypoxia. Hypoxia also causes endothelial cell apoptosis, endothelial to mesenchymal transition as well as damage to the vascular pericytes, further promoting loss of peritubular capillaries and creating a vicious cycle typical for chronic progressive renal disease [2]. Although the underlying mechanisms are not completely elucidated, some investigations have reported that vascular endothelial growth factor (VEGF) may play a critical role in the cellular response to hypoxia [3].

VEGF is constitutively expressed in podocytes, proximal tubular cells, and medullary thick ascending limb cells in the juxtamedullary region of the normal kidney [4]. Altered VEGF expression has been observed in renal fibrosis as well as cystic tubular and glomerular diseases. There is some evidence that hypoxia-induced VEGF expression contributes to early hyperfiltration and microalbuminuria in diabetic rats and mice, which may play a central role in tubulointerstitial injury and renal fibrosis [5, 6]. Two major mechanisms have been found to stimulate VEGF expression, i.e., hypoxia and various cytokines, notably nuclear factor of kappaB (NF- κ B) and transforming growth factor (TGF) [7]. Nam et al. have found that the NF- κ B/HIF-1/VEGF pathway can activate under hypoxic conditions [8]. It has also been reported that TGF- β 1-induced VEGF expression in rat proximal tubular cells is mediated by Smad3 activation [6]. Furthermore, hypoxia-inducible factor could bind to Smad3, resulting in synergistic effects on VEGF expression [9]. Therefore, NF- κ B and TGF- β may play an important role on VEGF regulation under hypoxic conditions.

All-trans retinoic acid (ATRA), an active metabolite of vitamin A, which can modulate various physiological events, including cell cycle, cellular differentiation, and embryonic development [10, 11]. Endogenous retinoid levels are altered in different diseases of the lung, kidney and central nervous system, and contribute to their pathophysiology. Several investigations have reported that ATRA could play an excellent protective role in some renal diseases [12], but the detailed mechanism of its action is not clear. Interestingly, ATRA-mediated protective effects in various cells is related to the inhibition of TGF- β activities [13, 14]. However, TGF- β 1 is not involved in the induction of VEGF in response to acute hypoxia in NRK52E cells [12]. In both early and advanced models of renal fibrosis, the expression of TGF- β 2 is also markedly increased. In addition, TGF- β 2 is hypoxia-sensitive and involved in the preservation of the chondrocyte phenotype under hypoxic conditions and renal fibrogenesis [15]. Thus, we drew up a hypothesis that ATRA-mediated protective effects in cultured renal epithelial cells could be involved in suppression of VEGF expression via inhibiting NF- κ B and TGF- β 2 pathway.

Materials and Methods

Cell culture

NRK52E renal tubular epithelial cells were purchased from American Type Culture Collection (Rockville, MD, USA) and cultured in DMEM containing 10% fetal bovine serum in a humidified incubator containing 5% CO₂ at 37°C.

MTT assay

Cells were plated at a density of 4×10^3 cells/well in 96-well plates. After overnight recovery, NRK52E cells were exposed to 1 μ M of ATRA for 24 h prior to a 24 h incubation with 100 μ M of CoCl₂. Then, 20 μ l of MTT solution (2 mg/ml) was added and incubated for 4 h. After removal of the MTT solution, 200 μ l of dimethyl sulfoxide was added, and the absorbance at 570 nm was measured.

Western blot

Whole-cell extracts were prepared. Protein samples were resolved in 10% SDS-PAGE gels, transferred onto PVDF membrane, and blocked with 5% skim milk. Subsequently, Primary antibody incubation was carried out at 4°C overnight followed by incubation with secondary antibody conjugated to horseradish peroxidase for 1.5 h. Protein bands were detected using the ECL detection system (Pierce, Rockford, USA).

Electrophoretic mobility shift assay (EMSA)

Nuclear proteins were extracted. The preformed NF- κ B recognized probe (Beyotime, China) (5'AGT TGA GGG GAC TTT CCC AGGC-3'; 3'-TCA ACT CCC CTG AAA GGG TCCG-5') was set as the positive control. The sample without nucleoprotein was the negative control. Nucleoproteins and probes were incubated under room temperature for 20 min. EMSA was performed using Light-Shift Chemiluminescent EMSA Kit (Pierce, Rockford, USA) according to the manufacturer's protocol.

Co-immunoprecipitation assay

Total cellular proteins were added with 50 μ L of agarose beads for 30 min and then incubated overnight with polyclonal serum. This was followed by addition of agarose beads and incubation for 4 h. Then agarose beads were collected by centrifugation. Complexes were eluted in SDS lysis buffer and subsequently analyzed by western blot using the indicated antibodies (anti-TGF β 2, anti-Scepep1).

Statistical analysis

Experiments were performed in triplicate and data were expressed as mean $\pm$ standard deviation (SD). Statistical significance was determined by Student's t test or one-way analysis of variance (ANOVA). A value of $P < 0.05$ was considered statistically significant.

Results

ATRA inhibited VEGF expression

For mimicking renal hypoxia, NRK52E cells were treated with 100 μ M of cobalt chloride (CoCl₂), which is known to prevent ubiquitination and thus degradation of HIF-1 α protein in similar manner as true hypoxia and is therefore widely accepted as a compound mimicking effects of hypoxia. As shown in Fig. 1A, VEGF protein expression was increased at various time points after CoCl₂ treatment and the maximum expression was obtained at 6 h. Results from MTT assay showed that CoCl₂ could significantly inhibit cell growth. In contrast, the addition of ATRA led to a remarkable increase in survival rate (Fig. 1B). Western blot analysis revealed that CoCl₂-induced VEGF elevation were partially abolished in the presence of ATRA (Fig. 1C). These results suggest that the possible involvement of VEGF in ATRA-mediated protective effects against hypoxia induced injury.

ATRA downregulated p65 expression and inhibited NF- κ B activity

Because the expression of VEGF was regulated by NF- κ B and p65 was the transcriptionally active subunit of NF- κ B, western blot was performed to assess the effects of ATRA on p65 expression. As shown in Fig. 2A, CoCl₂ treatment significantly increased the protein expression of p65 in NRK52E cells, which were partially abolished in the presence of ATRA. Then, the effects of ATRA on the NF- κ B activities were assessed by EMSA. CoCl₂ induced NF- κ B activity remarkably. However, ATRA treatment significantly inhibited NF- κ B activity induced by CoCl₂ (Fig. 2B). Furthermore, we examined whether CoCl₂ increased VEGF production after blocking NF- κ B activation with pyrrolidine dithiocarbamate (PTDC). Treatment with PTDC partially abolished VEGF induced by CoCl₂ (Fig. 2C). In addition, ATRA can increase PTDC-mediated inhibition. Taken together, these data suggest that ATRA inhibits CoCl₂-induced VEGF expression by inhibiting NF- κ B activation and there may be other molecular mechanism involved in ATRA-mediated protective effects against hypoxia induced injury.

Fig. 1. ATRA inhibited VEGF expression induced by CoCl₂. After NRK52E cells were treated with CoCl₂ (100 μ M) for 0, 1, 3, 6, 9, and 12 h, VEGF protein levels were examined by western blot (A). Cells were pretreated for 24 h with ATRA (1 μ M) before incubation with CoCl₂ (100 μ M) for 24 h. Effect of ATRA on survival rate was assessed by MTT assay (B). Cells were pretreated for 24 h with ATRA (1 μ M) before incubation with CoCl₂ (100 μ M) for 6 h. VEGF expression was assessed by western blot (C). Data are expressed as mean $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 vs the control group; # P < 0.05, ## P < 0.01 vs the CoCl₂ group.

ATRA inhibited TGF- β 2 expression

As shown in Fig. 3, the protein expression of TGF- β 2 was increased significantly in CoCl₂-treated group compared with the control group. However, ATRA treatment significantly downregulated the expression of TGF- β 2 induced by CoCl₂. These results indicate that ATRA attenuates hypoxia-induced injury by inhibition of TGF- β 2.

ATRA stimulated Scape1 expression

Serine carboxypeptidase 1 (Scape1), a retinoid-inducible gene, have been found to be involved in kidney homeostasis. To determine the mechanism of ATRA against hypoxia induced injury, the effects of ATRA on Scape1 expression were studied. As shown in Fig. 4, there was no obvious change in Scape1 expression between CoCl₂-treated and control cells. ATRA pretreatment remarkably increased Scape1 expression. These data suggest that ATRA attenuates hypoxia-induced injury by stimulation of Scape1.

TGF- β 2 interacted with Scape1

To further determine whether TGF- β 2 could interact with Scape1 and led to antagonistic effect. As shown in Fig. 5, co-immunoprecipitation of Scape1 with antibodies against TGF- β 2 brought down Scape1, confirming the association of TGF- β 2 with Scape1.

Fig. 2. Effect of ATRA on p65 expression was assessed by western blot (A). EMSA was performed to determine NF- κ B/DNA binding activity (B). Cells were pretreated for 30 min with PDTC (30 μ M) before incubation with CoCl₂ (100 μ M) for 6 h. VEGF expression was assessed by western blot (C). Data are expressed as mean $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 vs the control group; # P < 0.05, ## P < 0.01 vs the CoCl₂ group.

Discussion

In this study, it was demonstrated that incubation of rat kidney tubular epithelial cells with CoCl₂ could result in marked elevation of VEGF expression, and this effect is attenuated by ATRA, suggesting the protective effects of ATRA against hypoxia induced injury.

NF- κ B constitutes one of the most important intracellular signaling pathways, of which p65 is the transcriptionally active subunit. Recent evidences suggest that NF- κ B activation may be under the control of oxidant/antioxidant balance [16]. In combination with HIF-1 α , which is regarded as a master regulator for cellular responses to hypoxia, the activated NF- κ B pathway enhances epithelial VEGF expression [8]. Specifically, in this study, it was found that hypoxia-induced NF- κ B activation was suppressed by ATRA, consistent with a previous observation in the mesangial cell system [17]. Treatment with PTDC can partially abolished VEGF elevation induced by CoCl₂ in NRK52E cells. However, ATRA can enhance PTDC-mediated inhibition. These results indicate that ATRA may inhibit CoCl₂-induced VEGF expression by inhibiting NF- κ B and there may be other molecular mechanism involved in ATRA-mediated protective effects against hypoxia induced injury.

Fig. 3. Effects of ATRA on the expression of TGF- β 2. The expression of TGF- β 2 was assessed by Western blot. Data are expressed as mean $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 vs the control group; ## P < 0.01 vs the CoCl2 group.

Fig. 4. Effects of ATRA on the expression of Scep1. The expression of Scep1 was assessed by Western blot. Data are expressed as mean $\pm$ SD (n = 3). * P < 0.05, ** P < 0.01 vs the control group; ## P < 0.01 vs the CoCl2 group.

Fig. 5. TGF- β 2 interacted with Scep1. Co-immunoprecipitation of Scep1 with TGF- β 2. The representative blot from 1 of 3 independent experiments.

TGF- β 2 is an important growth factor involved in the development of renal fibrosis. Previous studies have shown that TGF- β 2 can upregulate VEGF mRNA expression and protein secretion in human retinal pigment epithelial cells and mouse embryonic stem cells [18, 19]. In this study, it was observed that CoCl2-mimicking hypoxia increased TGF- β 2 expression, which was decreased in rat proximal tubular epithelial cells after pretreatment with ATRA. Thus, these data suggest that regulation of hypoxia-induced VEGF by ATRA may be partially driven by TGF- β 2 in addition to the classical NF- κ B-mediated pathway.

Scep1, initially named retinoid-inducible serine carboxypeptidase, has been detected strongly in renal proximal convoluted tubular epithelium and may be involved in kidney homeostasis [18]. In this study, it was demonstrated that ATRA can stimulate Scep1 expression under normoxic and hypoxia condition, suggesting its role in protection against hypoxia-induced kidney injury. In addition to direct transcriptional control, many target genes were indirectly regulated by ATRA through secondary events, such as induction and activation of TGF- β 2. Then, it was confirmed that TGF- β 2 interacted with Scep1 in NRK52E cells by Co-immunoprecipitation assay.

In conclusion, the present study demonstrates that the reduction of VEGF by addition of ATRA might be regulated by suppressing NF- κ B activation and decreased TGF- β 2 production.

Further research is needed to confirm the protective effects of ATRA against hypoxia-induced renal injury *in vivo* and to clarify other molecular mechanisms, so as to pave the way for its ultimate application in the clinic to benefit patients of renal diseases.

Abbreviations

VEGF (vascular endothelial growth factor); NF- κ B (nuclear factor of kappaB); TGF (transforming growth factor); ATRA (all-trans retinoic acid); EMSA (Electrophoretic mobility shift assay); SD (standard deviation); ANOVA (one-way analysis of variance); PDTC (pyrrolidine dithiocarbamate); Scep (Serine carboxypeptidase 1); CoCl₂ (cobalt chloride).

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Disclosure Statement

The authors indicate no potential conflicts of interest.

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