All-trans retinoic acid regulated prohibitin by retinoic acid receptor α in hypoxia-induced renal tubular epithelial cell injury

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Abstract: All-trans retinoic acid (ATRA) is a critical component in cell processes such as cell growth, differentiation and apoptosis, and it is also crucial in the regulation of extracellular matrix (ECM) deposition. Prohibitin (PHB) can regulate cell proliferation, apoptosis and differentiation. The current study investigated whether ATRA regulated PHB is induced by hypoxia/reoxygenation injury in renal tubular epithelial cells (RTEC), using gene interference treatments (knockdown or overexpression of RARα). Our results indicate that ATRA can augment the expression of RARα and PHB proteins and reduce the expression of TGF-β1, FN and Col-IV proteins. PHB expression was reduced in an ATRA treated RARα group, and TGF-β1, FN and Col-IV were up-regulated compared to the ATRA treated RARα+ group. We postulate that ATRA can induce the PHB expression by RARα in hypoxia/reperfusion related RTEC injury.

Key words: All-trans retinoic acid (ATRA); RARα; Prohibitin; Transforming growth factor beta 1 (TGF-β1); Renal tubular epithelial cells (RTEC); Oxidative stress; Extracellular matrix (ECM).

Introduction

Renal interstitial fibrosis (RIF) is a common condition involved in the progression of end-stage renal failure which is characterized by the excessive deposition of extracellular matrix (ECM) (1, 2). The transition of renal tubular epithelial cells (RTEC) to a mesenchymal phenotype is widely accepted as the underlying mechanism of RIF (3). Oxidative induced injury is accompanied by an increase of transforming growth factor β1 (TGF-β1) that can induce ECM accumulation. TGF-β1 is a key cytokine in the process of ECM deposition, promoting renal fibrosis (4).

All-trans retinoic acid (ATRA) is the main bioactive derivative of vitamin A, mediates cell apoptosis, differentiation and proliferation, and is also crucial for the regulation of ECM deposition (5-7). RARα modulates the regulatory actions of ATRA. Prohibitin (PHB) is an evolutionarily conserved protein which is involved in numerous cellular activities and also plays vital roles in the regulation of cell apoptosis, differentiation and proliferation (8). PHB overexpression protects mitochondrial respiratory mechanisms from oxidative stress injury(9). In our previous studies, we found that ATRA can increase PHB expression in a RIF rat model (10). However, the potential molecular mechanism of how ATRA regulates PHB is unclear. In the current study, we assess whether ATRA regulates PHB through the RARα.

Materials and Methods

Cell culture and treatment

The NRK-52E cells, also known as the normal rat renal proximal tubular epithelial cells (RTEC), were purchased from the Shanghai Cell Bank of the Chinese Academy of Sciences (Shanghai, China). The cells were cultured in DMEM/F12 with 5% fetal bovine serum, and placed in an incubator with a humidified atmosphere (37°C; 5% CO2).

Cells were divided into seven groups: (i) Control group, cells were cultured with normal atmosphere. (ii) H-R (hypoxia/reoxygenation) group, cells were cultured with 5% CO2 and 95% N2. (iii) RARα- group, cells were transfected by RARα-siRNA lentivirus for 48h. (iv) RARα+ group, cells were transfected by RARα+ lentivirus for 48h. (v) ATRA-RARα- group, RARα-cells were treated with 0.1mM ATRA(Sigma, USA) for 48h. (vi) ATRA-RARα+ group, RARα+ cells were treated with 0.1mM ATRA for 48h. (vii) Negative control group, cells were treated with empty lentivirus, n=6, respectively. The cells were then harvested for further detection.

mRNA expressions of RARα, PHB, and TGF-β1

The mRNA expression of RARα, PHB and TGF-β1 were determined with the following methods. RNA was extracted from each group with TRIzol (Beijing Tiangen, Co., China). ExScript RT reagent kit (Fermentas, MBI) was used for the reverse transcription of RNA. Primers
(designed by primer premier 5.0) for RARα, PHB and TGF-β1 were used with SYBR Premix Ex Taq (Roche Inc., Basel, Switzerland) for amplification. β-actin was used as the internal standard, and experiments were repeated three times. Gene expression was determined by the average Ct (threshold cycle), then analyzed with the comparative CT method (2−ΔΔCt method)(11).

Western-blot analysis
Total protein of each group was extracted with RIPA lysis buffer containing PMSF (Protease and phosphatase inhibitors, Sigma-Aldrich Corp., St. Louis, MO, USA). The modified Bradford assay was used to quantify the protein concentration. 40mg of total protein was loaded on gels for electrophoresis. The primary antibodies of RARα (Abcam, USA), PHB, TGF-β1, FN and Col-IV were incubated overnight at 4°C. Images were collected with an Odyssey Infrared Imaging System Scan (Li-Cor, Lincoln, NE, USA). Li-Cor Odyssey 3.0 analytical software was used to assess band intensity (12).

Oxidation and antioxidant product measurement
Malonyldialdehyde (MDA), reactive oxygen species (ROS), superoxide dismutase (SOD), and glutathione (GSH) were determined as previously described (13).

Statistical analysis
All data are presented as mean ± SD. One-way analysis of variation (ANOVA) with post-hoc Fisher’s LSD was applied for Gaussian distributed data. Conversely, Kruskal-Wallis with post-hoc Mann-Whitney was for parameters inconsistent with a Gaussian distribution. P < 0.05 was accepted as statistically significant. SPSS version 13.0 (SPSS Inc., Chicago, IL, USA) was used for all statistical analysis.

Results
mRNA expression
In the H-R treatment group, the mRNA expression of RARα and PHB were decreased, and the mRNA expression of TGF-β1 was increased (Figure 1). RARα and PHB mRNA expression was increased in the RARα+ group when compared with the RARα- group. Furthermore, TGF-β1 mRNA expression was reduced in RARα+ group when compared with that in RARα- group (Figure 1). RARα and PHB expression in the ATRA treated RARα+ group was increased, and TGF-β1 expression was reduced, when compared with levels in the ATRA treated RARα- group (Figure 1).

Protein expression
In the H-R treatment group, the protein expressions of RARα and PHB were reduced, and the protein expression of TGF-β1, FN and Col-IV were increased compared to those in the control group (each P<0.01, Figure 2). RARα and PHB proteins were increased in the RARα+ group when compared with those in the RARα- group. Furthermore, levels of TGF-β1, FN and Col-IV proteins were reduced in the RARα+ group when compared with those in the RARα- group (each P<0.01, Figure 2). Protein levels of RARα and PHB in the ATRA treated RARα+ group were increased, and TGF-β1, FN and Col-IV levels were reduced, when compared with those in the ATRA treated RARα- group (each P<0.01, Figure 2).

Redox status determinations
Compared with the control (normal group), H-R treatment increased the expression of MDA and ROS, and reduced GSH and SOD (each P<0.01, Figure 3). SOD and GSH expression levels were increased in the RARα+ group when compared with those in the RARα- group. Furthermore, levels of ROS and MDA were reduced in the RARα+ group when compared with that in RARα- group (each P<0.01, Figure 3). SOD
and GSH expression levels in the ATRA treated RARα+ group were increased, and ROS and MDA levels were reduced, when compared with those in the ATRA treated RARα- group (each \( P<0.01 \), Figure 3).

### Correlation analysis

Correlation analysis indicated that RARα protein level was positively correlated with PHB, SOD, and GSH \((r= 0.728, 0.816, 0.822; \text{each } P<0.05)\), but positively correlated with TGF-β1, FN, Col-IV, ROS, MDA \((r= 0.734, 0.808, 0.815, 0.793, 0.823; \text{each } P<0.05)\).

### Discussion

In the current investigation, ATRA treatment induced the expression of PHB by RARα. RARα protein level was positively correlated with PHB, GSH, and SOD, but positively correlated with TGF-β1, FN, Col-IV, ROS, and MDA. In our previous studies (10, 14), we confirmed that ATRA could induce the PHB expression in RTEC induced by hypoxia/reperfusion and in the renal tissue of renal interstitial fibrotic rats. In this study, we focused on the potential mechanism of ATRA effects on PHB. We found that ATRA may increase PHB expression through RARα in a RTEC injury model.

Previous investigations have reported that ATRA is capable of regulating RARα expression. Zhong et al (15) investigated the mechanism of ATRA’s effects on innate immunity signalling pathways in mouse liver injury induced by ischemia/reperfusion, and reported that ATRA may regulate innate immunity through the RARα/Akt/Foxo1 pathway to protect liver from the injury. Kalitin et al (16) investigated the role of ATRA in A549 lung cancer cells and reported that ATRA can increase both ligand and receptor expression and was correlated with RARα expression. Zhang et al (17) reported that treatment with ATRA at a concentration of 4 \( \mu \text{mol/l} \) can suppress cell apoptosis of PC12 cells following oxygen glucose deprivation injury, potentially through regulation of the RARα signalling pathway.

However, there have been no reports assessing the role of ATRA on RARα expression in RTEC induced by hypoxia/reperfusion.

In conclusion, we demonstrate that ATRA can induce PHB expression by RARα in a hypoxia/reperfusion induced RTEC injury. RARα protein levels were positively correlated with PHB, GSH, and SOD levels, but positively correlated with the TGF-β1, FN, Col-IV, ROS, and MDA levels. However, this hypothesis should be confirmed in vivo in future studies. We intend to perform these experiments in rat and inhibit or over-express RARα expression in renal tissue to test whether ATRA regulates PHB through RARα.

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### Competing financial interests

The authors have declared that no competing interests exist.

### References

ATRA regulated PHB by RARα.


