Effects of $A_{2B}R$ on the biological behavior of mouse renal fibroblasts during hypoxia

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Abstract. Fibroblasts are the effector cells of collagen secretion in renal interstitial fibrosis (RIF), and their proliferation and activation are essential for the development of RIF. Hypoxic ischemia in local tissues has been identified in chronic kidney diseases (CKDs), with adenosine (ADO) as a key signaling molecule. The current study investigated the association between ADO and the biological behavior of renal fibroblasts by establishing an *in vitro* hypoxia cell model. This aimed to provide experimental evidence for the prevention and treatment of RIF. NIH3T3 fibroblasts were exposed to hypoxia, and the subtypes of the ADO receptor (AR) on the cell surface were identified by a TaqMan probe-based assay. Cells were divided into the following four groups: i) Control; ii) 5'-N-ethylcarboxamidoadenosine (NECA); iii) PT, NECA + 8-phenyltheophylline (PT); and iv) MRS, NECA + N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide (MRS1754). The mRNA levels of transforming growth factor-β1 (TGF-β1), procollagen α1 (I) and α-smooth muscle actin (α-SMA) were measured following 24, 48, and 72 h of hypoxia. Cell proliferation was evaluated by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay at 0, 12, 24, 48 and 72 h. The results demonstrated that $A_{2B}R$ was the predominant AR subtype present in hypoxia-stimulated fibroblasts. NECA significantly induced fibroblast proliferation and upregulated the expression of TGF-β1, procollagen α1 (I) and α-SMA mRNA, while 8-PT and MRS1754 inhibited fibroblast proliferation and downregulated the expression of TGF-β1, procollagen α1 (I) and α-SMA mRNA. The blockage of $A_{2B}R$ in hypoxia significantly inhibited the proliferation and activation of fibroblasts, and reduced the production of profibrotic cytokines, thus preventing the generation and development of fibrosis.

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Introduction

Renal interstitial fibrosis (RIF) is considered to be the outcome of all types of chronic kidney disease (CKD) and the common pathway that leads to end-stage renal failure (1). It has been demonstrated that in the process of RIF, renal fibroblasts, the effector cells for collagen secretion, underwent excessive proliferation and phenotypic transformation into myofibroblasts (2,3), resulting in abnormal proliferation of fibroblasts and accumulation of extracellular matrix (ECM) (4-6).

It is widely accepted that the loss of peritubular capillaries leads to local hypoxic ischemia during RIF. Adenosine (ADO) is a key factor in hypoxic ischemia-induced signal transduction via binding to the ADO receptor (AR) on the cell surface (7,8). A previous study in the mouse unilateral ureteral obstruction (UUO) model demonstrated that in addition to occlusion, the kidney exhibited hypoxic aggravation, ADO elevation and interstitial collagen accumulation, finally resulting in RIF (9). It was hypothesized that the binding of ADO with AR under hypoxia triggered a series of pathological alterations in fibroblasts, further inducing the progression of RIF. In the present study, an *in vitro* mouse fibroblast model was used to investigate the effects and related mechanism of the ADO signaling pathway in RIF development.

Materials and methods

Cell culture. Cells from the mouse renal fibroblast cell line NIH3T3 (third/fourth passage) (Promab Biotechnologies, Inc., Changsha, China) were divided into the following four groups: i) Control; ii) 5'-N-ethylcarboxamidoadenosine, NECA (cat. no. 1691, Tocris Bioscience, Minneapolis, MN, USA); iii) PT, NECA + 8-phenyltheophylline (PT) (cat. no. 2795, Tocris Bioscience); and iv) MRS, NECA + N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)phenoxy]-acetamide (MRS1754) (cat. no. 2752, Tocris Bioscience). NECA was used as an analogue of ADO with *in vitro* instability. The cells were detected following incubation for 12, 24, 48 and 72 h. The drug concentrations were as follows: NECA, analogue of ADO, 20 μM; 8-PT, AR blocker, 20 μM, and MRS1754, $A_{2B}R$ antagonist, 20 μM.

Reverse transcription-quantitative polymerase chain reaction (RT-qPCR). RT-qPCR primers were designed by Premier, version 3.0 software (Premier Biosoft International, Palo Alto,

Table I. Primer sequences.

Gene	Primer sequence (5' to 3')	Amplified fragment length (bp)	Annealing temperature (˚C)
α_1 (I) procollagen-F	GTTCTCCTGGCAAAGACGGA	199	58
α_1 (I) procollagen-R	CGGCCACCATCTTGAGACTT		
TGF-β1-F	AGGGCTACCATGCCAACTTC	168	58
TGF-β1-R	CCACGTAGTAGACGATGGGC		
α-SMA-F	GGACTCTGGAGATGGTGTGAC	167	58
α-SMA-R	CAATCTCACGCTCGGCAGTA		
GAPDH (mouse)-F	AACTTTGGCATTGTGGAAGG	132	58/59
GAPDH (mouse)-R	GGATGCAGGGATGATGTTCT		

TGF-β1, transforming growth factor-β1; α-SMA; α-smooth muscle actin; bp, base pairs.

Table II. AR primer and probe sequences.

Gene	Primer sequence (5' to 3')	Annealing temperature (˚C)
A_1R	F-GTTTGGCTGGAACAACCTGA	
	R-ACACTTGATCACGGGCTCC	57
	Probe: FAM-AACAAGCCTGGATAGCCAACGGCA	
$A_{2A}R$	F-CCCCTTCATCTACGCCTACAG	
	R-GTGGGTTCGGATGATCTTCC	56
	Probe: FAM-TCCGGGAGTTCCGCCAGACCT	
$A_{2B}R$	F-GCGAGAGGGATCATTGCTG	
	R-CAGGAACGGAGTCAATCCAA	56
	Probe: FAM-CCTCTGGGTCCTTGCCTTTGGC	
A_3R	F-ATACCAGATGTCGCAATGTGC	
	R-GCAGGCGTAGACAATAGGGTT	56
	Probe: FAM-CATGGAGTTCGCGTGGGACAACAG	
β-actin	F-GCTCTGGCTCCTAGCACCAT	
	R-CCACCGATCCACACAGAGTAC	60
	Probe: FAM-ATCAAGATCATTGCTCCTCCTGAGCGC	

AR, adenosine receptor.

CA, USA) and synthesized by ProMab Biotechnologies, Inc. The primer sequences are listed in Table I.

Fibroblast total RNA was isolated by the single-step method using TRIzol (15596-026; Invitrogen Life Technologies, Carlsbad, CA, USA). RiboLock™ Ribonuclease Inhibitor (EO0381; Thermo Fisher Scientific, Pittsburgh, PA, USA) was used to remove genomic DNA. The reverse transcription reaction was performed using the RevertAid H Minus First Strand cDNA Synthesis kit (K1631; Thermo Fisher Scientific). SYBR® Green master mix (4309155; Applied Biosystems, Life Technologies, Foster City, CA, USA) was used for the RT-qPCR assay of target genes.

Fibroblast cell surface AR types were identified under hypoxia (1% O_2, 5% CO_2 and 94% N_2) by TaqMan probe-based analysis using JumpStart Taq Ready Mix kit (P2893; Sigma-Aldrich, St. Louis, MO, USA). Primer and probe sequences of A_1R, $A_{2A}R$, $A_{2B}R$, A_3R and β-actin mRNA are shown in Table II (10-12).

The PCR cycling conditions were as follows: 40 cycles of 94˚C (20 sec), 58˚C (20 sec) and 72˚C (30 sec), using an ABI Prism® 7900HT sequence detection system (Applied Biosystems Life Technologies). PCR quantification was conducted as previously described (13).

Cell proliferation assay. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay (MTT Cell Proliferation and Cytotoxicity Detection kit; Keygen Biotech Co., Ltd., Nanjing, China) was performed to determine the cell growth curve. Cells were seeded into a 96-well plate with $1x10^4$ cells/well and were subjected to hypoxia (1% O_2, 5% CO_2 and 94% N_2) for 24 h. Following incubation with the drugs described above, MTT and dimethyl

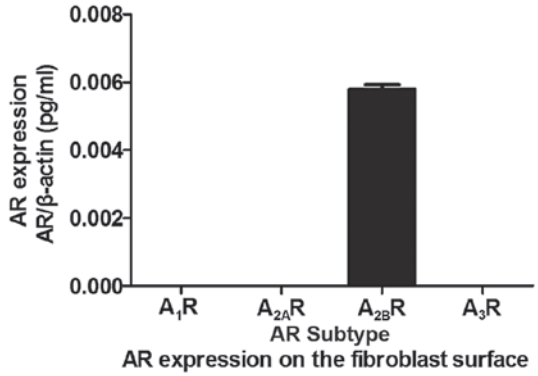

Figure 1. AR expression on the surface of renal fibroblasts under hypoxia. $A_{2B}R$ is the predominant receptor subtype (n=4). AR, adenosine receptor.

sulfoxide were added sequentially and the optical density (OD) was measured at 570 nm (DNM-9606 Enzyme Mark Analyzer; Perlong Medical, Beijing, China).

Cell-survival rate calculation: Cell-survival rate (%) = $(OD_{sample} - OD_{blank})/(OD_{control} - OD_{blank}) \times 100$. OD_{blank} refers to the value of culture medium mixed with MTT without cells. In addition, the cells were visualized microscopically (GX51; Olympus Corporation, Tokyo, Japan).

Statistical analysis. Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA), and all data are presented as the mean ± standard error of the mean. To analyze the differences between groups, Student's t-test for two groups, analysis of variance for multiple group comparisons, and Tukey's test for repeated measures were conducted. P<0.05 was considered to indicate a statistically significant difference.

Results

AR expression under hypoxia. The TaqMan probe-based assay demonstrated that the predominantly expressed AR on mouse renal fibroblasts under conditions of hypoxia was the $A_{2B}R$ subtype (Fig. 1).

Expression of transforming growth factor-β1 (TGF-β1), procollagen α1 (I) and α-smooth muscle actin (α-SMA).
TGF-β1 mRNA expression. Fig. 2A demonstrates that a significant increase in the levels of TGF-β1 mRNA was observed in fibroblasts under hypoxia in the NECA group (P<0.01). However, the PT and MRS groups were identified to have significantly lower expression levels than the NECA group (P<0.05) at days 2 and 3. No significant differences were observed between the PT and MRS treatment groups (P>0.05).

Procollagen α1 (I) mRNA expression. Fig. 2B demonstrates that NECA significantly increased the levels of procollagen α1 (I) mRNA expression (P<0.01). Compared with NECA, procollagen α1 (I) mRNA expression was significantly reduced following PT or MRS treatment on days 2 and 3 (P<0.05). PT and MRS however, were not identified to significantly influence procollagen α1 (I) mRNA expression levels (P>0.05), and no differences were observed between the PT and MRS groups (P>0.05).

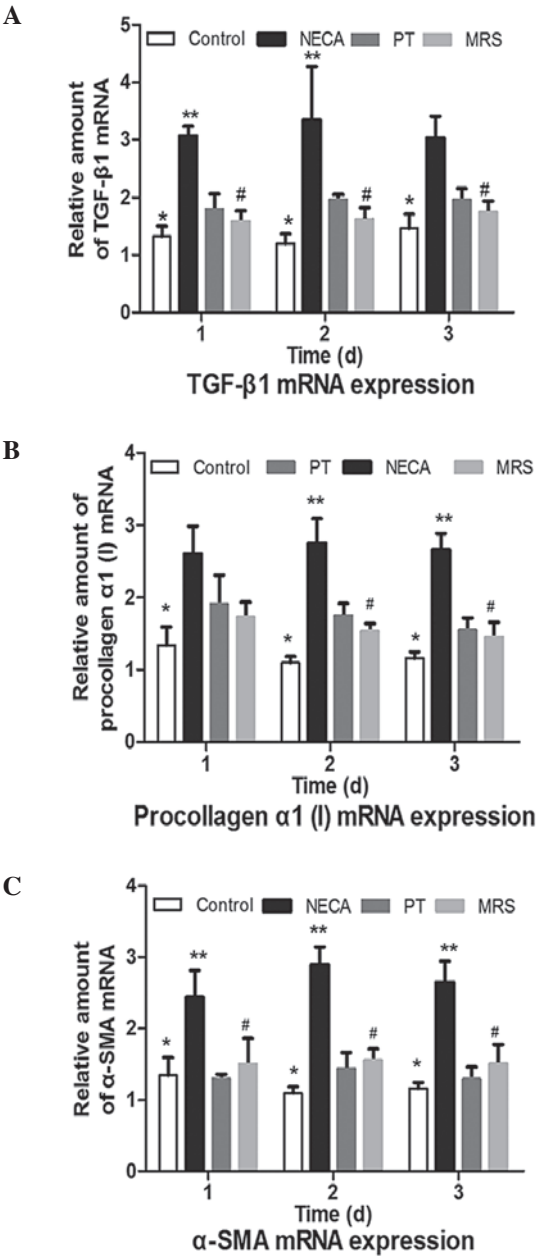

Figure 2. Expression of TGF-β1, procollagen α1 (I) and α-SMA mRNA expression levels in the control, PT, NECA and MRS groups. (A) TGF-β1 mRNA expression. ADO significantly upregulated TGF-β1 mRNA expression, while blocking $A_{2B}R$ reversed TGF-β1 mRNA overexpression. (B) Procollagen α1 (I) mRNA expression. ADO significantly upregulated procollagen α1 (I) mRNA expression, while blocking $A_{2B}R$ reversed procollagen α1 (I) mRNA overexpression. (C) α-SMA mRNA expression. ADO significantly upregulated α-SMA mRNA expression, while blocking $A_{2B}R$ reversed the α-SMA mRNA overexpression. *P<0.01 vs. the NECA group; **P<0.01 vs. the PT group; $^{\#}$P<0.05 vs. the NECA group. TGF-β1, transforming growth factor-β1; α-SMA, α-smooth muscle actin; ADO, adenosine; NECA, 5'-N-ethylcarboxamidoadenosine; PT, 8-phenyltheophylline; MRS, MRS1754.

α-SMA mRNA expression. As presented in Fig. 2C, the stimulation of NECA significantly induced the expression of α-SMA (P<0.01). Compared with the control group, there was no effect of PT or MRS on α-SMA mRNA expression levels (P>0.05), which were significantly lower than in the NECA group (P<0.05). No significant differences were identified between the PT and MRS treatment groups (P>0.05).

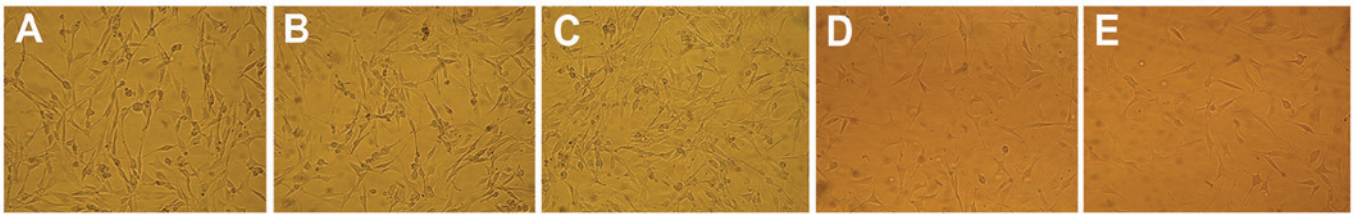

Figure 3. Proliferation of fibroblasts in each group. Proliferation of fibroblasts at (A) 0 h, and at 72 h in the (B) control group, (C) NECA group, (D) PT group and (E) MRS group. (Magnification, x200). NECA, 5'-N-ethylcarboxamidoadenosine; PT, 8-phenyltheophylline; MRS, MRS1754.

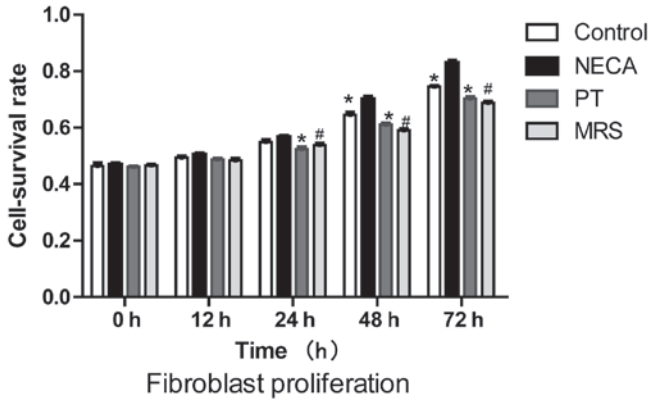

Figure 4. Renal fibroblast proliferation. ADO significantly increased renal fibroblast proliferation, while blocking $A_{2B}R$ reduced ADO-induced proliferation. *P<0.001 vs. the NECA group; #P<0.01 vs. the NECA group. ADO, adenosine; NECA, 5'-N-ethylcarboxamidoadenosine; PT, 8-phenyltheophylline; MRS, MRS1754.

Proliferation of fibroblasts. Proliferation of renal fibroblasts was measured following 0, 12, 24, 48 and 72 h of hypoxia in triplicate (Fig. 3).

The cell survival rates of each group are presented in Fig. 4. Compared with the control group, NECA significantly induced renal fibroblast proliferation at 48 and 72 h (P<0.001). The addition of 8-PT or MRS 1754 was able to reverse the NECA-induced increase in renal fibroblast proliferation at 24, 48 and 72 h (P<0.01).

Discussion

Fibroblast proliferation and activation are closely associated with RIF development. Fibroblasts embedded within the renal interstitium synthesize various components of the ECM, including type I collagen, type III collagen and fibronectin (14). The majority of fibroblasts exist in a quiescent state and a small proportion remain active in order to repair damaged tissues (15). However, in pathological conditions, including hypoxic ischemia, inflammation and tissue damage, the excretion of cytokines and ECM results in excessive fibroblast proliferation and transdifferentiation to activated myofibroblasts. As a result of the enhanced collagen production by myofibroblasts, the ECM accumulation hinders normal renal function and results in the development of RIF. In this pathological process, fibroblast proliferation and activation are key to the development of RIF.

ADO is a chemical that is naturally present in all cells and an increase in the cytoplasmic ADO concentration under hypoxic ischemia is considered as a response to stress (16-18). There are two key sources of cellular ADO: The degradation of ADO triphosphate, and the dephosphorylation of adenosine monophosphate, which is produced by the extracellular adenine nucleotide metabolism (19). Extracellular ADO exerts its biological effects by binding with AR on the cell surface. Three types and four subtypes of AR have been identified, including A_1R, $A_{2A}R$, $A_{2B}R$ and A_3R (20). ADO has been reported to have vasodilatory effects in the majority of organs (21), and protects against tissue damage during acute ischemic injury in the heart (22), brain (23), liver (24) and kidney (25). However, extended exposure to a high concentration of ADO has been demonstrated to lead to tissue damage and increased organ dysfunction (26). In previous studies, it has been identified that extracellular ADO continually increases during RIF, and ADO serves an important function in RIF development and kidney dysfunction (9,27). AR inhibitors, such as PT, have been demonstrated to protect kidney function in UUO mice (9,27,28). In order to further understand the function of the AR in RIF development, a hypoxia mouse renal fibroblast model was generated in the current study, in order to dynamically investigate the function of ADO and the associated AR. In the present study, it was observed that $A_{2B}R$ was the predominant receptor type on the surface of the fibroblasts under hypoxia. The specific $A_{2B}R$ inhibitor MRS1754 produced similar effects to the global AR inhibitor 8-PT in renal fibroblast proliferation inhibition (P>0.05), indicating that $A_{2B}R$ was the predominant AR type during hypoxia and ADO increased fibroblast proliferation by binding with $A_{2B}R$.

During the development of RIF, fibroblasts exhibit smooth muscle cell-like characteristics following a specific transformation, producing myofibroblasts (29). In addition, α-SMA is a known marker of this fibroblast phenotypic transformation (30). Data obtained from animals and humans have clarified the common process of phenotypic transformation of fibroblasts expressing α-SMA into myofibroblasts during CKD development (31-35). As the active form, myofibroblast are important in the progression of fibrosis, by inducing excessive ECM accumulation (36,37). The data from the current study demonstrated that compared with the control group, NECA significantly increased α-SMA mRNA expression levels (P<0.01). Compared with the NECA group, NECA combined with 8-PT or MRS1754 reversed the NECA-induced upregulation of α-SMA mRNA (P<0.05). The inhibition of global AR or $A_{2B}R$ was able to reduce transformation of the fibroblast phenotype, leading to the delay of RIF development and protection of kidney function. No significant differences were observed between 8-PT and

MRS1754 in α-SMA expression, indicating that $A_{2B}R$ was the predominant AR type responsible for fibroblast transdifferentiation during hypoxia.

A previous study investigated the involvement of the cytokine regulation network in the development of RIF (38). Under hypoxic ischemia, large quantities of cytokines and chemotactic factors are excreted from stressed cells, resulting in structural alterations and dysfunction of the renal tissue. In the current study, two verified profibrotic cytokines, TGF-β1 and procollagen α1 (I), were investigated. The results demonstrated that compared with the control group, NECA significantly increased TGF-β1 mRNA expression levels ($P<0.01$), while 8-PT and MRS1754 were able to reverse NECA-induced upregulation of TGF-β1 mRNA ($P<0.05$). Similar results were observed in the evaluation of procollagen α1 (I) levels. The results suggested that under hypoxia, ADO may accelerate RIF development by inducing the excretion of pro-fibrotic cytokines. However, the inhibition of global AR or $A_{2B}R$ was only able to effectively alleviate disease by inhibiting cytokine synthesis. No significant differences were observed between 8-PT and MRS1754 treatment in the levels of TGF-β1 and procollagen α1 (I) regulation, indicating that $A_{2B}R$ was the predominant receptor type for fibroblast profibrotic cytokine excretion under hypoxia.

In conclusion, the current study demonstrated that ADO was important in the regulation of biological behavior in fibroblasts, and $A_{2B}R$ was identified as the predominant receptor type on the surface of fibroblasts under conditions of hypoxia. The inhibition of $A_{2B}R$ significantly reduced the proliferation and activation of fibroblasts, and reduced the excretion of profibrotic cytokines, thus preventing RIF development. The current study suggests that $A_{2B}R$ may be a novel target in the treatment of RIF.

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