Aldose reductase regulates platelet-derived growth factor-induced proliferation through mediating cell cycle progression in rat mesangial cells

LIPING ZOU[1*], WENJUAN WANG[1*], ZUDE XU[1,2], NONG ZHANG[2] and TAO JIANG[2]

[1]Department of Pathology, Huashan Hospital, Fudan University, Shanghai 200040;
[2]Department of Pathology, Shanghai Medical College, Fudan University, Shanghai 200032, P.R. China

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Abstract. Aldose reductase (AR), the first and the rate-limiting enzyme of the polyol pathway, has been implicated in platelet-derived growth factor (PDGF)-induced proliferation of rat mesangial cells (MsCs). It is well known that AR plays an important role in various chronic diabetic complications, for example, diabetic nephropathy. Moreover, our previous studies have demonstrated that an AR inhibitor (ARI) significantly reduced the proliferation of rat MsCs induced by PDGF, however, the mechanism remains unclear. The aim of the present study was to elucidate the molecular mechanisms through which AR regulates PDGF-induced rat MsC proliferation. It was demonstrated that PDGF-induced MsC proliferation was significantly inhibited by pretreatment with ARI. Cell cycle analysis by flow cytometry revealed that ARI prevented the entry of cells from the G1 into the S phase. Furthermore, the effect of the PI3K/Akt signaling pathway on the cell cycle was analyzed. The PI3K/Akt pathway was activated with PDGF treatment. However, ARI blocked Akt activation in response to PDGF. Moreover, PDGF increased the levels of $p21^{Cip1}$ cyclin kinase inhibitor protein in MsC, which was markedly inhibited by pretreatment with ARI. Conversely, PDGF significantly reduced the levels of the $p27^{Kip1}$ cyclin kinase inhibitor protein, which was also restored by pretreatment with ARI. In conclusion, AR is involved in PDGF-induced rat MsC proliferation, and may serve as a potential target for the inhibition of MsC proliferation in several types of glomerulonephritis.

Correspondence to: Dr Tao Jiang, Department of Pathology, Shanghai Medical College, Fudan University, 138 Yixueyuan Road, Shanghai 200032, P.R. China
E-mail: taojiang@fudan.edu.cn

*Contributed equally

Abbreviations: AKR, aldo-keto reductase; AR, aldose reductase; ARI, aldose reductase inhibitor; CDK, cyclin-dependent kinase; CKI, cyclin-dependent kinase inhibitor; ECM, extracellular matrix; GSH, glutathione; GS-DHN, glutathionyl-1,4-dihydroxynonane; HNE, 4-hydroxy-trans-2-nonenal; MAPK, mitogen-activated protein kinase; MsC, mesangial cell; PDGF, platelet-derived growth factor; PDGFR, platelet-derived growth factor receptor; PI3K, phosphoinositide 3-kinase; ROS, reactive oxygen species; TGF-β1, transforming growth factor-β1; VSMC, vascular smooth muscle cell

Key words: aldose reductase, platelet-derived growth factor, PI3K/Akt, $p21^{Cip1}$, $p27^{Kip1}$, mesangial cell

Introduction

Aldose reductase (AR), a member of the aldo-keto reductase (AKR) superfamily, is responsible for conversion of glucose to sorbitol of the polyol pathway in glucose metabolism (1). AR is a cytosolic enzyme and is widely distributed in various tissues and organs such as eye lens, retina, kidney, and reproductive organs. During the past several decades the studies of AR focus on the pathology of long term diabetic complications, including nephropathy, as it plays a pivotal role in the glucose metabolism under hyperglycemia (2-4). However, recent studies indicate that AR functions as not only a metabolizing glucose enzyme but also an enzyme catalyzing the reduction of a wide array of substances including various endogenous and exogenous aldehydes and their glutathione (GSH)-conjugates, phospholipids and steroids (5-7). In addition, it has been shown that AR is associated with cardiac disorders, inflammation, mood disorders and cancers (8-10). Our previous studies also demonstrated that AR was one of the responsive genes for transforming growth factor-β1 (TGF-β1) in cultured mesangial cells. AR has also been confirmed to play a role in extracellular matrix (ECM) deposition and MsC proliferation mediated by TGF-β1 which is a crucial growth factor in glomerulonephritis and glomerulosclerosis, and other growth factors, including platelet-derived growth factor (PDGF) (11).

Abnormal proliferation of MsCs contributes to the pathogenesis of renal fibrosis and glomerulosclerosis (12). PDGF is generally approved to be a major mitogen for MsCs and has a high transcription level during both experimental and human glomerulonephritis. Besides, PDGF receptor (PDGFR) mRNA and protein expression are upregulated. Several study reports showed the involvement of PDGF and PDGFR in the proliferation and migration of MsCs (13). Activation of the intrinsic

tyrosine kinase activity of PDGFR facilitates recruitment of several SH2 domain-containing molecules and associated proteins including the p85 subunit of phosphoinositide 3-kinase (PI3K), RasGAP and PLCγ1. In glomerulonephritis, MsCs are activated, followed by increased production and release of PDGF into the extracellular space, which activates proliferation of MsCs again as a feedback loop (14,15). Specific antagonism of PDGF suppresses MsC proliferation *in vitro*, and in experimental glomerulosclerosis (16). Since the effects of ARI on the inhibition of MsC proliferation induced by PDGF have been shown, it is possible that AR could be a potential target for alleviating MsC proliferation, and then ECM deposition and glomerulosclerosis.

Although PDGF has a critical role in regulating cell proliferation in several cell types, the related signaling pathways vary among different cells (17). It is known that PDGF binds to a tyrosine kinase receptor which activates a number of downstream pathways, including the mitogen-activated protein kinase (MAPK) family members, PI3K/Akt and many other kinases in MsC (18,19). ARI was recently reported to reduce the phosphorylation of MAPK1/2 in metaplasia of airway epithelial cells and PI3K/Akt in vascular smooth muscle cells (VSMCs) (20,21). However, the PI3K/Akt signaling pathway is one of the most common downstream pathways of PDGF in regulating cell proliferation. Overexpression of dominant negative Akt resulted in complete inhibition of PDGF-induced DNA synthesis in MsC. On the other side, inhibition of MAPK only partially attenuated DNA synthesis (22). In our current study, we confirmed the involvement of the PI3K/Akt pathway in modulating AR-induced inhibition of MsC proliferation.

The mammalian cell cycle is a tightly controlled nuclear event positively regulated by cyclin-dependent kinases (CDKs) and their cyclin-regulatory subunits, and negatively by cyclin-dependent kinase inhibitors (CKIs). Among the CKIs both $p21^{Cip1}$ and $p27^{Kip1}$ contain binding domains for CDK which may intercept the ability of CDK to form active complexes with cyclins, leading to interference with the proliferation of MsC (23,24).

In this study, we investigated the mechanism by which ARI inhibits PDGF-induced MsC proliferation. We demonstrated that ARI was coupled to attenuation of PI3K/Akt pathway activity in response to PDGF. In addition, our results indicate that ARI arrested PDGF-induced MsC proliferation in the G1 phase through mediating the levels of $p21^{Cip1}$ and $p27^{Kip1}$.

Materials and methods

Cell culture and reagents. Rat MsCs were obtained by culturing glomeruli isolated from the kidneys of 200-250 g Sprague-Dawley rats by conventional sieving methods as previously described (25). The cells were cultured in RPMI-1640 medium containing 10% FBS (Gibco-BRL), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.3 g/ml sodium pyruvate at 37˚C in an atmosphere containing 5% CO_2. All experiments were performed using cells between passages 4 and 10. When the cells reached 60-70% confluence, the medium was changed to fresh serum-free RPMI-1640 containing 10 μM zopolrestat. After 24 h, the cells were stimulated with 20 ng/ml PDGF-BB for the further investigation. The RPMI-1640 medium was purchased from Invitrogen (San Diego, CA, USA). The ARI zopolrestat was a gift from Pfizer. PDGF-BB was purchased from Sigma Chemical (St. Louis, MO, USA). The wild (HA-Akt) and dominant-negative (HA-Akt-K179A) were generously provided by Dr Boudewijn Burgering (University Medical Center, Utrecht, Netherlands).

MTT assay. The MTT reduction assay was used as a qualitative index of cell viability. After incubation with different compounds as described above, 20 μl MTT (5 mg/ml) (Invitrogen Corp., Carlsbad, CA, USA) was added and cells were cultured for an additional 4 h. Subsequently, cells were lysed using dimethylsulfoxide (150 μl/well) (Pierce Biotechnology, Inc., Rockford, IL, USA). When the formanzan crystals were completely dissolved, the optical density (OD) was measured at 490 nm using an ELx800 multiwell plate reader (Bio-Tek Instruments, Winooski, VT, USA).

DNA synthesis assay. A colorimetric immunoassay kit Cell proliferation ELISA, BrdU (colorimetric) (Boehringer Mannheim GmbH, Mannheim, Germany) was used for quantification of cell proliferation. This assay is based on the measurement of BrdU incorporation during DNA synthesis. Briefly, the cells were seeded in 96-well plates; pre-incubated for 24 h with zopolrestat (10 μM), and then stimulate with PDGF for another 24 h. They were then labeled with BrdU for 3 h at 37˚C, washed, and fixed and stained with anti-BrdU antibody for 90 min at 37˚C. After three washes, the substrate, tetramethylbenzidine, was added, followed by incubation for 30 min. A blocking solution (1 M H_2SO_4) was then added, and the absorbance of the samples was measured at 450 nm with a reference wavelength of 690 nm using an ELx800 multiwell plate reader (Bio-Tek Instruments).

Flow cytometry. Cell cycle analysis was performed using flow cytometry. After 24 h of treatment with different compounds, cells were washed twice with phosphate-buffered saline (PBS) and resuspended in 70% ethanol for 1 h at 4˚C. Fixed cells were collected by centrifugation, treated with RNase (25 μg/ml) at 37˚C for 30 min and stained with propidium iodide (50 μg/ml) at 4˚C for 30 min in the dark. The number of cells in the G1, S and G2/M phases was analyzed by flow cytometry using a FACSCalibur Flow Cytometer (Becton-Dickinson, San Jose, CA, USA).

Western blot analyses. Cells were cultured and treated as described above, grown to 60-70% confluence, replaced with serum-free medium for 24 h and then subjected to PDGF (20 ng/ml) in the presence or absence of zopolrestat (10 μM). Cell lysis was performed on ice with fresh lysis buffer [1 M Tris (pH 8.0), 2 M NaCl, 10% NaN_3, 10% SDS, 10% NP-40, and 1% sodium deoxycholate]. All lysates were centrifuged at 15,000 x g for 10 min at 4˚C followed by bicinchoninic acid assay (BCA assay; Pierce Biotechnology, Inc.) to determine protein concentrations. Protein (40 μg) was loaded onto 8 or 10% sodium dodecyl sulfate polyacrylamide gel (SDS-PAGE) and electrophoretically transferred to polyvinylidene fluoride (PVDF). Membranes were blocked for a minimum of 1 h at room temperature in 5% bovine serum albumin (BSA) in Tris-buffered saline with 1 ml Tween-20 per liter. The membranes were incubated overnight at 4˚C with primary antibodies for

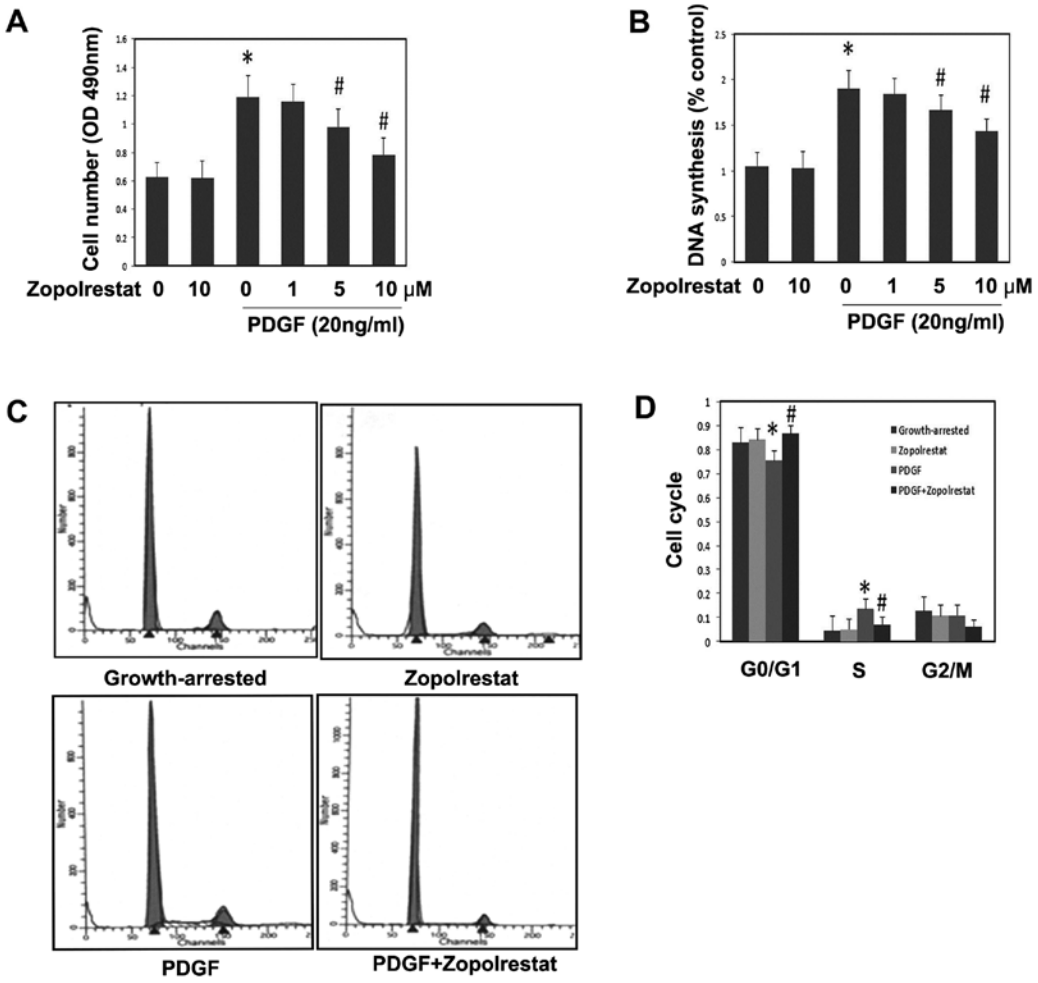

Figure 1. Zopolrestat inhibits G1 cell cycle progression in PDGF-induced mesangial cells. (A) Zopolrestat concentration-dependently suppressed PDGF-induced mesangial cell proliferation. Cells were treated with the indicated concentration of zopolrestat for 24 h prior to treatment with PDGF (20 ng/ml) for another 24 h. Cell proliferation was determined by the MTT assay. Values are the mean ± SD (n=3 independent experiments each in triplicate; *P<0.05 vs. control; #P<0.05 vs. PDGF). (B) Zopolrestat inhibits PDGF-induced DNA synthesis in mesangial cells. Zopolrestat inhibits PDGF-induced DNA synthesis in a concentration-dependent manner in mesangial cells. DNA synthesis was measured by ELISA according to the manufacturer's instructions. Values are the mean ± SD. (n=3 independent experiments each in triplicate; *P<0.05 vs. control; #P<0.05 vs. PDGF). (C and D) Zopolrestat inhibits G1-to-S progression in PDGF-stimulated mesangial cells. Synchronized cells were treated with or without zopolrestat (10 μM) 24 h prior to stimulation with PDGF (20 ng/ml) for another 24 h. DNA was stained with propidium iodide and assayed by flow cytometry. (n=3 independent experiments each in triplicate; *P<0.05 vs. growth-arrested; #P<0.05 vs. PDGF).

p21 (1:500), p27 (1:1,000; all from Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA), mouse anti-β-actin (1:1,000; Sigma Chemical), phospho-Akt (1:1,000), Akt (1:1,000) (all from Cell Signaling Technology), PDGF β-receptor subunit (1:1,000; Santa Cruz Biotechnology, Inc.). After being incubated with the respective secondary antibody, immune complexes were detected with ECL Plus (Amersham Biosciences, Piscataway, NJ, USA) on Kodak X-ray film.

Statistical analysis. All the experiments were repeated at least three times independently. Differences were assessed using ANOVA. All values are expressed as mean ± SD, and statistical significance was defined at P<0.05.

Results

Effect of zopolrestat on cell proliferation induced by PDGF in rat MsC. Mesangial cell proliferation was measured by the MTT assay. Growth-arrested MsCs were treated with or without the ARI zopolrestat in different doses for 24 h prior to stimulation with PDGF (20 ng/ml) for another 24 h. The results show that PDGF significantly induced MsC proliferation (Fig. 1A) (P<0.05 vs. control). Pretreatment with zopolrestat alone had no effects on MsC proliferation (Fig. 1A). However, it inhibited PDGF-induced MsC proliferation in a dose-dependent manner (Fig. 1A) (P<0.05 vs. PDGF group). To further assess the effect of zopolrestat on DNA synthesis in MsC proliferation, a BrdU cell proliferation assay was performed. PDGF (20 ng/ml) resulted in an increase in the amount of DNA synthesis, but zopolrestat decreased the DNA synthesis in a dose-dependent manner (Fig. 1B). In contrast, zopolrestat alone did not affect DNA synthesis in MsCs. Our results indicate that zopolrestat treatment inhibited the PDGF-stimulated BrdU incorporation into DNA in MsCs.

In order to further evaluate the effect of ARI zopolrestat treatment upon cell cycle profiles, we then performed flow cytometry. The data showed that PDGF decreased the proportion of cells in the G1 phase from 82.8 to 75.4%, (Fig. 1C) (P<0.05 vs. control)

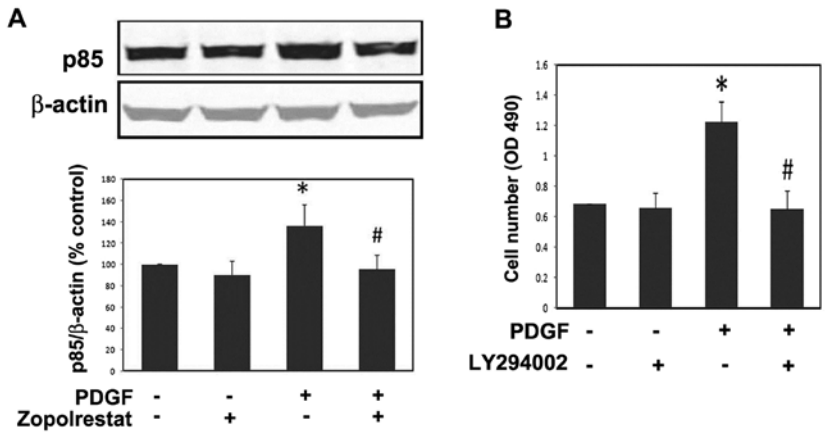

Figure 2. Effects of zopolrestat on p85 protein expression in mesangial cells. Mesangial cells were treated with or without zopolrestat (10 μM) 24 h prior to stimulation with PDGF (20 ng/ml). (A) The p85 protein expression was measured by western blotting. (B) Inhibition of PI3K blocks PDGF-induced cell proliferation. MTT analyses were performed. (n=3 independent experiments each in triplicate; *P<0.05 vs. control; #P<0.05 vs. PDGF).

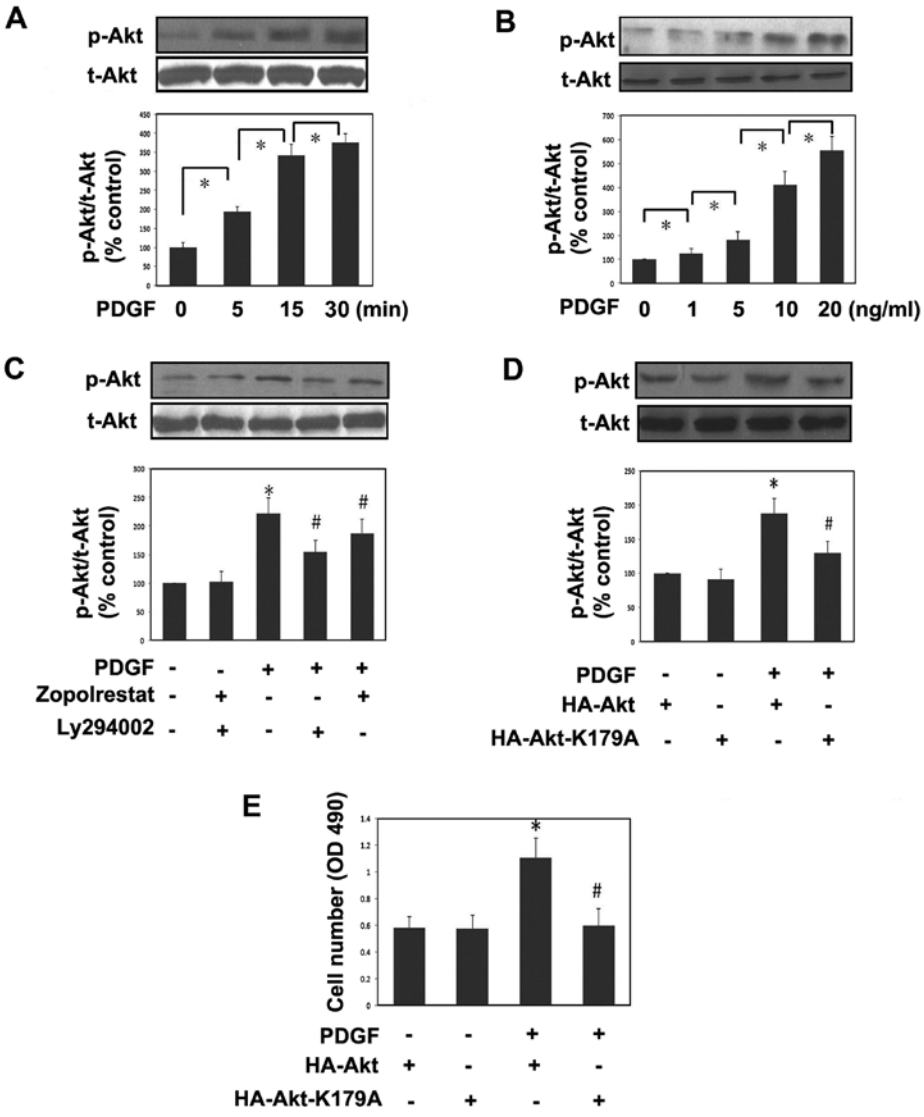

Figure 3. Zopolrestat inhibits the activation of Akt in PDGF-induced mesangial cells. (A and B) Effect of PDGF on Akt activity. (A) Mesangial cells were treated with PDGF (20 ng/ml) for the indicated periods of time. (B) Mesangial cells were stimulated with the indicated concentration of PDGF for 15 min. (C) Zopolrestat (10 μM) reduced the increase of the phosphorylation levels of Akt induced by PDGF (20 ng/ml). (D) Mesangial cells were transfected with either wild-type (HA-Akt) or dominant-negative form (HA-Akt-K179A) of Akt, serum-starved for 24 h, and then stimulated with or without PDGF for 15 min. HA-Akt-K179A blocked the phosphorylation level of Akt induced by PDGF (20 ng/ml). Western blot analyses were performed from (A) to (D). (E) Dominant-negative form (HA-Akt-K179A) blocks PDGF-induced cell proliferation. MTT analyses were performed (n=3 independent experiments each in triplicate; *P<0.05 vs. control; #P<0.05 vs. PDGF).

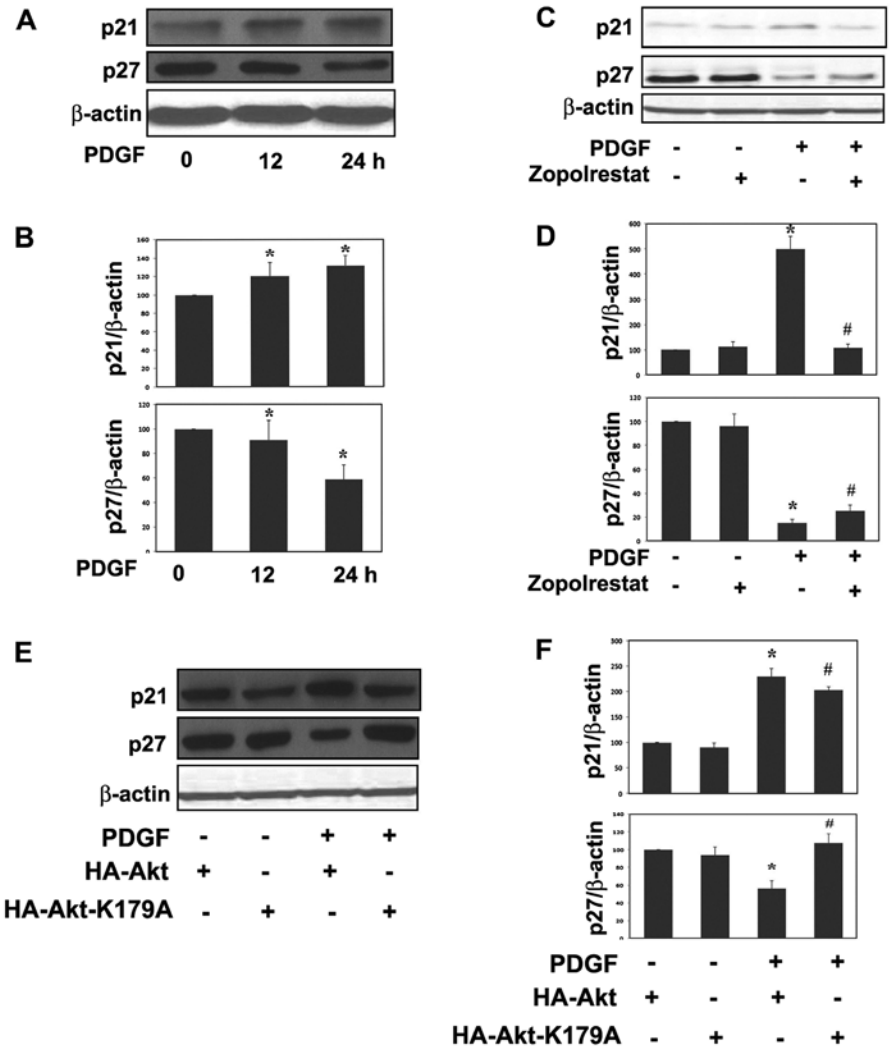

Figure 4. Zopolrestat reverses the expression level of the cell cycle proteins $p21^{Cip1}$ and $p27^{Kip1}$ induced by PDGF in mesangial cells. (A and B) Time course response of PDGF-induced $p21^{Cip1}$ and $p27^{Kip1}$ protein expression in mesangial cells. (C and D) Zopolrestat reversed the changes of the $p21^{Cip1}$ and $p27^{Kip1}$ protein expression induced by PDGF. (E and F) HA-Akt-K179A reversed the changes of $p21^{Cip1}$ and $p27^{Kip1}$ protein expression induced by PDGF. The protein expression $p21^{Cip1}$ and $p27^{Kip1}$ were measured by western blotting. (n=3 independent experiments each in triplicate; $^{*}P<0.05$ vs. control, $^{\#}P<0.05$ vs. PDGF).

and increased that in the S phase from 4.5 to 13.6%, indicating that PDGF could promote cell cycle progression. In contrast, the pretreatment of zopolrestat increased the number of cells in the G1 phase (from 75.4 to 86.7%, P<0.05) and decreased that in the S phase (from 13.6 to 7.25%, P<0.05). These results suggest that zopolrestat could block PDGF-induced cell cycle progression by inhibiting the G1-S phase transition and arresting cells in G1.

Effects of zopolrestat on the activation of PI3K induced by PDGF in rat MsCs. Previous studies showed that PI3K activity is necessary for PDGF-induced MsC proliferation (18). Therefore, we examined the effect of ARI on PI3K activity in MsC. PDGF-activated PI3K activity was assessed by measuring the levels of the p85 regulatory subunit of PI3K. PDGF obviously increased the protein expression of the p85 subunit, while pre-incubation of ARI significantly reduced the p85 subunit activity (Fig. 2A). We then observed that LY294002 (Calbiochem), a specific inhibitor of PI3K, could suppress cell proliferation induced by PDGF (Fig. 2B). We conclude from the above results that PI3K is involved in the inhibition of ARI on PDGF-induced MsC proliferation, and that further studies are warranted.

Effects of zopolrestat on the phosphorylation levels of Akt kinase induced by PDGF in rat mesangial cell. Since the activation of the PI3K/Akt pathway is a key step in the proliferation process of a variety of cell types including MsCs (18,22), western blot analysis was next performed to evaluate the phosphorylation levels of this signaling pathway. The phosphorylation levels of Akt were rapidly induced with time and reached their highest level at about 15-30 min after PDGF stimulation (Fig. 3A). The representative western blots demonstrate that PDGF led to Akt pathway activation in a dose-dependent manner (Fig. 3B). Pre-incubation of the ARI zopolrestat markedly attenuated the induction of PDGF on phospho-Akt, whereas zopolrestat alone had no effects on Akt phosphorylation levels. Meanwhile, we found that LY294002 could downregulate the phosphorylation levels of Akt (Fig. 3C). To closely confirm the role of Akt in PDGF-induced cell proliferation, a dominant-negative mutant of Akt, HA-Akt-K179A, was transfected into MsCs. This

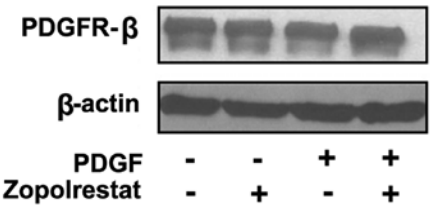

Figure 5. Effects of zopolrestat on PDGFR-β receptor protein expression in mesangial cells. Mesangial cells were treated with or without zopolrestat (10 μM) 24 h prior to stimulation with PDGF (20 ng/ml). PDGFR-β receptor expression was measured by western blotting (n=3 independent experiments each in triplicate).

mutant significantly reduced the phosphorylation levels of Akt induced by PDGF (Fig. 3D). In agreement with these findings, we found that HA-Akt-K179A significantly reduced cell proliferation induced by PDGF, indicating a role of Akt in this effect. These results show that the effects of AR inhibition on the PDGF-induced stimulation of mesangial cell proliferation are mediated by the Akt signaling pathways.

Effect of zopolrestat on protein expression of p21^{Cip1} and p27^{Kip1} induced by PDGF in rat MsC. The levels of the G1 phase CKI p21^{Cip1} and p27^{Kip1} in quiescent control and proliferating MsCs were determined by western blot analysis (Fig. 4). Growth-arrested MsCs were treated with or without ARI zopolrestat (10 μM) 24 h prior to stimulation with PDGF (20 ng/ml). Much more appreciable expression of p27^{Kip1} than p21^{Cip1} was observed in quiescent control mesangial cells (Fig. 4A). No change in their protein expression levels was observed in zopolrestat-treated MsCs. PDGF-treated cells showed an apparent increase in levels of p21^{Cip1} in a time-dependent manner. The increase of p21^{Cip1} was obviously suppressed by co-treatment with zopolrestat or by HA-Akt-K179A mutant cells. In contrast, incubation with PDGF caused an apparent decrease in detectable levels of p27^{Kip1} in a time-dependent manner; however, the reduction was restored by ARI zopolrestat or by HA-Akt-K179A mutant cells.

Effects of zopolrestat on PDGFR-β receptor protein expression. To test whether zopolrestat affects the PDGF-mediated mitogenic signaling cascade at the level of the PDGF receptor, we examined the effects of the ARI zopolrestat on the protein expression of the PDGFR-β receptor. Detectable levels of PDGFR-β receptor protein expression were expressed in quiescent control cells and no change in their levels was observed in PDGF-treated MsCs with or without pretreatment of zopolrestat (Fig. 5).

Discussion

In the present study, we demonstrated that ARI partially inhibited the rat MsC proliferation stimulated by PDGF through modulating the levels of p21^{Cip1} and p27^{Kip1}. We also confirmed that ARI suppressed the Akt signal pathway in response to PDGF. From these results it was suggested that ARI may has a beneficial role in inhibiting the deterioration of renal function featured with MsC proliferation and ECM deposition.

AR, the rate-limiting enzyme in the polyol pathway, was first found to be implicated in the etiology of the diabetic complications apart from its physiological role as osmoregulator in the kidney and supplier for sperm energy (25,26). However, recent reports have demonstrated that, under normal glucose levels, AR may be in involved in various pathological processes other than hyperglycemia as it is a broad-specificity aldo-keto reductase with wide species and tissue distribution (8). Our previous studies showed that AR is one of the responsive genes for TGF-β1, regulates TGF-β1-induced production of fibronectin and type IV collagen, and participates in PDGF-induced mesangial cell proliferation in cultured rat mesangial cells (11,27). The details of this anti-proliferative mechanism of ARI in mesangial cells are not clear. In this study, we studied the mechanism by which ARI inhibits PDGF-induced MsC proliferation under normal glucose concentrations. Our study confirmed the role of AR in regulating MsC proliferation induced by PDGF, which is consistent with other reports in different cell types induced by distinct growth-related factors (10,25,28).

In our previous studies, we observed that MsC with AR overexpression proliferated faster than the controls, which indicated the important role of AR in MsC proliferation. We next determined the role of ARI, the specific AR inhibitor, in MsC proliferation. Consistent with the increased proliferation due to AR overexpression, pretreatment of ARI significantly inhibited MsC proliferation, which confirmed the regulation of AR on MsC proliferation (11). However, ARI did not show obvious inhibition for serum-induced MsC proliferation, which is likely due to the fact that there are other growth factors besides PDGF in serum. Previous reports showed that ARI had no effect on PDGF-induced rat aortic smooth muscle cell proliferation under normal glucose conditions (29). Taken together, the effect of ARI on PDGF-induced cell proliferation is cell type-dependent. In this study we further elucidated the molecular mechanism by which ARI arrested PDGF-induced MsC proliferation. It was confirmed that PDGF-induced cell proliferation was significantly suppressed by pretreatment of ARI while ARI itself had no effects on MsC proliferation. Our results suggest that AR plays a role in PDGF-induced cell proliferation and ARI may be available to be used to protect renal function against mesangial cell abnormally proliferative associated disorders. In addition, our data showed that ARI had no effects on PDGFR-β receptor protein expression which is consistent with previous reports (30,31).

The PI3K pathway has been reported to mediate PDGF-induced MsC proliferation (18). One of the major downstream signaling pathways of PI3K is the serine/threonine kinase Akt/PKB. Akt kinase activity is thought to be stimulated by a variety of extracellular signaling factors, including many growth factors and cytokines. The role of Akt related to inhibition of apoptosis has been extensively reported. For example, angiopoietin-1 inhibits endothelial cell apoptosis via the Akt/survivin pathway (32). Similarly, in RGC-5 retinal ganglion cells imatinib diminishes PDGF-induced Akt phosphorylation to induce apoptosis (33). However, in our study we confirmed the Akt kinase-mediated PDGF-induced DNA synthesis, and more importantly that pretreatment of ARI suppressed it, which is consistent with a previous report in colon cancer cells (34). Our data showed that PDGF activates Akt phos-

phorylation in a time- and dose-dependent manner, and Akt phosphorylation was significantly reduced after co-treatment with AR or being transfected with HA-Akt-K179A. We demonstrated that ARI blocked Akt phosphorylation triggered by PDGF in MsCs. These data directly link AR to the induction of Akt without hyperglycemia in rat MsC. Although the precise role of Akt in regulating proliferation is unclear, our results indicate that during the process of AR-modulated cell proliferation the altered expression of $p21^{Cip1}$ and $p27^{Kip1}$ levels that were observed in PDGF-stimulated cells are coupled to increased levels of Akt kinase phosphorylation. In this study, we confirmed that the AR modulated cell proliferation through the Akt pathway mediating PDGF signaling in MsC, which is in agreement with reports in other studies (14,35,36).

Cell cycle progression CDK inhibitors, in particular those members of the Cip/Kip family contribute to cell-cycle regulatory functions. The Cip/Kip family comprise of $p21^{Waf1/Cip1}$ (p21), $p27^{Kip1}$ (p27) and $p57^{Kip2}$ (p57), which inhibit cyclin-CDK complexes both in the G1 and S phases of the cell cycle (37,38). Former reports have demonstrated that $p21^{Cip1}$ and $p27^{Kip1}$ are critically involved in cell cycle arrest in MsC (23). In our experiments PDGF induced the expression of $p21^{Cip1}$, and this was apparently blocked by ARI or after being transfected with HA-Akt-k179A, which was in agreement with the previous study (39). Increased expression of $p21^{Cip1}$ during proliferation is observed because it acts as a scaffold to facilitate the assembly of cyclins and CDKs required for DNA synthesis (24). Our data indicate that ARI negatively regulates $p21^{Cip1}$ expression in MsC. On the other hand, it has been reported that the onset of MsC proliferation is associated with a reduction in $p27^{Kip1}$ levels, and in addition, $p27^{Kip1}$ safeguards against inflammatory glomerular injury in anti-GBM glomerulonephritis (40). To confirm this observation, we found that PDGF greatly downregulated $p27^{Kip1}$ protein level, and the effect was reversed by pretreatment of ARI or after being transfected with HA-Akt-k179A. It is suggested that $p27^{Kip1}$ is regulated by ARI in a positive manner. In our experiments PDGF showed contrary effects on $p21^{Cip1}$ and $p27^{Kip1}$ expression. It is interesting to note that overexpression of YB-1 led to rat MsC proliferation by decreasing $p21^{Cip1}$ expression, but increasing $p27^{Kip1}$ expression (41). The underlying mechanism of the p21 and p27 diverse roles in response to different growth factors in the same cell type remains to be explored.

Accumulating evidence has suggested that under normal glucose conditions AR may be upregulated by factors other than hyperglycemia and therefore participate in pathological conditions in non-diabetic disorders. For instance, growth factors cause oxidative stress via generation of reactive oxygen species (ROS) which forms toxic lipid aldehydes such as 4-hydroxy-trans-2-nonenal (HNE) by lipid peroxidation. The final products of HNE catalyzed by AR, glutathionyl-1,4-dihydroxynonane (GS-DHN), transduce inflammatory signaling via a cascade of protein kinases, such as PI3K (8,9). Growth factors-stimulated PI3K may utilize its major downstream Akt kinase signaling pathway for induction of DNA synthesis in MsCs. Our observation would support the importance of the AR/PI3K/Akt/cell cycle protein cascade for the PDGF-induced proliferation of MsCs.

In summary, our data demonstrate that ARI inhibits PDGF-stimulated MsC proliferation via modulation of the PI3K/Akt pathway which results in alterations in the levels of $p21^{Cip1}$ and $p27^{Kip1}$. Further studies to elucidate the precise molecular mechanisms by which AR mediates PDGF-induced cell proliferation are currently underway. Future investigation will be needed to determine how AR affects the PI3K/Akt signal pathway and the expression level of p21 and p27. Understanding this mechanism is potentially important in developing therapeutic strategies to ameliorate mesangial cell proliferative disorders as well as to prevent renal fibrosis.

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