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Inhibition of PTEN Expression and Activity by Angiotensin II Induces Proliferation and Migration of Vascular Smooth Muscle Cells

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ABSTRACT

PTEN (phosphatase and tensin homolog deleted on chromosome 10) is a tumor suppressor and has been suggested recently to be involved in the regulation of cardiovascular diseases. The molecular mechanisms of this regulation are however poorly understood. This study shows that down regulation of PTEN expression and activity by angiotensin II (Ang II) increased proliferation and migration of vascular smooth muscle cells (VSMCs). The presence of Ang II induced rapid PTEN phosphorylation and oxidation in accordance with increased AKT and FAK phosphorylation. The Ang II-mediated VSMC proliferation and migration was inhibited when cellular PTEN expression was increased by AT1 inhibitor losartan, PPARγ agonist rosiglitazone, NF-κB inhibitor BAY 11-7082. Over expression of PTEN in VSMCs by adenovirus transduction also resulted in inhibition of cell proliferation and migration in response to Ang II. These results suggest that PTEN down-regulation is involved in proliferation and migration of VSMCs induced by Ang II. This provides insight into the molecular regulation of PTEN in vascular smooth muscle cells and suggests that targeting the action of PTEN may represent an effective therapeutic approach for the treatment of cardiovascular diseases. J. Cell. Biochem. 114: 174–182, 2013.

KEY WORDS: PTEN; ANGIOTENSIN II; VASCULAR SMOOTH MUSCLE CELLS; PROLIFERATION; MIGRATION

Vascular smooth muscle cell (VSMC) is the only cellular component of vascular middle layer. Under physiological conditions, vascular smooth muscle regulates angiotasis by contraction and relaxation, and smooth muscle cells release some regulatory molecules for maintaining the normal functions of blood vessels. Abnormal proliferation of VSMC, resulting in VSMC migration from media to intima, is a critical event in the development of some vascular hypertrophy diseases such as coronary artery disease, percutaneous transluminal coronary angioplasty restenosis, and vascular hypertrophy in hypertension. So finding the mechanisms and inhibiting abnormal proliferation of VSMC are important for the treatment of vascular hypertrophy diseases.

PTEN is a dual phosphatase tumor suppressor which expressed in central nervous system, heart, liver, kidney, gastrointestinal tract, lungs, and skin as a constitutive secretion protein. PTEN is known to be involved in the regulation of a variety of physiological and pathological processes and particularly in cancer cells proliferation, differentiation, apoptosis, adhesion, and migration [Kotelevets et al., 2001; Steelman et al., 2004]. Recently, it has been reported that abnormal action of PTEN is liked with cardiac myocyte hypertrophy [Schwartzbauer and Robbins, 2001; Oudit and Penninger, 2009].

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High growth rate of the smooth muscle cells (SMCs) during vascular development and after vascular injury in vivo are associated with PTEN inactivation [Mourani et al., 2004]. Increasing activity and expression of PTEN could inhibit SMC proliferation [Garl et al., 2004].

In the present study, we investigated the relationship between the expression and activity of PTEN and VSMC proliferation and migration in response to angiotensin II (Ang II), an important member of angiotensin family. Ang II plays a critical role in the pathogenesis of several cardiovascular diseases associated with VSMC proliferation and migration [Wildroudt and Freeman, 2006; Tristano et al., 2007; Kyotani et al., 2010]. We showed that PTEN level was low in VSMCs, and the presence of Ang II induced rapid increase of the expression of the inactive forms of PTEN (p-PTEN and ox-PTEN). The presence of AT1 inhibitor losartan, PPARγ agonist rosiglitazone or NF-κB inhibitor BAY 11-7082 increased the PTEN expression in VSMCs, resulting in inhibition of cell proliferation and migration. Furthermore, over expression of PTEN by adenovirus-PTEN transduction also inhibited VSMCs proliferation and migration.

MATERIALS AND METHODS

MATERIALS

Dulbecco's modified eagle medium (DMEM) was purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) and 3,(4,5-dimethylthiazole-2-yl)2,5-diphenyltetrazolium bromide (MTT) were obtained from Solarbio Science and Technology (Beijing, China). CCK-8 was purchased from Jingmei Biotech (Jinan, China). Ang II was purchased from Sigma–Aldrich (St. Louis, MO). BAY 11-7082 and 2′,7′-dichlorofluorescein diacetate (DCFH-DA) were obtained from Beyotime Biotechnology (Jiangsu, China). Rabbit monoclonal antibodies (mAbs) against caspase-3, PTEN, and antibodies against phospho-PTEN (Ser-380/Thr-382/-383), phospho-Akt (Ser473), and phosphor-FAK (Tyr397) were purchased from Cell Signaling Technology (Boston, MA). Mouse mAb against β-actin was obtained from Abcam (Cambridge, UK). Peroxidase-conjugated goat anti-rabbit (mouse) antibodies were from Jackson ImmunoResearch (Pennsylvania). PTEN and GAPDH primer were synthesized by BioSune (Shanghai, China). Trizol total RNA extraction kit was obtained from Sangon (Shanghai, China). First strand cDNA synthesis kit and SYBR Green realtime PCR master mix were ordered from Toyobo (Osaka, Japan). Losarta and rosiglitazone were obtained from Cayman Chemical (Michigan). Ad-PTEN and Ad-GFP were kindly provided by Department of Biochemistry and Molecular Biology, Louisiana State University Health Sciences Center.

Adult male SD rats with body weight ranged from 180 to 200 g were purchased from the Animal Center of Shandong University (animal accreditation no.: SCXK-00-0004). All animal works were performed in accordance to the guidelines by the animal care procedures of Shandong University.

CELL CULTURE AND TREATMENT

The thoracic aorta of rats was carefully excised, cut into small pieces (~1 mm^3), and attached to the wall of 100 ml culture bottle for 2 h before cultured in DMEM with 10% FBS under standardized condition. Characterization of VSMCs was conducted by morphology examination as well as by immunocytochemistry with anti-α-smooth muscle actin antibody. All the cells were used from passage 3 to 8.

Cells were treated with Ang II (0.1, 1, or 10 μmol/L) for different time courses according to the experimental design.

AD-PTEN TRANSDUCTION IN VSMCs

The adenovirus encoding the green fluorescence protein (Ad-GFP) and PTEN protein (Ad-PTEN) were generated using the ViraPower Adenoviral Expression System (Invitrogen) as the manufacture's instruction and the viruses were amplified and titrated as described [Wang et al., 2010]. In briefly, adenovirus amplification was propagated in 293A cells to obtain high-titer stocks. When multiplicity of infection (MOI) was 100, VSMCs were co-cultured with Ad-PTEN or Ad-GFP (as a control) for 72 h. The transduction was examined by immunoblotting with anti-PTEN antibody.

CELLS PROLIFERATION

After appropriate treatments, cell proliferation was determined by MTT assay, CCK-8 assay, and cell counting. Briefly, VSMCs were seeded in 96-well plates with 1×10^4 cells/well in DMEM supplemented with 10% FBS. After adhesion of cells to the plate, the medium was changed to serum-free DMEM and cultured for 24 h before incubation of the cells with Ang II. For MTT assay, 20 μl MTT solution (5 mg/ml) was then added to each well for 4 h at 37°C. The supernatant was removed and the cell pellet was re-suspended in 0.1 ml DMSO and the absorbance (A value) was measured using a microtiter plate reader Spectra Rainbow (Tecan, Austria) at a wavelength of 570 nm. For CCK-8 assay, 10 μl CCK-8 solution was added to each well for 2 h at 37°C and then absorbance (A value) was measured using the microtiter plate reader at a wavelength of 450 nm. Cell proliferation was also measured by direct cell counting.

CELL MIGRATION

VSMC migration was determined using a scratch wound assay. Briefly, VSMCs were grown in 60-mm dishes until confluence. The cell monolayer was disrupted with a sterile rubber policeman to create a cell-free zone of 1 mm before washed with PBS and treated with or without Ang II (1 μmol/L) for 2, 4, 8, 12, and 24 h. Cells were visualized on an Olympus IX51 inverted microscope (Olympus, Japan). Cell migration was quantified by measuring the width of the cell-free zone (distance between the edges of the injured monolayer) at eight distinct positions with a digitally calibrated micrometer.

WESTERN BLOT ANALYSIS

After appropriate treatments, cells were lysed with extraction buffer (50 mM Tris–Hcl pH 7.5; 1 mM EDTA; 150 NaCl; 50 mM NaF; 1 mM Na_3VO_4; 1 mM PMSF, 1% NP-40) and centrifuged at 13,000 rpm for 15 min. The resulting supernatant was fractionated on 10% SDS–PAGE and transferred to nitrocellulose membranes. Membranes were blocked with 5% milk in TBST before incubation with primary antibodies against caspase-3 (1:1,000), PTEN (1:1,000), phospho-PTEN (1:1,000), phospho-Akt (1:1,000), phospho-FAK (1:1,000), or β-actin (1:10,000) for 2 h and followed by horseradish peroxidase-conjugated secondary antibodies. The

membranes were developed by enhanced chemiluminescence and exposed to X-ray films. The bands were quantified by densitometry analysis with AlphaEaseFC (Alpha Innotech Company).

MEASUREMENT OF REACTIVE OXYGEN SPECIES (ROS) GENERATION

Intracellular formation of ROS was assessed as described previously using oxidation sensitive dye DCFH-DA as a substrate [Qian et al., 2008]. Briefly, serum-starved cells were treated with Ang II for the indicated time. After washing with PBS, 10 μM 2′,7′-DCFH-DA in serum-free DMEM was introduced in dark for 30 min at 37°C. After washing with PBS, the fluorescence intensity was measured with a VICTOR Wallac 1420 Mutilable Counter (Perkin Elmer Life Science, USA) at the excitation wavelength of 485 nm and emission wavelength of 535 nm.

ANALYSIS OF OXIDIZED FORMS OF PTEN BY NONREDUCING SDS–PAGE AND IMMUNOBLOTTING

After appropriate stimulation, the cells were lysed in buffer (50 mM Tris–Hcl pH 7.5; 1 mM EDTA; 150 mM NaCl; 50 mM NaF; 1 mM Na_3VO_4; 1 mM PMSF, 1% NP-40; 50 mM *N*-ethylmaleimide) and centrifuged at 13,000 rpm for 15 min. The resulting supernatant was fractionated on 10% nonreducing SDS–polyacrylamide gels followed by Western blot using anti-PTEN antibody [Lee et al., 2002; Kim et al., 2008]. Cells treated with 1 mmol/L H_2O_2 for 15 min as a positive control.

REAL-TIME PCR

Total cellular RNA was extracted from VSMCs with trizol total RNA extraction kit. Reverse transcription was carried out with 2 μg extracted RNA using first strand cDNA synthesis kit. PCR reaction was performed using SYBR Green realtime PCR master mix. The following primers were used for specific amplification of PTEN and GAPDH [Hao et al., 2009]: for PTEN (F: 5′-ATACCAGGACCAGAG-GAAACC-3′; R: 5′-TTGTCATTATCCGCACGCTC-3′); for GAPDH (F: 5′-TCCCTCAAGATTGT CAGCAA-3′; R: 5′-AGATCCACAACGGA-TACAT T-3′). RT-PCR conditions were: 2 min at 95°C, followed by 40 cycles of denaturing at 95°C for 15 s, annealing and extension for 15 s at 60°C and 20 s at 72°C. Threshold cycle (Ct), the cycle at which emission rises above baseline, was determined for the target gene in each sample, and relative quantization of target transcript levels was determined by evaluating the expression $2-\Delta\Delta C_t$ (Eppendorf, Germany).

STATISTICAL ANALYSIS

Statistical analysis was performed by one-way ANOVA followed by Fisher's protected least significance difference (PLSD) test. A probability value <0.05 was considered significant.

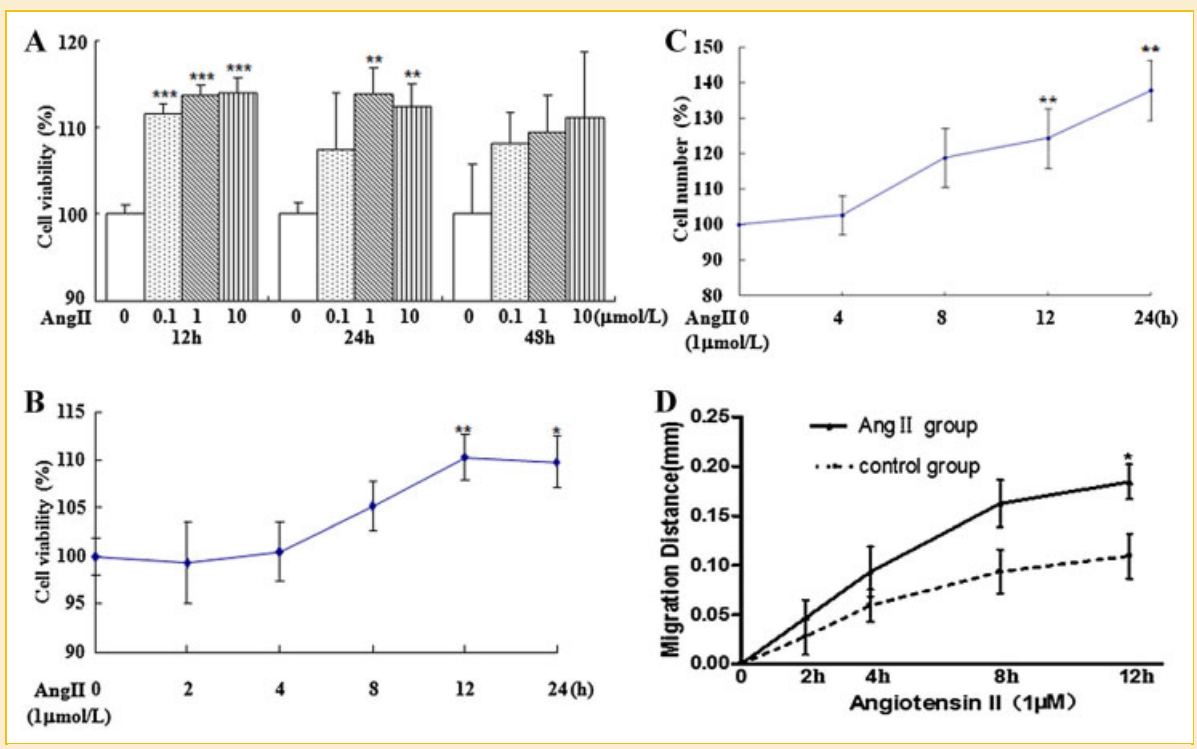

Fig. 1. Ang II promotes VSMC proliferation and migration. A: Cells were treated with 0.1, 1, and 10 μmol/L Ang II, respectively for 12, 24, and 48 h before the cell viability was examined by MTT assay. Values are mean ± SE (n = 6). $^{**}P < 0.01$, $^{***}P < 0.001$ compared with cells unstimulated with Ang II. B,C: Cells were treated with 1 μmol/L Ang II for 0, 2, 4, 8, 12, and 24 h before the cell viability was examined by CCK-8 assay (B) or cell counting (C). Data are expressed as mean ± SE (n = 6). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with cells treated with Ang II for 0 h. D: Cells were treated with 1 μmol/L Ang II for 2, 4, 8, and 12h, DMEM as control. Cell migration was examined by scratch wound assay and quantified by measuring the width of the cell-free zone at eight distinct positions. Data are expressed as mean ± SE (n = 8). $^{*}P < 0.05$ compared with control group.

RESULTS

ANG II INDUCES VSMC PROLIFERATION AND MIGRATION

The presence of Ang II induced dose- and time-dependent increase of VSMC proliferation (Fig. 1A–C) when assessed either by MTT (Fig. 1A), CCK8 (Fig. 1B), or cell counting (Fig. 1C). At 0.1, 1, and 10 μmol/L for 12 h, Ang II induced 11.6% ($P < 0.001$), 13.6% ($P < 0.001$), and 14% ($P < 0.001$) increase of VSMC proliferation when assessed by MTT assay (Fig. 1A). The CCK-8 assay showed the similar result with MTT assay, which was 1 μmol/L Ang II promoted VSMC proliferation within 24 h, the optimal time point was 12 h (Fig. 1B). At 1 μmol/L for 12 and 24 h, Ang II increased VSMC proliferation at 24.3% ($P < 0.01$) and 37.8% ($P < 0.01$) on the basis of initial cell numbers at 1×10^4 cells/well at time 0 by cell counting (Fig. 1C). The presence of Ang II also caused significant increase of VSMC migration in the scratch wound assay. Nearly twofolds ($P < 0.05$) increase of cell migration was observed after incubation of the cells with 1 μmol/L Ang II for 12 h (Figs. 1D and 2).

ANG II-MEDIATED CELL PROLIFERATION AND MIGRATION IS ASSOCIATED WITH INHIBITION OF PTEN TRANSCRIPTION, EXPRESSION AND ACTIVITY IN VSMC

To test whether PTEN was involved in Ang II-mediated VSMC proliferation and migration, we determined the expression and activity of PTEN in VSMC response to Ang II. Results showed that the expression of total PTEN protein in VSMCs reduced in the presence of Ang II in a concentration- (Fig. 3A) and time- (Fig. 3B,C) dependent manner. The mRNA level of PTEN in VSMCs decreased to nearly half of control group by treatment with 1 μmol/L Ang II for 2 h (Fig. 3D). These results indicated that 1 μmol/L Ang II caused obvious inhibition of both mRNA level and protein expression of PTEN.

We further investigated the alteration of PTEN activity in VSMCs response to Ang II. It is known that PTEN localization, activity and stability are associated with its phosphorylation status (p-PTEN) in the C-terminal tail. PTEN phosphorylation at its C-terminus (residues 380–385) results in interaction of its C2 domain and

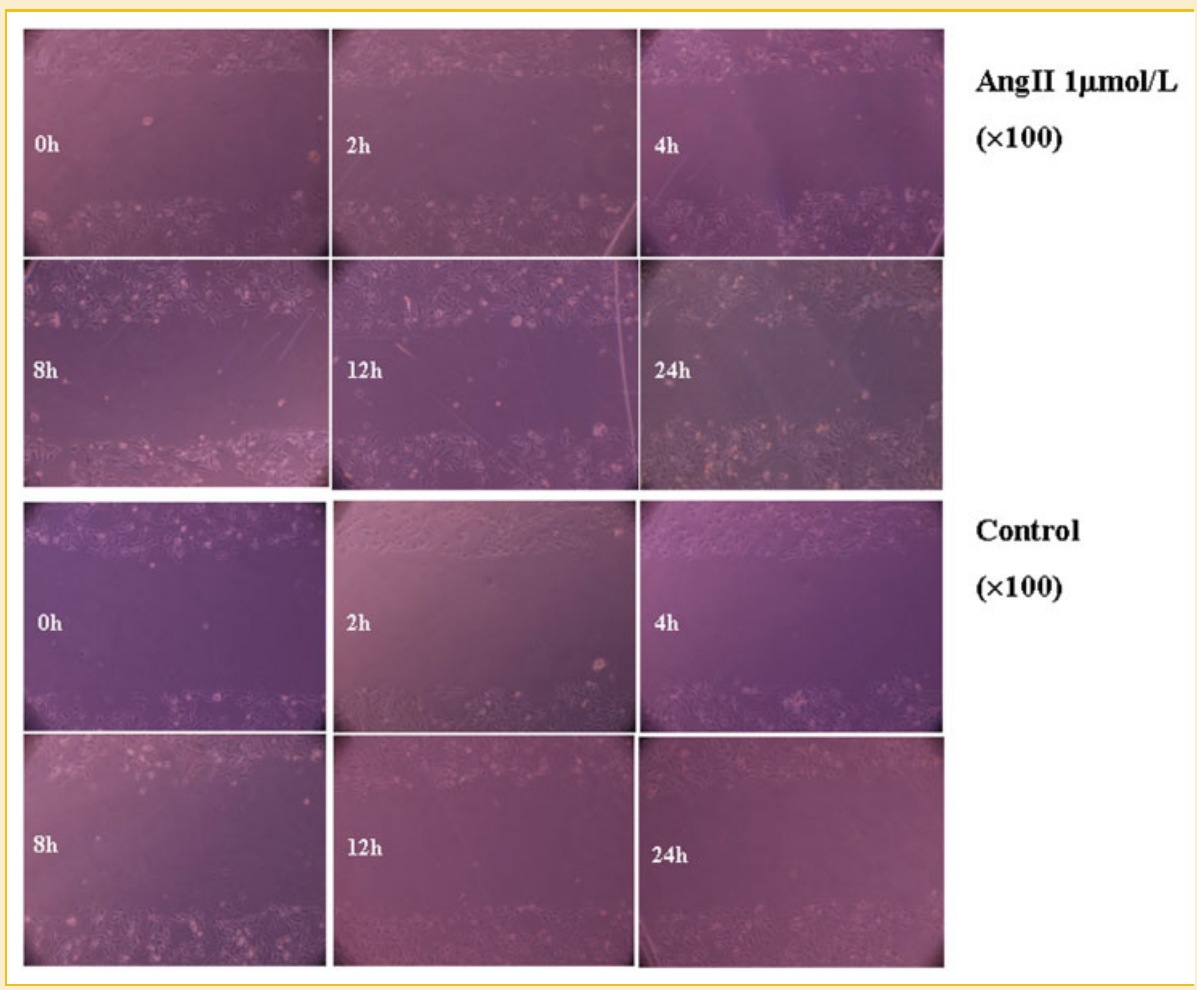

Fig. 2. The representative images of the scratch assay. VSMCs were grown in 60-mm dishes until confluence and were disrupted with a sterile rubber policeman to create a cell-free zone of 1 mm. Then cells treated with 1 μmol/L Ang II for 2, 4, 8, 12, and 24 h, DMEM as control. At each time point, cells were visualized and pictures taken under microscope at 100× magnification.

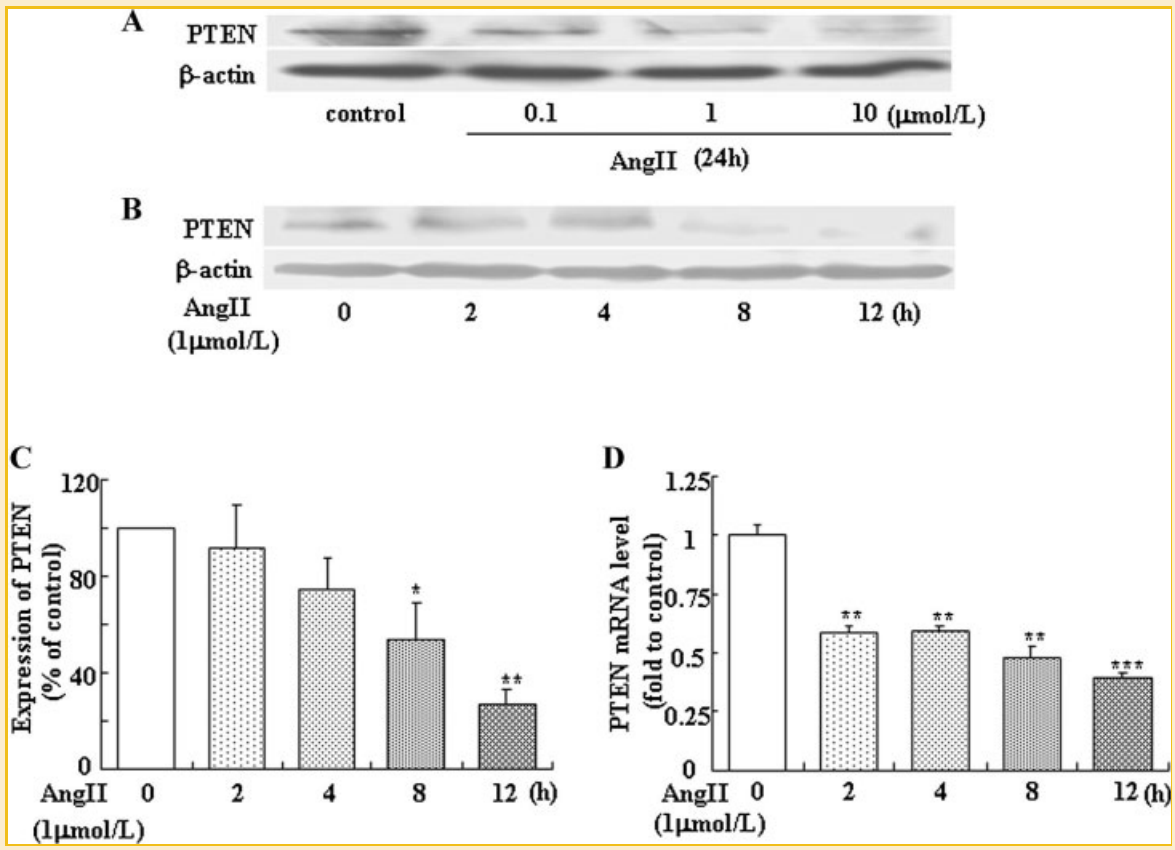

Fig. 3. Ang II increases PTEN protein and mRNA level in VSMC. A,B: Representative blots of PTEN expression in VSMCs treated with 0.1, 1, and 10 μmol/L Ang II for 24 h (A) or with Ang II (1 μmol/L) for 0, 2, 4, 8, and 12 h (B). Quantification of the band intensity by densitometry is shown in Figure 3C. Data are expressed as mean ± SE (n = 3). D: Cells were treated with Ang II (1 μmol/L) for 0, 2, 4, 8, and 12 h before the PTEN expression was measured by real time-PCR. Data are expressed as mean ± SE (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with cells treated with Ang II for 0 h.

phosphatase domain, serving as a pseudosubstrate and causing an auto-inhibited conformation [Tamguney and Stokoe, 2007]. Therefore we determined the expression of p-PTEN to reflex the activity of PTEN indirectly. We found that the presence of Ang II induced rapid PTEN phosphorylation, which started from 5 min and lasted for 30 min (Fig. 4A,B).

Apart from being regulated by its phosphorylation status, PTEN activity is also known to be regulated by reactive oxygen species (ROS). Oxidation of catalytic site cysteine 124 residue of PTEN by ROS to form an intramolecular disulfide bond with cysteine 71 could down-regulate PTEN activity [Lee et al., 2002; Tamguney and Stokoe, 2007]. We found that the presence of Ang II induced rapid increase of ROS formation (fluorescence intensity) in VSMC which reached a maximal level within 60 min before gradually returning to its basal level (Fig. 4C). Coincided with the increase of intracellular ROS level was the increase of oxidized PTEN (ox-PTEN) expression, while non-ox-PTEN level was not affected (Fig. 4D,E).

Collectively, these results suggested that the presence of Ang II inhibits PTEN expression and activity in VSMCs and its effect on PTEN activity was associated with its stimulation of PTEN phosphorylation and intracellular ROS formation.

ANG II-MEDIATED CELL PROLIFERATION AND MIGRATION IS ASSOCIATED WITH PTEN SIGNALING IN VSMC

Akt and FAK are down stream signaling of PTEN, which were negative regulated by PTEN. It is becoming apparent that Akt/PKB and FAK signaling pathways play various roles in cell survival, promotion, and migration. We found that the presence of Ang II rapidly increased the phosphorylation of Akt and FAK (p-Akt and p-FAK) in VSMCs in a time dependent manner during 12 h (Fig. 5A,C). After 12 h incubation with Ang II, VSMC showed to have 4.5- and 3.5-fold higher levels of p-Akt and P-FAK, respectively (Fig. 5B,D). These results suggested that Akt and FAK signaling were activated in PTEN inhibition induced by Ang II.

ANG II-MEDIATED PROLIFERATION, MIGRATION AND PHOSPHORYLATION OF Akt AND FAK ARE PREVENTED BY PTEN REGULATORS

It was found that Ang II-mediated down-regulation of PTEN expression in VSMCs was blocked significantly by the presence of AT1 inhibitor losartan (20 μmol/L), peroxisome proliferator-activated receptor γ (PPARγ) agonist rosiglitazone (100 μmol/L) and NF-κB inhibitor BAY11-7082 (10 μmol/L) (Fig. 6A,B). Furthermore,

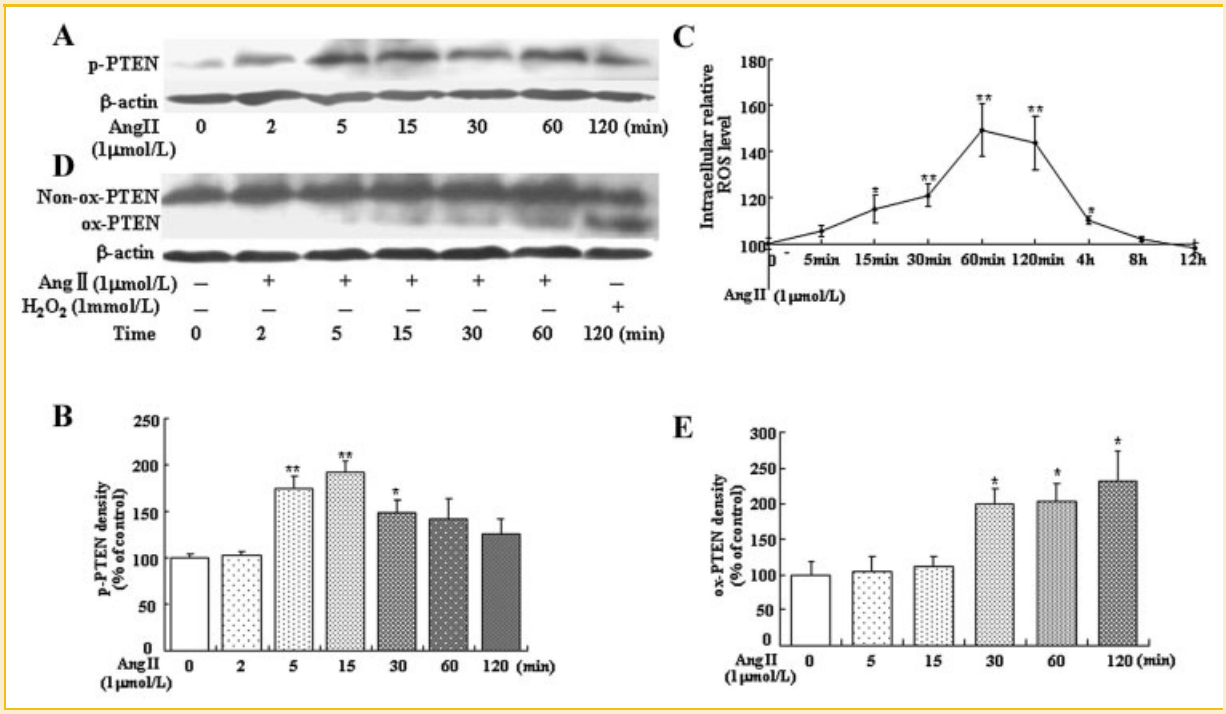

Fig. 4. Effect of Ang II on PTEN phosphorylation, oxidation and ROS level in VSMC. A,B: Cells were treated with Ang II (1 μmol/L) for 0–120 min before PTEN phosphorylation was assessed by anti-p-PTEN blotting. Representative blots and quantification are shown. Data are expressed as mean ± SE (n = 3). C: After treatment with Ang II, cells were loaded with DCFH-DA and the cell-associated fluorescent intensities was determined (Ex = 485 nm and Em = 535 nm). All data are expressed as mean ± SE (n = 6). D,E: Cells were treated with Ang II (1 μmol/L) for 0–120 min or H_2O_2 (1 mmol/L) for 15 min before PTEN oxidation was assessed by immunoblotting. Representative blots of ox-PTEN expression and quantification by densitometry are shown. Data are expressed as mean ± SE (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$ compared with cells treated with Ang II for 0 min.

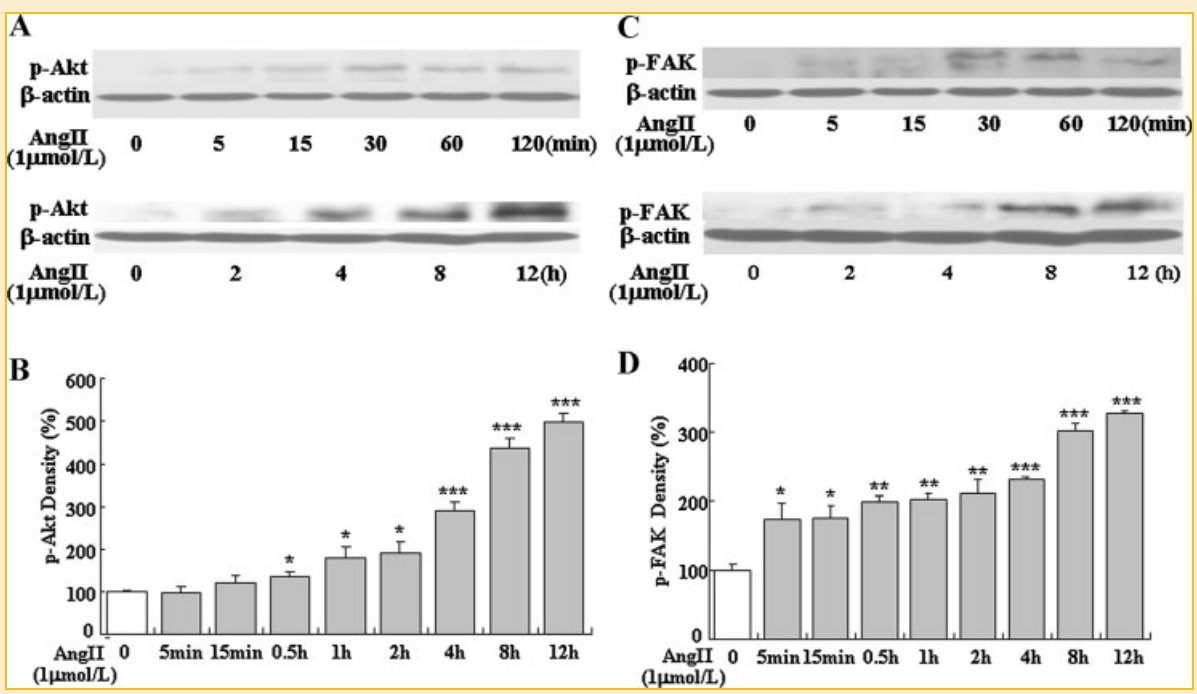

Fig. 5. Effect of Ang II on cellular-p-Akt and p-FAK. Cells were treated with Ang II (1 μmol/L) for the indicated period before cellular p-Akt (A) and p-FAK (C) were assessed by immunoblotting. Quantification of the band intensity by densitometry is shown in Figure 5B,D. Data are expressed as mean ± SE (n = 3). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ compared with cells treated with Ang II for 0 min.

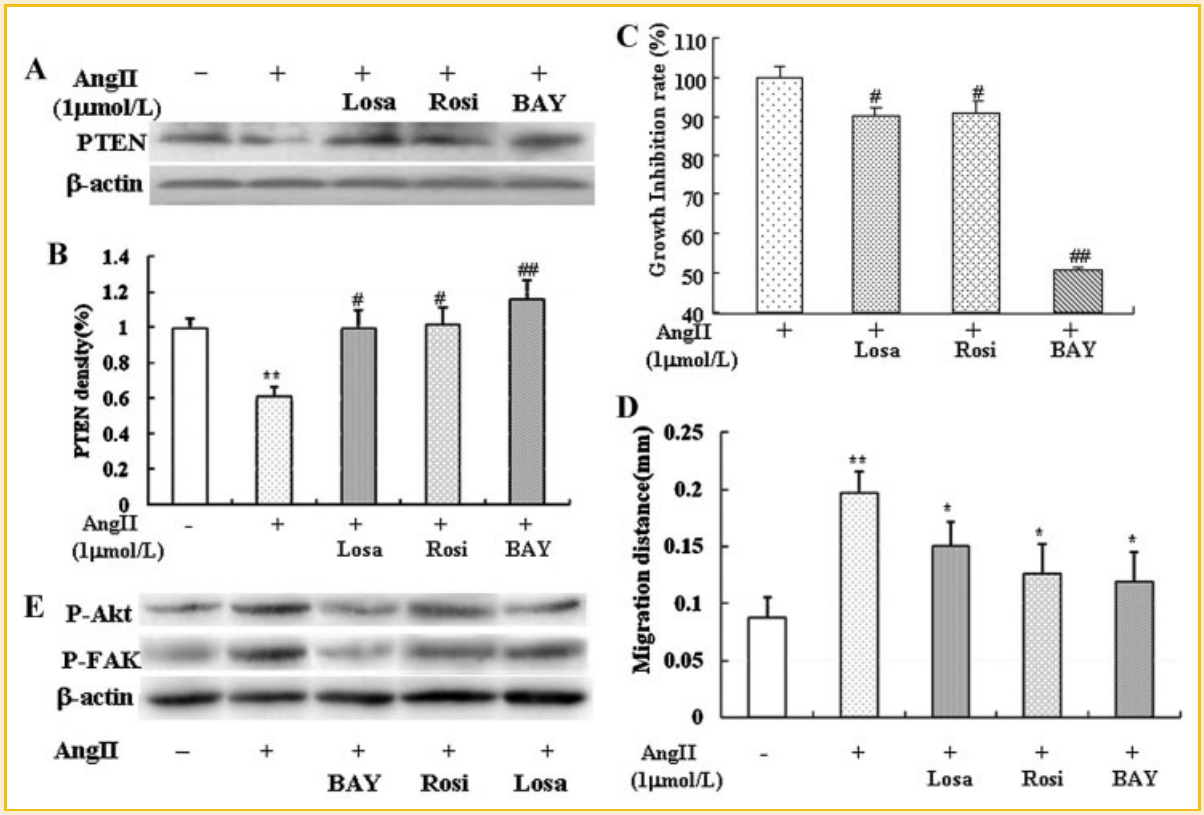

Fig. 6. Effect of losartan, rosiglitazone, and BAY11-7082 on Ang II-mediated PTEN expression, p-Akt and p-FAK and cell proliferation and migration. VSMC were pre-incubated with losartan (Losa), rosiglitazone (Rosi), and BAY11-7082 (BAY) for 1 h before introduction of 1 μmol/L Ang II, then cellular PTEN expression was examined by immunoblotting (A,B), cell viability by CCK8 (C), cell migration by scratch wound healing assay (D) or p-Akt and p-FAK expression by immunoblotting (E). Representative blots and quantification density are shown in Figure 6A,B,E. Data are expressed as mean ± SE (n = 6). * $P < 0.05$, ** $P < 0.01$ compared with Ang II group.

Ang II-mediated VSMCs proliferation, migration (Fig. 6C,D) and phosphorylation of Akt and FAK (Fig. 6E) were also inhibited by these three agents.

PTEN OVEREXPRESSION INHIBITS ANG II-MEDIATED VSMCs PROLIFERATION AND MIGRATION

After Ad-GFP or Ad-PTEN transduction for 72 h, the expression of PTEN in VSMC was measured by Western blotting. Results showed that transduction with Ad-PTEN resulted in 2.5-fold ($P < 0.01$) increase of PTEN expression in VSMCs (Fig. 7A,B). PTEN over expression resulted in significantly reduced effect of Ang II on VSMC proliferation and migration (Fig. 7C,D).

DISCUSSION

Abnormal proliferation of VSMC is the pathologic basis of vascular hypertrophy diseases such as coronary artery disease, percutaneous transluminal coronary angioplasty restenosis, and vascular hypertrophy in hypertension. Previous studies have reported that Ang II regulates VSMC proliferation and migration by unknown mechanism. This study shows that Ang II-mediated VSMC proliferation and migration is associated with inhibition of PTEN expression and activity.

PTEN was initially identified as a tumor suppressor in 1997 and is now known to involve in many human diseases, such as cancer, diabetes and cardiovascular diseases. As an inositol phosphatase, PTEN dephosphorylates the phospholipid products (e.g., phosphatidylinositol 3,4,5-trisphosphate, PIP3) of PI3K with high specificity, and also dephosphorylates the protein substrates of FAK.

It is known that the expression and activity of PTEN can be positively or negatively regulated at the transcriptional as well as post-translational levels, for example, by phosphorylation and oxidation. Phosphorylation of the PTEN C-terminal (residues 380–385) has shown to serve as a pseudosubstrate and induce PTEN auto-inhibition. Phosphorylation of PTEN can also inhibit PTEN action by inducing its translocation from plasma membrane to cytosol [Tamguney and Stokoe, 2007]. It has been reported that the decrease of PTEN expression and increase of p-PTEN (Ser-380) expression reduce PTEN activity in saphenous vein smooth muscle cells of human coronary artery bypass grafting conduits [Mitra et al., 2009]. PTEN activity is also known to be regulated by ROS, in which ROS oxidizes the PTEN cysteine residue (C124) for the formation of an intramolecular disulfide bond involving its C71 residue [Tamguney and Stokoe, 2007]. Exposure of Hela cells to H_2O_2 could increase ox-PTEN expression and induce PTEN inactivation in concentration and time dependent manners [Lee et al., 2002]. Connor et al. [2005] has reported that alterations in the steady state

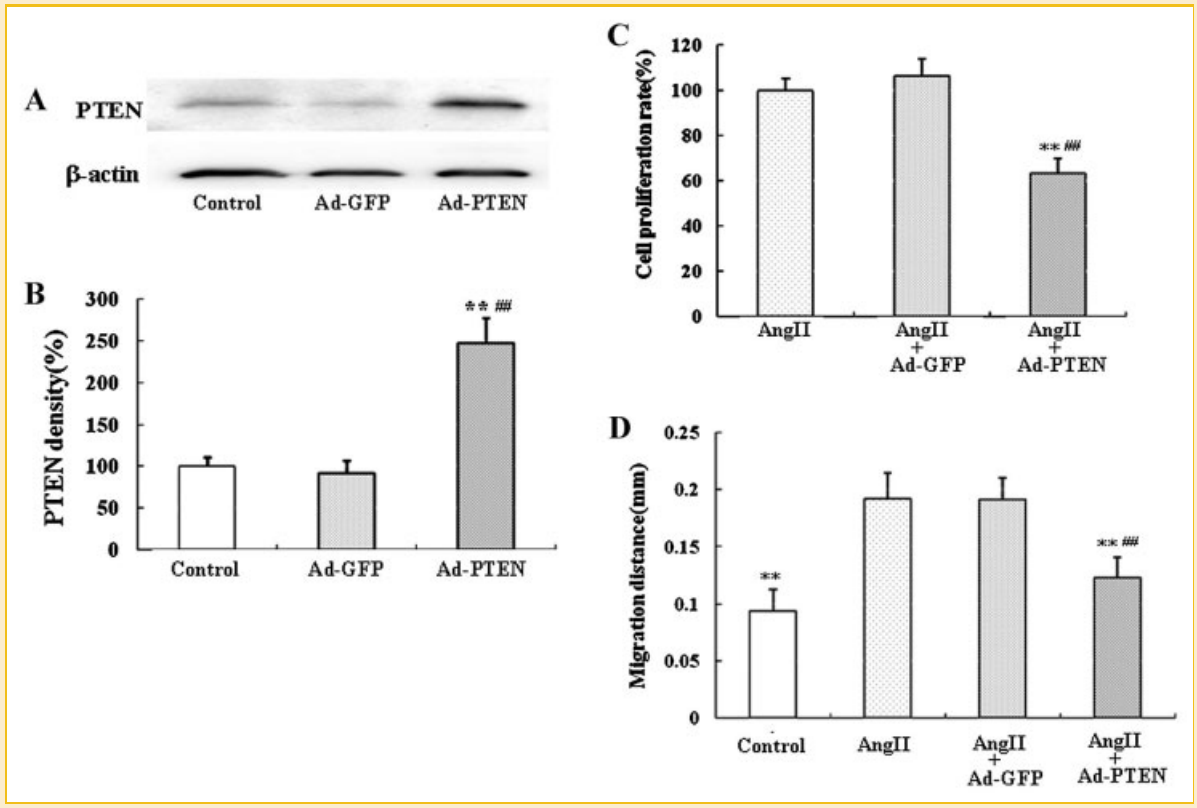

Fig. 7. Effect of PTEN over expression on VSMC proliferation and migration. VSMC were transfected with Ad-PTEN or Ad-GFP for 72 h, the expression of PTEN was measured by Western blotting. Representative blots of PTEN expression are shown in (A) and quantification in (B; n = 3). $^{**}P < 0.01$ compared with control (untransfected VSMC) group, $^{\#\#}P < 0.01$ compared with Ad-GFP group. After transfection of VSMC with Ad-PTEN or Ad-GFP for 60 h, Ang II was added for another 12 h and the cell viability was then examined by MTT assay (C) or cell migration was examined by scratch wound assay (D). Data are expressed as mean ± SE (n = 6). $^{**}P < 0.01$ compared with Ang II group, $^{\#\#}P < 0.01$ compared with Ang II +Ad-GFP group.

of mitochondrial H_2O_2 production by over expression of antioxidant enzyme modulated the redox state of PTEN, and PI3K/Akt signaling. In the present study, we found that the presence of Ang II caused rapid increase of p-PTEN expression, suggesting that inhibition of PTEN activity likely contributes to the initial action of Ang II-mediated VSMC proliferation and migration. Furthermore, Ang II also increased the intracellular ROS production and ox-PTEN expression within 2 h, indicating that ROS accumulation and PTEN oxidation may be involved in Ang II-mediated decrease of PTEN activity in early stage of the Ang II action. After Ang II treatment for 2 h, the total protein expression and mRNA level of PTEN began to decrease gradually, indicating PTEN expression decrease may be contributed to the later stage of the Ang II action.

To further confirm the role of PTEN in the cell proliferation and migration induced by Ang II, Losartan (AT1 inhibitor), rosiglitazone (PPARγ agonist), and BAY11-7082 (NF-κB inhibitor) were used to change PTEN expression in VSMCs. It was found that preincubation of the cells with Losartan, rosiglitazone or BAY11-7082 prevented Ang II-mediated down-regulation of PTEN expression and VSMC proliferation and migration. Losartan has previously shown to antagonize AT1 action and reverse the effect of Ang II. Rosiglitazone, the selective ligand of PPARγ, has been reported to increase PTEN expression by binding to PPAR response elements (PPREs) on PTEN promoter [Patel et al., 2001; Zhang et al., 2006]. BAY11-7082 can bind to NF-κB on 1,574–1,565 and the PTEN promoter region 1,450–1,441 [Xia et al., 2007], thus increase PTEN expression. The importance of PTEN expression in Ang II-mediated VSMCs proliferation and migration was further demonstrated by the reduction of Ang II action in VSMCs that over express PTEN.

Previous studies have shown that the phosphatase activity of PTEN regulates the signal transduction of phosphatidylinositol-3-kinase (PI3K) [Cantley and Neel, 1999; Maehama and Dixon, 1999], extracellular signal-regulated kinase (ERK) [Chung et al., 2006] and focal adhesion kinase (FAK) [Chatzizacharias et al., 2007], and is critical for its tumor suppressor function. The demonstration in this study that the expressions of p-Akt and p-FAK were rapidly induced following introduction of Ang II in a time dependent manner during 12h suggests that phosphorylation of Akt and FAK likely contributes to the reduction of PTEN activity induced by Ang II, hence to Ang II-mediated VSMC proliferation and migration.

In conclusion, inhibition of PTEN expression or activity induced by Ang II in VSMCs increases cell proliferation and migration, an effect that involves activation of cellular Akt and FAK signaling. When PTEN expression was increased in VSMCs either activating by losartan, rosiglitazone, NF-κB inhibitor, or overexpression by

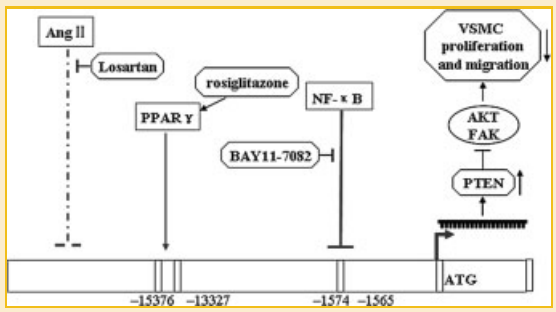

Fig. 8. Proposed relationship between PTEN expression and cell proliferation and migration.

transduction, the VSMCs abnormal proliferation and migration induced by Ang II could be inhibited. We proposed the relationship between PTEN expression and cell proliferation and migration in Figure 8. This provides insight into the molecular regulation of PTEN in VSMCs and may have important implications for the development of therapeutic strategies to prevent restenosis and atherosclerosis.

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