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Losartan Inhibits Vascular Smooth Muscle Cell Proliferation through Activation of AMP-Activated Protein Kinase

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Losartan is a selective angiotensin II (Ang II) type 1 (AT_1) receptor antagonist which inhibits vascular smooth muscle cells (VSMCs) contraction and proliferation. We hypothesized that losartan may prevent cell proliferation by activating AMP-activated protein kinase (AMPK) in VSMCs. VSMCs were treated with various concentrations of losartan. AMPK activation was measured by Western blot analysis and cell proliferation was measured by MTT assay and flowcytometry. Losartan dose- and time-dependently increased the phosphorylation of AMPK and its downstream target, acetyl-CoA carboxylase (ACC) in VSMCs. Losartan also significantly decreased the Ang II- or 15% FBS-induced VSMC proliferation by inhibiting the expression of cell cycle associated proteins, such as p-Rb, cyclin D, and cyclin E. Compound C, a specific inhibitor of AMPK, or AMPK siRNA blocked the losartan-induced inhibition of cell proliferation and the G_0/G_1 cell cycle arrest. These data suggest that losartan-induced AMPK activation might attenuate Ang II-induced VSMC proliferation through the inhibition of cell cycle progression.

Key Words: AMP-activated protein kinase (AMPK), Angiotensin II type 1 (AT_1) receptor antagonist, Losartan, Vascular smooth muscle cells (VSMCs), Proliferation

INTRODUCTION

The proliferation of vascular smooth muscle cells (VSMCs) plays a vital role in hypertension. In addition, abnormal VSMC growth contributes to vascular diseases such as atherosclerosis and restenosis following angioplasty [1]. On the other hand, in a normal artery, VSMCs exist in a non-proliferative quiescent state and show a well-differentiated contractile phenotype.

AMP-activated protein kinase (AMPK) is an important cellular fuel sensor. The enzyme is a heterotrimeric complex consisting of a catalytic α subunit and regulatory β and γ subunits with multiple genes encoding each subunit. AMPK is activated by stress such as physical exercise, hypoxia, and nutrient depletion that increase the cellular AMP/ATP ratio [2]. Activation of AMPK requires phosphorylation at Thr172, which resides in the activation loop of the α1- and α2-subunits [3]. Several AMPK upstream kinases are capable of phosphorylating AMPK at Thr172, including LKB1 [4] and calcium calmodulin-dependent kinase kinase [5]. AMPK has been implicated in the regulation of physiological signals, such as inhibition of cholesterol, fatty acid, and protein synthesis, and enhancement of glucose uptake and blood flow [6,7]. AMPK activation also exhibits several salutary effects on vascular function and improves vascular abnormalities. For example, AMPK activation improves endothelial function [8-10] and suppresses VSMC proliferation [11]. Thus, AMPK activation might inhibit VSMC proliferation in vascular tissues.

Angiotensin II (Ang II) receptor blockers (ARBs) inhibit the effects of Ang II by antagonizing the Ang II type 1 (AT_1) receptor. ARBs interfere with the unfavorable effects of Ang II, and reduce of blood pressure [12]. ARBs have beneficial effects in the treatment of heart failure, renal failure, and myocardial infarction. Studies of cultured VSMCs show that ARBs inhibit DNA synthesis, VSMC proliferation, and protein synthesis [13-17]. Moreover, olmesartan ameliorates insulin resistance and decreases triglyceride production in fructose-fed rats [18]. Telmisartan has a clear antihypertensive effect but may also be effective for metabolic syndrome, with a peroxisome proliferator-activated receptor-γ agonistic effect [19].

In the present study, we investigated whether the anti-proliferative effect of losartan, an ARBs, is mediated through AMPK activation in VSMCs and the precise mechanisms underlying its action.

METHODS

Materials and cell culture

Dulbecco's modified eagle medium (DMEM) and fetal bo-

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ABBREVIATIONS: VSMC, vascular smooth muscle cell; AMPK, AMP-activated protein kinase; ACC, acetyl-CoA carboxylase; Ang II, Angiotensin II; ARB, Angiotensin II receptor blockers.

vine serum (FBS) were purchased from Thermo Scientific (South Logan, UT, U.S.A.). Pro-prep protein extract solution was purchased from Intron Biotechnology (Sungnam, Korea). Anti-AMPK, anti-phospho-AMPK, anti-phospho-acetyl CoA carboxylase (ACC), anti-phospho-LKB1, anti-Rb, anti-phospho-Rb, and anti-p53 antibodies were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Losartan, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI), and anti-β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Ang II and compound C were purchased from Calbiochem (San Diego, CA, U.S.A.). AMPK siRNA, anti-cyclin E, and anti-p21 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). AICAR (5-Aminoimidazole-4-carboxamide-1-β-D-ribofuranoside) was purchased by Toronto Research Chemicals (North York, Ontario, Canada). Lipofectamine 2000 was purchased from Invitrogen (Carlsbad, CA, U.S.A.). Anti-cyclin D antibody was purchased from Upstate (Billerica, MA, U.S.A.) Anti-p27 antibody was purchased from BD biosciences (San Jose, CA, U.S.A.).

Aortic VSMCs were isolated from 11-weeks-old male Sprague-Dawley rats and were grown in DMEM with 10% FBS and 1% antibiotic-antimycotic (penicillin 10,000 U/ml, amphotericin B 25 μg/ml, streptomycin 10,000 μg/ml). Cells were maintained at 37°C and in a 5% CO_2 incubator. We used VSMCs from 4 to 8 passages at 70~90% confluence in 10 cm dishes and cell growth was arrested by incubation of the cells in serum-free DMEM for16~24 hrs prior to use.

Western blot analysis

Whole cell extracts were prepared by lysing the cells in pro-prep protein extract solution. The protein concentration was quantified with protein assay reagent from Bio-Rad (Hercules, CA, U.S.A.). Equal amounts of protein were mixed with sodium dodecyl sulfate (SDS) sample buffer and incubated for 5 min at 100°C before loading. Total protein samples (30 μg) were subjected to 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) for 1 hr 30 min at 100 V. The separated proteins were electrophoretically transferred onto a PVDF membrane for 1 hr at 30 mA. The membranes were blocked with 5% non-fat milk in phosphate buffered saline containing 0.05% Tween 20 (PBS-T) for 2 hrs at room temperature. The membranes were then incubated with the primary antibodies at a dilution of 1 : 1,000 in 5% skim milk in PBS overnight at 4°C. The membranes were then washed with four changes of wash buffer (0.05% Tween 20 in PBS) and incubated for 1 hr at room temperature in PBS-T containing anti-rabbit (Stressgen, Ann Arbor, MI, U.S.A.), anti-mouse IgG or anti-goat IgG (Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) antibodies. After four times rinses with wash buffer, the membranes were exposed to ECL western blot detection reagents.

Cell proliferation assay

Cell proliferation was analyzed using the MTT assay. VSMCs were seeded on 24-well plates at a density of 1×10^4 cells per well in DMEM supplemented with 10% FBS. After treatments with Ang II, losartan, and inhibitors, 50 μl of 1 mg/ml MTT solution was added to each well (0.1 mg/well) and incubated for 4 hrs. The supernatants were aspirated, and the formazan crystals in each well were solubilized with 200 μl dimethyl sulfoxide (DMSO). An aliquot of this solution (100 μl) was placed in 96-well plates. Cell proliferation was assessed by measuring the absorbance at 570 nm using a microplate reader. The experiments were repeated 3 times.

Experiments using RNA interference

Transfections of VSMCs with siRNA were performed. VSMCs were seeded at 60~70% confluence on 24-well or 6-well dishes on the day before transfection. The cells were then transfected with AMPK siRNA using lipofectamine 2000 reagent, according to the manufacturer's instructions. Following an incubation period of 48 hrs, the AMPK protein level was measured using western blot analysis, while the cell proliferation was analyzed using the MTT assay.

Flow cytometric analysis for apoptosis and cell cycle

Apoptosis was examined by Annexin V-fluorescein isothiocyanate (FITC) staining (BD Biosciences, San Jose, CA, U.S.A.) according to the manufacturer's instructions. Cells were seeded on 6-well plates and incubated for 2 days. Cells were treated with various concentrations of losartan and Ang II for 24 hrs. The FITC fluorescence intensity of 10,000 cells was measured using a Becton-Dickinson FACS Caliber flow cytometer (BD Biosciences). Cell cycle profiles were analyzed by propidium iodide (PI) staining. A minimum of 10,000 cells in each sample was detected according to intracellular PI fluorescence intensity by flow cytometry, and cell cycle was analyzed by Cell Quest software (BD Biosciences).

Statistical analysis

All data are represented as the mean±S.E.M. Differences between data sets were assessed by analysis of variance (ANOVA) followed by Bonferroni's *t*-test. p values <0.05 were considered as significant.

RESULTS

Losartan activates AMPK and suppresses cell proliferation in VSMCs

We examined the effect of losartan on AMPK phosphorylation in VSMCs. Losartan increased AMPK phosphorylation in a time- and dose-dependent manner. Losartan also increased ACC phosphorylation, a major downstream target protein in the AMPK signaling cascade, and LKB1 phosphorylation, which is an upstream kinase of AMPK (Fig. 1A and 1B). We next examined the effect of losartan on VSMC proliferation. Treatment of VSMCs with Ang II (1 μM) or 15% FBS increased cell proliferation. Losartan decreased VSMC proliferation induced by Ang II or 15% FBS in a dose-dependent manner (Fig. 1C and 1D).

Inhibition of AMPK activity reverses the anti-proliferative effect of losartan

To further demonstrate the inhibitory effect of losartan caused by AMPK activation, we examined the effects of AMPK inhibitors on VSMCs proliferation. Losartan decreased Ang II-induced VSMCs proliferation (from 163.8± 0.9% to 125.1±3.9%). Compound C, a specific inhibitor of

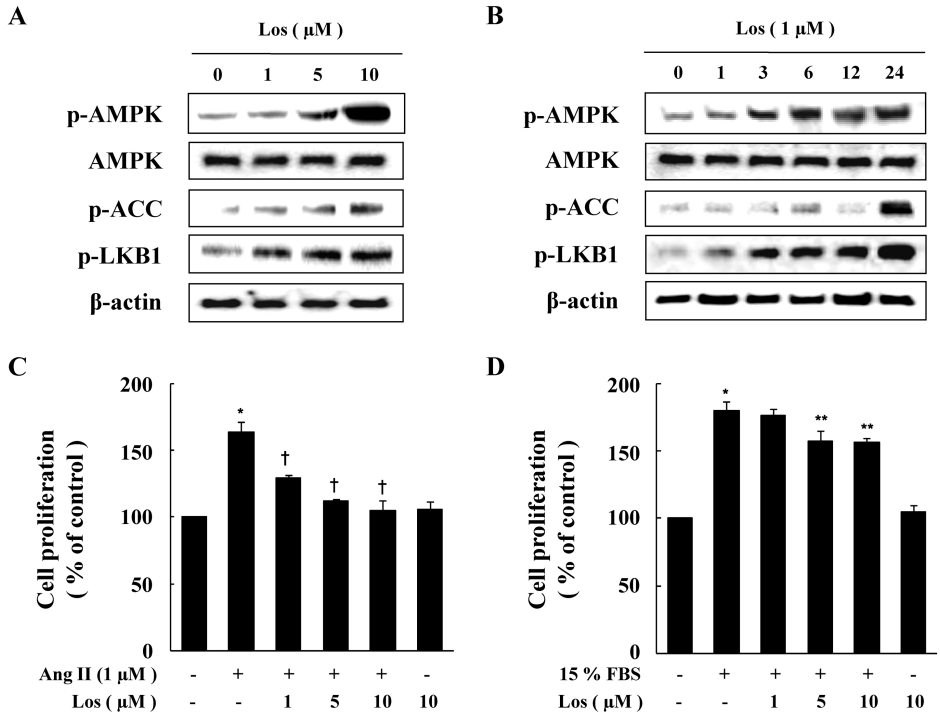

Fig. 1. Effect of Losartan on the Phosphorylation of AMPK and Cell Proliferation in VSMCs. Cells were treated with the indicated concentration of losartan for 1 hr (A) or for the indicated periods (B). Protein expression of p-AMPK, p-ACC, and p-LKB1 were determined by western blot analysis. Representative blots from three independent experiments are shown. Cells were treated with Ang II (C) or 15% FBS (D) in the presence or absence of indicated concentration of losartan for 48 hrs. Cell proliferation was determined by the MTT assay. Data are represented as the mean±S.E.M (n=4). *p value <0.05 compared with control, $^{\dagger}p<0.05$ compared with Ang II, **$p<0.05$ compared with 15% FBS.

AMPK, restored the losartan-induced inhibition of VSMC proliferation (from 125.1±3.9% to 149.7±3.5%). Genetic inhibition of AMPK with siRNA also restored the losartan-induced anti-proliferative effect (from 125.1±3.9% to 147.5±5.1%). AICAR, a well-known activator of AMPK, also inhibited Ang II-induced VSMC proliferation (from 163.8±0.9% to 145.1±3.1%) (Fig. 2A). The losartan-induced AMPK phosphorylation in VSMCs was significantly inhibited by pretreatment of compound C or AMPK siRNA (Fig. 2B). These results indicate that losartan-induced AMPK activation inhibits cell proliferation in VSMCs.

Losartan induces p53 and p21 expression but not apoptosis

The reduction in cell number induced by losartan could result from either an increase in cell death or inhibition of proliferation. To distinguish these possibilities, we first examined whether losartan could induce apoptosis. Apoptosis was measured by flow cytometric analysis with annexin V staining after losartan treatment. Losartan-treated cells display no apoptotic cell death (Fig. 3A). We then examined the effect of losartan on the expression of p53, a key regulator of the cell cycle, as well as p21 and p27, downstream targets of p53. Losartan increased p53 and p21 expression in a time-dependent manner, whereas the levels of p27 were not changed (Fig. 3B).

Ang II-induced cell cycle progression in G_2/M phase associated with phospho-Rb, cyclin D, and cyclin E is reversed by losartan through AMPK

We examined the effect of losartan on the phosphorylation of Rb, a cell cycle regulator that acts between the G1 and S phase. The Rb phosphorylation induced by Ang II was inhibited by losartan. Other proteins involved in cell

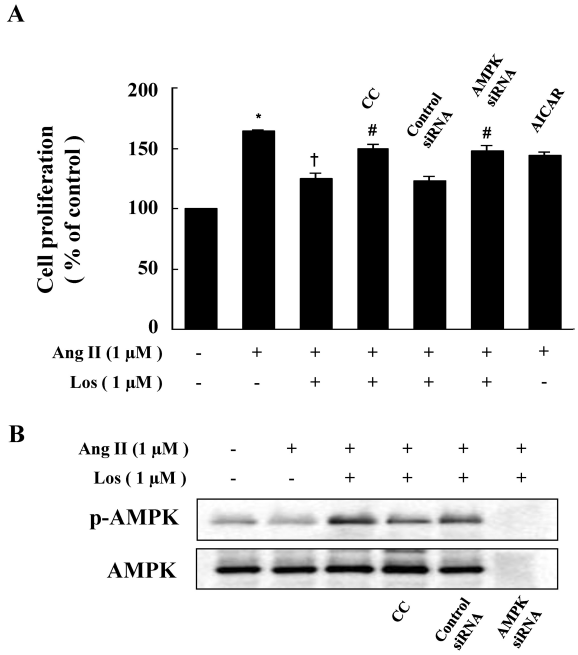

Fig. 2. Inhibitory Action of Compound C and AMPK siRNA on the Anti-Proliferative Effect of Losartan. (A) Cells were pretreated with compound C (CC) (1 μM) or transfected with control siRNA or AMPK siRNA in the presence of losartan, and then stimulated with Ang II for 48 hrs. Cell proliferation was determined by the MTT assay. Data are represented as the mean±S.E.M (n=4). *$p<0.05$ compared with control, $^{\dagger}p<0.05$ compared with Ang II, $^{\#}p<0.05$ compared with Ang II+losartan. (B) Cells were pretreated with CC (1 μM) or transfected with AMPK siRNA in the presence of losartan, and then stimulated with Ang II for 48 hrs. Protein expression of p-AMPK was determined by western blot analysis. Representative blots from three independent experiments are shown.

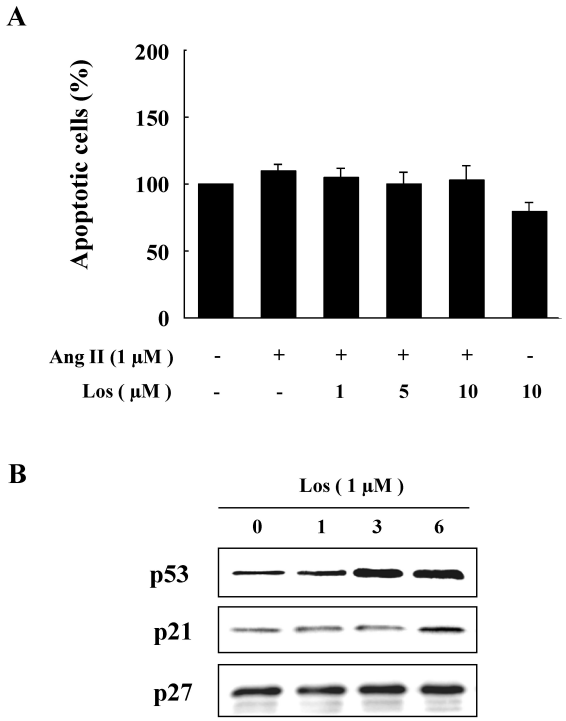

Fig. 3. Losartan-induced Expressions of p53 and p21. (A) Cells were treated with Ang II in the presence or absence of losartan for 48 hrs. Apoptosis was assessed by Annexin V-fluorescein isothiocyanate (FITC) staining by flow cytometric analysis and the percentage of apoptotic cells was then determined. (B) Cells were treated with losartan for the indicated periods of time. Protein expressions of p53, p21, and p27 were determined by western blot analysis. Representative blots from three independent experiments are shown.

cycle regulation, such as cyclin D and cyclin E were also inhibited by losartan. These effects of losartan were restored by compound C or AMPK siRNA (Fig. 4A). We further examined the effect of AMPK on cell cycle progression using flow cytometric analysis with PI staining. Compared with Ang II-treated cells, losartan significantly increased the number of cells in the G_0/G_1 phase. Pretreatment with compound C and AMPK siRNA blocked the inhibitory effect of losartan on cell cycle progression. These results indicate that losartan blocks VSMC proliferation via AMPK by increasing G_0/G_1 arrest and decreasing G_2/M phase (Fig. 4B~4D).

DISCUSSION

The renin-angiotensin-aldosterone system (RAAS), an important regulator of blood pressure as well as fluid and electrolyte balance, plays an important role in the pathophysiology of cardiovascular diseases. Blockade of RAAS with angiotensin-converting enzyme inhibitors and ARBs lowers blood pressure and decreases morbidity and mortality in patients with chronic heart failure [20]. A direct approach to blocking this system is to antagonize Ang II at the level of its receptor. Metabolically stable and orally effective ARBs are therefore therapeutically desirable, losartan was the first discovered and approved ARBs.

Recent studies have suggested that Ang II impairs the action of insulin [21,22] and inhibition of Ang II could improve insulin sensitivity. ARBs ameliorates insulin resistance and hyperleptinemia in sucrose-fed, spontaneously hypertensive rats [23]. Similarly, irbesartan was reported to improve insulin sensitivity in obese Zucker rats with severe insulin resistance [24]. The effects of ARBs on glucose metabolism have been shown in several studies on animals

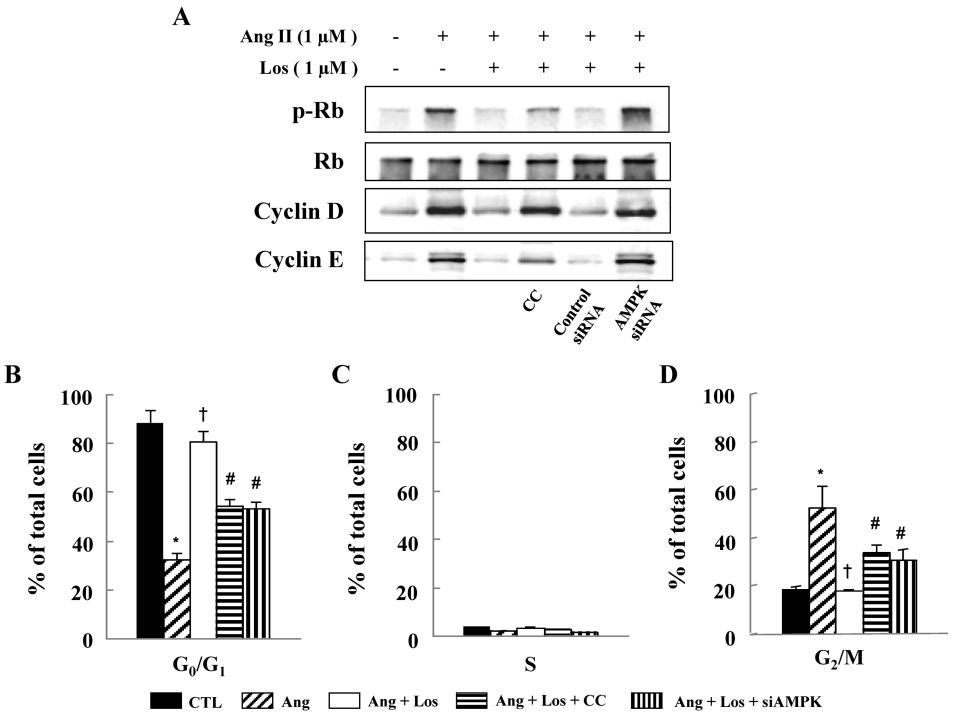

Fig. 4. Losartan Inhibits Cell Cycle Progression via AMPK activation. Cells were pretreated with compound C (CC) (1 μM) or transfected with control siRNA or AMPK siRNA in the presence of losartan, and then stimulated with Ang II for 24 hrs. (A) Protein expressions of p-Rb, cyclin D, and cyclin E were determined by western blot analysis. Representative blots from three independent experiments are shown. Cell cycle analysis was assessed by PI staining and the percentage of cells in G_0/G_1 phase (B), S phase (C), and G_2/M phase (D). Data are represented as the mean± S.E.M (n=4). *$p<0.05$ compared with control, $^{\dagger}p<0.05$ compared with Ang II, $^{\#}p<0.05$ compared with Ang II+losartan.

and cell lines, for example, telmisartan administration increases the uptake of glucose and GLUT4 protein expression [25]. In addition, blockade of the renin-angiotensin system by ARBs increased the plasma adiponectin level [26]. These findings indicate that the ARBs can induce AMPK activation, because ARBs elicited improvement of metabolic function.

In the present study, we first demonstrate that losartan activates AMPK by phosphorylating AMPK at Thr172 in VSMC (Fig. 1). The extent of AMPK phosphorylation at Thr172 strongly reflects its activity [27]. Losartan also increased phosphorylation of ACC, a downstream substrate of AMPK, at Ser79. AMPK activation mediates the beneficial effects of metformin [28], adiponectin [29], and statin [30]. AMPK is a serine/threonine protein kinase, that serves as an energy sensor in eukaryotic cells. Several studies revealed that AMPK activation strongly suppressed cell proliferation in normal cells as well as in tumor cells [31]. These effects of AMPK are mediated through multiple mechanisms including cell cycle regulation and inhibition of protein synthesis. AMPK activation by AICAR or constitutively activated AMPK induces a G_0/G_1 cell cycle arrest via AMPK-dependent Ser15 phosphorylation of p53 in human VSMCs [32].

In this study, we found that inhibition of Ang II-induced VSMC proliferation by losartan was mediated by AMPK activation and AT_1 receptor blockade. Losartan decreased Ang II-induced VSMCs proliferation (from 163.8±0.9% to 125.1± 3.9%, net inhibition 38.7%). The effect of losartan could be divided to two parts, growth inhibition by AMPK activation was estimated as about 24% (calculated from AMPK inhibition with Compound C and AMPK siRNA) and growth inhibition by AT_1 receptor blockade was estimated as about 15%. Compound C and AMPK siRNA restored the losartan-induced inhibition of VSMC proliferation (from 125.1±3.9% to 149.7±3.5% and 147.5±5.1%, respectively). For a growth inhibition by AMPK activation, effect of AICAR, a well-known activator of AMPK, was compared. AICAR inhibited Ang II-induced VSMC proliferation (from 163.8±0.9% to 145.1±3.1%) (Fig. 2A).

Losartan increased p21 through the up-regulation of p53. Losartan suppressed Ang II-induced Rb phosphorylation, as well as cyclin D and cyclin E expression which are required for cell cycle progression. Compound C and AMPK siRNA reversed the losartan-induced suppression of Rb phosphorylation, cyclin D, and cyclin E. The mechanism of growth suppression by losartan is therefore G_0/G_1 cell cycle arrest which is reversed by AMPK inhibition, such as compound C or AMPK siRNA, but not by apoptosis (Fig. 4). These findings suggest that the anti-proliferative effect of losartan might be in part via an AMPK-p53-p21 signaling pathway.

In conclusion, losartan-induced AMPK activation inhibited the Ang II-induced VSMC proliferation through cell cycle arrest. Our observations also indicate that AMPK activation could be a novel target for the prevention of vascular proliferative disorders such as atherosclerosis.

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