Biomedical Research 2015; 26 (4): 744-749 *ISSN 0970-938X*
www.biomedres.info

Pentoxifylline inhibits angiotensin II-induced proliferation in rat vascular smooth muscle cells.

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Abstract

We tested the ability of the drug pentoxifylline to inhibit the activity of the vasoactive hormone angiotensin II in vascular smooth muscle cells. Cell proliferation, intracellular cAMP, and expression of cell cycle proteins were measured in cells isolated from male Sprague-Dawley rats. Angiotensin II significantly induced proliferation in VSMCs ($p < 0.01$). Pentoxifylline significantly blocked this induction in a dose-dependent manner ($p < 0.05$), and a dose of 0.5 mM was sufficient to completely reverse the effect of angiotensin II. Angiotensin II reduced production of cAMP from 34.62 ± 0.59 pmol/mg protein in untreated cells to 17.49 ± 3.30 pmol/mg protein ($p < 0.05$). cAMP production was restored to 40.68 ± 0.49 and 41.50 ± 1.78 pmol/mg protein in the presence of 1.0 and 2.0 mM pentoxifylline, respectively. In addition, the same treatments increased cAMP production in untreated cells to 54.82 ± 4.40 and 67.68 ± 4.29 pmol/mg protein, respectively ($p < 0.05$). Angiotensin II significantly upregulated ($p < 0.05$) cyclin D1 mRNA 2-fold, but not cyclin E, cyclin A, CDK2, CDK4, or P27. Pretreatment with pentoxifylline prevented this effect. Similarly, the drug blocked the ability of angiotensin II to increase the abundance cyclin D1 protein. *Conclusion:* Pentoxifylline attenuated angiotensin II-induced proliferation by stimulating cAMP production and partially regulating the cell cycle. However, studies are required to investigate the effects of the drug on the hypertensive vessel wall.

Keywords: Pentoxifylline, Angiotensin II, vascular smooth muscle, cell proliferation, cAMP,

Accepted August 07 2015

Introduction

The renin-angiotensin system is a pathologic in vascular injury [1,2]. In this system, the vasoactive hormone angiotensin II constricts and enhances resistance in blood vessels to increase blood pressure [3]. In addition, the hormone stimulates DNA synthesis, cell division, and proliferation in vascular smooth muscle cells (VSMCs) [4], which maintain vascular integrity and tone. Under pathologic conditions, these cells also remodel arteries [5,6], especially when stimulated by mitogens such as angiotensin II, platelet-derived growth factor, and transforming growth factor-β1 [7]. While vascular injury is complex and attributable to many factors such as cell migration, growth, apoptosis, calcification, and inflammation [7,8], the activities of angiotensin II indicate that it is a major catalyst of hypertensive vascular damage due to tissue remodeling [9].

Pentoxifylline, a non-selective inhibitor of cyclic-3',5'-phosphodiesterase, is used to treat peripheral vascular disease [10]. The drug inhibits cell proliferation, inflammation, and fibrosis [11,12] via the cAMP pathway [13]. Although cAMP is well known to modulate vascular proliferation, it is unclear whether pentoxifylline could block angiotensin II-induced proliferation in VSMCs, or whether its anti-proliferative activity is linked to cell cycle proteins. Therefore, we analyzed the effect of the drug on proliferation and the cell cycle in angiotensin II-stimulated cells.

Materials and Methods

Cell culture

VSMCs were isolated from the thoracic aorta of male Sprague-Dawley rats (weight, 180–200 g). Cells were grown to subconfluence at 37 °C in a humidified atmosphere of 95% air and 5% CO_2 in Dulbecco's modified Eagle's media supplemented with 10% fetal

bovine serum, 25 mM HEPES pH 7.4, 100 U/mL penicillin, and 100 µg/mL streptomycin. After 4–8 passages, cells were cultured for 24 h in 0.1% fetal bovine serum prior to experiments, in which cells were cultured in 5.5 mM normal glucose, pretreated for 30 min with pentoxifylline, and stimulated with 1 µM angiotensin II (Sigma-Aldrich, St. Louis, USA). Cells were harvested 24 h thereafter for analysis of mRNA and protein expression. Data were collected from triplicate experiments.

Proliferation assay

Cells were seeded in 96-well plates at 1×10^5 cells/well, and grown to confluence, at which point the medium was replaced by fresh serum-free medium. Cultures were then incubated at 37 °C for 4 h with 100 µL 0.5 mg/mL 3-(4, 5-di-methylthizol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT), washed with phosphate-buffered saline, lysed with 100 µL dimethyl sulfoxide, and shaken for 10 min. Finally, absorbance was measured at 570 nm using an automatic microplate reader to determine the amount of MTT reduced to formazan (Molecular Devices, Sunnyvale, CA)

cAMP assay

Media were removed from cultures, and cells were incubated for 20 min at room temperature in 500 µL cold 0.1 N HCl. Cells were then scraped and pelleted by centrifugation at 1000 ×*g* for 10 min. Extracts were acetylated, and cAMP content was measured by an enzyme immunoassay kit (Cayman Chemical Company, Ann Arbor, USA), following the manufacturer's instructions. cAMP levels were normalized to protein concentrations, which were determined using Advanced Protein Assay Reagent (Bio-Rad, Hercules, USA).

Real time reverse transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted using TRIzol and was reverse-transcribed using a cDNA synthesis kit (Fermentas, Burlington, Canada) as previously described [14]. Gene expression was measured by real time RT-PCR using the SYBR Green Master mix and standard three-step cycles. Data were normalized to those of the housekeeping gene GAPDH. Primers were designed using Primer 3 software and had the following sequences: cyclin A1, sense 5' CAGTACTTAAGGCGGCAAGG 3' and anti-sense 5' TAAGCTGCTGCTGCTACCAA 3'; cyclin D1, sense 5' AGGGGATTCAGGACGACTCT 3' and anti-sense 5' GGGCAACCTTCCCAATAAAT 3'; cyclin E1, sense 5' ATGTCCAAGTGGCCTACGTC 3' and anti-sense 5' GCGAGGACACCATAAGGAAA 3'; P27, sense 5' CAGAATCATAAGCCCCTGGA 3' and anti-sense 5' TCTGACGAGTCAGGCATTTG 3'; cyclin-dependent kinase 4 (CDK4), sense 5' GAAGACGACTGGCCTCGAGA 3' and anti-sense 5' ACTGCGCTCCAGATTCCTCC 3'; CDK2, sense 5' TGACCAACTCTTCCGGATCT 3' and anti-sense 5' ATAACAAGCTCCGTCCGTCT 3'; and GAPDH, sense 5' TGCACCACCAACTGCTTAGC 3' and anti-sense 5' GGCATGGACTGTGGTCATGAG 3'.

Western blot

Cells were lysed in buffer containing 150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, and 1 mM phenylmethylsulfonylfluoride, and total protein concentrations in extracts were determined with Advanced Protein Assay Reagent (Bio-Rad, Hercules, USA). Samples containing 40 µg protein were separated on 6 or 10% SDS-polyacrylamide gels under denaturing conditions and blotted onto polyvinylidene difluoride (Immobilon-P, Millipore, USA) for 120 min at 250 mA. Blots were blocked for 1 h at room temperature with 0.15% Tween-20 and 5% non-fat milk in phosphate-buffered saline, and probed overnight at 4 °C with monoclonal antibodies against rat cyclin D (Cell Signaling, USA, 1:500), and β-actin (Santa-Cruz, USA, 1:3000). Membranes were then washed 4 times with Tween-20 in phosphate-buffered saline, and labeled for 30 min at room temperature with a 1:3000 dilution of horseradish peroxidase-conjugated secondary antibody. Blots were visualized by the ECL method (Amersham, Buckinghamshire, UK).

Statistical analysis

Data were analyzed in the statistical program SPSS 15.0 using non-parametric Mann-Whitney Room corporate law. $p < 0.05$ was considered statistically significant.

Results

Anti-proliferative activity of pentoxifylline

Angiotensin II significantly induced ($p < 0.0001$) proliferation in VSMCs, as measured by the amount of MTT reduced to formazan, the formation of which is quantified by absorbance at 570 nm (Figure 1). The absorbance at 570 nm was 0.29 ± 0.02 in untreated cells, and 0.40 ± 0.05 in hormone-stimulated cells. Pentoxifylline significantly blocked ($p < 0.05$) this induction in a dose-dependent manner, and a dose of 0.5 mM was sufficient to completely reverse the effect of angiotensin II, and reduce the absorbance to 0.29 ± 0.03 (Figure 1).

Effect of pentoxifylline on intracellular cAMP

Angiotensin II reduced production of cAMP from 34.62 ± 0.59 pmol/mg protein in untreated cells to 17.49 ± 3.30 pmol/mg protein ($p < 0.05$). cAMP production was restored to 40.68 ± 0.49 and 41.50 ± 1.78 pmol/mg protein in the presence of 1.0 and 2.0 mM pentoxifylline, respectively. In addition, the same treatments increased .

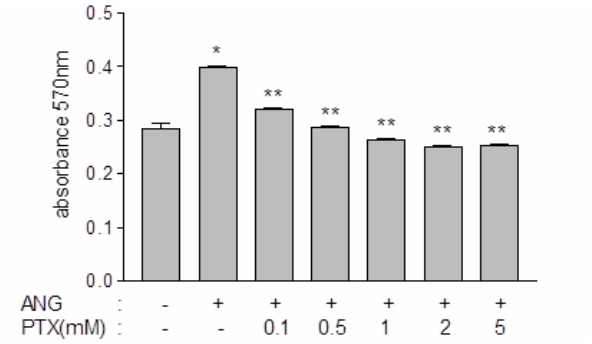

Figure 1. *Effect of pentoxifylline on VSMCs proliferation stimulated by angiotensin II. Pentoxifylline inhibits, in a dose-dependent manner, the proliferation of vascular smooth muscle cells stimulated with 1 μM angiotensin II. Data are mean ± S.E. *, p < 0.05 vs. control; **, p < 0.05 vs. angiotensin II.*

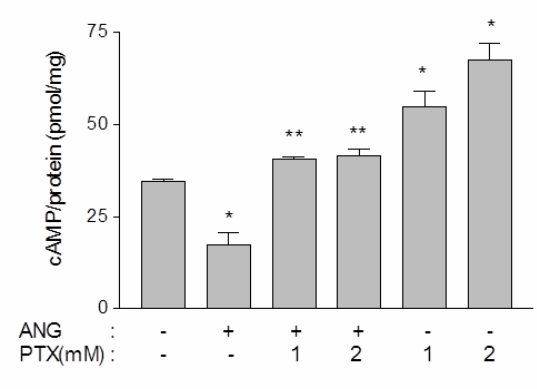

Figure 2. *Effect of pentoxifylline on production of cAMP in vascular smooth muscle cells exposed to 1 μM angiotensin II. Data are mean ± S.E. *, p < 0.05 vs. control; **, p < 0.05 vs. angiotensin II.*

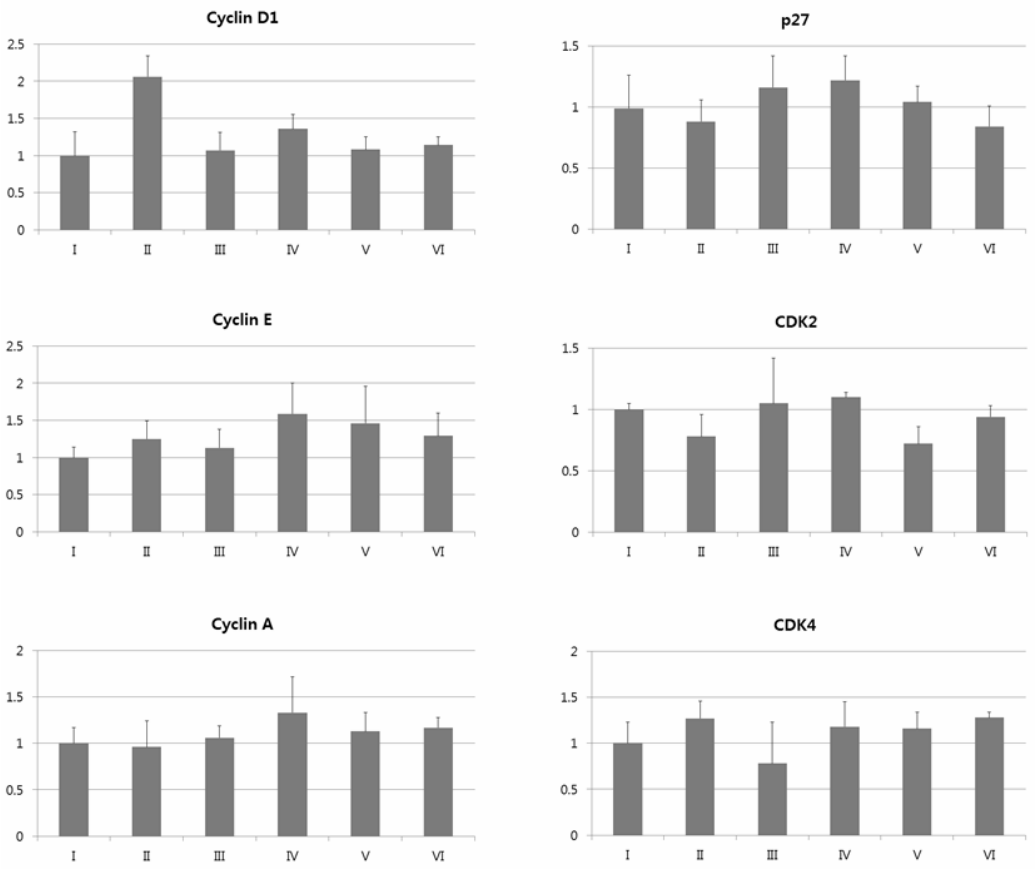

Figure 3. *The mRNA expression of cell cycle related proteins by RT-PCR. Angiotensin II upregulated cyclin D1 mRNA 2-fold, but not cyclin E, cyclin A, CDK2, CDK4, and P27. Pretreatment with 0.1–2.0 mM pentoxifylline blocked this effect. I, control; II, angiotensin II; III-VI, 0.1 mM (III), 0.5 mM (IV), 1.0 mM (V), and 2.0 mM (VI) pentoxifylline. Data are mean ± S.E. *, p < 0.05 vs. control; **, p < 0.05 vs. angiotensin II.*

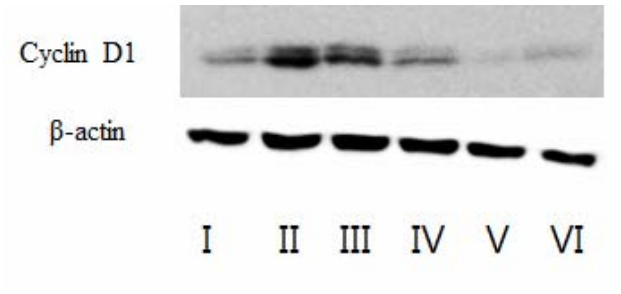

Figure 4. *Cyclin D1 protein expression by Western blotting. Angiotensin II (ANG) significantly stimulated protein expression of cyclin D1, an effect reversed by pentoxifylline (PTX) in a dose-dependent manner. I, control; II, angiotensin II; III-VI, 0.1 mM (III), 0.5 mM (IV), 1.0 mM (V), and 2.0 mM (VI) pentoxifylline.*

cAMP production in untreated cells to 54.82 ± 4.40 and 67.68 ± 4.29 pmol/mg protein, respectively ($p < 0.05$) (Figure 2).

Cell cycle regulation by pentoxifylline

Angiotensin II significantly upregulated ($p < 0.05$) cyclin D1 mRNA 2-fold, but not cyclin E, cyclin A, CDK2, CDK4, or P27 (Figure 3). Pretreatment with pentoxifylline prevented this effect. Similarly, the drug blocked the ability of angiotensin II to increase the abundance cyclin D1 protein (Figure 4).

Discussion

Accelerated proliferation in vascular smooth muscle cells is an important factor in the development of hypertension [15]. The vasoactive hormone angiotensin II elicits such acceleration via several mechanisms. For instance, the mitogenic factors platelet-derived growth factor and transforming growth factor-β1 enhance angiotensin II activity [16,17]. The hormone also induces production of mitochondrial reactive oxygen species, which are associated with hypertension and atherosclerosis [18]. Moreover, angiotensin II stimulates NADPH oxidase [19], which contributes to cell growth and inflammation in atherosclerosis [20]. Finally, the hormone inhibits phosphate and tensin homolog to promote cell proliferation and migration [21] and upregulates extracellular signal-regulated kinase in response to high amounts of sodium [22].

In contrast, intracellular cAMP maintains vascular smooth muscle cells in the quiescent state under physiological conditions and facilitates injury repair by inhibiting proliferation [13,23,24]. Thus, a specific inhibitor of PDE 3 and 4 induces vascular relaxation and prevents proliferation by increasing cAMP [25,26]. Accordingly, we hypothesized that pentoxifylline, a drug that also increases intracellular cAMP, may block the ability of angiotensin II to stimulate vascular cell proliferation. Indeed, our data demonstrate that pentoxifylline attenuates proliferation in hormone-stimulated vascular smooth muscle cells via cAMP production. This finding is consistent with previous results [27,28].

In particular, cAMP blocks cell cycle progression by inhibiting cyclin D, and c-myc [23,29]. Cyclin D1 regulates progression from the G1 to S phase [30,31]. In contrast, angiotensin II stimulates cyclin D1 and cyclin E and inhibits p27 [32,33], ultimately inducing proliferation. On the other hand, studies have demonstrated that pentoxifylline prevents platelet-derived growth factor from stimulating cyclin D1 expression in mesangial cells and airway smooth muscle cells [11,34]. In line with these data, we found pentoxifylline to attenuate expression of cyclin D1 after exposure to angiotensin II, but not other cell cycle proteins.

In summary, pentoxifylline attenuates proliferation in angiotensin II-stimulated vascular smooth muscle cells by increasing cAMP and partially regulating the cell cycle. Similarly, the drug has been reported to inhibit proliferation in other cells, including astrocytes, fibroblasts, hepatic stellate cells, and mesangial cells [35-38]. *In vivo*, the drug inhibits vascular proliferation in models of carotid arterial anastomosis model and angioplasty [39,40]. However, further studies are required to define pentoxifylline activity in the hypertensive vessel wall. Nevertheless, the data imply that administration of pentoxifylline may be an alternative strategy to prevent vascular proliferation and constriction in pathological conditions.

Declaration of interest: *The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.*

Acknowledgement: This work was supported by the Postdoctoral Research Program of Inje University 2007.

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