

*Full Length Research Paper*

# Suppression of vascular smooth muscle cells' proliferation and migration by valsartan via MAPK pathway

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**This study aimed to determine the effects of valsartan on the proliferation and migration of isolated rat vascular smooth muscle cells (VSMCs) and the expression of phospho-p42/44 mitogen-activated protein kinase (MAPK) promoted by angiotensin II (Ang II). VSMCs from the rat thoracic aorta were cultured by attachment-block culture. VSMCs' proliferation and migration were measured by MTT assay and migration chamber system, respectively. Phospho-p42/44 MAPK expression was determined by western blot. Our results reveal that valsartan inhibits Ang II-induced VSMCs' proliferation and migration via suppression of Ang II-induced phospho-P42/44 MAPK expression.**

**Key words:** Vascular smooth muscle, valsartan, angiotensin II, mitogen-activated protein kinase.

## INTRODUCTION

Percutaneous coronary intervention (PCI) and stenting has become one of the major therapies for coronary heart disease (CHD). In-stent restenosis (ISR) is a potential complication following PCI, and often leads to fatal consequences if not promptly recognized and treated (Haude et al., 1993; Mehran et al., 1999). The pathogenesis of ISR is complex, but proliferation and migration of vascular smooth muscle cells (VSMCs) in the intima seem to play a critical role. VSMCs may form the bulk of the neointimal hyperplasia tissue by inducing neointimal hyperplasia and extracellular matrix formation (Mitra and Agrawal, 2006). The stimulation of angiotensin II type 1 receptor (AT1R) is closely related to ISR (Langeveld et al., 2005), and phospho-p42/44 mitogen-activated protein kinase (MAPK) may be a critical regulatory factor for Ang II-mediated proliferation and migration in VSMCs (Xi et al., 1999).

Some recent clinical trials have shown that selective AT1R blocker and valsartan, may be effective in preventing ISR in patient receiving PCI with complex coronary lesions (Ribichini et al., 2005; Peters et al.,

2001, 2005), but the signaling pathways by which valsartan preventing ISR are unclear. Therefore, the aims of the study were to investigate the effects of valsartan on the proliferation and migration of isolated rat VSMCs, and also the effects of valsartan on the expression of phospho-p42/44 MAPK.

## MATERIALS AND METHODS

### Animal and reagents

This study was approved by the institutional review board of the Beijing Friendship Hospital. Healthy male Wistar rats (weight 200 ± 20 g) were purchased from the Chinese Academy of Medical Sciences, Beijing. Valsartan was purchased from Beijing Second Pharmaceutical Co. Ltd. (Beijing, China). Ang II and PD98059 were purchased from Sigma Chemical Co. Ltd. (St. Louis, USA). Phospho-p42/44 MAP kinase antibody was purchased from Cell Signaling Technology Co. Ltd (Danvers, USA).

### Cell culture of VSMCs

VSMCs from the thoracic aorta of the animals were cultured by attachment-block culture. Before the culture, the external connective tissues, the adventitial layer and the endothelium were removed from the aorta. The remaining tissues were cut into 1×1 mm<sup>2</sup> sections, which were attached to the bottom of the culture

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**Table 1.** Effect of Ang II on phospho-p42/44 MAPK expression.

Time (min)	Control	Ang II ( $10^{-7}$ mmol/L)	P
5	0.10 ± 0.05	0.43 ± 0.03	<0.01
15	0.12 ± 0.05	0.35 ± 0.04	<0.01
30	0.19 ± 0.05	0.25 ± 0.04	<0.01

flask. The cells were cultured in Dulbecco's modified eagle medium (DMEM) containing 20% fetal bovine serum and incubated at 37°C in an incubator containing 5% CO<sub>2</sub>. When cells covered over 60% of the bottom, they were digested in 0.25% trypsin for passage. The passaged VSMCs were maintained in DMEM medium containing 10% fetal bovine serum. The identity of the VSMCs was assessed by immunostaining with an antibody against  $\alpha$ -smooth muscle actin. The VSMCs of 5th to 10th passages were used for the experiments.

#### MTT DMEM assay

For proliferation assays, VSMCs were plated at  $5 \times 10^4$  cells/ml in 96-well plates, 100  $\mu$ L per well. Cells growth was arrested by incubating the cells in DMEM medium containing 0.5% fetal bovine serum for 24 h. Subsequently, the respective testing drugs were added to the culture medium, which was further cultured in DMEM medium containing 1% fetal bovine serum for 24 h. 20 h into this culture, 0.5% MTT (20  $\mu$ L per well) was added to the 96-well plates, with continued incubation for an additional 4 h. The culture medium was removed and dimethyl sulfoxide (DMSO) was added to the 96-well plates (150  $\mu$ L per well). The optical density (OD) value was evaluated with enzyme mark instrument (Bio-Rad, CA, USA). Five different concentrations of Ang II ( $10^{-10}$  to  $10^{-6}$  mmol/L) were used to stimulate quiescent VSMCs for 24 h (Table 1). When valsartan or PD98059 were used, they were applied 30 min prior to the addition of Ang II. Experiments were repeated at least three times.

#### Migration chamber system

VSMCs growth was arrested by incubating the cells in DMEM medium containing 0.5% fetal bovine serum for 24 h. The culture media was then changed to DMEM medium containing 1% fetal bovine serum. Cells were subsequently plated at  $1 \times 10^5$  cells/ml, 1 ml per upper chamber. Then, 3.5 ml DMEM medium containing 10% fetal bovine serum was added to lower chamber. After a 24-h migration period, transwell chambers were fixed by methyl alcohol and stained by hematoxylin, and then the non-migrating cells were removed with a cotton swab from the upper surface. The number of cells that had migrated to the lower surface of the membrane was determined per  $\times 200$  high power field. 10 randomly chosen HPFs were counted per membrane. When valsartan or PD98059 were used, they were applied 30 min prior to the addition of Ang II. Experiments were repeated at least three times.

#### Western blot analysis

Western blot analysis was carried out following standard procedure. Briefly, equal amounts of proteins from different groups were separated on 9% sodium dodecyl sulfonate-polyacrylamide gel electrophoresis (SDS-PAGE) and electro-transferred to polyvinylidene difluoride (PVDF) membrane. Non-specific binding sites were blocked in PBS containing 5% nonfat milk. The blots were incubated overnight at 4°C with primary antibodies and washed three times with polybutylene succinate-co-butylene terephthalate

(PBST) before probing with horseradish peroxidase-conjugated secondary antibodies for 2 h at room temperature. The blots were then visualized with enhanced chemiluminescence (ECL) according to the manufacture's instruction. The density of bands was scanned and quantified by GEL-DOC 2000 UV gel imaging system. When valsartan or PD98059 were used, they were applied 30 min prior to the addition of Ang II. Experiments were repeated at least three times.

#### Statistical analysis

All data were analyzed by SPSS 11.5 software. Descriptive data were expressed as mean  $\pm$  standard deviation (SD). Analysis of variance (ANOVA) was used in comparison between the groups. Statistic significance was set at a level of  $P < 0.05$ .

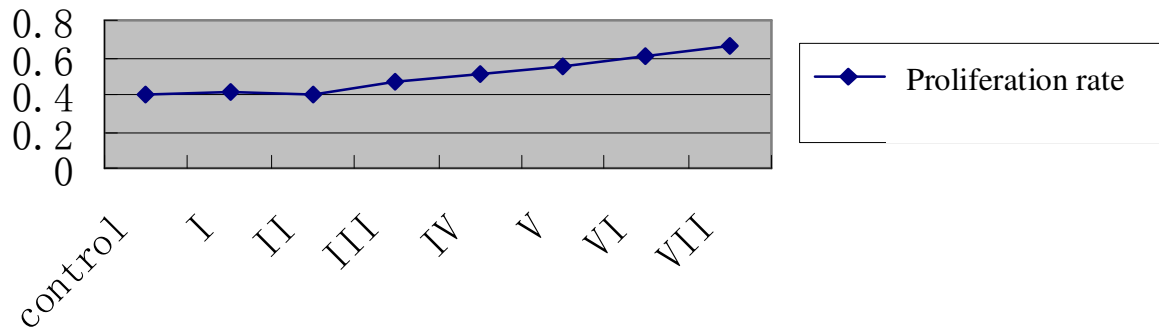
## RESULTS

### Effect of valsartan and PD98059's on VSMCs proliferation induced by Ang II

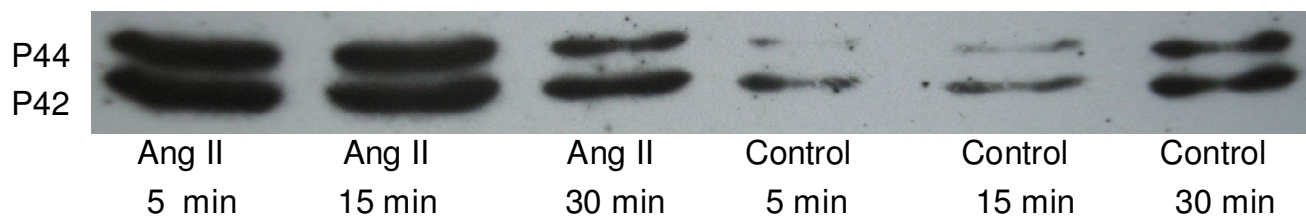
The VSMCs proliferation rate in the control group was  $0.42 \pm 0.03$  mM. The cell proliferation rate in the five Ang II groups ( $10^{-10}$ ,  $10^{-9}$ ,  $10^{-8}$ ,  $10^{-7}$  and  $10^{-6}$  mM) was  $0.46 \pm 0.03$ ,  $0.46 \pm 0.04$ ,  $0.48 \pm 0.02$  ( $P < 0.05$ ),  $0.54 \pm 0.05$  ( $P < 0.01$ ), and  $0.59 \pm 0.06$  ( $P < 0.01$ ), respectively. The cell proliferation rate in the Ang II  $10^{-6}$  group was also greater than in the  $10^{-8}$  and  $10^{-7}$  mM groups ( $P < 0.01$ ). Figure 1 shows the effect of valsartan on the Ang II ( $10^{-7}$  mM) induced VSMCs proliferation rate. Compared with the Ang II ( $0.66 \pm 0.03$ ) group only, the groups pretreated with valsartan showed poor cell proliferation rates. The cell proliferation rates in five different valsartan ( $10^{-3}$ ,  $10^{-4}$ ,  $10^{-5}$ ,  $10^{-6}$  and  $10^{-7}$ ) + Ang II groups were  $0.40 \pm 0.02$ ,  $0.47 \pm 0.03$ ,  $0.51 \pm 0.03$ ,  $0.55 \pm 0.22$  and  $0.61 \pm 0.05$ , respectively. These entire proliferation rate were significantly different with Ang II stimulated group alone ( $P < 0.05$  or  $0.01$ ). Moreover, the proliferation rate in the cells pretreated with PD98059 ( $10^{-5}$  mM) and Ang II ( $10^{-7}$  mM) was  $0.45 \pm 0.05$ , which was less than in the cells treated with Ang II ( $10^{-7}$  mM) only ( $0.61 \pm 0.06$ ,  $P < 0.01$ ).

### Effect of valsartan and PD98059's on Ang II induced VSMCs migration

Ang II ( $10^{-7}$  mM) was used to stimulate quiescent VSMCs for 24 h, and migration chamber system was used to evaluate VSMC migration. The cell migration of the Ang II alone and the control group was  $57.2 \pm 9.51$  (mM) and



**Figure 1.** Effect of valsartan on Ang II induced VSMCs proliferation. Group I, [Val( $10^{-5}$ )]; II, Val( $10^{-3}$ ); III, Val( $10^{-4}$ ) + Ang II( $10^{-7}$ ); IV, Val( $10^{-5}$ ) + Ang II( $10^{-7}$ ); V, Val( $10^{-6}$ ) + Ang II( $10^{-7}$ ); VI, Val( $10^{-7}$ ) + Ang II( $10^{-7}$ ); VII, Ang II( $10^{-7}$ ).



**Figure 2.** Representative autoradiogram showing the expression of phospho-p42/44 MAPK at different time points following Ang II stimulation.

11.6 ± 2.0 (mM), respectively ( $P < 0.01$ ). The migration of cells treated with valsartan alone was  $10.7 \pm 1.57$  ( $P > 0.05$  compared with the control group). The migration in the groups treated with both valsartan ( $10^{-5}$  and  $10^{-4}$  mmol/L) and Ang II ( $10^{-7}$  mM) was  $23.4 \pm 1.71$  and  $32.3 \pm 4.24$  mM, respectively ( $P < 0.01$  compared with Ang II alone group). The cell migration in the group treated with both PD98059 ( $10^{-5}$  mmol/L) and Ang II ( $10^{-7}$  mM) was  $27.5 \pm 2.07$ , which was lower than with the Ang II alone group ( $P < 0.01$ ).

#### Effects of valsartan and PD98059's on Ang II induced phospho-p42/44 MAPK expression

Phospho-p42/44 MAPK expression of VSMCs was evaluated in three different times following the addition of Ang II ( $10^{-7}$  mM) for 5, 15 and 30 min (Figure 2). As shown in Table 1, the greatest MAPK expression was found for 5 min following the addition of Ang II. Therefore, this time point was chosen for the following studies on valsartan and PD98059.

The phospho-p42/44 MAPK expression in cells treated with PD98059 ( $10^{-5}$  mM) + Ang II ( $10^{-7}$  mM), or valsartan ( $10^{-5}$  mM) + Ang II ( $10^{-7}$  mM), was  $0.45 \pm 0.03$  and  $0.43 \pm 0.04$  mmol/L, respectively. Both were significantly lower than in the Ang II alone group ( $0.52 \pm 0.03$ ,  $P < 0.01$ ), but higher than that in the control ( $0.38 \pm 0.04$ ,  $P < 0.05$ ) or the valsartan alone group ( $0.37 \pm 0.03$ ,  $P < 0.01$ ).

#### DISCUSSION

ISR is an excessive response to vascular injury by stent implantation. Stent implantation causing the denudation of the endothelium initiates blood clotting, promotes inflammatory response, releases various kinds of enzymes, growth factors and cell factors, and finally results in neointimal hyperplasia (Mitra and Agrawal, 2006). Renin-angiotensin-aldosterone system (RAAS) plays a fundamental role in maintaining vascular function. Ang II is the important production of RAAS. Ang II has two selective receptors AT1R and AT2R, which play the adverse role in VSMCs' growth and proliferation. Some studies have indicated that the activation of AT1R by Ang II could result in several deleterious effects on the cardiovascular system, namely cell migration and proliferation, extracellular matrix deposition, inflammation, promotion of thrombosis, and production of reactive oxygen species (Langeveld et al., 2005). The blockade of RAAS may decrease the rates of restenosis.

In recent studies, angiotensin converting enzyme inhibitor (ACEI) has been shown to reduce the recurrence of acute myocardial infarction and unstable angina, but could not decrease the rate of ISR (Ribichini et al., 2005) although angiotensin receptor blocker (ARB) might decrease the rate of ISR by selectively blocking AT1R and promoting AT2R's stimulation. The Val-PREST trial in 2001 by Peters et al. (2001) and the VALVACE trial in 2005 by Peters et al. (2005) both have manifested that

valsartan is effective in preventing of ISR. In 2007, Iwata et al. compared valsartan with losartan in the prevention of ISR, and the study also reveals valsartan as effective. The relative experiments are few. In addition, Wang et al. (2006a, b) found that valsartan could reduce the neointimal hyperplasia, and the potential mechanisms might be upregulating AT2R's expression and decreasing collagen deposition. Furthermore, Lin et al. (2005) found that valsartan could antagonize the VSMCs' proliferation induced by Ang II, and could raise the VSMCs' apoptosis by potentially upregulating the expression of the Bax and caspase 3. Similarly, Hu et al. (2002) found that valsartan could antagonize the proliferation and migration of VSMCs induced by Ang II.

In this experiment, we evaluated the VSMCs' proliferation and migration by MTT assay and migration chamber system, respectively. Our results indicate that valsartan could obviously inhibit the VSMCs' proliferation and migration induced by Ang II, which was concurrent with the present studies.

MAPK family is an important extracellular signal transduction pathway, including P42/44 MAPK, c-jun N-terminal kinase and P38 MAPK. The present studies attested to the fact that many kinds of enzymes, cytokines, and growth factors participated in the process of ISR, mainly by enhancing the VSMCs' proliferation and migration, and MAPK might be the common extracellular signal transduction pathway (Yu et al., 2007). In addition, the proliferation and migration of VSMCs induced by Ang II might also be related with upregulating P42/44 MAPK expression (Xi et al., 1999).

Furthermore, we evaluated the effects of PD98059 on VSMCs' proliferation and migration, and found that PD98059 could obviously inhibit the VSMCs' proliferation and migration induced by Ang II.

This result reveals that the proliferation and migration of VSMCs induced by Ang II might be related with upregulating P42/44 MAPK expression. Subsequently, we evaluated the effects of valsartan and PD98059 on Phospho-p42/44 MAPK expression in VSMCs by western blot. We found that Ang II-induced Phospho-p42/44 MAPK expression was maximal in 5 min and the effect could be obviously inhibited by valsartan and PD98059. This result therefore revealed that valsartan could inhibit Ang II-induced Phospho-p42/44 MAPK expression.

In conclusion, our experiment revealed that the mechanism of valsartan in preventing ISR might be related with its inhibiting Ang II-induced VSMCs' proliferation and migration and phospho-P42/44 MAPK expression. This experiment could support the use of valsartan in preventing ISR afterward.

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