Valsartan Decreases Platelet Activity and Arterial Thrombotic Events in Elderly Patients with Hypertension

Fang Wu[1], Hong-Yan Wang[1], Fan Cai[1], Ling-Jie Wang[2], Feng-Ru Zhang[1], Xiao-Nan Chen[1], Qian Yang[3], Meng-Hui Jiang[3], Xue-Feng Wang[4], Wei-Feng Shen[2]

[1]Department of Geriatrics, Ruijin Hospital, Jiao Tong University School of Medicine, Shanghai 200025, China
[2]Department of Cardiology, Ruijin Hospital, Jiao Tong University School of Medicine, Shanghai 200025, China
[3]Institute of Health Sciences, Jiao Tong University School of Medicine and Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, Shanghai 200025, China
[4]Department of Laboratory Medicine, Ruijin Hospital, Jiao Tong University School of Medicine, Shanghai 200025, China

Abstract

Background: Angiotensin type 1 receptor (AT^1R) antagonists are extensively used for blood pressure control in elderly patients with hypertension. This study aimed to investigate the inhibitory effects of AT_1R antagonist valsartan on platelet aggregation and the occurrence of cardio-cerebral thrombotic events in elderly patients with hypertension.

Methods: Two-hundred and ten patients with hypertension and aged > 60 years were randomized to valsartan ($n = 140$) or amlodipine ($n = 70$) on admission. The primary endpoint was platelet aggregation rate (PAR) induced by arachidonic acid at discharge, and the secondary endpoint was the rate of thrombotic events including brain infarction and myocardial infarction during follow-up. Human aortic endothelial cells (HAECs) were stimulated by angiotensin II (Ang II, 100 nmol/L) with or without pretreatment of valsartan (100 nmol/L), and relative expression of cyclooxygenase-2 (COX-2) and thromboxane B_2 (TXB_2) and both p38 mitogen-activated protein kinase (p38MAPK) and nuclear factor-kB (NF-kB) activities were assessed. Statistical analyses were performed by GraphPad Prism 5.0 software (GraphPad Software, Inc., California, USA).

Results: PAR was lower after treatment with valsartan (11.49 ± 0.69% vs. 18.71 ± 2.47%, $P < 0.001$), associated with more reduced plasma levels of COX-2 (76.94 ± 7.07 U/L vs. 116.4 ± 15.89 U/L, $P < 0.001$) and TXB_2 (1667 ± 56.50 pg/ml vs. 2207 ± 180.20 pg/ml) (all $P < 0.001$). Plasma COX-2 and TXB_2 levels correlated significantly with PAR in overall patients ($r = 0.109$, $P < 0.001$). During follow-up (median, 18 months), there was a significantly lower thrombotic event rate in patients treated with valsartan (14.3% vs. 32.8%, $P = 0.002$). Relative expression of COX-2 and secretion of TXB_2 with concordant phosphorylation of p38MAPK and NF-kB were increased in HAECs when stimulated by Ang II (100 nmol/L) but were significantly decreased by valsartan pretreatment (100 nmol/L).

Conclusions: AT_1R antagonist valsartan decreases platelet activity by attenuating COX-2/TXA_2 expression through p38MAPK and NF-kB pathways and reduces the occurrence of cardio-cerebral thrombotic events in elderly patients with hypertension.

Key words: Angiotensin Type 1 Receptor Antagonist; Elderly; Hypertension; Platelet Activity; Thrombosis

Introduction

A large body of evidence has demonstrated that activation of renin-angiotensin system (RAS) plays a key role in the development and progression of hypertension and cardiovascular disease.[1] Angiotensin II (Ang II), the major effector of RAS,[2] exerts its biological effects mainly through specific receptors, namely angiotensin type 1 receptor (AT_1R) and type 2 receptor.[3] Since AT_1R antagonists have been shown to improve endothelial function and vascular remodeling in addition to their predominant vasodilatation effects,[4,5] these agents are extensively used for blood pressure control in elderly patients with hypertension.[6]

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Activation of platelets bears the brunt of complicate cascades of thrombogenesis. Among the physiological activators of platelet, thromboxane A_2 (TXA_2) is the primary one which is conversed from arachidonic acid in the platelets and catalyzed by cyclooxygenase (COX).[7-9] TXA_2 is regulated by two isoforms of COX. COX-1 is usually found in the physical condition, whereas COX-2 is induced by lipopolysaccharide and some cytokines.[10-12] Clinical studies have demonstrated a close relationship between plasma levels of TXA_2 or COX-2 and platelet activities.[13] AT_1R antagonists are known to exhibit potent anti-platelet properties which may differ from other anti-platelet agents.[14] However, the impact of these agents on arterial thrombotic events in elderly patients with hypertension remains largely unclear.

Address for correspondence: Dr. Wei-Feng Shen, Department of Cardiology, Ruijin Hospital, Jiao Tong University School of Medicine, Shanghai 200025, China
E-Mail: rjshenweifeng@126.com

In this randomized trial, we assessed the effect of valsartan-an AT_1R antagonist on platelet aggregation rate (PAR) and the occurrence of cardio-cerebral thrombotic events including brain and myocardial infarction in hypertensive patients aged >60 years. To further elucidate the potential mechanisms of its anti-platelet property, relative expression of COX-2 and TXB_2 and p38 mitogen-activated protein kinase (p38MAPK) and nuclear factor-kB (NF-kB) activities in human aortic endothelial cells (HAECs) stimulated by Ang II with or without pretreatment of valsartan were also determined.

Methods

Study population

Two hundred and thirty-six consecutive elderly (>60 years in age) patients with hypertension who were admitted to the Department of Geriatrics, Ruijin Hospital, Shanghai Jiao Tong University School of Medicine between April 2011 and August 2013 were screened. Hypertension was diagnosed as systolic blood pressure ≥140 mmHg and/or diastolic pressure ≥90 mmHg before recruitment.[15] For the purpose of research, patients with acute or chronic infection, hepatic or at least moderate renal insufficiency (estimated glomerular filtration rate [eGFR] <60 $ml \cdot min^{-1} \cdot 1.73\ m^{-2}$) ($n$ = 17), trauma or surgery within 2 months (n = 2), and other blood diseases (e.g. hemophilia, and leukemia, aplastic anemia) (n = 5) were excluded. We also excluded patients with resistant hypertension (n = 2).[16] The remaining 210 eligible elderly hypertensive patients (191 men and 19 women, mean age 79.2 ± 1.0 years) were randomized to valsartan (Beijing Novartis Pharma Ltd., China) (AT_1R group; n = 140) or amlodipine (Beijing Novartis Pharma Ltd.) (non-AT_1R group; n = 70) in a 2:1 ratio [Figure 1] by random group design. For each group, the initial dose of valsartan (80 mg, once daily) or amlordipine (5 mg, once daily) was titrated to achieve target blood pressure (<140/90 mmHg for patients under 80 years and < 150/90 mmHg for those older than 80 years). A small dose of diuretics like hydrochlorothiazide (Shanghai Xinyi Pharma Ltd., China) (12.5–25 mg, once daily) may be added if maximum daily dose of valsartan (160 mg) or amlodipine (10 mg) was insufficient for optimal blood pressure control. Other medications including β-blockers, anti-platelet agents, and statins were prescribed at the discretion of the physicians.

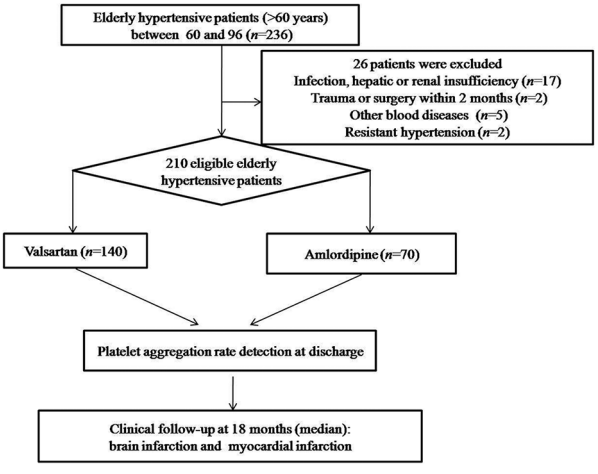

Figure 1: Flowchart of patient enrollment.

The study protocol was approved by the Ethics Review Committees of Shanghai Jiao Tong University and Ruijin Hospital, and informed consent was given by each subject.

Biochemical investigation

Platelets aggregation in platelet-rich plasma was tested at discharge among the patients. Light transmission aggregometry through Chrono-Log platelet aggregometer (Chrono-Log Corp., USA) induced by 0.5 μmol/L arachidonic acid described previously.[17] Plasma levels of COX-2 (MyBiosource, USA) and TXA_2 (Enzo Life Sciences, USA) were determined by ELISA. TXA_2 were represented by its metabolite TXB_2 because it is unstable in common condition.

Follow-up

All patients were followed-up in a special outpatient clinic or by telephone conversation with patients or their relatives every 3 months after discharge. The occurrence of arterial thrombotic events, including brain and myocardial infarctions were recorded. Brain infarction was defined by neurologic examination, head magnetic resonance imaging and/or computed tomography. Myocardial infarction was defined by the presence of typical chest pain, electrocardiographic ST-segment elevation with or without Q waves, and serum cardiac enzyme elevations at least twice the upper limit of the normal range. In order to guarantee rigorous data quality, all thrombotic events were reviewed by two experienced interventional cardiologists.

Cell culture

Human aortic endothelial cells were cultured in Dulbecco's Modified Eagle's Medium (Life Technologies Corporation, USA) supplemented with 10% v/v fetal bovine serum (Life Technologies Corporation) and 1% penicillin-streptomycin (Life Technologies Corporation), and incubated at 37°C in humidified atmosphere containing 5% CO_2. Valsartan (Melonepharina, China), SB203580 (Beyotime, China), JSH-23 (Beyotime) and NS-398 (Sigma, USA) was preadded into the medium 30 min before Ang II treatment.

Real-time polymerase chain reaction

Total RNA prepared with RNAprep Pure Cell/Bacteria Kit (Tiangen biotech, China) was reverse transcribed to cDNA using SuperScript™ Preamplification system (TaKaRa Biotech, China). Prime used in the reaction were as follows: COX-2: 5'-CCCACCCATGTCAAAACCGA-3' (forward), 5'-CCGGGTACAATCGCACTTATACT-3' (reverse); GAPDH: 5'-ATGGGGAAGGTGAAGGTCG-3' (forward), 5'-GGGGTCAT-TGATGGCAACAATA-3' (reverse). Real time-polymerase chain reaction (7900 HT by Applied Biosystems, USA) was performed using SYBR Green

Master Mix (Roche, Switzerland) with the following conditions: 94°C for 5 min, followed by 40 cycles at 94°C for 30 s and 60°C for 30 s. The relative gene expression between treatment and control was calculated using the $2^{-\Delta Ct}$ method with GAPDH as the reference gene.

Western blotting assay

Protein was extracted with RIPA lysis buffer (Beyotime, China), separated with 10% SDS-PAGE gel, and transferred onto nitrocellulose membrane. After blocking with 5% nonfat milk in TBST (tris-buffered saline, Tween 20), the membrane was probed with diluted primary antibodies at 4°C overnight. The membrane was washed 10 min for three times by TBST and then incubated with horseradish peroxidase-conjugated secondary antibody for 2 h at room temperature, washed three times again, and exposed by enhanced chemiluminescence. All antibodies were purchased from Cell Signaling Technology (USA).

Statistical analysis

The primary endpoint was PAR induced by arachidonic acid at discharge, and the secondary endpoint was the rate of thrombotic events including brain infarction and myocardial infarction during follow-up. All statistical analyses were performed by GraphPad Prism 5.0 software (GraphPad Software, Inc., California, USA). Student's *t*-test or one-way analysis of variance (ANOVA) was used for comparison of quantitative data between different groups. Chi-square test was used for comparing qualitative data. Data are expressed as mean ± standard deviation (SD) or frequency. A two-sided $P < 0.05$ was considered to be statistically significant.

Results

Baseline characteristics

Both AT_1R and non-AT_1R groups were well-matched with respect to age, sex, risk factors for coronary artery disease, blood pressure control, hepatic and renal function, and nonantihypertensive medications including β-blockers, anti-platelet agents, and statins [Table 1].

Table 1: Baseline characteristics of elderly hypertensive patients

Variables	AT_1R group (n = 140)	Non-AT_1R group (n = 70)	P
Gender (male/female)	128/12	63/7	0.734
Age (years)	78.59 ± 0.82	79.83 ± 1.17	0.964
Diabetes mellitus (n, %)	49 (35.0)	26 (37.1)	0.760
Coronary artery disease (n, %)	50 (35.7)	26 (37.1)	0.839
Transient ischemic attack (n, %)	38 (27.1)	22 (31.4)	0.420
Systolic blood pressure (mmHg)	130.70 ± 1.33	127.50 ± 1.94	0.617
Diastolic blood pressure (mmHg)	74.45 ± 0.55	73.23 ± 1.12	0.345
White blood cell ($\times 10^9$/L)	5.97 ± 0.15	6.13 ± 0.21	0.616
Platelets ($\times 10^9$/L)	196.40 ± 3.53	203.60 ± 8.51	0.606
Alanine transaminase (IU/L)	22.68 ± 0.92	23.53 ± 2.02	0.927
Aspartate aminotransferase (IU/L)	23.57 ± 0.73	24.86 ± 1.61	0.525
Alkaline phosphatase (IU/L)	56.83 ± 1.74	56.28 ± 1.85	0.986
Urea nitrogen (mmol/L)	6.18 ± 0.25	6.07 ± 0.41	0.759
Creatinine (μmol/L)	88.53 ± 1.95	87.78 ± 3.61	0.608
Uric acid (μmol/L)	384.60 ± 23.41	346.10 ± 10.65	0.189
Triglyceride (mmol/L)	1.49 ± 0.11	1.52 ± 0.17	0.694
Cholesterol (mmol/L)	4.13 ± 0.09	4.18 ± 0.13	0.740
High-density lipoprotein (mmol/L)	1.31 ± 0.03	1.20 ± 0.04	0.260
Low-density lipoprotein (mmol/L)	2.37 ± 0.07	2.52 ± 0.11	0.159
Fasting blood-glucose (mmol/L)	5.18 ± 0.09	5.37 ± 0.17	0.122
β-blockers (%)	16 (11.4)	9 (12.9)	0.763
Anti-platelet (n, %)	130 (92.9)	67 (95.7)	0.418
Statins (n, %)	56 (40.0)	30 (42.9)	0.691

Data are shown as mean ± SD or frequency (%). AT_1R: Angiotensin type 1 receptor; SD: Standard deviation.

Platelets activity and plasma levels of cyclooxygenase-2 and thromboxane B2

Arachidonic acid-induced PAR and plasma levels of COX-2 and TXB_2 were significantly lower in the AT_1R group than in the non-AT_1R group (for all such comparisons, $P < 0.001$) [Table 2]. PAR correlated significantly with COX-2 ($r = 0.109$, $P < 0.001$) and TXB_2 ($r = 0.070$, $P < 0.001$), and COX-2 was closely related to TXB_2 ($r = 0.251$, $P < 0.001$).

Table 2: Platelet aggregation rate and plasma level of COX-2 and TXB_2

Variables	AT_1R group (n = 140)	Non-AT_1R group (n = 70)	P
Platelet aggregation rate (%)	11.49 ± 0.69	18.71 ± 2.47	<0.001
COX-2 (U/L)	75.94 ± 7.07	116.4 ± 15.89	<0.001
TXB_2 (pg/ml)	1667 ± 56.50	2207 ± 180.20	<0.001

Data are shown as mean ± SD. SD: Standard deviation; AT_1R: Angiotensin type 1 receptor; COX-2: Cyclooxygenase-2; TXB_2: Thromboxane B_2.

Thrombotic events

During follow-up (median 18 months), 9 patients in the AT_1R group and 11 in the non-AT_1R group developed brain infarction ($P = 0.031$), and 11 patients in the AT_1R group and 12 in the non-AT_1R group experienced myocardial infarction ($P = 0.042$). This resulted in a significantly lower overall arterial thrombotic event rate in the AT_1R group compared with that in the non-AT_1R group ($P = 0.002$) [Table 3].

Angiotensin II up-regulated expression of cyclooxygenase-2 via phosphorylation of p38 mitogen-activated protein kinase and nuclear factor-kB in human aortic endothelial cells

The expression of COX-2 mRNA in HCACEs was dose-and time-dependently increased after stimulation with Ang II, with maximum expression (4 times higher than control, $P < 0.001$) 1 h after stimulation with Ang II (100 nmol/L) [Figure 2a and 2b]. At protein level, the peak expression of COX-2 occurred 2 hours after Ang II stimulation (100 nmol/L) [Figure 2c]. Ang II also increased the phosphorylation of p38MAPK (p-p38MAPK) and NF-kB (p-NF-kB) [Figure 2d]; when pretreating HAECs

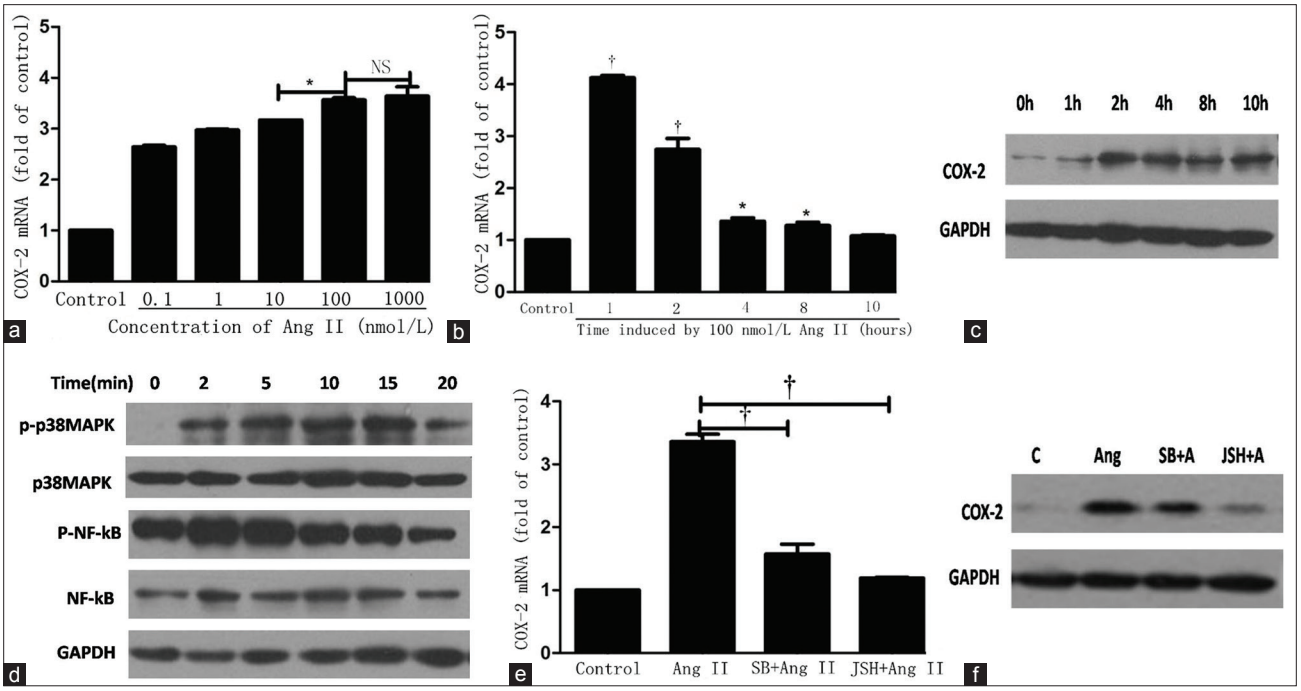

Figure 2: Angiotensin II (Ang II) induced the expression of cyclooxygenase-2 (COX-2) and phosphorylation of p38 mitogen-activated protein kinase (p38MAPK) and nuclear factor-kB (NF-kB) in human aortic endothelial cells. (a) mRNA expression of COX-2 according to the concentration of Ang II. *$P < 0.01$. NS: not significant. (b) mRNA expression of COX-2 according to the time induced by Ang II (100 nmol/L). *$P < 0.01$ vs. control. †$P < 0.001$ vs. control. (c) Protein expression of COX-2 induced by Ang II (100 nmol/L). (d) Protein expression of p38 mitogen-activated protein kinase (p38MAPK), phosphorylation of p38MAPK, NF-kB and phosphorylation of NF-kB induced by Ang II. (e) The effect of SB203580 (25 nmol/L) and JSH-23 (25 nmol/L) on COX-2 mRNA expression induced by Ang II for 1 h; †$P < 0.001$. (f) The effect of SB203580 (25 nmol/L) and JSH-23 (25 nmol/L) on COX-2 protein expression induced by Ang II for 2 h. C means control group, SB + Ang means SB203580 + Ang II group, JSH + Ang means JSH-23 + Ang II group. Data represent the mean ± standard deviation ($n = 3$).

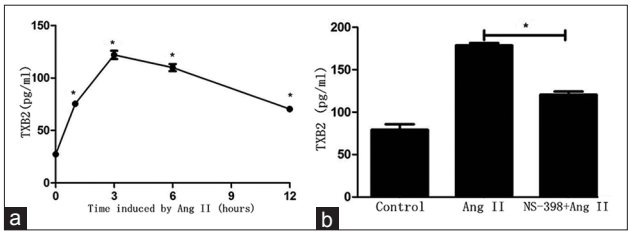

Figure 3: Angiotensin II (Ang II) induced the expression of thromboxane B_2 (TXB_2) in human aortic endothelial cells (HAECs). (a) Ang II's effect on TXB_2 expression; *$P < 0.001$ vs. 0 h. (b) HAECs were pretreated with NS-398 (2.5 μmol/L) and then incubated by Ang II for 3 h. *$P < 0.001$. Data represent the mean ± standard deviation ($n = 3$).

with p38MAPK inhibitor SB203580 (25 μmol/L) and NF-kB inhibitor JSH-23 (25 μmol/L), the expression of COX-2 was significantly decreased at both mRNA and protein levels [Figure 2e and f].

Cyclooxygenase-2 inhibitor suppressed angiotensin II-induced secretion of thromboxane B2 in human aortic endothelial cells

Ang II (100 nmol/L) led to a rapid increased secretion of TXB_2 in HAECs with peak secretion of 122.09 pg/ml at 3 h after its stimulation (4.47 times compared with 0 h, $P < 0.001$) [Figure 3a] and this effect could be suppressed by COX-2 specific inhibitor NS-398 ($P < 0.001$) [Figure 3b].

Table 3: Comparison of thrombotic event rate during follow-up (n (%))

Variables	AT_1R group ($n = 140$)	Non-AT_1R group ($n = 70$)	P
Brain infarction	9 (6.43)	11 (15.71)	0.031
Myocardial infarction	11 (7.86)	12 (17.14)	0.042
Overall thrombotic events	20 (14.3)	23 (32.8)	0.002

AT_1R: Angiotensin type 1 receptor.

Valsartan decreased phosphorylation of p38 mitogen-activated protein kinase and nuclear factor-kB, as well as expression of cyclooxygenase-2 induced by angiotensin II in human aortic endothelial cells

After pretreatment with valsartan (100 nmol/L), HAECs were stimulated with Ang II (100 nmol/L) for 10 min to detect the p-p38MAPK and p-NF-kB, 1 h to detect COX-2 mRNA and 3 h to detect COX-2 protein. Valsartan (100 nmol/L) significantly inhibited Ang II-induced COX-2 mRNA and protein expression ($P < 0.001$) [Figure 4a and 4b], with parallel decrease in p-p38MAPK and p-NF-kB [Figure 4c].

Discussion

This randomized trial demonstrates that AT_1R antagonist valsartan reduces cardio-cerebral thrombotic events in

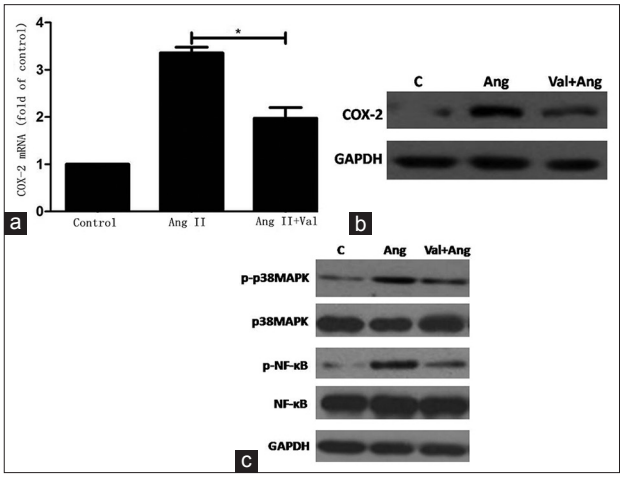

Figure 4: Valsartan inhibited angiotensin II (Ang II) induced cyclooxygenase-2 (COX-2) and phosphorylation of p38 mitogen-activated protein kinase (p38MAPK)/nuclear factor-kB in human aortic endothelial cells. (a) Valsartan's effect on COX-2 mRNA expression induced by Ang II (100 nmol/L); $*P < 0.01$. (b) Valsartan's (100 nmol/L) effect on COX-2 protein expression induced by Ang II (100 nmol/L); (c) Valsartan's (100 nmol/L) effect on p38 mitogen-activated protein kinase (p38MAPK), phosphorylation of p38MAPK, phosphorylation of nuclear factor-kB (NF-kB), NF-kB expression induced by Ang II (100 nmol/L). C means control group, Ang means Ang II group, Val + Ang means Valsartan + Ang II group. Data represent the mean ± standard deviation ($n = 3$).

elderly patients with hypertension, by inhibiting platelet activity through attenuating Ang II-induced COX-2/TXA_2 expression via p38MAPK and NF-kB pathways.

The results of this study show that platelet aggregation induced by arachidonic acids and plasma levels of COX-2 and TXA_2 were elevated in elderly hypertensive patients. Although the majority of these patients were receiving anti-platelet therapy, around 20% of them still developed brain or myocardial infarction during follow-up. This is consistent with previous reports that elderly hypertensive patients are at high risk for cardio-cerebral vascular thrombosis.[18] Interestingly, anti-hypertensive treatment with valsartan inhibits platelets activity and is associated with a lower rate of brain infarction in the AT_1R group. The nonsignificant decrease in the occurrence of myocardial infarction after valsartan treatment may be, at least partly, due to a smaller number of study patients and a shorter period of follow-up for addressing such an outcome issue. Nevertheless, our observations substantiate a notion that AT_1R antagonists exert a beneficial effect on the prevention of cardio-cerebral thrombotic events in elderly patients with hypertension.

It is well-recognized that Ang II regulates vascular structure and function and participates in the inflammatory response in vascular tissue.[19] *In vitro* experiments demonstrated that Ang II stimulation significantly increases platelet-free calcium concentration, intracellular pH, and thrombin-induced platelet aggregation.[20] Animal models have shown that this molecule increases platelet activity and accelerates microvascular thrombosis, whereas these processes are suppressed in AT_1R deficient mice.[21] Similar to the findings in vascular smooth muscle cells and aortic fibroblasts,[22] we found that Ang II up-regulates the expression of COX-2 in human endothelial cells, which subsequently catalyzes arachidonic acid into prostaglandin and leads to an increased level of TXA_2. These observations suggest that AT_1R antagonists may retard the process of thrombus formation by inhibiting platelet aggregation.[23]

Our *in vitro* study indicates that the effect of Ang II on the expression of COX-2 was at least partially through the modulation of p38MAPK and NF-kB pathways. The activity of transcription factor NF-kB is a hallmark of cellular inflammation response and has already been demonstrated in the expression of VCAM-1 regulated by ROS induced by Ang II.[24,25] However, the role of classic G protein, receptor tyrosine kinases (EGFR, platelet-derived growth factor and insulin receptor), NADPH oxidases and serine/threonine kinases such as PKC and MAPKs (JNK and p42/44MAPK) in the action of Ang II need further studies.[25,26]

We recognized several limitations in the present study. First, the number of patients in this study had insufficient power to detect the difference in terms of clinical outcome, thus further prospective studies with larger sample size are needed to confirm the beneficial effects of AT_1R antagonists in the antithrombotic treatment of elderly hypertensive patients. Second, we could not completely eliminate the effect of other factors on the platelets activity, such as coronary artery disease and diabetes, which are common co-morbidities in these patients.

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