Valsartan reduces AT1-AA-induced apoptosis through suppression oxidative stress mediated ER stress in endothelial progenitor cells

Z.-C. WANG$^{1,2}$, J. QI$^2$, L.-M. LIU$^2$, J. LI$^2$, H.-Y. XU$^2$, B. LIANG$^{1,3}$, B. LI$^{1,3}$

$^1$Shanxi Medical University, Taiyuan, Shanxi, China
$^2$Department of Cardiology, Shanxi Cardiovascular Hospital, Taiyuan, Shanxi, China
$^3$Department of Cardiology, The Second Hospital of Shanxi Medical University, Taiyuan, Shanxi, China

Abstract. – OBJECTIVE:Valsartan has been reported to have the function of treating hypertension and improving the prognosis of patients. Many studies indicated that valsartan can also increase angiotensin II, andosterone and plasma renin activity (PRA). Autoantibodies against the angiotensin II type 1 receptor (AT1-AA) have been showed to increase reactive oxygen species (ROS) and calcium (Ca$^{2+}$) and result in apoptosis in vascular smooth muscle cells. In this study, we attempted to explore the effect of valsartan on AT1-AA-induced apoptosis in endothelial progenitor cells.

MATERIALS AND METHODS: Endothelial progenitor cells (EPCs) were cultured. The cytotoxicity was determined by MTT assay. EPCs apoptosis was determined by DAPI staining and flow cytometry. Reactive oxygen species, intracellular calcium concentration and calpain activity were measured using Fluostar Omega Spectrofluorimeter. The expression of p-ERK, p-eIF-2α, CHOP, Bcl-2 and caspase-3 were detected by Western blot.

RESULTS: MTT assays showed valsartan significantly inhibited AT1-AA-induced decline of the viability of EPCs. DAPI staining and flow cytometry results indicated valsartan inhibited AT1-AA-induced decline of the viability of EPCs via inhibiting AT1-AA-induced apoptosis. Furthermore, the increasing of reactive oxygen species, intracellular calcium and calpain activity induced by AT1-AA in EPCs were also recovered after pre-treated with valsartan. Meanwhile, the upregulation of p-ERK, p-eIF-2α and CHOP, downregulation of Bcl-2, and activation of Caspase-3 caused by AT1-AA were reversed after pre-incubated with valsartan.

CONCLUSIONS: Valsartan could inhibit AT1-AA-induced apoptosis through inhibiting oxidative stress mediated ER stress in EPCs.

Key Words:
Valsartan, AT1-AA, Apoptosis, Oxidative stress, ER stress.

Introduction

Coronary heart disease (CHD) is one of the main diseases to harm human health. CHD, which usually damages the dynamic imbalance between repairmen and impairment of the endothelium, results from the excessive proliferation of neo-endothelium and the dysfunction of endothelium after the impairment of the vessel. Endothelial progenitor cells (EPCs) are a kind of cells that can proliferate and differentiate from endothelial cell and yet do not express mature vascular cells, which is precursor cell of vessel. EPCs involve in repairing endothelial injury and the disorder of this process is one of the reasons for coronary heart disease. Autoantibodies against the angiotensin II type 1 receptor (AT1-AA), which belongs to the G-protein-coupled receptor, are a membrane receptor and have all features of hormone receptors. Many reports showed that AT1-AA has been found in refractory hypertension, preeclampsia and renal-allograft rejection patients. AT1-AA has been detected by the chronotropic responses to AT1-AA-mediated stimulation of cultured rat cardiomyocytes. Previous studies have found that AT1-AA can increase reactive oxygen species (ROS) and calcium (Ca$^{2+}$) and result in apoptosis in vascular smooth muscle cells. Although AT1-AA has been recognized to induce apoptosis in vascular smooth muscle cells, little is known about its effects on EPCs and underlying mechanism. It is necessary to look for effective drugs to antagonism the effect of AT1-AA on EPCs. Angiotensin II receptor blockers (ARBs) is a member of drugs for hypertension and the mechanism of ARBs is lowering blood pressure by blocking angiotensin II effect. Valsartan has been reported to have the function of treating hypertension and improving the prognosis of patients.
been used to initialize and maintain the therapy of antihypertensive for the five classes of antihypertensive drugs\textsuperscript{13}. ARBs have recently been reported to suppress atrial fibrillation (AF) recurrence in both paroxysmal and persistent AF\textsuperscript{14}.Valsartan is one of the ARBs, which has been reported to treat hypertension and improve the prognosis of patients. Valsartan is a selective antagonist of the angiotensin II type 1 receptor\textsuperscript{15,16}. Many reports have shown a significant decrease of caspase-3 and BAX in valsartan-treated group compared to active control group in myocardial level. Meanwhile, the myocardial apoptosis index also has a significant decrease in a valsartan-treated group. Valsartan has been shown to inhibit apoptosis and reduce caspase-3 activity to treat hypertension and improve the prognosis of patients\textsuperscript{17-19}. Also, it has been shown to have the abilities to increase angiotensin II, aldosterone and plasma renin activity (PRA)\textsuperscript{20}. Given the function of valsartan, we try to explore whether valsartan could inhibit AT1-AA-induced apoptosis in EPCs and the underlying mechanism. In this study, we verified the effect of valsartan on AT1-AA-induced apoptosis on EPCs. The results showed valsartan could inhibit AT1-AA-induced apoptosis through inhibiting oxidative stress mediated ER stress in EPCs. Therefore, valsartan has great potential as a drug against coronary heart disease.

Materials and Methods

Reagents and Chemicals
EGM-2 medium and fetal bovine serum (FBS) were obtained from Gibco (Grand Island, NY, USA). 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were purchased from Cell Signaling (Beverly, MA, USA). DAPI staining was from Pharmingen-Becton Dickinson (San Diego, CA, USA). Annexin-V-FITC/PI apoptosis detection kit was obtained from Sigma-Aldrich (St. Louis, MO, USA). Western lysis buffer, BCA protein kit and enhanced chemiluminescence kit were from Beyotime Institute of Biotechnology (Shanghai, China). p-eIF-2α and eIF-2α antibodies were purchased from Abcam (Cambridge, MA, USA). CHOP antibodies were from Cell Signaling (Beverly, MA, USA). Antibodies against Bcl-2, caspase-3 and α-tubulin were purchased from Beyotime Institute of Biotechnology (Nantong, China). p-ERK and ERK were obtained from Bioword (New York, NY, USA). AT1-AA was obtained from Abcam (Cambridge, MA, USA). Valsartan was purchased from Sigma-Aldrich (St. Louis, MO, USA).

Cell Culture
EPCs were obtained from the Institute of Cell Research (Shanghai, China). EPCs were cultured in EGM-2 medium, which was supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Beiyotime, Beijing, China). Cells were incubated at 37°C in a humidified atmosphere with 5% CO\textsubscript{2}.

MTT Assay
The cell viability was detected by MTT assay. EPCs (8000 cells/well) were seeded in flat-bottomed 96-well plates. After 24 h, cells were exposed in AT1-AA (2.5, 5 and 10 μM) for 24 h or 10 μM for 3, 6, 9, 12 and 24 h. Based on previous dose-response studies, effective AT1-AA (10 μM for 12 h) was chosen as optimum for further studies. Then valsartan (0.5, 1 and 2 μM) for 24 or 1 μM for 2, 4, 6, 8, 12 and 24 h were added to the wells to determine pre-treatment efficacy\textsuperscript{20}. Control group was cultured with phosphate-buffered saline (PBS) in medium. 20 μL MTT (0.5 mg/ml in PBS) was added to each well and the plates were incubated for 4 h at 37°C. Then the medium was removed and 150 μL DMSO was added to extract intracellular formazan in EPCs cells for 10 min to solute the purple formazan crystals. Absorbance was detected with a microplate reader Uscn Kit Inc (Wuhan, China) at 570 nm. The percentage of cell growth inhibition was calculated based on the following formula: (A\textsubscript{570 Control}-A\textsubscript{570 Experiment}) / A\textsubscript{570 Control} × 100%. All assays were executed in three independent experiments.

DAPI Staining of Apoptotic Cells
The apoptosis of EPCs was detected by DAPI staining. Briefly, EPCs were seeded at a density of 2×10\textsuperscript{5} cells/well in flat-bottomed 12-well plates with glass slides. After 24 h, EPCs were treated with AT1-AA (2.5, 5 and 10 μM), valsartan (1 μM) in medium alone or composite processing. Control group was treated with PBS in medium. EPCs were washed with PBS twice and stained with DAPI in the dark at room temperature for 20 min. Then, EPCs were washed with PBS and cell nuclei were observed under laser confocal scanning microscope (LCSM) (Hiobio, Beijing, China). The apoptosis rate was measured by calculating the number of cells with nuclear phenotypic changes in 20 different microscopic fields.
Flow Cytometric Analysis

AT1-AA-induced apoptosis in EPCs were measured by flow cytometry using the annexin V-FITC and propidium iodide (PI) staining method. Briefly, EPCs were plated at a density of 1x10^6 cells/dish into 60 mm dishes. After 24 h, EPCs were exposed in AT1-AA (2.5, 5 and 10 μM), valsartan (1 μM) in medium alone or composite processing. Control group was treated with PBS in medium. Then cells were trypsinized, washed with cold PBS and re-suspended in binding buffer containing annexin V-FITC (5 μL) and of PI (2.5 μL). Cells were cultured at room temperature in the dark for 30 min. Finally, apoptosis was detected with a FAC Sort flow cytometer (Becton, Franklin Lakes, NJ, USA).

Measurement of Reactive Oxygen Species

Reactive oxygen species was detected by cell permeant probe CM-H2DCFDA. Briefly, EPCs were seeded in 6-well plates overnight and pre-treated with valsartan (1 μM) for 4 h and incubated with AT1-AA (10 μM) for 12 h. Cells were incubated with fluorescence probe 2', 7'-di-chlorofluorescein diacetate (DCF-DA) 20 μM for 30 min. Then cells were trypsinized, washed with PBS (pH 7.4) and monitored at an excitation wavelength of 488 nm and emission of 530 nm by Fluostar Omega Spectrofluorimeter (Bio-gene, Hong Kong, China).

Measurement of Intracellular Calcium Concentration

The level of intracellular calcium in EPCs was determined by Fluo-4 AM staining. Briefly, cells were plated in 96-wells and incubated with Fluor-4 AM fluorescent at 25°C for 30 min. The Hanks’ Balanced Salt Solution (HBSS), which was used in experiments, included (mM): NaCl, 142; KCl, 5.6; MgCl2, 1; CaCl2, 2; Na2HPO4, 0.34; KH2PO4, 0.44; HEPES, 10; glucose, 5.6; buffered to pH 7.4 with NaOH. The Ca2+-free HBSS contained (mM): NaCl, 142; KCl, 5.6; MgCl2, 1; Na2HPO4, 0.34; KH2PO4, 0.44; HEPES, 10; glucose, 5.6; EGTA, 1. Fluorescence measurements were determined at 488 nm for excitation and 522 nm for emission by Fluostar Omega Spectrofluorimeter (Bio-gene, Hong Kong, China).

Calpain Activity Assays

EPCs were seeded on 24-well plates and pre-treated with Tetrakis (acetoxyethyl) 1,2-Bis(2-aminophenoxy) ethane-N,N,N’,N’-tetraacetate (BAPTA), a calcium chelator or calpeptin and an inhibitor of calpain for 1 h. Then cells were loaded with Suc-Leu-Leu-Val-Tyr-AMC calpain protease substrate (40 M) and incubated with shikonin to the indicated time under a humidified atmosphere with 5% CO2 at 37°C. The fluorescent was detected by Fluostar Omega Spectrofluorimeter (Bio-gene, H.K, China) at 360 nm for excitation and 460 nm for emission.

Western Blotting

Treated cells were washed with PBS (4°C) and lysed with Western lysis buffer containing 1% PMSF for 15 min and centrifuged at 13000 rpm at 4°C for 15 min to precipitate the insoluble material. Protein concentration was measured with a BCA protein kit. Eighty micrograms of each protein were loaded on 12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. The membrane was blocked in TBS (Tris-buffered saline containing Tween 20 and 5% milk) for 1 h and incubated with anti-p-ERK, anti-ERK, anti-p-eIF-2α, anti-eIF-2α, anti-CHOP, anti-Bcl-2, anti-caspase-3 and anti-α-tubulin at 4°C overnight. Flowing this, the membranes were washed with Tris-buffered saline containing Tween 20 (TBST) and incubated with the appropriate HRP-conjugated secondary antibody (Abcam, Cambridge, UK). Finally, the amount of protein was detected by chemiluminescence.

Statistical Analysis

Results were analyzed using SPSS 17.0 software (SPSS Inc., Chicago, IL, USA) for Windows and expressed as the mean ± standard error of the mean. Statistical significance of differences was analyzed with one-way analysis of variance (ANOVA) with Tukey’s multiple comparison tests. A value of less than 0.05 (p < 0.05) and 0.01 (p < 0.01) were considered significant and highly significant, respectively.

Results

Valsartan Inhibits AT1-AA-Induced Decline of Cell Viability in EPCs

To investigate whether valsartan could inhibit AT1-AA-induced decline of cell viability in EPCs, we firstly detected the effect of AT1-AA on the viability of EPCs. EPCs were treated with AT1-AA (2.5, 5 and 10 μM) for 24 h or 10 μM for 3, 6, 9, 12 and 24 h. Results showed AT1-AA inhibited growth of EPCs in dose- and time-dependent (Figure 1A and B). Meanwhile, effective AT1-AA (10 μM) inhibited cell viability in a dose- and time-dependent manner (Figure 1C and D).
μM for 12 h) was chosen as optimum for further studies. Then valsartan (0.5, 1 and 2 μM) for 24 or 1 μM for 2, 4, 6, 8, 12 and 24 h was added to the wells to determine the pre-treatment efficacy. The results indicated the decline of cell viability in EPCs, which was induced by AT1-AA, was elevated significantly by valsartan and the effective dose and time is 2 μM for 4 h (Figure 1C and D). All above results suggested valsartan could inhibit AT1-AA-induced decline of viability of EPCs.

**Valsartan inhibits AT1-AA-Induced Cell Apoptosis in EPCs**

To verify whether valsartan inhibited AT1-AA-induced decline of cell viability in EPCs is related to apoptosis, EPCs were treated with AT1-AA (2.5, 5 and 10 μM for 12 h), valsartan (1 μM for 4 h) alone or composite processing. The apoptosis was determined by DAPI staining and flow cytometric analysis. As shown in Figure 2A and B, EPCs exhibited the apoptotic characteristics of chromatin condensation with typical apoptotic bodies after treated with AT1-AA and the percentage of apoptotic cells was significantly increased in a dose-dependent manner. After pre-treated with valsartan, the apoptosis rate of EPCs was significantly reverse compared with AT1-AA alone. The apoptosis of EPCs was further determined by flow cytometry using the annexin V-FITC and propidium iodide (PI) staining method (annexin V/PI double-staining). As suggested in Figure 2C and D, the apoptosis rate of EPCs induced by

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**Figure 1.** Effect of valsartan on AT1-AA induced decline of cell viability in EPCs. (A) EPCs were treated with various concentrations of AT1-AA (2.5, 5 and 10 μM) for 24 h and cell viability was determined by MTT assay; (B) EPCs were treated with AT1-AA (10 μM) for 3, 6, 9, 12, 24 h and then cell viability was detected by MTT assay; (C) EPCs were pre-treated with various concentrations of valsartan (0.5, 1 and 2 μM) for 24 h and then incubated with AT1-AA (10 μM) for 12 h. The cell viability was detected by MTT assay; (D) EPCs were pre-incubated with valsartan (1 μM) for 2, 4, 6, 8, 12, 24 h and then treated with AT1-AA (10 μM) for 12 h. Cell viability was determined by MTT assay. In (A), (B), (C) and (D), values are percent as the mean ± SD of three independent experiments. 0.01 < *p < 0.05 and **p < 0.01 vs. control; 0.01 < #p < 0.05 and ##p < 0.01 vs. AT1-AA alone.
AT1-AA (10 μM) alone increased to 30.3% but reversed to 13.7% after pre-treated with valsartan. These results indicated valsartan inhibited AT1-AA-induced apoptosis in EPCs.

Valsartan inhibits AT1-AA-Induced Cell Apoptosis Through Decreasing ROS Generation and Intracellular Calcium in EPCs

Both ROS and Ca\(^2+\) have been reported to involve in apoptosis signal transduction pathways and they have a close relationship in this pathway\(^21\). To determine whether valsartan inhibited AT1-AA-induced apoptosis in EPCs via decreasing ROS generation and intracellular calcium, EPCs were pre-treated with valsartan (1 μM) for 4 h and incubated with AT1-AA (10 μM) for 12 h or treated with AT1-AA (10 μM) for 6, 12 and 24 h alone. Results showed AT1-AA increased ROS generation (Figure 3A) and intracellular calcium as early as 90 min (Figure 3B and C) and the effect of AT1-AA on intracellular calcium in EPCs in time-dependent manner (Figure 3B). Meanwhile, after EPCs pre-treated with valsartan, the increasing of ROS and intracellular calcium

![Figure 3](image_url)

**Figure 2.** Protective effect of valsartan on AT1-AA induced apoptosis in EPCs. (A) EPCs were pre-treated with valsartan (1 μM) for 4 h and incubated with various concentrations of AT1-AA (2.5, 5 and 10 μM) for 12 h. Then cell nuclei were stained with DAPI and images were captured under a fluorescence microscope; (B) The percentage of apoptotic cells was measured; (C) EPCs were pre-treated with valsartan (1 μM) for 4 h and incubated with various concentrations of AT1-AA (2.5, 5 and 10 μM) for 12 h and then cell apoptosis were determined by flow cytometry analysis using annexin V/propidium iodide double-staining; (D) Apoptosis ratio from flow cytometry were shown in the bar graph. The data in (B) and (D) were expressed as mean ± SD and acquired from three biologically independent experiments. 0.01 < *p < 0.05 and **p < 0.01 vs. control; 0.01 < #p < 0.05 and ##p < 0.01 vs. AT1-AA alone.
was rescued (Figure 3A and C). Calcium-activated neutral protease (Calpain) activity was also detected after exposed with AT1-AA and results suggested a marked increase. After pre-treated with valsartan, AT1-AA-induced increase of calpain activity was reversed (Figure 3D). All these results indicated valsartan inhibited AT1-AA-induced cell apoptosis through decreasing ROS generation and intracellular calcium in EPCs.

**Figure 3.** Valsartan inhibits AT1-AA-induced ROS generation, increase in intracellular Calcium in EPCs. (A) Valsartan inhibits AT1-AA-induced ROS generation in EPCs. EPCs were seeded in flat-bottomed 6-well plates overnight and pre-treated with valsartan (1 μM) for 4 h and incubated with AT1-AA (10 μM) for 12 h. Then, EPCs were incubated DCF-DA (50 μM) for 30 min and washed with PBS (4°C) three times. Finally, the generation of ROS was detected by fluorimetry according to standard protocol. (B) AT1-AA induces increase in intracellular calcium in EPCs. EPCs were plated in 96-well plates and exposed with AT1-AA (10 μM) for 12 h. Then EPCs were stained with Fluo-4AM (50 μM/mL) dye and the level of intracellular calcium measured by Fluostar Omega Spectrofluorimeter. (C) Valsartan inhibits AT1-AA-induced increase in intracellular calcium in EPCs. EPCs were seeded in 96-well plates and pre-treated with valsartan (1 μM) for 4 h and incubated with AT1-AA (10 μM) for 12 h. The level of intracellular calcium in EPCs was also determined by Fluostar Omega Spectrofluorimeter. (D) Valsartan inhibits AT1-AA-induced calpain activity in EPCs. EPCs were pre-treated with valsartan (1 μM) for 4 h and incubated with AT1-AA (10 μM) for 12 h and calpain activity analyzed, as described in Material and Methods. The data were expressed as mean ± SD and acquired from three biologically independent experiments. 0.01 < *p < 0.05 and **p <0.01 vs. control; 0.01 < #p < 0.05 and ##p < 0.01 vs. AT1-AA alone.

**Valsartan inhibits AT1-AA-induced Cell Apoptosis Through Oxidative Stress Mediated ER Stress**

ROS-mediated free intracellular Ca²⁺ is known to be involved in ER stress-induced apoptotic signaling. Many reports have shown drug-induced increase in ROS generation, intracellular calcium and activation of ER leads to apoptosis in cells. As the previous results suggested, valsartan inhib-
imited AT1-AA-induced increase of ROS generation and intracellular in EPCs. Therefore, we explored whether valsartan inhibited AT1-AA-induced apoptosis via inhibiting ROS dependent ER stress and examined ER stress-associated proteins in EPCs. The key factors of the ER stress include p-ERK, p-eIF-2α, CHOP, Bcl-2 and caspase-3. EPCs were pre-treated with valsartan (1 μM) for 4 h and incubated with AT1-AA (10 μM) for 12 h or exposed with AT1-AA (10 μM) for 0.5, 1, 3, 6, 9 and 12 h alone. Results revealed AT1-AA stimulated ERK1/2 phosphorylation in time-dependent manner and had a marked increase as early as 6 h (Figure 4A and B). As showed in Figure 4C, D and Figure 5A, B, C, AT1-AA effectively increased p-ERK, p-eIF-2α, CHOP, and caspase-3 protein levels along with decreased Bcl-2 expression. After pre-treated with valsartan, the increase of p-ERK, p-eIF-2α, CHOP and Caspase-3 and decrease of Bcl-2 induced by AT1-AA were all reversed. These data indicated valsartan inhibited AT1-AA-induced apoptosis through inhibiting oxidative stress mediated ER stress in EPCs.

**Discussion**

EPCs play an important role in coronary heart disease. EPCs can proliferate and differentiate from endothelial cell and involve in repairing endothelial injury4,5. EPCs injury is one reason for coronary heart disease. AT1-AA has been...
showed to induce apoptosis in endothelial progenitor cells. Furthermore, valsartan can reduce drugs-induced apoptosis by regulating oxidative stress mediated ER stress. Apoptosis is an important reason in drugs-induced cell death. Therefore, we explore whether valsartan decreased AT1-AA-induced decline of EPCs viability by apoptosis. MTT results revealed AT1-AA inhibited growth of EPCs in dose- and time-dependent and valsartan could inhibit AT1-AA-induced decline of viability of EPCs. Results of DAPI staining and flow cytometry showed apoptotic bodies and apoptosis ratio were both significantly decreased after pre-treatment with valsartan compared with AT1-AA alone. Many studies showed both ROS and Ca\textsuperscript{2+} involve in apoptosis signal transduction pathways and they have a close relationship in this pathway. Therefore, we explored the relationship between ROS, Ca\textsuperscript{2+} and AT1-AA- induced apoptosis. Results indicated AT1-AA increased ROS generation and intracellular calcium and the level of ROS generation and intracellular calcium were decreased after pre-treated with valsartan in EPCs. These results showed valsartan inhibited AT1-AA-induced cell apoptosis through decreasing ROS generation and intracellular calcium in EPCs. Oxidative stress mediated ER stress have been reported to play an important role in apoptosis process. Therefore, we investigated the effect of oxidative stress mediated ER stress on AT1-AA-induced cellular apoptosis and the effect of valsartan was further explored. The activation of ERK, which mainly depends on the stimuli and cell types involved, has been showed to cause cell apoptosis. EIF2α and CHOP are important proteins in ER stress apoptotic pathway. Bel-2 protein family is the central regulator of cell apoptosis. Anti-apoptosis protein Bel-2 is a
Valsartan effect on AT1-AA-induced apoptosis in endothelial progenitor cells

Conclusions

Valsartan inhibits AT1-AA-induced apoptosis in EPCs by regulating ROS and Ca\(^{2+}\) and inhibiting ER stress-dependent apoptosis pathway. Valsartan decreases AT1-AA-induced activation of ERK and in turn modulates p-ERK, p-eIF2α, CHOP, Bcl-2 and caspase-3 and decreased the down-regulating of Bcl-2 after pre-treatment with valsartan. All these results indicated valsartan inhibited AT1-AA-induced apoptosis inhibiting oxidative stress mediated ER stress in EPCs.

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Conflict of Interest

The Authors declare that they have no conflict of interests.

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