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Roles of angiotensin II, Olmesartan and PD123319 on proliferation, oxidative stress and TGF-β1 in HUVEC culture

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ABSTRACT

Aims: Angiotensin II (Ang II) causes endothelial cell damage. Oxidative stress is involved in the pathophysiology of cardiovascular diseases via transforming growth factor-beta 1 (TGF-β1). In most studies increases in Ang II and TGF-β1 levels in several cell types are bidirectional. The present study investigated the effects of Ang II on oxidative stress, cell proliferation, and TGF-β1 levels in human umbilical vein endothelial cells (HUVECs).

Methods: HUVECs were treated with Ang II (0.1 μM), Ang II type 1 receptor (ATR1) antagonist Olmesartan (1 μM), and Ang II type 2 receptors (ATR2) antagonist PD123319 (1 μM) for 24 hours. Cell proliferation and viability were evaluated by the tetrazolium salt (MTT) assay. Total antioxidant capacity (TAC) and total oxidant capacity (TOC) were measured by spectrophotometer intracellularly and in the culture medium. The TGF-β1 level was measured by enzyme-linked immunosorbent assay (ELISA).

Results: The addition of 1 μM, 0.1 μM, and 0.01 μM Ang II increased proliferation in HUVECs. Cell proliferation increased significantly in both Ang II and Ang II+Olmesartan+PD123319 groups. However, Ang II+Olmesartan tended to decrease cell proliferation. In the control group TAC and TOC levels remained in the normal range in HUVEC extracts. In other all groups, TOC values increased compared to control. In HUVECs medium, TAC level was higher in the control, Ang II and Ang II+Olmesartan groups, but normal and tolerable in other groups whereas, TOC levels were elevated in control and other all groups. In HUVECs extracts, compared with the control, TGF-β1 level was significantly lower in the Ang II group, but increased in the Ang II+Olmesartan group. There was no significant difference in TGF-β1 levels between medium groups.

Conclusions: Ang II shows its proliferative effects through ATR1 activation, whereas stimulation of ATR2 seems to have a key role in the pathophysiology of oxidative stress.

Introduction

Endothelial cells lay the inner surface of all blood vessels as a single layer, and play an important role in the regulation of vascular homeostasis (1). The resting endothelial cells prevent thrombus formation in the blood-tissue interface and play a crucial role in the regulation of inflammation (2). They are also involved in the growth and proliferation of various cell types, especially the smooth muscle cells (3). Endothelial dysfunction is associated with adverse clinical outcomes since vascular wall injury is responsible for vascular diseases (4). Smooth muscle cell proliferation and matrix synthesis can lead to damaged vessel wall repair, but can also cause occlusion in the same lumen (5).

Angiotensin II (Ang II), the main effector molecule of the Renin-Angiotensin System, has physiological and pathological effects on the cardiovascular system (6). Ang II affects the vascular system via Ang II type 1 (ATR1) and type 2 receptors (ATR2) (7). It activates the phospholipase C enzyme by binding

to ATR1s (7,8). However, previous studies have reported that the vascular effects of Ang II may be related to the balance between ATR1-mediated nitric oxide (NO) function and reactive oxygen species (ROS) (8). ROS play role in the physiopathology of many diseases, including hypertension, diabetes and their long-term chronic complications (9).

It has been reported that oxidative stress occurs due to an imbalance in the production of reactive oxygen derivatives and antioxidant systems (10). Free radicals or ROS are the metabolic products that are formed by oxygen spent during the conversion of energy from nutrients in the body (11). Oxidative stress increases the proliferation of vascular cells and accelerates plaque formation (12). Besides, with increasing levels of ROS, organelle damage (e.g., mitochondria), DNA damage, and other disorders including wrong folding of proteins occur (13).

Transforming growth factor-beta (TGF-β) is a cytokine with many subtypes that has regulatory and physiological effects on cells (14). The role of TGF-β on cell growth, development, proliferation, extracellular matrix synthesis, immune system modulation, apoptosis, cell cycle, migration, and angiogenesis was also reported (15). TGF-β inhibits cell proliferation in some cell types (endothelial, epithelium, immune cells) depending on the environment in which it lives (16). TGF-β has also been shown to play a role in the transformation of mesenchymal cells into endothelial cells. TGF-β has many subtypes, including TGF-β1, TGF-β2 and TGF-β3 (16).

Some *in vivo* and *in vitro* studies found that Ang II increases TGF-β1 levels in several cells and they have a relation with each other (17,18). It has also been reported that Ang II and TGF-β subtypes play significant roles in the pathophysiology of many cardiovascular diseases. For instance, oxidative stress and TGF-β1 are important in the pathophysiology of hypertension (19,20). Various drugs such as Angiotensin Converting Enzyme and Ang II type 1 receptor blockers that inhibit RAS components are used in the treatment of many RAS-related cardiovascular diseases (6). Considering the information given above, we investigated the effects of Ang II, and its blockage with Olmesartan and PD123319 on proliferation, total antioxidant capacity (TAC), total oxidant capacity (TOC), and TGF-β1 levels in human umbilical vein endothelial cells (HUVECs).

Methods

Cell culture and treatments

HUVEC culture line was obtained from ATCC, USA. The cells were cultured in Dulbecco's Modified Eagle Medium (DMEM, D6046, Sigma-Aldrich) with 20% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific), penicillin-streptomycin (PSA, P4333-100 mL, Sigma-Aldrich) at 37 °C in humid air with 5% CO_2. The culture medium was replaced every 72 h. The cells were obtained by passaging for the proliferation and were incubated with different doses of Ang II, ([Val5]-Ang II acetate salt hydrate, A2900-50 mg, Sigma-Aldrich, 1000, 100, 10, 1, 0.1, 0.01, and 0.001 μM), ATR1 blocker Olmesartan (Olmesartan, SML1394-50 mg, Sigma-Aldrich, 1 μM) and ATR2 blocker PD123319 (PD123319 di (trifluoroacetate) salt hydrate, P186-10 mg, Sigma-Aldrich, 1 μM) for 24 h. Chemicals were dissolved in a cell medium (DMEM+10% FBS+1% PSA). Cell proliferation, TAC, TOC, and TGF-β1 levels were measured in all groups.

Experimental groups were; 1: Control, 2: Ang II (0.1 μM), 3: Olmesartan (1 μM), 4: Ang II+Olmesartan, 5: PD123319 (1 μM), 6: Ang II+PD123319, 7: Olmesartan+PD123319, 8: Ang II+Olmesartan+PD123319.

Evaluation of cell proliferation

Cell proliferation, cytotoxicity, and viability were evaluated using a tetrazolium salt (MTT) assay. It is based on the measurement of cell metabolic activity. Briefly, HUVECs were plated in a 96-well plate with $5x10^3$-$1x10^4$ cells in each well. Cells were incubated for 24 h at 37 °C in a 5% CO_2 environment with Ang II, Olmesartan, and PD123319. The cell medium was changed and 100 uL fresh medium was added. After adding 10 μL MTT (5 mg/mL, Thiazolyl Blue Tetrazolium Bromide, M5655-1G, Sigma-Aldrich) solution to each well, the cells were incubated for 3-4 h. Then the solution was replaced with 100 μL DMSO (dimethyl sulfoxide, EMPLURA, Merck, M116743100) and incubated again for an additional 20-30 min. The pale yellow MTT dye was formed. The average absorbance for each group was calculated using a microplate reader (A570 nm-A630 nm, Molecular Devices Filter Max F5) (21).

Preparation of cell and medium extract

The cells were seeded in six-well culture plates following trypsinization. After waiting for 24-72 h. for cell proliferation, Ang II (0.1 μM), Olmesartan (1 μM), and PD123319 (1 μM) were applied. After 24 h, cell and medium extraction were performed. Cell media were taken into falcon tubes and the supernatants were taken after centrifuging 200xg at +4 °C for 10 min. For cell extraction, 100 μL 140 mM KCl solution was added to the wells, and the bottom of the wells was scraped. The cells were then broken down in the sonicator for a few minutes and centrifuged at 10.000xg for 15 min and supernatants were collected. Samples were kept at -80 °C until studied.

Lowry method

Standards and samples (50 μL) were pipetted into 96-well dishes and left at room temperature for 45 min. The C reagent (150 μL) was added to the wells, which consists of a 100:1 mixture of A and B reagents. A reagent was 2% Na_2CO_3, 0.4% NaOH, 0.16% Na-tartrate; B reagent was 4% $CuSO_4.5H_2O$. Folin-Ciocalteu's reagent (3 μL) was added to all tubes after incubation. The absorbance values of the samples and standards were measured using a spectrophotometer at a wavelength of 660 nm. The protein amount of each sample was

obtained using BSA absorbances at the doses of 4, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.031 and 0 mg/mL. The total amount of protein in cell extract and cell medium was calculated using the standard curve. The standard curve was created with bovine serum albumin absorbances and the total amount of protein in the cell extract and medium was calculated (22,23).

Biochemical analysis

TAC in cell and medium extracts was measured by Rel Assay commercial kit (Diagnostics Rel Assay Kit, Turkey). Results were expressed as mmol Trolox equivalent/L. TOC in cell and medium extracts was also measured by Rel Assay commercial kit (Diagnostics Rel Assay Kit, Turkey) and results were expressed as μmol H_2O_2 equivalent/L. The oxidative stress index (OSI), which is expressed as the percentage of the ratio of TOC levels to TAC levels, was calculated and the results were expressed as "arbitrary unit" (24).

Measurement of TGF-β1 level

The levels of TGF-β1 in the cell and medium supernatant were measured using an enzyme-linked immunosorbent assay kit (Invitrogen Immunoassay Kit/KAC1689, United States). The kit was used in accordance with the protocols specified. The plate was evaluated at 450 nm absorbance with a microplate reader (Molecular Devices Filter Max F5, USA).

Statistical Analysis

Data were analyzed using the Statistical Package for the Social Sciences Statistics for Windows software, version 21.0 (IBM Corp., Armonk, NY). The results were expressed as mean±standard deviation. The normality of distribution was tested using the Shapiro-Wilk test. Dunnett and Tukey tests were performed as ANOVA post-hoc after ensuring homogeneous distribution. The statistically significant level was <0.05.

Results

Total protein measurement

Measurements of total protein in HUVECs cell and medium supernatant are shown in Table 1.

Effects of Angiotensin II doses on HUVEC proliferation

HUVEC proliferation was expressed as absorbance (Figure 1). We examined the viability of HUVECs at the concentrations of 1000, 100, 10, 1, 0.1, 0.01, and 0.001 μM of Ang II. Results showed that cell proliferation increased at concentrations of 1 μM (14%, p=0.008), 0.1 μM (14%, p=0.006), and 0.01 μM (25%, p<0.001) compared to the control group.

Effects of Angiotensin II, Olmesartan, and PD123319 on HUVEC proliferation

Ang II 0.1 μM administration increased cell proliferation by 12% compared to controls (p=0.006) (Figure 2). There was a decreasing trend (15%) in Ang II+Olmesartan group bu the difference was not significant. Compared with the Ang II+Olmesartan treated group, cell proliferation was significantly elevated in Ang II+Olmesartan+PD123319 treated group (73%), (p<0.001).

TOC and TAC levels

Concerning the TAC level (mmol Trolox Equiv./L), the following instructions were provided by the manufacturer of the

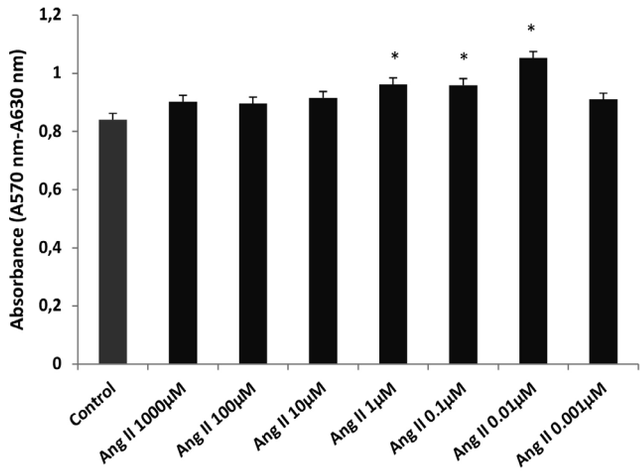

Figure 1. Angiotensin II dose study in human umbilical vein endothelial cells. The data are expressed as mean±standard deviation

*p<0.05 according to the control group. One-way ANOVA post-hoc Dunnett test (n=8-12)

Table 1. The total protein amount in cell and medium extract

Groups	Cell extract (mg/mL)	Cell medium (mg/mL)
1. Control	1.71	4.90
2. Ang II	2.71	5.15
3. Olmesartan	3.56	5.06
4. Ang II+Olmesartan	1.50	4.49
5. PD123319	3.46	4.24
6. Ang II+PD123319	2.37	6.22
7. Olmesartan+PD123319	1.22	6.81
8. Ang II+Olmesartan+PD123319	1.35	6.17

Ang II: Angiotensin II

kit used in the analysis; >2.0 very good, 1.45 to 2.00 normal, 1.20 to 1.45 tolerable, 1.00 to 1.20 low, and <1.20 very low antioxidant level. Concerning the TOC level (μmol H_2O_2 Equiv./L) the following instructions were provided; <5.00 very good, 8.00-5.00 normal, 12.00-8.00 high, and >12.00 very high oxidant level. In our measurements, in the control group TAC (1.75 mmol Trolox Equiv./L) and TOC levels (7.2 μmol H_2O_2 Equiv./L) remained in the normal range in HUVEC extracts. In other HUVEC extracts groups (Ang II+Olmesartan, Olmesartan+PD123319, Ang II+Olmesartan+PD123319), TAC values were normal and tolerable (1.3-1.7 mmol Trolox Equiv./L) and Ang II, Olmesartan, PD123319, and Ang II+PD123319 groups TAC values were higher (2.4-3.6 mmol Trolox Equiv./L). In cell extracts TOC values increased in Ang II, Olmesartan, Ang II+Olmesartan, PD123319, Ang II+PD123319, Olmesartan+PD123319, Ang II+Olmesartan+PD123319 groups compared to control (9.5-14.2 μmol H_2O_2 Equiv./L).

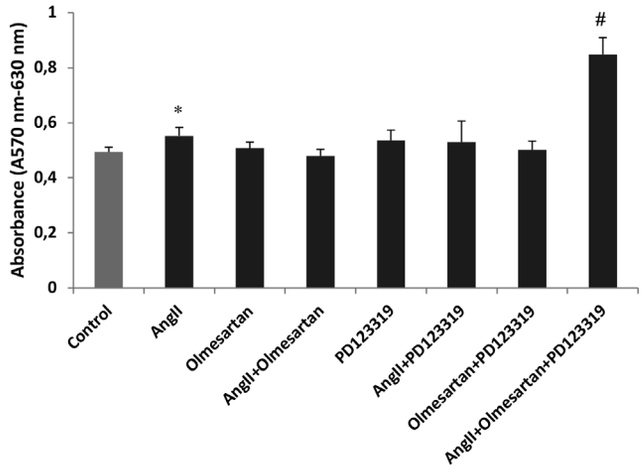

Figure 2. Angiotensin II, Olmesartan, and PD123319 proliferation study in human umbilical vein endothelial cells culture. Data are expressed as mean±standard deviation

*p<0.05 according to the control group, #p<0.05 compared to Ang II+Olmesartan group. One-way ANOVA post-hoc Tukey test (n=8-16)

Ang II: Angiotensin II

In the HUVECs medium, TAC levels were higher in the control (4.8 mmol Trolox Equiv./L), Ang II and Ang II+Olmesartan groups (2.8-2.86 mmol Trolox Equiv./L) but normal and tolerable in Olmesartan, PD123319, Ang II+PD123319, Olmesartan+PD123319 and Ang II+Olmesartan+PD123319 medium groups (1.41-1.68 mmol Trolox Equiv./L). Whereas, TOC levels increased in control (11.4 μmol H_2O_2 Equiv./L) and other all groups in HUVECs medium groups (10.8-14.4 μmol H_2O_2 Equiv./L). TAC, TOC, and OSI (TOS/TAC) values measured in the samples obtained from HUVEC extract groups are shown in Table 2 and 3.

Table 2. TOC, TAC, and OSI in HUVECs extracts

HUVEC groups (cell extracts)	TAC (mmol Trolox Equiv./L)	TOC (μmol H_2O_2 Equiv./L)	OSI (arbitrary unit)
1. Control	1.75	7.2	4.11
2. Ang II	2.8	9.5	3.39
3. Olmesartan	3.6	10.2	2.83
4. Ang II+Olmesartan	1.7	13.3	7.82
5. PD123319	3.5	11.4	3.25
6. Ang II+PD123319	2.4	14.2	5.91
7. Olmesartan+PD123319	1.3	12.6	9.69
8. Ang II+Olmesartan+PD123319	1.4	11.3	8.07

Equiv.: Equivalent, OSI: Oxidative stress index, TAC: Total antioxidant capacity, TOC: Total oxidant capacity, HUVECs: Human umbilical vein endothelial cells, Ang II: Angiotensin II

Table 3. TOC, TAC, and OSI in HUVECs medium extracts

HUVECs groups (medium extract)	TAC (mmol Trolox Equiv./L)	TOC (μmol H_2O_2 Equiv./L)	OSI (arbitrary unit)
1. Control	4.8	11.4	2.37
2. Ang II	2.8	10.8	3.85
3. Olmesartan	1.41	12.1	8.58
4. Ang II+Olmesartan	2.86	13.2	4.61
5. PD123319	1.53	11.9	7.77
6. Ang II+PD123319	1.33	14.4	10.82
7. Olmesartan+PD123319	1.42	12.6	8.87
8. Ang II+Olmesartan+PD123319	1.68	11.3	6.72

Equiv.: Equivalent, OSI: Oxidative stress index, TAC: Total antioxidant capacity, TOC: Total oxidant capacity, HUVECs: Human umbilical vein endothelial cells, Ang II: Angiotensin II

Table 4. TGF-β1 level in HUVECs extract and medium

Groups	Cell extract (pg/mg) (mean±SD)	Cell medium (pg/mg) (mean±SD)
1. Control	318±25.06	61.58±4.09
2. Ang II	206±14.88*	40.93±2.83
3. Olmesartan	162±14.35*	41.42±6.00
4. Ang II+Olmesartan	374±19.52#	40.55±6.46
5. PD123319	181±10.36*	42.85±9.57
6. Ang II+PD123319	231±17.66	31.58±6.11
7. Olmesartan+PD123319	396±32.93	28.17±3.92
8. Ang II+Olmesartan+PD123319	356±36.10#	24.84±2.65

Data are expressed as mean±SD. *p<0.05 relative to control, #p<0.05 relative to Ang II group. One-way ANOVA post-hoc Tukey test (each group worked 5 replicates). SD: Standard deviation, TGF-β1: Transforming growth factor-beta 1, HUVECs: Human umbilical vein endothelial cells, Ang II: Angiotensin II

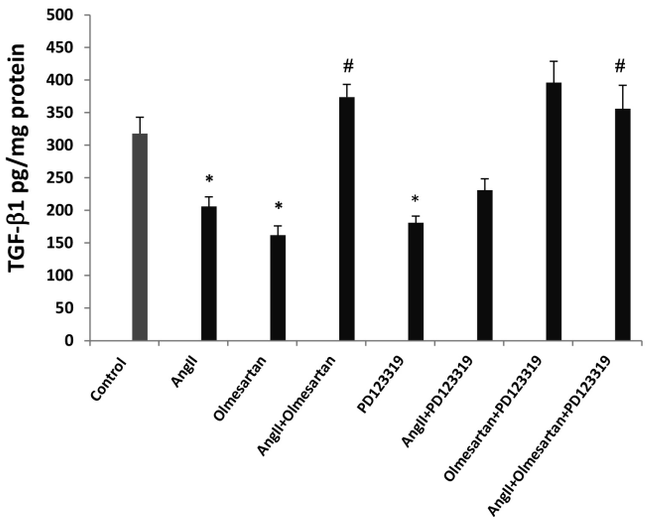

Figure 3. TGF-β1 levels in Angiotensin II, Olmesartan, and PD123319 treated groups in human umbilical vein endothelial cells culture. Data are expressed as mean±standard deviation

*p<0.05 compared to the control group, #p<0.05 compared to Ang II group. One-way ANOVA post-hoc Tukey test (n=5)

TGF-β1: Transforming growth factor-beta 1, Ang II: Angiotensin II

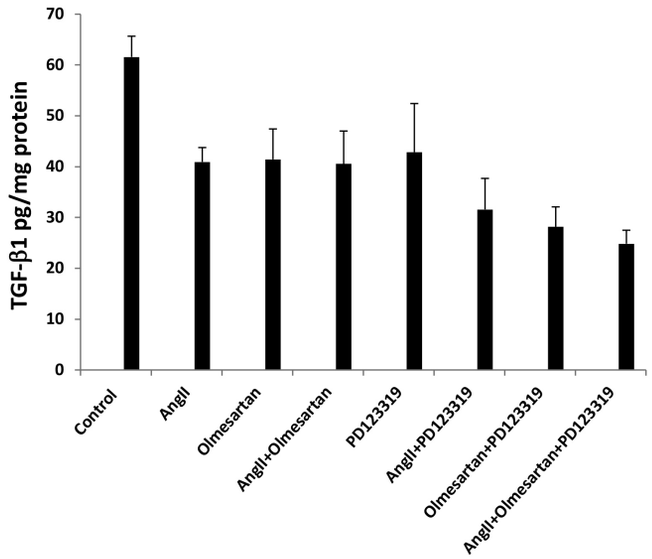

Figure 4. TGF-β1 levels in human umbilical vein endothelial cells medium in Angiotensin II, Olmesartan, and PD123319 applied groups. Data are expressed as mean±standard deviation

One-way ANOVA post-hoc Tukey test (n=6)

TGF-β1: Transforming growth factor-beta 1, Ang II: Angiotensin II

TGF-β1 levels in HUVECs extract and medium

TGF-β1 levels measured in cell extracts and medium in HUVEC groups are shown in Table 4. Compared with all HUVECs medium groups, TGF-β1 level in all HUVECs extract groups was significantly higher (p<0.001). In HUVECs extracts, compared with the control, TGF-β1 level was significantly lower in the Ang II group (p=0.030). Compared to the Ang II group, TGF-β1 level was significantly higher in the Ang II+Olmesartan group (p<0.001) and Ang II+Olmesartan+PD123319 group (p=0.001) (Figure 3). There was no significant difference in TGF-β1 levels between medium groups (Figure 4).

Discussion

Hypertension is one of the most important health problems worldwide (25). While many of the physiological mechanisms and intracellular molecular pathways that regulate blood pressure are known, some of the pathological mechanisms that lead to hypertension are still unknown (7,25). It was indicated that RAS components and receptors may be responsible for the physiopathology of hypertension (26). Drugs that inhibit RAS components and receptors are frequently used for treating this disease (6).

Ang II, the most important potent molecule of RAS, increases proliferation and oxidative stress in endothelial cells (8). 1 μM, 0.1 μM, and 0.01 μM Ang II treatment increased HUVEC proliferation in the current study. As the administration dose of Ang II reduces, cell proliferation appears to decrease in a dose-dependent manner. Therefore, the dose of Ang

II for maximum proliferative effect was chosen as 0.1 μM for combined applications with ATR blockers in our study. This finding is consistent with the studies performed with different cell types (27,28). In our previous study, enhanced proliferation was observed also with the same dose of Ang II in primary culture of vascular smooth muscle cells obtained from rat thoracic aorta (29). Dose-dependent cellular effects of Ang II are complex. Additionally, plasma and tissue levels of Ang II (differences in physiological dose) in humans and different species may differ (8). There is no adequate and comprehensive study on intracellular and extracellular Ang II measurement.

It is already known that Ang II may have some different cellular effects and responses (8). Studies have shown that ATR1 blockers reduce cell proliferation caused by Ang II in some different cell cultures (28,30). However, Olmesartan did not inhibit the proliferation of Ang II in HUVECs in our study. The reason may be the insufficient dose of Olmesartan (1 μM). However, the doses of Olmesartan (1 μM) and PD123319 (1 μM) were determined in accordance with the literature and chosen for co-administration (31,32). PD123319 was found to increase endothelial cell proliferation in this study. Forrester et al. (8) reported that ATR2 blockers increase cell proliferation compared to ATR1 blockers. It was considered that stimulation of ATR2s affects vascular cell physiology and exposes these effects by increasing NO release. Contrary to the current study, while 1 μM PD123319 increased proliferation, 1 μM Olmesartan reduced proliferation in vascular smooth muscle cells in our previous study (29). As a possible explanation, PD123319 and Olmesartan might activate different intracellular proliferative and non-proliferative signaling mechanisms in the endothelial cells.

TAC and TOC levels in the cell extract control group were determined within normal limits. Concerning OSI we observed that the oxidant and antioxidant processes were balanced in the control group, whereas both oxidants and antioxidants were augmented in the cell extract groups (Ang II, Olmesartan, PD123319, and Ang II+PD123319). This finding may indicate a potential cause of vascular diseases. Moreover, there was very high oxidative stress level in Ang II+Olmesartan, Olmesartan+PD123319, and Ang II+Olmesartan+PD123319 cell extract groups. These findings may be considered more serious and many pathologies can be suspected. In the literature, it has been reported that Ang II increases oxidative stress, while ATR1 blockers decrease oxidants on the vascular structure (8).

As Ang II can cause endothelial cell damage, inflammation, and even cell death (33), and ATR1 blockers can reverse these effects (6). However, there was no elevation in TAC levels in the Olmesartan group in our study. The reason for this finding may be the insufficient dose of Olmesartan. we also found that when the medium and cell extract control groups were compared, both the oxidant and antioxidant capacity of the medium were increased. This may be explained by the maintanece of balance by the cells as a physiological process. Except for the Ang II and Ang II+Olmesartan groups, higher oxidative stress was found in all treatment groups. Ang II may induce oxidative stress on the cardiovascular system through ATR2s. Nitric oxide is a free radical and plays a potent role in the physiological mechanisms of the cardiovascular system (34). Also, the stimulation of ATR2s increases nitric oxide synthesis (8). In addition, increased nitric oxide levels in cardiac cells may decrease the expression of ATR1. It can be thought that the blockade of ATR2s may reduce oxidative stress.

Cellular TGF-β1 level was higher inside the cell compared to the medium in our study. At the same time, Ang II and ATR1 blocker Olmesartan reduced TGF-β1 synthesis in endothelial cells. However, in an *in vivo* study, it was reported that Ang II administration to rat cardiovascular endothelial cells has augmented TGF-β1 levels and another ATR1 blocker losartan decreased plasma level of TGF-β1 (35). Although it is known that the TGF-β protein family increases cell proliferation and migration, its effects on some cell types may be the opposite (19). Our results showed that TGF-β1 may have proliferative effects on vascular cells and Ang II administration may reduce TGF-β1 levels. The proliferative effects of Ang II on cardiovascular cells are known and the induced TGF-β1 levels are reported to be related to proliferation (36,37). It has been suggested that TGF-b1 can affect intracellular signaling through cell type-dependent mechanisms (38,39). Therefore, the relationship between Ang II and TGF-β1 is important, which has been postulated in the physiopathology of cardiovascular diseases such as hypertension (19). In this respect, when all cellular mechanisms are evaluated as a whole, the fact that Ang II reduces TGF-β1 levels in our study and this result cannot be associated with proliferation. We expected an increase in TGF-β1 levels in correlation with the proliferation-enhancing effects of Ang II. Our fındings suggest that Ang II may affect different TGF-β subtypes that may be more effective in endothelial cell proliferation, and may activate different proliferation pathways.

Several limitations of the current study should be acknowledged. In contrast to other studies in different cell types, Olmesartan application did not decrease HUVEC proliferation in our study. This might have been caused by the use of only a single and low dose of Olmesartan (1 μM), as well as by the measurement of cell proliferation with a single method. As another limitation, only TGF-β1 could be measured as one of the cell proliferation pathways. Oxidative stress could be evaluated by using superoxide dismutase, glutathione peroxidase, catalase, and malondialdehyde, which lacked in our analyses.

Conclusion

The present study showed that Ang II and ATR2 blocker PD123319 increased HUVEC proliferation. While, Ang II

demonstrated most of its proliferative effects through ATR1 activation, stimulation of ATR2 seemed to have a more prominent role in oxidative stress processes in endothelial cells.

Ethics

Ethics Committee Approval and Informed Consent: Ethics committee approval was not obtained from the patient and a consent form was not obtained because it was a cell culture study. The HUVEC cell culture line used in the study was obtained from the Health Sciences University Stem Cell Research Center.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Z.Ç., K.A., A.Ş.İ., Concept: Z.Ç., K.A., A.Ş.İ., Design: Z.Ç., K.A., A.Ş.İ., Data Collection or Processing: Z.Ç., K.A., A.Ş.İ., Analysis or Interpretation: Z.Ç., K.A., A.Ş.İ., Literature Search: Z.Ç., K.A., A.Ş.İ., Writing: Z.Ç., K.A., A.Ş.İ.

Conflict of Interest: No conflict of interest was declared by the authors.

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References

1. Galley HF, Webster NR. Physiology of the endothelium. British Journal of Anaesthesia. 2004;93:105-113.
2. Du J, Leng J, Zhang L, Bai G, Yang D, Lin H, et al. Angiotensin II-Induced Apoptosis of Human Umbilical Vein Endothelial Cells was Inhibited by Blueberry Anthocyanin Through Bax- and Caspase 3-Dependent Pathways. Med Sci Monit. 2016;22:3223-3228.
3. Bierhansl L, Conradi LC, Treps L, Dewerchin M, Carmeliet P. Central Role of Metabolism in Endothelial Cell Function and Vascular Disease. Physiology. 2017;32:126-140.
4. Medina-Leyte DJ, Dominguez-Perez M, Mercado I, Villarreal-Molina MT, Jacobo-Albavera L. Use of Human Umbilical Vein Endothelial Cells (HUVEC) as a Model to Study Cardiovascular Disease: A Review. Appl Sci-Basel. 2020;10.
5. Xu J, Shi GP. Vascular wall extracellular matrix proteins and vascular diseases. Biochim Biophys Acta. 2014;1842:2106-2119.
6. Atlas SA. The renin-angiotensin aldosterone system: pathophysiological role and pharmacologic inhibition. J Manag Care Pharm. 2007;13(8 Suppl B):9-20.
7. Mehta PK, Griendling KK. Angiotensin II cell signaling: physiological and pathological effects in the cardiovascular system. Am J Physiol-Cell Ph. 2007;292:C82-C97.
8. Forrester SJ, Booz GW, Sigmund CD, Coffman TM, Kawai T, Rizzo V, et al. Angiotensin II Signal Transduction: An Update on Mechanisms of Physiology and Pathophysiology. Physiol Rev. 2018;98:1627-1738.
9. Wang M, Liu Y, Liang Y, Naruse K, Takahashi K. Systematic Understanding of Pathophysiological Mechanisms of Oxidative Stress-Related Conditions-Diabetes Mellitus, Cardiovascular Diseases, and Ischemia-Reperfusion Injury. Front Cardiovasc Med. 2021;8:649785.
10. Sena CM, Leandro A, Azul L, Seica R, Perry G. Vascular Oxidative Stress: Impact and Therapeutic Approaches. Front Physiol. 2018;9:1668.
11. Phaniendra A, Jestadi DB, Periyasamy L. Free Radicals: Properties, Sources, Targets, and Their Implication in Various Diseases. Indian J Clin Bioche. 2015;30:11-26.
12. Munzel T, Camici GG, Maack C, Bonetti NR, Fuster V, Kovacic JC. Impact of Oxidative Stress on the Heart and Vasculature Part 2 of a 3-Part Series. J Am Coll Cardiol. 201711;70:212-229.
13. Redza-Dutordoir M, Averill-Bates DA. Activation of apoptosis signalling pathways by reactive oxygen species. Biochim Biophys Acta. 2016;1863:2977-2992.
14. Massague J, Cheifetz S, Laiho M, Ralph DA, Weis FM, Zentella A. Transforming growth factor-beta. Cancer Surveys. 1992;12:81-103.
15. Tian M, Schiemann WP. The TGF-beta paradox in human cancer: an update. Future Oncol. 2009;5:259-271.
16. Kubiczkova L, Sedlarikova L, Hajek R, Sevcikova S. TGF-beta - an excellent servant but a bad master. Journal of Translational Medicine. 2012;10:183.
17. Kim SK, Ohta K, Hamaguchi A, Omura T, Yukimura T, Miura K, et al. Angiotensin-II Type-I Receptor Antagonist Inhibits the Gene-Expression of Transforming Growth-Factor-Beta-1 and Extracellular-Matrix in Cardiac and Vascular Tissues of Hypertensive Rats. J Pharmacol Exp Ther. 1995;273:509-515.
18. Crawford DC, Chobanian AV, Brecher P. Angiotensin II induces fibronectin expression associated with cardiac fibrosis in the rat. Circ Res. 1994;74:727-739.
19. Rosenkranz S. TGF-beta1 and angiotensin networking in cardiac remodeling. Cardiovasc Res. 2004;63:423-432.
20. Taniyama Y, Griendling KK. Reactive oxygen species in the vasculature: molecular and cellular mechanisms. Hypertension. 2003;42:1075-1081.
21. Riss TL, Moravec RA, Niles AL, Duellman S, Benink HA, Worzella TJ, et al. Cell Viability Assays. In: Markossian S, Grossman A, Brimacombe K, Arkin M, Auld D, Austin CP, et al., editors. Assay Guidance Manual. Bethesda (MD)2004.
22. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein measurement with the Folin phenol reagent. J Biol Chem. 1951;193:265-275.
23. Waterborg JH, Matthews HR. The Lowry method for protein quantitation. Methods Mol Biol. 1994;32:1-4.
24. Esen C, Alkan BA, Kirnap M, Akgul O, Isikoglu S, Erel O. The effects of chronic periodontitis and rheumatoid arthritis on serum and gingival crevicular fluid total antioxidant/oxidant status and oxidative stress index. J Periodontol. 2012;83:773-779.

25. Oparil S, Acelajado MC, Bakris GL, Berlowitz DR, Cifkova R, Dominiczak AF, et al. Hypertension. Nature reviews Disease primers. 2018;4:18014.

26. Van Haaster MC, McDonough AA, Gurley SB. Blood pressure regulation by the angiotensin type 1 receptor in the proximal tubule. Curr Opin Nephrol Hypertens. 2018;27:1-7.

27. Xu XP, He HL, Hu SL, Han JB, Huang LL, Xu JY, et al. Ang II-AT2R increases mesenchymal stem cell migration by signaling through the FAK and RhoA/Cdc42 pathways in vitro. Stem Cell Res Ther. 2017;8:164.

28. Tambelline N, Oliveira K, Olchanheski LR, Sordi R, Otuki MF, Favero GM, et al. The Effect of Losartan on Angiotensin II-Induced Cell Proliferation in a Rat Aorta Smooth Muscle Cell Line. Braz Arch Biol Techn. 2012;55:263-268.

29. Cicek Z, Yasar ZG, Akillioglu K, Dogan A. Effect of Intracellular Angiotensin II on Diabetic Rat Vascular Smooth Muscle Cell Proliferation. Acta Physiol. 2018;225:45.

30. Hwang YJ, Park JH, Cho DH. Activation of AMPK by Telmisartan Decreases Basal and PDGF-stimulated VSMC Proliferation via Inhibiting the mTOR/p70S6K Signaling Axis. Journal of Korean Medical Science. 2020;35:e289.

31. Feng LH, Sun HC, Zhu XD, Zhang SZ, Li XL, Li KS, et al. Irbesartan inhibits metastasis by interrupting the adherence of tumor cell to endothelial cell induced by angiotensin II in hepatocellular carcinoma. Annals of Translational Medicine. 2021;9:207.

32. Gong X, Shao L, Fu YM, Zou Y. Effects of Olmesartan on Endothelial Progenitor Cell Mobilization and Function in Carotid Atherosclerosis. Med Sci Monit. 2015; 21:1189-1193.

33. Wen H, Gwathmey JK, Xie LH. Oxidative stress-mediated effects of angiotensin II in the cardiovascular system. World J Hypertens. 2012;2:34-44.

34. Zhao Y, Vanhoutte PM, Leung SW. Vascular nitric oxide: Beyond eNOS. J Pharmacol Sci. 2015;129:83-94.

35. Miguel-Carrasco JL, Beaumont J, San Jose G, Moreno MU, Lopez B, Gonzalez A, et al. Mechanisms underlying the cardiac antifibrotic effects of losartan metabolites. Scientific Reports. 2017;7:41865.

36. Wenzel S, Taimor G, Piper HM, Schluter KD. Redox-sensitive intermediates mediate angiotensin II-induced p38 MAP kinase activation, AP-1 binding activity, and TGF-beta expression in adult ventricular cardiomyocytes. FASEB J. 2001;15:2291-2293.

37. Moriguchi Y, Matsubara H, Mori Y, Murasawa S, Masaki H, Maruyama K, et al. Angiotensin II-induced transactivation of epidermal growth factor receptor regulates fibronectin and transforming growth factor-beta synthesis via transcriptional and posttranscriptional mechanisms. Circulation Research. 1999;84:1073-1084.

38. Robertson IB, Rifkin DB. Regulation of the Bioavailability of TGF-beta and TGF-beta-Related Proteins. Cold Spring Harb Perspect Biol. 2016;8:a021907.

39. Derynck R, Budi EH. Specificity, versatility, and control of TGF-beta family signaling. Sci Signal. 2019;12.