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Intracellular Angiotensin-II Measurement in Streptozotocin-Induced Rat Vascular Smooth Muscle Cells and Its Relationship with Angiotensin-II Receptors

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ABSTRACT

Objectives: Angiotensin II (Ang-II) is vital constituent of renin angiotensin aldosterone system and increases in some cardiovascular diseases such as diabetes. However, there are not enough studies related to intracellular and extracellular Ang-II levels and its interaction with Ang-II type 1 and 2 receptors (ATR1, ATR2) in vascular smooth muscle cells (VSMCs). We aimed to investigate healthy and diabetic rat model of VSMCs (H-VSMCs, D-VSMCs) proliferation and Ang-II levels.

Methods: VSMCs were isolated from the aorta of healthy and diabetic Wistar rats. Diabetic model was achieved by intravenous administration of 45 mg/kg streptozotocin. Firstly, different Ang-II (0-1000 µM) concentration was performed for dose study. Ang-II (0.1 µM), Ang-II type 1 receptor (ATR1) antagonist (Olmesartan, 1 µM) and Ang-II type 2 receptor (ATR2) antagonist (PD123,319, 1 µM) were practiced together, and thereafter cell proliferation was evaluated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) method. Intracellular and extracellular Ang-II levels were measured by ELISA kit.

Results: While H-VSMCs proliferation increased in Ang-II 0.1, 0.01, 0.001 and 0.0001 µM, D-VSMCs proliferation increased Ang-II 0.1 and 0.01 µM applications ($P<0.05$). Olmesartan 1 µM inhibited proliferation in H-VSMCs. Ang-II detected intracellular and extracellular groups of VSMCs, but no significant difference was found between H-VSMCs and D-VSMCs groups ($P>0.05$).

Conclusions: Ang-II enhances proliferation of H-VSMCs and D-VSMCs. There is no relationship that could be established between intracellular and extracellular Ang-II levels, H-VSMCs and D-VSMCs proliferation and Ang-II receptors.

Keywords: Angiotensin II, Diabetes, Smooth Muscle Cell, Vascular

One of the most important components of the vascular structure is vascular smooth muscle cells (VSMCs). Numerous physiological (growth, vasoconstriction, extracellular matrix formation, vasodilatation) and pathological processes (hypertrophy, fibrosis, migration, proliferation, inflammation) occur in the media layer of the vascular tissue. Moreover, proliferation of VSMCs causes

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pathologies such as atherosclerosis, hypertension and diabetes mellitus [1, 2].

Primary cell culture of the VSMCs is constantly used in vitro studies by researchers. Different techniques are also used to obtain primary VSMCs culture. However, achieving pure VSMCs can be quite difficult, and it has some complex stages [3, 4].

VSMCs may also undergo phenotypic changes in vascular diseases [5, 6]. Many specific proteins are expressed in early (SMα-actin, myocardin, SM22-α, h-caldesmon and SM-calponin) and late (desmin, meta-vinculin, SM-1, SM-2 and smoothelin) phases from the early stages of the differentiation of VSMCs [7, 8].

The systemic or extracellular renin angiotensin aldosterone system (RAS) has various roles in regulating water and electrolyte homeostasis, blood pressure, proliferation and growth of the VSMCs, migration and inflammation in cardiovascular system [9, 10].

Local or intracellular RAS components have been identified in many different types of tissues and cells. But, role of the nuclear components of intracellular RAS is still unknown [11]. The major components of RAS are (1-) Angiotensinogen synthesized from the liver, (2-) Renin, produced from the juxtaglomerular apparatus (JGA) cells in kidneys and converts angiotensinogen to Angiotensin I (Ang-I), (3-) Angiotensin-converting enzyme (ACE), converts Ang-I to Ang-II, synthesized in lung capillaries (4-) Ang-II receptors (ATR1s, ATR2s) are responsible for Ang-II's cellular effects [12-14].

It is also known that the component of RAS is synthesized in different cells and secreted to the outside of the cell membrane [11, 15]. The secretion of Ang-II from vascular layer can be inhibited by ACE, ATR and renin inhibitors. Some studies demonstrate local production was controlled by secretion of Ang-II [11].

RAS is directly involved in physiology and physiopathology of the cardiovascular system. However, some of RAS components, signaling pathways, and cellular effects of local RAS are cited as unexplained aspects of this system [16].

Ang-II is locating center of RAS and has physiological and pathological effects in the cardiovascular system. While, Ang-II has acute effects such regulation blood pressure by regulating water, salt homeostasis and vasoconstriction, its chronic effects cause hyperplasia, hypertrophy and migration in vascular smooth muscle cells [17].

Ang-II shows its effects on VSMCs through ATR1 and ATR2. Inositol-3 phosphate (IP3) and diacylglycerol (DAG) are formed by binding to ATR1 and activate number of signaling pathways that induce vasoconstriction, cell proliferation, growth, fibrosis and inflammation [14, 18]. The formation of mitogen activated protein kinase (MAPK), protein kinase C (PKC), reactive oxygen products (ROS) and NADPH oxidase increase with the activation of tyrosine kinase receptors [19]. But binding to ATR2 results in increased nitric oxide (NO) release, leading to vasodilatation and decreased cell proliferation [9, 20].

It has been suggested that local RAS components can be selectively activated in many specific conditions and cell types that induce various cardiovascular pathological problems [12, 14]. Local RAS components also increased in diseases such as diabetes mellitus (DM), atherosclerosis and hypertension emerge in cardiovascular and many other tissues [21]. For all that, it is understood that it may play a key role in its development and progress of these diseases. However, the intracellular effects of local RAS components on cardiovascular tissue and the mechanism of these pathways have not been fully elucidated [15, 22]. When its effects on VSMCs mechanisms are figured out, intracellular RAS can be used to treat cardiovascular diseases [23].

Many in vivo and in vitro studies have been performed to compare the effects of systemic and local RAS components in normal and diabetic disease conditions. It has been indicated that VSMCs may have changes in their individual behaviors and interaction with each other, under physiological and pathological conditions. But there are differences in the cellular effects (proliferation, migration) of Ang-II and ATR in pathological conditions such as diabetes and hyperglycemia and it may lead several differences in cellular responses to ATR blockers that constantly used in the treatment of hypertension [24-26].

In the light of these aspects, we aim to investigate the effects of Ang-II, ATR1 antagonist (Olmesartan) and ATR2 antagonist (PD123,319) on proliferation, and determine the intracellular and medium Ang-II levels in healthy and streptozotocin (STZ)-induced

diabetic rat model VSMCs (H-VSMCs and D-VSMCs) in this study.

METHODS

Animals

We used Wistar albino male rats (8 weeks old, body weight 180-200 g) in the present study. Healthy and diabetic animals were housed under a 12-hour light/12-hour dark cycle in care rooms, fed with standard rat chow (MBD Feed Trade, protein 19%, Türkiye) and were allowed to water with ad libitum. Rats were anaesthetized with ketamine/xylazine (Ketasol 10%, richterpharma ag, Rompun 2%, Bayer, 100/10 mg/kg) was administered intraperitoneally. The diabetic rat model (n=5) was performed by intravenous injection of 45 mg/kg streptozotocin (STZ) (Cat-No: S0130, Sigma-Aldrich) in citrate buffer (10 mM, pH 4.5) through tail vein and the animals in the control group (n=5) were administered the same amount of citrate buffer. The blood glucose levels of the animals were measured with a glucometer (Gluco Dr). Healthy and diabetic rats blood glucose level evaluated before and three days after STZ application. Animals with blood glucose levels above 250 mg/mL were included in the diabetic model group (Figure 1). Primary cell culture of VSMCs were done 8 weeks after induction of diabetes.

Isolation of VSMCs from Healthy and Diabetic Model Rats

Firstly, abdomen of the anaesthetized rat was opened from the upper middle region under sterile conditions. The thoracic aorta was removed and transferred to a culture dish with cold transfer medium (Hank's Balanced Salt Solution-HBSS, Cat-No: L2055, Biochrom Merck, Calcium chloride dihydrate, Cat-No: C7902, Sigma-Aldrich, Penicillin-streptomycin-PSA, Cat-No: P4333, Sigma-Aldrich) (Figure 2).

The isolated tissue was cleaned from connective tissue under a stereo microscope and endothelial layer was scraped slowly and lightly along the vessel. After

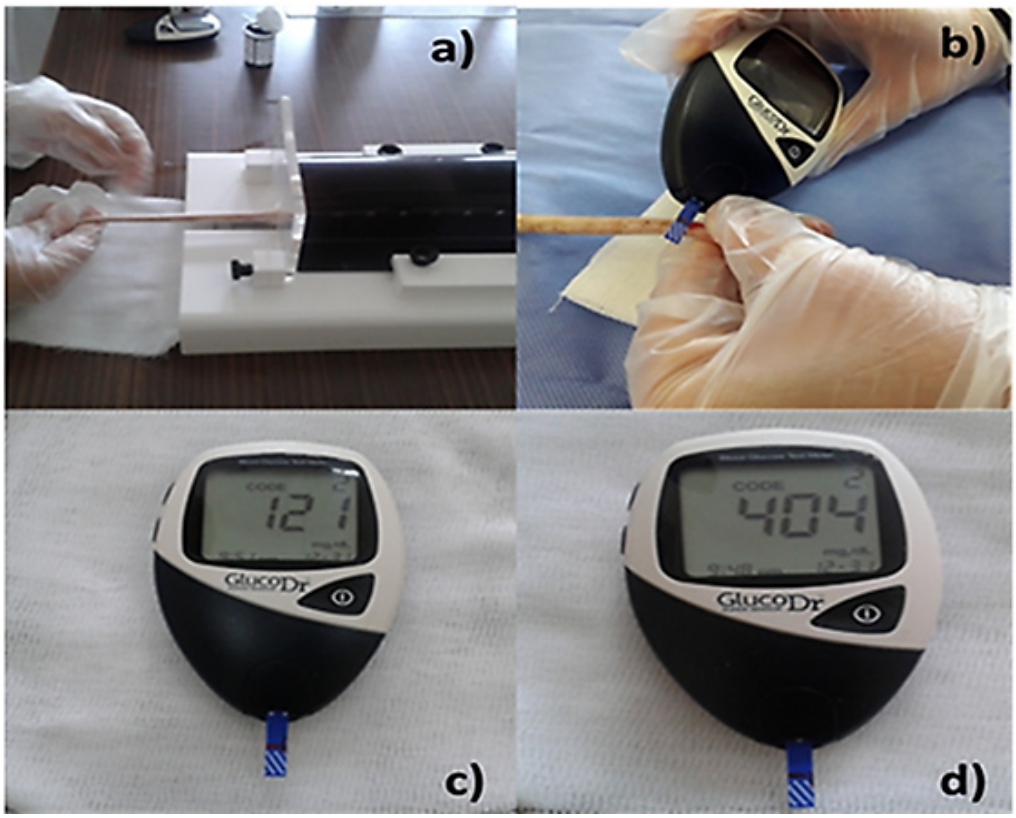

FIGURE 1. Diabetic animal. a) Placing the animal in the handle, b) Measurement of blood glucose levels with glucometer, c) Normal rat blood glucose, and d) Diabetic rat blood glucose.

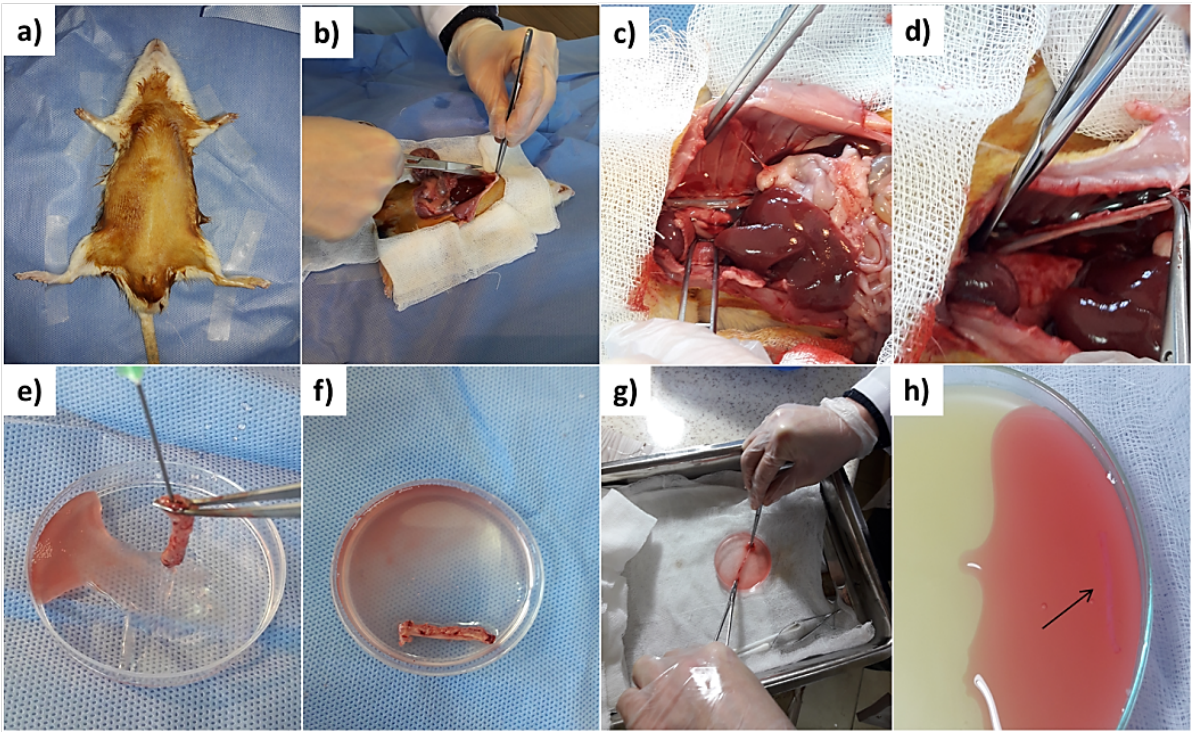

FIGURE 2. Isolation of the aorta. a) Sterilizing the abdomen, b) Cutting the midline of the abdomen, c) Isolation of the thoracic aorta, d) Holding the end of the vein with forceps, e) Cleaning the inner section of aorta with transfer solution, f) Isolated aorta, g) Cleaning the tissue surrounding the vessel, and h) Isolated aorta indicated by arrow.

separating the media layer from the adventitia, it was taken into a petri dish containing enzyme dissociation solution (HBSS, HEPES, Bovine serum albumin (BSA, Cat-No: A3156, Sigma-Aldrich), Trypsin inhibitor from glycine (STI, Cat-No: T6522, Sigma-Aldrich), Elastase (Cat-No: E7885, Sigma-Aldrich), Collagenase (Cat-No: C2674, Sigma-Aldrich), Calcium chloride dihydrate. It was cut into small pieces and incubated 45 min at 37°C in Dulbecco's Modified Eagle Medium (DMEM, Cat-No: D6046, Sigma-Aldrich) containing 20% Fetal Bovine Serum (FBS, Gibco, Thermo Fisher Scientific) and 1% Penicillin-Streptomycin (PSA, Cat-No: P4343, Sigma-Aldrich). It was centrifuged at 300 g for 5 min (Figure 3). The pellet was seeded in a 25 cm2 cell culture dish with medium. When the cells were examined with inverted microscope on day 5, it was seen that the cells adhered to the culture plate basement and started to proliferate. The culture cell medium was changed every 72 hours and incubated in a 37°C incubator with 5% CO_2, 95% air mixture and humidity. When cells were grown to 70 to 80% confluence passaging was performed.

The primary culture of VSMCs exhibited typical spindle-shaped appearance with characteristic 'hill and valley' patterns [27, 28]. All experiments were performed in VSMCs between passages 4 and 8. It was observed that in our study isolated thoracic aorta of the diabetic animals was very fragile and tended to fragmentation. The probability of culturing VSMCs from diabetic rats was at a very low rate compared to healthy rats. While rate of successful culture from diabetic animals is ~10%, in which vascular cells are obtained and attached to the base, ~90% in healthy animals.

Cell Purity and Identification

Immunocytochemistry (IHC) method performed to show expression of SMα-actin, caldesmon and calponin proteins. Firstly, VSMCs are seeded in polylysine-coated slides. They were placed in petri dishes containing cell media and incubated for 3-5 days. IHC staining confirmed positive for α-SMA, caldesmon and calponin. Our primary VSMCs culture method showed higher purity incidence and morphology similarity.

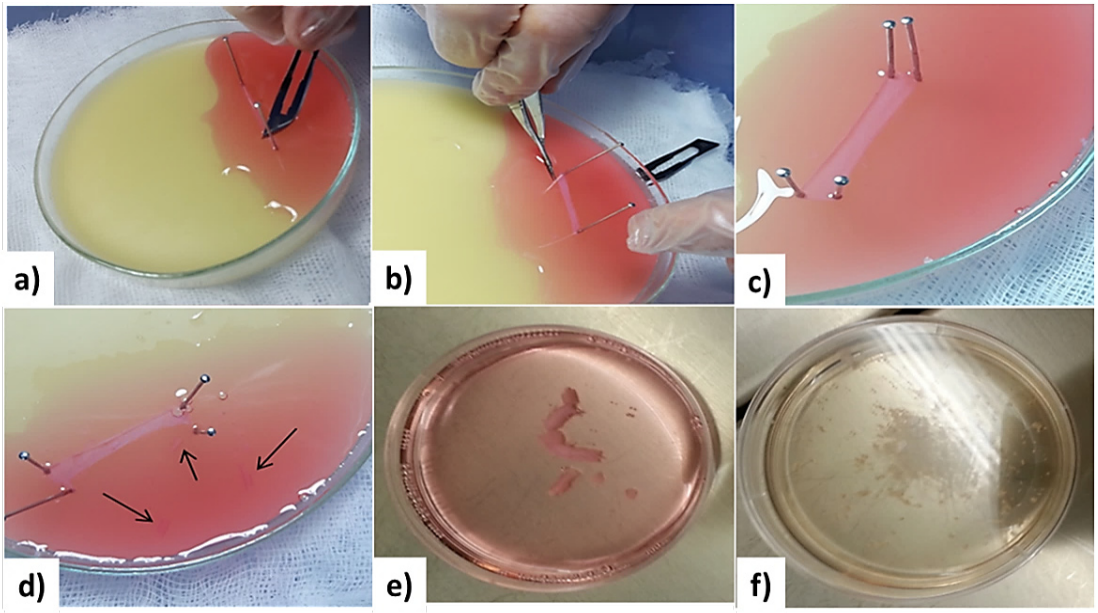

FIGURE 3. VSMCs isolation. a) Cleaning the adventitia layer with scalpel, b) Cutting the aorta with microsurgical scissors, c) Stretching the aorta from the inner surface with the needles, d) Isolated media layer indicated black arrows. Enzyme dissociation stage, e) Isolated smooth muscle layer, and f) Smooth muscle layer divided into pieces. VSMCs, vascular smooth muscle cells.

MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) Assay

H-VSMCs and D-VSMCs were seeded at a density of 5×103-10x103 cells/100 µL per well and incubated for 48 hours before drug treatment. Then, cells were incubated for 24-48 hours with Ang-II concentrations (0-Control-1000 µM, [Val5]-Angiotensin II acetate salt hydrate, Cat-No: A2900, Sigma-Aldrich) for dose study. Maximum proliferative Ang-II dose was determined for 24 hours and administered with Olmesartan (1 µM, Cat-No: SML1394, Sigma-Aldrich) and PD123,319 (1 µM, Cat-No: P186, Sigma-Aldrich). After removing solutions 100 uL fresh medium and 10 µL Thiazolyl Blue Tetrazolium Bromide-5 mg/mL (MTT, Cat-No: M5655, Sigma-Aldrich) was added to each well and incubated 3-4 hours. Then, MTT solution is removed and 100 µL dimethyl sulfoxide (DMSO, Cat-No: M116743100, Merck) was added for dissolving formazan. After 20 min of incubation, the absorbance values of the wells at 570 nm wavelength were measured on the plate reader (Eon, Biotek). The average absorbance value of each group was determined, and the percentage viability of the cells was calculated.

Preparation of Vsmcs and Medium Extracts

H-VSMCs and D-VSMCs (~0.5×106) were seeded on 6-well plates. Then, Ang-II, (0.1 µM), Olmesartan, (1 µM) and PD123,319, (1 µM) were applied for 24 hours. Mediums in wells were collected and centrifuged at 300 g for 10 min. Cell extracts prepared with RIPA lysis buffer (Cat-No:SC-24948A, Santa Cruz Biotechnology) according to protocol. RIPA buffer-450 µL added to wells. Cells were removed from the surface with a plastic cell scraper and centrifuged at 10.000 g for 15 min at +4°C. Supernatant was stored at -80°C until the study carried out.

Total Protein Measurement by Lowry Method

The protein amount of samples between 0.01-1.0 mg/mL can be determined with this method [29]. BSA doses (0-4 mg/mL), blank and unknown samples (50 µL) were pipetted into wells and incubated at room temperature for 45 min. C reagent (150 µL, 100:1 mixture of A (2% Na2CO3, 0.4% NaOH, 0.16% Na-tartrate) and B (4% CuSO4.5H2O) reagents) incubated 20 min and following Folin-Ciocalteau's reagent (3 µL) was added and incubated 30 min. Absorbance values of the samples and standards were measured at

660 nm wavelength. The standard curve created with BSA absorbance values and protein concentration of the cell extracts were calculated.

ELISA

Ang-II levels in H-VSMCs, D-VSMCs and mediums was measured with a competitive ELISA kit (Angiotensin II EIA Kit, Cat-No: RAB0010, Sigma-Aldrich). Standards were prepared and other experimental steps were performed in accordance with the specified protocols. The absorbance values of the samples at 450 nm were measured. The medium Ang-II level was determined in pg/mL. H-VSMCs and D-VSMCs Ang-II level pg Ang-II/mg protein was calculated based on the protein amount of the samples.

Statistical Analysis

The data were analyzed using SPSS statistics 21.0 program (SPSS Inc, Chicago, 9 Illinois, USA) and expressed as mean±SE. One-way ANOVA was used as a parametric test in the case of homogeneous distributions. Post hoc Dunnett's and Tukey test were used to show the difference between groups. Mann Whitney U test was used as non-parametric Kruskal Wallis Post Hoc for data that were not homogeneously distributed. $P<0.05$ value was considered as significant.

RESULTS

Animal Blood Sugar and Urine Levels

Blood glucose levels of healthy and diabetic animals were monitored by glucometer before and on the 3rd day after STZ administration. Measurement of blood glucose levels followed for eight weeks. The urine of healthy and diabetic animals was measured with a strip. It was observed that levels of blood glucose in healthy rats were between 120-140 mg/dL, STZ-induced rats glucose changed between 330-430 mg/dL in 8 weeks. While urine glucose levels were <50 mg/dL in healthy rats, it was measured in diabetic

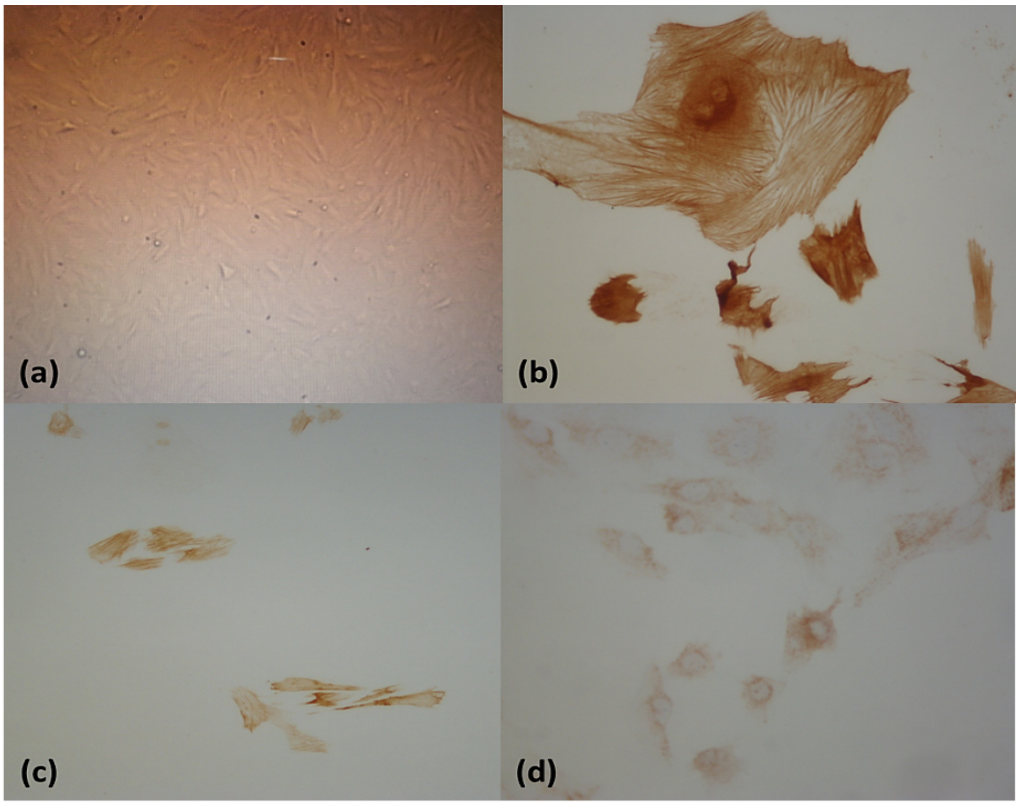

FIGURE 4. Evaluation of VSMCs. (a) View of VSMCs in light microscopy before being stained (×200), (b) VSMCs stained with α-SMA (×400), (c) VSMCs stained with caldesmon (×200), and (d) VSMCs stained with calponin (×400). VSMCs, vascular smooth muscle cells; α-SMA, α-smooth muscle actin.

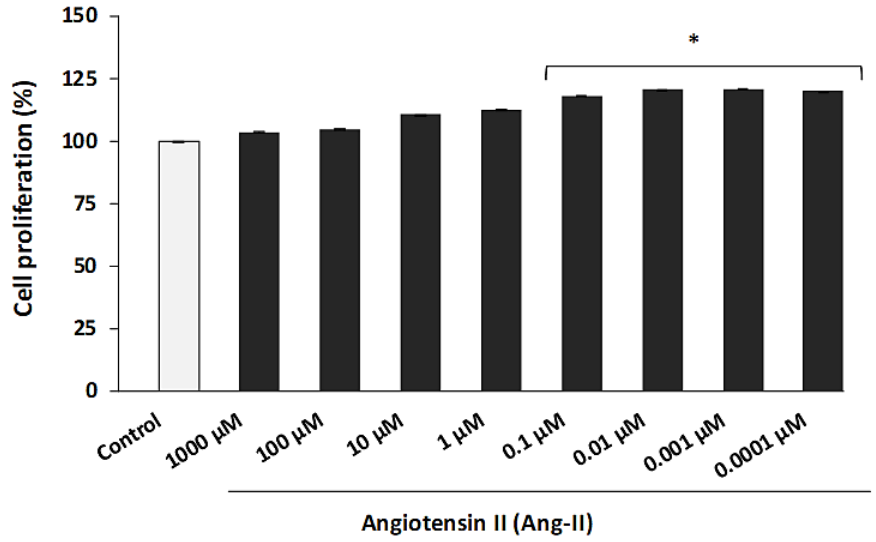

FIGURE 5. Effect of Ang-II doses on H-VSMCs proliferation for 24 h. *Compared with the control group (P<0.05). Results shown as mean±SE for the three experimental replicates (n=6-12). H-VSMCs= healthy rat model vascular smooth muscle cells, SE=standard error.

rats as 500-1000 mg/dL. However, proteinuria was detected in diabetic model rats, while proteinuria was not observed in healthy rats with urine strips.

Evaluation of VSMCs

The VSMCs were adhered to the glass coverslip surface and multiplied before the staining and VSMCs proliferation was evaluated with the inverted microscope (Figure 4a). After VSMCs covered the slide surface entirely, the specimens were incubated with α-SMA, caldesmon and calponin antibodies by IHC (immunohistochemical) method protocol (Figures. 4b, 4c and 4d). The cells used in the study were verified to be VSMCs.

Effect of Ang-II on H-VSMCs Proliferation for 24 and 48 Hours

It was determined that the Ang-II doses (0-1000

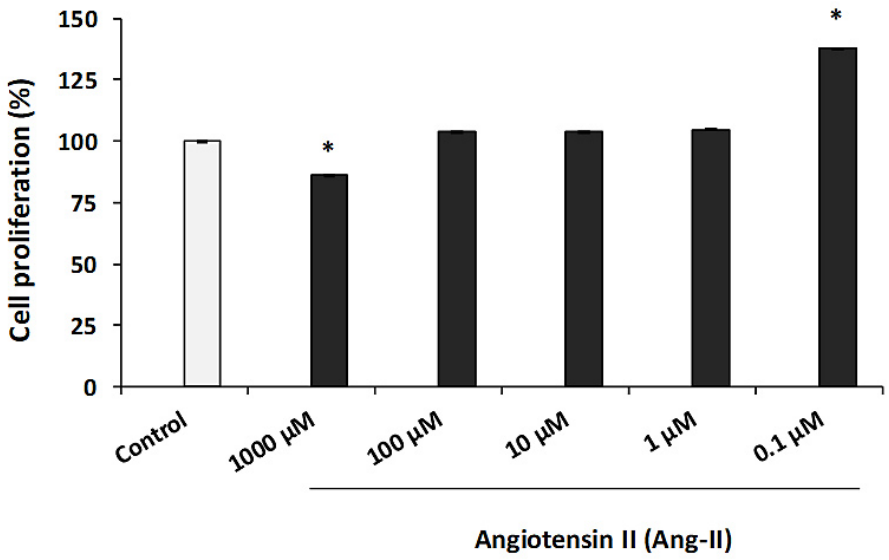

FIGURE 6. Effect of Ang-II doses on H-VSMCs proliferation for 48 h. *Compared with the control group (P<0.05). Results shown as mean±SE for the three experimental replicates (n=12-15). H-VSMCs, healthy rat model vascular smooth muscle cells; SE, standard error.

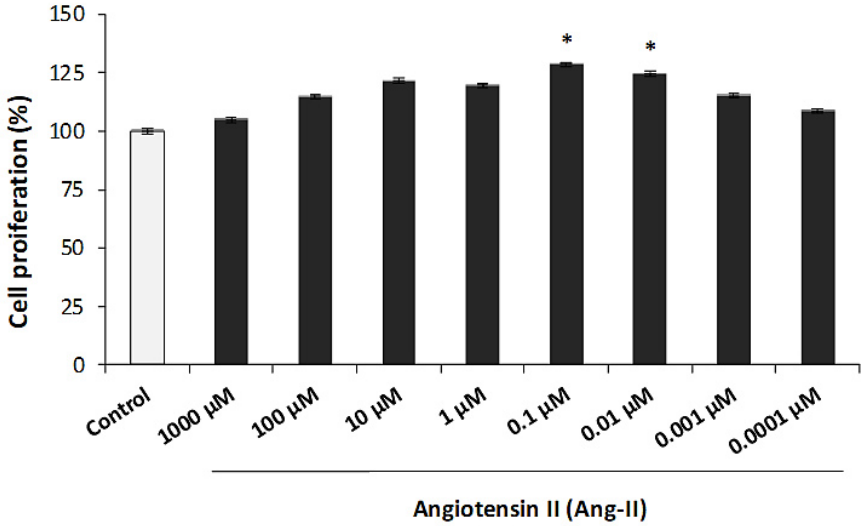

FIGURE 7. Effect of Ang-II doses on D-VSMCs proliferation for 24 h. *Compared with the control group (P<0.05). Results shown as mean±SE for the three experimental replicates (n=6-7). D-VSMCs, diabetic rat model vascular smooth muscle cells; SE, standard error.

µM) effect H-VSMCs proliferation for 24 h and 48 h. The H-VSMCs proliferation increased in Ang-II (0.1 µM) by 18%, Ang-II (0.01 µM) by 20%, Ang-II (0.001 µM) by 20% and Ang-II (0.0001 µM) by 20% (P<0.001). It was observed that Ang-II elevated H-VSMCs proliferation in a dose-dependent manner. Maximum cell proliferation was seen in Ang-II (0.1 µM) dose. Consequently, it was selected for subsequent combined applications (Figure 5).

While Ang-II (0.1 µM) increased cell proliferation in H-VSMCs for 48 h by 37%, Ang-II (1000 µM) dose reduced cell proliferation by 14% (P<0.001) (Figure 6).

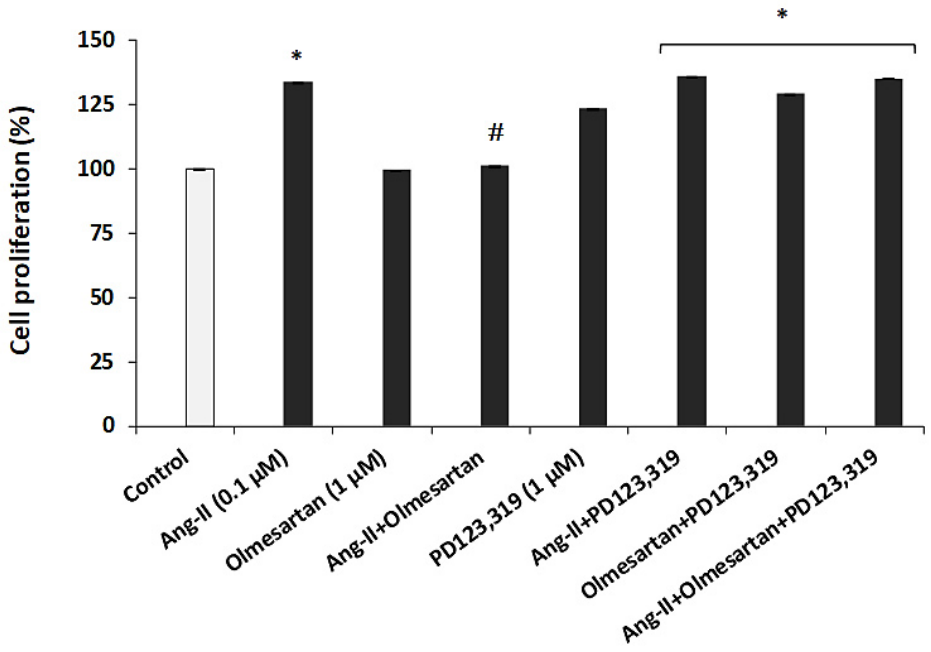

FIGURE 8. Effect of Angiotensin II (Ang-II), Olmesartan and PD123,319 on H-VSMCs proliferation for 24 h. *Compared with the control group, ✓Compared with the Ang-II group (P<0.05). Results shown as mean±SE for the three experimental replicates (n=12). H-VSMCs, healthy rat model vascular smooth muscle cells; SE, standard error.

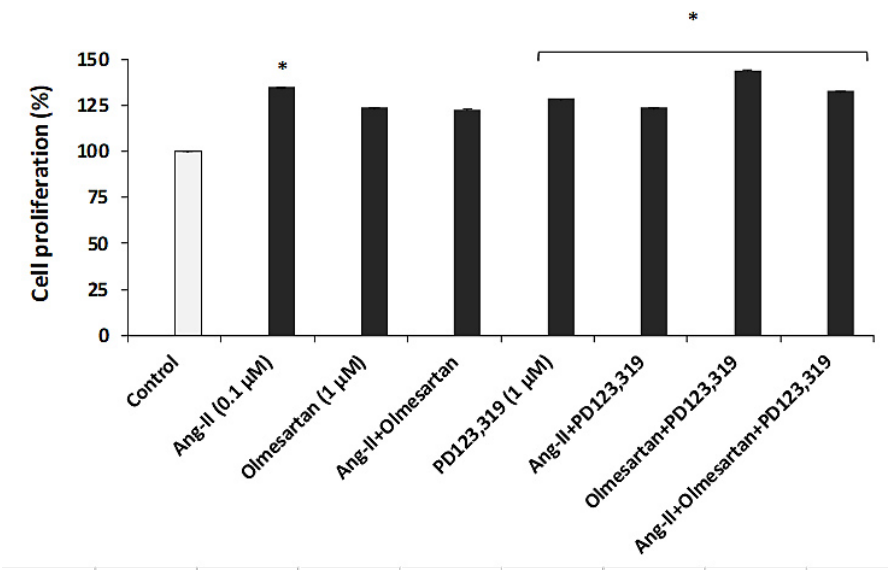

FIGURE 9. Effect of Angiotensin II (Ang-II), Olmesartan and PD123,319 on D-VSMCs proliferation for 24 h. *Compared with the control group (P<0.05). Results shown as mean±SE for the three experimental replicates (n=12). D-VSMCs, diabetic rat model vascular smooth muscle cells; SE, standard error.

Effect of Ang-II on D-VSMCs Proliferation for 24 Hours

D-VSMCs proliferation increased in the application of Ang-II (0.1 µM) by 28% (P=0.010) and Ang-II (0.01 µM) by 24% (P=0.030) (Figure 7).

Effect of Angiotensin II, Olmesartan and PD123,319 on H-VSMCs Proliferation for 24 Hours

There was a significant difference between the groups of H-VSMCs in single and combined applications of Ang-II (1 µM), Olmesartan (1 µM) and

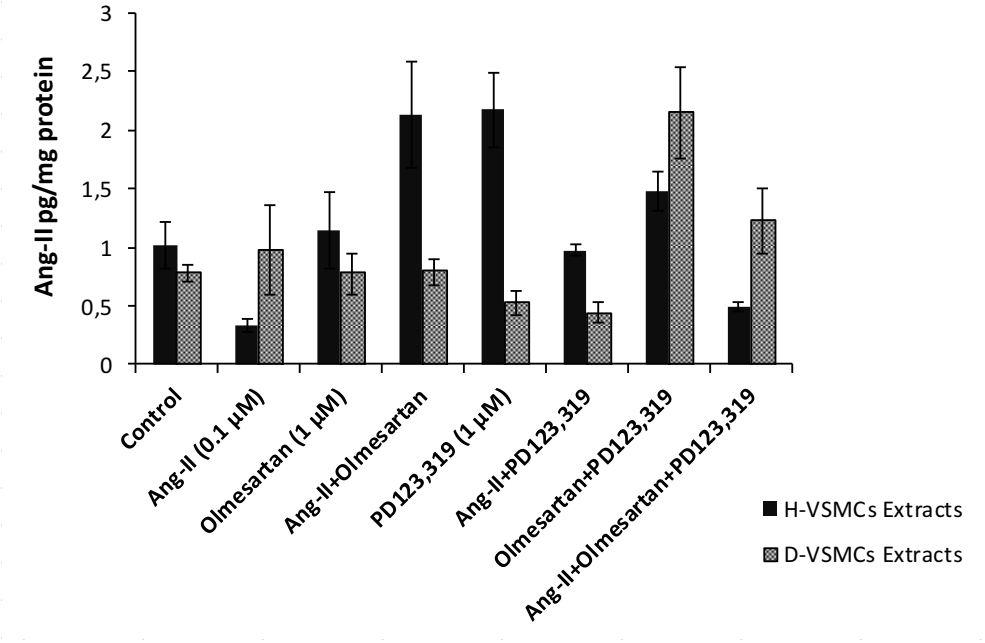

FIGURE 10. Ang-II levels in H-VSMCs and D-VSMCs extracts groups. Results shown as mean±SE for the experimental replicates (n=4). H-VSMCs, healthy rat model vascular smooth muscle cells; D-VSMCs, diabetic rat model vascular smooth muscle cells; SE, standard error.

PD123,319 (0.1 µM). Cell proliferation increased in Ang-II (0.1 µM) by 33% (P=0.007), Ang-II+PD123,319 by 36% (P=0.001), Olmesartan +PD123,319 by 29% (P=0.008), Ang-II+Olmesartan +PD123,319 by 35% (P=0.001) compared with the control group. But no significant difference was observed when Olmesartan (P=1.000) and PD123,319 compared with the control group (P=0.071). When Ang-II+Olmesartan group compared with the Ang-II (0.1 µM) group it was determined that cell proliferation decreased by 32% (P=0.028) (Figure 8).

Effect of Angiotensin II, Olmesartan and PD123,319 on D-VSMCs Proliferation for 24 Hours

Significant differences were found in D-VSMCs proliferation in single and combine treatment of Ang-II (0.1 µM), Olmesartan (1 µM) and PD123,319 (1 µM). Cell proliferation increased significantly in Ang-II (0.1 µM) by 34% (P=0.002), PD123,319 by 28% (P=0.011), Ang-II+PD123,319 by 23% (P=0.049), Olmesartan+PD123,319 by 44% (P<0.001), Ang-II+Olmesartan+PD123,319 groups by 32% (P<0.001) compared with the control group (Figure 9).

Ang-II Levels in H-VSMCs and D-VSMCs Extracts

Ang-II levels in H-VSMCs and D-VSMCs extract (intracellular) groups expressed as pg/mg protein. We didn't find significant difference between the groups of H-VSMCs and D-VSMCs extracts groups. Intracellular Ang-II levels decreased in Ang-II (0.1 µM) (P=0.338), Ang-II+PD123,319 (P=0.999) and Ang-II+Olmesartan+PD123,319 groups in H-VSMCs compared with the control group (P=0.953). However, it was detected that Ang-II increased in Olmesartan (1 µM), Ang-II+Olmesartan, PD123,319 (1 µM) and Olmesartan+PD123,319 groups compared with the control group in H-VSMCs (P=0.766).

Contrary to these findings, it was determined that Ang-II levels increased in Ang-II (0.1 µM) (P=1.000), Ang-II+Olmesartan (P=0.998), Olmesartan+PD123,319 (P=0.089) and Ang-II+Olmesartan+PD123,319 groups compared with the control group in D-VSMCs (P=0.945). Ang-II levels declined in Olmesartan (1 µM) (P=1.000), PD123,319 (1 µM) (P=0.926) and Ang-II+PD123,319 groups in compared with the control group in D-VSMCs (P=0.954) (Figure 10).

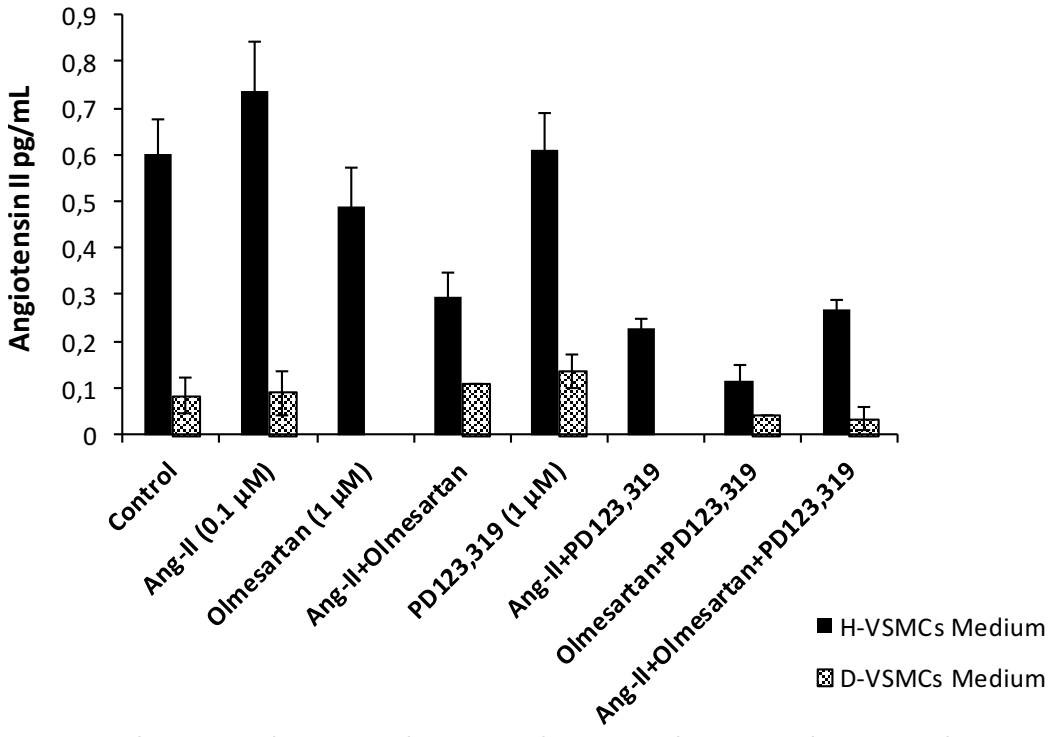

FIGURE 11. Ang-II levels in H-VSMCs and D-VSMCs medium groups. Results shown as mean±SE for the experimental replicates (n=4). H-VSMCs, healthy rat model vascular smooth muscle cells; D-VSMCs, diabetic rat model vascular smooth muscle cells; SE, standard error.

Ang-II Levels in H-VSMCs and D-VSMCs Medium

Ang-II levels in H-VSMCs and D-VSMCs medium (extracellular) groups were presented as pg/mL. Ang-II levels in H-VSMCs groups higher than all the D-VSMCs groups. But there is no significant difference between the groups of H-VSMCs and D-VSMCs.

Ang-II levels increased in Ang-II (0.1 µM) (P=0.822) and PD123,319 (1 µM) groups compared with the control group in H-VSMCs (P=0.992). On the other hand, Ang-II levels attenuated in Olmesartan (1 µM), Ang-II+Olmesartan, Ang-II+PD123,319, Olmesartan+PD123,319 and Ang-II+Olmesartan+PD123,319 groups compared with the control group in D-VSMCs (P=0.438).

We could not detect Ang-II in Olmesartan and Ang-II+PD123,319 groups in D-VSMCs (Figure 11). Ang-II levels increased in Ang-II+Olmesartan and PD123,319 (1 µM) groups compared with the control group in D-VSMCs. However, Ang-II levels decreased in Olmesartan+PD123,319 and Ang-II+Olmesartan+PD123,319 groups compared with the control group in D-VSMCs (P=0.825).

DISCUSSION

Intracellular components of RAS and its effects of signaling pathways have many undisclosed aspects. Local RAS can have also selective effects in many specific conditions and cell types, that lead to inducing various cardiovascular pathological problems. It has been stated that local RAS components as well as systemic RAS are increased in cardiovascular and many other tissues where diseases such as diabetes, atherosclerosis and hypertension occur [22].

The intracellular effect of local RAS components on cardiovascular tissue and the mechanism of these pathways has not been fully elucidated. It may play a key role in the development and progression of cardiovascular diseases [30]. However, there are differences in the cellular effects (proliferation, migration) of Ang-II and ATRs in pathological conditions such as diabetes and hyperglycemia [31]. These changes may lead to differences in cellular responses to agents such as ATR1 blockers being used in the treatment of cardiovascular pathologies [30].

In the dose study of Ang-II in H-VSMCs and D-VSMCs, we found a dose-dependent response curve. The highest proliferating dose of Ang-II in 24- and 48-hours applications was determined as 0.1 µM and this dose was used in combined applications. Ang-II (1000 µM) application also significantly reduced cell proliferation. Our result showed that, high doses of Ang-II caused cell death. In a study of primary VSMCs, 100 nM Ang-II application for 24 hours showed an increase of 80% in protein synthesis and 45% in cell proliferation. In the same study, it was stated that 100 nM Ang-II was the most increased dose of hypertrophy in VSMCs [32]. This dose is like the Ang-II dose (0.1 µM) used in our study.

Moreover, Ang-II has been reported to be a potent stimulant for VSMCs and has been investigated to cause an increase in cell volume and protein content [33]. But in another study, Ang-II (100 µM) administration for 24 and 48 hours significantly increased cell proliferation. The proliferative dose of Ang-II was 1000 times higher than our dose [34]. It was considered that may be occurred due to the use of a different cell culture line such as A7r5 is the embryonic rat smooth muscle cell line. Moreover, A7r5 cells may have the ability to reproduce more rapidly and actively due to their embryonic origin. For this reason, changes in the proliferation responses of different doses of Ang-II can be seen.

In current study, Ang-II is a potent hypertrophic agent but has no sufficient evidence regarding with its mitogenic activity compared D-VSMCs to H-VSMCs. When the groups were compared in terms of proliferation in Ang-II applications, D-VSMCs proliferation tended to increase compared to H-VSMCs. It has also been suggested that the cells undergo hypertrophy along with proliferation under pathological conditions. When we examined H-VSMCs and D-VSMCs under inverted microscope, some morphological changes (increase in diabetic cell size) between H-VSMCs and D-VSMCs were observed. These morphological changes in VSMCs may clarify why we cannot see significant proliferation increase in our study.

In a study, administration of Ang-II to rats for 2 weeks has been shown to cause hypertension and hypertrophy of the vascular smooth muscle layer [35]. In other study has indicated that activation Ang-II

synthesis increases vascular wall and intima thickness of large arteries in diseases such as atherosclerosis, hypertension and diabetes. In addition, it may cause hypertrophy, hyperplasia and migration in VSMCs and it has been known that Ang-II has these effects mainly on ATR1 and ATR2 [6].

It was observed that in our study, Ang-II, Ang-II+PD123,319, Olmesartan+PD123,319 and Ang-II+Olmesartan+PD123,319 groups caused an increase in cell proliferation, while a decline was seen in Ang-II+Olmesartan group in H-VSMCs. Olmesartan 1 µM also inhibits cell proliferation in H-VSMCs but not detected a significant decrease in D-VSMCs. This may be thought to be due to Olmesartan dose not being sufficient to protect against vascular dysfunction and proliferation caused by diabetes mellitus. But in an in vivo study, 0.1 µM Ang-II showed that cell proliferation in both obese and non-obese Wistar rats and it was significantly attenuated with application of 0.1 µM Ang-II+0.1 µM Olmesartan. In this study, the dose of 0.1 µM Olmesartan was sufficient to decrease cell proliferation [36]. However, higher dose of Olmesartan was also used to inhibit proliferation and migration in another study [37]. It has been reported in the literature that ATR blockers inhibit VSMCs proliferation both in vitro and in vivo research [24]. In addition, it has been shown that, 10 µmol/L and 100 µmol/L Losartan application in A7r5 cells significantly reduced Ang II-induced cell proliferation in co-administration with 100 µmol/L Ang-II. It was stated that dose of 100 µmol/L Losartan significantly attenuated cell proliferation. This finding had been associated with intracellular Ang-II production by researchers [34].

Likewise, it was determined that, while the number of cells increased, application of 0.1 µM PD123,319 with 0.1 µM Ang-II attenuate apoptosis significantly in VSMCs. It has been reported that ATR2 blockade has increased cell proliferation [38]. However, the effect of ATR2 on cell proliferation could not be determined in our study when 0.1 µM Ang-II and 1 µM PD123,319 were applied together.

We have thus shown in our study that rat aortic smooth muscle cells synthesize Ang-II. And we found that intracellular Ang-II levels in H-VSMCs extracts and D-VSMCs extracts were higher than the extracellular Ang-II levels in H-VSMCs and D-VSMCs medium. But we did not detect any differences between groups in intracellular and extracellular Ang-II levels compared in H-VSMCs and diabetic D-VSMCs.

There are also some studies in which the Ang-II level is measured differently. In a review study, it is indicated that the plasma value of Ang-II in humans has been measured as 5-35 fmol/mL and 8.7 fmol/mL [17]. Ang-II levels in human arterial blood were determined by Catt *et al*. [39] 0.5-4.7 mµg/100 mL, Boyd *et al*. [40] 0.8-5.6 mµg/100 mL in venous plasma, Gocke *et al*. [41] (1968) stated it as 1.8-11.0 mg/100 mL [42]. Campbell *et al*. [43] measured Ang-II level in plasma as 50 fmol/mL [43]. It is stated that the plasma levels of RAS components in different species such as rats may be lower than humans [17].

RAS components are found in small amounts inside of the cells, which can be changed in pathological conditions. Due to the increase of Ang-II levels in various cardiovascular pathologies, it has been seen that the determination of Ang-II levels is very substantial. It is also necessary to fully analyze both the intracellular and extracellular molecular mechanism linkages of this system.

Strengths and Limitations

It was crucial to isolate and then produce smooth muscle cells from aorta of diabetic rats. Our chances of success were low because of the fragility of diabetic animal aorta. The process of isolating vascular smooth muscle cells had become very sensitive due to the damage caused by diabetes in the vascular structure. Ang-II measurement was quite difficult in consequence of low intracellular Ang-II levels among species and different tissues. Furthermore, short half-life and instability of Ang-II were also among the limitations of our study.

CONCLUSION

In current study, Ang-II increased healthy and diabetic VSMCs proliferation. When Ang-II and Olmesartan, an ATR1 antagonist, were applied together, proliferation decreased in healthy VSMCs. A decrease was also seen in diabetic VSMCs, but it was not significant. However, when Ang-II and PD123,319, an

ATR2 antagonist were administered together to healthy and diabetic VSMCs, no effect on proliferation was determined. In addition, since there was no meaning difference in intracellular and extracellular Ang-II levels compared in healthy and diabetic VSMCs, no relationship could be established between Ang-II levels, cell proliferation and Ang-II type 1 and 2 receptors in diabetes. Furthermore, it has been considered that further studies and methods with high accuracy and sensitivity were needed to measure Ang-II levels. Thus, comparisons of Ang-II levels between groups can be made more clearly and accurately. Additionally, it may enable the determination of the relationship between Ang-II levels and their effect on vascular smooth muscle cells proliferation.

Ethics Approval and Consent to Participate

The present study was approved by Çukurova University Animal Experiments Local Ethics Committee (Decision No: 2016/11-1 and date: 22.12.2016, Adana, Türkiye). All experimental procedures were carried out accordance with the U.K. Animals (Scientific Procedures) Act, 1986 and associated guidelines, EU Directive 2010/63/EU for animal experiments, or the National Institutes of Health guide for the care and use of Laboratory animals (NIH Publications No. 8023, revised 1978).

Data Availability

All data generated or analyzed during this study are included in this published article. The data that support the findings of this study are available on request from the corresponding author, upon reasonable request.

Authors' Contribution

Study Conception: ZÇ, KA, ZGY, AD; Study Design: ZÇ, KA, ZGY, AD; Supervision: ZÇ, KA, ZGY, AD; Funding: CÜBAP-TTU No. 2017-8071; Materials: N/A; Data Collection and/or Processing: ZÇ, KA, ZGY; Statistical Analysis and/or Data Interpretation: ZÇ, KA; Literature Review: ZÇ, KA, AD; Manuscript Preparation: ZÇ, KA, AD; and Critical Review: ZÇ, KA, AD.

Conflict of Interest

The author(s) disclosed no conflict of interest during the preparation or publication of this manuscript.

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Generative Artificial Intelligence Statement

The author(s) declare that no artificial intelligence-based tools or applications were used during the preparation process of this manuscript. The all content of the study was produced by the author(s) in accordance with scientific research methods and academic ethical principles.

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