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THE EFFECTS OF ANGIOTENSIN II, OLMESARTAN AND PD123,319 ON PROTEIN KINASE C IN STZ INDUCED RAT VASCULAR SMOOTH MUSCLE CELL CULTURE

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Abstract: Protein kinase C (PKC) is a promoter enzyme that plays a vital role in signal transduction of vascular smooth muscle cells (VSMCs). It has numerous vascular functions, such as vascular cell growth, cytokine activation and angiogenesis. But, these mechanisms are deteriorating in diabetes mellitus. Angiotensin II (Ang II) effects on vascular structure binding Ang II type-1 and type-2 receptors (ATR1 and ATR2) and stimulates PKC mostly through ATR1s. We aim to investigate the effects of Ang II, Olmesartan and PD123,319 on PKC levels of healthy and streptozotocin (STZ) induced rat VSMCs (H-VSMCs and STZ-VSMCs) in this study. The primary culture of VSMCs were isolated from thoracic aorta of healthy and STZ (45 mg/kg, given via the tail vein) induced Wistar rats. PKC levels were measured in H-VSMCs and STZ-VSMCs by enzyme-linked immunosorbent assay (ELISA). In H-VSMCs, Ang II group compared to the control group, PKC levels decreased significantly (P = 0.000); whereas, Ang II group compared to control, PKC levels were higher, but not significantly in STZ-VSMCs (P = 0.088). PKC levels were increased in Ang II+Olmesartan (P = 0.000) and Ang II+PD123,319 (P = 0.000) groups compared to Ang II group in H-VSMCs, but in STZ-VSMCs, PKC levels in Ang II+Olmesartan (P = 0.001) and Ang II+PD123,319 (P = 0.000) groups compared to Ang II group were decreased significantly. Ang II, its receptors and PKC seem to modulate each other and may have a relationship in hyperglycemic conditions. Also, considering that, ATR1 blocker (ATR1B) Olmesartan and ATR2 blocker (ATR2B) PD123,319 may be protective against vascular injury by reducing PKC levels in STZ-VSMCs.

Keywords: Ang II, PKC, Vascular smooth muscle, Cell culture

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1. Introduction

Diabetes mellitus is a metabolic syndrome that characterized with the lack of insulin and insulin insensitivity that leads to hyperglycemia (Daryabor et al., 2020). Over the last decades, diabetes has rising incidence throughout the world. It has been known that hyperglycemia is the main reason for the development of macrovascular and microvascular complications of diabetes (American Diabetes, 2009; Daryabor et al., 2020). For all that, it has been reported, advanced glycation end products, polyol pathway and renin angiotensin aldosteron system (RAS) activation, reactive oxygen products (ROS) formation and PKC increases as a result of hyperglycemia in studies (Forrester et al., 2018; Sharma and Sharma, 2013). Diabetes mellitus has adverse long term effects that involves in the evolution and progression of vascular injury. In vitro and in vivo researches stated that, these complications are associated with the complex mechanisms that leading to initiate a large number of intracellular signaling pathway changes (American Diabetes, 2009; Naudi et al., 2012).

VSMCs are the fundamental components of vascular structure and play a substantial role in consist of the vascular physiology and pathology. Primary culture of the VSMCs has been widely used as a precious vehicle to investigate the molecular mechanisms of vascular structure (Rameshrad et al., 2016; Jaminon et al., 2019).

The RAS has many roles in the cardiovascular system pathophysiologies such as heart attack, atherosclerosis and hypertension (Paz Ocaranza et al., 2020). Systemic RAS components are secreted into circulation and its substrates are transformed through some enzymes. The most important effector component of this system is Ang II and it has substantial roles in many physiopathological processes such as regulation of blood pressure, aldosterone release, vasoconstriction, growth, proliferation and inflammation of vascular cells (Pacurari et al., 2014; Forrester et al., 2018).

Moreover, Ang II is formed by destruction of Angiotensin I with Angiotensin Converting Enzyme. Ang II shows effects binding ATR1 and ATR2 that activates G protein receptors. Ang II enhanced cell proliferation, growth of new vascular structures and vasoconstriction via ATR1, but inhibited cell proliferation and growth through ATR2.

When Ang II stimulates ATR1, phospholipase C enzyme is activated and hydrolyzed phosphoinositol 4,5-bisphosphate (PIP2) in seconds. After that, diacil glycerol (DAG) activates protein kinase C with calcium and causes activation of various intracellular proteins (Forrester et al., 2018; Touyz and Schiffrin, 2000).

ATR1B (Olmesartan) and ATR2B (PD123,319) were used in our study. Olmesartan is an ATR1B that inhibits RAS by blocking mostly ATR1s and it is widely used to treat hypertensive patients. In addition to its inhibitory effects on the RAS, it attenuates vascular inflammation, fibrosis and proliferation (Agata et al., 2006). But, ATR2 activation increases nitric oxide (NO) and ROS production (Forrester et al., 2018).

PKC is a regulating enzyme is associated with the serine threonine kinase family and reported has many vascular functions. PKC has many isoforms such as PKC α, β1, β2, δ (Kizub et al., 2014). Although, PKC is activated by intracellular calcium and DAG. It was indicated as an important transport pathway in many cellular systems that plays prominent roles in signal transduction and cell signal physiology (Steinberg, 2008). Furthermore, PKC is involved in dispose of vascular smooth muscle contractility, cytokine activation, extracellular matrix synthesis, cell permeability and growth and angiogenesis in vascular tissue. It has been shown that situations in cells are associated with diabetic cases (Lien et al., 2021; Das Evcimen and King, 2007).

In relation to these information, the purpose of this study was to examine the effects of Ang II, Olmesartan and PD123,319 on PKC levels in H-VSMCs and STZ-VSMCs.

2. Materials and Methods

Primary rat VSMCs culture was used in our study. The VSMCs were isolated from the thoracic aorta of healthy and STZ induced diabetic Wistar albino male rats (180-200 g, 8-week old). The animals were obtained from Cukurova University Health Sciences Experimental Application and Research Center (Adana, Türkiye). All experimental applications on the animals were carried out in accordance with the decision of no. 3 dated 22.12.2016 of the local ethics committe of the Cukurova University Animal Experiments Council Meeting. Animals were housed under 12-hours of light/12 hours of night cycles in care rooms. They were fed on standard rat chow and water with ad libitum. The animals were divided into healthy and STZ induced group for the chemical applications. The diabetic model of VSMCs culture was done 8 weeks after STZ application. There were sixteen experimental groups in our study (PKC levels were measured in Control, Ang II, Olmesartan, Ang II+Olmesartan, PD123,319, Ang II+PD123,319, Olmesartan+PD123,319, Ang II+Olmesartan+PD123,319 groups in H-VSMCs and the same groups of STZ-VSMCs). The study was funded by CÜBAP-TSA No. 2017-8133 Cukurova University with a scientific research project (Adana, Türkiye).

2.1. Diabetic Animal Model

Ketamine/xylazine (Ketasol 10%, richterpharma ag and Rompun 2%, Bayer, 100/10 mg/kg) was injected to intraperitoneally. Diabetes was induced in rats with a single STZ dose (Streptozocin, S0130-1G, Sigma-Aldrich, 45 mg/kg) given via the tail vein (n=5). The evaluation of blood glucose in healthy and STZ induced rats was detected before and 3 days after STZ application. Blood glucose measured with calibrated glucometer (Gluco Dr). Glucose levels were higher in STZ treated groups. Blood glucose higher than 250 mg/dL were included in the diabetic experimental group.

2.2. Primer Vascular Smooth Muscle Cell Culture and Treatments

VSMCs were isolated from thoracic aorta of Wistar albino rats. Briefly, under sterile conditions, the endothelium and connective tissue were removed in cold (+4 °C) transfer medium under a stereo microscope. After the isolation of the media layer, the tissue put into a pedri dish containing 4 mL of enzyme digestion solution and cut into several small pieces of 1-2 mm^2. After 45 minutes, 8 mL of cell medium was added and centrifuged at 300 g for 5 min. The pellet was resuspended with 5 mL fresh cell medium and seeded to T-25 flasks.

VSMCs began to grow out at about 3-5 days and were examined with inverted phase contrast microscope. Cells were confluent relatively after 10 days. It was seen that the VSMCs spread and multiplied by holding on the bottom of the culture flask (Chi et al., 2017; Chamley-Campbell et al., 1979).

H-VSMCs were cultured in low glucose Dulbecco's Modified Eagle Medium (DMEM, D6046, Sigma-Aldrich) and STZ-VSMCs were cultured in high glucose DMEM (F0435, Biochrom Merck) containing 20% fetal bovine serum (FBS, Gibco, Thermo Fisher Scientific), penicillin-streptomycin antibiotic (PSA, P4333-100 mL, Sigma-Aldrich) at 37 °C with 5% CO_2 in a humidified incubator. The culture medium was refreshed every 72 hours. We used passages of 4-8 VSMCs in our experiments. They were confirmed positive with immunostaining (99%) for alpha-smooth muscle cell actin (α-SMA), caldesmon and calponin. It was confirmed that 90% of cell viable with trypan blue staining. After that, they were incubated with Ang-II, ([Val5]-Angiotensin II acetate salt hydrate, Sigma-Aldrich, A2900-50 mg, 0.1 μM), Olmesartan (Olmesartan, Sigma-Aldrich, SML1394-50 mg, 1 μM) and PD123,319 (PD123319 di (trifluoroacetate) salt hydrate, P186-10 mg, Sigma-Aldrich, 1 μM) for 24 h. The chemicals were dissolved in cell medium. PKC levels were measured in experimental groups of H-VSMCs and STZ-VSMCs extract.

2.3. Immunohistochemical (IHC) Assay

VSMCs were seeded to glass polylysine slides and incubated in a pedri dish containing 20 mL cell culture medium for 3-5 days. The cells were labeled with α-SMA, caldesmon and calponin antibody by Department of Pathology, Cukurova University.

2.4. Preparation of Cell Extracts

Cell extracts prepared with RIPA buffer (RIPA Lysis

Buffer System, Santa Cruz Biotechnology, Inc., SC-24948A) according to the kit protocol. The VSMCs (~0.3x106) were seeded into 6-well plates and 0.1 µM Ang II, 1 µM Olmesartan and 1 µM PD123,319 were administered for 24 h. After, 450 µL of RIPA buffer was added to wells and the cells were removed from the surface with a cell scraper. The cells were then broken down in the sonicator for a few minutes and centrifuged at 10.000 g for 15 min. at +4 °C and supernatants collected. The cell extract samples were kept in -80 °C deep-freezer until studied.

2.5. Lowry Method

The dilution series of bovine serum albumin (BSA) doses (4, 2, 1, 0.5, 0.25, 0.125, 0.062, 0.03, 0 mg/mL), blank and cell extract samples (50 µL) were pipetted into 96-well culture dish and incubated at room temperature for 45 min. C reagent (150 µL) was added to wells. C reagent consists of 100:1 mixture of A and B reagents. A reagent is 2% Na2CO3, 0.4% NaOH, 0.16% Na-tartrate; B reagent is 4% CuSO4.5H2O prepared. A while after, the Folin-Ciocalteau's reagent (3 µL) was added to all wells. The absorbance values of the samples and standards were measured at a wavelength of 660 nm with a microplate reader (Eon, Biotek). Protein concentration of the cell extracts was calculated with the standard curve (Lowry et al., 1951; Waterborg and Matthews, 1994).

2.6. ELISA

The level of PKC in VSMCs was measured by a sandwich ELISA kit (E-EL-R0815, Elabscience Biotechnology Inc.) according to manufacturer's instructions. The optical density of plate was evaluated at an absorbance wavelength of 450 nm with a microplate reader (Eon, Biotek). The levels of PKC were measured in cell extracts of H-VSMCs and STZ-VSMCs groups (Table 1).

Table 1. Protein Kinase C levels were shown in Healthy and STZ Induced VSMCs groups

	Healthy VSMCs PKC Levels		STZ Induced VSMCs PKC Levels	
Groups	pg/mg protein ($\bar{x}$) ± SD	n	pg/mg protein ($\bar{x}$) ± SD	n
Control	1039.09±82.75	5	448.44±9.86	5
Ang II	327.83±14.75*	5	750.49±16.27	5
Olmesartan	1119.51±38.97	5	435.06±12.71	5
Ang II+Olmesartan	1734.00±120.95$^{\#}$	5	347.14±21.81*	5
PD123,319	1864.51±89.75	5	313.01±25.50	5
Ang II+PD123,319	972.08±59.82$^{\#}$	5	225.50±13.55*	5
Olmesartan+PD123,319	1518.82±101.30	5	1315.40±90.97	5
Ang II+Olmesartan+PD123,319	385.71±10.44	5	546.81±41.63	5

2.7. Statistical Analysis

All experimental groups were studied in 5 repetitions. The data were expressed as mean ± SD). SPSS 21.0 package program (SPSS Inc, Chicago, 9 Illinois, USA) was used for statistical analysis. The variances were analyzed with Levene Test. The variances were homogeneous distributions and one-way and two-way ANOVA was used as a parametric test. Post-hoc Tukey test were used to show the difference between groups. Statistical significance level was $p<0.05$ in all tests.

3. Results

3.1. Evaluation of VSMCs

The VSMCs were seeded on the polylysinized slide with positive charged before staining. Cell morphology and proliferation was evaluated with an inverted microscope (Figure 1A). After 2-4 days VSMCs covered the slide surface and after reaching sufficient confluence numbers, the preparations were stained with α-SMA, caldesmon and calponin by IHC method (Figure 1B, 1C and 1D). The purity of cells was test by multiple immunstaining. VSMCs proved characteristic hill and valley trial and the cells used in our study were confirmed 100 % positive VSMCs.

3.2. Protein Kinase C Determination in H-VSMCs and STZ-VSMCs by ELISA

We examined the effects of Ang II, Olmesartan and PD123,319 applications for 24 h on PKC levels in H-VSMCs and STZ-VSMCs. PKC levels in STZ-VSMCs groups (Control, Olmesartan, Ang II+Olmesartan, PD123,319, Ang II+PD123,319, Olmesartan+PD123,319) were lower than H-VSMCs groups except Ang II and Ang II+Olmesartan+PD123,319 groups in our study ($p<0.05$). While, Ang II group compared to the control group, PKC levels decreased significantly in H-VSMCs ($P = 0.000$); whereas, Ang II group compared to control PKC levels increase, but not significantly in STZ-VSMCs ($P = 0.088$). PKC levels in Ang II+Olmesartan ($P = 0.000$) and Ang II+PD123,319 ($P = 0.000$) groups compared to Ang II group were increased in H-VSMCs, but in STZ-VSMCs, PKC levels in Ang II+Olmesartan ($P = 0.001$) and Ang II+PD123,319 ($P = 0.000$) groups compared to Ang II group were decreased significantly (Figure 2, Figure 3).

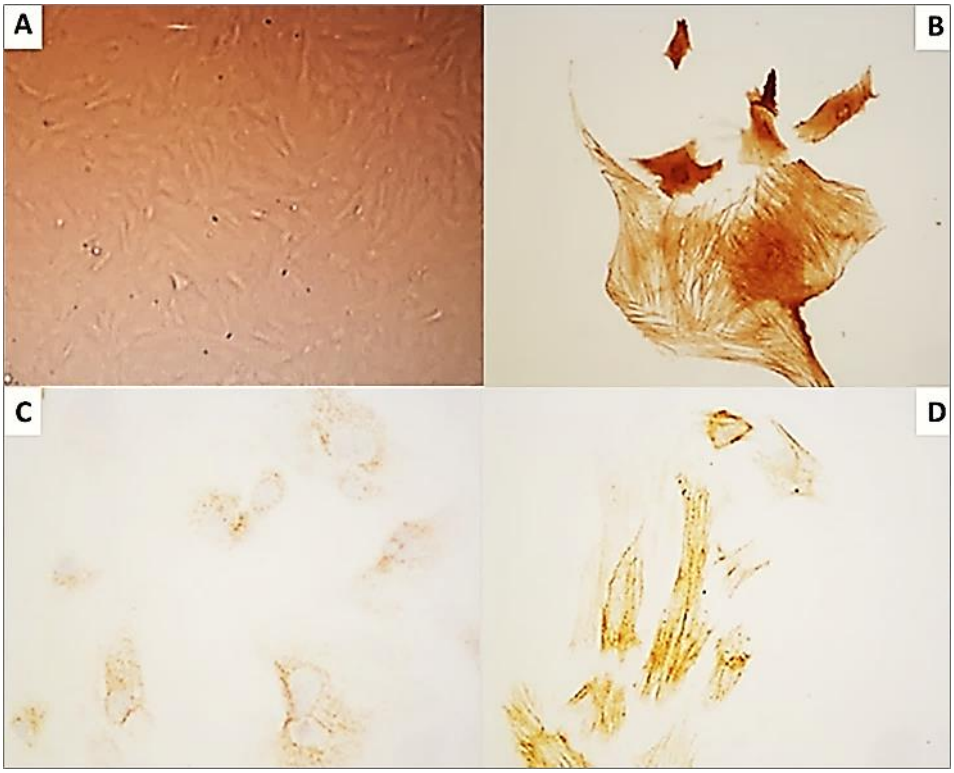

Figure 1. Evaluation of VSMCs. (A) Appearance of VSMCs in light microscopy before being stained (×200) (B) VSMCs stained with α-SMA by IHC method (×400) (C) VSMCs stained with caldesmon by IHC method (×200) (D) VSMCs stained with calponin by IHC method (×400). α-SMA= alfa-smooth muscle actin, IHC= immunohistochemistry, VSMCs= vascular smooth muscle cells.

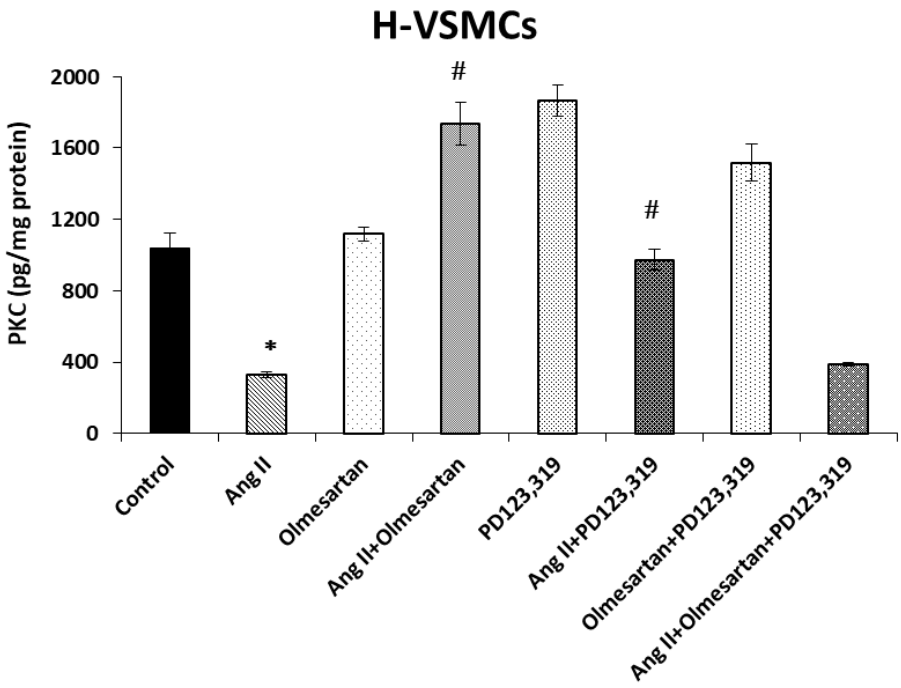

Figure 2. Protein Kinase C Levels in Angiotensin II (Ang II), Olmesartan and PD123,319 applications on H-VSMCs for 24 h. The data are shown mean±SD. ∗P<0.05 according to the control group, #P<0.05 according to Ang II group. One-way ANOVA post-hoc Tukey test, (n=5). Ang II= angiotensin II, H-VSMCs= healthy vascular smooth muscle cell, SD= standard deviation.

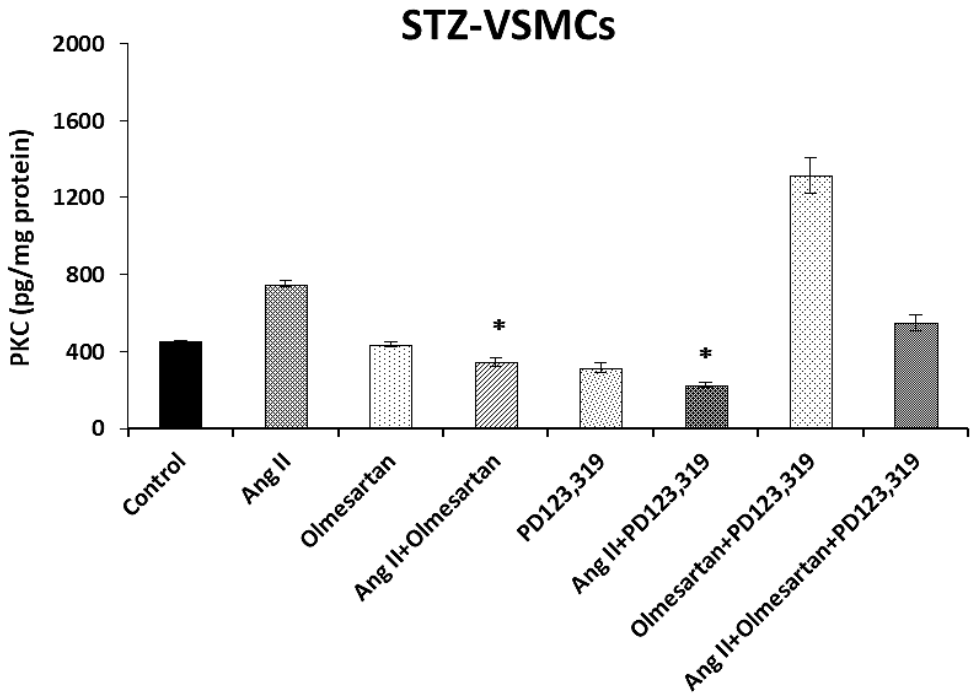

Figure 3. Protein Kinase C Levels in Ang II, Olmesartan and PD123,319 applications on STZ-VSMCs for 24 h. The data are shown mean±SD. ∗P<0.05 according to Ang II group, One-way ANOVA post-hoc Tukey test, (n=5). STZ-VSMCs= STZ induced vascular smooth muscle cell, Angiotensin II= Ang II, SD= standard deviation

4. Discussion

In our study, we detected expression of α-SMA in VSMCs in this study, but α-SMA expression can also be found in many cells such as fibroblasts (Hinz et al., 2001). Consequently, VSMCs were also double-staining with specific markers for VSMCs such as calponin and caldesmon. It was confirmed 100 % positive with VSMCs. After proving that, we cultured cells and used 4-8 passages in all experiments of our study.

Previous studies showed that, in diabetic conditions the vascular structure damage induces various intracellular pathways (Rask-Madsen and King, 2013) and Ang II activation is one of them and plays crucial roles in vascular pathologies such as hypertension (Forrester et al., 2018). In addition, it has been reported that, diabetes induces PKC pathway activation. But, several studies demonstrated that the distinct roles of PKC isoforms in regulation of the cell signaling processes in hyperglycemic states and it has been shown that, PKC levels increase in pathological conditions in the different cell types (Das Evcimen and King, 2007; Geraldes and King, 2010). Also, hyperglycemia increases PKC, and many related different intracellular signaling pathways are activated (Kizub et al., 2014). From this point of view, Ang II increases PKC via mostly ATR1s. Consequently, it can be thought that Ang II receptors and PKC can be associated with each other.

In our study, PKC levels were decreased in control group of STZ-VSMCs compared to H-VSMCs and Ang II application increased PKC levels in STZ-VSMCs but decreased in H-VSMCs. It has shown that prolonged exposure of 100 nm Ang II induces down regulation of intracellular DAG-IP3 pathway in a study (Forrester et al., 2018). This result suggested that, Ang II inhibited PKC signaling pathway in H-VSMCs and it is correlated with our present results, but in STZ-VSMCs, we investigated that, Ang II administration would increase PKC signaling pathway whether in diabetic condition. The reason for this result may be impaired VSMCs response under high glucose medium environment. On the other hand, PKC-mediated effects of Ang II may be activated through different receptors or it may be showing its effects by activation of different PKC subtypes in hyperglycemia. It has been considering that, PKC has different suptypes in various species and also, the effect of different types of PKC and its receptors have also not been fully elucidated.

Ang II has been found PKC-enhancing effects and the inhibitory effect of Olmesartan administration on ATR1 in STZ-VSMCs is obvious in our present study. This result we found is compatible with the studies in the literature (Malhotra et al., 2001). Furthermore, it was reported that in another study, ATR1 may be associated with the activation of the PKC-ε subtype in diabetic rat cardiomyocytes (Malhotra et al., 1997). However, Olmesartan administration again counteracted the PKC-reducing effect of Ang II in H-VSMCs, but this time it increased the PKC levels in the diabetic state.

In our current study, the application of PD123,319 decreased PKC levels in STZ-VSMCs, but PKC levels increased in H-VSMCs. It has been shown that, ATR2s are less common than ATR1s in the vascular structure. In addition, ATR2s cause opposite intracellular changes

compared to ATR1s (Mehta and Griendling, 2007). Therefore, considering of these studies, the effectiveness of ATR2s through PKC signaling pathway is less than ATR1s. However, it was observed that ATR2 blocker PD123,319, like ATR1 blocker Olmesartan, reduced the PKC level of STZ-VSMCs in our study. Our findings also suggest that, the effects of these two Ang II receptors on the PKC pathway may be greater than we expected and literature knowledge.

5. Conclusion

In conclusion, our study shows that, Ang II and PKC seem to affect each other and can have a tight relationship in hyperglycemic conditions. The elevated Ang II and PKC levels can play significant roles in vascular complications of diabetes. Furthermore, the decrease of PKC levels in the application of Olmesartan also suggests that the PKC pathway may be a pivotal indicator of VSMCs damage in our current research. It is also thought that ATR1s and ATR2s may have very prominent roles by affecting PKC levels and ATR1 blocker Olmesartan and ATR2 blocker PD123,319 may prevent vascular injury by reducing PKC levels in diabetes. And, these results show that, it may have been activated several different intracellular signaling pathway responses in physiological and pathological occasions in VSMCs. However, different subtypes of PKC may be activated in normal and diabetic vascular tissue by ATRs and measuring these PKC subtypes separately will be obtaining more accurate and valuable outcomes. Moreover, diabetes mellitus and its vascular complications are very important public health problems and new drug therapy strategies are needed to control hyperglycemia. Given the strong connection of RAS and PKC pathway, ATR1s and ATR2s may also be promising target for the treatment of the disease.

Author Contributions

Percentages of the author(s) contributions is present below. All authors reviewed and approved final version of the manuscript.

%	Z.Ç.	K.A.	A.D.
C	34	33	33
D	34	33	33
S	34	33	33
DCP	34	33	33
DAI	34	33	33
L	34	33	33
W	34	33	33
CR	34	33	33
SR	34	33	33
PM	34	33	33
FA	34	33	33

C= concept, D= design, S= supervision, DCP= data collection and/or processing, DAI= data analysis and/or interpretation, L= literature search, W= writing, CR= critical review, SR= submission and revision, PM= project management, FA= funding acquisition.

Conflict of Interest

The authors declared that there is no potential conflict of interest with respect to the research, authorship, and/or publication of this article.

Ethical Approval/Informed Consent

This study was conducted with the permission of the Çukurova University Faculty of Medicine Ethics Committee (approval date: 22.12.2016, number: 3).

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