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Article

The direct effects and molecular mechanisms of azilsartan, a new angiotensin AT1-receptor blocker, on the function of high-glucose treated endothelial progenitor cells

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Abstract:

Background:

Angiogenesis is impaired in the presence of diabetes mellitus (DM) or hyperglycemia. Endothelial progenitor cells (EPCs), which may play a critical role in vascular repair as well as angiogenesis, have been shown impaired in the presence of clinical DM and hyperglycemia. EPCs could also serve as a potential material for cell therapy in DM related cardiovascular disease. Azilsartan, a new angiotensin AT1-receptor blocker (ARB), was recently approved and is expected to exert a more potent, sustained for 24 h BP-lowering effect compared to existing ARBs. However, the vasculoprotective effects and detailed mechanisms have not been clarified.

Aims:

This project was conduct to investigate the effects and potential mechanisms of angiotensin AT1-receptor blocking by azilsartan on the function and angiogenesis capability of EPCs. It should be clarified that whether and how azilsartan could improve the function of high-glucose treated EPCs and the EPCs from patients with type 2 DM.

Methods:

Blood samples were obtained from the peripheral veins of the healthy volunteers or patients with type 2 DM (T2DM) in the morning hours after an overnight fasting. Total mononuclear cells were isolated and cultured with or without high glucose condition to generate late EPCs. Late EPCs from diabetic patients were treated with azilsartan (0.1 and 10μM) or olmesartan, another ARB (0.1 and 10 μM) for 24 hours. Late EPCs from healthy volunteers were treated with high glucose (25mM) for 3 days, and then treated by azilsartan (0.1 or 10μM) with high glucose for one day. The expression of vascular endothelial growth factor (VEGF) and stromal cell-derived factor -1 (SDF-1) were evaluated by Western blotting in cultured EPCs. The effects of azilsartan (0.1 and 10μM) were also examined on human coronary artery endothelial cell (HCAEC) lines.

Results:

Olmesartan (0.1 and 10μM), but not azilsartan (0.1 and 10μM), dose-dependently increased the expression of VEGF and SDF-1 on late EPCs from T2DM patients. There were also no effects of azilsartan (0.1 and 10μM) on high glucose treated late EPCs from healthy volunteers or on high glucose treated HCAEC.

Conclusions:
The direct effects of azilsartan on VEGF/ SDF-1 expression of EPCs were different from that of olmesartan, another ARB. Olmesartan but not azilsartan might have better angiogentic effects on EPCs, which should be further proven in in vivo animal studies. However, our findings did provide a potential rational to clinical consideration of ARB therapy for T2DM patients with cardiovascular complications.

Keywords: Angiogenesis, azilsartan, endothelial olmesartan, progenitor cell, diabetes mellitus, peripheral artery disease, vascular endothelial growth factor.

1. Introduction

It has been shown that angiogenesis, a type of blood vessel formation from preexisting ones, plays a critical role in physiological and pathological condition. Clinically, patients with Type 2 diabetes mellitus (DM) have long-term complications that involve in angiogenesis including an attenuated angiogenic response in wound healing and ulcers [1]. Moreover, the formation of capillaries and the blood flow of the ischemic hindlimb are both decreased in diabetic versus non-diabetic mice in murine hindlimb ischemic model [2]. Thus, improving local angiogenesis and enhancing neovasculogenesis via activating vascular progenitors to improve perfusion of ischemic tissues may be one of the most attractive therapeutic strategies for DM related cardiovascular diseases [3–5].

It was shown that reduced levels of circulating endothelial progenitor cells (EPCs), firstly defined by Dr. Asahara [6,7], could independently predict atherosclerotic disease progression and development of cardiovascular events [8–12], thus supporting an important role for endogenous vascular repair of EPCs to modulate the clinical course of atherosclerotic diseases [13–15]. Animal and clinical studies of cell therapy further showed that transplantation of autologous EPCs or other cellular pools enriched with vascular progenitors are feasible in both coronary and peripheral atherosclerotic diseases [16–20]. However, almost all cardiovascular risk factors such as aging, hypertension, DM, and hypercholesterolemia have been shown to exert detrimental effects on EPC number and function [21,22].

Thus, one of the major determinants of vascular repair as well as angiogenesis is the quality of EPCs [23,24]. There are many atherosclerosis risk factors including DM and high concentration/level of glucose that may also impair EPCs function [22,25,26]. Unfortunately, in clinical condition, the patients who may potentially benefit from cell therapy usually have multiple atherosclerosis risk factors that may also impair the EPC number and function [22]. It is then critical to preserve the EPC quality and enhance the EPC function from the diseased patients as possible as we can for autologous cell therapy [27]. It is especially critical to diabetic patients [28].

Although the clinical importance of EPCs have been well recognized, there are diverse definition and serial arguments about the characteristics of "true" circulating EPCs [29–37]. It is then important to culture these early EPCs and confirm their angiogenesis capability in vitro and even in vivo [38]. In the past few years, we have established an experiment platform with primarily cultured late EPCs, which could simulate the morphology, nature and characteristics of mature human endothelial cells [25]. With this platform, we have demonstrated the critical role of endothelial nitric oxide synthesis (eNOS) for the impairment of high-glucose treated EPCs [25]. On the other hand, both our team and several other investigator teams showed the possibility of different interventional strategies to improve EPC function in vitro, in vivo, and even in human [25,28,39**? ?** ,40].

The renin-angiotensin-aldosterone system (RAS) can regulate blood pressure, hydroelectrolyte balance and cell function, which has been shown excessively activated in the presence of DM. Superabundant RAS activity leads to pathological conditions such as hypertension, atherosclerosis,

thrombosis, cardiac and renal diseases [**? ? ?**], which is particularly in the presence of type 2 DM [**?**]. Renin is secreted by kidney and catalyzes angiotensinogen to Angiotensin 1, which is the rate-limiting step in RAS [**?**]. Angiotensin 2 produced from Angiotensin 1 by multiple enzymatic pathways including Angiotensin 2 converting enzyme (ACE) and other chymases, impairs endothelial function by inhibiting eNOS, increasing leukocyte infiltration and increasing adhesion to vascular wall, inducing oxidative stress, and promoting atherosclerosis [**? ? ?**]. RAS inhibitors such as direct renin inhibitor, ACE-1 and angiotensin AT1-receptor blockers (ARBs) have been used for hypertension, DM and related cardiovascular diseases including tissue ischemia [**? ? ?**]. It was also shown that some ARBs such as olmesartan and irbesartan could increase EPC number and improve EPC function in type 2 DM patients [25].

Azilsartan, a new ARB, was recently approved and is expected to exert a more potent, sustained 24-hour blood pressure-lowering effect compared to existing ARBs (candesartan cilexetil, olmesartan, telmisartan, valsartan, and irbesartan). In the in vitro study, azilsartan was shown with higher affinity for and slower dissociation from AT1 receptors and with stronger inverse agonism [**?**]. These effects of azilsartan on the AT1 receptor may underlie its superior blood pressure-lowering properties (compared to other ARBs) and may be beneficial in diabetic vascular remodelling [**? ?**]. However, it was not known whether this new ARB could also improve neovasculogenesis especially in the presence of DM or high glucose.

Thus, in this study, we evaluated the direct effects and potential molecular mechanisms of azilsartan, compared with olmesartan, another ARB, on late EPCs from type 2 DM patients. Further experiments were also done to evaluate the potential effects of azisartan on high-glucose treated late EPCs from healthy subjects and high-glucose treated human aortic endothelial cell (HAEC) and human coronary artery endothelial cell (HCAEC) lines. Our findings might provide a potential rational for clinical implication of different ARBs on type 2 DM patients especially those patients with vascular complications.

2. Materials and Methods

Both vascular endothelial growth factor (VEGF) and stromal cell-derived factor-1 (SDF-1) are associated with clinical atherosclerosis, which play the critical role in vascular repair and angiogenesis especially in diabetic condition [**? ? ? ? ?**]. We have previously shown that aliskiren, a direct renin inhibitor as one of the family of RAS inhibitors, could improve neoangiogenesis in diabetic animals [2] and directly improve diabetic EPC function via the activation of VEGF and SDF-1 in vitro [40]. The following Figure.1 showed the working hypothesis that we generated originally for this study.

2.1. Patient selection and blood sampling

Only stable type 2 DM patients without insulin treatment were enrolled. Patients with other significant systemic diseases, receiving major operation in the past 6 months, or currently under medical treatment for other diseases were excluded. Another group of healthy subjects was also enrolled for comparison.

Peripheral blood samples (20 mL) were obtained in heparin-coated tubes from the peripheral veins of the healthy volunteers or patients with type 2 DM in the morning hours after an overnight fasting.

2.2. Culture for late EPCs

Total mononuclear cells were isolated by density gradient centrifugation with Histopaque-1077 (Sigma), and the serum was preserved. Briefly, Mononuclear cells (5×106) were plated in 2–mL of endothelial growth medium (EGM-2 MV Cambrex, East Rutherford, NJ, USA), with 15% individual serum on fibronectin-coated, 6-well plates. After 4 days of culturing, the medium was changed, and nonadherent cells were removed; attached early EPCs appeared elongated with spindle shapes. Some

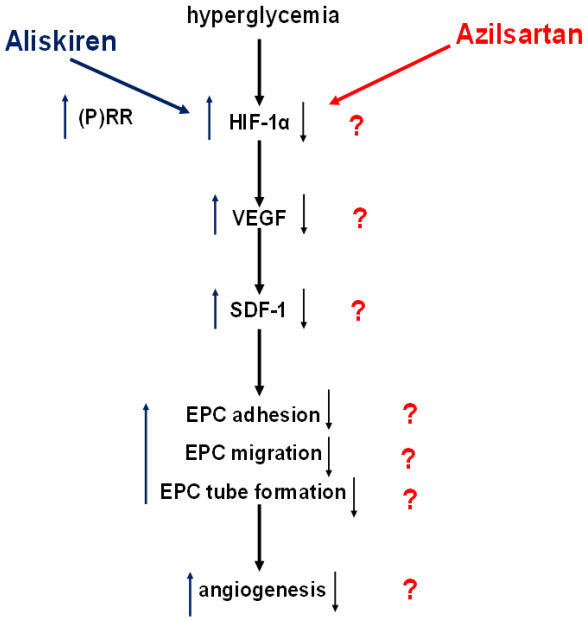

Figure 1. Working hypothesis

MNCs were allowed to grow into colonies of late (out-growth) EPCs, which emerged 2–3 weeks after the start of MNC culture. The late EPCs exhibited a 'cobblestone' morphology and monolayer growth pattern typical of mature endothelial cells at confluence.

Late EPCs from diabetic patients were treated with azilsartan (Sigma, USA) (0.1 and 10 μM) or olmesartan (Sigma, USA), another ARB (0.1 and 10 μM) for 24 hours. Late EPCs from healthy volunteers were treated with high glucose (25mM) for 3 days, followed by azilsartan treatment (0.1 or 10μM) with high glucose for 24 hours. Then, the expression of VEGF/SDF-1 and functional studies were separately conducted accordingly.

2.3. Culture for cell lines

Either human aortic endothelial cells (HAECs, purchased from Lonza, Switzerland) or human coronary artery endothelial cells (HCAECs, purchased from Lonza, Switzerland) were cultured in growth medium ECM containing 5% fetal bovine serum on coated fibronectin 6-well plates, incubated at a temperature of 37C and 5% carbon dioxide to induce cell proliferation. After the cells were replicated, HAECs or HCAECs were cultured with glucose (25mM) for 3 days. Then, they were treated with olmesartan or azilsartan for 24 hours (each of treatment for 0.1 and 10μM).

Late EPCs from diabetic patients were treated with azilsartan (Sigma, USA) (0.1 and 10μM) or olmesartan (Sigma, USA), another ARB (0.1 and 10 μM) for 24 hours. Late EPCs from healthy volunteers were treated with high glucose (25mM) for 3 days, followed by azilsartan treatment (0.1 or 10μ) with high glucose for 24 hours. Then, the expression of VEGF/SDF-1 and functional studies were separately conducted accordingly.

2.4. Migration of late EPCs

The migration was evaluated by a chamber assay. EPCs (1x104 cells) were resuspended in VEGF-free EBM-2 before or after pretreatment with olmesaratn (0.1 or 10μM) or azilsartan (0.1 or 10μM) in high glucose for a day after high glucose conditioning for 3 days. The cells were added to the upper chamber of 24-well transwell plates with polycarbonate membrane. EBM-2 supplemented with fetal bovine serum was added to the lower chamber. The chambers were incubated for 18 hours. After incubation, the membrane was fixed with 4% paraformaldehyde and stained using hematoxylin

solution. The numbers of migrated cells were counted in 6 random high-power (x100) microscopic fields.

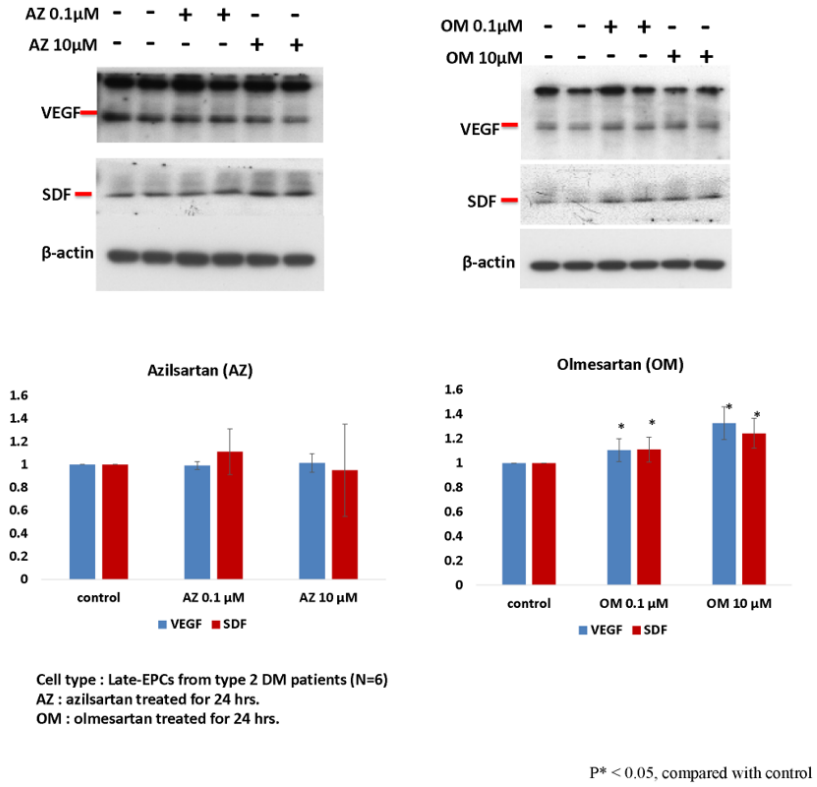

Figure 2. Effects of azilsartan or olmesartan on the expression of VEGF and SDF-1 in late EPCs from type 2 DM patients.

2.5. Tube formation of late EPCs

In vitro tube formation assay was performed with an angiogenesis assay kit (Invitrogen). ECMatrix gel solution was mixed with ECMatrix diluent buffer, and placed in a 96-well plate. Then 1×104 late EPCs were placed on a matrix solution with EGM-2 and incubated for 18 hours. Tubule formation was inspected under an inverted light microscope (×40). Four representative fields were taken, and the average of the total area of complete tubes formed by cells was compared by using computer software, Image-Pro Plus.

2.6. Western Blot assay for VEGF and SDF-1 expression

Western Blot assay was conducted to investigate the expression of VEGF/SDF-1 in late EPCs or cell lines (HAECs/HCAECs) with or without high glucose and/or azilsartan/olmesartan treatment. After the incubation, cells were washed, scraped, collected, and centrifuged at 12,000 × g at 4C for 1 hour to yield the whole cell extract. Samples were denatured, subjected to SDS-polyacrylamide gel electrophoresis, and transferred to PVDF membrane. Membranes were incubated with an anti- VEGF and anti-SDF-1 for 24 hours, and then incubated with an anti- rabbit for 1 hour. The immunoreactive bands detected by enhanced chemiluminescence reagents were developed by Hyperfilm-ECL.

2.7. Transfection of VEGF siRNA into late EPCs

Transfection of VEGF siRNA into late EPCs was not done due to the absent effects of azisartan on SDF-1 expression in EPCs.

2.8. Statistics

Results were given as means ± standard errors of the mean (SEM). Statistical analysis was done by unpaired Student's t test or analysis of variance, followed by Scheffe's multiple-comparison post hoc test. SPSS software (version 14; SPSS, Chicago, IL, USA) was used to analyze data. A p value of <0.05 was considered statistically significant.

3. Results

3.1. Effects of azilsartan or olmesartan on the expression of VEGF and SDF-1 in late EPCs from type 2 DM patients

Olmesartan (0.1 and 10μM), but not azilsartan (0.1 and 10μM), dose-dependently increased the expression of VEGF and SDF-1 on late EPCs from T2DM patients (Figure.2).

3.2. Effects of azilsartan on the function of late EPCs from type 2 DM patients

Further experiments were conducted to evaluate the effects of azilsartan on the functions of late EPCs from type 2 DM patients. There were no differences among controls and azilsartan treatment (0.1 and 10μM), indicating no effects of azilsartan treatment on the migration and tube formation of late EPCs from type 2 DM patients. (data not shown)

3.3. Effects of azilsartan on the expression of VEGF and SDF-1 in high-glucose treated late EPCs from healthy subjects

Further experiments were conducted to evaluate the effects of azilsartan on high glucose-treated late EPCs from healthy volunteers. There were no differences among controls and azilsartan treatment (0.1 and 10μM), indicating no effects of azilsartan treatment on the expression of VEGF and SDF-1 in high glucose-treated late EPCs from healthy volunteers (Figure.3).

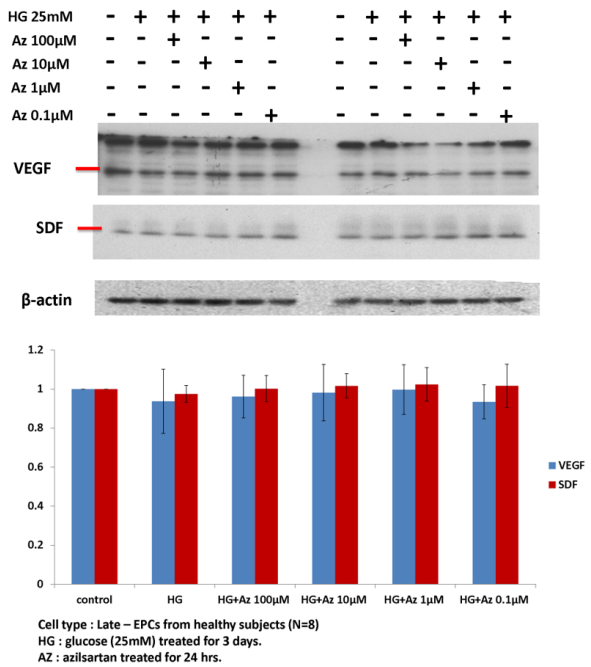

Figure 3. Effects of azilsartan on the expression of VEGF and SDF-1 in high-glucose treated late EPCs from healthy subjects.

3.4. Effects of azilsartan or olmesartan on the expression of VEGF and SDF-1 in high-glucose treated HAECs

Similar to the findings on late EPCs from T2DM patients, olmesartan (0.1 and 10μM), but not azilsartan (0.1 and 10μM), dose-dependently increased the expression of VEGF and SDF-1 on high-glucose treated HAECs (Figure.4).

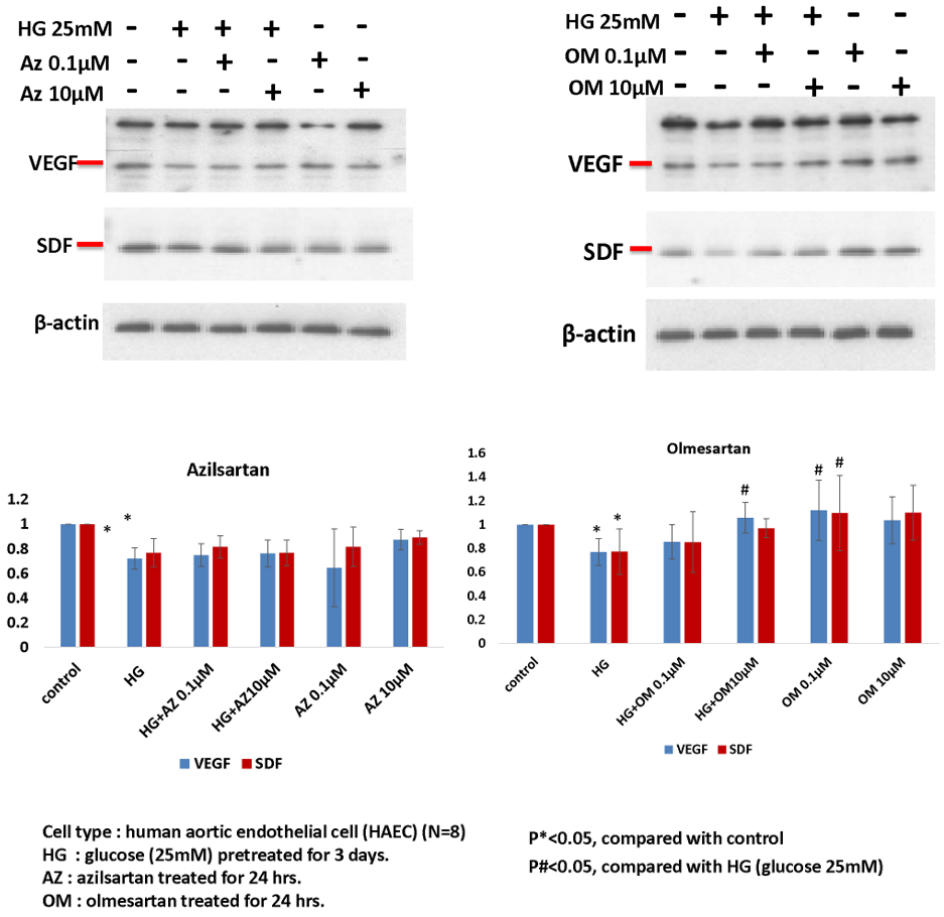

Figure 4. Effects of azilsartan or olmesartan on the expression of VEGF and SDF-1 in high-glucose treated HAECs.

3.5. Effects of azilsartan or olmesartan on the expression of VEGF and SDF-1 in high-glucose treated HCAECs

Similar to the findings on high-glucose treated HAECs, there were no differences between controls and azilsartan treatment (0.1 and 10μM), indicating no effects of azilsartan treatment on the expression of VEGF and SDF-1 in high glucose treated HCAECs. Interestingly, different from the findings on high-glucose treated HAECs, there were no effects of olmesartan treatment (0.1 and 10μM) on high-glucose treated HCAECs (Figure.5).

4. Discussion and Conclusions

Olmesartan, an old ARB, but not azilsartan, a new ARB, increased the expression of SDF-1 VEGF in late EPCs from type 2 DM patients, suggesting the potential angiogenesis capacity of olmesartan rather than azilsartan. It seems that the beneficial effects on EPCs are different among different ARBs, which may not be a class effect of ARBs. On the other hand, olmesartan but not azilsartan could increase the expression of SDF-1 VEGF in high-glucose treated HAECs. However, both olmesartan and azilsartan had no effects on high-glucose treated HCAECs. While the beneficial effects on human

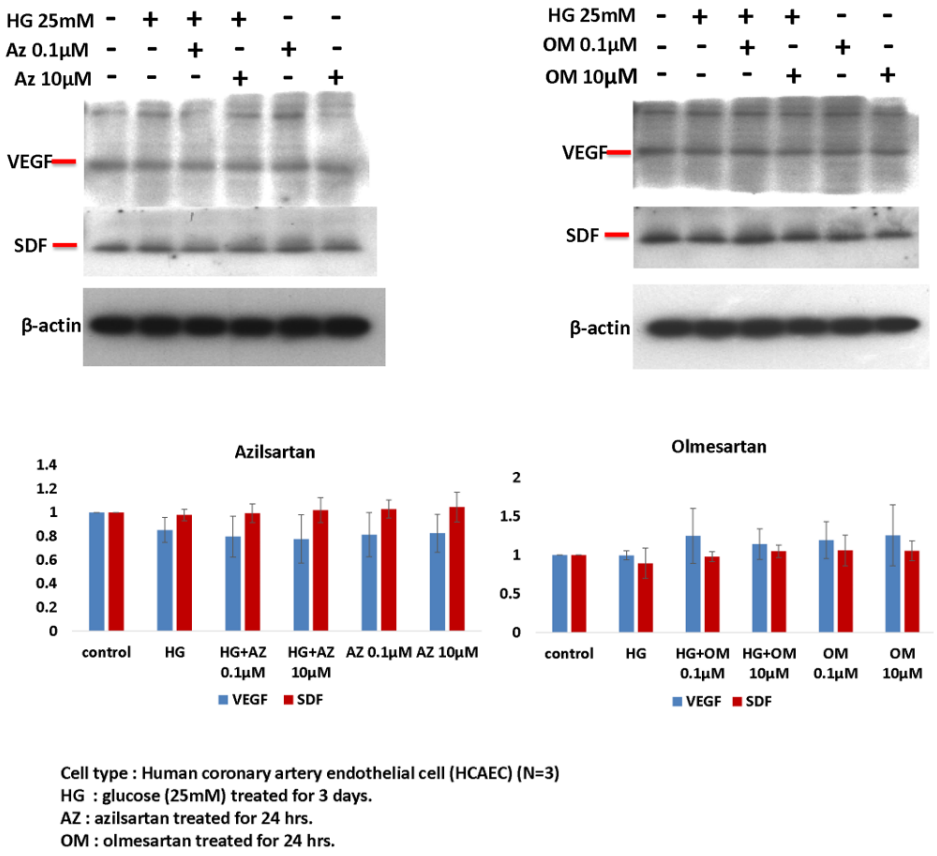

Figure 5. Effects of azilsartan or olmesartan on the expression of VEGF and SDF-1 in high-glucose treated HCAECs.

endothelial cells seem different among different ARBs, they might be also different between different types and sites of human endothelial cells.

The above might be important since ARBs have been widely used for hypertensive treatment and suggested for vascular protection in type 2 DM patients. In such case, The present results indicated the absence of effects of azilsartan on SDF-1 VEGF expression in different EPCs as well as in different human artery endothelial cells, suggesting the disapproval of this hypothesis. However, the current study only elucidated a part of our original working hypothesis. Future studies were still required to investigate other in vitro as well as in vivo effects of azilsartan on vascular protection and angiogenesis.

On the other hand, our findings on olmesartan are line with that of the previous human study [39]. More interestingly, it is indicated that different from azilsartan, olmesartan, another ARB, may directly increase the VEGF/ SDF-1 expression in late EPCs as well as in HAECs but not in HCAECs. The above findings suggested the selective effects of some ARBs (such as olmesartan) on some selective types of vascular cells (such as EPCs and HAECs). One may then speculate that olmesartan rather than azilsartan might improve in vitro and in vivo angiogenesis.

Our previous data indicated that high glucose may inhibit plasma SDF-1 VEGF levels and impair angiogenesis in diabetic animals, which could be improved by aliskiren, a direct renin inhibitor, treatment in vivo [2]. Given the critical role in angiogenesis and EPC homing, plasma SDF-1 VEGF levels were significantly reduced in diabetic animals, which could be improved by aliskiren in a dose-dependent fashion in vivo [2]. However, the in vivo effects of azilsartan, a new ARB, on plasma SDF-1 VEGF levels were not known. Further studies may be still worthy to investigate the in vivo effects of azilsartan though its in vitro effects might not be significant.

While providing significant blood pressure reduction than other ARBs did, azilsartan might give even less effects on angiogenesis capacity of either EPCs or vascular endothelial cells. On the

other hand, olmesartan might improve angiogenesis capacity of diabetic EPCs and of some vascular endothelial cells especially HAECs. Taken together, our findings might provide a potential rational for the selection of different ARBs for clinical implication to type 2 DM patients especially to those patients requiring aggressive vascular protection for vascular complications.

Conflicts of Interest:
The authors declare no conflict of interest.

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