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BRIEF COMMUNICATION

# Aliskiren decreases oxidative stress and angiogenic markers in retinal pigment epithelium cells

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**Abstract** There is growing evidence on the role of ocular renin–angiotensin system (RAS) in the development of diabetic retinopathy (DR), particularly due to the trigger of oxidative stress and angiogenesis. Despite this there is no effective RAS-based therapy in DR capable of preventing retinal damage induced by RAS activation. We recently described that retinal pigment epithelium (RPE) cells express the main components of the RAS. We here propose to investigate the role of glucose upon the retinal RAS and whether aliskiren, a direct renin inhibitor, protects RPE cells from angiogenesis and oxidative stress. RPE cells were chosen as target since one of the first events in DR is the dysfunction of the RPE retinal layer, which as a key function in maintaining the integrity of the retina. We found that the RAS present in the RPE cells was deregulated by hyperglycemic glucose concentrations. Exposure of RPE cells to angiotensin II increased the levels of the main pro-angiogenic factor, vascular endothelial growth factor (VEGF) in a concentration-dependent manner. Additionally, angiotensin II also stimulated the production of reactive oxygen species in RPE cells. Treatment of RPE cells with aliskiren decreased the levels of oxidative stress and promoted the expression of anti-angiogenic factors such as the pigment epithelium-derived factor and the $VEGF_{165}b$ isoform. Our findings demonstrate that the RAS is deregulated in hyperglycemic conditions and that aliskiren successfully protected RPE cells from RAS over activation. These anti-angiogenic and antioxidant properties described for aliskiren over RPE cells suggest that this drug has potential to be used in the treatment of diabetic retinopathy.

**Keywords** Retinal pigment epithelium · Aliskiren · Renin–angiotensin system · Angiogenesis · Oxidative stress

**Abbreviations**

| | |
|---|---|
| ACE | Angiotensin II-converting enzyme |
| ARB | Angiotensin II receptor blocker |
| BRB | Blood retinal barrier |
| DR | Diabetic retinopathy |
| DRI | Direct renin inhibitor |
| PEDF | Pigment epithelium-derived factor |
| RAS | Renin–angiotensin system |
| ROS | Reactive oxygen species |
| RPE | Retinal pigment epithelium |
| VEGF | Vascular endothelial growth factor |

## Introduction

Retinal diseases are serious visual threatening disorders and persist as the leading cause of acquired blindness worldwide. Diabetic retinopathy (DR) is considered a

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microvascular complication derived from sustained hyperglycemia. How hyperglycemia induces changes in the retina in DR is not entirely defined, but it is known that glucose triggers angiogenesis and oxidative stress in retinal cells contributing to cellular damage [1, 2]. Recently, we demonstrated that the equilibrium between angiogenic and anti-angiogenic factors was disrupted by both hyperglycemia and reactive oxygen species (ROS) in the retina of a diabetic mouse model [3]. We also demonstrated that the retinal pigment epithelium (RPE) cells that constitute the outer blood retinal barrier (BRB) were particularly susceptible to both glucose and ROS concentrations [3], highlighting the importance of studying the RPE in the context of DR.

Of all the different pathways activated by glucose in the retina, the renin–angiotensin system (RAS) has been described as a key contributor to the development of retinal diseases including DR [2, 4]. The finding of a specific RAS within the retina, namely in RPE cells, and the fact that deregulation of this system triggers hallmarks of DR such as angiogenesis and oxidative stress, support a role for RAS in this pathology [4]. These effects are mainly attributed to angiotensin II, which induces oxidative stress in the retina and in parallel elicits angiogenesis and neovascularization, a feature of late-stage DR [5]. Studies based on RAS inhibition using angiotensin II receptor blockers (ARBs) or angiotensin-converting enzyme (ACE) inhibitors have shown attenuation of some features of DR, but none was completely effective in halting DR. The reason for this failure is that these inhibitors target very downstream components of the RAS cascade. The direct renin inhibitors (DRIs) are the most recent class of RAS inhibitors, blocking the RAS at the very beginning of the cascade. These seem promising to establish new and effective RAS-based treatments for DR. Due to the importance of the RPE in DR and the role of RAS in this pathology, the aim of the present study is to explore how glucose modulates the RAS in these cells. Additionally, it is investigated the potential of aliskiren, the only DRI clinically approved, in decreasing the expression of DR markers in the RPE cells. Our data will contribute to clarify the potential of aliskiren as an efficient alternative drug to protect RPE cells from damage induced by RAS over activation and to be used in the treatment of DR.

## Materials and methods

### Reagents

Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), trypsin–EDTA, glutamine, penicillin/streptomycin, antibody against β-actin and renin assay kit (MAK157) were purchased from Sigma-Aldrich (USA). Antibodies against (pro)renin (sc-22752), goat anti-mouse HRP and goat anti-rabbit HRP were purchased from Santa Cruz Biotechnology (USA). Antibodies against (pro)renin receptor (ab40790), vascular endothelial growth factor (VEGF) isoforms 165 and 165b were purchased from Abcam (UK). Antibody against the pigment epithelium-derived factor (PEDF) was purchased from Merck Millipore (Germany). Aliskiren was purchased from Selleckchem (USA).

### Cell culture

D407 cells, a human RPE cell line, were kindly provided by Dr. Jean Bennett (University of Pennsylvania, USA). Cells were maintained in 5 % $CO_2$, 95 % air at 37 °C and were grown in DMEM medium with 1 % penicillin/streptomycin, 1 % glutamine and 5 % FBS. Cells were dissociated with trypsin–EDTA, split 1:5 and cultured in culture plates with 21 $cm^2$ growth areas (Orange Scientific, Belgium). Culture medium was changed every 2 days, and cells reached confluence after 3 days of incubation. For the experiments regarding glucose effects, cells were cultured either in DMEM with 5.5 mM glucose, to mimic a physiological concentration or in DMEM with 25 mM glucose, a hyperglycemic condition.

### Extraction of proteins

Confluent cells were grown in FBS-free culture medium during 48 h, with the appropriate glucose concentration (5.5 or 25 mM). The conditioned media were collected and concentrated (Amicon Ultra-2, Merck Millipore). To obtain cellular lysates, cells were washed with PBS and homogenized in cold RIPA buffer (50 mM Tris–HCl pH 7.4, 1 % NP-40, 0.25 % Na-deoxycholate, 150 mM NaCl and 1 mM EDTA) with a protease inhibitor cocktail (Roche, Germany). Cells were incubated on ice for 20 min, centrifuged (16,200 g, 20 min, 4 °C) and the supernatant was collected. Protein concentration was determined in both cellular lysates and concentrated conditioned media using the Bradford reagent (BioRad, USA).

### Immunoblotting

Protein samples were mixed with 4× SDS sample buffer, denatured and equal amounts were loaded and subjected to electrophoresis in a 12 % SDS acrylamide gel. After proteins were electrotransferred onto PVDF membranes, the transblot sheets were blocked with 5 % non-fat dry milk in Tris-buffered saline 0.1 % Tween-20 (TBS-T) for 1 h at room temperature. Membranes were incubated with antibodies against (pro)renin (1/500), (pro)renin receptor (1/

2000), VEGF (1/1000), $VEGF_{165}b$ (1/500) or PEDF (1/1000), overnight at 4 °C in 5 % non-fat dry milk in TBS-T. The membranes were subsequently incubated with the respective secondary antibody (1/5000) for 1 h at room temperature and with the enhanced chemiluminescence detection reagent for 5 min. To ensure that samples were evenly loaded the membranes were stripped and re-probed for β-actin (1/10,000). Membranes were visualized in a GelDoc system (BioRad).

### Renin activity

Renin activity was measured in cells grown in DMEM culture medium with 5.5 or 25 mM glucose by means of a fluorometric assay kit. Cellular lysates were collected and processed for the measurement of renin activity according with the manufacturers' instructions. Fluorescence was recorded every 5 min during 1 h at 540/590 nm (Ex/Em) using a microplate reader (InfiniteM200, TECAN). Values were normalized to protein concentration in each condition and expressed as percentage of control, with control assumed to be the condition with 5.5 mM of glucose.

### Measurement of ROS levels

ROS levels were measured using the oxidative stress probe CM-$H_2$DCFDA (Life Technologies). Cells were grown at 10,000 cells/well in 96-well plates and were exposed to either aliskiren or angiotensin II (details in Results section). Cells were washed with PBS and loaded with 10 μM CM-$H_2$DCFDA for 30 min at 37 °C. Fluorescence intensity was measured in a microplate reader (InfiniteM200, TECAN) at 494/522 nm (Ex/Em) during 60 min. ROS levels were expressed as percentage of control, with control assumed to be the condition without treatment.

### Statistical analysis

Arithmetic means are given with standard error of the mean. Statistical analysis was performed using an unpaired *t* test and two-way analysis of variance followed by the Dunnett's multiple comparison test for multiple comparisons. A value of $P < 0.05$ was considered to be statistically significant.

## Results

### Glucose modulates (pro)renin and the (pro)renin receptor in RPE cells

(Pro)renin and its receptor are key players in the RAS due to its actions at the rate-limiting step of the system. Expression of (pro)renin was evaluated in cellular lysates and supernatants of RPE cells exposed to 5.5 mM (normoglucose) or 25 mM of glucose (high glucose). The levels of (pro)renin expressed in lysates of RPE cells were more elevated in high glucose than in normal glucose conditions (Fig. 1a, b), while the levels of (pro)renin secreted to the extracellular space were higher in normal glucose medium compared with high glucose medium (Fig. 1a, b). Renin activity followed an increasing trend when RPE cells grown in 25 mM of glucose, although statistical significance was not reached (Fig. 1c). The levels of the (pro)renin receptor were higher when RPE cells were grown in the hyperglycemic medium than in the normoglycemic, as shown in Fig. 1d, e.

### Activation of the renin–angiotensin system induces angiogenesis and oxidative stress in RPE cells

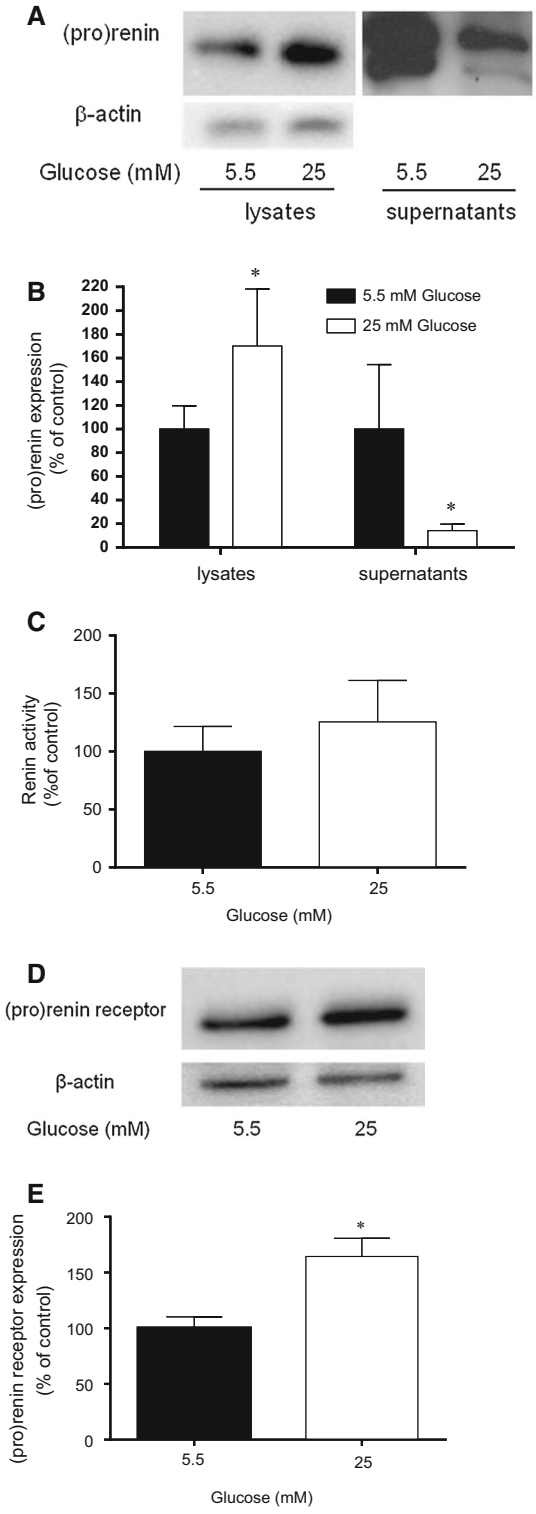
VEGF is a potent inducer of angiogenesis, but oxidative stress also contributes to this process. To assess if activation of the RAS contributes to deleterious effects in RPE cells via VEGF and oxidative stress activation, these were exposed to angiotensin II. It was observed following stimulation of RPE cells with angiotensin II that the levels of VEGF increased in a concentration-dependent manner (Fig. 2a, b). Additionally, exposure of RPE cells to angiotensin II increased the production of ROS in a time-dependent manner (Fig. 2c).

### Aliskiren prevents angiogenesis and oxidative stress in RPE cells

Aliskiren is a direct renin inhibitor acting at the beginning of the RAS cascade. RPE cells were exposed to aliskiren (100 μM) up to 24 h followed by the measurement of ROS levels, angiogenic and anti-angiogenic factors (Fig. 3). Aliskiren significantly decreased the production of ROS (Fig. 3a) and the levels of VEGF in a time-dependent manner (Fig. 3b, c), an effect that reached statistic significance after a 24 h exposure with aliskiren (Fig. 3b, c). On the other hand, the levels of two relevant anti-angiogenic factors increased in RPE cells following aliskiren treatment: the expression of $VEGF_{165}b$ significantly increased after a 12 h exposure with aliskiren, while the expression of PEDF increased in a time-dependent manner with aliskiren treatment, reaching statistical significance after a 24 h of exposure (Fig. 3b, c).

## Discussion

RPE cells have a crucial role in the retina as they are part of the BRB, which is largely impacted in DR. In DR patients, lesions in the RPE near the macula caused fluid leakage




◀**Fig. 1** Glucose modulates the expression of RAS in RPE cells. **a** Representative immunoblots and **b** densitometric analysis of (pro)renin expression in the presence of 5.5 and 25 mM of glucose in cell lysates and supernatants of RPE cells. β-actin is used as loading control. Values are expressed as percentage of control, with control being the condition with 5.5 mM of glucose. $*P < 0.05$ compared with control values, determined by Dunnett's multiple comparison test. Results are representative of 6 independent experiments. **c** Renin activity in the presence of 5.5 and 25 mM of glucose in cellular lysates of RPE cells. Values are normalized to protein concentration in each condition and expressed as percentage of control. Control is assumed to be the condition with 5.5 mM of glucose. Results are representative of 5 independent experiments. **d** Representative immunoblots and **e** densitometric analysis of (pro)renin receptor expression in the presence of 5.5 and 25 mM of glucose in cell lysates of RPE cells. β-actin is used as loading control. Values are expressed as percentage of control, with control being the condition with 5.5 mM of glucose. $*P < 0.05$ compared with control values, determined by unpaired *t* test. Results are representative of 3 independent experiments

and were responsible for macula edema, a major cause of vision loss in DR [6]. The precise mechanisms contributing to RPE dysfunction in DR are not completely clarified, but it is known that angiogenesis and oxidative stress are two major contributors [7, 8]. We recently demonstrated that the balance between angiogenic and anti-angiogenic factors is finely modulated by glucose and ROS levels in RPE cells [3], emphasizing the importance of these factors to the development of DR [1]. The present study was designed to further explore intracellular targets of glucose in RPE cells in order to better understand the mechanisms underlying DR. In this study, our attention was focused on the renin–angiotensin system, an important hormonal system in the regulation of blood pressure that is also present in the eye. Recently, we found that RPE cells express the main components of the RAS [9]. This system has been described as being strongly influenced by glucose, and evidences show a correlation between the over activation of RAS and DR [4, 10, 11]. The RAS can be activated by two different pathways: an angiotensin II-dependent and an angiotensin II-independent pathway. The former involves the secretion of (pro)renin and its binding to the (pro)renin receptor present at the cellular membrane. This binding activates different intracellular signaling pathways, such as the mitogen-activated protein kinases which lead to organ damage [12]. The angiotensin II-dependent pathway takes place in the intracellular compartment and involves the conversion of angiotensinogen to angiotensin I by renin. Angiotensin I is then converted to angiotensin II by the action of ACE. Our results show that high glucose increases the levels of (pro)renin in the intracellular milieu of RPE cells, but the levels of (pro)renin secreted to the extracellular medium were significantly decreased by excess of glucose. These results demonstrate that hyperglycemia promotes the overexpression and retention of (pro)renin in RPE cells. Similar results were obtained with rat mesangial cells where high glucose decreased the levels of (pro)renin in the culture medium, resulting in its intracellular accumulation [11]. Although statistical significance was not reached, a trend was observed for the

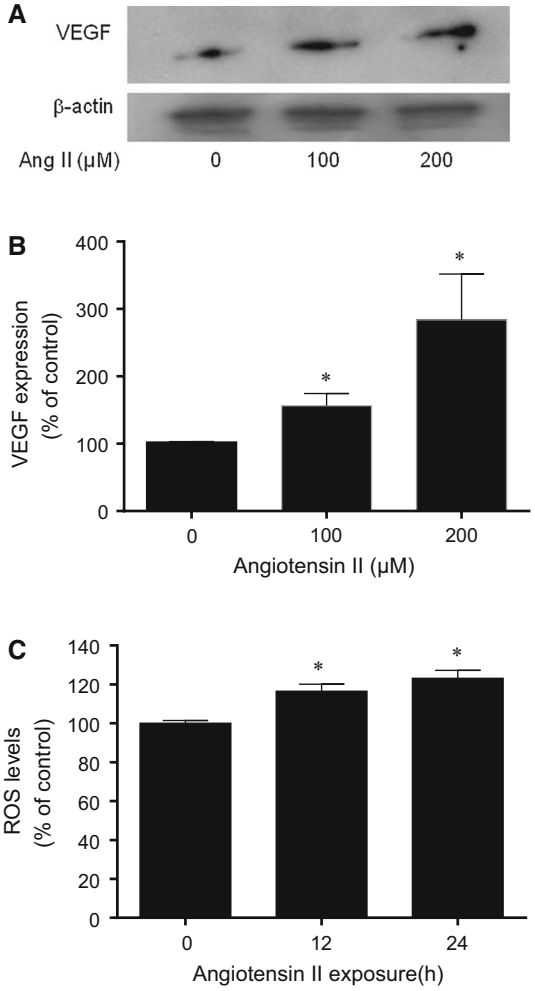



**Fig. 2** Activation of RAS induces angiogenesis and oxidative stress in RPE cells. **a** Representative immunoblots and **b** densitometric analysis of VEGF expression in the presence of angiotensin II (0, 100, 200 μM; 24 h) in RPE cells. β-actin is used as the loading control. Values are expressed as percentage of control with control being the condition without angiotensin II. $*P < 0.05$ compared with control values, determined by Dunnett's multiple comparison test. Results are representative of 5 independent experiments. **c** Levels of ROS in the presence of angiotensin II (0, 12, 24 h; 200 μM) in RPE cells. Values are expressed as percentage of control with control being the condition without angiotensin II. $*P < 0.05$ compared with control values, determined by Dunnett's multiple comparison test. Results are representative of 4 independent experiments

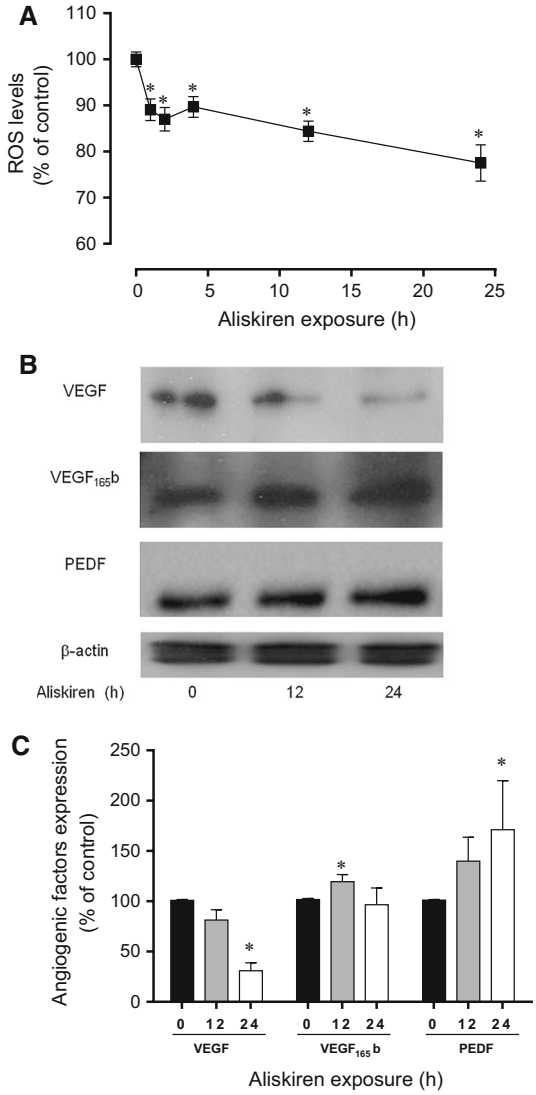



**Fig. 3** Aliskiren protects RPE cells from oxidative stress and angiogenesis. **a** Levels of ROS in the presence of aliskiren (0, 1, 2, 4, 12, 24 h; 100 μM) in RPE cells. Values are expressed as percentage of control with control being the condition without aliskiren. $*P < 0.05$ compared with control values, determined by Dunnett's multiple comparison test. Results are representative of 6 independent experiments. **b** Representative immunoblots and **c** densitometric analysis of VEGF, $VEGF_{165}b$ and PEDF expression in the presence of aliskiren (0, 12, 24 h; 100 μM) in RPE cells. β-actin is used as the loading control. Values are expressed as percentage of control with control being the condition without aliskiren. $*P < 0.05$ compared with control values, determined by Dunnett's multiple comparison test. Results are representative of 6 independent experiments

activity of renin in the presence of high glucose in cellular lysates of RPE cells. Lack of statistical significance might be related to sample size. Nevertheless, the retention of (pro)renin in the intracellular compartment induced by excess of glucose will activate the angiotensin II-dependent pathway, ensuing angiotensin II synthesis. The (pro)renin receptor was also modulated by high glucose in RPE cells. This receptor has a predominant subcellular localization [12], and its translocation to the cellular membrane takes place only following a certain stimulus. In the present study, the levels of the (pro)renin receptor were measured in total lysates and not in membrane and cytosolic fractions. Since high glucose promoted the overexpression and retention of (pro)renin in the intracellular space, we hypothesize that this condition might also favor the retention of the (pro)renin receptor within the RPE cells.

In the present study, we verified that following RAS activation, simulated by angiotensin II treatment, the levels of VEGF and ROS increased in RPE cells. Due to the complexity of RAS pathways, several inhibitors have emerged to block this system at different steps. Recently, it was shown that captopril (an ACE inhibitor) and candesartan (an ARB) decreased the production of ROS in the retinas of ischemic rats [13]. Perindopril (an ACE inhibitor) and candesartan decreased the levels of VEGF in the retinas of streptozotocin-induced diabetic rats [14, 15]. Despite these results, the RAS was not effectively blocked, since these drugs promote a compensation feedback mechanism that increases renin [16, 17]. In fact, it was previously shown that administration of enalapril (an ACE inhibitor) increased the expression of renin in the mouse retina [18]. These results highlight the need of alternative drugs to target the RAS in a sustained manner for the treatment of retinal diseases. Comparing with the traditional RAS inhibitors, the DRIs have the advantage of acting directly on renin, the rate-limiting step of the cascade, and for that reason these drugs might be more effective candidates to block the RAS. Aliskiren is the only DRI clinically approved and Wilkinson-Berka et al. [19] demonstrated that in comparison with an ACE inhibitor, aliskiren conferred similar or higher retinal protection in two models of retinal disease. Nevertheless, the direct effects of DRIs on RPE cells are unknown, namely in pathological conditions. Recently, we demonstrated that aliskiren inhibit the RAS present in the RPE cells and we described the mechanism of action associated with this inhibition [9]. In the present study, we demonstrate for the first time that aliskiren reduced the production of ROS in RPE cells in a sustained and time-dependent manner, suggesting that this drug has antioxidant capacity.

It is well established that in patients with retinal diseases the balance between angiogenic and anti-angiogenic factors is shifted toward the former, promoting angiogenesis and neovascularization, and this is crucial to the progression of the disease [20]. In the retina, RPE cells are one of the sources of VEGF [21, 22] and inhibition of this factor constitutes the main therapy in order to decrease ocular neovascularization. On the other hand, $VEGF_{165}b$, originated by alternative splicing of the VEGF mRNA, has anti-angiogenic properties and is down-regulated in pathologies associated with abnormal angiogenesis, such as retinopathies [23, 24]. PEDF, synthesized by RPE cells, is known to suppress angiogenesis and is also down-regulated in retinal diseases [20]. Our findings demonstrate that aliskiren decreases the levels of VEGF while up-regulates $VEGF_{165}b$ and PEDF, successfully contributing to restore the balance between angiogenic and anti-angiogenic factors in RPE cells. Overall, our findings suggest that aliskiren combines antioxidant and anti-angiogenic functions over RPE cells. To the best of our knowledge, none of the classical RAS blockers used in the treatment of retinopathies gather these properties and for that reason aliskiren might offer advantages over those RAS inhibitors for the treatment of these diseases. Interestingly, in a recent systematic review aimed to evaluate the effect of RAS inhibitors on diabetic retinopathy, it was found that ACE inhibitors were better than ARBs for the treatment of this disease [25]. We hypothesized that this finding might be related to the fact that the ACE is a preceding component in the RAS cascade than the angiotensin II receptor. This supports our theory that upstream components of the RAS might be more adequate targets to promote effective RAS inhibition.

In conclusion, we demonstrated that the RAS is deregulated by high glucose in RPE cells, inducing angiogenesis and oxidative stress, specific markers of diabetic retinopathy. Aliskiren was shown to have antioxidant and anti-angiogenic properties by effectively inhibiting these pathological features. Therefore, it is suggested that aliskiren might be a promising drug to target the RAS in diabetic retinopathy improving the disease profile.

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**Compliance with ethical standards**

**Conflict of interest** The authors declare that they have no conflict of interest.

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