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Aliskiren inhibits the renin-angiotensin system in retinal pigment epithelium cells

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ABSTRACT

Observations of increased angiotensin II levels and activation of the (pro)renin receptor in retinopathies support the role of ocular renin-angiotensin system (RAS) in the development of retinal diseases. While targeting RAS presents significant therapeutic potential, current RAS-based therapies are ineffective halting the progression of these diseases. A new class of drugs, the direct renin inhibitors such as aliskiren, is a potential therapeutic alternative. However, it is unclear how aliskiren acts in the retina, in particular in the retinal pigment epithelium (RPE), the structure responsible for the maintenance of retinal homeostasis whose role is deeply compromised in retinal diseases. We firstly analyzed the expression and activity of the main RAS components in RPE cells. Time- and concentration-dependent treatments with aliskiren were performed to modulate different pathways of the RAS in RPE cells. Our data demonstrate that RPE cells express the main RAS constituents. Exposure of RPE cells to aliskiren inhibited the activity of renin and consequently decreased the levels of angiotensin II. Additionally, aliskiren reduced the translocation of the (pro)renin receptor to the cellular membrane of RPE cells preventing the activation of ERK1/2.

Our findings of the RPE well-defined RAS, together with the demonstration that aliskiren effectively blocks this system at different steps of the cascade, suggest that aliskiren might be an alternative and successful drug in preventing the deleterious effects derived from the overactivation of the RAS, known to contribute to the pathogenesis of different retinal diseases.

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1. Introduction

The renin-angiotensin system (RAS) is a set of intricate pathways with a well defined function in the regulation of blood pressure and body fluid homeostasis. The RAS was initially identified in the kidney but currently it is well established that it is also present in other tissues such as the retina. (Fletcher et al., 2010; Giese & Speth, 2013; White et al., 2015) Although the function of the ocular RAS is still not fully understood, its deregulation is associated with the pathogenesis of diabetic retinopathy (DR) and age-related macular degeneration (AMD). (Fletcher et al., 2010; Funatsu et al., 2002a,b) Abnormal levels of angiotensin II and the activation of the angiotensin II receptor were associated with vascular dysfunction and neovascularisation in the retina. (Giese & Speth, 2013; Funatsu et al., 2002a,b) Therefore, the use of RAS blockers [angiotensin II receptor blockers (ARB) and angiotensin II converting enzyme (ACE) inhibitors] has been tested in experimental models of DR and AMD. (Nagai et al., 2007; Nagai et al., 2006) Clinical trials using ARBs or ACE inhibitors to prevent or decrease the progression of DR (Chaturvedi et al., 2008; Sjolie et al., 2008; Mauer et al., 2009) have shown these to be mildly protective. This limited success may be related with the fact that these conventional RAS inhibitors act in downstream steps of the RAS, promoting a compensation feedback mechanism that increases renin (Muller and Luft, 2006) and compromises the effectiveness of the treatment.

A new generation of RAS inhibitors are the direct renin inhibitors (DRI). Renin is the rate-limiting step of the RAS and its direct inhibition promotes high and sustained RAS blockage. Presently the only DRI available for medical use is aliskiren, an antihypertensive drug. (Wood et al., 2003) Aliskiren was shown to be effective in decreasing retinal ischemic damage in an experimental model. (Tenkumo et al., 2014) Wilkinson-

Abbreviations: ACE, angiotensin II converting enzyme; AMD, age-related macular degeneration; ARB, angiotensin II receptor blocker; BRB, blood-retinal barrier; DR, diabetic retinopathy; DRI, direct renin inhibitor; RAS, renin-angiotensin system; RPE, retinal pigment epithelium.

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Berka et al. compared the effectiveness of an ACE inhibitor with aliskiren in preventing pathological features in models of retinopathy (Wilkinson-Berka et al., 2011) and concluded that aliskiren conferred a similar or greater level of protection than the ACE inhibitor, suggesting that aliskiren has potential for the treatment of retinopathies. Despite that, studies on the effect of aliskiren upon retinal cells are scarce and the mechanism of action of this drug upon the retinal RAS has not been described so far. Batenburg et al. showed that aliskiren has anti-inflammatory properties in Müller cells (Batenburg et al., 2014), but no studies were conducted on the retinal pigment epithelium (RPE). The RPE has a fundamental role in the homeostasis of the retina, in maintaining the integrity of the outer blood retinal barrier (oBRB), and its function is deeply compromised in DR and AMD (Kozlowski, 2012; Simo et al., 2010; Cunha-Vaz et al., 2011). How RAS might contribute to the impairment of the role of the RPE is unknown. Therefore the aim of the present study is to better characterize the RAS and to determine how a direct renin inhibitor, aliskiren, influences the different components of this system in RPE cells. The obtained data will contribute to elucidate the interplay of the RAS and RPE cells and to assess if aliskiren is a potential drug for the treatment of retinopathies.

2. Materials and methods

2.1. Reagents

(Pro)renin, p-ERK1/2, t-ERK, β-tubulin, anti-mouse HRP and anti-rabbit HRP antibodies were purchased from Santa Cruz Biotechnology, Inc. (USA). (Pro)renin receptor antibody was purchased from Abcam (UK). The Alexa Fluor® 488 and calcein-AM were purchased from Thermo Fisher Scientific (USA). Aliskiren was purchased from Selleckchem (USA). All the other reagents were purchased from Sigma-Aldrich (USA).

2.2. Cell culture

D407 cells, a human RPE cell line, were kindly provided by Dr. Jean Bennett (University of Pennsylvania, USA). HEK293 cells were used as control cells for the presence of the RAS. Cells were maintained in 5% CO_2–95% air at 37 °C and grown in DMEM culture medium supplemented with 1% penicillin/streptomycin, 1% glutamine and FBS (5% for D407 and 10% for HEK293). For sub-culturing, cells were dissociated with a trypsin-EDTA solution, split 1:5 and cultured in 21 cm^2 culture plates (Orange Scientific, Belgium). Culture medium was changed every 2 days, and cells reached confluence after 3 days of incubation.

2.3. Immunocytochemistry

60,000 cells/well were grown overnight in glass coverslips at 37 °C. Cells were fixed with 4% paraformaldehyde for 15 min at room temperature, permeabilized and blocked with 10% normal serum in PBS-T (0.2% TritonX-100) for 30 min at room temperature. Cells were incubated with primary antibodies for (pro)renin and (pro)renin receptor both diluted at 1/500 in PBS-T for 1 h at room temperature. After three washes cells were incubated with the secondary antibodies (1/2000) for 1 h at room temperature. DAPI was included in the secondary antibody solution to stain the nuclei. Cells were visualized using a fluorescence microscope at a 630× magnification (Axio Observer Z2, Zeiss).

2.4. Extraction of proteins

Confluent cells were grown in FBS-free culture medium for 48 h. The conditioned medium of each cell line was collected and concentrated (Amicon Ultra-2 filters, Merck-Millipore). To obtain cellular lysates cells were washed with PBS and homogenized in RIPA buffer (50 mM Tris-HCl pH 7.4, 1% NP-40, 0.25% Na-deoxycholate, 150 mM NaCl and 1 mM EDTA) supplemented with protease inhibitors. Cells were incubated on ice for 20 min, centrifuged (16,200g, 20 min, 4 °C) and the supernatant was collected. Protein concentration was determined using the Bradford reagent (BioRad, USA) in cellular lysates and concentrated conditioned media. To obtain cytosolic and membrane fractions, cells were homogenized in lysis buffer (20 mM Hepes pH 7.4, 1 mM EDTA, 250 mM sucrose and protease inhibitors). Lysates were centrifuged to pellet nuclei and cellular debris. Supernatants were centrifuged (100,000g, 1 h at 4 °C) and the obtained supernatants correspond to the cytosolic fraction. The remaining pellet was resuspended (in 10 mM Hepes pH 7.4, 250 mM sucrose and protease inhibitors) to obtain the membrane fraction.

2.5. Immunoblotting

Protein samples were mixed with 4× SDS sample buffer, heated for 5 min at 95 °C, and equal amounts of protein were loaded in a 12% SDS acrylamide gel. After proteins were electrotransferred the membranes 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 the primary antibodies against (pro)renin (1/500) and (pro)renin receptor (1/1000), in 5% non-fat dry milk in TBS-T overnight at 4 °C. The membranes were subsequently incubated with the secondary antibodies conjugated with HRP (1/5000) for 1 h at room temperature, incubated with the chemiluminescence detection reagent and visualized in a GelDoc system (BioRad). The membranes were stripped and re-probed for β-tubulin (1/1000). In the aliskiren experiments RPE cells were exposed to aliskiren during 4 h and collected for detection of (pro)renin and (pro)renin receptor expression. RPE cells were subjected to a time-course to aliskiren (10, 30, 60, 120 and 240 min) to determine the activation of ERK1/2. Cells were collected and analyzed for phospho-ERK1/2 (1/1000) and total-ERK (1/200) expression.

2.6. Cell viability

RPE cells were seeded at 10,000 cells/well in 96-multiwell plates and incubated with aliskiren [50 and 100 μM (30 and 60 μg/ml in absolute dose)] for 2 h and 4 h. Cells were also incubated with ethanol as the positive control for cell death and DMSO as the vehicle for aliskiren. Cells were incubated with calcein-AM (5 μM) for 30 min at 37 °C and the fluorescence was measured (Ex. 493/Em. 530 nm; InfiniteM200, TECAN). Data were expressed as percentage of control, with control being the condition without aliskiren.

2.7. Renin activity

RPE cells were incubated with aliskiren [50 and 100 μM (30 and 60 μg/ml in absolute dose)] for 2 h and 4 h. Cellular lysates were collected and processed according with the manufacturers' instructions. Fluorescence was recorded every 5 min during 60 min (Ex. 540/Em 590 nm; InfiniteM200, TECAN). Values were normalized to protein concentration in each condition and expressed as percentage of control, with control being the condition without aliskiren.

2.8. Measurement of angiotensin II levels

RPE cells were incubated with aliskiren [100 μM (60 μg/ml in absolute dose); 4 h] and cellular lysates were collected and processed according with the manufacturers' instructions (Abs. 450 nm; InfiniteM200, TECAN). Values were normalized to protein concentration in each condition and expressed as percentage of control, with control assumed to be the condition without aliskiren.

2.9. Statistical analysis

Arithmetic means are given with SEM. Statistical analysis was performed using an unpaired *t*-test and two-way analysis of variance

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