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Direct renin inhibition with aliskiren improves ischemia-induced neovascularization: Blood pressure-independent effect

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ABSTRACT

Background: Renin is the rate limiting step for the activation of the renin-angiotensin-aldosterone system, which is linked to the development of endothelial dysfunction, hypertension and atherosclerosis. However, the specific role of renin during physiological responses to tissue ischemia is currently unknown. Aliskiren is the only direct renin inhibitor that is clinically used as an orally active antihypertensive drug. Here we tested the hypothesis that aliskiren might improve neovascularization in response to ischemia.

Methods and results: At a dose that did not modulate blood pressure (10 mg/kg), aliskiren led to improved blood flow recovery after hindlimb ischemia in C57BL/6 mice (Doppler flow ratios 0.71 ± 0.07 vs. 0.55 ± 0.03; $P < 0.05$). In ischemic muscles, treatment with aliskiren was associated with a significant increase of vascular density, reduced oxidative stress levels and increased expression of VEGF and eNOS. Aliskiren treatment also significantly increased the number of bone marrow-derived endothelial progenitor cells (EPCs) after hindlimb ischemia. Moreover, the angiogenic properties of EPCs (migration, adhesion, integration into tubules) were significantly improved in mice treated with aliskiren. In vitro, aliskiren improves cellular migration and tubule formation in HUVECs. This is associated with an increased expression of nitric oxide (NO), and a significant reduction of oxidative stress levels. Importantly, the angiogenic properties of aliskiren in vitro and in vivo are completely abolished following treatment with the NOS inhibitor L-NAME.

Conclusion: Direct renin inhibition with aliskiren leads to improved ischemia-induced neovascularization that is not dependant on blood pressure lowering. The mechanism involves beneficial effects of aliskiren on oxidative stress and NO angiogenic pathway, together with an increase in the number and the functional activities of EPCs.

1. Introduction

The capacity of the organism to counteract the negative effects of vascular occlusions depends in large part on its ability to develop new vessels (neovascularization) [1]. Postnatal neovascularization necessitates angiogenesis, a process that involves activation, proliferation and migration of mature endothelial cells [2]. The endothelial cell specific mitogen Vascular Endothelial Growth Factor (VEGF) is essential for the induction of angiogenesis [3]. In addition, nitric oxide (NO) is recognized as a critical factor for endothelial cell migration, VEGF-induced angiogenesis [4,5], and ischemia-induced neovascularization [6]. On the other hand, it has recently been demonstrated that postnatal neovascularisation not only depends on the extension of the pre-existing vasculature, but also necessitates the action of bone marrow-derived endothelial progenitor cells (EPCs) [7,8]. EPCs have been shown to reach sites of neovascularization where they can differentiate into mature ECs and/or secrete angiogenic growth factors [9].

Unfortunately, the conditions leading to the development of atherosclerosis in patients are also often associated with impaired neovascularization in response to ischemia [1]. Several cardiovascular diseases have been linked to an increased activation of the renin-angiotensin-aldosterone system (RAS). Activation of RAS leads hypertension, endothelial dysfunction and atherosclerosis [10]. Chronic stimulation of RAS is also associated with impaired blood flow recuperation following ischemia [11], and a reduction in EPC number and function [12]. Clinically, inhibition of RAS has

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classically been performed using angiotensin converting enzyme (ACE) inhibitors and angiotensin II receptor blockers (ARBs). Although the beneficial effects of these therapies in patients with atherosclerotic diseases and heart failure are well established [10], their potential role for the modulation of ischemia-induced neovascularization in the context of vascular occlusions is unclear. For example, while treatment with the ACE inhibitor quinaprilat improved neovascularization and angiogenesis in a rabbit model of hindlimb ischemia, the ACE inhibitor captopril did not have any significant effect compared to placebo in the same model [11]. Published studies regarding ARB effects on angiogenesis in cardiovascular disease models have also shown conflicting results. Some studies reported pro-angiogenic effects of ARBs while other studies documented reduced angiogenesis following treatment with ARBs [13].

Renin can be considered the rate limiting step involved in the activation of RAS, since it catalyzes the formation of angiotensin I from angiotensinogen with very high substrate specificity. This characteristic makes it an ideal target for the therapeutic blockade of RAS. Aliskiren is the only direct renin inhibitor that is clinically used as an orally active antihypertensive drug. In addition to its beneficial effect on blood pressure, recent evidence suggests that aliskiren improves endothelial function and might prevent the development and/or the progression of atherosclerotic diseases [14,15]. However, the effect of aliskiren on blood flow recuperation and neovascularization in the setting of vascular occlusion and tissue ischemia is currently unknown. In the present report, we studied the effect of aliskiren on postnatal neovascularization using a mouse model of hindlimb ischemia. We also investigated potential mechanisms that could be involved in that physiopathology including the role of NO, oxidative stress and EPCs.

2. Methods

2.1. Experimental animals and treatments

The protocol was approved by the Comité Institutionnel de Protection des Animaux (CIPA) of the Centre Hospitalier de l'Université de Montréal (CHUM). 6 to 8 week-old C57BL/6 mice were obtained from Charles River (St. Constant, Canada). Mice were maintained in 12 h light–dark cycle and fed ad libitum. Aliskiren (Novartis, Dorval, Canada) was administered in drinking water to achieve a dose of 10 or 50 mg/kg/day. The treatment was started two weeks prior to surgery and maintained throughout the whole study. In some experiments, mice were also treated with the NOS inhibitor NG-nitro-L-arginine methyl ester (L-NAME 1 mg/kg) in combination with Aliskiren in drinking water.

2.2. Murine ischemic hindlimb model and monitoring of blood flow

Unilateral hindlimb ischemia was surgically induced and hindlimb blood flow was monitored with a Laser Doppler perfusion imager (LDPI) system (Moor Instrument Ltd., Axminster, UK) as previously described [16]. To account for variables such as ambient light and temperature, the results are expressed as the ratio of perfusion in the left (ischemic) vs. right (non-ischemic) hindlimb [16]. Ambulatory impairment was evaluated using a scale from 1 to 4 [17]. Blood pressure was measured using a tail-cuff pressure instrument (BP-2000, Visitech Systems, Apex, NC).

2.3. Tissue preparation and immunochemistry

Whole ischemic hindlimb muscles were immediately fixed in tissue-fix overnight or preserved in OCT. Identification of endothelial cells was performed by immunostaining for mouse platelet endothelial cells adhesion molecule-1 (PECAM-1 or CD31) with a rat monoclonal antibody (Pharmingen, San Diego, CA) [16]. Arterioles were identified using an elastin stain kit (Sigma). To evaluate local oxidative stress levels in ischemic muscles, dihydroethidium staining (DHE, Molecular probes, Eugene, OR) was used as previously described [18].

2.4. Plasma NO release

NO production was determined indirectly using a commercial kit that measures the concentration of the stable end products nitrate and nitrite based on the Griess reaction (R&D systems Inc., Minneapolis, MN) [18].

2.5. Western blot analysis

Hindlimb muscles or HUVECs were rinsed in PBS, frozen in liquid nitrogen and stored at −80 °C. Protein extracts were obtained after homogenization of muscles or HUVECs in ice-cold lysis buffer [16]. The membranes were probed with the following antibodies: 1:500 VEGF (Santa Cruz Biotechnology), 1:1000 phospho eNOS (Cell Signaling Technology, Boston, MA), 1:1000 eNOS (Santa Cruz Biotechnology, Santa Cruz, CA), 1:2000 NOX2/GP91 (Santa Cruz Biotechnology, Santa Cruz, CA), and 1:2000 beta-Actin (Santa Cruz Biotechnology). Protein expression was quantified by high-resolution optical densitometry (image J software). Results are expressed as density values normalized to the loading control.

2.6. Endothelial progenitor cells isolation and characterization (early outgrowth EPCs)

Mouse bone marrow mononuclear cells were isolated from the femora and tibiae, plated on 0.005% fibronectin and cultured in complete medium 200 with 20% FBS as previously described [18]. After 4 days in culture, non-adherent cells were removed by washing with PBS. Adherent cells were then stained with 1,1'-dioctadecyl-3,3,3′,3′ tetramethylindocarbocyanine perchlorate-acetylated low-density lipoprotein (DiI-acLDL, Invitrogen, OR, USA) and FITC-labeled lectin BS-1 (Bandeiraea simplicifolia, Sigma). Bone marrow EPCs were characterized as adherent cells positive for both DiI-acLDL uptake and lectin binding. Quantification was performed by examining several random microscopic fields [18].

2.7. Flow cytometry analysis of EPCs

The percentages of EPCs contained in the bone marrow or in the total viable cell population derived from the spleen was measured by flow cytometry (FACSCalibur flow cytometer, Becton Dickinson, Oakville, Ontario, Canada) using the fluorescence-coupled cell markers CD34-FITC and VEGFR-2 (Flk1)-PE (eBioscience, CA, USA) [19]. Cell phenotypes were determined by the analysis of 300,000 events.

2.8. Matrigel tubule assay

Growth factor reduced Matrigel Matrix (Becton Dickinson Labware, Bedford, MA) was thawed and placed in 96-well plates at 37 °C to allow solidification. DiI-labeled EPCs (3000) were co-plated with HUVECs (12,000) and cultured at 37 °C for 8 h with 50 ng/ml of VEGF. The number of incorporated EPCs in tubules was determined in 10 random fields [20]. Total number of tubules, tubule length and tubule branching were similar between groups in all the experiments (data not shown).