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Aliskiren: Preclinical evidence for the treatment of hyperproliferative skin disorders

Priscila Lúcia Pawloski[a], Camila Guimarães Moreira[a], Cintia Delai Silva Horinouchi[a], Daniel Fernandes[c], Luiz Renato Olchanheski Júnior[c], Willian Machado[c], Daniela Almeida Cabrini[a], Michele Dietrich[b], Kátia Paludo[b], Michel Fleith Otuki[a,c,*]

[a] Laboratory of Inflammation, Department of Pharmacology, Universidade Federal do Paraná, PO Box 19031, CEP 81530-900, Curitiba, Paraná, Brazil
[b] Department of Biological Sciences, Universidade Estadual de Ponta Grossa, Avenida Carlos Cavalcanti, 4748, CEP 84030-900, Ponta Grossa, Paraná, Brazil
[c] Department of Pharmaceutical Sciences, Universidade Estadual de Ponta Grossa, Avenida Carlos Cavalcanti, 4748 (Room – M47), CEP 84030-900 Ponta Grossa, Paraná, Brazil

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ABSTRACT

Psoriasis is a complex inflammatory and hyperproliferative skin disease. The pathogenesis and mechanisms involved are not completely understood, which makes treatment a difficult issue. Angiotensin II, the most active peptide of the renin-angiotensin system, seems to be involved in processes related to psoriasis pathogenesis, such as inflammation and cell proliferation. The aim of this study was to investigate the influence of renin inhibition on inflammation parameters and keratinocyte proliferation in a mouse model of chronic skin inflammation induced by croton oil. Aliskiren had anti-inflammatory effects by reducing levels of tumor necrosis factor-α and interleukin -6, and by inhibiting myeloperoxidase activity. Aliskiren also showed antiproliferative activity by reducing epidermal hyperplasia and proliferating cell nuclear antigen levels. Aliskiren treatment did not induce alterations in the cardiovascular system, normal skin thickness, and organ weight. These results suggest that aliskiren could be a valuable tool to be incorporated in the treatment of hyperproliferative and inflammatory skin disorders such as psoriasis.

1. Introduction

Psoriasis is a common chronic inflammatory idiopathic skin disease which affects 1–3% of the global population [1] and greatly compromises patient quality of life [2]. The main symptom is erythematous plaques on the skin, sharply demarcated, with silvery scales, which are the result of epidermal hyperproliferation, with incomplete maturation and cornification of keratinocytes [3]. It is a complex inflammatory autoimmune disease with a genetic basis [4]. However, the exact mechanisms are not completely understood, which has hampered the selection of correct therapeutic interventions, especially for treatment-resistant patients [5].

The renin-angiotensin system (RAS) is one of the most studied endocrine systems due to its involvement in cardiovascular regulation [6]. RAS is present in most body systems, including the skin, which can synthesize and store RAS components [7,8]. Angiotensin II (Ang II) is considered the most important active peptide of RAS and acts through AT1 and AT2 receptors. The presence of these receptors in human and rat skin suggests the possible involvement of this system in skin processes such as inflammation, wound healing, cell proliferation and tissue repair [9]. Thus, the aim of this study was to investigate the effect of renin inhibition on skin inflammation and keratinocytes proliferation in a mouse ear edema model.

2. Methods

2.1. Animals

Experiments were conducted using female Swiss mice (25–35 g) housed at 22 ± 2 °C under a 12 h light/dark cycle and with access to food and water ad libitum. Animals were allowed to adapt to the laboratory for at least 24 h before testing and were used only once. Experiments were performed in accordance with guidelines specified by the Ethics Committee on Animal Experimentation, and the

Abbreviations: Ang II, angiotensin II; BSA, bovine serum albumin; INF, interferon; IL, interleukin; MPO, myeloperoxidase; NAG, *N*-acetyl-β-D-glucosaminidase; PCNA, proliferating cell nuclear antigen; RAS, renin-angiotensin System; TNF, tumor necrosis factor

* Corresponding author at: Departamento de Farmacologia, Setor de Ciências Biológicas, Universidade Federal do Paraná, Av. Francisco Hoffman dos Santos, s/n, POBox:19031, 81530-900, Curitiba, PR, Brazil.
E-mail address: otuki@ufpr.br (M.F. Otuki).

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experimental protocol was approved by the Ethics Committee for Animal Use of Federal University of Paraná (protocol number 726).

2.2. Croton oil-induced chronic skin inflammation

The chronic inflammatory process was induced by multiple topical application of croton oil (0.4 mg/ear) on alternate days for nine days. Aliskiren (30, 100 and 300 mg/kg) and dexamethasone (3 mg/kg) were administered orally by gavage from the fifth day of the trial for the last four days (twice a day). Control animals were treated with TPA and the vehicle used to dilute Aliskiren (saline by gavage). Ear thickness (μm) was evaluated every day with a digital micrometer (Electronic Outsides Micrometer, Digimess). On the ninth day of the experiment, the animals were euthanized and biopsies of ear tissue (6 mm) were collected and submitted to analyses.

2.3. Systolic pressure measurement

During the croton oil-induced edema experiment, blood pressure was measured at the beginning and at the end of the treatments using a tail-cuff system [10]. Animals were placed in a restrainer for 5 min, a cuff was attached to their tail and BP was then recorded. After the established period, the values of cardiac frequency and systolic pressure were registered through integrative software (ChartPro7® AD Instruments Pty Ltd., Castle Hill, Australia).

2.4. Aliskiren safety evaluation

Aliskiren (30, 100 and 300 mg/kg) and dexamethasone (3 mg/kg) were orally administered by gavage for seven days (twice a day). Ear thickness (μm) was evaluated before and eight days after the initiation of treatment with a digital micrometer (Electronic Outsides Micrometer, Digimess). On the eighth day of the experiment, the animals were euthanized and the organs spleen, thyme, adrenal and lymph nodes were obtained and weighed.

2.5. MPO and NAG enzyme activity assay

To assess enzyme activity, the methodology of Bradley et al. modified by De Young et al. was used [11,12]. The biopsies (6 mm diameter tissue samples) were added to 80 mM (phosphate-buffered saline) PBS pH 5.4 containing 0.5% of Hexadecyltrimethylammonium bromide (HTAB) and were homogenized in a motor-driven homogenizer. The samples were placed in microfuge tubes and centrifuged. Myeloperoxidase (MPO) activity was achieved with triplicates of the supernatant placed on 96-well plates, where a mixture containing PBS and hydrogen peroxide was subsequently added into each well. The addition of TMB in dimethylformamide promoted the start of the reaction. The plate was then incubated at 37 °C for 3 min and the reaction was stopped by the addition of sodium acetate. *N*-acetyl-β-D-glucosaminidase (NAG) activity was reached with triplicates of supernatant placed on 96-well plates, followed by the addition of citrate buffer. The reaction was initiated by the addition of NAG. The plate was incubated at 37 °C for 1 h and the reaction was stopped by the addition of glycine buffer. The enzymatic activity was determined colorimetrically using a plate reader (EL808; BioTech Instruments, Inc.) set to measure absorbance at 620 nm for MPO or 405 nm for NAG. The results are expressed as mOD/mg tissue.

2.6. Measurement of IL-6 and TNF-α levels

Levels of interleukin (IL)-6 and tumor necrosis factor (TNF)-α in ear tissue samples homogenates were quantified using a mouse ELISA kit (eBioscience, San Diego, USA) according to the manufacturer's instructions. Cytokines levels were normalized to the total protein content of each sample, which was determined using a Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA, USA).

2.7. Histology and immunohistochemical evaluation of PCNA levels

Sections (5 μm) of tissue previously fixed in ALFAC and embedded in paraffin were cut onto gelatinized glass slides and deparaffinized two times with xylene and rehydrated through a graded alcohol bath. To block radical aldehydes, each section was treated with glycine (0.1 M) and with 3% hydrogen peroxide in methanol to block endogenous peroxidase. Slices were treated with bovine serum albumin (BSA) 1% in PBS to diminish non-specific staining. For the detection of proliferating cell nuclear antigen (PCNA), slides were incubated with a 1:50 dilution of a polyclonal anti-PCNA antibody (Santa Cruz Biotechnology, Inc, USA) in PBS/BSA 1% at room temperature in a humid chamber for 2 h and washed with PBS/BSA 1%. Subsequently, the sections were incubated using a secondary antibody IgG HRP (Santa Cruz Biotechnology, Inc., USA) diluted 1:50 in PBS/BSA 1% at room temperature in a humid chamber for 1 h. The peroxidase-binding sites were detected by staining with DAB substrate Kit (BD Bioscience, California, USA), incubated for 15 min. Finally, slices were counterstained with Mayer's hematoxylin and then dehydrated and mounted. PCNA-positive cells and epidermis thickness were assessed in representative areas at 400× magnification using an optical microscope. The quantification was performed by analyzing the number of positive cells or epidermis thickness (μm) per field of five fields from three different histological sections of three animals per group.

2.8. Evaluation of PCNA levels by western blot

Tissue samples were prepared as previously described by Medeiros et al. [13] with some modifications. Briefly, samples were homogenized in ice-cold RIPA buffer plus protease inhibitors with mechanical homogenizer. The samples were submitted to centrifugation and the supernatant containing whole protein extracts were recovered and the protein concentration was determined using a Bradford Protein Assay (Bio-Rad Laboratories, USA). Equal amounts of protein extracts (30 μg) were boiled in Laemmli buffer for 5 min, resolved by a 12.5% SDS–PAGE and analyzed by western blot using polyclonal anti-PCNA (Santa Cruz). The reaction was developed using chemiluminescent substrate (West Pico, Pierce). The blots corresponding to PCNA were measured using ImageJ Software (National Institutes of Health, Bethesda, MD) and values were used for statistical analysis.

2.9. Statistical analysis

The results are expressed as mean ± S.E.M. Data were evaluated by one-way or two-way analysis of variance (ANOVA) followed by the Newman-Keuls or Bonferroni post-hoc test when appropriate. $P < 0.05$ was considered to indicate statistical significance. The values were obtained using the statistical software GraphPad Prism version 6.00, San Diego California, USA.

3. Results

3.1. Aliskiren reduces inflammatory parameters in a chronic inflammation model

Ear edema induced by multiple applications of croton oil mimics skin chronic inflammatory processes, showing not only edema but other characteristics such as epidermal hyperproliferation and leukocyte infiltration. As observed in Fig. 1A, after the establishment of the inflammatory process, neither of the tested doses of aliskiren was able to reverse the induced increase in ear thickness. However, the anti-edematogenic effects of dexamethasone were noted from the 6th day, with maximum inhibition of 62.0% ± 4.5% in the 8th day when compared with the control group, which did not receive treatment after challenge