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Drug Discovery and Resistance

Effect of the bradykinin 1 receptor antagonist SSR240612 after oral administration in *Mycobacterium tuberculosis*-infected mice

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ABSTRACT

The role, if any, played by the kinin system in tuberculosis infection models, either *in vivo* or *in vitro*, was investigated. The effects of *Mycobacterium tuberculosis* infection on C57BL/6 wild type, $B_1R-/-$, $B_2R-/-$ and double B_1R/B_2R knockout mice were evaluated. Immunohistochemistry analysis was carried out to assess B_1R and B_2R expression in spleens and lungs of *M. tuberculosis*-infected mice. In addition, *in vitro* experiments with *M. tuberculosis*-infected macrophages were performed. The *in vivo* effects of HOE-140 and SSR240612 on the mice model of infection were also evaluated. Infected $B_2R-/-$ mice exhibited increased splenomegaly, whereas decreased spleen weight in infected double B_1R/B_2R knockout mice was observed. The bacterial load, determined as colony-forming units, did not differ in the spleens and lungs of the studied mouse strains. Importantly, immunohistochemical analysis revealed that B_1R was upregulated in both spleens and lungs of infected mice. *M. tuberculosis*-infected macrophages incubated with SSR240612, alone or in combination with des-Arg^9-BK, for four days, displayed a marked inhibitory effect on CFU counts. However, the pre-incubation of the selective B_1R (des-Arg^9-BK and SSR240612) and B_2R (BK and HOE-140) agonists and antagonists, respectively, did not significantly affect the bacterial loads. A statistically significant reduction in the CFU of *M. tuberculosis* in lungs and spleens of animals treated with SSR240612, but not with HOE-140, was observed. Further efforts should be pursued to clarify whether or not SSR240612 might be considered an option for the treatment of tuberculosis.

1. Introduction

Tuberculosis (TB), caused by *Mycobacterium tuberculosis*, is a fatal infectious disease that resulted in more deaths than HIV in 2015, an estimated 1.4 million people [1]. Despite some progress in the pipeline for new drug candidates and regimens, there is still a critical need for the development of new drugs to treat TB [1], and improvements in our understanding of *M. tuberculosis* metabolism can aid the development of innovative strategies to combat this disease. Kinins and their receptors (B_1R and B_2R) are implicated in physiological and pathological processes, including infections [2–11]. Importantly, constitutive B_2R and inducible B_1R were found to play an important role in modulating the inflammatory process induced by injection of *Mycobacterium bovis* bacillus Calmette-Guérin *in vivo* [12]. Accordingly, the use of kinin antagonists has been considered an interesting alternative to treat inflammatory and infectious conditions [2,13–15]. However, our understanding about the role of kinin system in the pathogenesis of intracellular organisms is still limited [3–7]. It was recently reported the quantification of circulating levels of B_2R and B_1R agonists, bradykinin (BK) and des-Arg^9-BK, in a cohort of 13 patients with active TB [16]. BK and des-Arg^9-BK concentrations seem to decrease as the patient shifts from pretreatment to early stage of therapy, to extended phase of dosing, and to treatment completion, suggesting a possible role of kinin system in human tuberculosis [16]. This work describes, to the best of our knowledge, the first study on the role of B_1R and B_2R during *M. tuberculosis* infection, and the assessment of effects of both B_1R and B_2R

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agonists and antagonists in models of TB infection.

2. Materials and methods

2.1. Agonists and antagonists

BK, des-Arg^9-BK, and HOE-140 were purchased from Bachem Americas Inc. (Bachem, USA); SSR240612 was donated by Sanofi (Sanofi Research, Paris, France).

2.2. Mice

C57BL/6 $B_1R^{-/-}$, $B_2R^{-/-}$, and $B_1B_2R^{-/-}$ (knockout) and wild-type C57BL/6 originated from breeding colonies donated by Dr. J. B. Pesquero (Unifesp, São Paulo, Brazil) were maintained in our animal facilities. For mice infections, male and female CF1 mice (25–30 g) obtained from the Central Biotery of PUCRS (CeMBE, Brazil) were employed. Mouse experiments were performed at Preclinical Tests Laboratory, located at INCT-TB-PUCRS. Animals were maintained at temperature- (22 ± 1 °C) and humidity-controlled room, with a 12/12 h light/dark cycle, and food and water available *ad libitum*. The current Brazilian guidelines for the care and use of animals for scientific and didactic procedures, from the National Council for the Control of Animal Experimentation (CONCEA, Brazil), was followed. The local Animal Ethics Committee approved the experimental protocol for infection (CEUA 14/00415). The ARRIVE Guidelines to report *in vivo* experiments [17] were followed.

2.3. Bacteria and macrophages

Virulent *M. tuberculosis* H37Rv laboratorial strain was maintained in Ogawa medium at 4 °C and subcultured in 7H9 media supplemented with tween 80 (0.05%) and albumin dextrose-catalase (ADC) liquid medium. *M. tuberculosis* inoculum was prepared as previously described [18]. The macrophage murine cell line RAW 264.7 (obtained from Cell Bank of Rio de Janeiro) was cultured in DMEM media (Dulbecco's Modified Eagle Medium) supplemented with 10% inactivated fetal bovine serum (FBS) and 1% antibiotics (penicillin-streptomycin). The cells were maintained in culture bottles at 37 °C in humidified atmosphere with 5% CO_2.

2.4. Infection of B_1 and B_2 receptor deleted mice

Knockout ($B_1R^{-/-}$, $B_2R^{-/-}$, and $B_1B_2R^{-/-}$) and wild-type C57BL/6 mice were anaesthetized by intraperitoneal injection of ketamine (100 mg/kg, Cristália) and xylazine (10 mg/kg, Vetbrands), and subsequently infected (through the retro-orbital venous plexus) with 10^6 viable *M. tuberculosis* H37Rv cells [18,19]. Four weeks after infection, mice were euthanized by isofluorane (Cristália) inhalation, and spleens and left lungs were removed under aseptic conditions. Spleens had their weights measured (in mg). The bacterial loads were determined by plating serial dilutions of lung and spleen homogenates on Middlebrook 7H10 agar (Difco) plates containing 10% OADC enrichment (Becton Dickinson). Plates were incubated at 37 °C for 3 weeks in a 5% CO_2 environment prior to counting colony forming units (CFU).

2.5. Effect of the agonists and antagonists in RAW 264.7 cell viability

The direct effect of the agonists and antagonists in RAW 264.7 viability was evaluated by the MTT method [20]. For this assay, RAW 264.7 cells (2000 cells/well, in a 96-well microtiter plate) were incubated with different concentrations of BK, des-Arg^9-BK, HOE-140, and SSR240612 [20], for 96 h. These cells were incubated with MTT (1 mg/mL) for 2 h. The formazan crystals were dissolved in DMSO, and the absorbance of this solution was measured at 595 nm (Spectra Max M2e, Molecular Devices, USA). Three different assays were performed, in triplicates.

2.6. M. tuberculosis susceptibility assay

Determination of *M. tuberculosis* susceptibility to the agonists and antagonists was carried out in 96-well plates. BK, des-Arg^9-BK, HOE-140, and SSR240612 were diluted in Middlebrook 7H9 medium containing 10% ADC to concentrations up to 500 nM, 500 nM, 50 μM, and 250 μM, respectively. The *M. tuberculosis* suspension was diluted in Middlebrook 7H9 broth containing 10% ADC to achieve an optical density value of 0.006 at 600 nm, and 100 μl was then added to each well. The plates were covered, sealed with parafilm and incubated at 37 °C. After 7 days of incubation, 60 μl of 0.01% resazurin solution was added to each well and incubated for additional two days at 37 °C [19]. At least three tests were independently carried out.

2.7. Infection experiments using macrophages

For the first *in vitro* infection test, macrophages were seeded in 24-well culture plates at a density of 10^5 cells per well in DMEM medium (with 10% FBS, without antibiotics) and incubated for 24 h at 37 °C with 5% CO_2. The cells were then washed with 0.9% NaCl to remove non-adherent cells and pre-incubated with BK (3 nM), des-Arg^9-BK (30 nM), HOE-140 (1 μM), and SSR240612 (10 μM), for 30 min, in DMEM medium. Infection of RAW 264.7 cells with *M. tuberculosis* H37Rv was performed at a multiplicity of infection of 1:1, for 3 h at 37 °C with 5% CO_2. Infected RAW 264.7 cells were washed three times with 0.9% saline solution to remove extracellular bacteria, followed by the addition of 1 ml 0.025% SDS dissolved in sterile 0.9% saline solution to lyse macrophages [21]. Lysates were serially diluted and plated on Middlebrook 7H10 Agar with 10% OADC. CFU was determined after incubation of plates for 21 days at 37 °C.

For the second experiment, RAW 264.7 cells were infected with *M. tuberculosis* H37Rv for 3 h, followed by washing extracellular bacteria. The cells were then treated with the tested substances in DMEM medium: BK (3 nM), des-Arg^9-BK (30 nM), HOE-140 (1 μM), and SSR240612 (10 μM). After 4 days, macrophages were then lysed with 0.025% SDS, and these suspensions were diluted and plated on Middlebrook 7H10 Agar containing OADC, prior to CFU counting.

2.8. Expression analysis of B_1R and B_2R in infected mice

Twenty-eight CF1 mice were used to assess B1R and B2R immunopositivity in spleens and lungs. Mice were divided into three groups: I. intravenously infected with *M. tuberculosis* H37Rv (10^2 bacteria in 200 μl), II. intravenously infected with *M. tuberculosis* H37Rv (10^4 bacteria in 200 μl), III. intravenous injection of 200 μl of NaCl 0.9% sterile solution. Mice were euthanized by isoflurane inhalation [18], 15 or 30 days post infection. Lungs and spleens were removed and fixed with 10% buffered formalin for 24 h and then processed for immunohistochemistry as described previously [22]. The antibodies employed were polyclonal rabbit anti-B1R (1:200, Cat#: ABR-011; Lot ABR011AN0150; Alomone Labs, Jerusalem, Israel), and polyclonal rabbit anti-B2R (1:200, Cat#: ABR-012; Lot ABR012AN0102; Alomone Labs, Jerusalem, Israel). To determine the B_1R- and B_2R-immunopositive cells, digitized TIFF images were transferred to a computer and analyzed using Image NIH Image J 1.36b Software (NIH, Bethesda, MD, USA). A specific plugin was used to determine the immunopositivity for B_1R or B_2R.

2.9. Treatment with SSR240612 or HOE-140 in vivo

Twenty CF1 mice were anaesthetized and intravenously injected with 10^5 *M. tuberculosis* H37Rv [18,19]. Mice were randomly divided into four groups with five animals each after six days post-infection. Three groups received treatments for three weeks (5 doses/week):