



Adenosine A_{2A} receptors are up-regulated and control the activation of human alveolar macrophages



Tiago M. Alfaro ^{a, b, c, *}, Diana I. Rodrigues ^c, Ângelo R. Tomé ^{c, d}, Rodrigo A. Cunha ^{a, c}, Carlos Robalo Cordeiro ^{a, b}

^a FMUC-Faculty of Medicine, University of Coimbra, Portugal

^b Pneumology Unit A, Centro Hospitalar e Universitário de Coimbra, Portugal

^c CNC - Centre for Neuroscience and Cell Biology, University of Coimbra, Portugal

^d Department of Life Sciences, Faculty of Sciences and Technology, University of Coimbra, Portugal

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ABSTRACT

Chronic inflammatory lung diseases remain a health concern and new anti-inflammatory treatments are needed. Targeting adenosine A_{2A} receptors (A_{2A}R) affords robust anti-inflammatory effects in animal models, but the translation of this promising strategy to humans has been challenging, possibly due to interspecies differences in receptor distribution and effects. Thus, we now assessed the efficiency of a selective A_{2A}R agonist to control the activation of fresh human alveolar inflammatory cells. We collected bronchoalveolar lavage fluid from patients with interstitial lung disease and loaded alveolar cells with the intracellular free calcium probe FURA-2/AM. Calcium transients were then recorded in response to superfusion with a proinflammatory peptide (N-formylmethionyl-leucyl-phenylalanine - FMLP), in the absence or presence of the selective A_{2A}R agonist CGS21680. In a second experiment, cells were continuously exposed to FMLP and A_{2A}R density was assessed by immunocytochemistry. Sixteen patients were included, nine for analysis of calcium transients, and seven for immunocytochemistry. When alveolar macrophages were exposed to 100 nM FMLP for 120 s, a peak elevation of intracellular free calcium levels (97.0% over baseline) was recorded; CGS21680 (100 and 300 nM) significantly reduced this peak to 89.5% and 81.5%, respectively. The immunofluorescence analysis revealed a time-dependent increase of A_{2A}R density in alveolar macrophage upon exposure to 1 μM FMLP, up to 148% of control at 6 h. These results show that pro-inflammatory stimuli up-regulate A_{2A}R and their activation dampens the impact of pro-inflammatory stimuli. This supports that targeting A_{2A}R is a promising therapy for human lung inflammatory diseases, especially for diseases with a strong inflammatory component.

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1. Background

Pulmonary inflammatory diseases are a major global cause of disability and early death [1]. Airway inflammatory diseases alone, such as asthma and chronic obstructive pulmonary disease (COPD), affect over 200 million people each, with the latter being currently the third leading cause of death [2]. Interstitial lung diseases are an extensive and heterogeneous group of inflammatory and/or fibrotic pulmonary diseases that can be idiopathic or associated to specific

exposures or systemic diseases. Although interstitial lung diseases have a relatively lower prevalence, a significant proportion of these patients progress to pulmonary fibrosis and respiratory failure, despite treatment [3].

The presently available anti-inflammatory strategies for pulmonary inflammatory disorders are largely based on the use of steroids, which are ineffective in delaying the evolution of COPD and have substantial side effects, including osteoporosis, hyperglycaemia and cataracts, when given systemically to manage interstitial lung diseases [3–5]. New anti-inflammatory therapies for pulmonary disease are urgently needed.

The modulation of the purinergic adenosine receptors is a promising strategy for the treatment of pulmonary inflammatory disorders [6]. Adenosine, a purine nucleoside, is kept at low

* Corresponding author. Centro de Pneumologia da UC, Hospitais da Universidade de Coimbra, 2º piso, Praceta Mota Pinto, 3000-075 Coimbra, Portugal.

E-mail address: alfarotm@gmail.com (T.M. Alfaro).

concentrations under physiological conditions, but its extracellular levels rise several fold upon cellular dysfunction as a result of increased ATP catabolism and adenosine release [5]. This increased extracellular concentration of adenosine acts as a retaliatory metabolite, reducing inflammation and promoting tissue repair [5]. Its effects are exerted through four G-protein coupled membrane receptors, A_1 , A_{2A} , A_{2B} and A_3 receptors, and adenosine A_{2A} receptors ($A_{2A}R$) play a major role in dampening the activation of neutrophils, macrophages and lymphocytes [5]. Animal models revealed that $A_{2A}R$ are dynamically engaged to control inflammation, since they can be up-regulated under inflammatory conditions to adapt their efficiency to a heightened cellular activity [6,7]. Accordingly, a robust protective role of $A_{2A}R$ agonists was shown in several preclinical models of asthma, COPD and acute lung injury, whereas genetic deletion or antagonism of $A_{2A}R$ led to disease worsening in the same models [7].

Unfortunately, the translation of these results to human studies has been challenging. Initial clinical studies using selective $A_{2A}R$ agonists for rhinitis, asthma and COPD were negative [8–10]. Some possibilities were considered to explain these results: one hypothesis proposed that the lower efficiency of $A_{2A}R$ agonists in humans could result from a significant inter-species variability in the presence and distribution of $A_{2A}R$ in the various tissues and cell types [11] or to an inability of human $A_{2A}R$ to alter their efficiency under inflammatory conditions. In fact, most of the studies on the anti-inflammatory effects of $A_{2A}R$ were performed in animal models or in human cell-cultures, which may not be representative of the human disease conditions [11,12].

We now aimed to assess the dynamics of $A_{2A}R$ and the efficiency of a selective $A_{2A}R$ agonist on fresh human alveolar inflammatory cells, which are representative of human disease conditions.

2. Methods

2.1. Patient and sample collection

We performed a prospective study including patients referred for bronchoscopy with bronchoalveolar lavage (BAL) for diagnosis of interstitial lung disease at the University Hospital of Coimbra in Portugal. The inclusion criteria were the presence or suspicion of an interstitial lung disease at diagnosis stage, informed consent and age over 18 years. The exclusion criteria were clinical or laboratory signs of infection, underage, pregnancy, non-consent and chronic treatment with steroids. All patients had a clinical indication for the exam, and no clinical exams were performed solely for this study. The protocol was approved by the local ethics committee and patients provided informed consent. The bronchoscopy and bronchoalveolar lavage were performed according to the department's general practice. The bronchoscope was introduced by the nares or mouth with the patient on dorsal decubitus, under local anaesthesia with lidocaine. After passing through the upper airway, the bronchoscope tip was wedged in one of the middle lobe segments and three 50 mL syringes of warmed saline were gently injected in the work channel and immediately aspirated. For this study, 10 mL of the pooled fluid was collected. The remaining fluid was sent for the central hospital laboratory for total and differential cell counts. For total cell count a Coulter cell counter was used. For the differential cell count, 50 mL of lavage fluid was spun at $500 \times g$ for 10 min at 4 °C. The supernatant was discarded, and the cells were re-suspended in 1 mL of Hank's buffered saline solution (HBSS). A slide was prepared on a cytospin 3 centrifuge using a 650 rpm speed with a low acceleration for 10 min. Staining was performed using May-Grünwald-Giemsa procedure [13]. A total of 500 cells

were counted and each cell type recorded as a proportion (%) of the total number of cells.

2.2. Reagents

All reagents were purchased from Sigma unless otherwise specified. The composition of the Hank's buffered saline solution (HBSS) was (mM): 136.9 NaCl, 4.2 NaHCO_3 , 0.34 NaH_2PO_4 , 0.44 KH_2PO_4 , 5.4 KCl, 1.3 CaCl_2 , 1 MgSO_4 , 5.6 glucose. The composition of the phosphate buffered saline (PBS) was (mM): 137 NaCl, 2.7 KCl, 8.1 Na_2HPO_4 , 1.47 KH_2PO_4 . For the immunocytochemistry analysis, the following antibodies and dilutions were used: goat polyclonal anti- $A_{2A}R$ (SC-7504, dilution 1:50; Santa Cruz Biotechnology, Santa Cruz, CA, USA), mouse monoclonal anti-CD68 (Novocastra NCL-L-CD68 no dilution; Menarini Diagnostics, Paço de Arcos, Portugal), donkey anti-goat IgG labelled with Alexa Fluor® 488 (ab150129, dilution 1:100; Abcam, Cambridge, UK), donkey anti-mouse IgG labelled with Alexa Fluor® 594 (ab150116, dilution 1:100; Abcam).

2.3. Intracellular free calcium analysis

The collected BAL was kept at 4 °C and transported to the laboratory within 4 h of collection. The remaining protocol was performed at controlled room temperature (20 °C). The cell viability was tested using a 0.2% solution of trypan blue in a Neubauer chamber. Samples with less than 50 cells/ μL or less than 90% viability were discarded. The BAL fluid was diluted (1:1) in HBSS with 1% penicillin/streptomycin (P/S), and the cells were plated in photoetched gridded coverslips with 12 mm diameter (Bellco glass, NJ, USA) during 60 min. The cells were then washed with HBSS and loaded with 5 μM FURA-2/AM with 1% bovine serum albumin and 0.4% pluronic F-127 in HBSS for 90 min. The coverslips were washed with HBSS and kept in the same medium until analysis. The intracellular free calcium analysis was performed using a Zeiss Axiovert 200 inverted microscope (Carl Zeiss, Jena, Germany). The coverslips were mounted in a RC-25 chamber (Warner Instruments, Harvard, UK) and placed under a continuous superfusion of 1 mL/min of HBSS. A Lambda DG4 xenon light (Sutter Instruments, Novato, CA, USA) was used to alternatively excite the cells with UV light centred at 340 and 380 nm with an exposure time of 1 s. The fluorescence was captured through a 40 $\times$ oil objective and a 510 nm band-pass filter (Carl Zeiss) connected to a digital camera (Cool SNAP; Roper Scientific, Trenton, NJ, USA). For each coverslip, the superfusion protocol consisted of 2 min of basal medium, 10 min of medium or $A_{2A}R$ agonist, followed by 2 min of formyl-methionyl-leucyl-phenylalanine (FMLP, final concentration of 100 nM), also without or with the $A_{2A}R$ agonist, CGS21680 (10–300 nM). At the end of the experiment, the cell coordinates were recorded. For cell type identification, the cells in the coverslips were fixed with ice-cold methanol for 10 min and stained with May-Grünwald-Giemsa. The analysed cells were located using the gridded coverslip coordinates and each cell type was identified by its staining pattern.

The offline image analysis was performed using the software ImageJ version 1.48v (NIH, USA). A region of interest (ROI) was manually selected for each cell and the average fluorescence intensity was measured for both series (340 and 380 nm image stacks). Previous experiments in cell-free coverslips established the background intensity (112 arbitrary units). A graph of the corrected ratio, R , the average fluorescence light intensity emitted upon alternated excitation at 340 and 380 nm ($R = F_{340}/F_{380}$) for each cell was created. Changes in R correspond to the changes in the levels of cytosolic free calcium [14,15]. For each cell, a baseline

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