



Modified expression of peripheral blood lymphocyte muscarinic cholinergic receptors in asthmatic children

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ARTICLE INFO

Article history:

Received 30 July 2014

Received in revised form 25 April 2015

Accepted 29 April 2015

Keywords:

Muscarinic receptors

Asthma

Western blot

Human

ABSTRACT

Lymphocytes possess an independent cholinergic system. We assessed the expression of muscarinic cholinergic receptors in lymphocytes from 49 asthmatic children and 10 age matched controls using Western blot. We demonstrated that CD4⁺ and CD8⁺ T cells expressed M2 and M4 muscarinic receptors which density were significantly increased in asthmatic children in comparison with controls. M2 and M4 receptor increase was strictly related with IgE and fraction of exhaled nitric oxide (FeNO) measurements and with impairment in objective measurements of airway obstruction. Increased lymphocyte muscarinic cholinergic receptor expression may concur with lung cholinergic dysfunction and with inflammatory molecular framework in asthma.

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1. Introduction

It has been recognized that asthma is an inflammatory disease of the airways (Bateman et al., 2008). There is now good evidence that many related traits of the disease may be largely attributable to inappropriate activation and cytokine production by CD4⁺ T cells (Corrigan and Kay, 1990; Gemou-Engesaeth et al., 1994; Machura et al., 2010). Patients with asthma display an exaggerated reactivity of airways in response to several certain indirect or direct stimuli (Amin et al., 2000). This airway hyperresponsiveness usually co-exists with airway inflammation (Busse, 2010). It is hypothesized that hyper-reactivity is associated with autonomic nervous system (ANS) dysfunction (Paggiaro et al., 1989; Costello et al., 1998; Fryer and Jacoby, 1998). In particular, the parasympathetic (vagal) component of the ANS appears to be implicated in the pathogenesis of asthma (Costello et al., 1998). Parasympathetic cholinergic system is the dominant neural broncho-constrictor system, and is involved in the regulation of airway caliber. It provides the dominant autonomic innervation of the upper and lower airways (Paggiaro et al., 1989). Acetylcholine (ACh) released from cholinergic

neuroeffector junctions activates postjunctional muscarinic cholinergic receptors located on airway smooth muscle, submucosal glands and blood vessels (Richardson and Béland, 1976; Barnes, 1986).

Muscarinic cholinergic receptor subtypes are expressed throughout the respiratory system, which mediate mucus secretion, smooth muscle constriction, vasodilatation and nitric oxide (NO) release (Costello et al., 1998). Prejunctional muscarinic M2 receptors located in parasympathetic neuroeffector junctions modulate neural ACh release. Stimulation of these receptors activates a cascade of intracellular events, including hydrolysis of phosphatidylinositol, increase of Ca²⁺, activation of mitogen-activated protein kinases, and inhibition of cAMP synthesis (Barnes, 2004). Today, five muscarinic receptor subtypes (M1–M5) related to the G-protein receptor super-family have been identified. In particular M1, M3, and M5 receptor subtypes couple preferentially to the Gq/11 protein, whereas M2 and M4 receptors are linked preferentially to Gi protein (Barnes, 2004; Felder, 1995; Caulfield and Birdsall, 1998).

ACh, in addition to being an important neurotransmitter in the central and peripheral nervous system, is also involved in the modulation of immune responses. Circulating lymphocytes possess the biosynthetic and catabolic enzymes of ACh, and express muscarinic cholinergic receptors (Rinner and Schauenstein, 1993). It's known that peripheral blood lymphocytes express different subtypes of muscarinic receptors with a relative density in normal subjects M3 > M5 > M4 > M2

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(Tayebati et al., 1999, 2002; Ricci et al., 2002). In adult patients with bronchial asthma and allergic rhinitis there is a modification of the expression of these receptors in lymphocytes with a marked increase in M2 and M5 receptor with a relative decrease of M3 receptors (Ricci et al., 2008). It was reported that the increase in M2 receptors correlates with bronchial hyper-responsiveness (Ricci et al., 2002).

Until now, there is very little direct evidence of the actual immunological mechanisms that operate in children suffering from asthma. Therefore, the purpose of this study was to detect if changes found in adults with asthma and/or allergic rhinitis, are also present in allergic children and if they are related with disease severity.

2. Methods

2.1. Patient characteristics

The study was carried out in the Pediatric Allergy Unit, Research Center, San Pietro Hospital. 49 children (35 male and 14 female; mean age 8.9) suffering from bronchial asthma were included (Table 1). 14/49 children were also suffering from allergic rhinitis (Table 1).

Patients did not receive any anti-asthmatic therapy or any other therapy for allergy since ten days preceding recruitment and were far from the acute phase of the disease. All study participants performed blood tests and all the patients and controls (10 age matched healthy children, 5 male and 5 female, mean age 10.1) performed spirometry. To explore aging dependent changes in the expression of muscarinic cholinergic receptors, we also enrolled 15 asthmatic adults (9 male and 6 female; mean age 35.6) to verify this hypothesis.

2.2. FeNO measurements

FeNO was measured by using the online single breath method with NIOX (Nitric Oxide Monitoring System; Aerocrine, Sweden) according to ATS guidelines (2011). Children younger than 10 years performed a 6-second exhalation, and FeNO was calculated during the last 2 s of the exhalation. Children ≥ 10 years performed an exhalation of 10 s, and FeNO was calculated during the last 3 s of the exhalation. Exhalations were approved if they did not deviate more than 2.5 ppb or 10% and were completed within a 15-minute period. The interval between exhalations was at least 30 s. Each subject performed no more than a total of 6 exhalations. FE_{NO} was calculated as the mean of 3 correct exhalations. All measurements were performed between 8:00 am and 5:00 pm.

2.3. Patients and peripheral blood lymphocyte preparation

Peripheral blood mononuclear cells (PBMC) were isolated from children affected by bronchial asthma and from children used as controls (Table 1). The procedures were detailed elsewhere (Ricci et al., 2002). Briefly, blood was drawn from the cubital vein, and cells

were separated from heparinized whole blood by “Ficoll–Hypaque” (Histopaque-1077, SIGMA-ALDRICH, St. Louis, USA) gradient centrifugation. This procedure allowed us to obtain a final concentration of 1×10^6 cells/ml. The PBMC layer was washed twice in phosphate-buffer saline (PBS) and re-suspended in fresh growth medium (RPMI-1640) (Sigma-Aldrich) supplemented with fetal calf serum (10%), penicillin (100 µg/ml) (Sigma-Aldrich), streptomycin (100 µg/ml) (Sigma-Aldrich) and L-glutamine (2 mM) (Sigma-Aldrich) at 37 °C in a 5% air atmosphere in a humidified incubator for 1 h to eliminate monocytes. Lymphocytes were also used for CD4 + and CD8 + T cell separation.

2.4. Purification of T lymphocytes (CD4 and CD8 positive)

CD4 + and CD8 + T lymphocytes were purified by magnetic cell sorting. PBMCs were labeled with BD IMag™ anti-human CD4 Particles-DM (BD Biosciences San Jose, CA USA). After 15 min of incubation at 20 °C, labeled cell suspension was then placed within the magnetic field of the BD IMagnet™. CD4 + T cells migrated toward the magnetic (positive fraction), leaving the unlabeled cells (CD4[−] T cells) in suspension so they can be drawn off (negative fraction). The tube was then removed from the magnetic field for resuspension of the positive fraction. The separation is repeated twice to increase the purity of the positive fraction. The positive fraction was then washed in PBS and evaluated in Flow Cytometry. The negative fraction was used for the subsequent CD8 + T cells purification.

A counting chamber was used to evaluate cell number. The purity of the preparations, assessed by flow cytometry, was usually >98–99%, and the viability, evaluated by the trypan blue exclusion test, was >95–98%.

2.5. Western blot analysis of muscarinic receptor

For the assessment of muscarinic M1r, M2r, M3r, M4r and M5r receptor protein expression the following antibodies were used. Goat polyclonal antibodies anti human M1–M5 receptors were purchased from Santa Cruz Biotechnology. Anti-M1 receptor antibody (C20-sc7470) was used at a dilution 1:300. It developed a specific single band at 52 kDa. Anti-M2 receptor antibody (C18-sc7472) was used at a dilution 1:500. It developed a specific single band at 75 kDa. Anti-M3 receptor antibody (C20-sc7474) was used at a dilution 1:500. It developed a specific single band at 75 kDa. Anti-M4 receptor antibody (C19-sc7477) was used at a dilution 1:500. It developed a specific single band at 70 kDa. Anti-M5 receptor antibody (C20-sc7478) was used at a dilution 1:400. It developed a specific single band at 60 kDa.

T lymphocytes (total, CD4 + and CD8 +) were suspended in lysis buffer (Tween 20, Glycerol, NaCl 2.5 M, Hepes 1 M pH 7.6, MgCl₂ 2 M, CaCl₂ 0.1 M, NaF 1 M, Na₂P₂O₇ 0.1 M, NaVO₃ 0.1 M, PMSF 0.1 M) (Roche) and then were cleared by centrifugation at 14,000 rpm for

Table 1
Baseline characteristics of patients enrolled for the study.

Patients (n = 49)	FEV1	FEV1/VC	IgE (kU/L)	FeNO (ppb)	
Mild intermittent (n = 10)	102 ± 10.2	82.4 ± 9.5	85 ± 15	8.5 ± 6	
Mild persistent (n = 24)	97.5 ± 15	80.1 ± 11.2	107.2 ± 18	14.4 ± 9	
Moderate persistent (n = 10)	75.6 ± 9.2	75.2 ± 2.8	325 ± 48	21.6 ± 10	
Severe persistent (n = 5)	58.8 ± 8.4	68.5 ± 3.2	638 ± 66	37.5 ± 15	
Healthy controls (n = 10)	108.1 ± 3.5	81.9 ± 8.8	12.2 ± 6.2	7.7 ± 5	
Patients (n = 49)	Prick responses	Sputum eosinophils%	Night symptoms weekly	Atopic dermatitis %	Allergic rhinitis
Mild intermittent (n = 10)	2 ± 1	<2	<1	6	2/10
Mild persistent (n = 24)	2 ± 1	<2	<1	8	8/24
Moderate persistent (n = 10)	2 ± 2	2–3	1–2	20	3/10
Severe persistent (n = 5)	5 ± 3	>3	>2	60	3/5
Healthy controls (n = 10)	Not tested	0	0	0	0

FEV1: forced expiratory volume in one second; FEV1/VC: forced expiratory volume in one second/vital capacity (Tiffeneau index); IgE: immunoglobulin E; FeNO: fraction of exhaled nitric oxide.

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