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Crosstalk between mAChRM3 and β2AR, via acetylcholine PI3/PKC/PBEP1/Raf-1 MEK1/2/ERK1/2 pathway activation, in human bronchial epithelial cells after long-term cigarette smoke exposure

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ABSTRACT

Background: Cigarette smoke extract (CSE) affects the expression of non-neuronal components of cholinergic system in bronchial epithelial cells and, as PEBP1/Raf-mediated MAPK1/2 and ERK1/2 pathway, promotes inflammation and oxidative stress.
Aims: We studied whether Acetylcholine (ACh) is involved in the mechanism of crosstalk between mAChRM3 and β2Adrenergic receptors (β2AR) promoting, via PI3/PKC/PBEP1/Raf/MEK1/2/ERK1/2 activation, β2AR desensitization, inflammation and, oxidative stress in a bronchial epithelial cell line (16HBE) after long-term exposure to cigarette smoke extract (LECSE).
Methods: We evaluated mAChRM3 and Choline Acetyltransferase (ChAT) expression, ACh production, PEBP1, ERk1/2, and β2AR phosphorylation, as well as NOX-4, ROS production and IL-8 release in 16HBE after LECSE. The inhibitory activity of Hemicholinium (HCh-3) (a potent choline uptake blocker), LY294002 (a highly selective inhibitor of PI3 kinase), Tiotropium (Spiriva®) (anticholinergic drug) and Olodaterol (β_2AR agonist), were tested in 16HBE after LECSE.
Results: mAChRM3, ChAT, ACh activity, pPEBP1, pβ2AR, pERK1/2, ROS, NOX-4 and IL-8 increased after LECSE in 16HBE LECSE compared to untreated cells. HCh-3 and LY294002 (alone or in combination) as well as Tiotropium (Spiriva®) or Olodaterol (alone or in combination) all reduced the levels of pPEBP1, pβ2AR, pERK1/2, ROS, NOX-4, and IL-8 in 16HBE LECSE compared to untreated cells.
Conclusions: LECSE promotes ACh production which enhances PI3/PKC/PEBP1/Raf-ERK1/2 pathway activation, heterologous β2AR desensitization, as well as release of inflammatory and oxidative mediators in bronchial epithelial cells. The use of anticholinergic drugs and long-acting β2-agonists, alone or in combination may be dampen these inflammatory mechanisms when used in combination in some epithelial cell types.

1. Introduction

The location, function, and crosstalk of β2-Adrenergic receptors (β2ARs) and muscarinic receptors (mAChRs) might be considered in the design, development, and use of these drugs to combat airway diseases. These receptors are typically expressed in the same cells and have the capacity to modulate the activity of each other controlling for the differentiation and inflammatory state of the cells [1–3]. Inflammation and oxidative/nitrosative stress, are considered of primary pathogenic importance in Chronic Obstructive Pulmonary Disease (COPD). The current therapeutic approach suggests the use of anticholinergic drugs and long-acting β2AR agonists in the treatment of COPD to maximize

Abbreviations: CSE, Cigarette Smoke Extract; ChAT, choline acetyltransferase; ACh, acetylcholine; mAChRs, Muscarinic Acetylcholine receptors; β2ARs, β2 Adrenergic receptors; PEBP1, phosphatidylethanolamine binding protein 1; ROS, Reactive oxigen species; NOX-4, NADPH oxidase 4; IL-8, Interleukin-8; MAPK1/2, Mitogen-Activated Protein Kinase 1/2; ERK1/2, extracellular signal-regulated kinase 1/2; PKC, protein kinase C; cAMP, cyclic 3,5 adenosine monophosphate; LY294002, 2-morpholin-4-yl-8-phenylchromen-4-one; HCh-3, Hemicholinium-3,2,2-(4,4_-biphenylene)bis(2-hydroxy-4,4-dimethylmorpholinium bromide, C24H34Br2N2O4; TIO, Tiotropium, Spiriva®: [1_, 2_, 4_, 5_, 7_-7-hydroxydi-2-thienylacetyl) oxy]-9,9-dimethyl-3-oxa-9-azoniatrcyclo[3.3.1.024], C19H22NO4S2Br_H2O; OLO, Olodaterol: 6-hydroxy-8-{(1R)-1-hydroxy-2-{[1-(4-methoxyphenyl)-2-methylpropan-2-yl]amino} ethyl}-4H-1,4-benzoxazin-3-one

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only bronchodilation. There has been little consideration of the importance of anti-inflammatory action of these drugs [4,5].

mAChRM3 activation stimulates phospholipase C (PLC), which generates the formation of diacylglycerol and inositol triphosphate (IP3), promoting release of Ca^{2+} from internal stores (1), while also activating protein kinase C (PKC) [6]. These two enzymes can then activate multiple signaling pathways in various cell types [7–10]. Activation of PLC via the intermediary heterotrimeric G protein Gq is the predominant pathway through which mAChRM3 regulates important airway cell functions aside from ASM contraction [11,12]. mAChRM3 itself is phosphorylated by GPCR kinases (GRKs) upon binding agonists such as ACh [13–15]. Stimulation of mAChRM3 activates the PKC, which is known to dissociate Raf kinase inhibitor protein-1 (RKIP also called PEBP1) from Raf-1, [16,17] promoting the activation of MEK/ERK1/2 cellular signaling often involved in inflammation and oxidative stress [18–20]. Under inflammatory conditions, conformational changes dissociated PEBP1 from Raf-1 to bind GRK2. The binding of GRK2 to PEBP1 prevents it from phosphorylating the β_2AR, allowing ongoing receptor activation [21,22].

β_2AR are coupled to inhibitory Gs proteins, and β_2AR agonist activates adenylate cyclase and increases 3,5 adenosine monophosphate (cAMP) levels. cAMP increases protein kinase A activity which is important for the bronchodilation response. The desensitization of β2AR is associated with reduced levels of cAMP in response to stimulation [1]. mAChRM3 activation promotes desensitization of the β2AR in a PKC-dependent manner [23]. In fibroblasts, β_2AR agonist mediate opposite effect of mAChRs reducing ACh induced endothelin-1 production [24]. Interestingly, in anesthetized guinea pigs and dogs, the long acting β_2AR agonist Olodaterol potently reverses contraction induced by ACh challenges [25]. The crosstalk between β2AR and mAChRs is competitive, and causes a reduction of the bronchodilator response. There is evidence that pharmacological approaches focusing on restoring the balance of affect mAChR-β2AR competition are effective in bronchodilation [26,27]. However, it is unknown whether a similar mechanism could possibly affect the inflammatory and oxidative processes by these drugs in other cell types, including epithelial cells.

The aim of the present study was to investigate whether long term exposure to cigarette smoke (LECSE) increases the mAChRM3 expression and the ACh production in bronchial epithelial cells. We analyzed whether ACh targeting mAChRM3 promotes via PKC/Raf/MEK1/2/ERK1/2 pathway activation, oxidative stress (ROS production and NOX-4 protein expression) as well as inflammation (IL-8 release) in bronchial epithelial cells. Furthermore, we studied whether ACh induces PEBP1 phosphorylation through a PKC -pPEBP1-GRK2 mechanism and further contributes to β2AR desensitization. Finally, with the aim to understand whether the LECSE might cause interferences between mAChRs and β2ARs reducing the pharmacological response to these drugs, we tested Tiotropium Bromide (Spiriva®) (anticholinergic drug with a higher selectivity for mAChRM3), and long-acting β2AR agonists Olodaterol, alone or in combination, on the mechanisms above mentioned.

2. Materials and methods

2.1. Epithelial cell cultures

The SV40 large T antigen-transformed 16HBE cell line (16HBE), an immortalized normal bronchial epithelial cell line was used in this study. The source and origin of 16HBE was kindly provided by Dr. D. Gruenert Laboratory (University of California, San Francisco, Calif) to IBIM-CNR Italy. The 16HBE cell line retains the morphology and functions of differentiated bronchial epithelial cells. The cells represent a clonal diploid (2n = 6) cell line isolated from human lung.

16HBE cells were cultured as adherent monolayers in Eagle's minimum essential medium (MEM, Euroclone) supplemented with 10% heat-inactivated (56 °C, 30 min) fetal bovine serum (FBS), 1% MEM (non-essential aminoacids, Euroclone), 2 mM L-glutamine and gentamicin 250 μg/ml at 37 °C in a humidified 5% CO_2 atmosphere.

2.2. Preparation of cigarette smoke extract

Commercially available cigarettes (Marlboro Red Label, Philip Morris International, Switzerland) were used in this study. Cigarette Smoke Extract (CSE) was prepared as previously described with minor modifications [28] and further diluted to the required concentration in serum-free medium.

2.3. Long term exposure to CSE treatment of 16HBE

The CSE was used to treat the cells. The cells were long term exposed to CSE (LECSE) 10% and 20%, for 3 cycles per day, to simulate the chronic treatment with cigarette smoke.

The cells were seeded in a T25 flask and grown to 60–70% confluence prior to treatment. In one treatment cycle, cells were stimulated for 15 min with LECSE 10% or 20% obtained in serum-free MEM, and then cultured for 1 h in CSE-free fresh growth medium (MEM 10% FBS). The viability of the cells to LECSE was analysed by trypan blue exclusion dye assay. The treatments were performed for 4 days consecutively as previously described to collect total protein, which were extracted from stimulated 16HBE cells using a lysis buffer (NaCl 50 mM, Tris-HCl 10 mM, EDTA 5 mM, NP-40 1%) containing a protease and phosphatase inhibitor [21]. Protein concentration was assessed using the Bradford method. The cell protein extracts were used to evaluate mAChRM3, ChAT, ACh, pβ2AR, pPEBP1, and pERK1/2 production.

2.4. Detection of ACh

We quantified ACh in protein extracts from 16HBE by a fluorimetric method using a commercial kit (BioVision Research Products, CA-USA, cat. #K615-100). The kit can detect choline (Ch), and total choline (TCh) (by adding acetylcholine esterase to the reaction, converting ACh to Ch), with a sensitivity until 50 pmol/well by plotting fluorescence readings (Ex/Em 535/587 nm) against the standard curve. This sensitivity corresponds to the concentration of 1 μM of TCh or Ch. ACh was evaluated as difference between TCh and Ch. Fluorescence intensity was read using a Wallac 1420 Victor^2 multilabel counter (Perkin-Elmer Life Sciences, Turku, Finland). Results were expressed as pmoli/μg protein.

2.5. Stimulation of 16HBE with ACh

After the selection of 20% CSE treatment, cells were pre-treated chronically (3 cycles per day for 4 days). At the end of the chronic CSE exposure, the cells were stimulated with and without ACh 1 μM (Sigma-Aldrich, Milan, Italy) for 30 min or for 48 h. The cell lysates obtained after 30 min of stimulation were used to detect pPEBP1, pβ2AR, pERK1/2 by western blot, and ROS production by flow cytometer. The cell lysates obtained after 48 h of stimulation were used to measure NOX-4 protein expression by western blot, and IL-8 release by ELISA.

2.6. Stimulation of 16HBE with drugs and inhibitors

Cells were treated with 1) Hemicholinium-3 (HCh-3) (a potent and selective choline uptake blocker, 25 μM) (Sigma-Aldrich, Milan, Italy) and/or, 2) LY294002 (a highly selective inhibitor of PI3 kinase) (10 μM, Sigma–Aldrich, Milan, Italy) and/or 3) Tiotropium bromide (Spiriva®) (100 nM) (anticholinergic drug with a higher affinity for mAChRM3) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany) alone or in combination with Olodaterol (10 nM) (long-long acting β2AR agonist) (Boehringer Ingelheim Pharma GmbH & Co. KG, Biberach, Germany). All were added to the cells after each daily treatment with LECSE (3 cycles per day for 4 days with CSE 20%) for

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