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A protective role for proteinase activated receptor 2 in airways of lipopolysaccharide-treated rats

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ABSTRACT

Proteinase activated receptor-2 (PAR2), a seven transmembrane domain G protein coupled receptor, is expressed on airway epithelium and smooth muscle cells and over-expressed in human airways under pathological conditions, such as asthma and chronic obstructive pulmonary disease (COPD). However, the role of PAR2 in airways has not yet been defined. Aim of the present study, was to evaluate the in vitro rat bronchial response to a synthetic peptide activating PAR2 (PAR2-AP; SLIGRL), following an in vivo treatment with bacterial lipopolysaccharide (LPS). Bronchi from LPS-treated animals showed an increased relaxant response to PAR2-AP, compared to naïve animals, the effect was maximum after 20-h pre-treatment and reduced by epithelium removal. Western blot analysis showed an increased PAR2 protein expression on bronchi removed 20 h after LPS treatment. PAR2-AP-induced bronchorelaxation was inhibited by ibuprofen, by the selective cyclooxygenase2 (COX-2) inhibitor, diisopropyl fluorophosphate (DFP) and partially by the calcitonin gene related peptide (CGRP) antagonist, rat-$CGRP_{[8–37]}$. Furthermore, there was a strong immunoreactivity for COX-2 on bronchial epithelium of LPS-treated rats. Prostaglandin E_2 (PGE_2) tissue release and CGRP tissue content were significantly increased following tissue incubation with PAR2-AP. The in vivo LPS treatment in rats strongly increases the bronchorelaxant effect of PAR2-AP, this effect correlates with an increased tissue protein receptor expression and the COX-2 localization on bronchial epithelium. Our work supports a role for PAR2 as a defence mechanism aimed to preserve bronchial functionality under systemic inflammatory conditions; both COX-2-derived PGE_2 and CGRP are involved in this effect.

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1. Introduction

Proteinase activated receptor 2 (PAR2), a seven transmembrane domain G protein coupled receptor activated by proteolytic cleavage which, together with other PARs, shows a unique mechanism of autoactivation, is expressed on several animal and human tissues, under physiological conditions [1]. Within the respiratory tract, PAR2 is expressed by airway epithelial and smooth muscle cells, as well as endothelial and vascular smooth muscle cells [1,2]. PAR2 is also expressed by lung macrophages, mast cells, granulocytes, lymphocytes and eosinophils [3,4]. The role of PAR2 in airways is still controversial. Studies performed on animal tissues have demonstrated a protective role for PAR2 in airways; indeed, activation of PAR2 receptors causes an epithelium-dependent relaxation of mouse isolated bronchi, that correlates with

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PAR2 immunoreactivity in cytoplasmic regions of airway epithelial cells [5], and also an epithelium-dependent relaxation of mouse and guinea pig tracheal rings [6,7]. In vivo, it has been demonstrated that activation of PAR2 produces a protective effect against 5HT-induced bronchoconstriction in rats [5] and histamine-induced bronchoconstriction in guinea pigs [8]. Conversely, it has also been reported that PAR2 activation leads to a sensory neuropeptide-dependent bronchoconstrictor response in vivo [9] and, in vitro, causes constriction of human bronchi [10] and hyperresponsiveness to histamine, in guinea pigs [11].

There is in vitro and in vivo evidence for an up-regulation of PAR2 by inflammatory stimuli, such as tumour necrosis factor-α (TNF-α) and interleukin-1α (IL-1α) [12], suggesting that the activation of PAR2 may be regulated by inflammatory mediators. Recently, it has been demonstrated an increased PAR2 expression in airways of mice during influenza A virus infection, coupled to a prolonged inhibition of methacoline-induced bronchoconstriction [13]. Similarly, within human airways, PAR2 is over-expressed under pathological conditions, such as asthma and chronic obstructive pulmonary disease (COPD) [2,14,15]. However, it is currently unclear the physiological significance of receptor up-regulation under certain diseases, both in animals and humans.

Injection of bacterial lipopolysaccharide (LPS) to experimental animals causes a systemic inflammatory response characterized by multiple organ dysfunction syndrome (MODS) [16,17]. Within the airways, the effect of LPS appears to be secondary to cytokine release, and it has been postulated that LPS exerts its effects on peripheral terminals of sensory afferents and, through cytokine release, regulates sensory nerves terminal release [18]. A relationship between PAR2 and non adrenergic non cholinergic (NANC) system was first proposed by Steinhoff et al. [19], who postulated a neurogenic mechanism at the basis of the pro-inflammatory effect of PAR2 agonist. Successively, it has been demonstrated that a neurogenic mechanism is also at the basis of colitis [20] and hyperalgesia [21] induced by PAR2 agonists in experimental animals.

Previously, we have shown that an increased protein expression of PAR2 on vascular endothelium and smooth muscle cells following injection of LPS, in rats, correlates with an increased hypothensive response to the injection of the synthetic peptide activating PAR2 (PAR2-AP) [22], suggesting that the increase in cellular PAR2 expression during inflammation might reflect an increased tissue sensitivity to endogenous PAR2 activators.

To further evaluate the role of PAR2 in airways, under inflammatory conditions, in the present study we have investigated the ex vivo effect of LPS on rat bronchial response to a synthetic peptide activating PAR2.

2. Materials and methods

2.1. Animals and LPS treatment

Male Wistar rats (200–250 g; Charles River, Milan, Italy) were slightly anaesthetized with enflurane and intravenously injected, through the caudal vein, with LPS from *Escherichia coli* (serotype 0127:B8; 14.0×10^6 U/kg) or with an equal volume of sterile saline (NaCl 0.9%; 1 mL/kg). The dose of LPS used was a threshold dose, chosen on the basis of a previous study performed on rats [22]. LPS or saline injected animals were randomized and sacrificed at different times after treatments.

2.2. Functional study

After 4, 12 and 20 h from treatment with LPS, animals were sacrificed by cervical dislocation, exsanguinated and lungs were removed and placed into a Petri dish containing Krebs solution of the following composition (nM): NaCl, 115.3; KCl, 4.9; $CaCl_2$, 1.46, $MgSO_4$, 1.2; KH_2PO_4, 1.2; $NaHCO_3$, 25.0 and glucose 11.1; termosted at 37 °C and oxygenated (CO_2, 5%; O_2, 95%). Main bronchi were dissected free of parenchyma and mounted in 2.5 mL isolated organ bath containing Krebs solution, at 37 °C, oxygenated (95% O_2 and 5% CO_2), and connected to an isometric force transducer (Ugo Basile) under a resting tension of 0.5 g. After about 60 min equilibration period, tissue reactivity was checked by evaluating the response to a single concentration of acetylcholine (Ach, 30 μM). After washing, a cumulative concentration response curve to PAR2-AP (1–100 μM), or to the control peptide (LSIGRL, 1–100 μM), was performed on tissue pre-contracted with carbachol (1 μM). The effect of PAR2-AP was also evaluated on bronchi after epithelium destruction, obtained by leaving tissue in contact with distilled water for 22 s. Epithelium destruction was then confirmed by histological examination. Furthermore, as positive control, to ascertain that distilled water challenge did not alter the ability of airway smooth muscle to relax, we tested relaxation in response to exogenous Prostaglandin E_2 (1 μM) before and after epithelium destruction.

2.3. Western blot analysis

Tissue samples removed as described above, were homogenised on ice in the following lysis buffer: Tris–HCl pH 7.5, 50 mM; NaCl, 150 mM; sodium ortovanadate, 1 mM; β-glycerophosphate, 20 mM; EDTA, 2 mM; DTT, 1 mM; PMSF 1 mM; leupeptin, 5 μg/mL; aprotinin, 5 μg/mL, pepstatin, 5 μg/mL. Protein concentration was measured by Bradford reagent using BSA as a standard. Protein samples (30 μg) were briefly boiled and subjects to electrophoresis on an SDS 10% polyacrylamide gel and transferred onto a nitrocellulose transfer membrane using standard procedure (Protran, Schleicher & Schuell, Germany). The membranes were placed in 5% non-fat milk for 1 h at room temperature and then incubated with rabbit anti PAR2 polyclonal antibody, 1:500, overnight at 4 °C. After three washes of 5 min each in PBS–Tween 20 (0.1%, v/v), membranes were incubated with the secondary antibody (1:5000) conjugated with horseradish peroxidase, antirabbit IgG for 2 h at 4 °C and then three 5-min washes were performed. The signal was detected with ECL (enhanced chemiluminescence) System according to the manufacturer's instructions (Amersham Pharmacia Biotech; Milan, Italy).

2.4. Effect of inhibitors

To investigate on mediators involved in the bronchorelaxant effect of PAR2-AP, functional experiments were also per-

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