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# Enhanced airway smooth muscle cell thromboxane receptor signaling via activation of JNK MAPK and extracellular calcium influx

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ABSTRACT

Thromboxane is a key inflammatory mediator and potent airway constrictor. It acts on thromboxane $A_2$ (TP) receptors and contributes to airway inflammation and airway hyperresponsiveness that is the characteristic feature of asthma. The present study was designed to study TP receptor signaling in airway smooth muscle cells by using an organ culture model and a set of selective pharmacological inhibitors for mitogen-activated protein kinase (MAPK) and calcium signal pathways. Western-blot, immunohistochemistry, myograph and a selective TP receptor agonist U46619 were used for examining TP receptor signal proteins and function. Organ culture of rat bronchial segments for up to 48 h induces a time-dependently increased airway contractile response to U46619. This indicates that organ culture increases TP receptor signaling in the airway smooth muscle cells. The enhanced bronchial contraction was attenuated by the inhibition of c-Jun N-terminal kinase (JNK) MAPK activity, chelation of extracellular calcium and calcium channel blocker nifedipine, suggesting that JNK MAPK activity and elevated intracellular calcium level are required for the TP receptor signaling. In conclusion, airway smooth muscle cell TP receptor signaling occurs via JNK MAPK activity and the elevation of extracellular calcium influx, which may provide knowledge for understanding the signaling pathway responsible for the modulation of TP receptor mediated airway hyperresponsiveness to thromboxane.



## 1. Introduction

Thromboxane $A_2$ ($TXA_2$) was identified in extracts of human platelets and shown to be capable of initiating the contraction of both vascular and airway smooth muscle (Armour et al., 1989; Hamberg et al., 1975). $TXA_2$ and other prostanoids are elevated in a number of lung diseases including asthma and chronic obstructive pulmonary disease (Barnes, 2001; Pratico et al., 1998; Robinson et al., 1985). It is involved in acute bronchoconstriction after antigen inhalation in asthmatic patients, as demonstrated by a markedly elevated level of thromboxane $B_2$ (Davi et al., 1997), a stable metabolite of $TXA_2$ (Iwamoto et al., 1988; Manning et al., 1991; Wenzel et al., 1989). A wealth of studies has shown the role of $TXA_2$ in the pathogenesis and pathophysiology of asthma (Devillier and Bessard, 1997; Dogne et al., 2002a; Wenzel, 1997). $TXA_2$ is generated in greater amounts in asthmatics than in normal subjects, and may participate in thickening and remodeling of the airway wall, subsequently contributing to airway hyperresponsiveness (Davi et al., 1997; Robinson et al., 1985).

$TXA_2$ mediates a number of cellular responses through binding to a specific receptor, the prostanoid thromboxane (TP) receptor (Alm et al., 2002), which signals through the activation of the Gq/11 family of G proteins (Kinsella, 2001). The TP receptor is activated by its natural agonist as well as by high levels of other eicosanoids such as prostaglandin $H_2$, prostaglandin F2α ($PGF_{2\alpha}$), and isoprostanes, all of which may play a role in asthma (Antczak et al., 2002; Dogne et al., 2002b). Many aspects of bronchial hyperresponsiveness are potentially mediated by the TP receptor, which associates the TP receptor with the pathophysiology of asthma. $TXA_2$ synthase inhibitors and TP receptor antagonists have been developed as anti-asthma drugs, and demonstrated to improve TP receptor-induced airflow limitation and bronchial hyperresponsiveness (Hanson et al., 2005; Ishimura et al., 2008; McKenniff et al., 1991). However, the signaling pathway responsible for the modulation of TP receptor mediated airway hyperresponsiveness to $TXA_2$ is not clear.

The mitogen-activated protein kinase (MAPK) pathway activation has been suggested to contribute to airway inflammation and airway hyperresponsiveness (Duan and Wong, 2006). The best-characterized of the mammalian MAPKs are 1) the 42- and 44-kDa extracellular signal-regulated kinases (ERKs) ERK2 and ERK1; 2) the c-Jun N-terminal kinase (JNK) or stress-activated protein kinase (SAPK); and 3) p38 MAPK. Among them, the JNK signaling is demonstrated to tightly regulate the TP receptor related inflammation (Bayat et al., 2008; Kumar et al., 2005) in vasculature and the JNK inhibitor SP600125 exerts effects on allergen-induced airway inflammation and remodeling (Eynott et al., 2004; Nath et al., 2005). A common

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feature associated with the regulation of airway smooth muscle contraction is a change in intracellular $Ca^{2+}$ concentration, the modification of calcium channel activity may predispose airway smooth muscle to hyperresponsiveness (Perez-Zoghbi et al., 2009).

Previously, we have demonstrated that organ culture induced airway hyperresponsiveness to bradykinin occurs via the up-regulated bradykinin receptors (Lei et al., 2010). The present study was designed to investigate if an organ culture affects TP receptor mediated airway contractile responses focusing on the roles of transcription and MAPK activity and calcium channel activities.

## 2. Materials and methods

### 2.1. Tissue preparation

Male Sprague Dawley rats (body weight 250–300 g, M&B, Denmark) were acclimatized for a week under standardized temperature (21–22 °C), humidity (50–60%) and light (12:12 light–dark) conditions in the Animal Department of Wallenberg Center in Lund. The rats were anesthetized by $CO_2$ inhalation and were exsanguinated. The lungs were immersed in cold buffer solution (NaCl 119 mM; $NaHCO_3$ 15 mM; KCl 4.6 mM; $MgCl_2$ 1.2 mM; $NaH_2PO_4$ 1.2 mM; $CaCl_2$ 1.5 mM and glucose 5.5 mM) and the bronchi were freed of adhering lung tissue down to the second generation by dissection under a microscope. Circular segments were cut from the bronchi with a diameter of 0.3 mm. The experimental protocol was approved by Lund University Animal Ethics Committee (M161-07).

### 2.2. Organ culture procedure

After the dissection, the segments were placed individually into wells of a 96-well plate with 200 µl serum-free DMEM culture medium containing L-glutamine (584 mg/l) and supplemented with penicillin (100 U/ml) and streptomycin (100 µg/ml). Incubation was performed at 37 °C in humidified 5% $CO_2$ in air for the required time intervals (12 h, 24 h or 48 h) in the presence or absence of intracellular signal inhibitors. The segments were transferred into new wells containing fresh media every 24 h.

### 2.3. In vitro pharmacology

Bronchial segments were immersed in temperature controlled (37 °C) myographs (Organ Bath Model 610 M, J.P. Trading, Aarhus, Denmark) containing 5 ml bicarbonate buffer solution (the same composition as referred in tissue preparation). The solution was continuously aerated with 5% $CO_2$ in $O_2$ resulting in a pH of 7.4. The bronchial segments were mounted for continuous recording of isometric tension by the Chart software (AD Instruments, Hastings, UK). A resting tone of 1.0 mN was applied to each segment, the pretension employed was chosen on the basis of pretension–contraction curves in $Ca^{2+}$-free and $Ca^{2+}$-containing solution as described earlier (Hogestatt et al., 1983) and as later modified for bronchial ring segments (Granstrom et al., 2006). The segments were allowed to stabilize at this tension for at least 1.5 h before being exposed to a potassium-rich (60 mM $K^+$) buffer solution with the same composition as the standard solution except that NaCl was replaced by an equimolar concentration of KCl. The potassium-induced contraction was used as a reference for the contractile capacity, and the individual segments were only used for further studies if two strong (>1 mN) reproducible contractions (variation<10%) could be elicited. Concentration–response curves for TP receptor agonist U46619 were obtained by a cumulative administration of the reagent. At a point 30 min before cumulative concentrations were administered, 3 µM of indomethacin and 100 µM of L-NG-monometylarginin (L-NMMA) were added to block the modifying effects of epithelial prostaglandin and NO release (Alm et al., 2002).

### 2.4. Western-blot

The epithelium-denuded segments of both fresh and organ cultured for 48 h groups were frozen in liquid nitrogen and homogenized in cell extract denaturing buffer (BioSource, Invitrogen, Carlsbad, CA) containing a phosphatase inhibitor cocktail and protease inhibitor cocktail (Sigma, St. Louis, MO). Protein concentration was measured with a Bio-Rad protein analysis kit and TECAN infinite M200. Proteins (40 µg) were loaded and separated on 4–15% Ready Gel Precast Gels (Bio-Rad, Life Science Research, Hercules, CA), followed by blocking with the 5% non-fat milk and washing with T-TBS buffer three times for 5 min, then incubated with a primary antibody overnight at 4 °C. After washing three times for 5 min, the membranes were incubated with a HRP conjugated secondary antibody for 1 h at room temperature, after washing five times for 5 min, the membranes were developed with SuperSignal® West Dura extended duration substrate (Thermo Scientific), visualized using a Fujifilm LAS-1000 luminescent image analyzer (Stamford, CT), and then analyzed with Image Gauge Ver. 4.0 (Fuji Photo Film Co., Ltd., Japan). The antibodies for TP receptor (Cayman Chemical, 1:500 dilution), phospho-SAPK/JNK kinase (Thr183/Tyr185, Cell Signaling Technology, 1:1000 dilution), SAPK/JNK kinase (Thr183/Tyr185, Cell Signaling Technology, 1:1000 dilution) and beta-actin (Santa Cruz, 1:5000 dilution) were used as the primary antibody; the anti-rabbit IgG, HRP-linked antibody (Cell Signaling Technology, 1:2000 dilution) and anti-mouse IgG, HRP-linked antibody (Cell Signaling Technology, 1:2000 dilution) were used as the secondary antibody.

### 2.5. Immunohistochemistry

The bronchial segments after organ culture were immersed in a fixative solution consisting of 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) for 3 h at 4 °C. After fixation, the specimens were dehydrated in 20% sucrose of phosphate buffer (0.1 M, pH 7.4) for 24 h at 4 °C, and then frozen in Tissue-Tek (Sakura Finetek Europe B.V., Zoeterwoude, Netherlands) and stored at −80 °C. Sections were cut at 10 µm thickness in a cryostat and mounted on SupperFrost Plus slides. Immunohistology staining with primary antibody against phospho-SAPK/JNK (Thr183/Tyr 185, monoclonal antibody, Cell Signaling) was performed. Briefly, the sections were incubated with the primary antibody (dilution: phospho-SAPK/JNK (Thr183/Tyr 185) 1:50) overnight at 4 °C, thereafter the secondary antibody donkey anti-rabbit IgG conjugated to Cy2™ was applied for 1 h at room temperature in dark. To identify the smooth muscle layer of the bronchial segments, immunohistology staining with the primary antibody against rat smooth muscle actin (Santa Cruz, 1:200 dilution) and the secondary antibody donkey anti-mouse IgG (H+L) conjugated to Texas Red (Jackson ImmunoResearch, 1:200 dilution) were also performed. In the control experiments, either the primary antibody or the secondary antibody was omitted. The stained bronchial segments were observed under a confocal microscope (Nikon, C1plus, Nikon Instruments Inc., NY, USA) and analyzed by Image J software (http://rsb.info.nih.gov/ij). The fluorescence intensity was measured on the smooth muscle cells. For each bronchial segment, six randomly selected sections were studied. In each section, the fluorescence intensity was measured at six preset areas.

### 2.6. Reagents

GR32191 ([1R-[1α(Z),2β,3β,5α]]-(+)-7-[5-([1,1′-biphenyl]-4-ylmethoxy)-3-hydroxy-2-(1-piperidinyl)cyclopentyl]-4-heptonoic acid) and SKF96365 (1-{b-[3-(4-Methoxyphenyl)propyl]-4-methoxyphenethyl}-1 H-imidazole, HCl) were purchased from TOCRIS bioscience; U46619 (9,11-dideoxy-9α,11α-methanoepoxy