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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 A₂ (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 kinases (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 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 elevation of extracellular calcium influx, which may provide knowledge for understanding signaling pathway responsible for modulation of TP receptor mediated airway hyperresponsiveness to thromboxane.

Key words: TP receptors, signaling, JNK MAPK, calcium influx, airway hyperresponsiveness.

1. Introduction

Thromboxane A₂ (TXA₂) 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). TXA₂ 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 B₂ (Davi et al.), a stable metabolite of TXA₂ (Iwamoto et al., 1988; Manning et al., 1991; Wenzel et al., 1989). A wealth of studies has shown the role of TXA₂ in the pathogenesis and pathophysiology of asthma (Devillier and Bessard, 1997; Dogne et al., 2002a; Wenzel, 1997). TXA₂ 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).

TXA₂ mediates a number of cellular responses through binding to a specific receptor, the prostanoid thromboxane (TP) receptor (Alm et al.), which signals through 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 H₂, prostaglandin F_{2α} (PGF_{2α}), 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. TXA₂ 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 modulation of TP receptor mediated airway hyperresponsiveness to TXA₂ is not clear.

The mitogen-activated protein kinases (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 amino-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 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 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 250-300g, 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 CO₂ inhalation and were exsanguinated. The lungs were immersed in cold buffer solution (NaCl 119 mM; NaHCO₃ 15 mM; KCl 4.6 mM; MgCl₂ 1.2 mM; NaH₂PO₄ 1.2 mM; CaCl₂ 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 Ethic's 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% CO₂ in air for the required time intervals (24 h, 48 h or 96 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 610M, 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% CO₂ in O₂ 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 Ca²⁺-free and Ca²⁺-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 cumulative administration of the reagent. At a point 30 minutes before cumulative concentrations were administered, 3 µM of indomethacin and 100 µM of L-NG-monomethylarginin (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 luminiscent 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). 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 CyTM2 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 analysed 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-[1 α (Z),2 β ,3 β ,5 α]]-(+)-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}-1H-imidazole, HCl,) were purchased from TOCRIS bioscience; U46619 (9,11-dideoxy-9 α ,11 α -methanoepoxy Prostaglandin F_{2 α} ; 9,11-dideoxy-9 α ,11 α -methanoepoxy PGF_{2 α}), SP600125 (1,9-Pyrazoloanthrone), SB203580 (4-(4-Fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1H-imidazole), PD98059 (2-(2-Amino-3-methoxyphenyl)-4H-1-benzopyran-4-one), nifedipine, atropine, indomethacin and L-NNA(NG-Nitro-L-arginine) were purchased from Sigma; MDL 105,221([(R)-1-[2-[3-(3,4-dichlorophenyl)-1-(3,4,5-trimethoxybenzoyl)-pyrrolidin-3-yl]-ethyl]-4-phenylpiperidine-4-carboxamide, hydrochloride) were purchased from ALEXIS Biochemicals.

2.7 Statistical analysis

All data are expressed as mean values $\pm$ S.E.M. Contractile responses to U46619 in each segment were expressed as percent of maximal contraction ($E_{\max}$) induced by 60 mM potassium buffer solution (the response is matched at $t = 0$). Each agonist concentration-effect curve was fitted to the Hill equation using an iterative, X is the logarithm of concentration, Y is the response, least square method (GraphPad Prism 4, San Diego, U.S.A), to provide estimates of $E_{\max}$ and pEC_{50} values (negative logarithm

of the agonist concentration that produces 50% of the maximal effect). Two-way analysis of variance (ANOVA) with Bonferroni post-test was used to compare the two corresponding data points at each concentration of the two curves, and unpaired Students' *t*-test with Welch's correction applied for the comparison of pEC₅₀ values (curve shift) and E_{max}. The data and statistical analysis were performed by Graph-Pad Prism 4 (San Diego, USA). *P* < 0.05 was considered as statistically significant.

3. Results

3.1 Enhancement of TP agonist U46619-induced contraction

Basal contractile responses to U46619 (TXA₂ analog) on isolated rat secondary bronchial ring segments were studied in fresh isolated segments. A weak contraction was elicited by U46619 in the fresh segments. The bronchial segments were cultured in serum-free medium for 12, 24 and 48 h in order to study the time course effects. Compared with control (fresh segments), organ culture time-dependently enhanced U46619-induced contraction. The E_{max} was reached at 24 h and the concentration effect curves were shifted to the left. The E_{max} for U46619 at 12 h increased from 56.51 ± 6.23 % to 67.85 ± 3.39 % (Table 1, Fig. 1A). The contractile responses were further enhanced when the culture periods extended to 24 h (E_{max} from 67.85 ± 3.39 % to 108.4 ± 7.73 %, Table 1, Fig. 1A.). 48 h of organ culture did not cause a further increase of U46619-induced contraction. We did not see a significant variation of the 60 mM potassium-induced contractions vary with time (fresh versus 24 h, 48 h and 96 h) in the present setup.

3.2 Pharmacological characterization of TP receptors

In order to investigate the TP receptors are responsible for the enhancement of U46619-induced contraction, a pharmacological characterization was performed in the bronchial segments cultured for 24 h. The TP receptor selective antagonist

GR32191 (10^{-7} M) (Armour et al., 1989; Zhang et al., 2009) was applied into the myograph bath 30 min before the U46619 concentration effect curves was obtained. Contraction to U46619 was antagonized by GR32191 with a pK_B value of 8.03 ± 0.07 , which shows the similar effects in human bronchi rings (Armour et al., 1989). GR32191 caused concentration-dependent, parallel rightward shift of the concentration-response curve to U46619 (Fig. 1B, pEC_{50} value from 6.87 ± 0.04 to 5.80 ± 0.05 , $P < 0.001$), without altering E_{max} (Fig. 1B, E_{max} : control 127.3 ± 3.43 % and GR32191 127.5 ± 7.42 %), suggesting that the U46619 response is mediated by the TP receptor (most likely as well as the time-dependent enhancement).

3.3 Role of transcriptional and translational mechanisms on enhancement of U46619-induced contraction

Actinomycin D (general transcriptional inhibitor, AcD, 4×10^{-6} M) and cycloheximide (general translational inhibitor, CHX, 10^{-5} M) were added to culture medium for 24 h, respectively, in order to block *de novo* transcription or translation of TP receptor induced by organ culture. The results showed that in comparison with control (vehicle), neither AcD nor CHX affected the elevated TP receptor-mediated responses elicited by organ culture (AcD E_{max} 96.55 ± 6.90 %, control E_{max} 115.1 ± 15.35 %; CHX E_{max} 128.8 ± 10.67 %, control E_{max} 109.3 ± 7.36 %). Thus, neither a transcriptional nor a post-transcriptional mechanism was involved in the organ culture-induced enhancement of U46619-induced contraction.

3.4 Role of nerve innervation in enhancement of U46619-induced contraction

A variety of chemical, physical and pharmacological stimuli may modulate cholinergic and sensory nerves in airways through activation of receptors on nerve terminals (Barnes, 2001). Muscarinic acetylcholine receptor antagonist atropine (1 μ M) and NK-1/NK-2 tachykinin receptor antagonist MDL 105,212 (1 μ M) were administrated into the myograph bath 30 min before the study of U46619-induced

concentration effect curves. There was no significant alteration of the concentration-effect curves of U46619 (atropine $E_{\max}$ 109.0 ± 2.67 %, control $E_{\max}$ 108.4 ± 1.90 %; MDL $E_{\max}$ 111.9 ± 6.86 %, control $E_{\max}$ 104.1 ± 3.49 %) in segments organ cultured for 24 h, suggesting that the airway parasympathetic and sensory innervation is not involved in the enhancement of U46619-induced airway contraction.

3.5 Role of epithelium in enhancement of U46619-induced contraction

In order to ascertain if epithelium is involved in the enhancement of U46619-induced contraction, the epithelium was removed before the bronchial segments were organ cultured. Denudation of epithelium was confirmed by immunohistochemistry (data not shown). The removal of epithelium did not affect the enhancement of contractile responses to U46619 (epithelium intact: $E_{\max}$ 105.1 ± 6.61 %, pEC_{50} 6.74 ± 0.06 ; epithelium denuded: $E_{\max}$ 109.8 ± 3.04 %, pEC_{50} 6.66 ± 0.02). This excludes a role for epithelial factors in the organ culture enhanced U46619-induced airway contraction.

3.6 Role of mitogen-activated protein kinase in enhancement of U46619-induced contraction

In order to investigate whether intracellular MAPK JNK, p38 and ERK 1/2 signal pathways were involved in the enhanced bronchial contractile response to U46619, a series of experiments were performed by using the MAPK subtypes inhibitors and vehicle (DMSO). The inhibitors were applied during the organ culture for 24 h or added into the myograph bath 30 min before administration of U46619.

Bronchial segments were cultured in the presence of SP600125 (10 μ M, JNK inhibitor) (Bennett et al., 2001), PD98059 (10 μ M, up-stream MEK1/2 inhibitor) (Alessi et al., 1995) and SB203580 (10 μ M, p38 inhibitor) (Cuenda et al., 1995) or vehicle (DMSO) for 24 h, respectively. SP600125 (10 μ M) exerted inhibitory effects on the enhancement of U46619-induced contraction at 24 h of organ culture

(SP600125 $E_{\max}$ 65.95 ± 3.36 %, $n = 8$, control $E_{\max}$ 95.28 ± 4.61 %, $n = 8$, $P < 0.01$). However, neither PD98059 (10 μ M) nor SB203580 (10 μ M) revealed a significant effect on the enhancement of TP receptor response (PD98059 $E_{\max}$ 96.92 ± 3.51 %, $n = 8$, control $E_{\max}$ 109.9 ± 5.63 %, $n = 8$; SB203580 $E_{\max}$ 110.9 ± 6.81 %, $n = 8$, control $E_{\max}$ 123.6 ± 9.28 %, $n = 8$).

The inhibitors of JNK (SP600125, 10 μ M), up-stream MEK1/2 (PD98059, 10 μ M) and p38 (SB203580, 10 μ M) or vehicle (DMSO) were applied 30 min prior to the first U46619 concentration in either fresh segments or segments cultured for 24 h, respectively. In fresh segments, the U46619 induced contraction was completely abolished by SP600125 (Fig. 2A and 2B) and partially inhibited by PD98059 (Fig. 2A and 2B, reduction by 25 %) and SB203580 (Fig. 2A and 2B, reduction by 55 %), respectively. In segments organ cultured for 24 h, the inhibitory pattern was altered, only SP600125 significantly suppressed the U46619-induced airway contraction (Fig. 2C and 2D, reduction by 70%), whereas, there is no significant effect of PD98059 (Fig. 2C and 2D) and SB203580 (Fig. 2C and 2D) on U46619-induced airway contraction. The present results show that the JNK pathway signaling was mainly involved after organ culture.

3.7 Role of extracellular Ca^{2+} influx in enhancement of U46619-induced contraction

To evaluate the role of intracellular Ca^{2+} signaling in mediating the enhancement of U46619-induced contraction, the inhibitory effects of calcium-free solution in the presence of the Ca^{2+} chelator EGTA (1 mM) (Parvez et al., 2006), the voltage-gated calcium channel (VGCC) blocker nifedipine (10 μ M) (Skogvall et al., 2007) and the receptor-operated calcium channel (ROCC) blocker SKF96365 (10 μ M) (Merritt et al., 1990) were determined in both fresh and organ cultured segments.

Calcium-free buffer (the same composition as the standard solution except the $CaCl_2$ is absent) in the presence of EGTA (1 mM) abolished the U46619-induced contraction in fresh bronchial segments (Fig. 3A and 3G) as well as in bronchial segments organ cultured for 24 h (Fig. 3B and 3G), indicating that enhancement of

U46619-induced contraction is extracellular calcium dependent. In the fresh segments, U46619-induced contraction was inhibited by nifedipine (reduction by 46%, Fig. 3C) or SKF96365 (reduction by 40%, Fig. 3E), respectively. In segments organ cultured for 24 h, the inhibitory pattern was altered, only nifedipine significantly suppressed the U46619-induced airway contraction (reduction by 60%, Fig. 3D and 3H), while there is no significant effect of SKF96365 (Fig. 3F and 3I) on U46619-induced airway contraction. These results show that the L-type voltage-gated calcium channel is required for enhancement of U46619-induced contraction.

To further determine if the participation of JNK and voltage-gated calcium channel in enhanced U46619-induced contraction via the same signal pathway, SP600125 (10 μ M) and nifedipine (10 μ M) were administrated together into the myograph bath 30 min before to obtain the U46619 concentration effect curve in bronchial segments cultured for 24 h. The combinatory use of SP600125 and nifedipine exerted a further inhibitory effect (reduction by 88%, Fig. 4A and 4B), when compare to the sole inhibitory effect by SP600125 (10 μ M, reduction by 74%, Fig. 4A and 4B) or nifedipine (10 μ M, reduction by 60%, Fig. 4A and 4B), suggests that JNK and L-type voltage-gated calcium channel also act through individual pathways besides the same pathway to enhance the TP receptor mediated airway hyperresponsiveness.

3.8 Effects of organ culture on TP receptor expression

The epithelium-denuded bronchial segments of both fresh and 24 h organ cultured (epithelium removed before the organ culture) were collected for determine the TP receptor expression in bronchial smooth muscle. We observed that the expression of TP receptor in bronchial smooth muscle was not affected by organ culture (Fig. 5A).

3.9 Enhancement of U46619-induced phosphorylation of JNK by organ culture

Since the JNK signaling was suggested to be mainly involved in the enhanced

U46619-induced bronchial contraction by organ culture, the alteration of U46619-induced phosphorylation of JNK were further determined in bronchial smooth muscle. After exposure the fresh and 24 h organ cultured bronchial segments (epithelium denuded) to U46619 (1 μ M) or vehicle, the tissues were immediately collected for the western blot procedure. In the fresh segments, the phospho-JNK/JNK value was increased by U46619 from 0.32 ± 0.03 to 0.53 ± 0.03 ($p < 0.01$, Fig. 5B); in the 24 h organ cultured segments, the phospho-JNK/JNK value was increased by U46619 from 0.43 ± 0.04 to 0.79 ± 0.04 ($p < 0.001$, Fig. 5B). The phospho-JNK/JNK value induced by U46619 was significantly enhanced by organ culture (fresh: 0.21 ± 0.01 vs. organ culture: 0.35 ± 0.02 , $p < 0.001$).

3.10 The role of calcium influx in U46619-induced JNK phosphorylation

The above studies suggested that U46619-induced JNK activation and extracellular calcium influx are involved in the enhanced bronchial contractile response to U46619. In order to test if the extracellular calcium influx is required for U46619-induced JNK phosphorylation, the JNK phosphorylation was examined by immunohistochemistry in both fresh bronchial segments and bronchial segments of 24 h organ culture in either normal buffer solution or calcium-free buffer solution. After exposure (the fresh or 24 h organ cultured bronchial segments) to U46619 (1 μ M) in either normal buffer (stable contraction seen) or calcium-free buffer, the tissues were immediately collected for the immunohistochemistry procedure.

Phosphorylated JNK protein was clearly observed in green color in the smooth muscle cell layer and in the epithelium layer in bronchial segments. There was a positive expression of phosphorylated JNK protein in bronchial smooth muscle in the fresh group (Fig. 6B), while it was increased at 24 h of organ culture (Fig. 6D); no significant change of phosphorylated JNK protein expressions was observed in the epithelium layer (Fig. 6B and 6D). The calcium-free buffer treatment did not modify the phosphorylated JNK protein expression in smooth muscle cell and epithelial layer in fresh (Fig. 6A) or organ culture group (Fig. 6C). Measurements of phosphorylated

JNK protein immunoreactivity showed that the phosphorylated JNK protein was significantly enhanced in bronchial smooth muscle cells after 24 h organ culture in normal buffer; while there is no change in epithelial layer (Fig. 6E). This data suggests that the U46619-induced JNK phosphorylation in bronchial smooth muscle cell was increased by organ culture for 24 h; this response is not calcium influx dependent.

4. Discussion

Airway hyperresponsiveness is characterized by an increased sensitivity of airway smooth muscle cells to constrictor agents, which can be demonstrated in almost all patients with current symptomatic asthma (Cockcroft and Davis, 2006). The increased sensitivity of the airways to constrictor agonists results in a steeper slope of the dose-response relationship and a greater maximal response to the agonists (O'Byrne and Inman, 2003). The present studies have for the first time demonstrated that organ culture of rat bronchial segments induces a time-dependent enhancement of TXA₂ analog U46619 induced airway contractions. This enhanced airway contraction to U46619 is downstream of TP receptor activation, which occurs via an increase of JNK activity and elevation of extracellular calcium influx.

U46619 is a chemical that mimics the effects of TXA₂. Exposure of human tracheal rings to U46619 results in cumulative, concentration-dependent contractions by stimulation of TP receptors (Armour et al., 1989) and inhaled U46619 is a potent bronchoconstrictor in human (Jones et al., 1992). In the present setup, organ culture of rat bronchial segments for 12 h to 48 h, contractile responses to U46619 was increased. Epithelium removal did not affect the enhancement of U46619-induced contractions, which suggests that the enhanced contractile response to U46619 is mediated selectively via bronchial smooth muscle cells. The increase in U46619-induced contraction was inhibited by the TP receptor antagonist GR32191, while there was no alteration of TP receptor protein expression; moreover, this elevation of contraction was not affected by general transcriptional inhibitor AcD or

general translational inhibitor CHX. Therefore, we provide an *in vitro* model of enhanced airway smooth muscle reactivity to U46619, which occurs through a mechanism downstream of the TP receptor, i.e. in the signal transduction.

MAPKs are a group of serine/threonine kinases that play an important role in the intracellular signalling in response to extracellular stimuli (Lewis et al., 1998; Schnaper, 1998). The different MAPKs are involved in such important cellular functions as proliferation, differentiation and survival. Studies involving intact animals as well as *in vitro* of tissues support that MAPK superfamily activation contribute to airway inflammation and airway hyperresponsiveness (Duan et al., 2005; Duan et al., 2004; Orsini et al., 1999). Especially, the G-protein coupled receptor signaling is tightly regulated by MAPK in airway smooth muscle (Citro et al., 2005; Zhang et al., 2007). In the present study, the acute administration of JNK, ERK 1/2 and p38 inhibitors significantly suppressed the U46619-induced contraction in fresh bronchial segments, which suggests that all three MAPK pathways are involved in the TP receptor mediated contraction in fresh bronchial segments. This is consistent with findings in human uterine smooth muscle cells (Miggin and Kinsella, 2001). After organ culture, only the JNK inhibitor was observed to reduce the enhanced U46619-induced contraction, while there was no effect of p38 inhibitor or MEK1/2 inhibitor. Furthermore, we found that the U46619-induced phosphorylated JNK protein expression was enhanced by organ culture. Studies of the JNK inhibitor SP600125 upon allergic airway challenges have demonstrated the involvement of JNK in airway inflammation, airway remodeling and airway hyperresponsiveness (Eynott et al., 2004; Nath et al., 2005). Here, we demonstrated that the affect of MAPK plays a vital role in the enhanced bronchial contractile responses to U46619. In addition, the contractile response to U46619 in organ cultured bronchial segments was not completely abolished by a JNK inhibitor, suggesting that other mechanisms may also be involved in regulating the contractile response to U46619.

Calcium ions have a central role in regulating airway smooth muscle contractile function, and many investigators have postulated that alterations in calcium regulatory mechanisms impair airway smooth muscle contractility (Bai et al., 2007; Jude et al.,

2008). Consequently, it has been hypothesized that the intracellular calcium concentration is abnormally elevated in asthmatic airway smooth muscle cells (Amrani and Panettieri, 1998; Amrani et al., 2004; Moynihan et al., 2008). The U46619-induced tonic contractions in rat bronchi is dependent on the extracellular calcium concentration, while it was not affected by intracellular calcium pools (Parvez et al., 2006). In the present study, calcium-free media in the presence of Ca^{2+} chelator EGTA resulted in complete suppression of the U46619-induced contraction in both fresh and organ cultured bronchial segments. This suggests that the enhanced U46619-induced contraction in bronchial smooth muscle is extracellular calcium dependent and could be enhanced by calcium influx. In fresh bronchial segments, the L-type VOCC Ca^{2+} channel blocker nifedipine and ROCC blocker SKF96365 showed similar suppressive effects on U46619-induced contraction, which is in concert with findings on TP receptor mediated contraction in rat aorta (Tosun et al., 1998). However, the inhibitory effects of two blockers were altered in organ cultured bronchial segments; nifedipine exerted a significant inhibitory effect on U46619-induced contraction, while SKF96365 had no significant effect on U46619-induced contraction. These findings imply that the affect of ROCC and L-type VOCC involved calcium influx may play an important role in the enhanced airway contraction to U46619.

The contractile response to U46619 in fresh bronchial segments could be abrogated by exposure to extracellular calcium-free medium or by SP600125 treatment, indicating that U46619-induced JNK activation and calcium signaling occur through the same or an associated pathway. After organ culture, chelation of extracellular calcium abrogated the contractile response to U46619, while SP600125 inhibited most of the contractile response, indicating that the U46619-induced JNK activation and a large part of the calcium signaling through the same pathway, a small part of calcium signaling through a 'JNK-unrelated' pathway. Combining use of SP600125 and nifedipine exerted a further inhibitory effect on U46619-induced contraction compared to the sole inhibitory effect by SP600125 in organ cultured bronchial segments, suggests that the L-type VOCC Ca^{2+} channel related calcium

influx may partially responsible for the 'JNK-unrelated' calcium signaling pathway. For the same pathway shared by JNK activation and calcium signaling, we confirm that the extracellular calcium influx is not required for the U46619-induced JNK activation both in fresh and in organ cultured bronchi; it is possible that the JNK activation is in the up-stream of the calcium signaling.

Thromboxane is a key inflammatory mediator and closely associated with airway hyperreactivity. The present study demonstrates that organ culture enhanced the bronchial contractile responses to thromboxane receptor agonist U46619 via a non-transcriptional mechanism through affect the MAPK and calcium influx. An in-vivo study shows that following antigen challenge, the influx of inflammatory cell eosinophila into the lung tissue was demonstrable at 4 h with a peak at 24 h and persisted for at least 8 days (Underwood et al., 1995). Similar time-dependent changes in the TP receptor mediated airway hyperresponsiveness is seen in the present study of organ culture of rat bronchi, suggesting that the organ culture model mimics some aspects of the in-vivo inflammatory responses and thus our findings may provide knowledge for understanding signaling pathway responsible for modulation of TP receptor mediated airway hyperresponsiveness to thromboxane (Fig.7).

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Table 1 Maximal contractile response (E_{max}) and pEC_{50} (negative logarithm of the agonist concentration that produces 50% of the maximal effect) values of rat bronchial ring segments in fresh and organ culture to U46619

Incubation time (h)	n	E_{max} (% of 60mM K^+)	pEC_{50}
0	8	56.51 ± 6.23	6.50 ± 0.08
12	8	67.86 ± 3.33	6.65 ± 0.04
24	8	109.6 ± 7.49^b	7.09 ± 0.09^b
48	7	124.4 ± 13.22^b	6.78 ± 0.12

Values are expressed as mean $\pm$ S.E.M. Statistical analysis was performed with one-way ANOVA and Dunnet post-test, $^bP < 0.01$ was compared with fresh segments, n = the number of experiments.

Legends for Figures

Fig. 1

Time course of organ culture on the contractile responses to U46619 in rat bronchial ring segments (A) and effects of TP receptor antagonist GR32191 (10^{-7} M) on U46619-induced contractions in rat bronchial segments at 24 h of organ culture (B). Each data point is derived from 7–8 experiments and presented as mean $\pm$ S.E.M.

Fig. 2

Effects of SP600125 (10 μ M), SB203580 (10 μ M) and PD98059 (10 μ M) on U46619-induced concentration-contraction curves (A), $E_{\max}$ (B) in fresh and concentration-contraction curves (C), $E_{\max}$ (D) in 24 h organ cultured rat bronchial segments. Each value was derived from 6-8 experiments and presented as mean $\pm$ S.E.M. Unpaired student's t-test with Welch's correction were used for statistic analysis. $**P < 0.01$ was compared with vehicle control, N.S. = not significant.

Fig. 3

Effects of calcium-free buffer in the presence of EGTA (1 mM, A,B and G), nifedipine (10 μ M, C,D and H) and SKF96365 (10 μ M, E,F and I) on U46619-induced contractions (A-F) and maximal contractile response ($E_{\max}$, G-I) in fresh (A, C and E) and 24 h organ cultured (B,D and F) rat bronchial segments, respectively. Each value was derived from 6-8 experiments and presented as mean $\pm$ S.E.M. Unpaired student's t-test with Welch's correction was used for statistic analysis. $*P < 0.05$, $***P < 0.001$ was compared with vehicle control, N.S. = not significant.

Fig. 4

Effects of SP600125 (10 μ M) and nifedipine (10 μ M) on U46619-induced concentration-contraction curves (A) and maximal contractile response ($E_{\max}$) (B) to U46619 in rat bronchial segments cultured for 24 h. Each value was derived from 6-8

experiments and presented as mean $\pm$ S.E.M. Unpaired student's t-test with Welch's correction were used for statistic analysis. $**P < 0.01$, $***P < 0.001$.

Fig. 5

Protein expression of TP receptors in bronchial smooth muscle are assessed by western blot in both fresh segments and 24 h organ cultured segments (A). Protein expression of phospho-SAPK/JNK (Thr183/Tyr185) in bronchial smooth muscle are assessed by western blot in both fresh segments and 24 h organ cultured segments after exposure to U46619 (1 μ M) or vehicle (B). Each data is derived from 5-8 experiments and presented as mean $\pm$ S.E.M. Unpaired student's t-test with Welch's correction was used for statistic analysis. $**P < 0.01$, $***P < 0.01$.

Fig. 6

Phospho-SAPK/JNK (Thr183/Tyr185) protein expression in bronchial epithelium and bronchial smooth muscle are assessed by immunohistochemistry in both fresh segments (A and B) and 24 h organ cultured segments (C and D) in calcium-free buffer solution (A and C) and normal buffer solution (B and D). The size bar corresponds to 100 μ m. SMC: smooth muscle cell, EP: epithelium. Semi-quantitation of phospho-SAPK/JNK (Thr183/Tyr185) protein in bronchial epithelium layer and bronchial smooth muscle layer (E), each data is derived from 6 experiments and presented as mean $\pm$ S.E.M, $n=6$. Unpaired student's t-test with Welch's correction was used for statistic analysis. $*P < 0.05$, $**P < 0.01$.

Fig.7

Schematic diagram of the hypothesis and experimental designs of present study. TP receptor: thromboxane A_2 receptor, JNK: c-Jun N-terminal kinase.

Figure 1

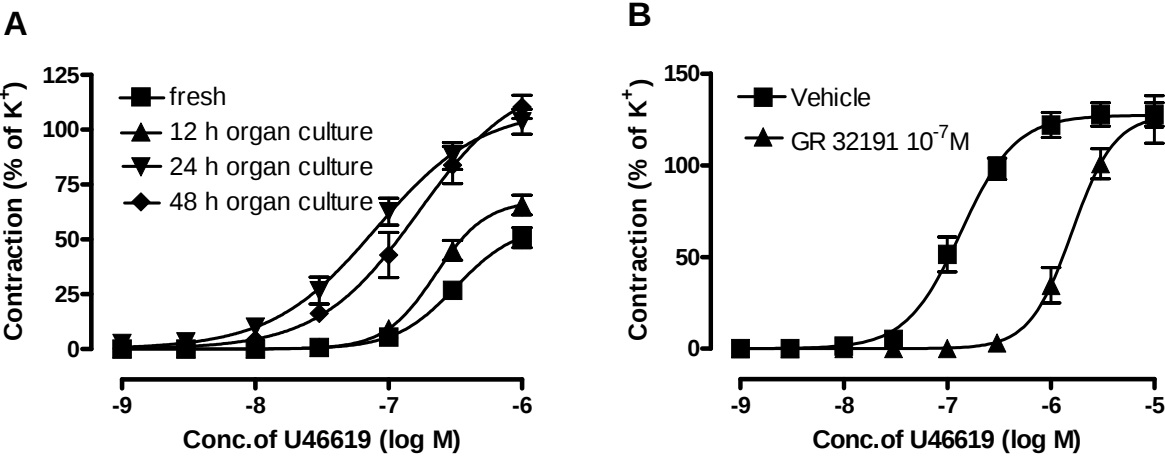


Figure 2

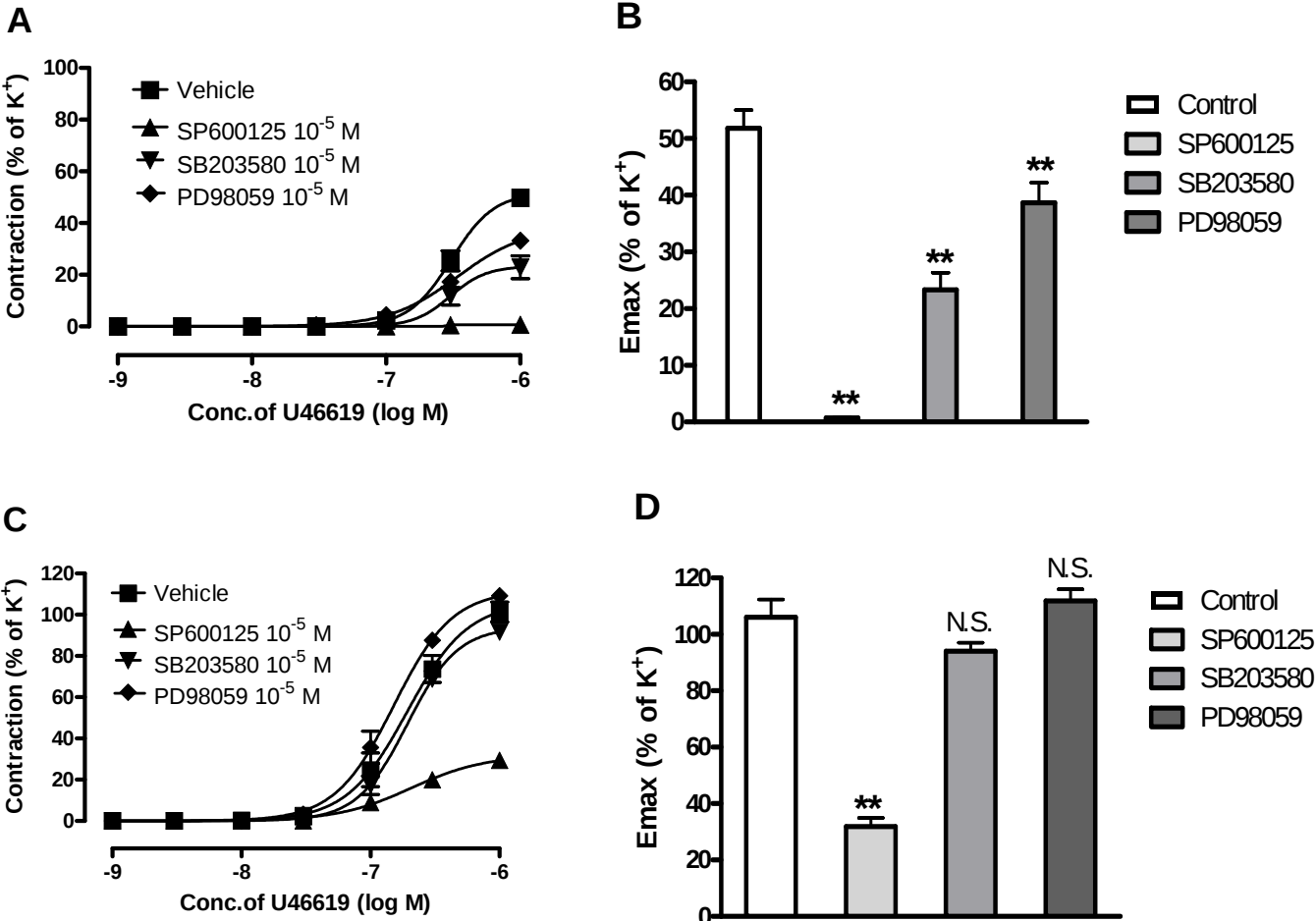
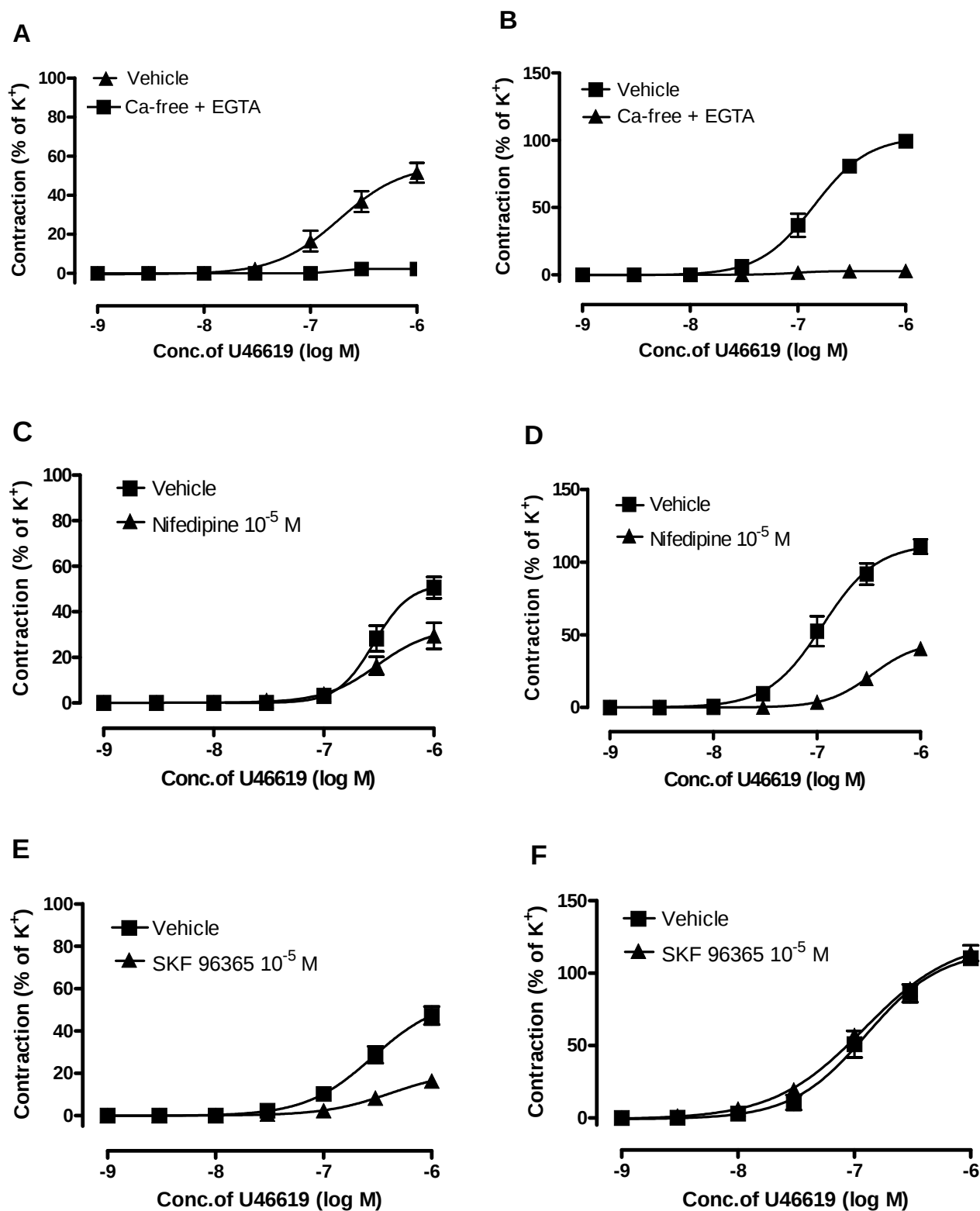


Figure 3



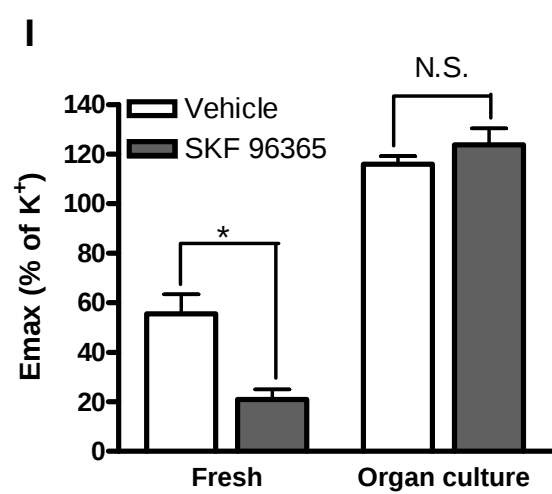
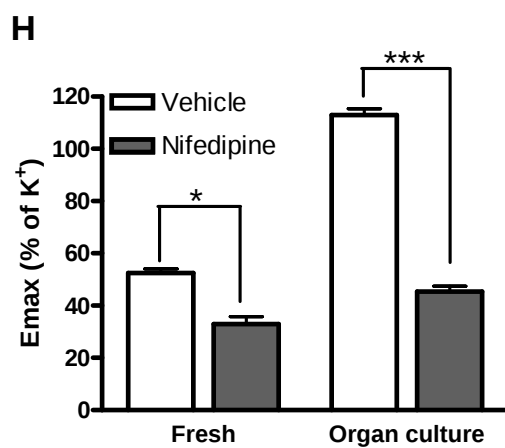
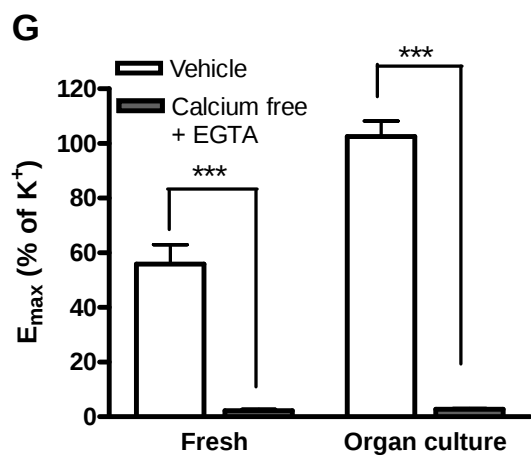


Figure 4

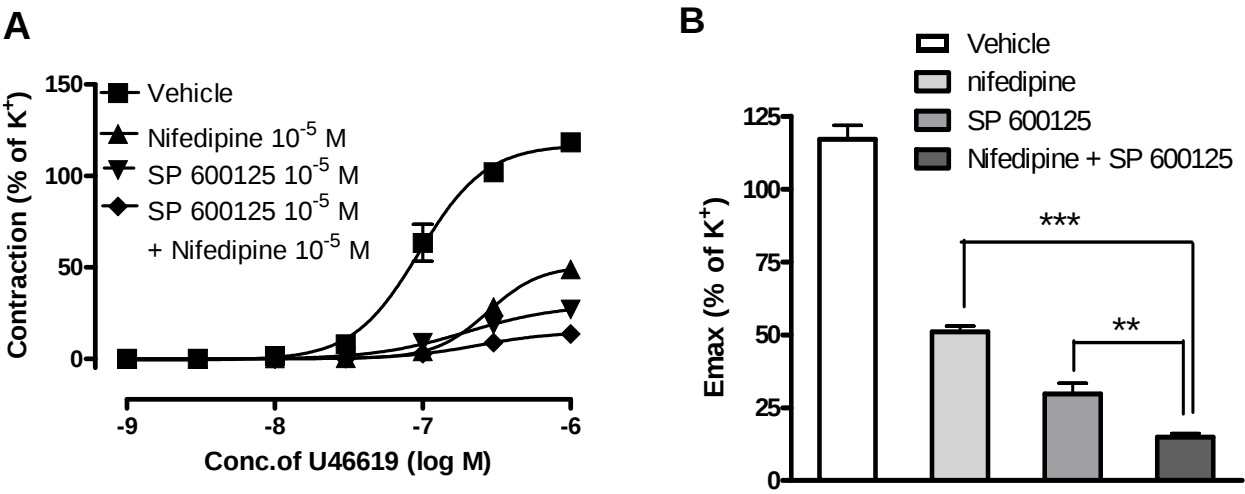
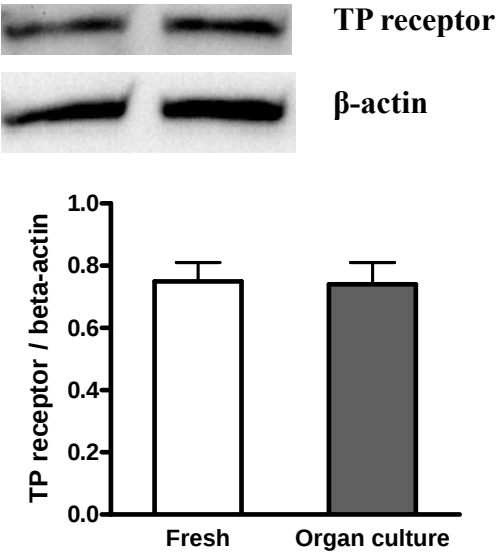


Figure 5

A



B

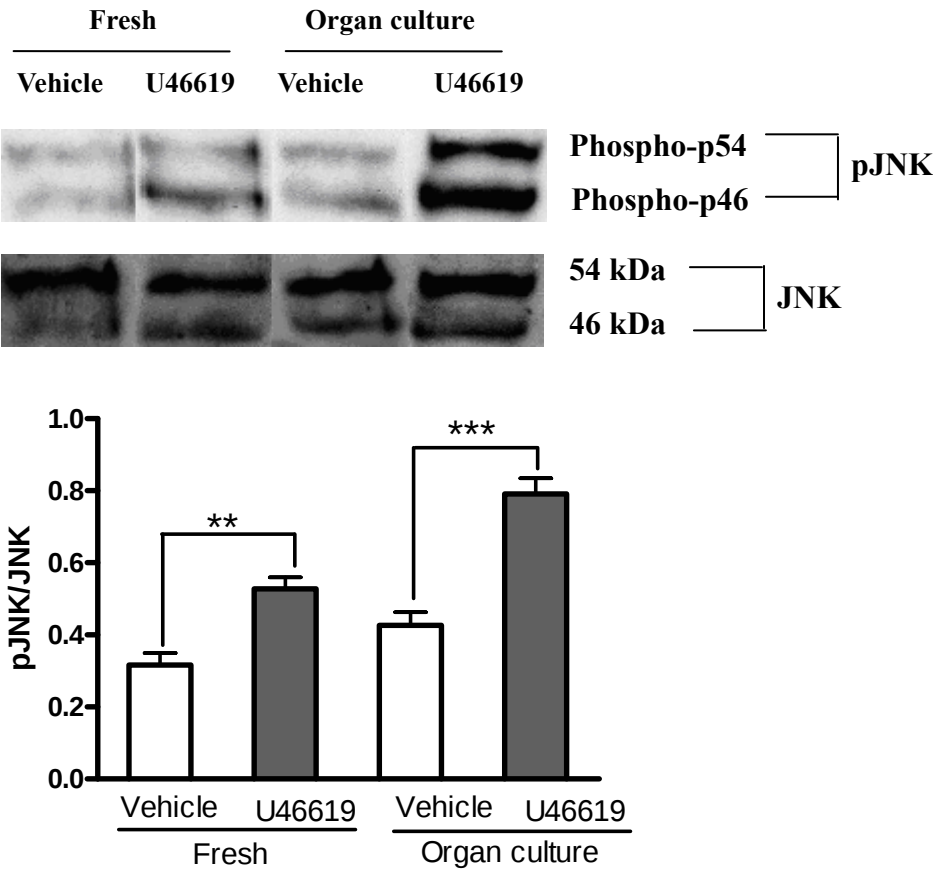


Figure 6

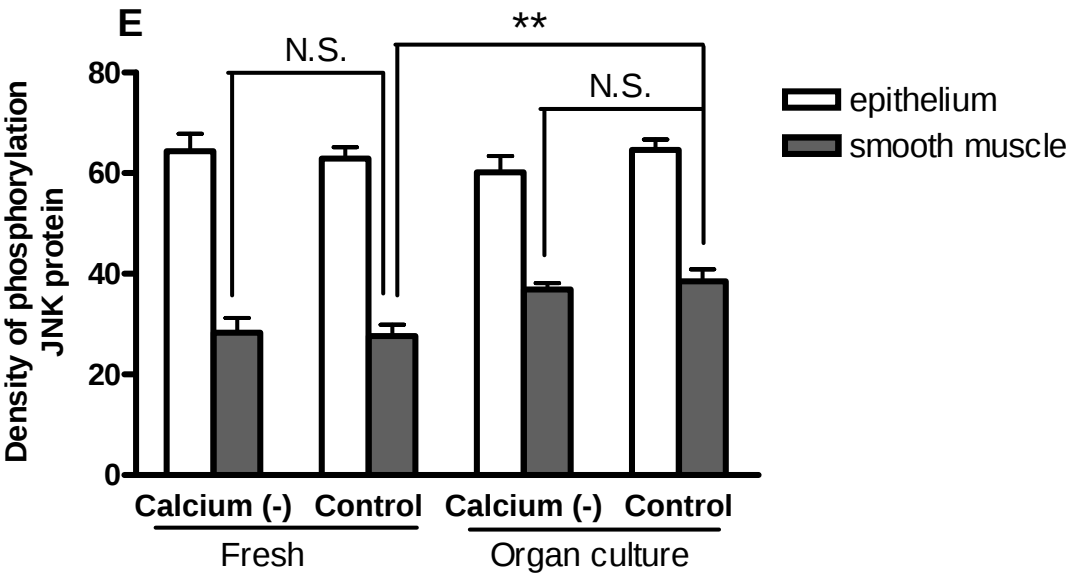
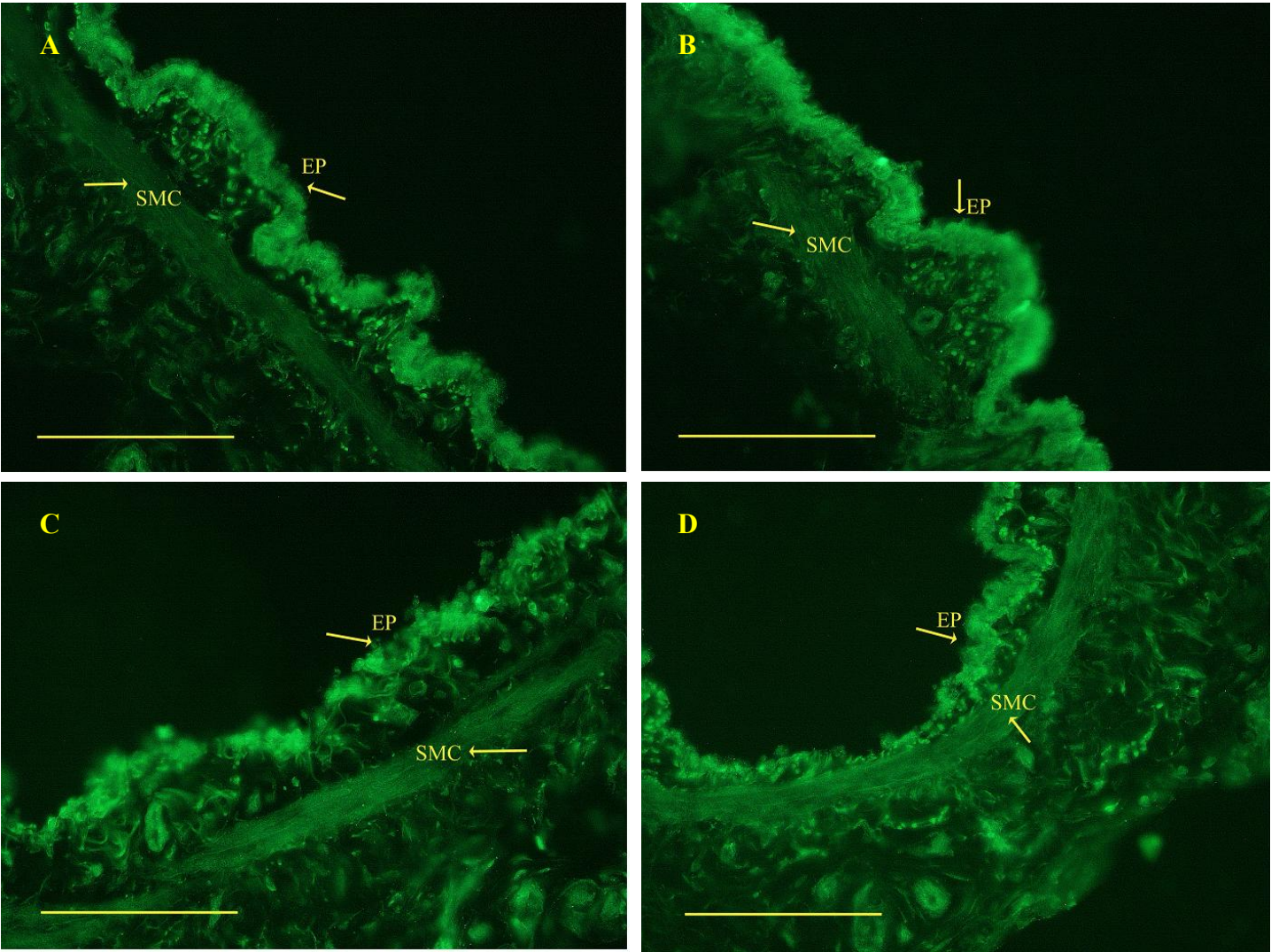


Figure 7

