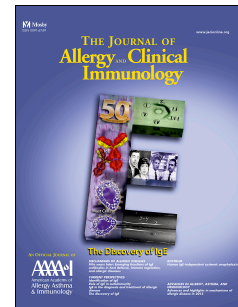


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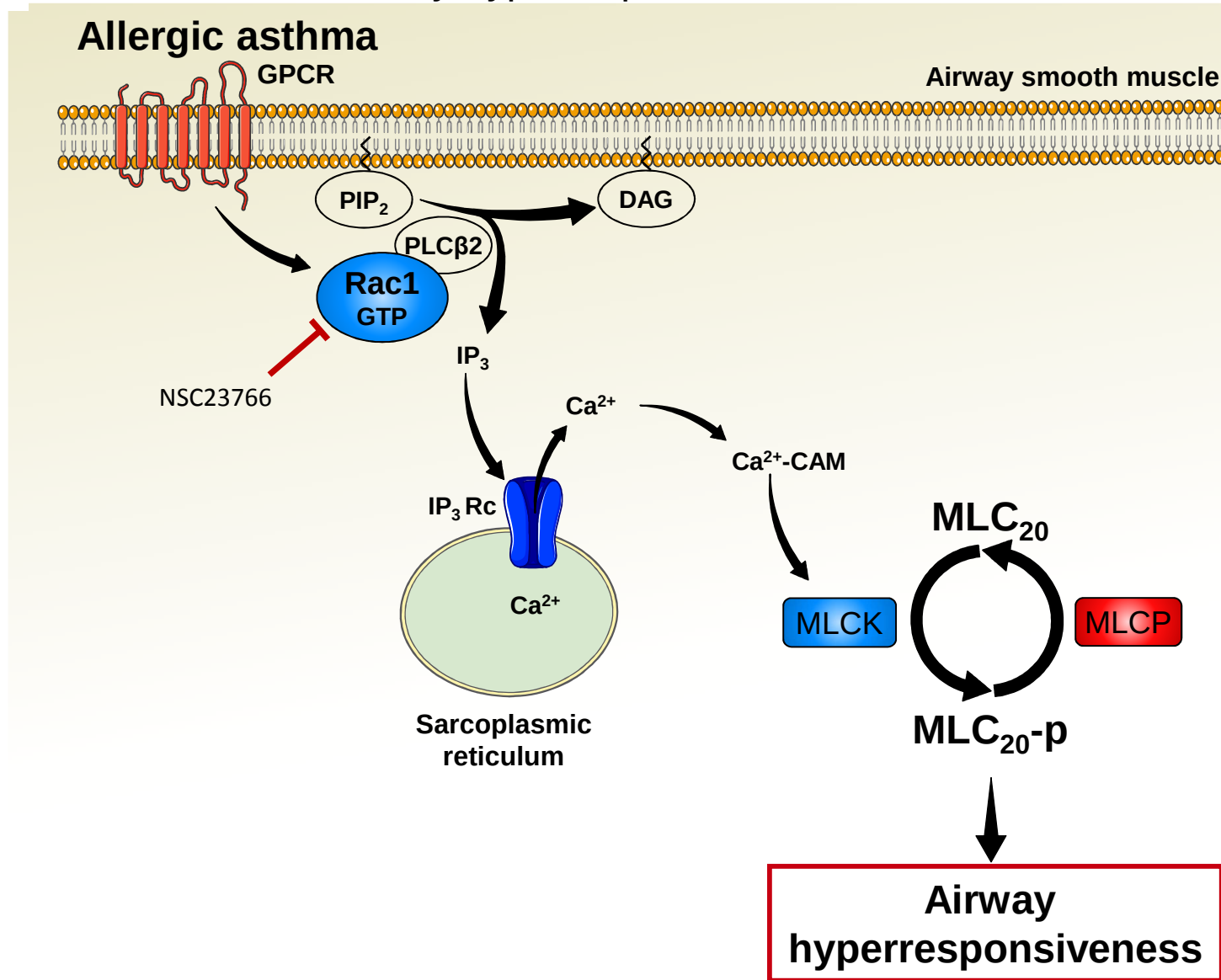
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Targeting of Rac1 prevents bronchoconstriction and airway hyperresponsiveness



Original article**Targeting of Rac1 prevents bronchoconstriction and airway hyperresponsiveness**

by

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ABSTRACT

Background: The molecular mechanisms responsible for airway smooth muscle cells (aSMC) contraction and proliferation in airway hyperresponsiveness (AHR) associated with asthma are still largely unknown. The small GTPases of the Rho family (RhoA, Rac1 and Cdc42) play a central role in SMC functions including migration, proliferation and contraction.

Objective: The objective of this study is to identify the role of Rac1 in aSMC contraction and to investigate its involvement in AHR associated with allergic asthma.

Methods: To define the role of Rac1 in aSMC, *ex-* and *in vitro* analyses of bronchial reactivity were performed on bronchi from smooth muscle (SM)-specific Rac1 knockout mice (SM-Rac1-KO) and human individuals. In addition, this murine model was exposed to allergens (ovalbumin or house dust mite extract) to decipher *in vivo* the implication of Rac1 in AHR.

Results: The specific SMC deletion or pharmacological inhibition of Rac1 in mice prevented the bronchoconstrictor response to methacholine. In human bronchi a similar role of Rac1 was observed during bronchoconstriction. We further demonstrated that Rac1 activation is responsible for bronchoconstrictor-induced increase in intracellular Ca^{2+} concentration and contraction both in murine and human bronchial aSMC, through its association with phospholipase C $\beta 2$ and the stimulation of inositol 1,4,5-trisphosphate production. *In vivo*, Rac1 deletion in SMC or pharmacological Rac1 inhibition by nebulization of NSC23766 prevented AHR in murine models of allergic asthma. Moreover, nebulization of NSC23766 decreased eosinophil and neutrophil populations in bronchoalveolar lavages from asthmatic mice.

Conclusion: Our data reveal an unexpected and essential role of Rac1 in the regulation of intracellular Ca^{2+} and contraction of aSMC, and the development of AHR. Rac1 thus appears

as an attractive therapeutic target in asthma, with a combined beneficial action on both bronchoconstriction and pulmonary inflammation.

“Capsule summary”

In this study, we unveiled an essential role of the protein Rac1 to initiate human and murine contraction of airway smooth muscle cells, and the development of airway hyperresponsiveness (AHR).

“Clinical Implications statements”

The nebulisation of Rac inhibitor prevents AHR in murine models of allergic asthma, with the additional advantage to also decrease pulmonary inflammation. Rac1 thus appears as a new attractive therapeutic target in asthma.

Key words: Rac1, airway smooth muscle, asthma, airway hyperresponsiveness, calcium, PLC, pulmonary inflammation.

Abbreviations/Acronyms :

AHR: airway hyperresponsiveness

aSMC: airway smooth muscle cells

CCh : carbachol

Der f : house dust mite extract-*Dermatophagoides farinae*

IP₃ : inositol 1,4,5-trisphosphate

OVA: ovalbumine

- 84 Pak1 : p21-activated kinase 1
- 85 PLC β 2: phospholipase C β 2
- 86 SM-Rac1-KO : smooth muscle cell-specific Rac1 knockout mice

ACCEPTED MANUSCRIPT

INTRODUCTION

Allergic asthma is a major public health problem characterized by inflammation and chronic bronchial obstruction associated with airflow alteration (1, 2). The major cause of morbidity and mortality from the disease is airway obstruction, that depends on both tissue remodeling (3, 4) and airways hyperresponsiveness (AHR) (5-7) due to excessive contraction of airway smooth muscle cell (aSMC) of the bronchial wall and inflammation (8). Indeed, AHR results from a complex crosstalk between immune and bronchial wall cells, driven by a network of cytokines, growth factors and bronchoconstrictors. The degree of AHR correlates with asthma severity and the need for therapy. Current treatments consist in inhaled combination of long acting beta-adrenergic receptor agonist for bronchodilation and anti-inflammatory corticosteroids. However, 5-10% of patients with severe asthma are refractory to these available medications and remain at high risk of serious morbidity and life-threatening asthma attacks (9).

As intracellular signalling molecules activated by extracellular stimuli, small GTPases of the Rho family (RhoA, Rac1 and Cdc42) act as molecular switches that regulate signalling cascades controlling SMC functions (10). Increasing evidence implicates the Rho protein Rac1 in the regulation of vascular SMC contraction (11, 12), and genetic deletion of Rac1 regulator (13) or effector (14) has been shown to modulate AHR in experimental allergic models. Furthermore, Rac1 has been identified last year as a potential link between pulmonary inflammation and bronchoconstriction (15).

Thus, we hypothesized that SM Rac1 could play an essential role in bronchoconstriction and during AHR associated with allergic asthma. To elucidate Rac1 functions in aSMC, we used a mouse model of SMC specific Rac1 deletion at adult stage (11). Notably, we observed that bronchi from these mice failed to develop contraction. We further demonstrate, in human and murine aSMC, that Rac1 is activated by bronchoconstrictor and plays a major role in the rise

in intracellular Ca^{2+} concentration through the control of PLC activity. In addition, we observed that inhibition of Rac1 activity prevents AHR-induced allergic asthma in mice, suggesting that Rac1 inhibition is a new therapeutic strategy against asthma.

METHODS

Mice. All experimental procedures and animal care were performed in accordance with the Regional Ethical Committee for Animal Experiments of the Pays de la Loire. C57Bl/6 *Rac1*^{lox/lox} and *SMMHC-Rac1*^{lox/lox} mice were obtained as previously described (11). *Rac1* deletion in smooth muscle cells was induced in 8-week-old *SMMHC-Rac1*^{lox/lox} males by intraperitoneal injection of tamoxifen (1 mg/day in sun-flower oil) for 5 consecutive days during two weeks. Tamoxifen-treated *Rac1*^{lox/lox} mice were used as control. Analyses were performed 1 month after tamoxifen treatment.

To validate *Rac1* deletion in airways smooth muscle cells, DNA *Rac1* status was analysed in trachea and liver by amplification of the wild type, floxed and deleted *Rac1* alleles using three primers simultaneously: Reverse: 5'-TCCAATCTGTGCTGCCCATC-3'; Forward1: 5'-CAGAGCTCGAATCCAGAACTAGTA-3'; and Forward2 5'-GATGCTTCTAGGGGTGAGCC-3'. All three primers were included in a single PCR and yielded PCR products of 115 bp (wild type), 140 bp (*Rac1* deleted), and 242 bp (*Rac1* floxed). Detection of *Rac1* mRNA was performed in tracheal and bronchial sections by *in situ* hybridization as described previously(11), using IHC of SM22a to detect SMC.

Human airways. Human bronchi were obtained from donor tissues. Tissues were collected in physiologic saline solution and used for primary airway SMC culture or airways reactivity *ex vivo*.

Cell culture. Primary airway SMC (aSMC) were isolated from primary bronchi of the indicated mouse strain or from human biopsies. Tissues were cleaned manually and the muscular layer was further digested 1h with collagenase II (1 mg/mL, Worthington) at 37°C under agitation. Cells grew up in DMEM (Gibco; Invitrogen) containing 10% foetal bovine serum, 100 units/mL penicillin and 100 µg/mL streptomycin at 37 °C and 5% CO₂. The culture medium was changed every 72 hours. All experiments were performed between

passages 1 and 2. Primary vascular smooth muscle cells (vSMC) were isolated and cultured as described previously (16).

Immunoblotting. Tissues or primary SMC in culture were incubated on ice with lysis buffer supplemented with proteases and phosphatases inhibitors cocktails (Sigma) and sodium orthovanadate. Lysates were subjected to SDS-PAGE, transferred to nitrocellulose membranes and incubated with specific antibodies. Rac1 antibody (610651) was from BD biosciences. PLC β 2 (129889) was from Abcam. MYPT1 (2634), p-MLC2 (3671), MLC2 (3672), p-Pak (2605), Pak1 (2602) antibodies were from Cell Signaling Technology. p-MYPT (sc17556) antibody was from Santa Cruz Biotechnology and tubulin from Beckman Coulter. Immune complexes were detected with appropriate secondary antibodies and enhanced chemiluminescence reagent (Clarity ECL BioRad). Protein band intensities were quantified using ImageJ Software (NIH software). Co-immunoprecipitation of PLC β 2 with Rac1 was carried out using anti-Rac1 or anti-PLC β 2 antibodies (1 μ g) incubated with Protein A magnetic beads 1h at 4°C according to the supplier's specifications (μ Macs Protein A/G Microbeads MultiMACS Protein A/G Kit, Miltenyi Biotec).

Analysis of Rac1 activity. In human or murine aSMC lysates, Rac1 activity was evaluated by active Rac immunoprecipitation using anti-Rac-GTP antibody (26903, NewEast Biosciences). The precipitated active Rac was subjected to SDS-PAGE and detected by immunoblot with anti-Rac1 antibody (BD biosciences). In pulmonary paraffin-embedded sections, Rac-GTP levels were detected by immunofluorescence. Sections were deparaffinized and permeabilized (PBS+0,1% Triton-X100) before incubation with anti-Rac-GTP antibody (1/500) overnight at 4°C. After three washes in PBS, sections were incubated 1h at room temperature with the secondary Alexa568-labeled anti-mouse antibody (1/1000).

Intracellular calcium measurements. Murine and human aSMC were seeded at 2.10^4 cells/well in 8-chamber borosilicate coverglass (Lab-Tek). *Rac1*^{lox/lox} cells were treated or not

with 4-hydroxy-tamoxifen (1 μ M, 48 h) to induce Rac1 deletion in SM-Cre positive cells (SMC-Rac1-KO). FluoForte probes were loaded using FluoForte calcium assay kit (Enzo Life Science) according to the supplier's specifications. When indicated, Rac1 inhibitor NSC23766 (10 μ M), IP3 receptor antagonist 2-ABP (10 μ M) and PLC inhibitor U73122 (10 μ M) were added 1 h before loading probes. After basal fluorescence recording, cells were stimulated with carbachol (30 μ M). The fluorescence was recorded using Metamorph software and quantified with ImageJ Software (NIH software).

IP3 assay. Murine and human aSMC were used to measure IP3 content after indicated treatments according to manufacturer's protocol (HitHunter® IP3 Fluorescence Polarization Assay, DiscoverX).

Airways reactivity ex vivo. Murine and human primary bronchi or murine trachea were cleaned, cut in rings and mounted on a multichannel isometric myograph in Krebs-Henseleit physiological solution (118.4 mM NaCl, 4.7 mM KCl, 2 mM CaCl₂, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃ and 11 mM glucose) at 37°C under oxygen. A pre-tension of 0.5 mN was applied. We constructed dose-response curves to methacholine (Sigma). When indicated, rings were pre-incubated 1h before contraction with PLC inhibitor U73122 (Tocris), IP3R inhibitor 2-ABP (Tocris) or PAK inhibitor IPA3 (Tocris) at 10 μ M. To inhibit Rac activity ex vivo, we observed that NSC23766 should be incubated 12h with bronchial rings. In contrast, the Rac inhibitor EHT1864 (Sigma) induced an efficient activity at 10⁻⁵M after 1h of incubation (Supplementary Fig. 2A). Thus, to limit incubation time, EHT1864 (Sigma) was selected to analyze airways reactivity ex vivo. The wire myograph was connected to a digital data recorder (MacLab/4e, AD Instruments) and recordings were analyzed using LabChart v7 software (AD Instruments).

Airways responsiveness measurement in vivo. Airway responsiveness was assessed in conscious, unrestrained mice using a barometric, whole-body plethysmography (EMKA

Technologies) by recording respiratory pressure curves in response to inhaled methacholine (Sigma) at concentrations of 0–40 mg/ml for 1 min. Airway responsiveness was expressed in enhanced pause (Penh) units. The Penh values measured after stimulation were averaged and expressed as the fold-increase over baseline values. Alternatively, dynamic airway resistance was measured using an invasive FlexiVent® apparatus (SCIREQ). In these analyses, mice were anesthetized (ketamine/xylazine), paralyzed (rocuronium bromide), tracheotomised and mechanically ventilated (150 breaths/min). Increasing concentrations of methacholine were administered via a nebulizer aerosol system (Aeroneb, Aerogen). When required, the Rac inhibitor NSC23766 (Tocris) and salbutamol were nebulised (300 µl at 5 mM) 10 min before methacholine challenge.

Respiratory parameters. Mice were placed in physiocages (Bioseb) to measure gas exchanges during 24h after a two-day acclimatizing period. The recording room was maintained with a 12-hour-light/12-hour-dark cycle.

Allergic asthma models. Allergic asthma was induced in mice using a total house dust mite extract (*Dermatophagoides Farinae*, Der f, from Stallergenes) as described previously(15). Briefly, mice were sensitized on days D0, D7, D14 and D21 by skin application of 500 µg Der f in 20 µL of dimethylsulfoxyde (DMSO, Sigma) onto the ears. Control mice were sensitized with DMSO. Intranasal challenges were performed with 250 µg of Der f in 40 µL of sterile PBS on D27 and D34. When indicated, allergic asthma was induced using ovalbumin (OVA) model. 10 µg OVA (A5503, Sigma) was administrated by intraperitoneal injections in alum vehicle (77161, Thermo scientific) on days D0, D14 and D21. Mice were then challenged through intranasal route with aerosolized OVA (50 µg in PBS) on D27, D28 and D29. Control mice were sensitized with PBS/alum. When indicated, allergic asthma mice were treated by repeated inhalations of NSC23766 or salbutamol (5 mM in PBS) before each

challenge and methacholine nebulization. All mice were sacrificed on 24h after last intranasal challenge for analysis.

Bronchoalveolar lavage fluid analysis. Mice were tracheotomised and 1 ml of sterile PBS was administrated intratracheally through a catheter. Cells and supernatants from recovered fluid were separated by centrifugation. Total cell number was count on Kova slides by optical microscopy. Identification of immune cells subpopulations were performed by flow cytometry analysis (15). Acquisition was performed on LSR II (BD Bioscience) and analyzed with FlowJo software.

Histology. Paraformaldehyde (4% in PBS, 1 mL) was administered intratracheally in the lungs through a flexible catheter, trachea was ligatured and lungs excised. Lungs were fixed in 4% paraformaldehyde for 48h and embedded into paraffin. 6 µm sections were stained with periodic acid-Schiff or hematoxylin/eosin for morphological studies. Histological grade was determined to assess inflammation and pulmonary remodelling (15).

Statistics. All data are expressed as the mean $\pm$ SEM of sample size n. For multiple comparisons, the non-parametric Kruskal-Wallis test was used followed by Dunns' post-test. For individual comparisons, statistical analysis was performed using non-parametric *t*-test (Mann-Whitney). Data analysis was performed using the GraphPad Prism software. The threshold for statistical significance was set at $P < 0.05$.

RESULTS

Inhibition of Rac1 expression or activity prevents bronchoconstriction in human and mice airways.

To elucidate the specific functions of Rac1 in SMC, we generated tamoxifen-inducible and SMC-specific Rac1 knockout mice (SM-Rac1-KO) (11). *In situ* hybridization and western-blot analysis confirmed the loss of Rac1 mRNA and protein expression in aSMC, which did not affect normal respiratory function under basal condition (Supplementary Fig.1). We first studied the functional impact of Rac1 deletion in aSMC by measuring the contractile response of bronchial rings from control (*Rac1*^{lox/lox}) and SM-Rac1-KO mice. The maximal contraction induced by the muscarinic receptor agonist methacholine was reduced by ~50% in bronchial rings from SM-Rac1-KO mice compared to *Rac1*^{lox/lox} mice (Fig.1A). This inhibitory effect of SMC Rac1 deletion on methacholine-induced bronchoconstriction was mimicked by the pharmacological Rac inhibitors (EHT1864 and NSC23766) in bronchial rings from control mice and humans (Fig. 1A and Supplementary Fig. 2A). Similarly, the bronchoconstriction induced by serotonin was reduced by ~70% in murine bronchial rings incubated with EHT1864 (Supplementary Fig. 2B).

To confirm *in vivo* the role of Rac1 in bronchoconstriction, we next measured the pulmonary resistance in control and SM-Rac1-KO mice. Although the baseline resistance was similar in the two groups, aerosolized methacholine-induced increase in pulmonary resistance was significantly smaller in SM-Rac1-KO mice than in control mice (Fig.1B). Consistent with this result, inhalation of the Rac inhibitor NSC23766 in control mice decreased methacholine-induced bronchoconstriction with an efficacy similar to salbutamol, a reference bronchodilator used by asthmatic patients (Fig.1B). In contrast, inhalation of NSC23766 in

SMC-Rac1-KO mice had no effect on methacholine-induced bronchoconstriction (data not shown), demonstrating the specific inhibition of NSC23766 on Rac activity. These results suggest that Rac1 plays a major role in aSMC contraction *in vivo*.

Bronchoconstrictors stimulate Rac1/PLC β 2 association to modulate intracellular Ca²⁺ concentration in aSMC. We next sought to identify the mechanism by which Rac1 controlled aSMC contraction by first analysing the activity of Rac1 and its main effector, p21-activated kinase 1 (Pak1). Stimulation of murine and human aSMC with the muscarinic cholinergic receptor agonist carbachol (CCh) increased Rac1 and Pak1 activity (Fig. 1C). NSC23766 treatment prevented Pak1 activation, indicating that muscarinic receptor stimulation turns on Rac1 and its downstream effector Pak1 in aSMC. The Pak inhibitor IPA3 had no significant effect on muscarinic receptor stimulation-induced contraction of mouse bronchial rings and only a weak inhibitory effect on tracheal rings (Fig.1D), as previously described (14). However, both bronchial and tracheal contractions were similarly reduced by pharmacological Rac1 inhibition (Fig. 1D), suggesting that the effect of Rac1 in bronchoconstriction is independent of Pak1.

We next examined the status of two essential signalling pathways involved in SMC contraction: Ca²⁺-sensitization and Ca²⁺ signalling pathways (10). Stimulation of murine and human aSMC by CCh, in the absence or in the presence of Rac1 inhibitor, did not significantly affect the level of phosphorylated myosin phosphatase target subunit (MYPT), suggesting that the activity of the myosin light chain phosphatase was not modified and the Ca²⁺-sensitization pathway not activated (Fig. 1C). In contrast, the CCh-induced rise in intracellular Ca²⁺ in control murine and human aSMC was abolished by Rac1 deletion or inhibition (Fig. 2A), suggesting that Rac1 regulates bronchial contraction by controlling intracellular Ca²⁺ signalling.

Activation of phospholipase C (PLC) β , production of inositol 1,4,5-trisphosphate (IP₃) and opening of IP₃ receptor-Ca²⁺ release channels of the sarcoplasmic reticulum are the main mechanisms activated by G-protein-coupled receptor agonists to induce cytosolic Ca²⁺ increase in SMC (17). To understand how Rac1 regulates intracellular Ca²⁺, we thus used inhibitors of PLC (U73122) and IP₃ receptor (2-ABP). Both U73122 and 2-ABP prevented muscarinic receptor activation-induced intracellular Ca²⁺ rise in aSMC and constriction of bronchial rings from control mice, but had no effect in aSMC and bronchial rings from SMC-Rac1-KO mice (Fig. 2B and Supplementary Fig. 2B). Consistent with this observation, we found that CCh promoted production of IP₃ in murine and human aSMC that was abolished by the PLC inhibition (U73122), and in a similar extend, by the Rac inhibitor NSC23766 (Fig. 2C). Interestingly, we observed that 5HT-dependent bronchoconstriction is also sensitive to inhibitors of PLC (U73122) and IP₃ receptor (2-ABP) (Supp. Fig. 2C). These results unveil a key role of Rac1 activity on the PLC/IP₃ signalling coupling bronchoconstrictor receptor to cytosolic Ca²⁺ rise in aSMC and contraction.

In recombinant systems and *in vitro* assays, active Rac1, 2 and 3 have been shown to interact with PLC β 2 isoform (18-20). While PLC β 2 expression was weak in murine vascular SMC, aSMC highly expressed the PLC β 2 isoform and CCh stimulation increased its association with Rac1 (Fig. 2D, E). This effect was blocked by NSC23766 suggesting that Rac1-PLC β 2 association required Rac1 activation (Fig. 2E). To confirm these results, we transiently expressed wild type Rac1 (Rac1^{WT}), a constitutively active (Rac1^{V12}) or a negative (Rac1^{N17}) Rac1 mutant in 3T3 cells and assessed their interaction with PLC β 2 (Figure 2F). The amount of Rac1^{WT} immunoprecipitated with PLC β 2 was increased by CCh stimulation. The Rac1^{V12} mutant was constitutively associated with PLC β 2 and CCh did not increase this association. By contrast, there was no interaction between PLC β 2 and Rac1^{N17} both in the absence or presence of CCh. These results demonstrate for the first time the association of endogenous

PLC β 2 with active Rac1 and its critical role in inducing IP₃ production, cytosolic Ca²⁺ rise and aSMC contraction in response to bronchoconstrictor.

Inhibition of Rac1 expression or activity protects from airway hyper-responsiveness.

We thus hypothesized that Rac1 could be causally involved in the excessive pathological bronchoconstriction associated with asthma. To assess this hypothesis, we first measured airway Rac1 activity in a mouse model of human allergic asthma, induced by percutaneous sensitization and intranasal challenge with house dust mite extract-*Dermatophagoides farinae* (Der f) (15). Exposure to Der f resulted in an increase in Rac1 activity in bronchi and peribronchial inflammatory cells that was inhibited by repeated NSC23766 nebulizations (Fig. 3A). Der f sensitization induced AHR that was prevented by acute NSC23766 nebulization (Fig. 3B) and SMC Rac1 deletion (Fig. 3C), suggesting a role of Rac1 activation in AHR. *Ex vivo* measurements of tension response to methacholine demonstrated the increased contraction of bronchial rings from Der f sensitized-mice compared to naive animals, and the inhibitory action of the Rac inhibitor EHT1864 confirmed that allergic asthma-associated excessive bronchoconstriction and AHR is dependent on Rac1 activity (Fig. 3D).

Rac inhibitor inhalation prevents pulmonary inflammation in murine allergic asthma models.

Histological analysis of lung sections showed that Der f-induced AHR was associated with massive mucus production and strong cellular infiltration of the airways that were similar in control and SMC-Rac1-KO mice (Fig. 4). These results indicate that aSMC Rac1 is not involved in lung inflammation associated to allergic asthma. However, we observed that repeated NSC23766 inhalations not only prevented AHR, but also reduced inflammatory cell infiltration and airway remodelling in Der f-sensitized mice (Fig. 5). Rac inhibition in Der f-sensitized mice decreased eosinophil and neutrophil influx in bronchoalveolar lavage (Fig.

5C). We observed the similar combined bronchodilator and anti-inflammatory effect of repeated NSC23766 treatment in ovalbumine (OVA)-induced asthmatic mice (Sup Fig. 3). The beneficial effect of NSC23766 on OVA-induced AHR was similar to that obtained with the reference bronchodilator salbutamol (Supplementary Fig. 3A) which had no action on inflammation (Supplementary Fig. 3D).

DISCUSSION

Taken together, our results greatly extend our understanding on the role of Rac1 in the control of murine and human aSMC contraction and have direct translational implications in asthma treatment. Our data establish that Rac1, acting through PLC β 2, links bronchoconstrictor G-protein coupled receptors to intracellular Ca²⁺ signaling and contraction. By a screen of 17 members of the Rho protein family in recombinant systems in COS-7 cells, only the active forms of Rac (Rac1, Rac2, and Rac3) have been identified to be able to bind PLC β 2 and stimulate PLC β activity, thus defining PLC β 2 as a Rac1 effector (19). This direct interaction between active GTP-bound Rac1 and PLC β 2 has been confirmed by the description of the crystal structure of the complex, demonstrating that Rac1 engages the pleckstrin-homology (PH) domain of PLC β 2 to optimize its orientation for substrate membranes (20). However, until now, the existence of such interaction of native proteins in endogenous systems and its role in the regulation of biological/physiological/pathophysiological processes have never been described. Here, we demonstrate that stimulation of murine and human muscarinic cholinergic receptor in aSMC increased Rac1 activity and promoted its association with PLC β 2. Activation of Rac1 leads to its translocation from the cytosol to the plasma membrane (21). Interestingly, a direct interaction between muscarinic receptor and PLC β leading to a close spatial proximity to both its substrate, PIP2, and its activator, G α q, has been recently described in CHO cells (22). This observation thus suggests that muscarinic receptor stimulation induces Rac1 activation and its translocation at the plasma membrane promoting Rac1/PLC β 2 interaction. Our results further show that this protein interaction is necessary to potentiate IP₃ production, and consequently intracellular Ca²⁺ rise and contraction in murine and human aSMC smooth muscle cell.

Studies focused on the role of Rac1 in SMC contraction by the use of pharmacological tools and genetic mouse models have produced conflicting data, reporting relaxing and contracting effects of Rac1 activity. Rahman et al. observed that pharmacological inhibition of Rac1 reduced or potentiated the contraction in different visceral SM tissues (urinary bladder and ileum) and arteries, depending on the contracting agonist used (23). For example, Rac inhibition by NSC23766 in mouse saphenous artery had no effect on KCl-induced contraction, decreased the contractile response to phenylephrine and potentiated the contraction induced by thromboxan A2 receptor stimulation (23). However, the specific depletion of Rac1 expression in vascular SMC demonstrates that the activation of Rac1 promotes vasorelaxation by positively regulating cyclic GMP-dependent signalling (11, 12). Inconsistent observations have also been reported in airway SMC since relaxing properties have been ascribed to Rac1 activity in guinea pig trachea (24), while activation of Pak1, the main effector of Rac1, has been associated with contraction of murine tracheal segments and human bronchial rings (14). These results suggest that the regulation of Rac1 activity by contracting agonists, its downstream target effectors, and consequently its functions in the regulation of SMC contraction, could be different according to SMC type and/or species. Different pattern of expression of intracellular signalling molecules in different SMC might support, at least in part, the inconsistent role of Rac1 in the regulation of SMC contraction. The essential role of PLC β 2 unveiled by our study fits with this hypothesis. We demonstrated that the connection between Rac1 and the PLC β 2/ Ca^{2+} signaling is responsible for the role of Rac1 in human and murine bronchial smooth muscle contraction. Accordingly, this new signaling pathway is expected to be essential particularly in SMC that strongly express the PLC β 2 isoform, as in human aSMC.

In addition to its role in aSMC contraction, we demonstrate that Rac1 is activated in airways from murine model of asthma. Deletion of the Rac1 gene in SM or pharmacological Rac1

inhibition prevented AHR, highlighting the potential of targeting Rac1 in asthma in humans. We observe that Rac inhibitors decreased basal pulmonary function (Fig. 1B) and airway contractility (Fig. 3D) but do not affect airway resistance (Fig. 3B). This apparent discrepancy is probably due to the experimental procedure. Indeed, the analysis of respiratory function was performed in conscious mice, while mice were anesthetized, paralyzed, tracheotomized and mechanically ventilated to evaluate airway resistance. All these procedures could alter airway reactivity and mask NSC23766 and EHT1864 effects.

Long-acting muscarinic antagonists (LAMAs) have been shown to produce beneficial effects on airway tone, smooth muscle contraction, mucus secretion, and vasodilation (25, 26), and on patients with symptomatic asthma (27-29). As we found that muscarinic receptor stimulation induced Rac1 activation, these positive actions of LAMAs could result at, least in part, from a reduced Rac1 activation and Rac1-mediated effects. However, despite the availability of therapies combining inhaled corticosteroids to reduce inflammation and long-acting β_2 -receptor agonist or LAMAs to limit AHR, asthma control remains suboptimal for many patients that are resistant to these treatments (30). Interestingly, inhalation of Rac inhibitors not only prevented AHR by inhibiting SMC Rac1, but also decreased pulmonary inflammation, probably by blocking Rac activity in immune/inflammatory cells. In fact, activation of Rac in leukocytes has been described to be involved in respiratory inflammation (13, 15, 31), and it is well-established that allergic asthma-associated airway inflammation is involved in AHR (15, 32).

Rac has also been reported to control epithelial barrier function and, although specific deletion of Rac1 in the epithelial cells did not affect the integrity of the airway epithelium *in vivo* in basal condition, it exacerbated inflammation and augmented Th2 cytokines in asthma models induced by house dust mite extract or ovalbumin as allergens (33). This effect has been ascribed to defective clearance of apoptotic cells by Rac1-deficient epithelial cells. One

would thus expect that Rac inhibitors, which inhibit Rac1 in all cell types, including bronchial epithelial cells, would also produce an aggravation of allergic inflammation. In fact, we did not observe such increase in allergic airway inflammation after treatment of allergic mice with pharmacological Rac1 inhibitors, which by contrast, reduced lung inflammation. However, our result is in agreement with a protective role of Rac inhibitors already described in pulmonary inflammation and asthma (13, 34).

In summary, our results in mice and human tissues reveal a new and fundamental role of Rac1 to promote aSMC contraction and AHR. Rac1 inhibition simultaneously prevents both AHR and pulmonary inflammation offering the opportunity to aggregate in a single molecule the desired anti-inflammatory and bronchodilatory actions of anti-asthma therapy. The development of Rac inhibitors could thus open up a new avenue for the treatment of pulmonary pathologies characterized by AHR and inflammation.

FIGURE LEGENDS

Figure 1: Rac1 controls human and murine aSMC contraction. (A) Contractile responses to methacholine in bronchi from control (*Rac1*^{lox/lox}), SMC-Rac1-KO mice (n=5-7) and humans (n=5). When indicated, human and murine bronchial rings were pre-treated with Rac1 inhibitor (EHT1864) before methacholine stimulation (n=5-7). (B) Analysis of airways reactivity to methacholine by a non-invasive plethysmography approach in *Rac1*^{lox/lox} and SMC-Rac1-KO mice (n=12) (left panel). Control mice were treated or not with NSC23766 or salbutamol (n=10) (right panel). A.U, arbitrary units. (C) Immunoblot analysis and quantification of Rac-GTP, Pak and MYPT expression and phosphorylation at different time of carbachol (CCh) stimulation in murine aSMC (upper panel) and after 5 min of CCh stimulation in human aSMC pre-incubated or not with NSC23766 for 1 h (lower panel). Corresponding quantifications are displayed on the right of the blots (n=3). (D) Contractile responses to methacholine in bronchi (upper panel) and trachea (lower panel) from *Rac1*^{lox/lox} mice (n=6-8). When indicated, rings were pre-treated with the Pak1 inhibitor IPA3 or the Rac inhibitor EHT1864. Data are expressed as mean $\pm$ SEM. **P*<0.05 and ***P*<0.01 versus control, ^{\$}*P*<0.05 vs CCh.

Figure 2: Rac1 interacts with PLC β 2 to modulate IP₃ production and intracellular Ca²⁺ concentration in aSMC. Representative images and quantification of intracellular Ca²⁺ level in aSMC- *Rac1*^{lox/lox}, aSMC-Rac1-KO and human aSMC loaded with the calcium probe FluoForte (scale bar: 50 μ m). Cells were pre-incubated for 1 h with NSC23766 (A), U73122 or 2-ABP (B). For each condition, pick of calcium was quantified at least in 25 cells (n=3-5 independent experiments). (C) Measurement of CCh-induced IP₃ production in murine (left panel) and human (right panel) aSMC pre-treated or not with Rac or PLC inhibitors (n=6). (D) Western blot analysis of PLC β 2 expression in human aSMC (HaSMC), vascular (vSMC)

and aSMC from control or SMC-Rac1-KO mice. **(E)** Co-immunoprecipitation of endogenous PLC β 2 with Rac1 in mouse aSMC under resting condition or stimulated with CCh in the absence or presence of NSC23766 (n=3). **(F)** Co-immunoprecipitation of endogenous PLC β 2 with the indicated Rac1 mutants expressed in 3T3 cells stimulated or not with CCh (n=3). TCL, total cell lysate. IP, immunoprecipitate. Data are expressed as mean $\pm$ SEM, * P <0.05, ** P <0.01 and *** P <0.001 versus control; $^{\$}$ P <0.05 and $^{\$\$}$ P <0.01 versus CCh-treated cells.

Figure 3: Airway hyperresponsiveness is dependent of Rac1 activity in murine allergic asthma model. **(A)** Representative images of Rac1-GTP immunofluorescence in lung sections from naive (DP) and Der f-sensitized (Der f) mice treated with NSC23766 or vehicle (NaCl) nebulization (n = 5-7). Nuclei were detected by DAPI staining. Scale bar, 100 μ m. **(B)** Analysis of airway reactivity to methacholine challenges by invasive methods (FlexiVENT) in naive (DP) and Der f-challenged mice (Der f) treated with NSC23766 or vehicle (NaCl) nebulization (n=5-8). Data are expressed as mean $\pm$ SEM. * P <0.05 versus DP NaCl; $^{\$}$ P <0.05 versus Der f NaCl. **(C)** Analysis of airway reactivity to methacholine challenges by invasive methods (FlexiVENT) in control Rac1^{lox/lox} and SMC-Rac1-KO mice challenged with Der f (n=5-8). Data are expressed as mean $\pm$ SEM; * P <0.05 versus control. **(D)** Contractile responses to methacholine in bronchi rings from naive (DP) and Der f-sensitized mice (Der f) (n=6) pre-incubated with the Rac1 inhibitor EHT1864. Data are expressed as mean $\pm$ SEM. * P <0.05 versus DP NaCl mice; $^{\$}$ P <0.05 versus Der f NaCl.

Figure 4: SM-Rac1 deletion has no effect on pulmonary remodelling associated to allergic asthma. **(A)** Hematoxylin/eosin (HE) and periodic acid-Schiff (PAS) staining of lung sections from naive (DP) and Der f-sensitized (Der f) mice of the indicated genotypes. Scale

bars, 100 μ m. Sections are representative of 4–6 mice of each genotype. **(B)** Histological grade evaluated on lung sections from naive and Der f-sensitized mice of the indicated genotypes. **(C)** Infiltrating cell concentration in BAL fluid from *Rac1*^{lox/lox} and SMC-*Rac1*-KO sensitized to Der f (n=6). Data are expressed as mean $\pm$ SEM. ** P <0.01 versus *Rac1*^{lox/lox} DP NaCl mice.

Figure 5: NSC23766 inhalations reduces pulmonary remodelling associated to allergic asthma. **(A)** Hematoxylin/eosin (HE) and periodic acid-Schiff (PAS) staining of lung sections from Der f-sensitized control mice treated with repeated NSC23766 or vehicle (NaCl) nebulizations. Scale bars, 100 μ m. Sections are representative of 5-7 lungs for each experimental condition. **(B)** Histological grades evaluated on lung sections from naive and Der f-sensitized control mice treated with NSC23766 or NaCl (n=6). **(C)** Infiltrating cells in BAL fluid of naïve (DP) and Der f-sensitized mice (Der f) submitted to repeated NSC23766, salbutamol or NaCl treatments (n=5 in each experimental group). Data are expressed as mean $\pm$ SEM. ** P <0.01 and *** P <0.001 versus DP NaCl mice; $^{\$}$ P <0.05 and $^{\$}$ P <0.01 versus Der f NaCl.

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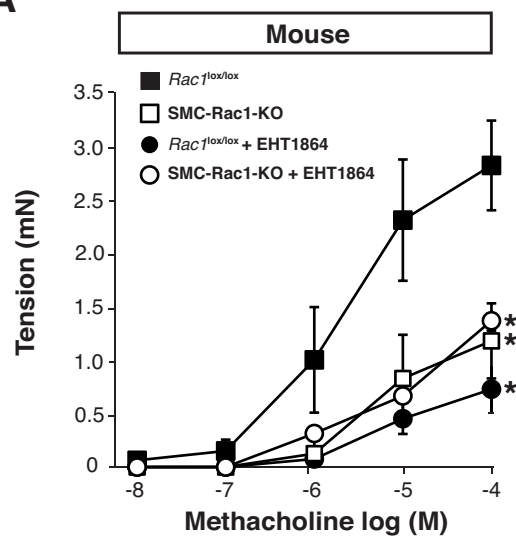
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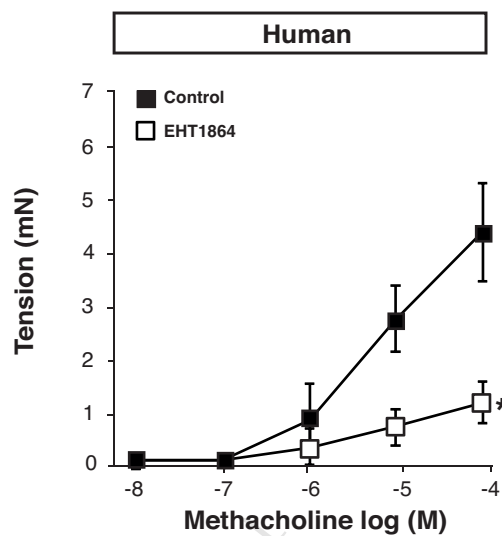
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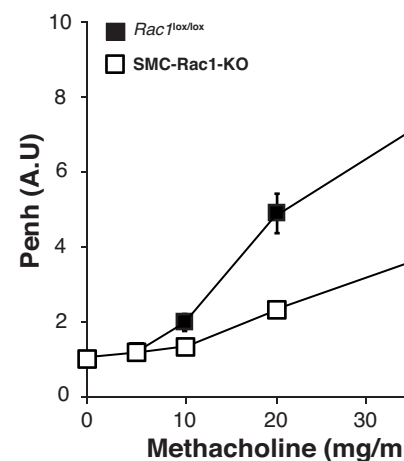
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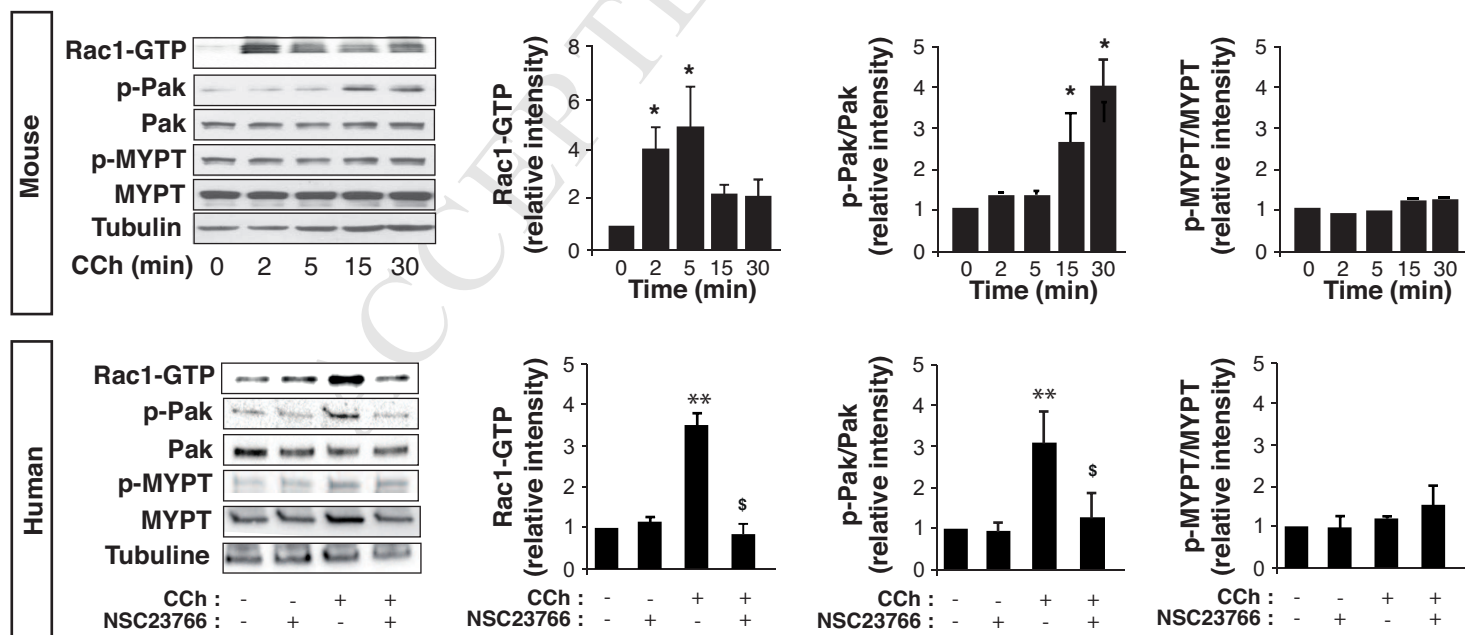
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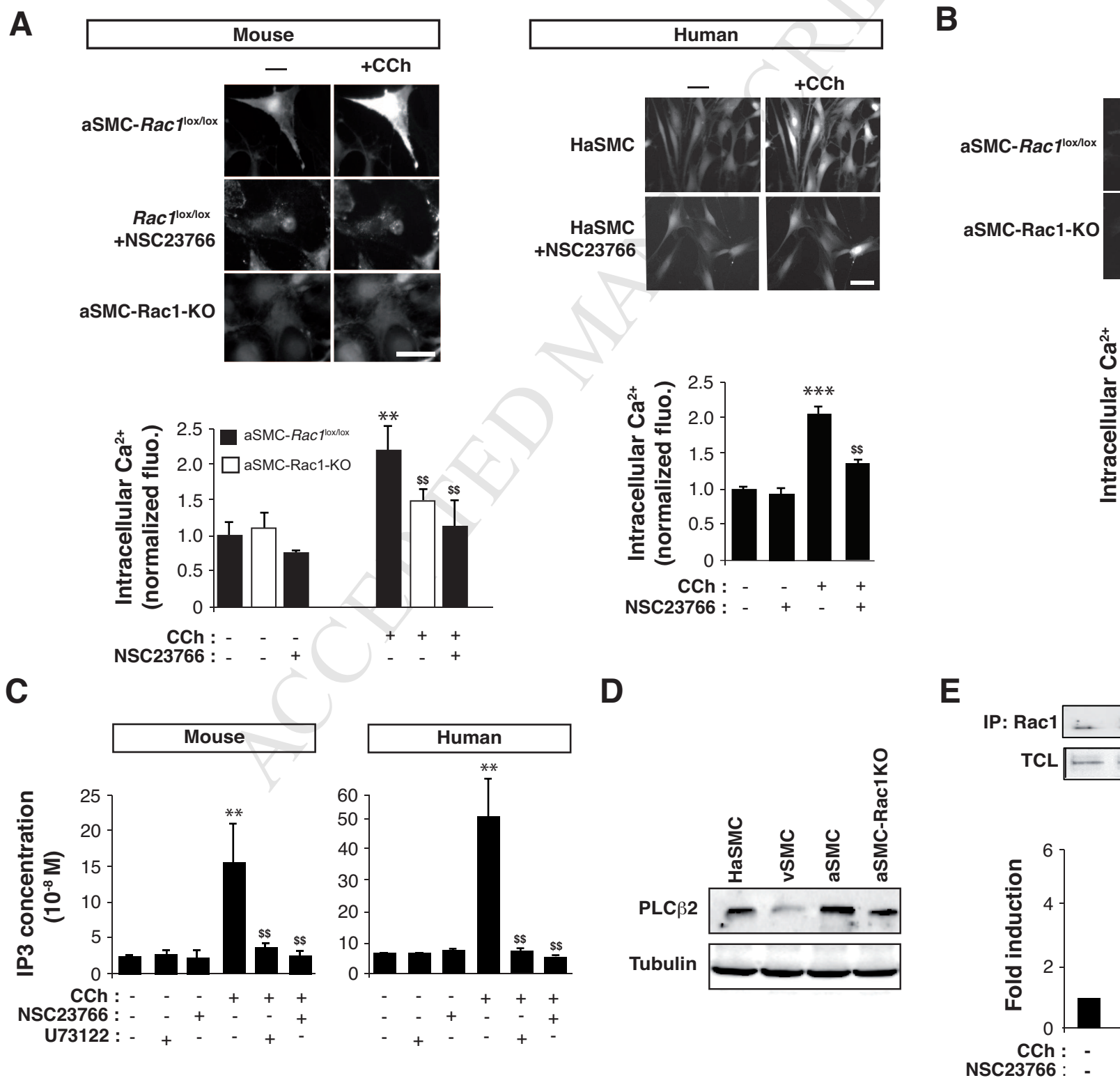


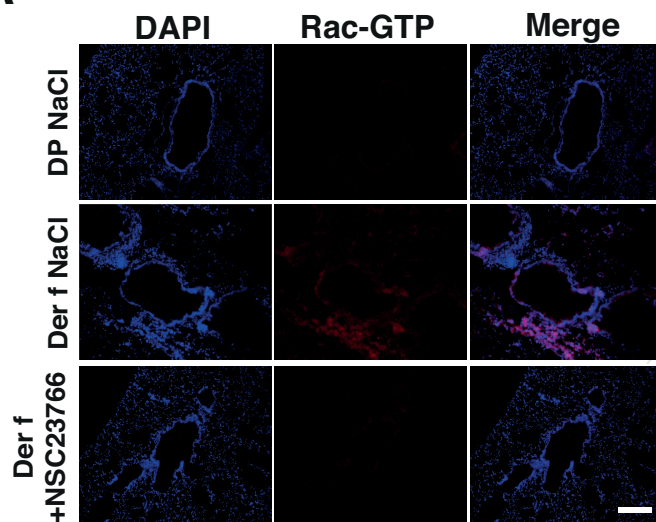
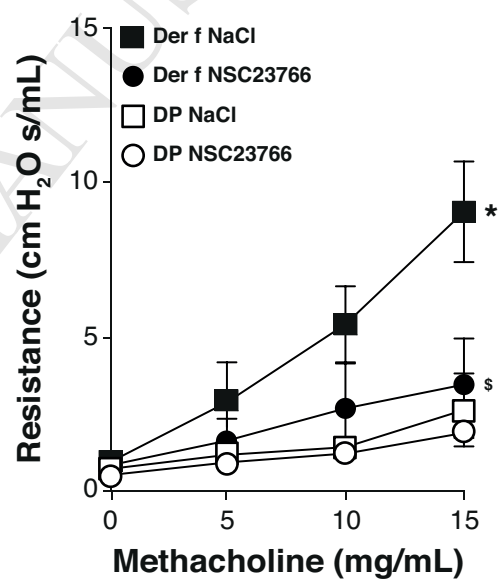
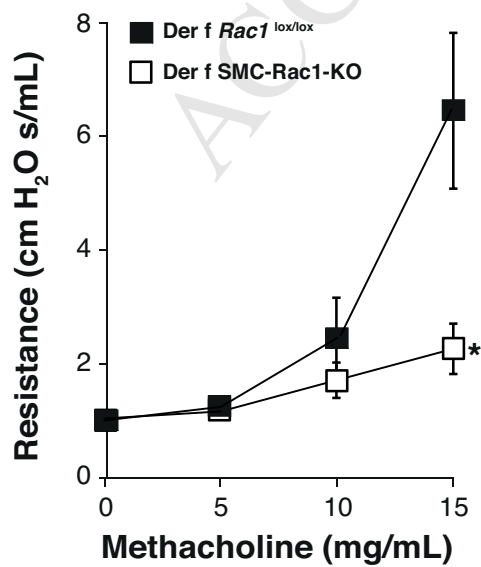
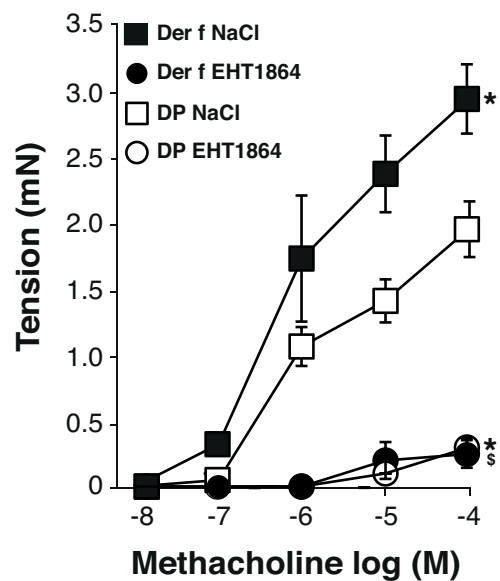
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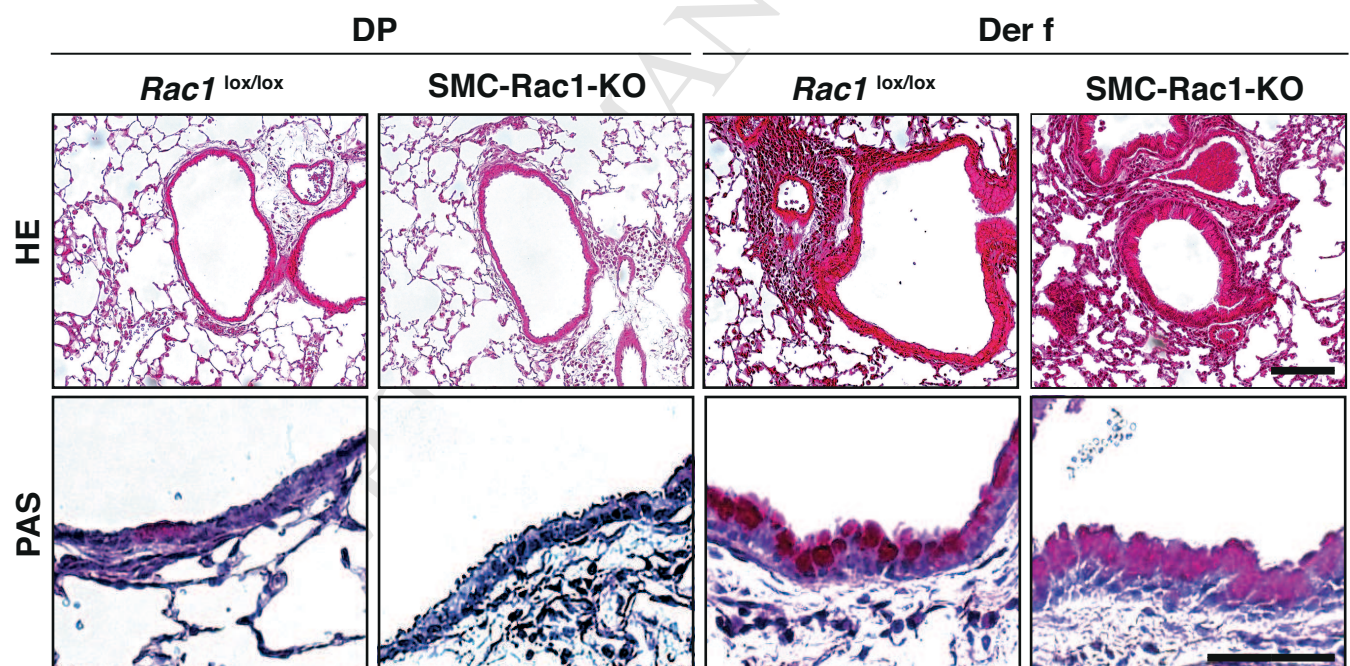
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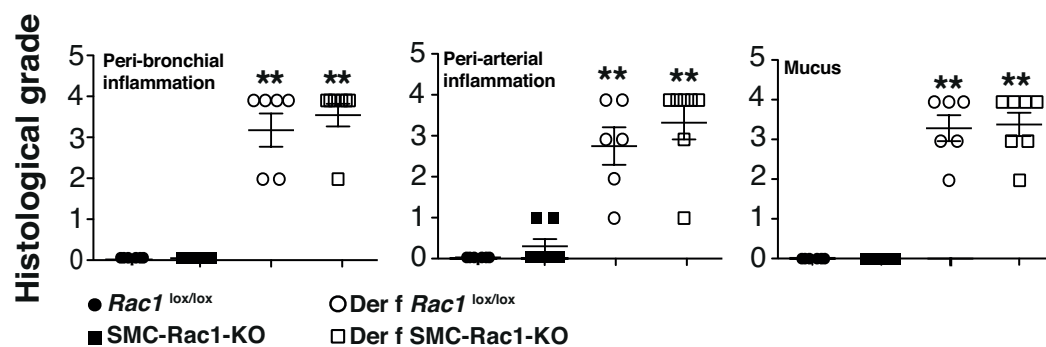


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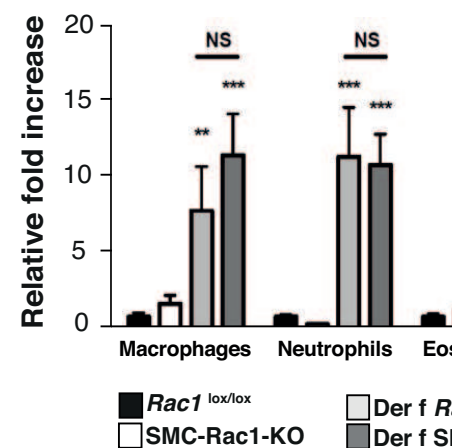
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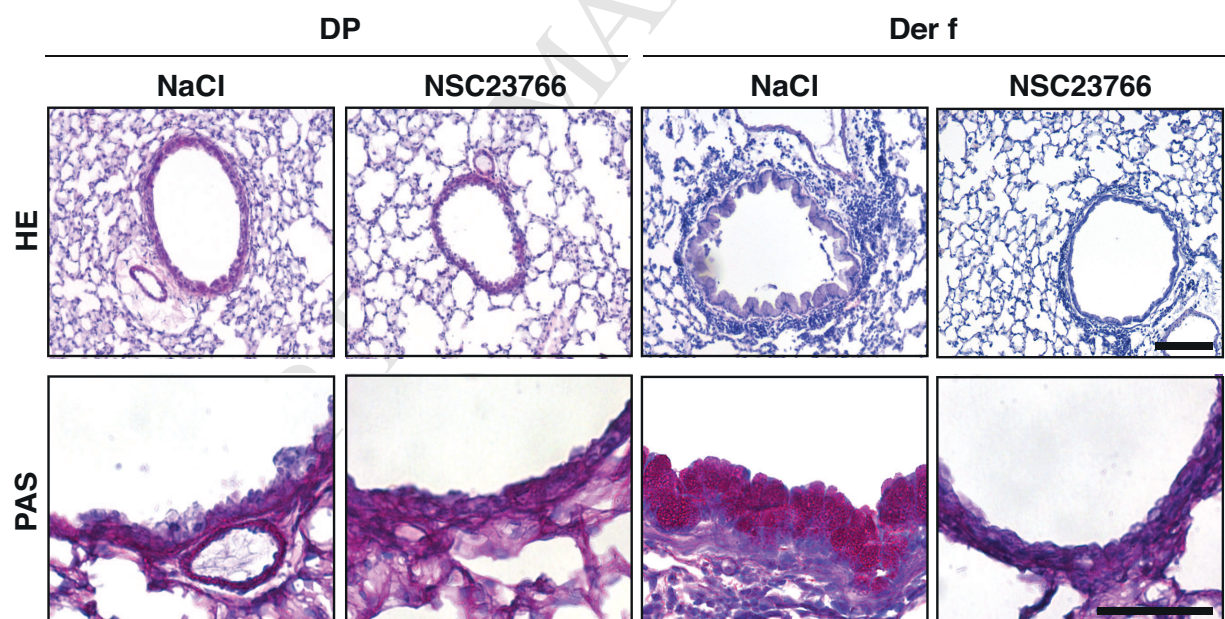
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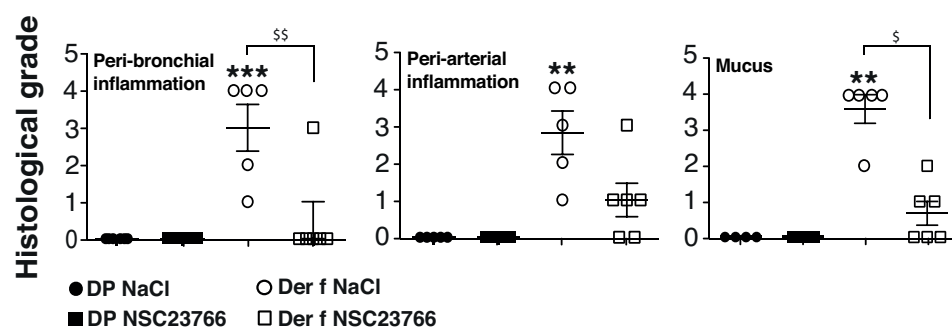
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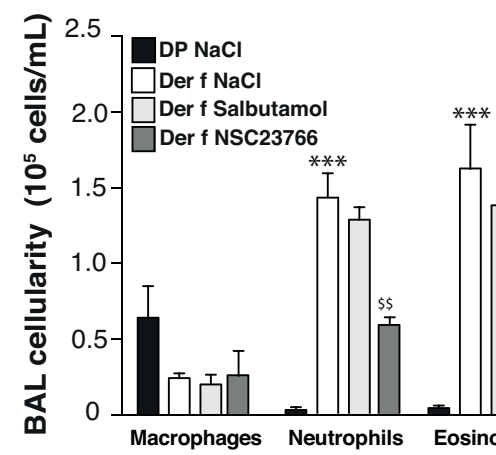
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Targeting of Rac1 prevents bronchoconstriction and airway hyperresponsiveness

by

Gwennan André-Grégoire^{1,2*}, Florian Dilasser^{1,2*}, Julie Chesné^{1,2}, Faouzi Braza^{1,2}, Antoine Magnan^{1,2,3}, Gervaise Loirand^{1,2,3†} and Vincent Sauzeau^{1,2,3†}

SUPPLEMENTARY INFORMATIONS**LEGENDS TO SUPPLEMENTARY FIGURES**

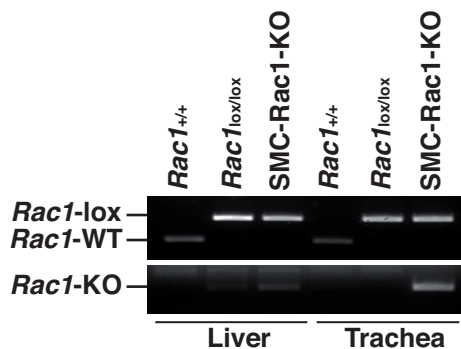
Supplementary Figure 1: Validation of Rac1 deletion in airway SMC and assessment of airway function in SM-Rac1-KO mice. (A) Genomic DNA status detected by PCR and **(B)** immunoblot analysis of Rac1 protein expression in tracheas from the indicated mice after tamoxifen treatment (n=4). Data are expressed as mean $\pm$ SEM. *** $P < 0.001$ versus SMC-*Rac1*^{lox/lox} mice. **(C)** Hematoxylin/eosin (HE) staining, immunohistochemical detection (IHC) of SM22a (SMC labelling) and *in situ* hybridization (ISH) analysis of *Rac1* mRNA expression in trachea (left panels) and bronchi (right panels) sections. Scale bars HE- 400 μ m, IHC/ISH- 40 μ m. **(D)** Respiratory function assessed by gas exchanges measurement in metabolic cages in *Rac1*^{lox/lox} and SMC-Rac1-KO mice (n=6). **(E)** Immunoblot analysis of indicated contractile protein expression and phosphorylation in *Rac1*^{lox/lox} and SMC-Rac1-KO bronchis (n=4).

Supplementary Figure 2: Rac1/PLC β 2/IP3 signalling pathway is involved in bronchoconstriction. (A) Contractile responses to methacholine in bronchi pre-incubated with Rac inhibitors, NSC23766 (12h) or EHT1864 (1h). **(B)** Contractile responses to

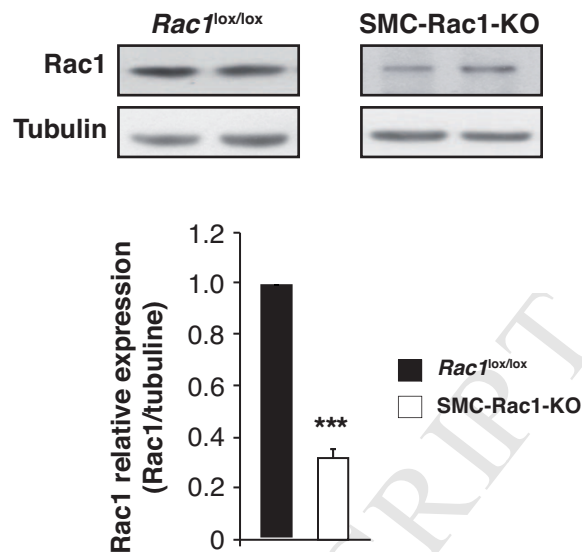
methacholine in bronchi from *Rac1*^{lox/lox} (left panel) and SMC-Rac1-KO (right panel) mice pre-treated or not with the PLC inhibitor U73122 or the IP₃ receptor inhibitor 2-ABP (n=4-6). Data are expressed as mean ± SEM. **P*<0.05 versus *Rac1*^{lox/lox}.

Supplementary Figure 3: Inhibition of Rac1 activity prevents airway hyper-responsiveness and pulmonary inflammation in OVA-sensitized mice. (A) Analysis of airway reactivity to methacholine challenges by non-invasive plethysmography in mice (n=9-10) challenged with ovalbumine (OVA) (o/o) or alum vehicle (ap/ap). When indicated, mice were treated with either salbutamol, the Rac inhibitor NSC23766, or the vehicle NaCl. **(B)** Airway reactivity to methacholine measured by FlexiVENT in naive and OVA-challenged mice pre-treated with NSC23766 or vehicle (NaCl) (n=5-6). **(C)** Histological sections and grades of lungs from naive (ap/ap) and OVA-sensitized (o/o) mice treated by repeated inhalations of NSC2366 or vehicle (NaCl). Sections are representative of 5–6 lungs in each experimental group. Upper panels, hematoxylin/eosin (HE) staining. Lower panels, periodic acid-Schiff (PAS) staining. Scale bars, 100 µm. **(D)** Infiltrating cells in BAL fluid of naive (ap/ap) and OVA-sensitized mice (o/o) submitted to NSC23766, salbultamol or NaCL (vehicle) treatments (n=5-6 in each experimental group). Data are expressed as mean ± SEM. **P*<0.05 and ***P*<0.01 versus ap/ap NaCl, ^{\$}*P*<0.05 versus o/o NaCl.

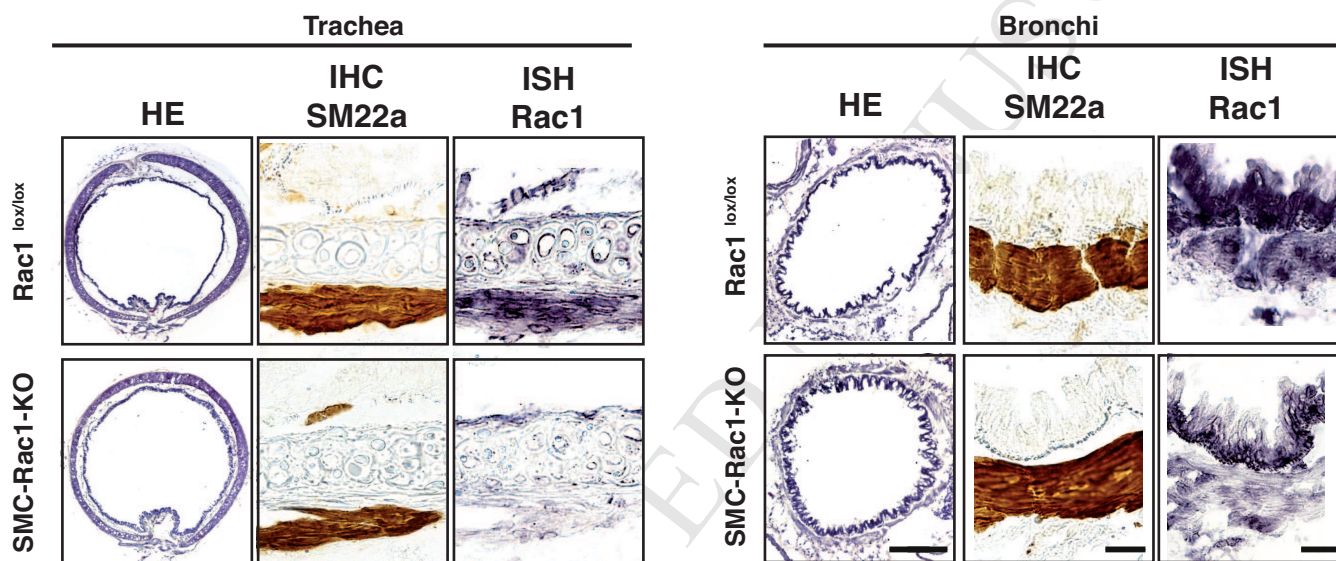
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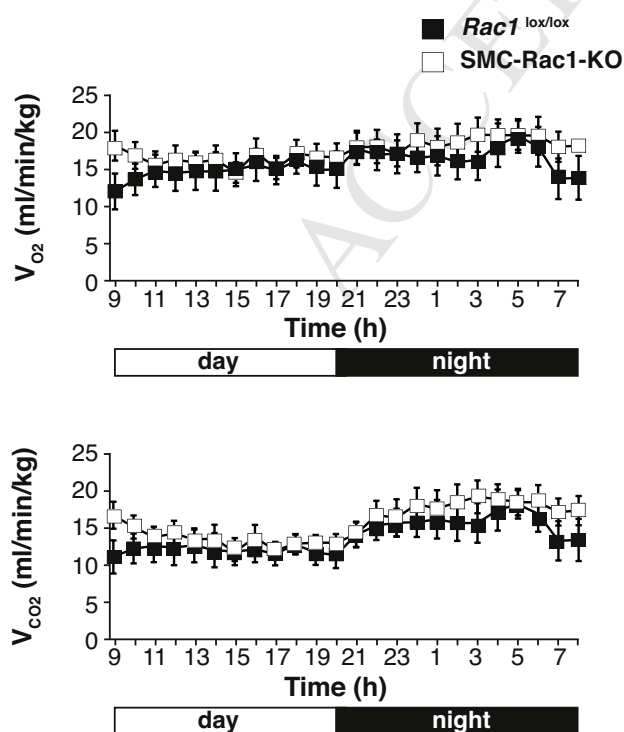
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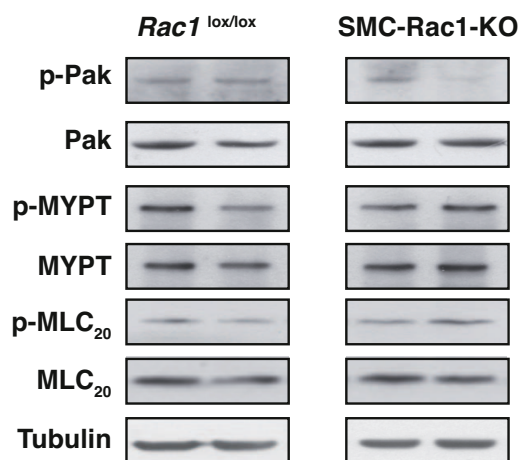
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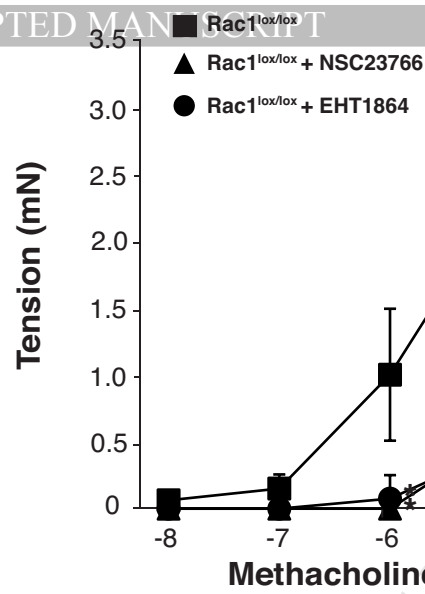


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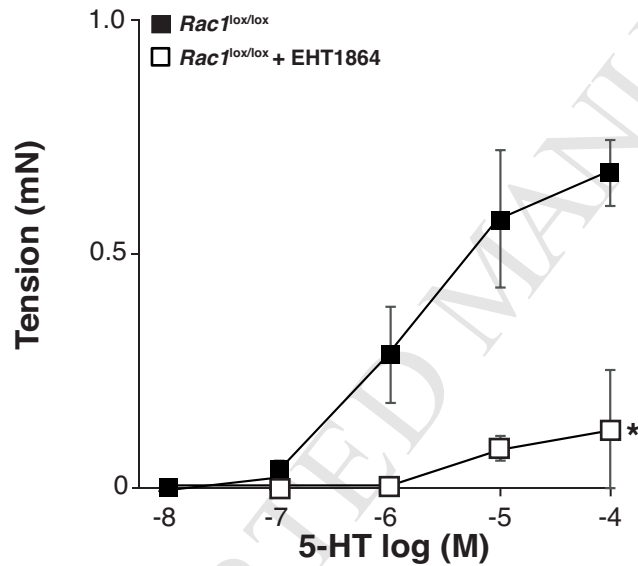


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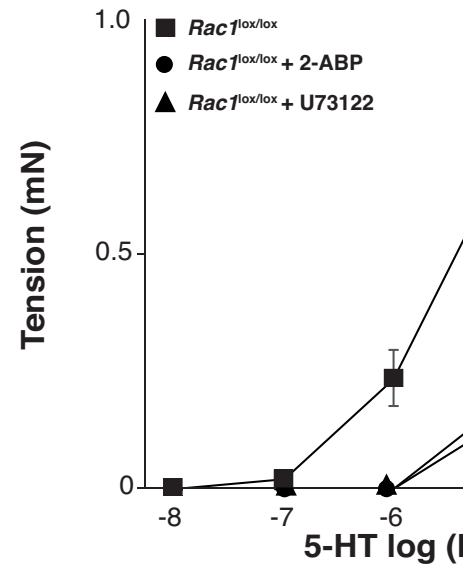




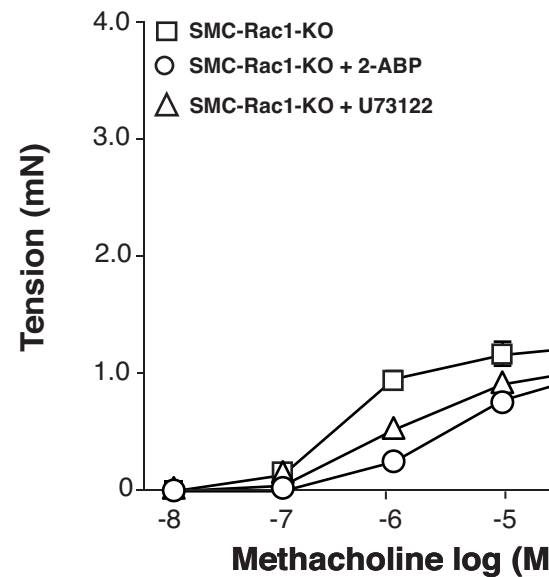
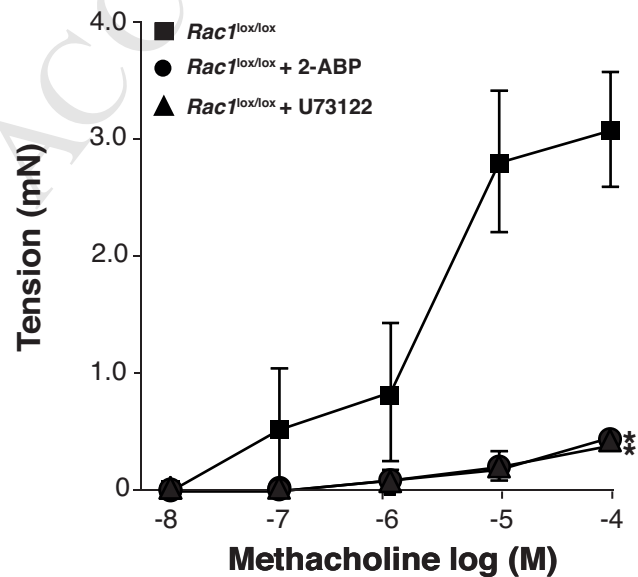
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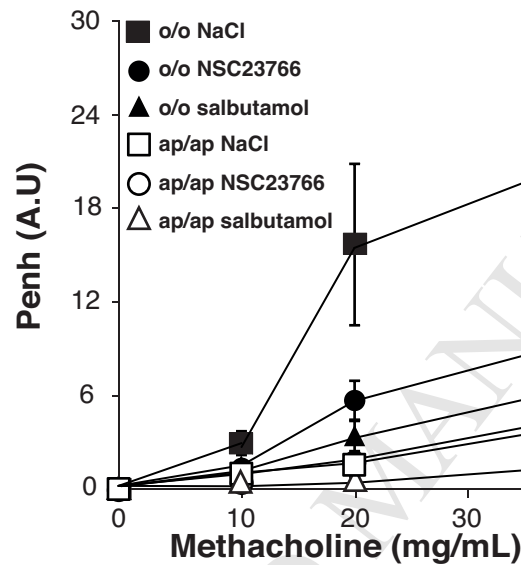
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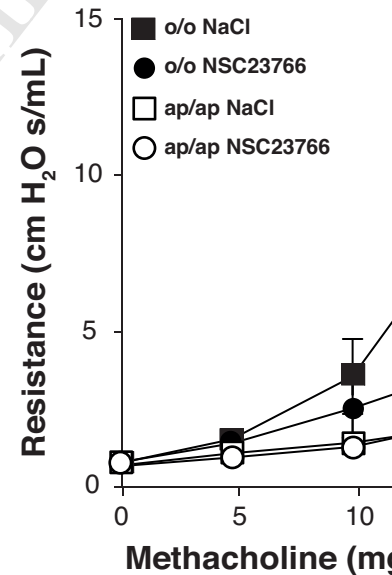
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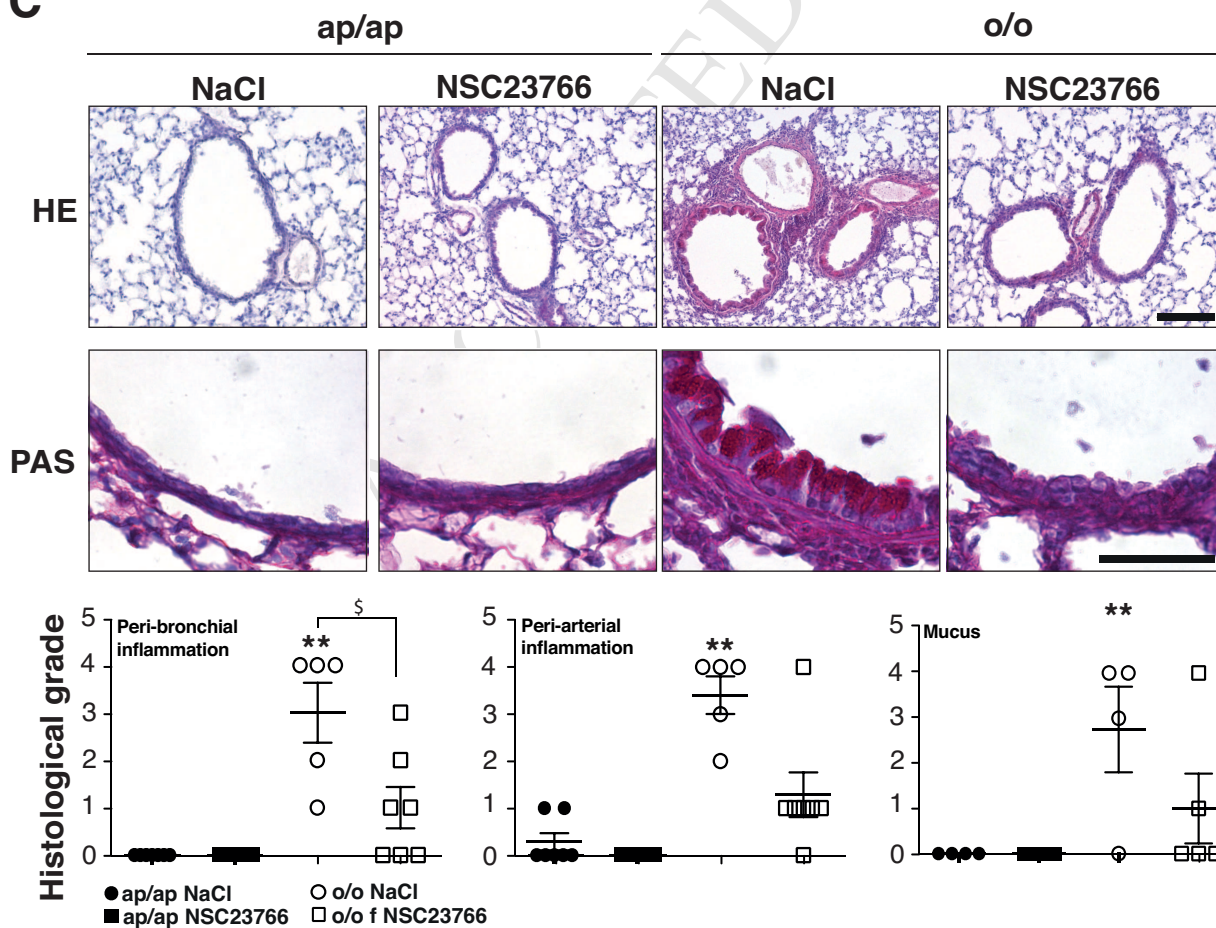
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