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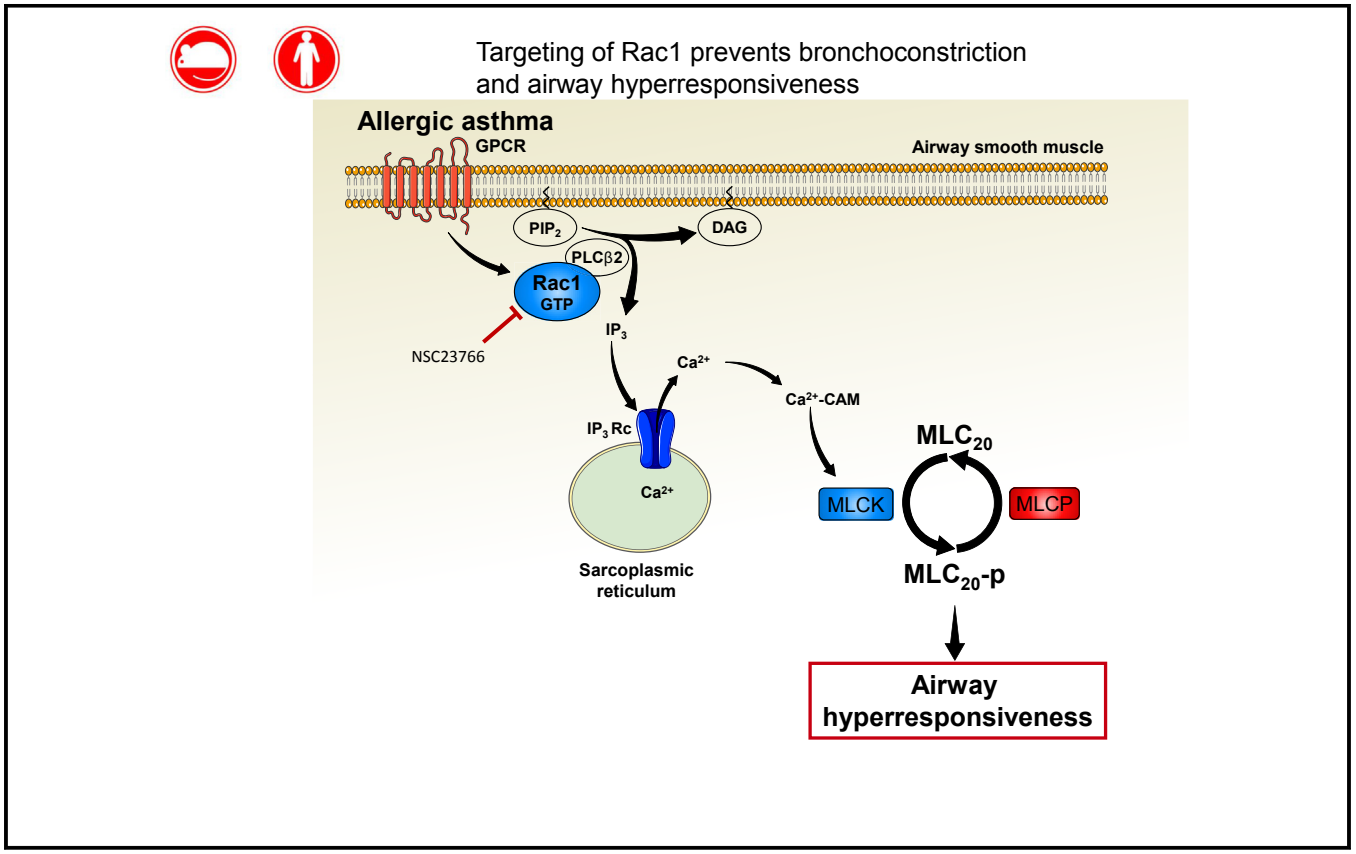
Gwennan André-Grégoire, PhD,[a]* Florian Dilasser, MSc,[a]* Julie Chesné, PhD,[a] Faouzi Braza, PhD,[a] Antoine Magnan, MD,[a,b] Gervaise Loirand, PhD,[a,b]‡ and Vincent Sauzeau, PhD[a,b]‡ *Nantes, France*

GRAPHICAL ABSTRACT



Background: The molecular mechanisms responsible for airway smooth muscle cells' (aSMCs) 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 was 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 and

From [a]NSERM, CNRS, UNIV Nantes, l'institut du thorax and [b]CHU Nantes, Nantes.
*These authors contributed equally to this work.
‡These authors contributed equally to this work.
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Corresponding author: Vincent Sauzeau, PhD, L'institut du thorax, Inserm 1087, 8 quai Moncousu, 44000 Nantes, France. E-mail: vincent.sauzeau@inserm.fr.
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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 in human bronchial aSMCs, through its association with phospholipase C β2 and the stimulation of inositol 1,4,5-trisphosphate production. *In vivo*, Rac1 deletion in SMCs 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 mice with asthma.
Conclusions: Our data reveal an unexpected and essential role of Rac1 in the regulation of intracellular Ca^{2+} and contraction of aSMCs, 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. (J Allergy Clin Immunol 2017;■■■:■■■-■■■.)

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

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, which depends on both tissue remodeling[3,4] and airways hyperresponsiveness (AHR)[5-7] due to excessive contraction of airway smooth muscle cells (aSMCs) 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% to 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 signaling molecules activated by extracellular stimuli, small GTPases of the Rho family (RhoA, Rac1, and Cdc42) act as molecular switches that regulate signaling 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 aSMCs, 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 aSMCs, 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.

Abbreviations used

AHR:	Airway hyperresponsiveness
aSMC:	Airway smooth muscle cell
CCh:	Carbachol
Der f:	House dust mite extract *Dermatophagoides farinae*
IP_3:	Inositol 1,4,5-trisphosphate
OVA:	Ovalbumine
Pak1:	p21-activated kinase 1
SMC:	Smooth muscle cell
SMC-Rac1-KO:	Smooth muscle cell–specific Rac1 knockout

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 (SMCs) was induced in 8-week-old *SMMHC-Rac1*$^{lox/lox}$ males by intraperitoneal injection of tamoxifen (1 mg/d in sunflower oil) for 5 consecutive days during 2 weeks. Tamoxifen-treated *Rac1*$^{lox/lox}$ mice were used as control. Analyses were performed 1 month after tamoxifen treatment.

To validate Rac1 deletion in aSMCs, DNA *Rac1* status was analyzed in trachea and liver by amplification of the wild-type, floxed, and deleted Rac1 alleles using 3 primers simultaneously: Reverse: 5′-TCCAATCTGTGCTGCC CATC-3′; Forward1: 5′-CAGAGCTCGAATCCAGAAACTAGTA-3′; and Forward2 5′-GATGCTTCTAGGGGTGAGCC-3′. All 3 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 immunohistochemical detection of SM22a to detect SMCs.

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 aSMCs 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 for 1 hour with collagenase II (1 mg/mL, Worthington Biochemical, Lakewood, NJ) at 37°C under agitation. Cells grew up in Dulbecco modified Eagle medium (Gibco; Invitrogen ThermoFisher Scientific, Waltham, Mass) containing 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin at 37°C and 5% CO_2. The culture medium was changed every 72 hours. All experiments were performed between passages 1 and 2. Primary vascular SMCs were isolated and cultured as described previously.[16]

Immunoblotting

Tissues or primary SMCs in culture were incubated on ice with lysis buffer supplemented with proteases and phosphatases inhibitor cocktails (Sigma Aldrich, Saint Quentin Fallavier, France) 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 (Le Pont de Claix, France) PLCβ2 (129889) was from Abcam (Paris, France). MYPT1 (2634), p-MLC2 (3671), MLC2 (3672), p-Pak (2605), and Pak1 (2602) antibodies were from Cell Signaling Technology (Leiden, The Netherlands). p-MYPT (sc17556) antibody was from Santa Cruz Biotechnology (Heidelberg, Germany) and tubulin from Beckman Coulter (Villepinte, France). Immune complexes were detected with appropriate secondary antibodies and enhanced chemiluminescence reagent (Clarity ECL BioRad, Marnes la Coquette, France). Protein band intensities were quantified using ImageJ Software (NIH software, Bethesda, Md). Coimmunoprecipitation of PLCβ2 with Rac1 was carried out using anti-Rac1 or anti-PLCβ2 antibodies (1 μg) incubated with Protein A magnetic beads for 1 hour at 4°C according to the supplier's specifications (μMacs Protein A/G Microbeads MultiMACS Protein A/G Kit, Miltenyi Biotec, Paris, France).

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, King of Prussia, Pa). 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 3 washes in PBS, sections were incubated for 1 hour at room temperature with the secondary Alexa568-labeled anti-mouse antibody (1/1000).

Intracellular calcium measurements

Murine and human aSMCs were seeded at 2×10^4 cells/well in 8-chamber borosilicate coverglass (Lab-Tek, ThermoFisher Scientific). *Rac1*$^{lox/lox}$ cells were treated or not with 4-hydroxy-tamoxifen (1 μM, 48 hours) to induce Rac1 deletion in SM-Cre positive cells (smooth muscle cell–specific Rac1 knockout [SMC-Rac1-KO] mice). FluoForte probes were loaded using FluoForte calcium assay kit (Enzo Life Science, Villeurbanne, France) according to the supplier's specifications. When indicated, Rac1 inhibitor NSC23766 (10 μM), IP3 receptor antagonist 2-ABP (10 μM), and PLC inhibitor U73122 (10 μM) were added 1 hour before loading probes. After basal fluorescence recording, cells were stimulated with carbachol (CCh) (30 μM). The fluorescence was recorded using Metamorph software and quantified with ImageJ Software (NIH software).

IP3 assay

Murine and human aSMCs were used to measure IP3 content after indicated treatments according to manufacturer's protocol (HitHunter IP3 Fluorescence Polarization Assay, DiscoverX, Birmingham, United Kingdom).

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 $CaCl_2$, 1.2 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, and 11 mM glucose) at 37°C under oxygen. A pretension of 0.5 mN was applied. We constructed dose-response curves to methacholine (Sigma-Aldrich, Paris, France). When indicated, rings were preincubated 1 hour before contraction with PLC inhibitor U73122 (Tocris, Lille, France), IP3R inhibitor 2-ABP (Tocris), or PAK inhibitor IPA3 (Tocris) at 10 μM. To inhibit Rac activity *ex vivo*, we observed that NSC23766 should be incubated for 12 hours with bronchial rings. In contrast, the Rac inhibitor EHT1864 (Sigma) induced an efficient activity at 10^{-5} mol after 1 hour of incubation (see Fig E2, *A*, in this article's Online Repository at www.jacionline.org). 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, Paris, France) 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, Paris, France) by recording respiratory pressure curves in response to inhaled methacholine (Sigma) at concentrations of 0 to 40 mg/mL for 1 minute. Airway responsiveness was expressed in enhanced pause (P_{enh}) units. The P_{enh} 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), tracheotomized, and mechanically ventilated (150 breaths/min). Increasing concentrations of methacholine were administered via a nebulizer aerosol system (Aeroneb, Aerogen, Dangan, Galway, Ireland). When required, the Rac inhibitor NSC23766 (Tocris) and salbutamol were nebulized (300 μL at 5 mM) 10 minutes before methacholine challenge.

Respiratory parameters

Mice were placed in physiocages (Bioseb, Vitrolles, France) to measure gas exchanges during 24 hours after a 2-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 (house dust mite extract *Dermatophagoides farinae* [Der f], from Stallergenes Greer, Antony, France) 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 dimethyl sulfoxide (Sigma) onto the ears. Control mice were sensitized with dimethyl sulfoxide. 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. Ten microgram of 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 24 hours after last intranasal challenge for analysis.

Bronchoalveolar lavage fluid analysis

Mice were tracheotomized 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 counted on Kova slides by optical microscopy. Identification of immune cell subpopulations was 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 were excised. Lungs were fixed in 4% paraformaldehyde for 48 hours and embedded into paraffin. Sections measuring 6 μm in size were stained with periodic acid-Schiff or hematoxylin/eosin for morphological studies. Histological grade was determined to assess inflammation and pulmonary remodeling.[15]

Statistics

All data are expressed as the mean ± SEM of sample size n. For multiple comparisons, the nonparametric Kruskal-Wallis test was used followed by Dunns' posttest. For individual comparisons, statistical analysis was performed using nonparametric *t* test (Mann-Whitney). Data analysis was performed using the GraphPad Prism software. The threshold for statistical significance was set at $P < .05$.

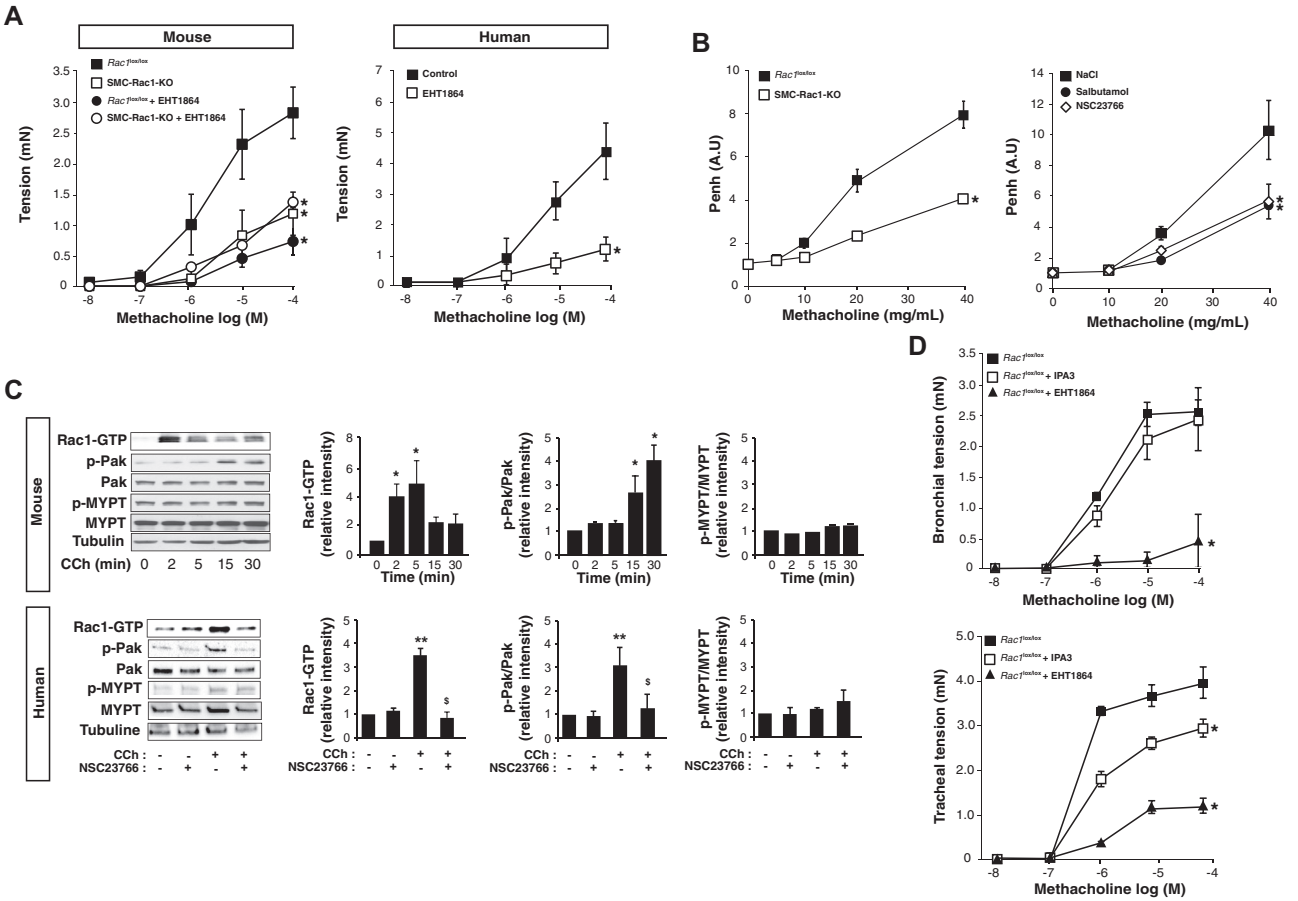

FIG 1. Rac1 controls human and murine aSMC contraction. **A,** Contractile responses to methacholine in bronchi from control (*Rac1*$^{lox/lox}$) and SMC-Rac1-KO mice (n = 5-7) and humans (n = 5). When indicated, human and murine bronchial rings were pretreated with Rac1 inhibitor (EHT1864) before methacholine stimulation (n = 5-7). **B,** Analysis of airways reactivity to methacholine by a noninvasive 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). **C,** Immunoblot analysis and quantification of Rac-GTP, Pak, and MYPT expression and phosphorylation at different time of CCh stimulation in murine aSMCs (upper panel) and after 5 minutes of CCh stimulation in human aSMCs preincubated or not with NSC23766 for 1 hour (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 pretreated with the Pak1 inhibitor IPA3 or the Rac inhibitor EHT1864. *AU,* Arbitrary units. Data are expressed as mean ± SEM. **P* < .05 and ***P* < .01 vs control, $^{\$}$*P* < .05 vs CCh.

RESULTS

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

To elucidate the specific functions of Rac1 in SMCs, we generated tamoxifen-inducible and SMC-Rac1-KO mice.[11] *In situ* hybridization and western blot analysis confirmed the loss of Rac1 mRNA and protein expression in aSMCs, which did not affect normal respiratory function under basal condition (see Fig E1 in this article's Online Repository at www.jacionline.org). We first studied the functional impact of Rac1 deletion in aSMCs by measuring the contractile response of bronchial rings from control (*Rac1*$^{lox/lox}$) and SMC-Rac1-KO mice. The maximal contraction induced by the muscarinic receptor agonist methacholine was reduced by approximately 50% in bronchial rings from SMC-Rac1-KO mice compared with *Rac1*$^{lox/lox}$ mice (Fig 1, *A*). 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 1, *A*, and Fig E2, *A*). Similarly, the bronchoconstriction induced by serotonin was reduced by approximately 70% in murine bronchial rings incubated with EHT1864 (Fig E2, *B*).

To confirm *in vivo* the role of Rac1 in bronchoconstriction, we next measured the pulmonary resistance in control and SMC-Rac1-KO mice. Although the baseline resistance was similar in the 2 groups, aerosolized methacholine-induced increase in pulmonary resistance was significantly smaller in SMC-Rac1-KO mice than in control mice (Fig 1, *B*). 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 patients with asthma (Fig 1, *B*). In contrast, inhalation of NSC23766 in SMC-Rac1-KO mice had

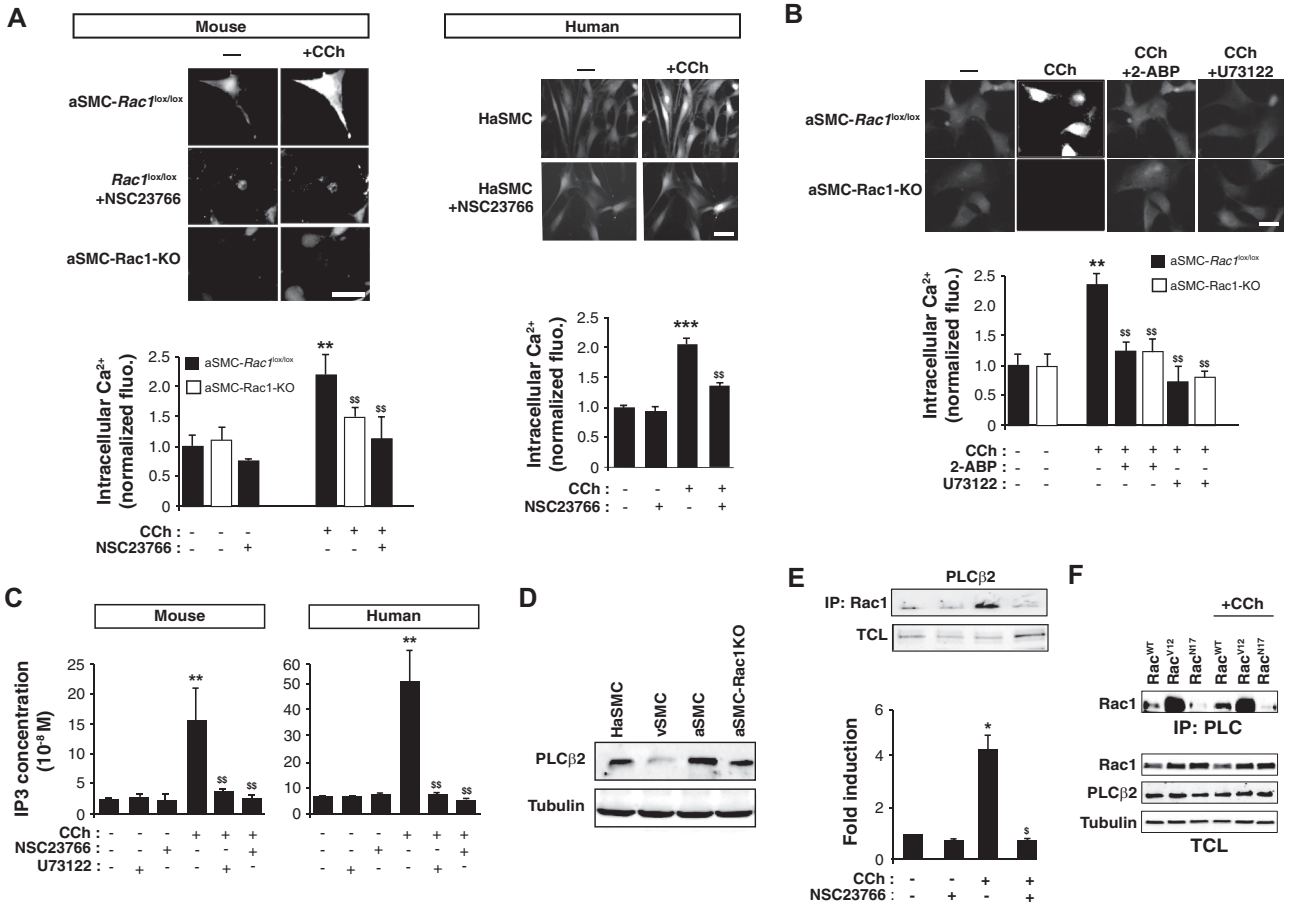

FIG 2. Rac1 interacts with PLCβ2 to modulate IP_3 production and intracellular Ca^{2+} concentration in aSMCs. Representative images and quantification of intracellular Ca^{2+} level in aSMC-*Rac1*$^{lox/lox}$, aSMC-Rac1-KO, and human aSMCs loaded with the calcium probe FluoForte (scale bar: 50 μm). Cells were preincubated for 1 hour with NSC23766 **(A)** and 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 IP_3 production in murine (left panel) and human (right panel) aSMCs pretreated or not with Rac or PLC inhibitors (n = 6). **D,** Western blot analysis of PLCβ2 expression in human aSMCs (HaSMC), vascular SMCs (vSMC), and aSMCs from control or SMC-Rac1-KO mice. **E,** Coimmunoprecipitation of endogenous PLCβ2 with Rac1 in mouse aSMCs under resting condition or stimulated with CCh in the absence or presence of NSC23766 (n = 3). **F,** Coimmunoprecipitation of endogenous PLCβ2 with the indicated Rac1 mutants expressed in 3T3 cells stimulated or not with CCh (n = 3). *IP,* Immunoprecipitate; *TCL,* total cell lysate. Data are expressed as mean ± SEM. $^{*}P < .05$, $^{**}P < .01$, and $^{***}P < .001$ vs control; $^{\$}P < .05$ and $^{\$\$}P < .01$ vs CCh-treated cells.

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/phospholipase C β2 association to modulate intracellular Ca^{2+} concentration in aSMCs

We next sought to identify the mechanism by which Rac1 controlled aSMC contraction by first analyzing the activity of Rac1 and its main effector, p21-activated kinase 1 (Pak1). Stimulation of murine and human aSMCs with the muscarinic cholinergic receptor agonist CCh increased Rac1 and Pak1 activity (Fig 1, *C*). NSC23766 treatment prevented Pak1 activation, indicating that muscarinic receptor stimulation turns on Rac1 and its downstream effector Pak1 in aSMCs. 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 1, *D*), as previously described.[14] However, both bronchial and tracheal contractions were similarly reduced by pharmacological Rac1 inhibition (Fig 1, *D*), suggesting that the effect of Rac1 in bronchoconstriction is independent of Pak1.

We next examined the status of 2 essential signaling pathways involved in SMC contraction: Ca^{2+} sensitization and Ca^{2+} signaling pathways.[10] Stimulation of murine and human aSMCs 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 Ca^{2+}-sensitization pathway not activated (Fig 1, *C*). In contrast, the CCh-induced rise in intracellular Ca^{2+} in control murine and human aSMCs was abolished by Rac1 deletion or inhibition

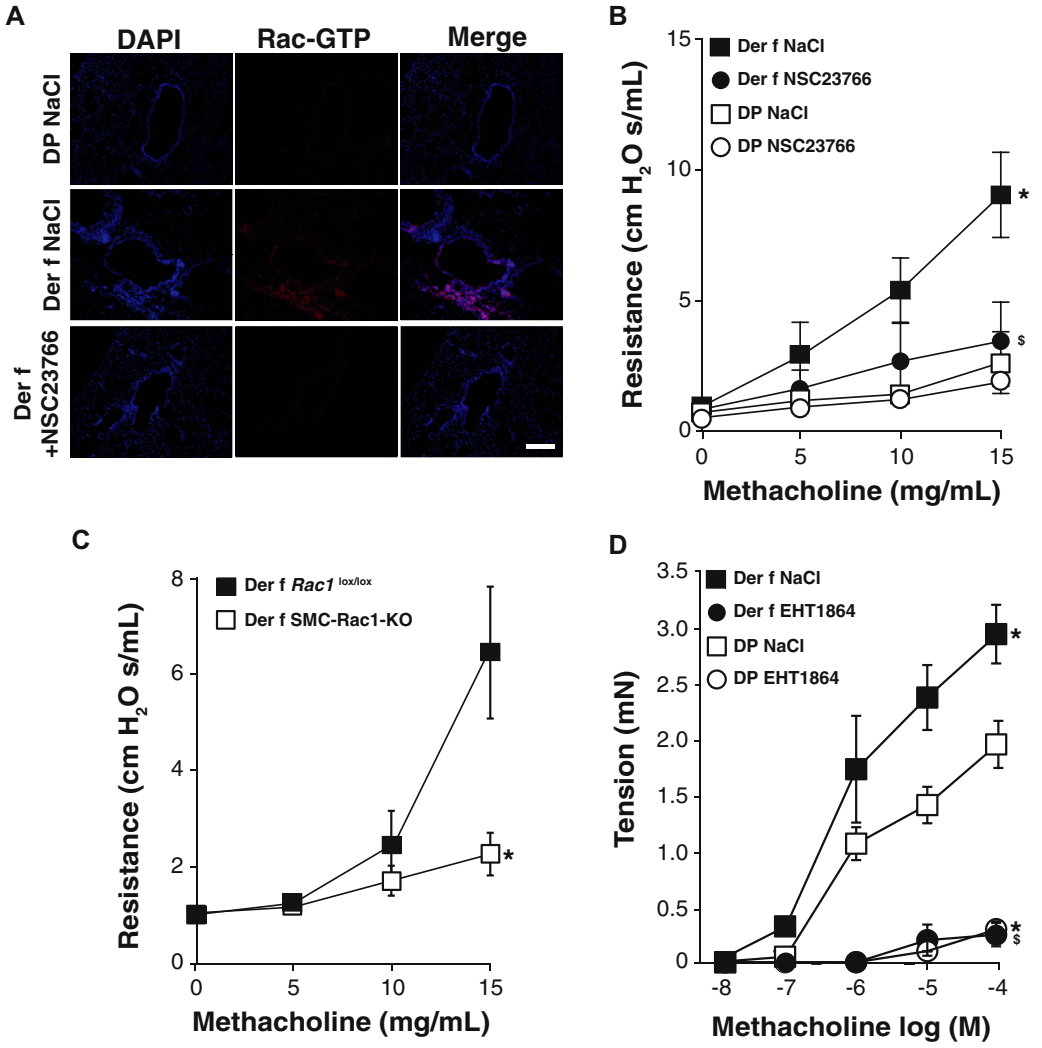

FIG 3. AHR is dependent of Rac1 activity in murine allergic asthma model. **A,** Representative images of Rac1-GTP immunofluorescence in lung sections from naive (DP) and Der f–sensitized (Der f) mice treated with NSC23766 or vehicle (NaCl) nebulization (n = 5-7). Nuclei were detected by DAPI staining. Scale bar, 100 μm. **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 ± SEM. **P* < .05 vs DP NaCl; $^{\$}$*P* < .05 vs 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 ± SEM; **P* < .05 vs control. **D,** Contractile responses to methacholine in bronchi rings from naive (DP) and Der f–sensitized mice (Der f) (n = 6) preincubated with the Rac1 inhibitor EHT1864. *DAPI,* 4′-6-diamidino-2-phenylindole, dihydrochloride. Data are expressed as mean ± SEM. **P* < .05 vs DP NaCl mice; $^{\$}$*P* < .05 vs Der f NaCl.

(Fig 2, *A*), suggesting that Rac1 regulates bronchial contraction by controlling intracellular Ca^{2+} signaling.

Activation of phospholipase C β, production of inositol 1,4,5-trisphosphate (IP_3), and opening of IP_3 receptor-Ca^{2+} release channels of the sarcoplasmic reticulum are the main mechanisms activated by G-protein–coupled receptor agonists to induce cytosolic Ca^{2+} increase in SMCs.[17] To understand how Rac1 regulates intracellular Ca^{2+}, we thus used inhibitors of PLC (U73122) and IP_3 receptor (2-ABP). Both U73122 and 2-ABP prevented muscarinic receptor activation-induced intracellular Ca^{2+} rise in aSMCs and constriction of bronchial rings from control mice, but had no effect in aSMCs and bronchial rings from SMC-Rac1-KO mice (Fig 2, *B*, and Fig E2, *B*). Consistent with this observation, we found that CCh promoted production of IP_3 in murine and human aSMCs that was abolished by the PLC inhibition (U73122), and in a similar extent, by the Rac inhibitor NSC23766 (Fig 2, *C*). Interestingly, we observed that 5HT-dependent bronchoconstriction is also sensitive to inhibitors of PLC (U73122) and IP3 receptor (2-ABP) (Fig E2, *C*). These results unveil a key role of Rac1 activity in the PLC/IP_3 signaling coupling bronchoconstrictor receptor to cytosolic Ca^{2+} rise in aSMC and contraction.

In recombinant systems and *in vitro* assays, active Rac1, 2, and 3 have been shown to interact with PLCβ2 isoform.[18-20] Although PLCβ2 expression was weak in murine vascular SMCs, aSMCs highly expressed the PLCβ2 isoform and CCh stimulation increased its association with Rac1 (Fig 2, *D* and *E*). This effect was blocked by NSC23766, suggesting that Rac1-PLCβ2

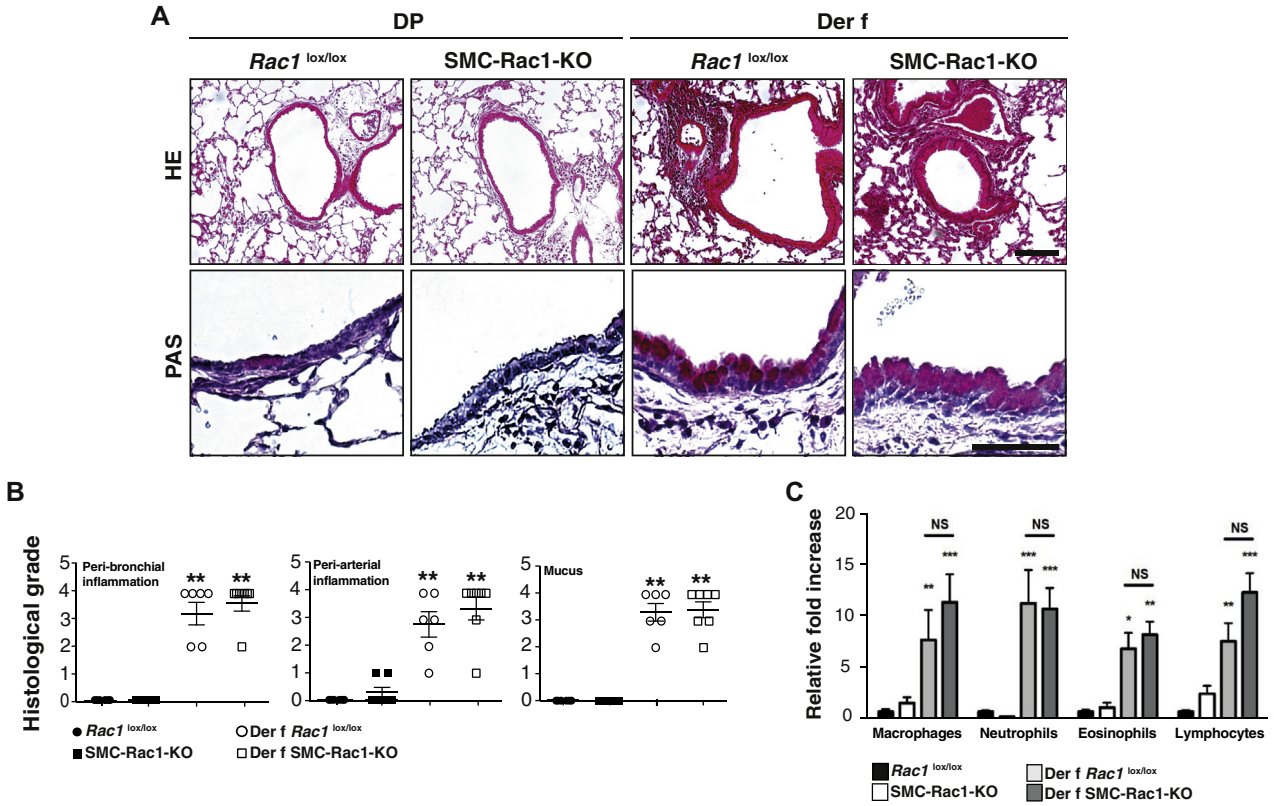

FIG 4. SMC-Rac1 deletion has no effect on pulmonary remodeling associated with allergic asthma. **A,** Hematoxylin/eosin (HE) and periodic acid-Schiff (PAS) staining of lung sections from naive (DP) and Der f–sensitized (Der f) mice of the indicated genotypes. Scale bars, 100 μm. Sections are representative of 4 to 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 bronchoalveolar lavage fluid from *Rac1*$^{lox/lox}$ and SMC-Rac1-KO sensitized to Der f (n = 6). *NS,* Not significant. Data are expressed as mean ± SEM. $^{**}P < .01$ vs Rac$^{lox/lox}$ DP NaCl mice.

association required Rac1 activation (Fig 2, *E*). 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 PLCβ2 (Fig 2, *F*). The amount of Rac1^{WT} immunoprecipitated with PLCβ2 was increased by CCh stimulation. The Rac1^{V12} mutant was constitutively associated with PLCβ2 and CCh did not increase this association. In contrast, there was no interaction between PLCβ2 and Rac1^{N17} both in the absence or in the presence of CCh. These results demonstrate for the first time the association of endogenous PLCβ2 with active Rac1 and its critical role in inducing IP_3 production, cytosolic Ca^{2+} rise, and aSMC contraction in response to bronchoconstrictor.

Inhibition of Rac1 expression or activity protects from AHR

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 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 3, *A*). Der f sensitization induced AHR that was prevented by acute NSC23766 nebulization (Fig 3, *B*) and SMC Rac1 deletion (Fig 3, *C*), 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 with 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 3, *D*).

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 with allergic asthma. However, we observed that repeated NSC23766 inhalations not only prevented AHR but also reduced inflammatory cell infiltration and airway remodeling in Der f–sensitized mice (Fig 5). Rac inhibition in Der f–sensitized mice decreased eosinophil and neutrophil influx in bronchoalveolar lavage (Fig 5, *C*). We observed the similar combined bronchodilator and anti-inflammatory effect of repeated NSC23766 treatment in OVA-induced mice with asthma (see Fig E3 in this article's Online Repository at www.jacionline.org). The beneficial effect

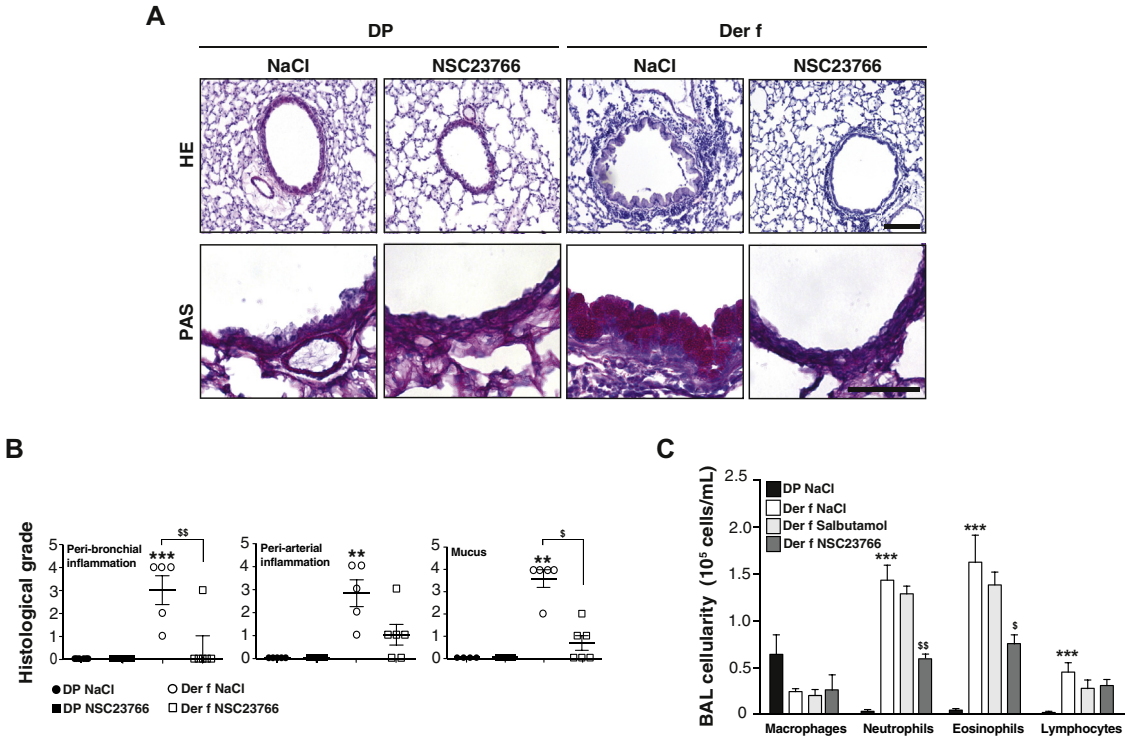

FIG 5. NSC23766 inhalations reduce pulmonary remodeling associated with allergic asthma. **A,** Hematoxylin/eosin (HE) and periodic acid-Schiff (PAS) staining of lung sections from Der f–sensitized control mice treated with repeated NSC23766 or vehicle (NaCl) nebulizations. Scale bars, 100 μm. Sections are representative of 5 to 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 bronchoalveolar lavage fluid of naive (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 ± SEM. $^{**}P < .01$ and $^{***}P < .001$ vs DP NaCl mice; $^{\$}P < .05$ and $^{\$\$}P < .01$ vs Der f NaCl.

of NSC23766 on OVA-induced AHR was similar to that obtained with the reference bronchodilator salbutamol (Fig E3, *A*), which had no action on inflammation (Fig E3, *D*).

DISCUSSION

Taken together, our results greatly extend our understanding of 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 PLCβ2, links bronchoconstrictor G-protein–coupled receptors to intracellular Ca^{2+} 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 PLCβ2 and stimulate PLCβ activity, thus defining PLCβ2 as a Rac1 effector.[19] This direct interaction between active GTP-bound Rac1 and PLCβ2 has been confirmed by the description of the crystal structure of the complex, demonstrating that Rac1 engages the pleckstrin-homology domain of PLCβ2 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 aSMCs increased Rac1 activity and promoted its association with PLCβ2. Activation of Rac1 leads to its translocation from the cytosol to the plasma membrane.[21] Interestingly, a direct interaction between muscarinic receptor and PLCβ leading to a close spatial proximity to both its substrate, PIP2, and its activator, Gαq, 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/PLCβ2 interaction. Our results further show that this protein interaction is necessary to potentiate IP_3 production, and consequently intracellular Ca^{2+} rise and contraction in murine and human aSMCs.

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[23] 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. However, the specific depletion of Rac1 expression in vascular SMCs demonstrates that the activation of Rac1 promotes vasorelaxation by positively regulating cyclic guanosine monophosphate-dependent signaling.[11,12] Inconsistent observations have also been reported in airway SMCs because

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 signaling molecules in different SMCs might support, at least in part, the inconsistent role of Rac1 in the regulation of SMC contraction. The essential role of PLCβ2 unveiled by our study fits with this hypothesis. We demonstrated that the connection between Rac1 and the PLCβ2/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 SMCs that strongly express the PLCβ2 isoform, as in human aSMCs.

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 1, *B*) and airway contractility (Fig 3, *D*) but do not affect airway resistance (Fig 3, *B*). 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, tracheotomied, and mechanically ventilated to evaluate airway resistance. All these procedures could alter airway reactivity and mask NSC23766 and EHT1864 effects.

Long-acting muscarinic antagonists 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 long-acting muscarinic antagonists 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 long-acting muscarinic antagonists to limit AHR, asthma control remains suboptimal for many patients who 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 T_H2 cytokines in asthma models induced by house dust mite extract or OVA 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, in 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 antiasthma therapy. The development of Rac inhibitors could thus open up a new avenue for the treatment of pulmonary pathologies characterized by AHR and inflammation.

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Clinical implications: The nebulization 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.

REFERENCES

1. Busse WW, Lemanske RF Jr. Asthma. N Engl J Med 2001;344:350-62.
2. To T, Stanojevic S, Moores G, Gershon AS, Bateman ED, Cruz AA, et al. Global asthma prevalence in adults: findings from the cross-sectional world health survey. BMC Public Health 2012;12:204.
3. Bento AM, Hershenson MB. Airway remodeling: potential contributions of subepithelial fibrosis and airway smooth muscle hypertrophy/hyperplasia to airway narrowing in asthma. Allergy Asthma Proc 1998;19:353-8.
4. Rydell-Tormanen K, Risse PA, Kanabar V, Bagchi R, Czubryt MP, Johnson JR. Smooth muscle in tissue remodeling and hyper-reactivity: airways and arteries. Pulm Pharmacol Ther 2013;26:13-23.
5. Opazo Saez AM, Seow CY, Pare PD. Peripheral airway smooth muscle mechanics in obstructive airways disease. Am J Respir Crit Care Med 2000;161:910-7.
6. Postma DS, Kerstjens HA. Characteristics of airway hyperresponsiveness in asthma and chronic obstructive pulmonary disease. Am J Respir Crit Care Med 1998;158:S187-92.
7. Cockcroft DW, Davis BE. Mechanisms of airway hyperresponsiveness. J Allergy Clin Immunol 2006;118:551-9; quiz 560-51.
8. Prakash YS. Airway smooth muscle in airway reactivity and remodeling: what have we learned? Am J Physiol Lung Cell Mol Physiol 2013;305:L912-33.
9. Barnes PJ. Immunology of asthma and chronic obstructive pulmonary disease. Nat Rev Immunol 2008;8:183-92.
10. Loirand G, Sauzeau V, Pacaud P. Small g proteins in the cardiovascular system: physiological and pathological aspects. Physiol Rev 2013;93:1659-720.
11. Andre G, Sandoval JE, Retailleau K, Loufrani L, Toumaniantz G, Offermanns S, et al. Smooth muscle specific rac1 deficiency induces hypertension by preventing p116rip3-dependent rhoa inhibition. J Am Heart Assoc 2014;3:e000852.
12. Fabbiano S, Menacho-Marquez M, Sevilla MA, Albarran-Juarez J, Zheng Y, Offermanns S, et al. Genetic dissection of the vav2-rac1 signaling axis in vascular smooth muscle cells. Mol Cell Biol 2014;34:4404-19.
13. Gong D, Fei F, Lim M, Yu M, Groffen J, Heisterkamp N. Abr, a negative regulator of rac, attenuates cockroach allergen-induced asthma in a mouse model. J Immunol 2013;191:4514-20.
14. Hoover WC, Zhang W, Xue Z, Gao H, Chernoff J, Clapp DW, et al. Inhibition of p21 activated kinase (pak) reduces airway responsiveness in vivo and in vitro in murine and human airways. PLoS One 2012;7:e42601.
15. Chesne J, Braza F, Chadeuf G, Mahay G, Cheminant MA, Loy J, et al. Prime role of il-17a in neutrophilia and airway smooth muscle contraction in a house dust mite-induced allergic asthma model. J Allergy Clin Immunol 2015;135:1643-5, e1645.

16. Gayard M, Guilluy C, Rousselle A, Viollet B, Henrion D, Pacaud P, et al. Ampk alpha 1-induced rhoa phosphorylation mediates vasoprotective effect of estradiol. Arterioscler Thromb Vasc Biol 2011;31:2634-42.
17. McGraw DW, Elwing JM, Fogel KM, Wang WC, Glinka CB, Mihlbachler KA, et al. Crosstalk between gi and gq/gs pathways in airway smooth muscle regulates bronchial contractility and relaxation. J Clin Invest 2007;117:1391-8.
18. Illenberger D, Schwald F, Gierschik P. Characterization and purification from bovine neutrophils of a soluble guanine-nucleotide-binding protein that mediates isozyme-specific stimulation of phospholipase c beta2. Eur J Biochem 1997;246: 71-7.
19. Snyder JT, Singer AU, Wing MR, Harden TK, Sondek J. The pleckstrin homology domain of phospholipase c-beta2 as an effector site for rac. J Biol Chem 2003;278: 21099-104.
20. Jezyk MR, Snyder JT, Gershberg S, Worthylake DK, Harden TK, Sondek J. Crystal structure of rac1 bound to its effector phospholipase c-beta2. Nat Struct Mol Biol 2006;13:1135-40.
21. Bustelo XR, Ojeda V, Barreira M, Sauzeau V, Castro-Castro A. Rac-ing to the plasma membrane: the long and complex work commute of rac1 during cell signaling. Small GTPases 2012;3:60-6.
22. Kan W, Adjobo-Hermans M, Burroughs M, Faibis G, Malik S, Tall GG, et al. M3 muscarinic receptor interaction with phospholipase c beta3 determines its signaling efficiency. J Biol Chem 2014;289:11206-18.
23. Rahman A, Davis B, Lovdahl C, Hanumaiah VT, Feil R, Brakebusch C, et al. The small gtpase rac1 is required for smooth muscle contraction. J Physiol 2014;592: 915-26.
24. Roscioni SS, Maarsingh H, Elzinga CR, Schuur J, Menzen M, Halayko AJ, et al. Epac as a novel effector of airway smooth muscle relaxation. J Cell Mol Med 2011; 15:1551-63.
25. Gosens R, Bos IS, Zaagsma J, Meurs H. Protective effects of tiotropium bromide in the progression of airway smooth muscle remodeling. Am J Respir Crit Care Med 2005;171:1096-102.
26. Kang JY, Rhee CK, Kim JS, Park CK, Kim SJ, Lee SH, et al. Effect of tiotropium bromide on airway remodeling in a chronic asthma model. Ann Allergy Asthma Immunol 2012;109:29-35.
27. Price D, Fromer L, Kaplan A, van der Molen T, Roman-Rodriguez M. Is there a rationale and role for long-acting anticholinergic bronchodilators in asthma? NPJ Prim Care Respir Med 2014;24:14023.
28. Wasilewski NV, Lougheed MD, Fisher JT. Changing face of beta2-adrenergic and muscarinic receptor therapies in asthma. Curr Opin Pharmacol 2014;16:148-56.
29. Wedzicha JA, Banerji D, Vogelmeier CF. Indacaterol-glycopyrronium for COPD. N Engl J Med 2016;375:899-900.
30. Moore WC, Peters SP. Severe asthma: an overview. J Allergy Clin Immunol 2006; 117:487-94; quiz 495.
31. Bahaie NS, Hosseinkhani MR, Ge XN, Kang BN, Ha SG, Blumenthal MS, et al. Regulation of eosinophil trafficking by swap-70 and its role in allergic airway inflammation. J Immunol 2012;188:1479-90.
32. Kudo M, Melton AC, Chen C, Engler MB, Huang KE, Ren X, et al. Il-17a produced by alphabeta t cells drives airway hyper-responsiveness in mice and enhances mouse and human airway smooth muscle contraction. Nat Med 2012; 18:547-54.
33. Juncadella IJ, Kadl A, Sharma AK, Shim YM, Hochreiter-Hufford A, Borish L, et al. Apoptotic cell clearance by bronchial epithelial cells critically influences airway inflammation. Nature 2013;493:547-51.
34. Yao HY, Chen L, Xu C, Wang J, Chen J, Xie QM, et al. Inhibition of rac activity alleviates lipopolysaccharide-induced acute pulmonary injury in mice. Biochim Biophys Acta 2011;1810:666-74.

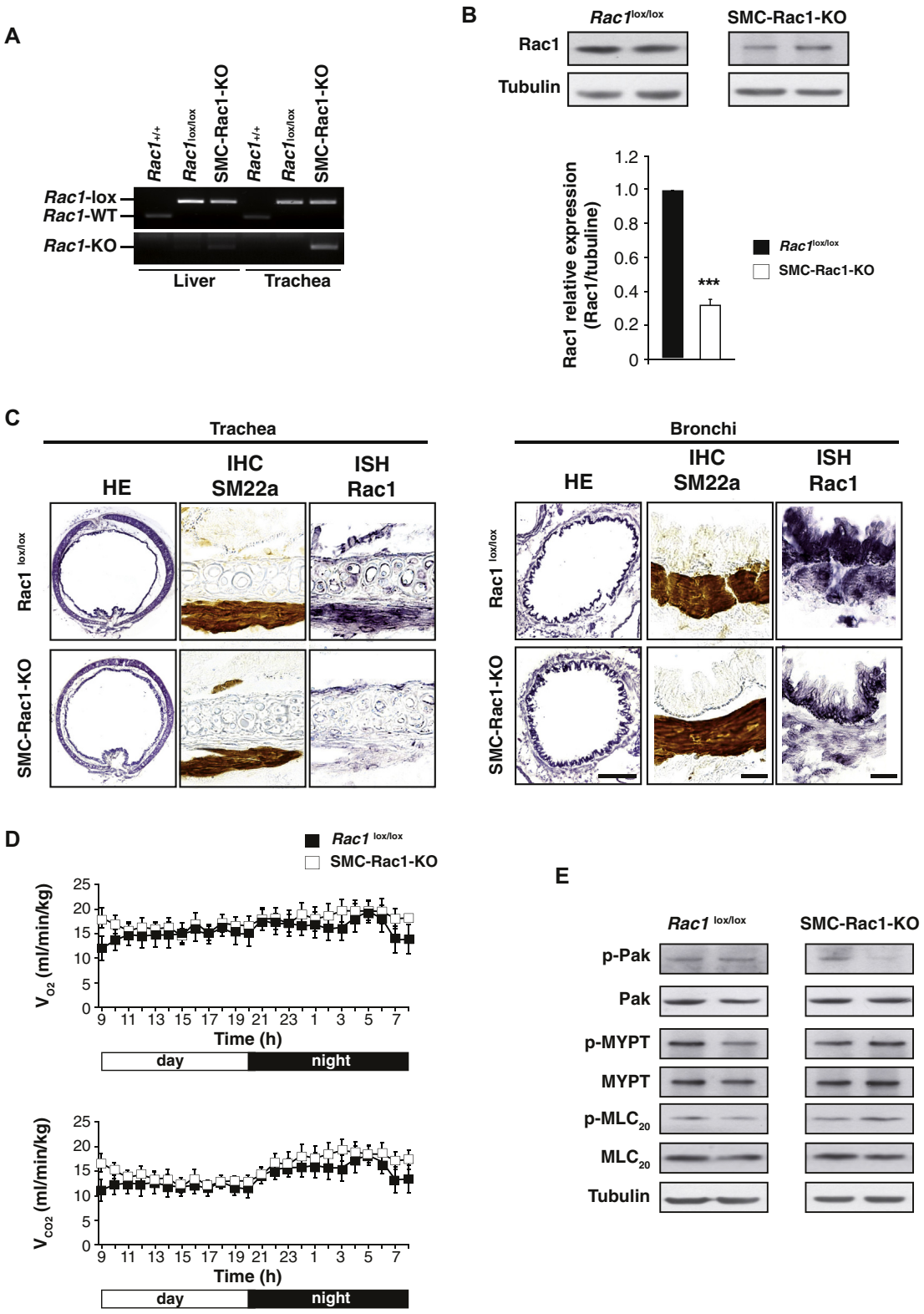

FIG E1. Validation of Rac1 deletion in aSMCs and assessment of airway function in SMC-Rac1-KO mice. **(A)** Genomic DNA status detected by PCR and **(B)** immunoblot analysis of Rac1 protein expression in tracheas from the indicated mice after tamoxifen treatment (n = 4). Data are expressed as mean ± SEM. ***P < .001 vs SMC-*Rac1*$^{lox/lox}$ mice. **C,** Hematoxylin/eosin (HE) staining, immunohistochemical (IHC) detection of SM22a (SMC labeling), and *in situ* hybridization (ISH) analysis of *Rac1* mRNA expression in trachea (left panels) and bronchi (right panels) sections. Scale bars: HE, 400 μm; IHC/ISH, 40 μm. **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).

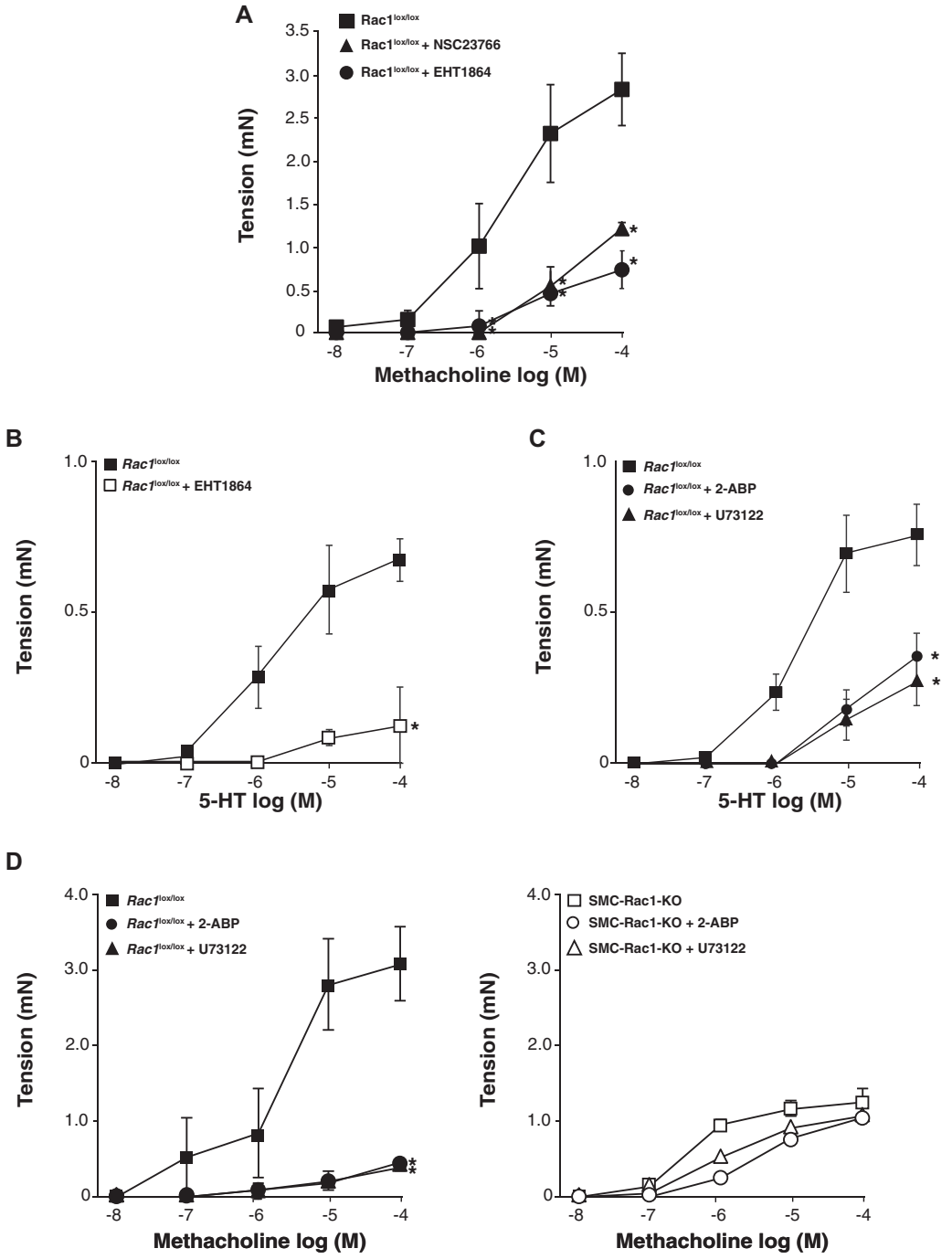

FIG E2. Rac1/PLCβ2/IP3 signaling pathway is involved in bronchoconstriction. **A,** Contractile responses to methacholine in bronchi preincubated with Rac inhibitors, NSC23766 (12 hours) or EHT1864 (1 hour). **B** and **C**, Contractile responses to 5-HT in bronchi preincubated with indicated inhibitors. **D,** Contractile responses to methacholine in bronchi from *Rac1*$^{lox/lox}$ (left panel) and SMC-Rac1-KO (right panel) mice pretreated or not with the PLC inhibitor U73122 or the IP_3 receptor inhibitor 2-ABP (n = 4-6). Data are expressed as mean ± SEM. **P* < .05 vs *Rac1*$^{lox/lox}$.

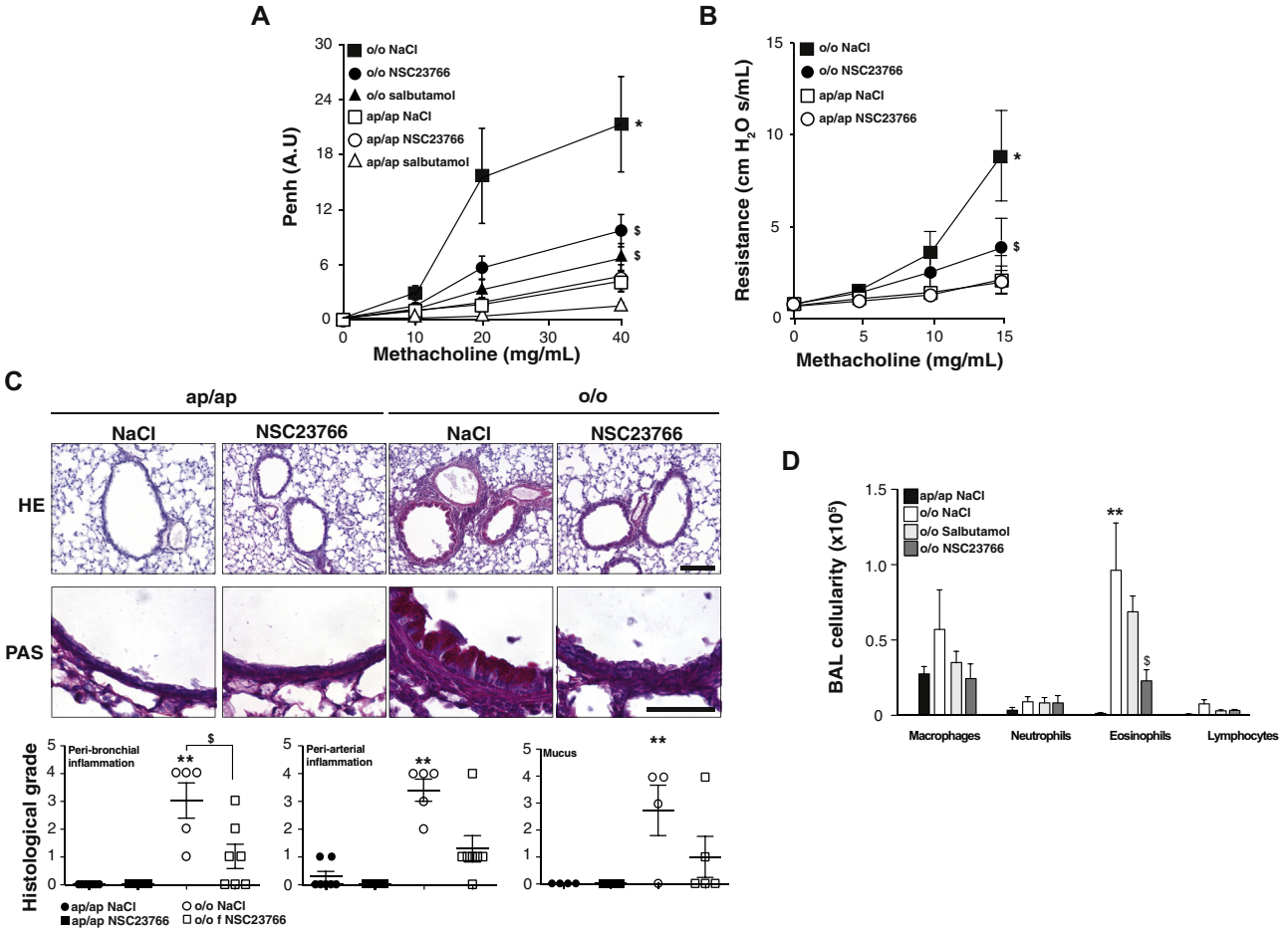

FIG E3. Inhibition of Rac1 activity prevents AHR and pulmonary inflammation in OVA-sensitized mice. **A,** Analysis of airway reactivity to methacholine challenges by noninvasive plethysmography in mice (n = 9-10) challenged with OVA (o/o) or alum vehicle (ap/ap). When indicated, mice were treated with either salbutamol, the Rac inhibitor NSC23766, or the vehicle NaCl. **B,** Airway reactivity to methacholine measured by FlexiVENT in naive and OVA-challenged mice pretreated 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 to 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). *BAL*, Bronchoalveolar lavage. Data are expressed as mean ± SEM. $^{*}P < .05$ and $^{**}P < .01$ vs ap/ap NaCl, $^{\$}P < .05$ vs o/o NaCl.