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Am J Physiol Lung Cell Mol Physiol 302:L140-L151, 2012. First published 7 October 2011;
doi: 10.1152/ajplung.00152.2011

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Allergic inflammation induces a persistent mechanistic switch in thromboxane-mediated airway constriction in the mouse

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Submitted 19 May 2011; accepted in final form 3 October 2011

Cyphert JM, Allen IC, Church RJ, Latour AM, Snouwaert JN, Coffman TM, Koller BH. Allergic inflammation induces a persistent mechanistic switch in thromboxane-mediated airway constriction in the mouse. *Am J Physiol Lung Cell Mol Physiol* 302: L140–L151, 2012. First published October 7, 2011; doi:10.1152/ajplung.00152.2011.—Actions of thromboxane (TXA₂) to alter airway resistance were first identified over 25 years ago. However, the mechanism underlying this physiological response has remained largely undefined. Here we address this question using a novel panel of mice in which expression of the thromboxane receptor (TP) has been genetically manipulated. We show that the response of the airways to TXA₂ is complex: it depends on expression of other G protein-coupled receptors but also on the physiological context of the signal. In the healthy airway, TXA₂-mediated airway constriction depends on expression of TP receptors by smooth muscle cells. In contrast, in the inflamed lung, the direct actions of TXA₂ on smooth muscle cell TP receptors no longer contribute to bronchoconstriction. Instead, in allergic lung disease, TXA₂-mediated airway constriction depends on neuronal TP receptors. Furthermore, this mechanistic switch persists long after resolution of pulmonary inflammation. Our findings demonstrate the powerful ability of lung inflammation to modify pathways leading to airway constriction, resulting in persistent changes in mechanisms of airway reactivity to key bronchoconstrictors. Such alterations are likely to shape the pathogenesis of asthmatic lung disease.

prostanoid; asthma; nerve; airway smooth muscle

CONSTRICTION OF THE AIRWAYS can be triggered both by the direct interaction of ligands with receptors directly on airway smooth muscle (ASM) or indirectly by activation of the nerves that regulate its tone. Thromboxane (TXA₂) is one of the most potent endogenous bronchoconstrictors, and this activity is conserved across several species, including humans, dogs, cats, guinea pigs, and rodents (4, 31, 45, 55).

TXA₂ is a prostanoid lipid mediator; its synthesis is initiated by the oxidation of arachidonic acid by either cyclooxygenase-1 or cyclooxygenase-2 into prostaglandin H₂ (PGH₂). The thromboxane synthase enzyme catalyzes the isomerization of PGH₂ to generate TXA₂, which has a very short half-life in aqueous solution and is rapidly hydrolyzed to the stable, inactive metabolite thromboxane B₂ (TXB₂). Although inactive, TXB₂ can be used as a marker for in vivo TXA₂ synthesis (19). TXA₂ exerts its biological functions through interactions with the heterotrimeric G protein-coupled thromboxane-prostanoid (TP) receptor (34, 43, 49, 52). All of the known

physiological functions of TXA₂ are dependent upon the expression of this receptor, which associates with the G_q family of proteins and mediates most of its physiological actions through the activation of phospholipase C and increases in intracellular calcium concentrations ([Ca²⁺]_i).

Enhanced TXA₂ biosynthesis is seen in the airways of atopic asthmatic subjects following allergen challenge (51, 61) and in patients with chronic obstructive pulmonary disease (15). Thromboxane has also been shown to be released after activation of human alveolar macrophages and mast cells (36, 47). In addition, inhibition of TP receptors attenuates allergic airway constriction in guinea pig and human precision-cut lung slices, further implicating TXA₂ in the asthmatic response (45, 65). Despite the overwhelming evidence demonstrating an important role for this mediator in modulating airway tone, the mechanism(s) of these actions are poorly defined. The TP receptor is broadly expressed in the lung (7, 12, 27, 30, 33, 38, 42, 56). Its expression by smooth muscle together with in vivo studies demonstrating altered intracellular Ca²⁺ levels in cultured smooth muscle cells exposed to TXA₂ agonists (29, 30) support a model in which TXA₂ acts directly on these cells to induce bronchoconstriction. However, a number of studies suggest more complex regulation, including indirect actions of TXA₂ to induce bronchoconstriction by stimulating neurons (31, 48, 50). However, the relative roles of these pathways in determining airway tone in the normal and diseased lung in the intact animal are not clear.

Airways are primarily innervated by sensory and parasympathetic fibers carried in the vagus nerve. The sensory neurons include myelinated stretch receptors and unmyelinated C fibers. Slowly and rapidly adapting stretch receptors (SARs and RARs, respectively) respond to mechanical forces caused by inflation and deflation during respiration. SARs respond to changes in tension across the airway wall and are thought to regulate changes in bronchial tone with inspiration. Activation of these receptors leads to inhibition of inspiration and parasympathetic activity, resulting in relaxation of ASM. RARs are stimulated by mechanical, chemical, and inflammatory stimuli. Activation of these receptors leads to increases in respiratory rate, inspiratory effort, and parasympathetic output, resulting in contraction of ASM, mucus secretion, and the cough reflex (13). However, the most abundant sensory nerves in the airways are C fibers (2). While typically quiescent during tidal breathing, C fiber activation can provide excitatory input to neuronal pathways driving autonomic output to the airways, resulting in bronchoconstriction, mucus secretion, and vasodilation (8, 37). The somata for these neurons are in the nodose and jugular ganglia. They send projections to nucleus tractus

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solitarius, relaying information from the lung and airways to the medullary respiratory centers. When stimulated, either mechanically or by irritants such as noxious substances or locally released mediators, these afferents can initiate a tachykinergic process that can influence activation of the efferent parasympathetic neurons. These parasympathetic neurons, also carried to the lung by the vagus, terminate on ganglia within the airway wall. Postganglionic fibers release acetylcholine, which acts on the smooth muscle, establishing tone in the healthy airway and causing constriction during cholinergic reflex (6).

TP receptors are broadly expressed in the nervous system, including on cell bodies in the nodose ganglia (5, 9, 58). A recent paper also suggests a role for TP receptors in stimulation of sensory neurons in the skin (5). Physiological studies show that bronchoconstriction initiated by direct physical stimulation of the parasympathetic pathways in the vagus can be augmented by TXA₂ (31, 48, 50), indicating that TXA₂ may act by enhancing ACh release in the postganglionic neurons. These findings support a model in which TXA₂ acts directly on TP receptors in neurons to stimulate a reflex activation of parasympathetic pathways. Involvement of neuronal pathways in TXA₂-mediated bronchoconstriction is also supported by our recent demonstration that vagotomy and anticholinergic agents dramatically attenuate TXA₂-induced bronchoconstriction (4).

Distinguishing between these possible models has proved difficult using traditional pharmacological and physiological approaches. To circumvent these obstacles we have generated mouse lines with a conditional thromboxane A₂ receptor (*Tbxa2r*) allele. Using this genetic approach, we show that pathways utilized by TXA₂ to modulate airway physiology are dynamic and susceptible to change, depending on the background of inflammation in the lung.

MATERIALS AND METHODS

Mice. 129/SvEv *Tbxa2r*^{-/-} mice were generated as previously described (52). Wild-type 129/SvEv mice were purchased from Jackson Laboratories. All animal studies were conducted in accordance with the National Institutes of Health *Guide for the Care and Use of Laboratory Animals* guidelines and were reviewed and approved by the Institutional Animal Care and Use Committee of the University of North Carolina at Chapel Hill.

Generation of mice carrying a *Tbxa2r* locus sensitive to Cre-mediated disruption. Cell-specific deletion of TP receptors was carried out using the Cre/loxP recombination system (Fig. 1). The strategy targeted exon 2 of *Tbxa2r*, which encodes six of the seven transmembrane domains of the TP receptor. Specifically, three fragments of the *Tbxa2r* gene were amplified using PCR, a 3,073-bp region upstream of exon 2, a 1,032-bp region containing exon 2, and a 4,424-bp region downstream of exon 2 using the following primer sets: 5'-ATAAGCTTGCGGCCGAGTTCCCTGGTGATG-3' and 5'-ATATCGATTAGCCCTAGCTGTCCTGGAA-3', 5'-GCTGCCTCAAAG AAAGGGTA-3' and 5'-GTGCGG CCGCTCTAAAGC-CCCAAAGACCT-3', and 5'-GTGGATCCCTGCAGGATGTAAC-AGGAAAGA-3' and 5'-GTGGTACCAGAACCCATCCCAGTTCTGA-3', respectively. These fragments were subcloned into the pCR 2.1 vector (Invitrogen) and sequenced to verify that the PCR amplification did not introduce mutations into essential sequences. The fragment corresponding to intron 2 was directly cloned into the pXenaLF² vector, 3' to the neomycin cassette and 5' to the pgk-tk cassette. The pXenaLF² vector contains the selectable marker gene for neomycin resistance (pgk-neo) flanked on both 5' and 3' sides by a loxP and frt site. The fragments corresponding to intron 1 and

exon 2 of *Tbxa2r* were subcloned 5' and 3' of the loxP cassette, respectively, in the pNebLox vector. This generated a fragment of *Tbxa2r* DNA with a loxP site placed in intron 1. This newly generated 5' arm-loxP-exon 2 cassette was subsequently removed from pNebLox and cloned into the pXenaLF² vector, 5' to its loxP cassette. The targeting plasmid was linearized and introduced into embryonic stem (ES) cells derived from 129/SvEv mice, and transformants were isolated using standard methodologies (40).

ES cells in which the plasmid integrated by homologous recombination and contained the loxP-exon2-loxP-flp-neo-flp cassette were used to generate chimeric animals, which in turn were bred to generate animals heterozygous for the floxed allele (F/+). The neomycin gene was then removed by breeding the heterozygous mice with C57BL/6J mice that express flp recombinase in the germline [B6;SJL-Tg(ACTFLPe)9205Dym/J; Jackson Laboratories].

Generation of mice with tissue-specific deletion of TP receptors. To generate mice with tissue-specific deletion in smooth muscle cells, *Tbxa2r* floxed homozygous mice (F/F) were crossed to *Tgln*-cre mice [STOCK Tg(Tagln-cre)1Her/J; Jackson Laboratory]. Mice expressing cre recombinase under the control of the Nestin promoter are also commercially available [B6.Cg-Tg(Nes-cre)1Kln/J; Jackson Laboratory] and were used to generate mice with tissue-specific deletion of TP receptors on neurons. The introduction of these transgenes onto the 129/SvEv background was initiated, and animals were backcrossed for seven generations. The resultant animals were bred to *Tbxa2r*^{-/-} mice to introduce the null (-) allele and to generate the following genotypes to be used for experiments: F/+ (wild type), F/+ cre (heterozygous), F/- (heterozygous), F/- cre (tissue-specific null).

Genotyping. To identify transformant ES cells, a DNA probe corresponding to the region immediately downstream of the targeted region (3' probe) was generated as previously described (52), and a probe corresponding to exon 2 of *Tbxa2r* (internal probe) was generated using the following primer sets: 5'-GCTGCCTCAAAG-AAAGGGTA-3' and 5'-GTGCGGCCGCTCTCTAAAGCCCCAAAGACCT-3'. These probes were used to identify targeted ES cells by Southern blot and were used to confirm the incorporation of loxP sites in successfully targeted ES cells. These probes were also used to genotype the resultant mice. Following digestion with BamHI, the exon 2 probe discussed above was used to confirm the removal of the neomycin cassette.

To identify tissue-specific deletion of *Tbxa2r* in the mice, a DNA probe corresponding to the region immediately 5' of the targeted region was generated by PCR (5'-AACCTGAGTCTGTGGGGTTG-3' and 5'-ACAAGCATCAAGGAGGGATG-3') and was used to assess exon 2 removal by cre/lox in selected tissues. The use of this probe in conjunction with a BamHI digestion of DNA resulted in the following DNA fragments corresponding to the various alleles: 11.2 kb (+), 4.5 kb (F), 4.1 kb (-), and 3.3 kb (removal of F).

Measurement of TP receptor expression in cultured ASM cells and peripheral nerves. ASM was isolated from tracheal explants. Briefly, the trachea between the larynx and the main stem bronchi was removed and placed in a sterile petri dish containing HBSS solution with a 2× concentration of antibiotic/antimycotic (penicillin/streptomycin/genomycin). The trachea was dissected free of surrounding tissues, and epithelium was removed by rubbing the luminal surface with a plastic blade. The trachea was then dissected into 2- to 3-mm segments and placed intima side down in a sterile culture dish. Explants were allowed to adhere to the dish and then were covered with 3 ml of DMEM + 20% FCS + 2× antibiotic/antimycotic and incubated at 37°C in a humidified chamber. After 3 days of cell growth, FCS was reduced to 10% and antibiotic/antimycotic to 1×. At confluent density, cells were harvested for RNA collection. Additionally, tracheas were excised from animals, cut longitudinally, and splayed open. Tissue was gently scraped to remove epithelial cells, rinsed briefly in PBS, and frozen on dry ice and homogenized before RNA isolation.

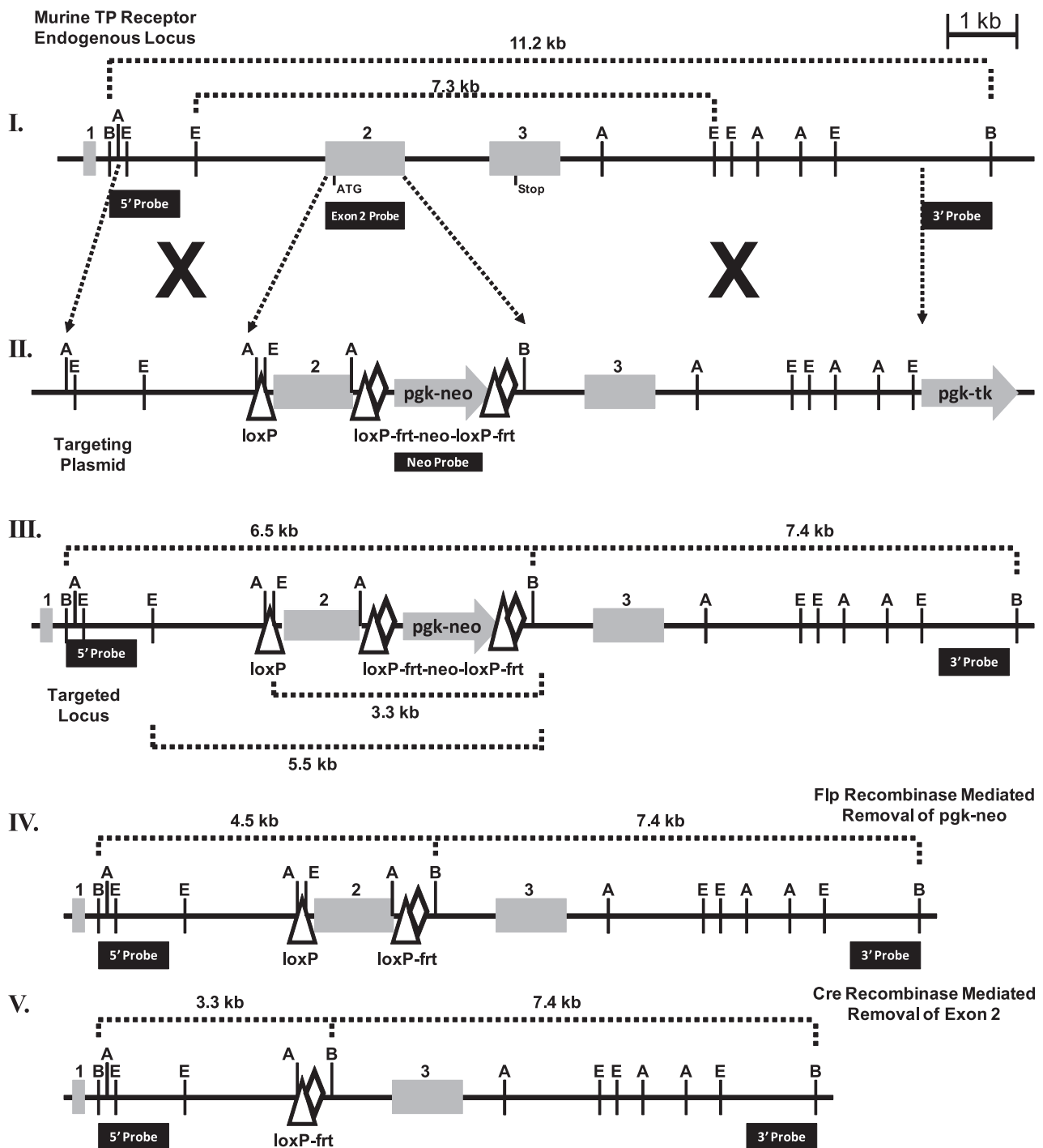


Fig. 1. Schematic depicting the generation of a floxed *Thxa2r* allele in embryonic stem (ES) cells. The organization of the endogenous locus, the targeting plasmid, and the *Thxa2r* allele generated after homologous recombination of the plasmid with the wild-type *Thxa2r* allele are shown. The DNA fragments required for assembly of the targeting plasmid were prepared from genomic DNA isolated from 129/SvEv-derived ES cells, and primers were designed using the sequence for the *Thxa2r* gene available in the Celera mouse genome. Fragments were cloned into the vector pXenaLF². LoxP sites are denoted with triangles, frt sites by diamonds, and the NEO gene is represented by the shaded arrow. Relevant restriction sites are abbreviated as follows: B, BamHI; A, ApaI; E, EcoRI. TP, thromboxane-prostanoid receptor. Gray boxes represent coding exons.

Nodose and superior cervical ganglia were collected from five to six mice and snap-frozen on dry ice. Pools of ganglia were homogenized using a glass pestle before RNA collection. RNA was collected using RNeasy reagent (Qiagen) and was converted to cDNA using a High Capacity cDNA RT kit (Applied Biosystems), and TP receptor expression was assessed by qPCR using a commercially available TP

probe and 18S probe as an internal control (Applied Biosystems). Data are shown relative to 129/SvEv *Thxa2r*^{+/+}.

Measurement of airway reactivity in intubated mice. Mice were anesthetized with 70–90 mg/kg pentobarbital sodium (American Pharmaceutical Partners, Los Angeles, CA), tracheostomized, and mechanically ventilated at a rate of 300 breaths/min, tidal volume of

6 ml/kg, and positive end-expiratory pressure of 3–4 cmH₂O with a computer-controlled small-animal ventilator (Scireq, Montreal, Canada). Once ventilated, mice were paralyzed with 0.8 mg/kg pancuronium bromide. Following baseline assessments, mice were exposed to aerosol challenges via an ultrasonic AeroNeb nebulizer (Scireq) connected through the inspiratory line of the ventilator circuit. Animals were ventilated at a rate of 200 breaths/min for 30 s with a tidal volume of 0.15 ml. Airway mechanics using the Forced Oscillatory Technique were determined every 10 s for the following 3 min, as previously described (59). Briefly, following passive expiration, a broadband (1–19.625 Hz) volume perturbation was applied to the lungs while the pressure required to generate the perturbations was assessed (25, 53, 59). The resultant pressure and flow data were fit into a constant-phase model as previously described (26, 59). Similar to other studies assessing forced oscillatory mechanics, we confined our analysis to R_{aw} (Newtonian resistance), which assesses the flow resistance of the conducting airways (53, 59). To assess TXA₂-mediated airway reactivity, mice were exposed to aerosol challenges of the stable thromboxane analog U-46619 (Cayman Chemical, Ann Arbor, MI) as previously described (4). Briefly, mice were exposed to a 30-s aerosol dose-response challenge of 10^{-5} , 10^{-4} , and 10^{-3} M. Following each aerosol challenge, airway responses were assessed for 3 min. In some cases, a bilateral vagotomy or surgical sham was performed immediately before baseline measurements as previously described (4, 14).

Because thromboxane has been suggested to be synergistic with ACh, we also assessed the increases in airway mechanics to methacholine (MCh), an ACh analog, following pretreatment with a mildly provoking dose of U-46619 in naïve mice. Airways were exposed to a 30-s aerosol dose of U-46619 (10^{-5} M) or vehicle (10% ethanol), and changes in airway mechanics were assessed for 3 min. Following this exposure, mice were challenged with a 30-s aerosol dose of MCh (3 mg/ml; Sigma Chemical, St. Louis, MO).

Induction of allergic airway disease. To evaluate the role of tissue-specific deletion of TP receptors in inflammatory cell recruitment and airway reactivity, mice were sensitized by intraperitoneal injection of 20 µg ovalbumin (OVA) emulsified in 2.25 mg aluminum hydroxide gel in a total volume of 200 µl on day 1. Mice were challenged (1 h) via the airways with OVA (1% in saline) for 3 days (days 14–16) using an ultrasonic nebulizer (DeVilbiss Health Care, Somerset, PA). On day 17, mice were anesthetized, placed on a ventilator, and challenged with increasing doses of U-46619 (10^{-5} to 10^{-3} M). In some experiments, mice were analyzed on day 38 to allow for resolution of airway inflammation. Airway measurements were taken every 10 s for 3 min. To assess lung inflammation, bronchoalveolar lavage was performed five times with 1.0 ml of sterile HBSS each time. The cellularity of the bronchoalveolar lavage fluid (BALF) was determined using a hemocytometer.

Statistical analysis. Data are presented as means $\pm$ SE. ANOVA followed by the Tukey-Kramer Honest Significant Difference for multiple comparisons was performed on complex data sets. Statistical significance for single data points was assessed by the Student's two-tailed *t*-test. A *P* value of <0.05 was considered statistically significant.

RESULTS

Generation of mice with tissue-specific loss of the TP receptor. The generation of a *Tbxa2r* allele sensitive to Cre-mediated deletion (F) is shown in Fig. 1. Loss of TP receptors in smooth muscle was verified by Southern blot (Fig. 2A) and qPCR (Fig. 2B). Additionally, the ability of the *Nestin*-cre to mediate loss of TP receptors in neurons was evaluated by Southern blot (Fig. 3A) and qPCR (Fig. 3B). Taken together, these results show that *Tgln*- and *Nestin*-driven cre transgenes

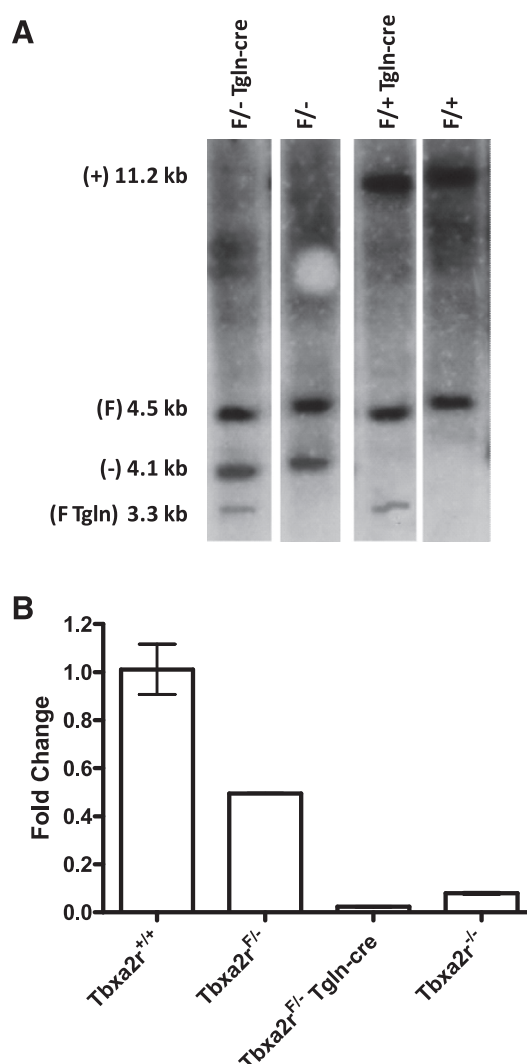


Fig. 2. Characterization of smooth muscle thromboxane-prostanoid (TP) receptor-deficient mice. **A:** to evaluate the efficiency of cre-mediated excision of exon 2 from the *Tbxa2r* allele in airway smooth muscle cells, three denuded tracheas were pooled together and DNA extracted and analyzed by Southern blot. As expected, an 11.2-kb BamH1 fragment corresponding to the wild-type allele was present in DNA prepared from *Tbxa2r*^{F/+}Tgln-cre and *Tbxa2r*^{F/+} littermates. In addition, the 4.5-kb BamH1 fragment generated from the *Tbxa2r*^F allele could also be detected. A 3.3-kb band in mice containing the transgene was also detected, consistent with cre-mediated loss of exon 2 in tracheal smooth muscle. To examine this further, the *Tgln*-cre was moved onto the *Tbxa2r*^{F/-} genetic background, generating *Tbxa2r*^{F/+} Tgln-cre animals. These were intercrossed with mice homozygous for the conditional allele generating *Tbxa2r*^{F/-} Tgln-cre animals and controls. In these animals, bands corresponding to the null allele (4.1 kb) and the floxed allele (4.5 kb) were observed. Again, a 3.3-kb band was also observed in samples from mice containing the transgene. **B:** primary cultures of tracheal smooth muscle cells were prepared from *Tbxa2r*^{F/-} mice, as well as littermates that express the *Tgln*-cre and evaluated by qPCR (*n* = 3). Additional cultures were established from wild-type mice and *Tbxa2r*^{F/-} mice. Expression of the TP receptor in smooth muscle cells derived from *Tbxa2r*^{F/-}Tgln-cre mice did not differ significantly from *Tbxa2r*^{F/-} mice, confirming the loss of TP receptor expression in airway smooth muscle of *Tbxa2r*^{F/-} Tgln-cre mice.

can be used to generate mice in which the loss of TP expression is restricted to either smooth muscle or neurons, respectively.

Airway constriction by TXA₂ agonist is unaffected in mice lacking TP receptors in neurons. Exposure of human and mouse airways to thromboxane results in airway constriction,

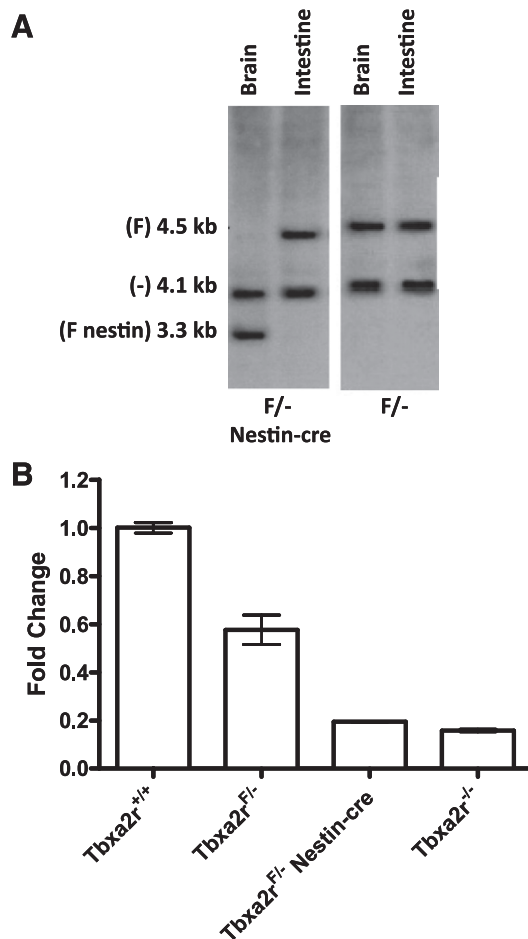


Fig. 3. Characterization of neural TP-deficient mice. **A**: the activity of the *Nestin-cre* in the brain and intestine was evaluated by Southern blot analysis. As expected, the 3.3-kb fragment indicative of cre-mediated excision of exon 2 of *Tbx2r* was observed only in DNA prepared from the brain of *Tbx2r^{F/-}* animals expressing the transgene, confirming neural-specific loss of the TP receptor. **B**: to determine whether expression of the nestin-cre transgene also results in loss of TP receptors in autonomic neurons, nodose/superior cervical (SCG) ganglia were isolated, and RNA was prepared and analyzed by qPCR ($n = 3$). Cre-mediated excision of the conditional allele resulted in TP expression in *Tbx2r^{F/-}* Nestin-cre mice that was not significantly different from that observed in ganglia prepared from *Tbx2r^{-/-}* animals, confirming loss of TP receptor expression in peripheral nerves.

which can be blocked by anti-cholinergic agents (4, 48). In the mouse, changes in airway mechanics in response to thromboxane can be measured in intubated, ventilated animals, and this response is lost in mice in which the vagus nerve has been severed (4). We therefore first asked whether this response to thromboxane was dependent on neuronal expression of TP receptors. TP null ($-/-$) mice were included in experiments to define the extent of TXA₂-mediated responses. Mice were anesthetized, tracheostomized, and exposed to increasing doses of the TP agonist U-46619, and changes in airway resistance (R_{aw}) were measured. As expected, a dose-dependent increase in R_{aw} was observed in response to U-46619 in control mice with heterozygous TP receptor expression ($F/-$). In comparison, U-46619 had no effect on airway resistance in mice homozygous for the null allele, indicating the dependence of this response on TP receptors. By contrast, despite the apparent requirement of the cholinergic reflex for U-46619-induced

constriction in the naïve lung (4), elimination of TP receptors from neuronal tissues ($F/-$ Nestin-cre) had no effect on this bronchoconstrictor response (Fig. 4). Thus, TP receptors on neurons are not required for TXA₂-dependent bronchoconstriction in the normal lung.

Elimination of TP receptors from smooth muscle attenuates airway reactivity after exposure to a TXA₂ agonist. Neural disruption of *Tbx2r* did not affect airway reactivity to U-46619, suggesting that TXA₂-mediated airway constriction may be mediated by other cell types in the lungs known to express this receptor. TP expression by smooth muscle cells is well documented, as is the ability of TP activation to mediate increases in $[Ca^{2+}]_i$ in cultured human primary ASM cells (46). To determine whether TXA₂ acts directly on ASM to mediate changes in airways resistance in vivo, we generated mice lacking TP receptors in smooth muscle, as described above. Mice were anesthetized, tracheostomized and exposed to increasing doses of U-46619. Once again, U-46619 induced a significant, dose-dependent increase in R_{aw} in heterozygous ($F/-$) mice, whereas TP receptor-deficient animals ($-/-$) were completely unresponsive (Fig. 5). Similarly, the R_{aw} response was significantly attenuated in the tissue-specific null ($F/-$ Tgln-cre) mice, indicating that this response depends primarily on activation of TP receptors in smooth muscle cells.

Synergy between TXA₂ and ACh in airway constriction. Previous reports indicate that exposure to low doses of TXA₂ can sensitize the airways to other bronchoconstricting agents, including ACh (32). To verify that this action occurs in the mouse and that it was mediated by TP receptors, wild-type and TP null mice were exposed to subconstrictor doses of U-46619 (10^{-5} M). The initial treatment with U-46619 was followed, 3 min later, by exposure to a nonprovoking concentration of MCh (3 mg/ml), a stable ACh analog that does not induce airway constriction by itself. Pretreatment with 10^{-5} M U-46619 induced a robust MCh response in wild-type animals, but this effect was abrogated in the TP receptor-deficient animals, indicating its

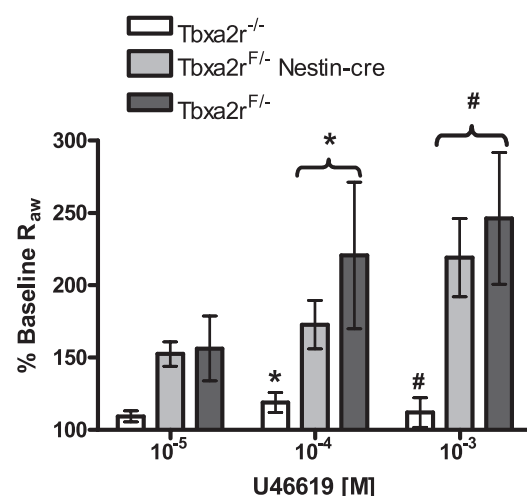


Fig. 4. U-46619-induced airway reactivity in mice deficient in neural TP receptors. *Tbx2r^{F/-}* mice and *Tbx2r^{F/-}* Nestin-cre littermates were anesthetized, tracheostomized, and exposed to increasing doses of the TP agonist U-46619, and changes in airway resistance were measured. *Tbx2r^{-/-}* mice were included in experiments to define TP-mediated responses. Neural-specific deletion of TP receptors does not significantly affect airway resistance induced by U-46619 aerosol challenge. *Tbx2r^{-/-}*, $n = 4$; *Tbx2r^{F/-}* Nestin-cre, $n = 8$; *Tbx2r^{F/-}*, $n = 4$. * $P < 0.05$ and # $P < 0.05$.

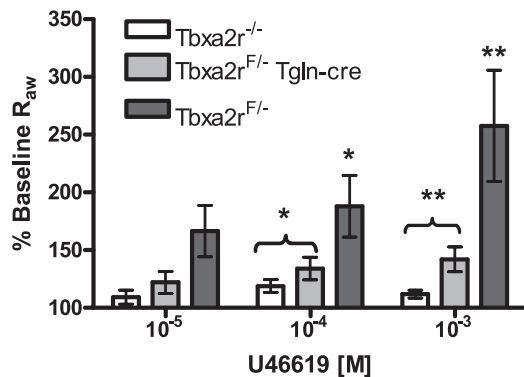


Fig. 5. U-46619-induced airway reactivity in mice deficient in smooth muscle TP receptors. *Tbx2r^{F/-}* mice and *Tbx2r^{F/-} Tgln-cre* littermates were anesthetized, tracheostomized, and exposed to increasing doses of the TP agonist U-46619, and changes in airway resistance were measured. *Tbx2r^{-/-}* mice were included in experiments to define TP-mediated responses. Smooth muscle-specific deletion of TP receptors significantly attenuates airway resistance induced by U-46619 aerosol challenge. R_{aw} , airway resistance. *Tbx2r^{-/-}*, $n = 5$; *Tbx2r^{F/-} Tgln-cre*, $n = 6$; *Tbx2r^{F/-}*, $n = 4$. * $P < 0.05$ and ** $P < 0.01$.

dependence on TP receptors (Fig. 6A). In contrast, U-46619 pretreatment did not enhance responsiveness to stimulation with noncholinergic agonists, including serotonin (5-HT), adenosine, PGD_2 , or $PGF_{2\alpha}$ (data not shown).

To determine whether actions of TXA_2 agonists to enhance the cholinergic responsiveness depend on TP receptors expressed on smooth muscle, mice with conditional loss of TP receptors on ASM and controls were exposed to low doses of U-46619 followed by administration of suboptimal doses of MCh. The enhanced airway constriction to MCh was significantly attenuated in the smooth muscle TP-deficient mice (Fig. 6B). Thus, sensitization of cholinergic responses by exposure to TXA_2 is primarily mediated by TP receptors in smooth muscle.

Pathways for thromboxane-mediated airway constriction are altered in the inflamed lung. Bronchoconstrictors mediate key clinical features of allergic lung disease, and we were therefore interested in testing the characteristics of TXA_2 -mediated airway constriction in the inflamed lung. Accordingly, we induced inflammation by sensitization and repeated aerosol challenges with the antigen OVA, a well-accepted model of robust allergic lung disease. We compared responses in control heterozygous, TP receptor-null, and smooth muscle TP-deficient mice. Animals of each genotype were sensitized to OVA followed by aerosol challenge with saline or OVA (Fig. 7, A and B, all mice were examined concurrently but separated in panels for clarity). Twenty four hours after the final antigen challenge, changes in airway mechanics in response to increasing doses of aerosolized U-46619 were measured. Altered TP receptor expression did not affect the extent of inflammation in the OVA-exposed animals (data not shown). The responses of heterozygous controls from each group that were OVA sensitized but challenged with saline were identical to the previous studies in nonsensitized mice (Figs. 7A and 5, respectively). As expected, U-46619 failed to induce constriction in TP null mice, either in the mice sensitized and challenged with OVA or saline. However, when mice with allergic airway disease were examined, there was brisk airway constriction to U-46619 in the mice lacking TP receptors only in smooth muscle that was virtually identical to the response seen in the heterozygous controls (Fig. 7B). Thus, in

the presence of lung inflammation, TP receptors in smooth muscle are not required for TXA_2 -dependent bronchoconstriction, indicating that this response must be mediated by receptors expressed by other cell lineages.

TP receptors in neuronal cells mediate bronchoconstriction in the inflamed airway. To determine the key pathways for TXA_2 -dependent bronchoconstriction in allergic lung disease, we tested a possible role for neural TP receptors. Allergic lung disease was induced in appropriate cohorts of mice as above, including groups of neural TP-deficient mice. To assess the neural pathways, we first analyzed the ability of wild-type mice to respond to U-46619 challenge following bilateral vagotomy or a surgical sham. The response to U-46619 challenge was abolished following vagotomy in both the naïve and inflamed airway (Fig. 8A), verifying the contribution of the cholinergic reflex in naïve mice (4) and indicating a neural dependence for U-46619-induced constriction in the inflamed airways. To determine if the neural contribution in the inflamed airways was dependent on TP receptors located on the nerves, we

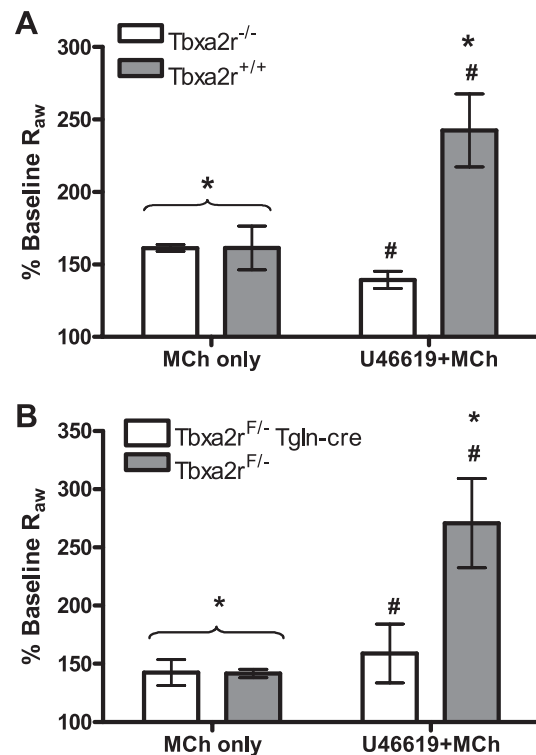


Fig. 6. Nonprovoking dose of U-46619 increases airway sensitivity to cholinergic stimuli. Airways were exposed to low levels of U-46619 (10^{-5} M) followed by subsequent cholinergic stimulation with low levels of methacholine (MCh, 3 mg/ml). Within 3 min of vehicle or U-46619 treatment, mice were exposed to an aerosol produced from a 3 mg/ml solution of MCh, which has been demonstrated to be only mildly provoking. A: *Tbx2r^{+/+}* mice exhibited increased sensitivity to cholinergic stimuli after challenge with a nonprovoking dose as demonstrated by an increase in R_{aw} . Sensitivity of *Tbx2r^{-/-}* mice to MCh did not change following U-46619 challenge. B: control *Tbx2r^{F/-}* heterozygous mice behaved similarly to wild-type animals; sensitivity of the airway MCh was significantly increased following U-46619 challenge. Mice lacking TP receptors in smooth muscle (*Tbx2r^{F/-} Tgln-cre*) failed to exhibit increased sensitivity to cholinergic stimuli following U-46619 challenge. MCh only: *Tbx2r^{+/+}*, $n = 4$; *Tbx2r^{-/-}*, $n = 3$; *Tbx2r^{F/-} Tgln-cre*, $n = 3$; *Tbx2r^{F/-}*, $n = 4$. U-46619 + MCh: *Tbx2r^{+/+}*, $n = 7$; *Tbx2r^{-/-}*, $n = 6$; *Tbx2r^{F/-} Tgln-cre*, $n = 7$; *Tbx2r^{F/-}*, $n = 8$. * $P < 0.05$ and # $P < 0.05$.

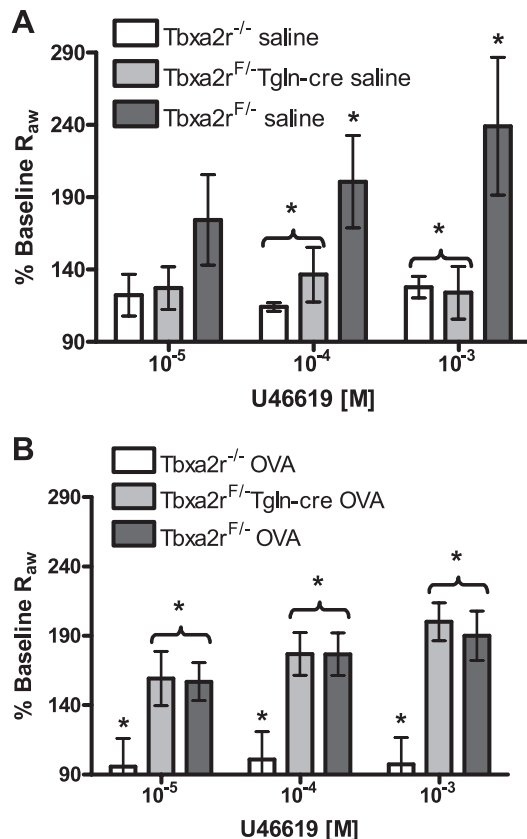


Fig. 7. Airway reactivity of mice lacking TP receptors in smooth muscle to U-46619 in an inflamed airway. Allergic lung disease was induced by sensitization and repeated aerosol with ovalbumin (OVA). Control animals were sensitized to OVA but challenged with saline. **A:** a dose-dependent increase in R_{aw} was observed in the saline-challenged *Tbxa2r*^{F/-} heterozygous mice in response to U-46619. Saline-challenged smooth muscle-deficient *Tbxa2r*^{F/-} *Tgln-cre* had no response to U-46619 at any dose, similar to *Tbxa2r*^{-/-} animals. *Tbxa2r*^{-/-}, $n = 3$; *Tbxa2r*^{F/-} *Tgln-cre*, $n = 5$; *Tbxa2r*^{F/-}, $n = 7$. * $P < 0.05$. **B:** OVA-challenged *Tbxa2r*^{F/-} *Tgln-cre* mice showed significantly increased airway reactivity compared with saline-challenged animals, similar to that observed in *Tbxa2r*^{F/-} heterozygous mice. *Tbxa2r*^{-/-}, $n = 3$; *Tbxa2r*^{F/-} *Tgln-cre*, $n = 5$; *Tbxa2r*^{F/-}, $n = 6$. * $P < 0.05$. All animals were evaluated in the same experiment; panels were separated for clarity.

assessed the response of neural TP-deficient mice to respond to U-46619 in the allergic setting. Loss of neuronal TP receptor expression did not alter the development of inflammation in the OVA-challenged animals (data not shown). Again, in the naïve animals and OVA-sensitized animals challenged with saline, the loss of neuronal TP expression did not alter the response to U-46619 (Figs. 4 and 8B, respectively). By contrast, OVA-challenged mice lacking TP receptors only in neurons failed to respond to U-46619 (Fig. 8C). Thus, in the inflamed lung, the pathway mediating TXA₂-dependent airway constriction is completely switched from TP receptors in smooth muscle to those in neuronal tissues. It can be envisioned that this mechanistic switch could be induced by changes in expression of the TP receptor on either smooth muscles and/or neurons. However, preliminary studies examining TP receptor expression by qPCR did not reveal any significant differences between cultured ASM, denuded trachea, or ganglia collected from naïve mice compared with mice with allergic lung inflammation (Fig. 9, A–C).

Altered TP receptor-mediated airway responsiveness persists after resolution of allergic inflammation. We next determined whether the characteristics of TXA₂-mediated bronchoconstriction were maintained after resolution of the inflam-

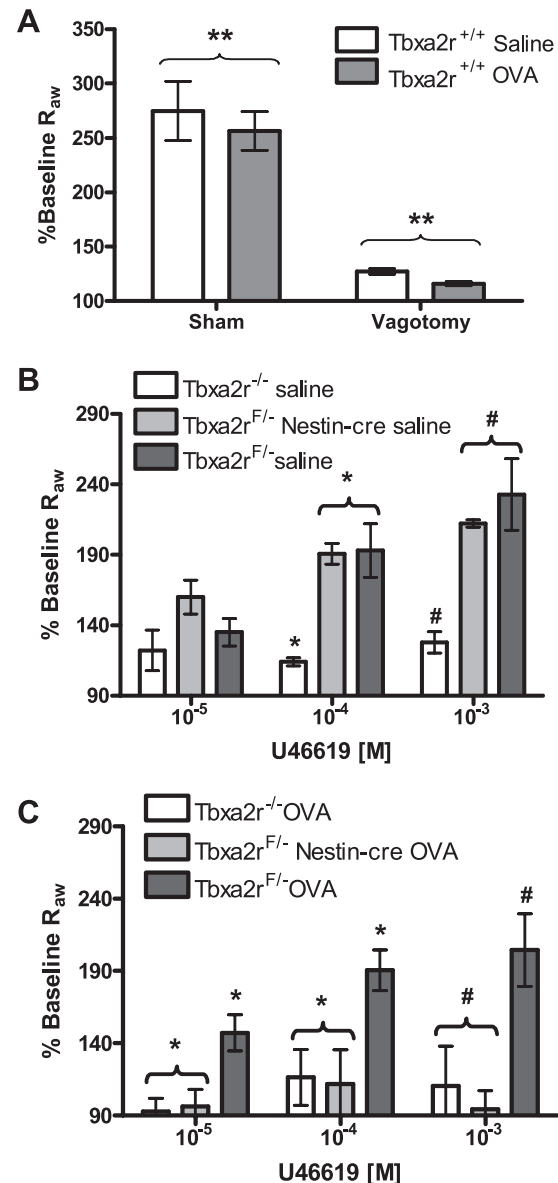


Fig. 8. U-46619-induced airway reactivity is dependent on neural TP receptors. Allergic lung disease was induced by sensitization and repeated aerosol with OVA. Control animals were sensitized to OVA but challenged with saline. **A:** naïve and inflamed wild-type (WT) mice received a single aerosol of U-46619 (10⁻³ M) following a surgical sham or bilateral vagotomy, and airway mechanics were assessed. Vagotomy abolished U-46619-induced increases in airway reactivity (R_{aw}) in both naïve mice and in those with OVA-induced airway disease. Saline: sham, $n = 3$; vagotomy, $n = 3$. OVA: sham, $n = 5$; vagotomy, $n = 5$. ** $P < 0.001$. **B:** following saline challenge, a dose-dependent increase in R_{aw} was observed in both *Tbxa2r*^{F/-} and *Tbxa2r*^{F/-} *Nestin-cre* mice in response to U-46619. No change in R_{aw} was seen in *Tbxa2r*^{-/-} animals. *Tbxa2r*^{-/-}, $n = 3$; *Tbxa2r*^{F/-} *Nestin-cre*, $n = 4$; *Tbxa2r*^{F/-}, $n = 4$. * $P < 0.05$ and # $P < 0.01$. **C:** following OVA challenge, an increase in airway reactivity following U-46619 challenge was seen in *Tbxa2r*^{F/-} mice but not in *Tbxa2r*^{F/-} *Nestin-cre* mice with inflamed airways. *Tbxa2r*^{-/-}, $n = 3$; *Tbxa2r*^{F/-} *Nestin-cre*, $n = 4$; *Tbxa2r*^{F/-}, $n = 6$. * $P < 0.05$ and # $P < 0.01$. All animals were evaluated in the same experiment; panels were separated for clarity.

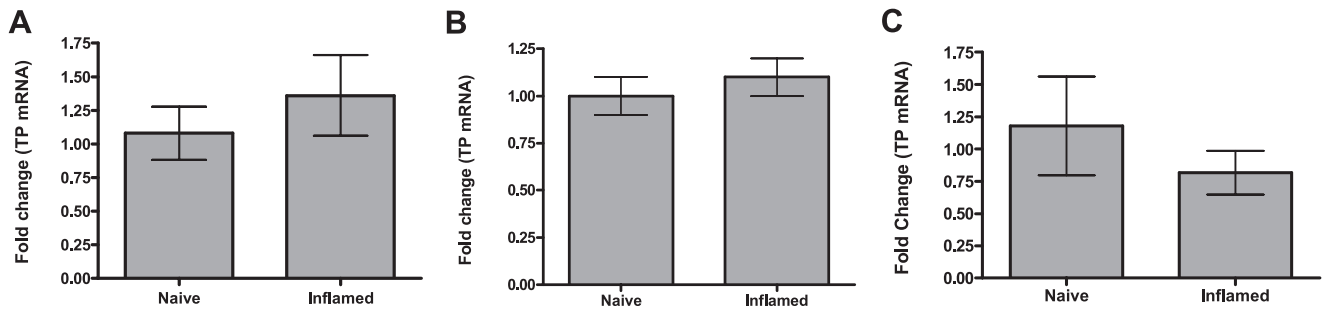


Fig. 9. Expression of TP receptors in naïve and OVA-treated mice. Allergic inflammation was induced in WT 129S6/SvEv mice by sensitization and repeated aerosol with OVA, and expression of TP receptors in cultured smooth muscle cells (A), trachea (B), and neurons (C) in mice with allergic airway disease relative to naïve controls was assessed via qPCR. After induction of allergic inflammation, TP receptor expression in cultured smooth muscle ($n = 3$), trachea ($n = 6$), and ganglia ($n = 3$ pools of 6 each) remained equal to expression in naïve animals.

matory response. Inflammation was induced using OVA sensitization as described above, in cohorts of mice lacking TP receptors in smooth muscle or neurons and in control littermates with heterozygous expression of TP receptors (Fig. 10, A and D). Consistent with the previous experiment, in animals tested 24 h after the final OVA challenge, smooth muscle TP null mice exhibited normal responsiveness to U-46619, whereas responses in neural TP null mice were substantially attenuated (Fig. 10, B and E). The remaining animals were housed with no further exposure to antigen for 3 wk after which their responsiveness to U-46619 was tested. Analysis of BALF from these animals showed markedly reduced cellularity, indicating resolution of their lung inflammation (Fig. 10, A and D). Nonetheless, the pattern of U-46619 responses was identical to that observed when the lung was inflamed, that is, dependence on smooth muscle TP receptors was lost, and airway constriction showed a persistent requirement for neural TP receptors, even after inflammatory cells were cleared from the airways (Fig. 10, C and F).

DISCUSSION

TXA₂ is a potent airway constrictor. Our studies show that this activity is mediated through complex pathways, which differ in the naïve and inflamed lung. In the mouse, inhaled TXA₂ analog (U-46619) resulted in a robust and consistent increase in central airway resistance, the specificity of which is demonstrated by the absence of this response in mice lacking TP receptor expression. Previous studies in our laboratory have shown that this response was lost in vagotomized mice or in mice lacking expression of the muscarinic acetylcholine receptor 3 (mAChR₃, M₃) (4). This dependence of the TXA₂ response on cholinergic innervation supports earlier studies that suggest that TXA₂ does not act directly on ASM but rather that it stimulates release of ACh from parasympathetic fibers that innervate the ASM (1, 3, 29, 30). However, the studies reported here show that the response to TXA₂ in normal lung requires expression of TP receptors by smooth muscle cells.

TP receptors are found on many types of smooth muscle cells, including ASM cells (10). Furthermore, experiments with cultured primary smooth muscle cells have demonstrated TXA₂-mediated increases in intracellular Ca²⁺ (10, 18, 23, 29, 30, 62), often accompanied by contraction, indicating a direct action of TXA₂ on smooth muscle cells. Our findings likewise suggest that TXA₂ mediates ASM contraction by acting di-

rectly on ASM cells. However, our studies reveal additional levels of complexity in this response. First, our previous studies have shown that action of TXA₂ in the airways is not itself sufficient to bring about bronchoconstriction: it is dependent on the presence of M₃ receptors. Second, this action of TXA₂ on the smooth muscle is lost after induction of allergic airway disease.

A number of models can be proposed to explain the dependence of TP-mediated smooth muscle constriction on the cholinergic pathways. The intrinsic tone of the ASM has been shown to be dependent on cholinergic innervations, reflecting constitutive release of ACh from parasympathetic fibers. It is possible that intracellular Ca²⁺ levels after TP receptor activation are insufficient to trigger constriction in completely relaxed ASM cells in which no M₃ receptors are occupied. In this model, M₃/ACh could be viewed as priming the smooth muscle, making it responsive to secondary stimulation by agents such as TXA₂. Alternatively, TXA₂ may sensitize the smooth muscle cells, rendering them responsive to the constitutive levels of local ACh present in the synaptic junctions typically required to maintain airway tone. Our finding that pretreatment with nonprovoking doses of TXA₂ sensitized the airway to constrict to a nonprovoking dose of MCh is consistent with this model. Moreover, we show that this effect is mediated by TP receptors in ASM. U-46619 did not sensitize the airway to 5-HT (data not shown), another potent airway constrictor in the mouse that has been shown to induce constriction in a similar muscarinic receptor-dependent fashion (14, 60). This observation does not support a simple model in which signaling by all G_q-coupled smooth muscle receptors is additive. Rather, it suggests a unique and necessary role for the ACh M₃ receptor in the function of other G protein-coupled receptors (GPCRs) that cannot be substituted by increased activity or combined activation of these receptors. We cannot, however, rule out the possibility that the attenuated R_{aw} observed in mice lacking smooth muscle expression of TP reflects not only the loss of functional receptor from ASM but also loss of the receptor from the vasculature of the lung. The Tgln promoter drives cre expression in both vascular and ASM cells. The potent airway constriction observed in response to this TP receptor agonist may reflect not only the ability of TP to constrict ASM but also its ability to constrict vascular beds and thus alter blood flow through the lung. This constriction could attenuate both the removal of ACh from the synapse and also decrease clearance of inhaled MCh. The mechanism(s) medi-

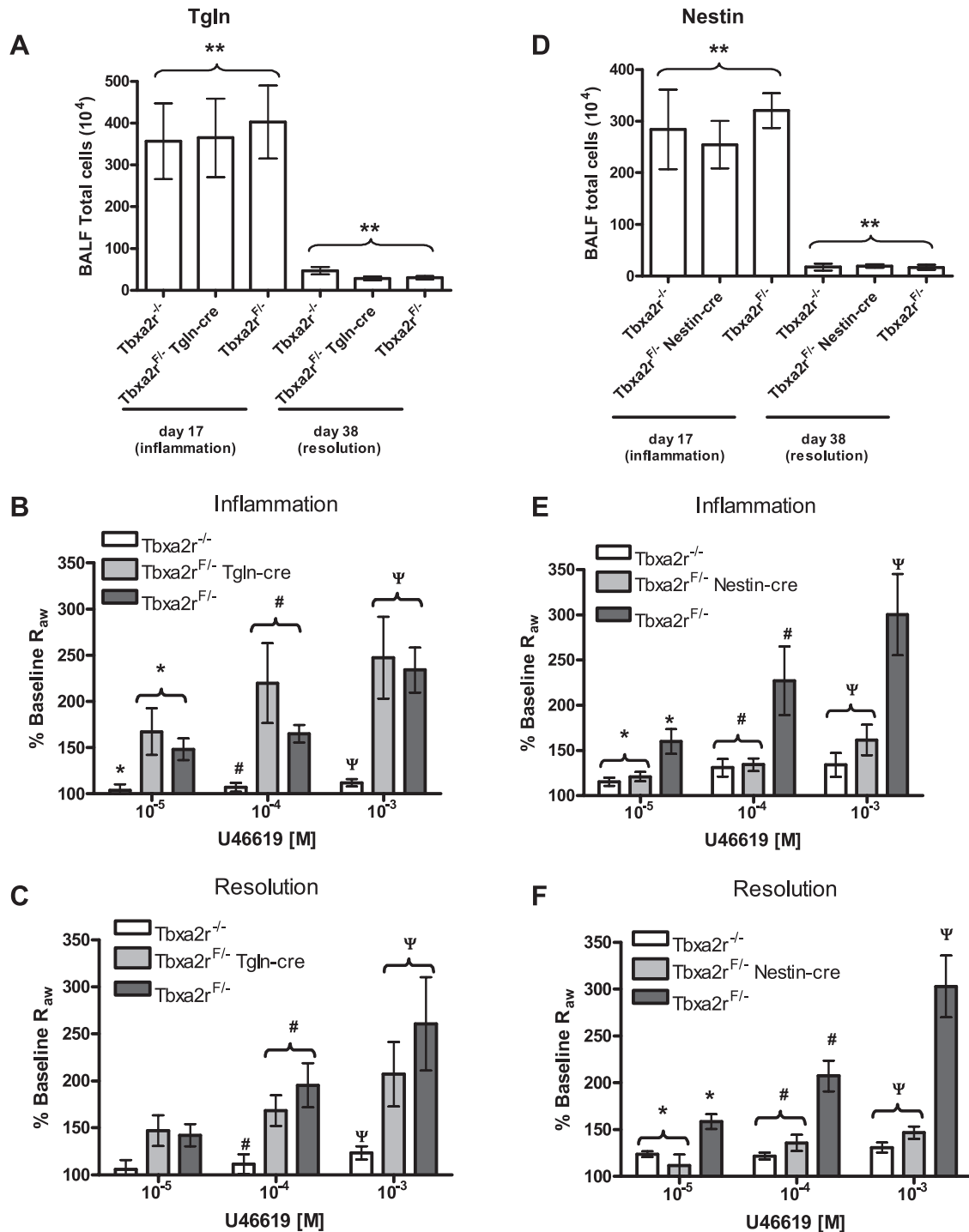


Fig. 10. Airway reactivity following resolution of allergic inflammation in neural TP-deficient mice. Allergic lung disease was induced by sensitization and repeated aerosol with OVA in smooth muscle (*Tgln*) TP-deficient mice (left), neural (*Nestin*) TP-deficient mice (right), and their heterozygous and TP-deficient controls. OVA aerosols resulted in significant inflammation on day 17 that was completely resolved by day 38 (A and D). Airway reactivity to U-46619 was evaluated on both day 17 during airway inflammation (B and E) and on day 38 following resolution of inflammation (C and F). Both during inflammation and after resolution, OVA-challenged *Tbxa2r*^{F/-} *Tgln*-cre mice showed significantly increased airway reactivity similar to that observed in *Tbxa2r*^{F/-} heterozygous mice, whereas OVA-challenged *Tbxa2r*^{F/-} *Nestin*-cre mice failed to respond to U-46619. BALF, bronchoalveolar lavage. Inflammation: *Tbxa2r*^{-/-}, *n* = 3; *Tbxa2r*^{F/-} *Tgln*-cre, *n* = 5; *Tbxa2r*^{F/-}, *n* = 6; *Tbxa2r*^{F/-} *Nestin*-cre, *n* = 7; *Tbxa2r*^{F/-}, *n* = 7. **P* < 0.05, #*P* < 0.05, and Ψ*P* < 0.05. Resolution: *Tbxa2r*^{-/-}, *n* = 3; *Tbxa2r*^{F/-} *Nestin*-cre, *n* = 6; *Tbxa2r*^{F/-}, *n* = 6. ***P* < 0.01, **P* < 0.05, #*P* < 0.05, and Ψ*P* < 0.05.

ating this synergy between the M_3 and TP receptors could reflect a number of interactions between these two G_q -coupled smooth muscle receptors, including heterodimerization, cross-reactivity between GPCR downstream effectors, and G protein redistribution (28). Indeed, several studies have documented such interactions between TP receptors and other GPCRs (16, 17, 28, 63, 64).

In the inflamed lung, the pathways mediating TXA_2 responses in the airway are completely modified. In this case, elimination of TP receptors from smooth muscle had surprisingly little impact on the response to TXA_2 . The mouse model of allergic inflammation is characterized by significantly increased mucus levels in the lung. Because mucus secretion is under cholinergic control, the constriction seen in smooth muscle-deficient TP mice could be simply due to mucus-induced airway obstruction in the inflamed lung. However, this is not supported by the fact that, although expression of mucin genes, specifically *Muc5ac*, returns to naïve levels after resolution of airway inflammation (data not shown), the U-46619-induced constriction remains dependent on expression of TP by neurons. The lack of a direct smooth muscle response to thromboxane in the allergic lung could reflect downregulation of the receptor on smooth muscle or an upregulation of the receptor on neurons, perhaps as an adaptive response, resulting in dominant neural regulation of this response. However, a cursory examination of the expression of TP mRNA on cultured smooth muscle cells, trachea denuded of epithelial cells, and ganglia from naïve mice and mice with allergic airway disease did not reveal significant change in expression. It is possible that such differences will be detected in future studies examining specific cell populations, however, at this time, antibodies specific for the mouse TP receptor are not available. Alternatively, it is possible that TP receptor expression is unaffected, but alterations in signal-effector coupling may impact the character of the downstream pathways activated in a background of inflammation. Recent studies have also suggested that, in addition to classical G protein pathways, GPCRs may signal through alternative pathways, such as those mediated by β -arrestin, and the utilization of these pathways may be modified in disease states (reviewed in Ref. 44). Such alterations in signaling may affect interactions between the TP receptor and the M_3 pathway. This may be because of effects of inflammatory mediators, perhaps through changes in either the expression or distribution of the M_3 receptors. Finally, changes in receptor and/or mediator expression within neuronal pathways after inflammation have been well documented, and evidence supports plasticity of both afferent and efferent neuronal pathways in both mice and other species. For example, Fryer and Jacoby (reviewed in Ref. 20) reported changes in function/expression of inhibitory mAChR₂ (M_2) receptors in guinea pig models of asthma. These studies suggested that release of major basic protein by eosinophils resulted in decreased M_2 receptors on cholinergic fibers and consequent loss of an essential ACh inhibitory feedback loop. Additionally, alteration in the synthesis of neuropeptides by sensory nerves has been reported in a number of inflammatory models (reviewed in Ref. 11). For example, allergic airway inflammation in guinea pigs results in the induction of substance P in airway nodose sensory neurons that were previously devoid of this neuropeptide (54). Furthermore, changes in sensory neurons have not been limited to tachykinins. A change in the expres-

sion pattern of TRPV1 was noted in rats before and after sensitization with allergen, with the sensitized animals showing expression on myelinated afferents in addition to its normal expression on C fibers (41).

Our studies clearly show a mechanistic switch in TXA_2 -mediated bronchoconstriction, from smooth muscle-dependent actions to neuronal-mediated actions. However, because nestin is expressed in both sensory and cholinergic neurons, we cannot at present determine which pathway is altered with respect to TP receptor activity in the inflamed airways. Models in which TXA_2 alters the activity of both efferent and afferent pathways have been proposed for both the lung and other organ systems (3, 5, 33, 57). For example, early ex vivo studies of canine tracheas showed that TXA_2 was able to increase the amplitude of smooth muscle contraction of canine trachea evoked using electrical field stimulation (3). Because these changes could be observed using concentrations of TXA_2 that alone could not constrict the smooth muscle, it was suggested that TXA_2 was capable of prejunctional stimulation of cholinergic neurons increasing release of ACh. Alternatively, TXA_2 may act directly on sensory neurons in the inflamed airways, since direct stimulation of pulmonary afferent nerves has been shown for other prostanoids. For example, using preparations of pulmonary primary sensory neurons isolated from the rat nodose ganglia, Kwong and Lee (35) showed that exposure to PGE_2 enhanced the response of these neurons to chemical stimuli. Likewise, expression of TP receptors has been detected in at least a subset of neurons with cell bodies located within the nodose ganglia (58). Furthermore, stimulation of TP receptors on mouse dorsal root ganglion neurons by U-46619 has been documented (5), and TXA_2 has been shown to contribute to myocardial ischemia through direct stimulation of TBXA₂ receptor located within the cardiac sensory nervous system (22).

Our observation that the switch from dependence of bronchoconstriction from TP receptors on smooth muscle to those on neurons persisted after removal of the inflammatory stimulus and resolution of much of the inflammation in the airways was perhaps our most unexpected finding. This indicates a long-term, adaptive change in the physiology of the ASM cells after exposure to allergic inflammation and is not consistent with various models of receptor desensitization or transient changes in receptor expression, although instances of receptor dysfunction in response to environmental exposures like ozone and viral infection have been reported and found to depend on inflammation and inflammatory cells such as leukocytes (21, 24). The reversibility of these signaling changes was not examined; only animals with active airway disease were studied. Another potential mechanism might be inflammation-induced epigenetic changes causing sustained changes to gene expression patterns. Several studies have suggested links between environmental exposures, even prenatal exposures, and asthma development and/or risk (reviewed in Ref. 39).

Our studies directly illustrate the profound impact of inflammation on the physiology of the lung, and particularly on the pathways regulating airway mechanics. The dramatic modification of pathways mediating airway reactivity in response to the potent endogenous bronchoconstrictor TXA_2 from dependence on smooth muscle to neuronal receptors indicates that the lung is capable of a broad range of adaptive measures to respond to pathological challenges. These adaptations will have significant consequences on the pathogenesis of lung

diseases such as asthma, including influences on responsiveness to bronchodilator therapies.

ACKNOWLEDGMENTS

We thank Kim Burns for assistance with histology and staining, Dr. Bob Bagnell for help with data analysis, and Dr. Stephen Tilley for helpful discussion.

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GRANTS

This work was supported by National Heart, Lung, and Blood Institute Grants NIH/NHLBI RO1HL-068141 and HL-080697 (to B. H. Koller).

DISCLOSURES

The authors have no conflicting financial interests.

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