ORIGINAL ARTICLE

# Effects of Cigarette Smoke Extract and Nicotine on Bronchial Tone and Acetylcholine-Induced Airway Contraction in Mouse Lung Slices

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## Abstract

*Background:* Tobacco smoke is a key risk factor for chronic obstructive pulmonary disease, but it may also alter the pathophysiology of asthma. In the present study, we analyzed whether tobacco smoke has acute or chronic effects on bronchial tone and whether it alters bronchial reactivity in vitro.
*Methods:* Airways in murine lung slices were digitally recorded and the change in cross-sectional area with time was quantified. T-bet KO mice served as a model for bronchial hyperreactivity. T-bet KO mice show a shift towards type 2 helper T lymphocytes and display histological as well as functional characteristics of asthma. Cigarette smoke extract (CSE) was obtained using commercially available cigarettes (Gauloise Blondes) by drawing cigarette smoke slowly through a water pump into a tube containing 10 mL of DMEM culture medium.
*Results:* Acute exposure to CSE led to relaxation of the airway. Acute exposure to nicotine resulted in a minor relaxation of the airway in Balb/C mice and in nonsignificant relaxation of the airway in T-bet KO mice. The nicotinic acetylcholine-receptor hexamethonium partially inhibited CSE-induced airway relaxation. Airway contraction in response to acetylcholine was stronger in T-bet KO mice than in Balb/C mice. After exposure to CSE or nicotine for 48 hours, acetylcholine-induced airway contraction was no longer different between the 2 types of mice.
*Conclusions:* Our data indicate that acute exposure to CSE leads to airway relaxation, which is partially mediated by nicotine. Chronic exposure to CSE reverses bronchial hyperreactivity in the airways of T-bet KO mice; this effect can be mimicked by chronic exposure to nicotine.

**Key words:** Tobacco smoke. Nicotine. Bronchial tone. Hyperreactivity. Asthma.

## Resumen

*Antecedentes:* El humo del tabaco es un factor de riesgo clave de la enfermedad pulmonar obstructiva crónica (EPOC), si bien también puede alterar la fisiopatología del asma. En este estudio se analizó si el humo del tabaco tiene efectos agudos o crónicos sobre el tono bronquial y si altera la reactividad bronquial in vitro.
*Métodos:* Se registraron digitalmente las vías respiratorias en secciones pulmonares de ratón y se cuantificó el cambio del área transversal con el tiempo. Se emplearon ratones KO T-bet como modelo de hiperreactividad bronquial. Los ratones KO T-bet muestran un cambio a linfocitos T cooperadores de tipo 2 y presentan las características histológicas y funcionales del asma. Se obtuvo extracto de humo de tabaco (EHT) a partir de cigarrillos comercialmente disponibles (Gauloise Blondes) extrayendo lentamente el humo de tabaco a través de una bomba de agua y transfiriéndolo a un tubo con 10 ml de medio de cultivo DMEM.
*Resultados:* La exposición aguda al EHT provocó una relajación de las vías respiratorias. La exposición aguda a la nicotina provocó una leve relajación de las vías respiratorias en los ratones Balb/C y una relajación no significativa de las vías respiratorias en los ratones KO T-bet. El hexametonio del receptor nicotínico de acetilcolina inhibió parcialmente la relajación de las vías respiratorias inducida por el EHT. La contracción de las vías respiratorias en respuesta a la acetilcolina fue mayor en los ratones KO T-bet que en los ratones Balb/C. Tras la exposición a EHT o a nicotina durante 48 horas, la contracción de las vías respiratorias inducida por acetilcolina ya no presenta diferencias entre ambos tipos de ratones.

*Conclusiones:* Los datos indican que una exposición aguda al EHT provoca una relajación de las vías respiratorias, mediada parcialmente por la nicotina. La exposición crónica al EHT invierte la hiperreactividad bronquial de las vías respiratorias de los ratones KO T-bet; este mismo efecto también puede producirse por una exposición crónica a la nicotina.

Palabras clave: Humo de tabaco. Nicotina. Tono bronquial. Hiperreactividad. Asma.

## Introduction

Tobacco smoke is a key risk factor for chronic obstructive pulmonary disease, lung cancer, and coronary heart disease, and it may also play a role in asthma. Lazarus et al [1] showed that the response to inhaled corticosteroids, which are the backbone of asthma therapy today, is diminished by smoking. Furthermore, children with asthma show worse symptoms if their parents smoke [2]. Nicotine, which is one of the most active pharmacological compounds in tobacco smoke, has anti-inflammatory effects in ulcerative colitis [3] and has been shown to decrease the release of interleukin 1ß and tumor necrosis factor in alveolar macrophages [4]. We performed an in vitro study to determine whether tobacco smoke has acute or chronic effects on bronchial tone and whether it alters bronchial reactivity to acetylcholine (ACH). We also evaluated whether possible effects are mediated by nicotine.

T-bet is a type 1 helper T cell ($T_H1$)–specific transcription factor that has the ability to convert $T_H2$ cells into $T_H1$ cells. Mice in whom the T-bet gene has been deleted (T-bet KO mice) spontaneously develop airway remodeling similar to that seen in asthma and have several functional and inflammatory features that are characteristic of this disease [5]. We recently showed that airways in lung slices from T-bet KO mice maintain bronchial hyperreactivity in vitro in that they have an elevated contractile response to ACH [6]. In the present study, we used this model to test the effects of tobacco smoke on bronchial hyperreactivity.

Acute exposure to cigarette smoke extract (CSE) leads to airway relaxation, which is partially mediated by nicotine. Chronic exposure to CSE reverses bronchial hyperreactivity in the airways of T-bet KO mice. This effect can be mimicked by chronic exposure to nicotine.

## Methods

Cell culture reagents were obtained from Life Technologies (Eggenstein, Germany); other reagents were from Sigma (Deisenhofen, Germany). Balb/C mice were purchased from Harlan-Winkelmann (Borchen, Germany) and T-bet KO mice on a Balb/C background were purchased from Charles River (Charles River Breeding Labs, Needham, Massachusetts, USA). All procedures were approved by the Ethics Committee of the Ludwig-Maximilians-University in Munich, Germany.

Lung slices were prepared as described elsewhere [6]. Briefly, 42 to 77-day-old mice were sacrificed by intraperitoneal injection of pentobarbital and the chest wall was removed. The trachea was cannulated using an intravenous catheter and the lungs were inflated with 2% agarose-sHBSS at 37°C. Subsequently, 0.1 to 0.2 mL of air was injected to flush the agarose-sHBSS out of the airways and the agarose was gelled by keeping the mouse preparation at 4°C. The lungs were removed and slices of approximately 200 µm were cut with an EMS-4000 Tissue Slicer (Electron Microscopy Sciences, Fort Washington, Pennsylvania, USA). The slices were maintained by floating them in DMEM supplemented with antibiotics and antimycotics at 37°C in 5% $CO_2$ for up to 5 days. In culture, lung slices maintain their contractile properties for up to 1 week [7]. Experiments were therefore performed on days 2 to 5 of cultivation and each slice was used for 1 experiment only. For each experimental group, slices from at least 3 different mice were used. To measure airway cross-sectional area, lung slices were placed in culture dishes, immersed in sHBSS, and held in position by a custom-made gold grid. Bright field images were recorded using a digital CCD camera (AxioCam MRm, Carl Zeiss Vision, Munich, Germany). Frames were captured in time-lapse (1 frame/s) and the cross-sectional area of the airway was measured by pixel summing using the image analysis software "Scion" (Scion Corporation, Frederick, Maryland, USA).

Cigarette smoke extract (CSE) was obtained using commercially available cigarettes (Gauloise Blondes, nominal nicotine 0.8 mg) as described previously [8]. Briefly, the smoke of 1 cigarette was drawn slowly through a water pump into a tube containing 10 mL of DMEM culture medium. Each cigarette was completely burned after 5 minutes. The CSE was then sterile filtered and kept at –32°C. The medium nicotine concentration in the CSE was found to be 168 µg/mL.

The Mann-Whitney rank sum test or analysis of variance by ranks (combined with pairwise multiple comparisons) was performed using Sigma Stat software (Jandel Scientific, Chicago, Illinois, USA). A *P* value of less than .05 was considered statistically significant.

## Results

Airways in lung slices (Figure 1) were monitored using phase-contrast microscopy and recorded in time-lapse at 1 frame/s. For each frame, the area of the airway was calculated and the change in airway area in response to agonists was monitored (Figure 2). Airways in lung slices from Balb/C and T-bet KO mice were exposed to 1 µM ACH with no prior incubation with CSE or nicotine. Contractions in lung slices from T-bet KO mice were significantly stronger (7±3% of starting value for Balb/C vs 45±6% for T-bet KO; $P<.001$; n=15 airways from 3 different mice; Figure 3).

Next, the acute effects of 5% CSE or 5% nicotine (10 µg/mL) on airway tone were investigated. CSE induced

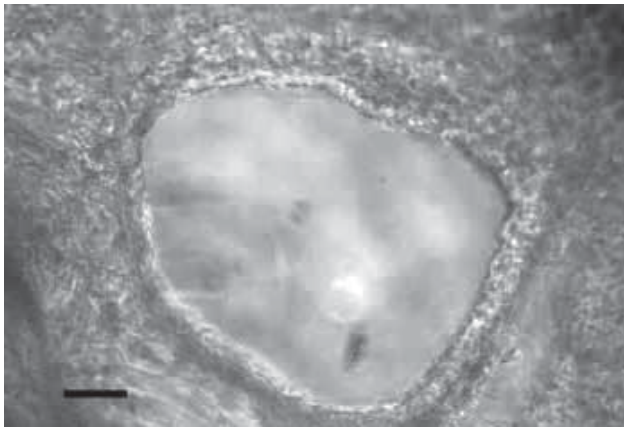


Figure 1. Phase-contrast image of an airway in a lung slice. The airway is cut in cross-section and has a clearly visible lumen. The scale bar represents 50 µm.

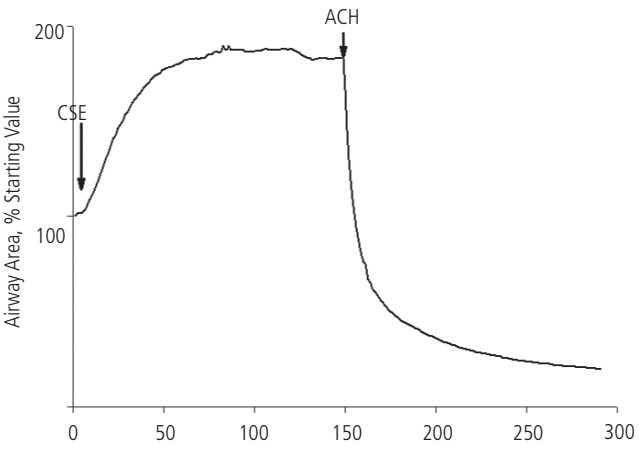



Figure 2. Airways were monitored using phase-contrast microscopy and recorded in time-lapse at 1 frame/s in response to cigarette smoke extract (CSE, 5%) and 1 µM acetylcholine. CSE led to relaxation (increase in airway area) while ACH induced contraction of the airway (decrease in airway area). ACH indicates acetylcholine; CSE, cigarette smoke extract.

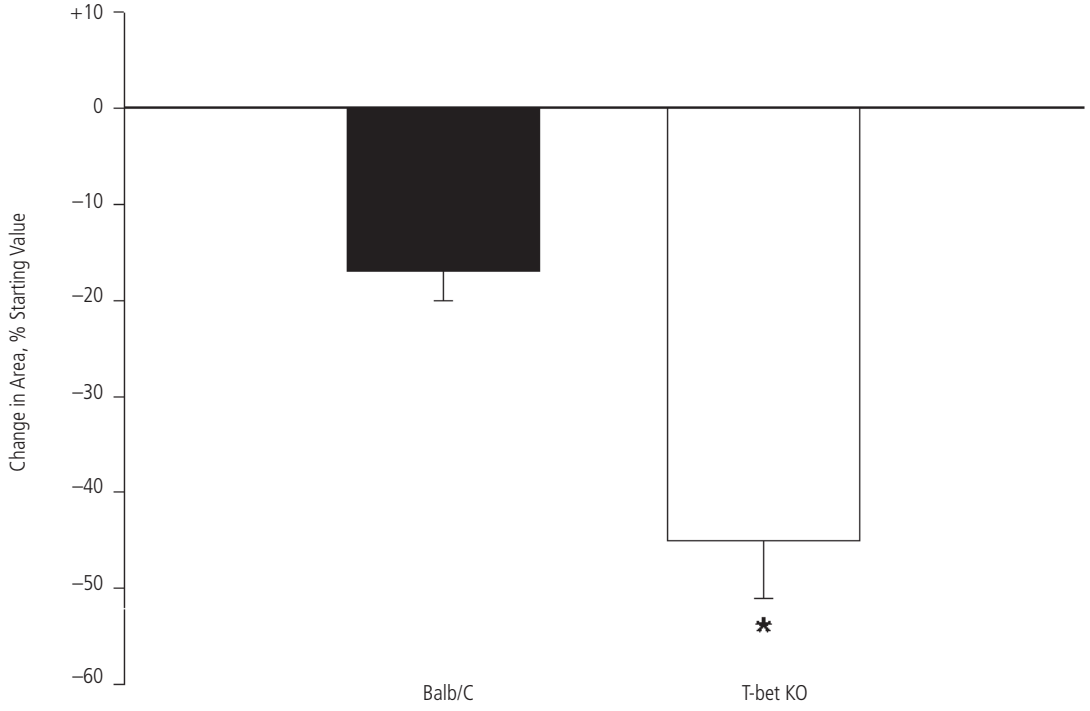



Figure 3. Airways in lung slices from Balb/C mice and T-bet KO mice—serving as an asthma model—were exposed to ACH and the change in airway area was quantified. Contractions in the airways of T-bet KO mice were significantly stronger (*$P$<.001, n=15 airways from 3 different mice). ACH indicates acetylcholine.

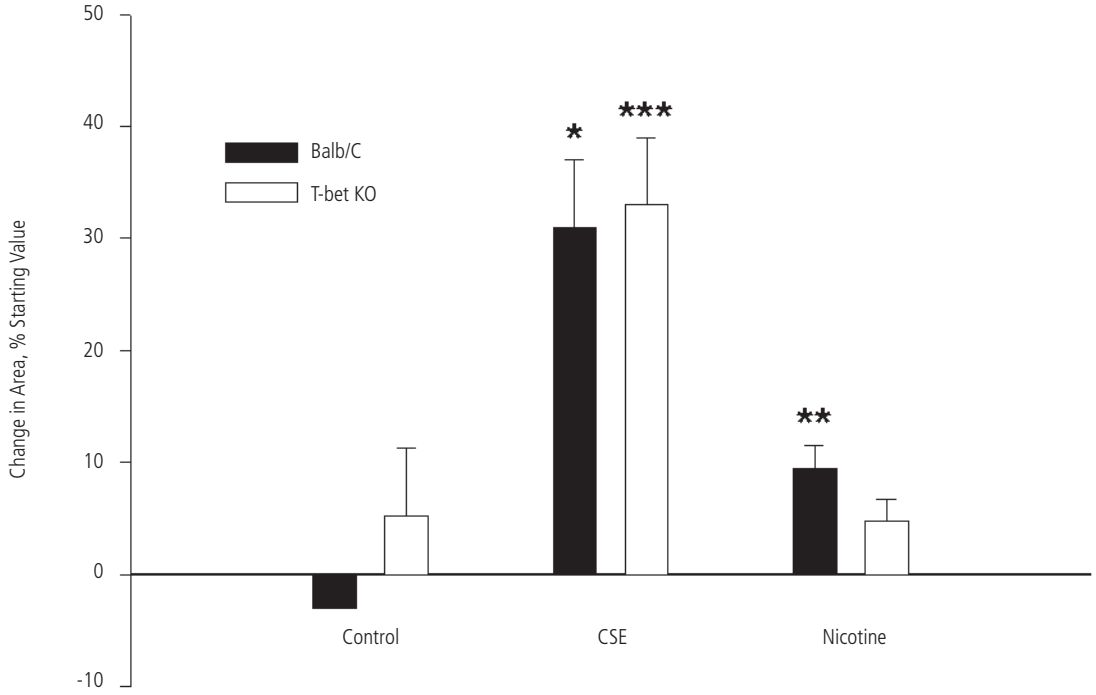
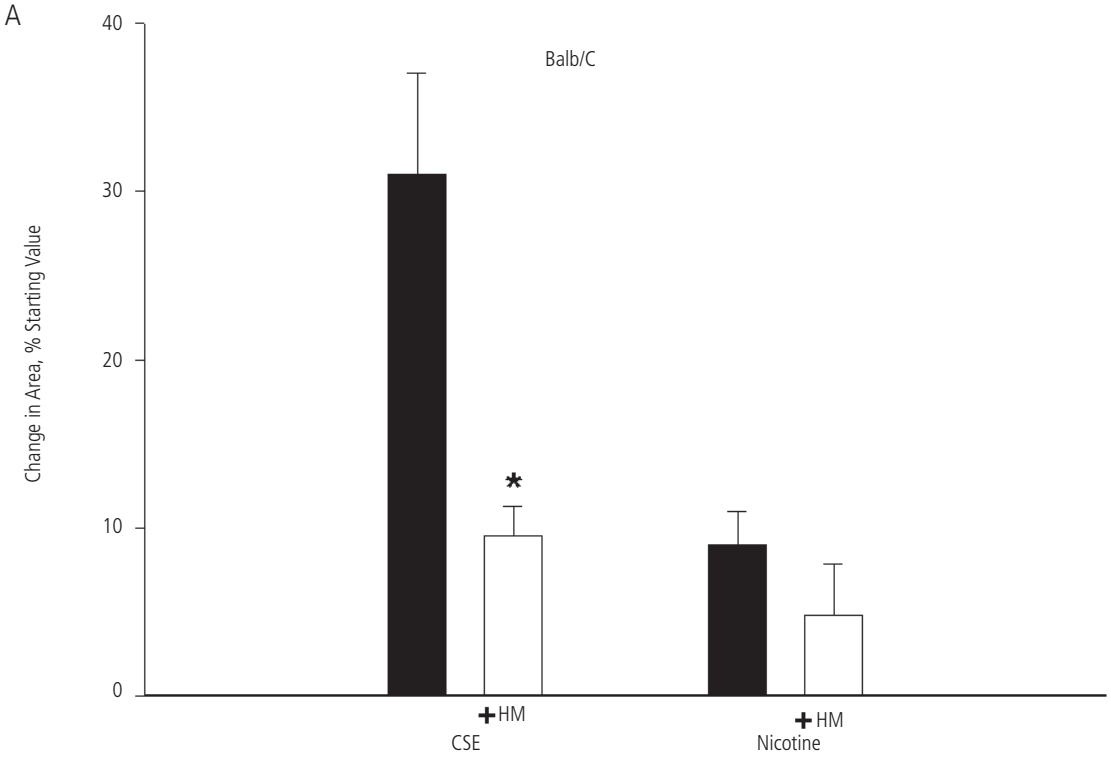



**Figure 4.** Airways in lung slices from Balb/C and T-bet KO mice were exposed to 5% CSE or 5% nicotine (10 µg/ml) and the change in airway area was quantified. Control slices were exposed to medium. In lung slices from Balb/C mice CSE and nicotine led to relaxation of the airways, with the relaxation being stronger for CSE (**P*<.05 vs control and nicotine, ***P*<.05 vs control, n=8-12 lung slices from 3 different mice). In lung slices from T-bet KO mice, only CSE caused significant airway relaxation (****P*<.05 vs control and nicotine, n=10 lung slices from 3 different mice). CSE indicates cigarette smoke extract.



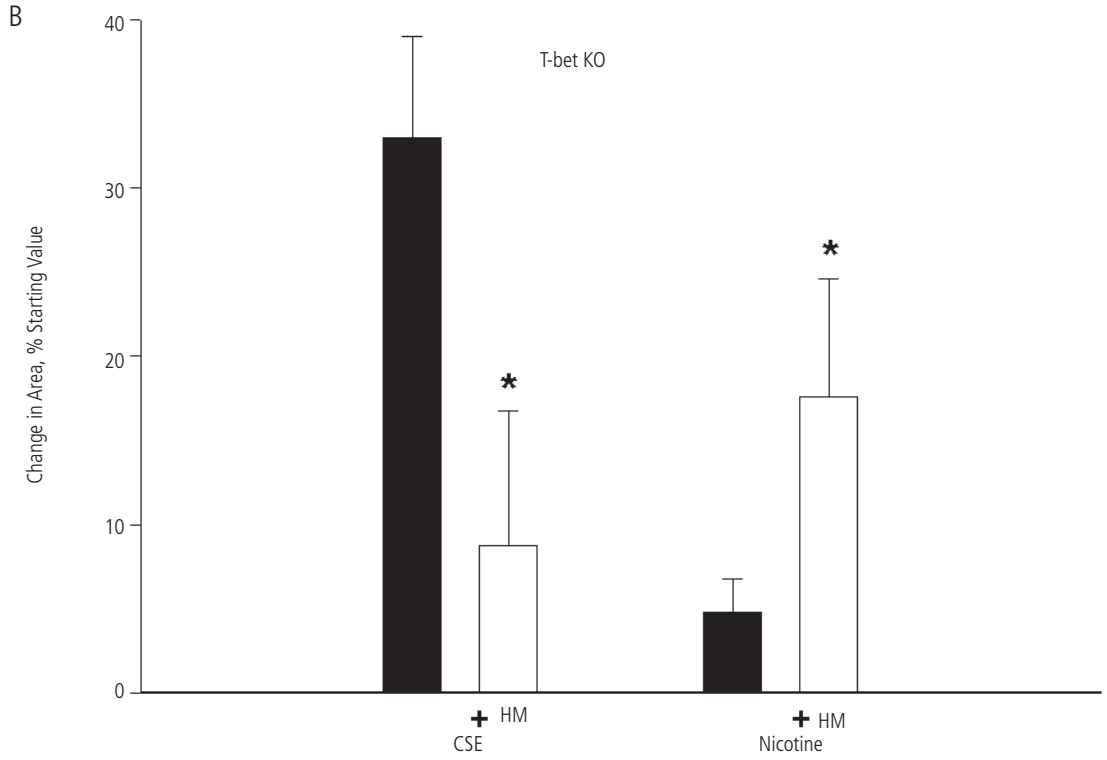



**Figure 5.** Airways in lung slices from Balb/C and T-bet KO mice were exposed to 5% CSE or 5% nicotine with or without 10 μM of hexamethonium to block nicotinic ACH-receptors. A, In lung slices from Balb/C mice, HM significantly reduced CSE-induced airway relaxation but did not alter the response to nicotine (**P*<.01, n=11 lung slices from 3 different mice). B, In lung slices from T-bet KO mice, HM also significantly reduced CSE-induced relaxation of the airway. Interestingly, HM increased nicotine-induced airway relaxation (**P*<.05, n=11 lung slices from 3 different mice). ACH indicates acetylcholine; CSE, cigarette smoke extract; HM, hexamethonium.

strong relaxation of the airway, which was comparable in Balb/C and T-bet KO lung slices (31±6% of starting value for Balb/C versus 33±6% for T-bet KO; n=8-12 lung slices from 3 different mice; *P*<.05 vs control and nicotine, Figure 4). Nicotine induced minor relaxation of the airway in lung slices from Balb/C mice, while relaxation in lung slices from T-bet KO mice was not statistically significant (9±2% of starting value for Balb/C; *P*<.05 vs control, 5±2% for T-bet KO; n=10 lung slices from 3 different mice; Figure 4).

To investigate whether the acute effects of CSE and nicotine were due to nicotinic ACH-receptor activation, lung slices were exposed to 5% CSE or 5% nicotine with or without 10 μM of the nicotinic ACH-receptor inhibitor hexamethonium. In lung slices from Balb/C mice, hexamethonium significantly reduced CSE-induced airway relaxation (31±6% without hexamethonium vs 10±2% with hexamethonium; *P*<.01, Figure 5A) but did not alter the response to nicotine (9±2% without vs 5±3 % with hexamethonium; n=11 lung slices from 3 different mice). In lung slices from T-bet KO mice, hexamethonium also significantly reduced CSE-induced airway relaxation (33±6% without hexamethonium vs 9±8% with hexamethonium; *P*<.01, Figure 5B). Interestingly, hexamethonium increased nicotine-induced relaxation of the airway (5±2% without hexamethonium vs 1±7% with hexamethonium; *P*<.05; n=11 lung slices from 3 different mice).

To test the long-term effects of CSE and nicotine on ACH-induced airway contraction, lung slices were cultured for 48 hours in medium containing 5% CSE or 5% nicotine and subsequently exposed to 1 μM ACH. In the control groups (cultivation in medium alone), contractions in the airways of T-bet KO mice were significantly stronger (7±3% of the starting value for Balb/C vs 45±6% for T-bet KO, *P*<.001; n=15 airways from 3 different mice, Figure 6). However, after culture with CSE or nicotine, no difference was observed between airways from Balb/C or T-bet KO mice (CSE, 20±3% for Balb/C vs 22±4% for T-bet KO; nicotine, 17±2% for Balb/C versus 17±2% for T-bet KO; n=15 airways from 3 different mice).

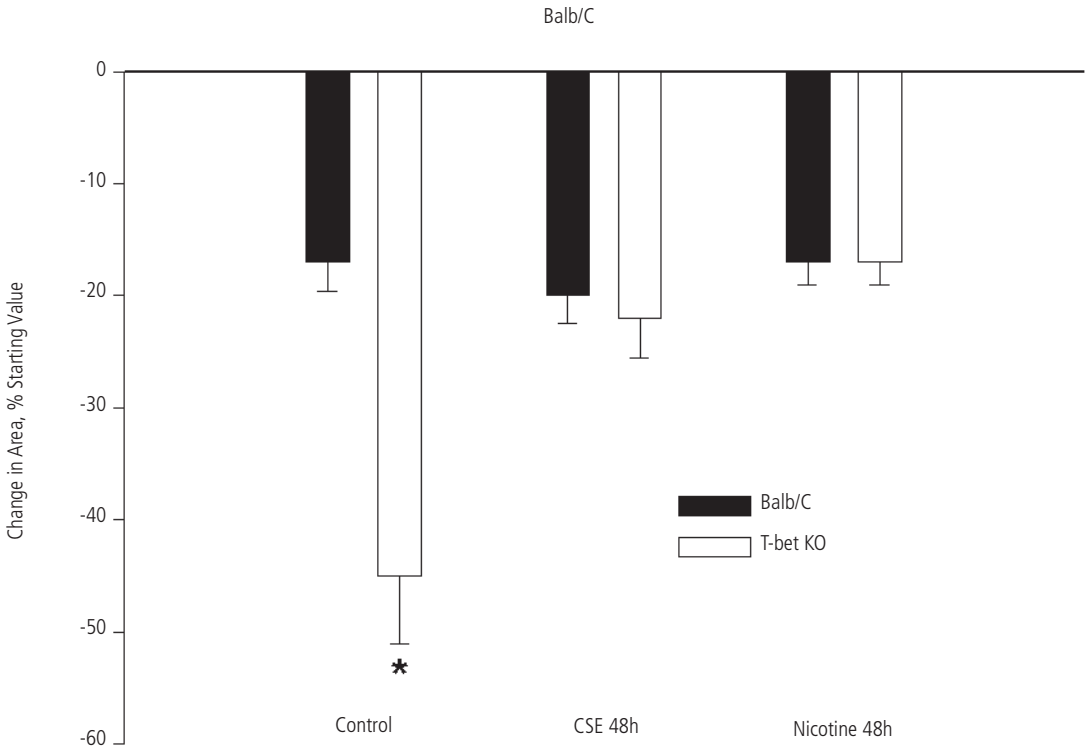



Figure 6. Lung slices from Balb/C mice and T-bet KO mice were cultured for 48 hours in medium containing 5% CSE or 5% nicotine and subsequently exposed to 1 μM ACH. In the control groups (culture in medium alone), contractions in the airways of T-bet KO mice were significantly stronger (*$P$<.001, n=15 airways from 3 different mice). However, after cultivation with CSE or nicotine no differences between airways from Balb/C or T-bet KO mice could be observed. ACH indicates acetylcholine; CSE, cigarette smoke extract.

## Discussion

We show that acute exposure to CSE led to relaxation of the airway. Acute exposure to nicotine resulted in minor relaxation in Balb/C and in nonsignificant relaxation in T-bet KO mice. The nicotinic ACH-receptor hexamethonium partially inhibited CSE-induced relaxation. Exposure of airways in lung slices from T-bet KO mice to CSE or nicotine for 48 hours completely reversed bronchial hyperreactivity.

Tobacco smoke is a mixture of solid and liquid components with a particle diameter of 0.1-1 μm and a particle concentration of 107-108/mL. Over 4000 different chemical compounds have been identified, and nicotine is the most pharmacologically active one [9]. For incubation with nicotine alone, we used 10 μg/mL of nicotine because this was the concentration we measured in 5% CSE. Therefore, the dissimilar effects of CSE and nicotine we observed were not due to different concentrations of nicotine, but to the complex composition of tobacco smoke.

Muscarinic ACH receptors are G protein–coupled and can be activated by muscarine and deactivated by atropine. To date, 5 subtypes (M1-M5) have been identified. Nicotinic ACH receptors are integral 252-kDa membrane proteins consisting of 5 subunits (2α, ß, γ, δ, binding site on α-subunits) and can be activated by nicotine. These receptors form unspecific cation channels and open to $Na^+$, K+, and $Ca2^+$ upon the binding of nicotine [10]. Chronic exposure to nicotine leads not only to inactivation but also to upregulation of nicotinic ACH receptors [11]. Hexamethonium unselectively blocks nicotinic ACH receptors.

We found that acute exposure to CSE led to relaxation of the airway. This was not because of toxic effects damaging airway smooth muscle cells, given that subsequent exposure to ACH resulted in strong airway contraction (Figure 2). Furthermore, hexamethonium partially reversed CSE-induced relaxation, thus indicating a specific effect via nicotinic ACH-receptors. However, even in the presence of hexamethonium, the airways still relaxed in response to CSE, and exposure to nicotine alone led to relaxation that was considerably smaller than that induced by CSE. Given the enormous number of chemical compounds in tobacco smoke, identification of the specific agents in CSE responsible for nicotine-independent relaxation seems unfeasible. In fact, it may be the complex composition of tobacco smoke, rather than specific compounds, which leads to nicotine-independent airway relaxation.

Addition of nicotine to lung slices from T-bet KO mice did not induce significant relaxation of the airway. However, in the presence of hexamethonium, the airways relaxed in response to nicotine. Although seemingly conflicting, these

data may make sense under the following 2 assumptions: First, different nicotinic ACH receptors can cause airway contraction or relaxation. And, second, hexamethonium does not block all nicotinic ACH receptors with the same affinity. If this were the case, nicotine could have induced relaxation of the airway with hexamethonium preferentially blocking contraction-inducing nicotinic ACH receptors. In this setting, expression of nicotinic ACH receptors is obviously different in Balb/C and T-bet KO airways. To the best of our knowledge, there are no studies on the effects of nicotinic ACH receptors on airway smooth muscle tone.

Incubation with CSE and nicotine for 48 hours did not alter ACH-induced airway contraction in lung slices from Balb/C mice. However, in lung slices from T-bet KO mice, incubation completely inhibited the increased contraction of airways, and there was no longer a difference between Balb/C and T-bet KO mice. Galzi et al [11] reported that chronic exposure to nicotine inhibited nicotinic ACH receptors. If this is also the case for muscarinic ACH receptors, then the decreased contractile response to ACH in T-bet KO airways could be explained. Alternatively, the decreased contractile response could be due to nonspecific effects of CSE or nicotine on the contractile apparatus of T-bet KO mice.

The negative impact of exposure to tobacco smoke in patients with asthma, regardless of whether exposure is active or passive, has been discussed in numerous studies [1,2]. Indeed, many asthma patients smoke, even though they are aware of the negative effects on their disease. Surprisingly, even patients who experience acute asthma attacks smoke. Our results indicated that this could be because patients have learned to benefit from the effect of smoke-induced relaxation of the airways.

We show that acute exposure to CSE leads to relaxation of the airway, and that this is partially mediated by nicotine. Chronic exposure to CSE reverses bronchial hyperreactivity in airways from T-bet KO mice; this effect can be mimicked by chronic exposure to nicotine.

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