Trichostatin A Abrogates Airway Constriction, but Not Inflammation, in Murine and Human Asthma Models

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Histone deacetylase (HDAC) inhibitors may offer novel approaches in the treatment of asthma. We postulate that trichostatin A (TSA), a Class I and II inhibitor of HDAC, inhibits airway hyperresponsiveness in antigen-challenged mice. Mice were sensitized and challenged with *Aspergillus fumigatus* antigen (AF) and treated with TSA, dexamethasone, or vehicle. Lung resistance (R_L) and dynamic compliance were measured, and bronchial alveolar lavage fluid (BALF) was analyzed for numbers of leukocytes and concentrations of cytokines. Human precision-cut lung slices (PCLS) were treated with TSA and their agonist-induced bronchoconstriction was measured, and TSA-treated human airway smooth muscle (ASM) cells were evaluated for the agonist-induced activation of Rho and intracellular release of Ca^{2+} . The activity of HDAC in murine lungs was enhanced by antigen and abrogated by TSA. TSA also inhibited methacholine (Mch)-induced increases in R_L and decreases in dynamic compliance in naive control mice and in AF-sensitized and -challenged mice. Total cell counts, concentrations of IL-4, and numbers of eosinophils in BALF were unchanged in mice treated with TSA or vehicle, whereas dexamethasone inhibited the numbers of eosinophils in BALF and concentrations of IL-4. TSA inhibited the carbacol-induced contraction of PCLS. Treatment with TSA inhibited the intracellular release of Ca^{2+} in ASM cells in response to histamine, without affecting the activation of Rho. The inhibition of HDAC abrogates airway hyperresponsiveness to Mch in both naive and antigen-challenged mice. TSA inhibits the agonist-induced contraction of PCLS and mobilization of Ca^{2+} in ASM cells. Thus, HDAC inhibitors demonstrate a mechanism of action distinct from that of anti-inflammatory agents such as steroids, and represent a promising therapeutic agent for airway disease.

Keywords: HDAC; asthma; allergen; mice; trichostatin A

Asthma manifests as reversible airway obstruction, hyperresponsiveness, and inflammation (1). Although most patients respond to conventional therapy, some exhibit severe or refractory asthma associated with frequent exacerbations, irreversible airflow limitation, and airway inflammation, despite maximal medical therapy (2). Patients with severe asthma also use healthcare resources disproportionately and experience more adverse effects from high doses of glucocorticoids (3). The need for improved therapeutic approaches to severe asthma continues, and the pursuit of epigenetic modifications of gene expression in asthma offers unique

CLINICAL RELEVANCE

This research demonstrates a novel role for inhibitors of histone deacetylase (HDAC) in abrogating airway constriction in response to contractile agonists in both animal and human models. Our findings suggest a therapeutic role for inhibitors of HDAC in asthma, and further suggest that airway smooth muscle contraction may be epigenetically modulated.

therapeutic opportunities. Accordingly, the acetylation and deacetylation of histone represent a potential therapeutic target (4).

Histone acetyl transferases (HATs) induce the acetylation of histone, whereas histone deacetylases (HDACs) remove the acetyl groups from histones to modulate gene transcription. Currently, 11 HDACs have been identified and grouped into three major categories by their sequence similarity to the *Saccharomyces cerevisiae* reduced potassium dependency-3 (RPD3) or histone-deacetylase 1 (Hda1) enzyme (5), and evidence suggests that HDACs differentially regulate genes (6). In addition to modulating gene activity by acetylating histones, HDACs also modulate nonhistone targets (7) that include transcription factors, cytokine receptors, cytoskeletal proteins, and nuclear hormone receptors (8). Although both HATs and HDACs may play a role in inflammatory lung disease and modulate steroid sensitivity (9), the roles of HATs and HDACs in the regulation of inflammatory and anti-inflammatory gene expression remain controversial. Airway cells derived from subjects with asthma demonstrate increased HAT activity and decreased HDAC activity (10), and the inhibition of HDAC improves airway hyperresponsiveness (AHR) and inflammation in some animal models of airway inflammation (11, 12).

Here, we characterize the expression of HDAC isoforms in murine lung tissue and in human airway smooth muscle (ASM) and epithelial cells. Further, we show that trichostatin A (TSA), a Class I and II inhibitor of HDAC, abrogates methacholine (Mch)-induced AHR without affecting leukocyte trafficking and concentrations of cytokines in bronchoalveolar lavage fluid (BALF) from antigen-challenged mice, human precision-cut lung slices (PCLS), and ASM cells.

MATERIALS AND METHODS

Mice

Female C57/BL6 mice, aged 8 weeks, were purchased from Charles River laboratories (Malvern, PA). All animal protocols were approved by the Animal Use and Care Committee at the University of Pennsylvania.

Antigen Sensitization and Challenge

As shown in Figure 1, mice were sensitized by intraperitoneal injections of 20 μ g antigen, a protein extract of the ubiquitous airborne fungus, *Aspergillus fumigatus* (AF; Bayer Pharmaceuticals, Spokane, WA) in 100 μ l PBS solution containing 2 mg of alum (Imject Alum; Pierce,

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Figure 1. Experimental design. Animals were sensitized with two intraperitoneal (IP) injections on Days 0 and 14 with 20 μ g of *Aspergillus fumigatus* antigen (AF). Three intranasal (IN) challenges of 25 μ g AF were performed, once a day for the 3 days before the animal was killed. Animals were treated with an HDAC inhibitor, trichostatin A (TSA), or DMSO (diluent) alone by IP injection once a day for the 3 days before being killed on Day 28.

Rockford, IL) on Days 0 and 14, and challenged on Days 25–27 with 30 μ l of AF extract in PBS (25 μ g) intranasally. This is a modification of our previously described protocol (13).

TSA Dosing

Mice received three intraperitoneal injections of 0.6 mg/kg of TSA (Sigma Aldrich) once daily on Days 25–27. Control animals received an equal volume of DMSO (carrier) without TSA by intraperitoneal injection.

Invasive Lung Function Measurements of Anesthetized, Cannulated Mice

Lung resistance (R_L), dynamic compliance, elastance, tissue damping, tissue elastance, and airway resistance were recorded using the FlexiVent system (SCIREQ Scientific Respiratory Equipment, Inc., Montreal, PQ, Canada), as described previously (14). Briefly, mice were anesthetized by an intraperitoneal injection of a ketamine (100 mg/kg) and xylazine (20 mg/kg) mixture. After anesthesia, a 0.5-cm incision was performed from the rostral to caudal direction. The flap of skin was retracted, the connective tissue was dissected away, and the trachea was exposed. The trachea was then cannulated between the second and third cartilage rings with a blunt-end stub adapter and secured with suture. The mouse was next connected to the FlexiVent system, and spontaneous respirations were terminated with an intramuscular injection of pancuronium bromide (3 mg/kg). Parameters of mechanical ventilation included a rate of 140 breaths/minute and a 0.25-ml tidal volume. The respiratory mechanics were measured as previously described (14). Airway responsiveness was measured after the inhalation of nebulized saline and increasing concentrations of nebulized Mch (1.25, 5, 10, and 20 mg/ml).

BAL Cell Count and Differential Cell Count

After measurements of R_L , lungs were lavaged with 1-ml aliquots of sterile saline through the tracheal cannula. After centrifuging ($500 \times g$ for 10 minutes at 4°C), the cell pellet was resuspended in RPMI medium. Differential cell counts were performed from cytospin preparations, as described elsewhere (15). Cells were identified as macrophages, eosinophils, neutrophils, and lymphocytes according to standard morphology, and a minimum of 300 cells was counted using a Nikon microscopic system (Nikon Instruments, Melville, NY) ($\times 400$ magnification). The percentages and absolute numbers of each cell type were then calculated. The supernatants were harvested and stored at -20°C for further analysis.

Determination of IL-4 and IL-6 in BALF

Concentrations of cytokines were determined by ELISA according to standard protocols, as previously described (16). The limits of detection were 5 pg/ml for IL-4 and IL-6 standards. We used the recombinant murine IL-4 and IL-6 included with the kits (R&D Systems, Minneapolis, MN) as control samples.

SDS-Polyacrylamide Gel Electrophoresis and Immunoblot Analysis

Tissue lysates were prepared in lysis buffer consisting of 20 mM Tris HCl (pH 7.5), 150 mM NaCl, 1 mM Na_2EDTA , 1 mM EGTA, 1% Triton

X-100, 1 μ g/ml leupeptin, 2.5 mM sodium pyrophosphate, 1 mM Na_3VO_4 , and 1 mM β -glycerophosphate. Phenylmethylsulfonyl fluoride (1 mM) was added before use. One hundred micrograms of each sample were separated by 4–12% SDS-PAGE (Invitrogen, Grand Island, NY) and transferred to polyvinylidene fluoride (PVDF) membranes. We used antibodies to HDAC1 (1:1,000 dilution; Cell Signaling), HDAC2 (1:1,000 dilution; Invitrogen), HDAC3 (1:1,000 dilution; Sigma Chemical Co., St. Louis, MO), HDAC4 (1:1,000 dilution; Cell Signaling, Danvers, MA), HDAC5 (1:1,000 dilution; Cell Signaling), HDAC6 (1:1,000 dilution; Santa Cruz Biotechnology, Santa Cruz, CA), HDAC8 (1:1,000 dilution; Santa Cruz Biotechnology), HDAC10 (1:1,000; Sigma), and anti-glyceraldehyde 3-phosphate dehydrogenase (1:5,000 dilution; Sigma). These antibodies are specific to both human and murine samples (17, 18). Primary antibody binding was visualized by using the Westernbreeze Kit (Invitrogen) as described previously, and according to the manufacturer's instructions (19, 20).

HDAC Activity Assay

The activity of HDAC was measured using a commercial fluorometric detection HDAC activity assay kit (Millipore/Upstate Cell Signaling, Lake Placid, NY), as described previously (19). In brief, total extract was prepared from freshly harvested lung tissue. Fifty micrograms of total extract were incubated with HDAC assay substrate and incubated at 30°C for 60 minutes. After the addition of reagent, samples were kept at room temperature for 15 minutes, and readings were taken with a fluorescent microplate reader at an excitation of 360 nm and emission of 450 nm. A standard curve was performed according to the manufacturer's protocol.

Cell Cultures

Epithelial cells were derived from protected brush specimens obtained from live human donors, in accordance with procedures approved by the University of Pennsylvania Committee on Studies Involving Human Beings. Specimens were centrifuged at 1,200 rpm for 5 minutes, and pelleted cells were seeded onto 100-mm collagen-coated tissue culture dishes and incubated at 37°C with 5% CO_2 . When cells reached 80% confluence, monolayers were transferred onto collagen-coated Transwell inserts, as previously described (21, 22). After the formation of monolayers, the apical surfaces of the Transwell inserts were raised to air-liquid interfaces (ALI), and cultured for an additional 2 weeks. Lysates were obtained on Days 15–16 at the ALI for immunoblot analyses.

Human ASM cells were derived from tracheas obtained from the National Disease Research Interchange (Philadelphia, PA). Human ASM cell culture was performed as described previously (23). Briefly, a segment of trachea just proximal to the carina was removed under aseptic conditions, and the tracheal muscle was isolated, centrifuged, and resuspended in buffer containing 0.2 mM CaCl_2 , 640 U/ml collagenase, 1.0 mg/ml soybean trypsin inhibitor, and 10 U/ml elastase. The tissue was enzymatically dissociated, filtered, and washed. Aliquots of the cell suspension were plated on plastic plates at a density of 1.0×10^4 cells/cm 2 . The cells were cultured in Ham's F-12 medium supplemented with 10% FBS, 100 U/ml penicillin, 0.1 mg/ml streptomycin, and 2.5 mg/ml amphotericin B, and this medium was replaced every 72 hours. Human ASM cells in subculture during passages 1–5 were used, because these cells retain the expression of native contractile protein, as demonstrated by immunocytochemical staining for smooth muscle actin and myosin (23).

Preparation of PCLS and Airway Function

The preparation of PCLS and airway function studies were performed as described previously (24). Briefly, healthy whole lungs were received from the National Disease Research Interchange and inflated with agarose solution. Lobes were then sectioned, and cores were made in which a small airway was visible. The cores were sliced into 250- μ m-thick PCLS, which were then placed in a 12-well plate and exposed to increasing doses of carbachol. After each dose of carbachol, images were collected and airway diameters were measured, as previously described (24).

Rho Activation Assay

Cultured human ASM cells were measured for the activation of Rho, using a Rho Activation Assay Kit (Upstate Cell Signaling Solutions) according to the manufacturer's protocol, as previously described (25). Total Rho was detected using an anti-Rho antibody (Upstate Cell Signaling Solutions).

Intracellular Ca^{2+} Assay

Cultured ASM cells were evaluated for the mobilization of Ca^{2+} in response to histamine, using fura-2 acetoxymethyl ester (AM) (Invitrogen). Approximately 30,000 cells were plated onto 25-mm coverslips. After 4 days, cells were serum-deprived and treated with TSA or vehicle. After 2 days of treatment with TSA or diluents, cells were washed in PBS. Measurements of fura-2 were performed as described elsewhere (26). Briefly, after incubation with fura-2 AM (1 μM) for 45–60 minutes at room temperature, cells were constantly perfused with a saline solution containing 130 mM NaCl, 4 mM KCl, 2 mM CaCl_2 , 2 mM MgCl_2 , 10 mM *N*-2-hydroxyethylpiperazine-*N'*-ethane sulfonic acid, and 15 mM glucose (pH 7.4). The emission signals in response to 340 and 380 nm were collected, and the 340 nm/380 nm signal ratio was analyzed after subtracting the background obtained in the presence of 10 mM MnCl_2 and 5 μM ionomycin in the absence of added Ca^{2+} .

RESULTS

TSA Inhibits the Antigen-Induced Activity of HDAC

As shown in Figure 2A, we characterized the expression of Class I and II HDACs in human and murine lung tissue by immunoblotting. HDAC isoforms are differentially expressed in human ASM cells, epithelial cells, and murine lung tissue. HDAC4 and HDAC6 are predominantly expressed in human ASM cells, whereas HDAC1 and HDAC3 are expressed by human airway epithelium. Given the spectrum of expression of HDAC isoforms in lung tissue, we examined the effects of a broad-spectrum Class 1 and 2 HDAC inhibitor, TSA, on antigen-

induced airway inflammation and function. We used a dose of TSA and a route of administration (0.6 mg/kg intraperitoneal, daily) previously shown to inhibit the activity of HDAC (27). Control mice were treated with DMSO alone. We then measured the activity of HDAC in lung tissue from naive and TSA-treated mice. As shown in Figure 2B, lung HDAC activity was significantly increased by exposure to antigen, whereas TSA effectively inhibited the activity of HDAC in both naive and antigen-exposed mice. In naive mice, the basal activity of HDAC was measured at $141 \times 10^4 \pm 7.7 \times 10^4$ relative light units (RLU). These results are presented relative to basal HDAC activity. In human ASM cells, treatment with TSA decreased the activity of HDAC in a dose-dependent manner (Figure 2C).

The Inhibition of HDAC Modulates Mch-Induced Changes in Lung Mechanics

The effects of TSA on lung resistance and compliance were determined after exposing mice to increasing doses of Mch. As shown in Figure 3A, Mch increased airway resistance in a dose-dependent manner ($E_{\text{max}} = 2.4 \pm 0.1 \text{ cm H}_2\text{O/ml}$, $P < 0.05$), whereas TSA substantially abrogated this response ($E_{\text{max}} = 1.0 \pm 0.01 \text{ cm H}_2\text{O/ml}$, $P < 0.05$) in non-antigen-exposed (naive) mice. Treatment with dexamethasone exerted little effect on baseline Mch-induced R_L in naive mice ($E_{\text{max}} = 2.3 \pm 0.07 \text{ cm H}_2\text{O/ml}$). In antigen-exposed mice, R_L increased to a greater degree in response to Mch, compared with that in naive mice ($E_{\text{max}} = 13.9 \pm 0.7 \text{ cm H}_2\text{O/ml}$), and treatment with TSA significantly blunted this response ($E_{\text{max}} = 7.8 \pm 0.1 \text{ cm H}_2\text{O/ml}$, $P < 0.05$) (Figure 3B). Dexamethasone also inhibited antigen-enhanced R_L to a similar degree as TSA ($E_{\text{max}} = 6.7 \pm 0.5 \text{ cm H}_2\text{O/ml}$, $P < 0.05$). As shown in Figure 4A, dynamic compliance was also decreased by Mch, with a maximum inhibition at $0.016 \pm 0.001 \text{ cm H}_2\text{O/ml}$. Treatment with TSA reversed this reduction in dynamic compliance with a maximum inhibition at

Figure 2. Expression and activity of histone deacetylase (HDAC) in human and murine tissue. (A) Immunoblot analyses of lysates from human airway smooth muscle (HASM) cells, lung epithelial cells, and wild-type murine lung tissue were performed using anti-HDAC antibodies. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was used to confirm equal protein loading. (B) Relative HDAC activity from lung tissue lysate was quantified. Data are reported as the ratio of measured HDAC activity to basal HDAC activity (Relative HDAC activity). Mice were sensitized and challenged with antigen or solvent (naive), and received vehicle treatment (DMSO) or TSA. Data represent means $\pm$ SD from five samples for each condition. $*P < 0.05$ (treatment versus basal concentrations). (C) Relative HDAC activity from HASM lysate was quantified. Data are reported as the ratio of measured HDAC activity to basal HDAC activity (Rel. HDAC activity). Cells were treated with TSA at concentrations of 1, 10, or 100 μM for 48 hours before lysis and assay. Data represent means $\pm$ SD from five samples for each condition. $*P < 0.05$ (treatment versus basal concentrations).

Figure 3. TSA inhibits lung resistance (R_L) in naive and antigen-exposed mice. (A) R_L of naive (non-antigen-exposed), vehicle, TSA, or dexamethasone (Dex)-treated mice (basal condition). Methacholine (Mch) increased R_L to a maximum of 2.46 ± 0.1 cm H₂O/mL. Treatment with Dex exerted little effect on basal Mch responsiveness. TSA markedly inhibited basal responsiveness to Mch. Data represent the mean $\pm$ SD for each condition derived from 12 measurements at each dose in three mice. $*P < 0.05$ (naive versus TSA-treated mice). (B) R_L of mice that were antigen-challenged and received vehicle (DMSO), TSA, or Dex. Maximum R_L in antigen-challenged mice was measured at 13.9 ± 0.7 cm H₂O/mL, that is, much higher than in naive mice. TSA was as effective as Dex in abrogating Mch-induced airway hyperresponsiveness (AHR). Data represent the means $\pm$ SD derived from 12 measurements at each dose in six mice for each condition. $*P < 0.05$ (AF + TSA versus AF). $^+P < 0.05$ (AF + Dex versus AF).

0.32 ± 0.001 cm H₂O/mL ($P < 0.05$). The maximum inhibition of dynamic compliance by Mch in dexamethasone-treated mice was measured at 0.10 ± 0.001 cm H₂O/mL. The maximum inhibition of dynamic compliance in antigen-exposed mice was 0.004 ± 0.0004 cm H₂O/mL (Figure 4B), and treatment with dexamethasone exerted little effect on dynamic compliance. Treatment with TSA significantly improved dynamic compliance in the mice, with a maximum inhibition at 0.007 ± 0.0009 cm H₂O/mL ($P < 0.05$). Together, these data demonstrate that treatment with TSA diminishes R_L and increases dynamic compliance in response to Mch at baseline and after exposure to antigen. By altering the response to multiple doses of Mch, TSA reduces AHR more effectively than dexamethasone under the conditions tested.

TSA Exerted Little Effect on Antigen-Induced Increases in BALF Cell Count

To address whether inhibiting HDAC modulated antigen-induced airway inflammation, BALF cell counts and concentrations of cytokines were determined in naive mice and in mice exposed to antigen and treated with TSA or dexamethasone (Figure 5A). Naive mice treated with dexamethasone, TSA, or DMSO showed no significant difference in basal BALF cell

counts. Exposure to antigen increased cell counts in BALF, compared with naive mice. Dexamethasone decreased the total cell counts in BALF of antigen-exposed mice, whereas TSA and DMSO exerted little effect. Mice exposed to antigen also exhibited increased eosinophil counts, as shown in Figure 5B. Although treatment with dexamethasone decreased the numbers of eosinophils in BALF, TSA and DMSO exerted little effect. Little difference was evident in the numbers of macrophages, neutrophils, or lymphocytes in untreated mice, mice exposed to antigen, or mice treated with TSA, dexamethasone, or DMSO. Regarding percentages of cells in BALF, the BALF from naive mice predominantly manifested macrophages, whereas exposure to antigen decreased the percentages of macrophages and increased the percentages of eosinophils (Figure 5C). Collectively, these data show that although both dexamethasone and TSA inhibit antigen-induced AHR, dexamethasone but not TSA inhibits antigen-induced airway inflammation.

Dexamethasone, but Not TSA, Inhibited Antigen-Induced Concentrations of Cytokines in BALF

IL-4 and IL-6 are inflammatory cytokines implicated in asthma. In naive mice, the concentrations of IL-4 and IL-6 in BALF were negligible, regardless of treatment with dexamethasone, DMSO, or TSA, as shown in Figure 6. Mice exposed to antigen exhibited increased concentrations of IL-4 and IL-6 in the BAL, and

Figure 4. TSA reversed antigen-induced effects on lung compliance. (A) Dynamic compliance was measured in naive mice and mice treated with vehicle (DMSO), TSA, or Dex in response to increasing doses of Mch measured by the Scireq FlexiVent system. TSA markedly inhibited Mch-induced decreases in dynamic compliance. Data represent the means $\pm$ SD derived from 12 measurements at each dose in three mice for each condition. $*P < 0.05$ (naive versus TSA-treated mice). (B) Dynamic compliance of mice challenged with antigen or solvent (naive), and of mice that received treatment (DMSO), TSA, or Dex. Data represent the means $\pm$ SD derived from 12 measurements at each dose in six mice for each condition. $*P < 0.05$ (AF + TSA versus AF).

Figure 5. Dex, but not TSA, inhibits antigen-induced increases in BALF cell counts. (A) The total number of cells was obtained from bronchoalveolar lavage fluid (BALF) of mice that were sensitized and challenged with antigen or diluent (naive) and mice that received vehicle (DMSO), TSA, or Dex. TSA exerted little effect on numbers of inflammatory cells in the BAL. Data represent the mean $\pm$ SD for each condition, derived from the BALF of three mice. * $P < 0.05$ (naive versus treatment cohort). (B) The cellular profile of BALF from mice that were sensitized and challenged with antigen or solvent (naive) and mice that received vehicle (DMSO), TSA, or Dex. Data represent the mean $\pm$ SD for each condition, derived from the BALF of three mice. * $P < 0.05$ (naive versus AF). + $P < 0.05$ (AF + Dex versus AF). (C) Percentages of cell subtypes. Data represent the mean $\pm$ SD for each condition, derived from the BALF of three mice. * $P < 0.05$ (baseline versus treatment cohort).

dexamethasone attenuated the increase in IL-4 but not IL-6. Treatment with DMSO or TSA, however, exerted little effect on concentrations of IL-4 or IL-6. These data suggest that treatment with dexamethasone abrogates concentrations of antigen-induced cytokines, whereas TSA exerts little effect on concentrations of cytokines.

TSA Inhibits Carbachol-Induced Contraction in PCLS

To determine whether TSA modulates human ASM function, PCLS were incubated with increasing doses of carbachol, and luminal narrowing was determined in untreated cells and compared with cells treated with 20 μ M or 4 μ M TSA. Carbachol abrogated airway luminal diameters, and treatment with TSA decreased luminal narrowing, with 4 μ M TSA inhibiting the luminal narrowing of PCLS by 55% $\pm$ 3.2%, and 20 μ M TSA inhibiting the luminal narrowing of PCLS by 68% $\pm$ 6% (Figure 7A).

TSA Inhibits the Release of Ca²⁺ in Human ASM without Affecting the Activation of Rho

To determine whether TSA modulates excitation-contraction coupling in humans, we evaluated the effects of TSA treatment on agonist-induced Ca²⁺ sensitization and mobilization in human ASM. Bradykinin and histamine were used as contractile agonists instead of carbachol, because muscarinic M3 receptors (M3R) responses are attenuated in cultured human ASM cells (28). Cultured human ASM cells treated with 10 μ M TSA did not show a decrease in the activation of Rho in response to bradykinin after incubation, as shown in Figure 7B, suggesting that Ca²⁺ sensitization

is not affected by treatment with TSA. In parallel, intracellular Ca²⁺ ([Ca²⁺]_i) was measured in human ASM cells in response to an application of histamine (100 μ M), to determine the effect of TSA treatment on the agonist-induced mobilization of Ca²⁺. Virtually every untreated cell exhibited a robust and consistent increase in [Ca²⁺]_i in response to histamine, but TSA inhibited the histamine-triggered increases in [Ca²⁺]_i in a dose-dependent manner without markedly altering the resting [Ca²⁺]_i level (Figure 7C). Together, these results suggest that TSA inhibits the contraction of ASM by disrupting the release of Ca²⁺ into ASM cells in response to stimulation with a contractile agonist.

DISCUSSION

We have shown that TSA, an inhibitor of HDAC activity, abrogates Mch-induced increases in airway resistance in both naive and antigen-exposed mice. Moreover, we show that TSA inhibits basal and antigen-induced sensitivity to Mch without altering numbers of leukocytes or concentrations of cytokines in BALF. Our experiments in human PCLS show a decrease in the carbachol-induced contraction after treatment with TSA, and a decrease in the agonist-induced intracellular release of Ca²⁺, with no effect on Ca²⁺ sensitization. Collectively, these data suggest that the inhibition of Mch responsiveness by TSA is not attributable to the anti-inflammatory activity of TSA, but is rather a direct effect on agonist-induced smooth muscle contraction. Further, the effects of TSA on AHR appear distinct from those of glucocorticoids, which modulate airway inflammation in response to antigen. Therefore, glucocorticoids and the inhibition of HDAC may modulate AHR by disparate mechanisms.

Figure 6. Dex, but not TSA, inhibits antigen-induced increases of IL-4 concentrations in BALF. Concentrations of (A) IL-4 and (B) IL-6 in BALF were measured from mice that were challenged with antigen or diluent (naive) and mice that received vehicle alone (DMSO), TSA, or Dex. TSA exerted little effect on the secretion of cytokines in mice sensitized and challenged with antigen. Data represent the mean $\pm$ SD for each condition, derived from the BALF of three mice. * $P < 0.05$ (naive versus AF). $^+P < 0.05$ (AF + Dex versus AF). # $P < 0.05$ (AF + Dex versus AF + TSA).

Our results diverge from those of Choi and colleagues (12), who reported that in antigen-exposed mice, TSA inhibited airway inflammation, including numbers of eosinophils and concentrations of IL-4 in BAL. The dose of TSA used and/or the timing of TSA treatment may explain the differential effects of TSA on these parameters of inflammation. Choi and colleagues treated mice with 1 mg/kg TSA on the first day of sensitization,

and the mice received 11 total doses of TSA over 22 days, whereas in our study, mice were treated with 0.6 mg/kg TSA on the day of antigen challenge, 26 days after the first sensitization treatment, and they received three doses of TSA over 3 days. A higher dose of TSA and/or a longer course of treatment may exert a direct effect on airway inflammation as well as smooth muscle contraction. However, our conclusion, that TSA can abrogate airway sensitivity to Mch in naive mice that exhibit no associated changes in inflammation, supports the hypothesis that TSA may directly inhibit bronchoconstriction, and our study of ASM showing a decrease in the intracellular release of Ca^{2+} after TSA treatment further supports this hypothesis. Our findings are in agreement with studies indicating that the contraction of isolated guinea pig tracheal rings in response to histamine, carbachol, and 5-hydroxytryptamine is abrogated by inhibitors of HDAC (11).

Contraction in ASM is regulated by multiple mechanisms, including release of Ca^{2+} stored in the sarcoplasmic reticulum, as well as the modulation of Ca^{2+} sensitivity by the activation of RhoA (29). Our finding that treatment with TSA inhibits the agonist-induced mobilization of calcium in human ASM in a dose dependent manner is novel, and elucidates a potential mechanism by which TSA inhibits contractile, agonist-induced AHR. Although our results demonstrate that TSA inhibits the activity of HDAC in the lung, the effects on chromatin structure and gene expression remain unclear. Expression profiling studies suggest that 2–10% of genes are modulated by HDACs (30), and that the inhibition of HDAC can both up-regulate and down-regulate gene expression (31). Mechanisms unrelated to chromatin remodeling and gene expression may also contribute to the effects of TSA on agonist-induced airway contraction. For example, the inhibition of HDAC hyperacetylates tubulin, decreases tubulin function, and impairs lysosome exocytosis (7). HDAC8, a Class I HDAC, associates with α -actin, and the inhibition of HDAC8 inhibits in turn the contraction, size, and spreading of smooth muscle (32). Further studies evaluating the inhibition of specific HDACs or the activity of specific genes after treatment with TSA will elucidate other mechanisms by which TSA inhibits ASM contraction in response to contractile agonists. We previously showed that augmenting the activity of HDAC by stimulating ASM cells with a combination of TNF- α and IFN- γ diminishes the acetylation of NF- κ B (33). Thus, TSA may inhibit AHR in mice and human ASM by modulating gene expression or by altering the acetylation of nonhistone targets, as demonstrated in the case of NF- κ B.

ASM has long been a target in asthma therapy (34), and newer therapies that disrupt ASM were recently approved

Figure 7. TSA inhibits the agonist-induced contraction of precision-cut lung slices (PCLS) by decreasing the agonist-induced mobilization of calcium in airway smooth muscle (ASM). (A) Carbachol induced contractions of PCLS after treatment with vehicle, TSA at 4 μ M, or TSA at 20 μ M. (B) Human ASM was treated with TSA (10 μ M) for 12, 24, 48, and 72 hours, and then treated with 1 μ M bradykinin for 1 minute, lysed, and assayed for active and total Rho by immunoprecipitation and immunoblot, respectively. (C) Fura-2 signal ratios from human ASM cells treated with TSA at 0 (untreated), 1, 5, and 10 μ M for 48 hours in response to histamine (100 μ M). In each group, approximately 20 cells were measured. In each graph, thin gray curves represent individual responses, and the thick dark curve represents the mean response.

(35). Patients with asthma demonstrate increased airway constriction in response to challenge with methacholine, which is commonly used to diagnose patients with suspected asthma (36). Our finding that the administration of TSA inhibits the activity of HDAC as well as the mobilization of calcium in response to contractile agonists in ASM suggests that the increased sensitivity to methacholine in patients with asthma may be an epigenetically regulated phenomenon, and that further elucidation of the mechanism by which inhibiting HDAC decreases the agonist-induced contraction of ASM could lead to novel asthma therapies.

In conclusion, our experiments show that in both human and murine models, TSA effectively inhibits lung HDAC activity and decreases agonist-induced lung resistance in both naive and antigen-challenged mice, and in human PCLS. Moreover, inhibitors of HDAC appear to modulate airway resistance by decreasing the release of Ca^{2+} in response to contractile agonists, a mechanism unrelated to any effects on inflammation. Thus, the inhibition of HDAC may offer a therapeutic approach that inhibits bronchoconstriction, apart from effects on airway inflammation.

Author disclosures are available with the text of this article at www.atsjournals.org.

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