Inhibition of histone deacetylase causes emphysema

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Mizuno S, Yasuo M, Bogaard HJ, Kraskauskas D, Natarajan R, Voelkel NF. Inhibition of histone deacetylase causes emphysema. Am J Physiol Lung Cell Mol Physiol 300: L402–L413, 2011. First published December 17, 2010; doi:10.1152/ajplung.00207.2010.—In patients with chronic obstructive pulmonary disease (COPD), histone deacetylase (HDAC) expression and activity are reduced in the lung tissue. However, whether HDAC activity controls the maintenance of the lung alveolar septal structures has not been investigated. To explore the consequences of HDAC inhibition and address the question of whether HDAC inhibition causes lung cell apoptosis and emphysema, male Sprague-Dawley rats and human pulmonary microvascular endothelial cells (HPMVEC) were treated with trichostatin A (TSA), a specific inhibitor of HDACs. Chronic TSA treatment increased the alveolar space area, mean linear intercept, and the number of caspase-3-positive cells in rat lungs. TSA suppressed hypoxia-inducible factor-1α (HIF-1α), VEGF, and lysyl oxidase (LOX) and increased microtubule-associated protein-1 light chain 3 (LC3), p53, and miR34a microRNA expression in both rat lungs and cultured HPMVEC. Gene silencing of HDAC2 using small interfering RNA (siRNA) in cultured HPMVEC resulted in the suppression of HIF-1α, VEGF, and LOX and an increase of p53 expression. These data indicate that HDAC inhibition causes emphysema and that HDAC-dependent mechanisms contribute to the maintenance of the adult lung structure. Our results also suggest that the increase in apoptosis, as a consequence of HDAC inhibition, is associated with decreased VEGF and HIF-1α expression.

vascular endothelial growth factor; hypoxia-inducible factor-1α; p53

EMPHYSEMATOUS LUNG TISSUE destruction together with chronic bronchitis are part of a clinical syndrome, chronic obstructive pulmonary disease (COPD), which is a global health problem, affecting mostly, but not exclusively, people who smoke or are exposed to biomass fuel smoke (34, 40). Chronic inflammation, proteolysis, and oxidative stress are considered key components in the pathogenesis of COPD/emphysema (1, 3, 38). In addition, it has been recognized, when tissue samples from patients with severe COPD/emphysema are investigated on a cellular level, that the homeostatic control and structure maintenance of the adult lung is impaired (47). Apoptosis, autophagy, and cellular senescence have all recently been reported to occur in COPD lung tissues (6, 11, 23, 48) and can contribute to lung tissue destruction. The fact that not all smokers develop severe COPD/emphysema has led to an intense search for genetic susceptibility factors (13, 36), and recent reports demonstrating the impact of cigarette smoke exposure on chromatin remodeling (51) and decreased histone deacetylase (HDAC) expression in the lungs from patients with COPD (25) now have provided a concept for epigenetic modifications that can explain alterations in lung tissue gene expression. Rahman et al. (39) reported that the HDAC inhibitor trichostatin A (TSA) increased NF-kB binding of H2O2-challenged lung alveolar cells, thus connecting oxidative stress, HDAC inhibition, and inflammation. In addition, Osoata et al. (33) reported reduced HDAC2 expression as a consequence of oxidative/nitrative stress. Although HDAC inhibition and COPD have been associated (2), it is unknown whether HDAC inhibition causes emphysema and, if so, by what mechanisms.

HDACs and histone acetyl transferases are two enzyme families whose enzymatic activities control the acetylation of lysine residues, in particular those of NH2-terminal extensions of core histones. HDACs are involved in the regulation of a large number of biological processes including cell survival and differentiation (50). HDAC inhibitors are used clinically to target tumor angiogenesis and the expression of the transcription factor hypoxia-inducible factor-1α (HIF-1α) and the angiogenesis factor VEGF (16). HIF-1α has been recognized as a fundamentally important controller of expression of a large number of genes (11, 43), including the expression of the collagen and elastin cross-linker lysyl oxidase (LOX), an enzyme essential for matrix stability (17, 18). We (52) have recently reported a decreased expression of both HDAC2 and HIF-1α protein in lung tissue samples from patients with COPD/emphysema and have wondered about possible HDAC-HIF-1α interactions.

Here, we demonstrate that the HDAC inhibitor TSA causes partially reversible emphysema in adult rats. We show that HDAC inhibition in this emphysema model affects the expression of lung tissue HIF-1α, VEGF, and LOX proteins. The importance of this novel emphysema model is twofold: our data suggest that treatment of patients with HDAC inhibitors may cause or worsen emphysema and also that alterations of chromatin remodeling likely will prevent the repair or regeneration of the destroyed lung.

MATERIALS AND METHODS

Animal experimental protocols. The protocol was approved by the animal care and use committee of the Virginia Commonwealth University. Adult male Sprague-Dawley rats (250–280 g) were injected subcutaneously with 0.5 mg/kg body wt of TSA once a day for 6 wk. Control rats received vehicle only (5% DMSO in PBS). This dose of TSA was comparable to the dose used in a preclinical rat cancer treatment study (49). In addition, we evaluated the effects of TSA treatment periods varying in length from hours to 6 wk. We also assessed the TSA treatment effects on lung tissue histone 3 acetylation for these various time points. To evaluate whether the effects of sequential TSA treatment on the lung structure were reversible, we included a group of rats that had been treated with our standard...
protocol for 6 wk, then TSA treatment was stopped, and the animals were killed 4 wk later. Six animals were used for each group.

Antibodies. The antibodies used in this study included rabbit anti-cleaved caspase-3 antibody and rabbit anti-acetyl histone 3 antibody (Cell Signaling Technology, Beverly, MA), rabbit anti-LOX antibody (Novus Biologicals, Littleton, CO), rabbit anti-LC3 antibody (Abgent, San Diego, CA), mouse anti-HIF-1α antibody, mouse anti-VEGF antibody, rabbit anti-HDAC2 antibody, mouse anti-HDAC3 antibody, goat anti-histone 3 antibody, mouse anti-p53 antibody, mouse anti-collagen 1A (COL1A1) antibody, rabbit anti-collagen 3A antibody, mouse anti-lamin A/C antibody, and goat anti-ribosomal protein L13A (RPL13A) antibody (Santa Cruz Biotechnology, Santa Cruz, CA), and mouse anti-β-actin antibody (Sigma, St. Louis, MO).

Morphometry. Lungs were inflated with 0.5% low-melting agarose at a constant pressure of 25 cmH2O, fixed in 10% formalin for 48 h, and paraffin-embedded by standard techniques. For further details regarding the selection of tissue sections for analysis and the computer-assisted quantification of the mean air space area, see the data supplement online at the AJP-Lung Cellular and Molecular Physiology web site. Sections (5 μm) were stained with hematoxylin and eosin. Images were acquired with a Carl Zeiss AxioCam color camera (Carl Zeiss Vision, Hallbergmoos, Germany) and analyzed using AxioVision Imaging System software (Carl Zeiss Vision). Ten lung fields per tissue section were captured at ×100 magnification, and then AxioVision Imaging System software was used to measure the mean alveolar air space areas (MAAA) and total length of alveolar perimeters (TLAP) in pixels per square micrometer (see detailed methods in online data supplement). Emphysematous changes were also assessed by measurement of the mean linear intercept (MLI). MLI is a measurement of mean interalveolar septal wall distance, which is widely used to examine alveolar space size. As previously described (5, 8), the MLI was measured by dividing the length of a line drawn across the lung section by the total number of intercepts counted within this line, at ×100 magnification. A total of 40 lines per rat lung were drawn and measured.

Immunohistochemical staining for active caspase-3. The slides with 5-μm paraffin sections were deparaffinized in xylene, rehydrated, and then submitted to microwave treatment in 10 mM citric acid monohydrate solution. After quenching of endogenous peroxidase with 3% H2O2 for 15 min, the slides were incubated overnight with anti-cleaved caspase-3 rabbit polyclonal antibody (1:200 dilution) at 4°C and subsequently incubated with biotinylated anti-rabbit IgG antibody for 30 min at room temperature. Following this secondary antibody application, sections were incubated with ABC complex (Vector Laboratories, Burlingame, CA) for 30 min at room temperature and developed with diaminobenzidine (DAB; Vector Laboratories) until reaching the desired stain intensity. A light hematoxylin counterstain was applied. Ten lung fields per tissue section were captured at ×400 magnification, and then the number of active caspase-3-positive cells was counted using the AxioVision Imaging System software using a color thresholding function. In each captured picture, the degree of caspase-3 positivity was indexed for the TLAP.

Normal rabbit serum in the absence of primary antibody was used as a control. All data are expressed as means ± SE (n = 6 animals for each group). *P < 0.05 vs. control.

Fig. 1. Trichostatin A (TSA) reduces lung tissue expression of histone deacetylase (HDAC) 2 and HDAC3 and inhibits HDAC activity of the lung tissue. A: RT-PCR analysis of class 1 HDAC expression in TSA-treated rat lungs. The bar graph shows the ratios of HDAC1, HDAC2, HDAC3, and HDAC8 mRNA expression relative to that of vehicle (control). B: representative Western blot analysis of HDAC2, HDAC3, and lamin A/C nuclear protein extracts from TSA-treated rat lungs. The bar graph shows the ratios of HDAC2 and HDAC3 protein expression relative to that of lamin A/C protein. C: HDAC activity of nuclear extracts from TSA-treated rat lungs. D: representative Western blot analysis of acetylated histone 3 (Acetyl H3; lysine 9) and total histone 3 nuclear protein extracts from TSA-treated rat lungs. The bar graph shows the ratios of acetylated histone 3 protein expression relative to that of whole histone 3 protein. Data are expressed as means ± SE (n = 6 animals for each group). *P < 0.05 vs. control.
a negative control, and the lung slides treated with SU5416 were used as a positive control (22).

**HDAC activity assay.** HDAC activity of lung nuclear extracts were measured using a colorimetric HDAC activity assay kit (BioVision, Mountain View, CA) according to the manufacturer’s protocol. HDAC activity was normalized to the activity in nuclear protein extracts of untreated control rat lungs. Each experiment was done in duplicate.

**Cell culture.** Human pulmonary microvascular endothelial cells (HPMVECs) were purchased from Lonza (Walkersville, MD) and cultured in endothelial cell growth medium (EGM) supplemented with 5% FBS (Lonza). The cells were cultured in 175-cm² tissue

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**Fig. 2. TSA causes alveolar septal cell loss.** A: representative photomicrographs of hematoxylin and eosin-stained lung sections from vehicle- (a) or TSA-treated (b) rats. The left bar graph shows the quantification of the mean alveolar air space area (MAAA), and the right bar graph shows the quantification of the mean linear intercept (MLI) in lung sections from rats treated with vehicle (control) or TSA. TSA causes alveolar septal cell apoptosis. B: representative photomicrographs of lung immunohistochemical staining for cleaved caspase-3 from vehicle- (a and c) or TSA (b and d)-treated rats. The bar graph shows the caspase-3 positivity index in lung sections from vehicle- or TSA-treated rats. The caspase-3 positivity index was calculated as described in the MATERIALS AND METHODS section. Data are expressed as means ± SE (n = 6 animals for each group). *P < 0.05 vs. control. Arrows indicate cleaved caspase-3- positive alveolar septal cells.
culture flasks in a cell culture incubator (37°C, 5% CO2-95% air) and used at the 6th passage (HPMVEC) after trypsinization in all experiments. HPMVEC were seeded in six-well culture plates or 10-cm culture dishes and cultured until confluence. After reaching confluence, media were changed, and after addition of different concentrations of TSA (0.01–10 μM), the cells were cultured for another 24 h. After incubation, the cells were harvested and used for RT-PCR and Western blot analysis.

Real-time RT-PCR analysis of mRNA and miRNA. Isolation of total RNA and microRNA (miRNA) from lung tissue and HPMVECs was performed using an miRNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer’s protocol. Total RNA (1 μg) was reverse-transcribed using a random primer and MultiScribe RT (High-Capacity cDNA Archive Kit; Applied Biosystems, Foster City, CA) for mRNA analysis and the miScript Reverse Transcription Kit (Qiagen) for miRNA analysis. PCR was performed with the resulting reverse transcription products using specific oligonucleotide primers. The sequence of these primers is shown in Supplemental Tables S1–S3. All PCR reactions were performed with a LightCycler 480 PCR system (Roche Diagnostics, Meylan, France) using DNA-binding SYBR Green dye (Applied Biosystems) for mRNA analysis and miScript SYBR Green dye (Qiagen) for miRNA analysis. The RPL13A gene (31) was used as reference mRNA, and miR191 was used as reference miRNA. Each PCR analysis was done in duplicate.

Western blot analysis. Cytoplasmic and nuclear proteins from lungs and cells were prepared using NE-PER Nuclear and Cytoplasmic Extraction Reagents based on the manufacturer’s protocol (Pierce, Rockford, IL). Proteins were extracted for protein content using a Bradford method. Each sample was quantified, and 40 μg of cytoplasmic protein or 20 μg of nuclear protein were loaded into each lane of a 4–12% Bis-Tris NuPAGE gel with MES SDS running buffer, according to the manufacturer’s protocol. The gel was transferred to a polyvinylidene difluoride (PVDF) membrane by electrophoresis, and the membrane was then probed with the primary and secondary antibodies. An enhanced chemiluminescence (ECL) system was used for the detection of the proteins. HeLa cell protein extracts treated with CoCl2 (Santa Cruz Biotechnology) were used as a positive control of HIF-1α, cytochrome c-treated Jurkat cell extracts (Cell Signaling Technology) were used as a positive control of cleaved caspase-3, and lysate of human VEGF-transfected 293T cells (Santa Cruz Biotechnology) was used as a positive control of VEGF. We used as a reference protein RPL13A because the expression of some genes has been shown to be affected by TSA treatment (31).
Analysis of HIF-1α mRNA and p53 protein stability. HPMVEC were seeded onto 6-cm dishes and cultured for the indicated periods with or without 1 μM TSA in the presence of the transcription inhibitor actinomycin D (ActD; 400 nM) or the protein synthesis inhibitor cycloheximide (CHX; 25 μg/ml). The cells were harvested, and HIF-1α mRNA and p53 protein stability were examined using real-time RT-PCR and Western blotting. β-Actin was used as a reference. Each assay was replicated in four to six independent experiments.

Transfection of small interfering RNA into cultured HPMVECs. HDAC2, p53, and control small interfering RNAs (siRNAs) were designed and synthesized by Invitrogen (Sunnyvale, CA). The HDAC2, HDAC3, p53, and negative control RNA target sequences were 5'-CCAAUGAGUUGCCAUAUAA-3', 5'-CCAAGAGUCUUAAUGCCUUTT-3', 5'-GCGCACAGAGGAAGAGAAU-3', and 5'-CCUAGAACCUAAGACCCUU-3', respectively. HPMVEC were seeded into 6-cm dishes and incubated until 60% confluence. After rinsing, the cells were incubated with a liposome solution comprising Opti-MEM medium, 10 μl/ml Lipofectamine 2000 (Invitrogen), and 100 nM siRNA with 10% FBS. After 8 h of incubation, the same amount of Opti-MEM medium containing 10% FBS was added to the dishes, and the incubation was continued for 16 h. After 24 h of transfection, the liposome solutions were replaced, and cells were cultured for 24 h. Then the cells were cultured with different concentrations of TSA, and RT-PCR and Western blot analysis were performed.

Statistical analysis. Results are expressed as means ± SE. Statistical analysis was performed using ANOVA with Bonferroni correc-

Fig. 4. Time course of lung structure alterations and expression of acetyl histone 3, HIF-1α, p53, and VEGF in TSA-treated rat lungs. A: the left bar graph shows the quantification of MAAA, and the right bar graph shows the quantification of MLI in lung sections from TSA-treated rats. B: representative Western blot analysis of acetyl histone 3, histone 3, HIF-1α, lamin A/C, p53, VEGF, and RPL13A protein from TSA-treated rat lungs. The top line graph shows the ratios of acetyl histone 3 protein expression relative to histone 3 protein. The bottom line graph shows the ratios of HIF-1α, VEGF, and p53 protein expression relative to control. Lamin A/C was used as a reference for HIF-1α, and RPL13A was used as a reference for VEGF and p53. Data are expressed as means ± SE (n = 6 animals for each group). *p < 0.05 vs. control (day 0).
tions for multiple comparisons. Comparisons were considered statistically significant at \( P < 0.05 \).

**RESULTS**

TSA reduces HDAC activity of lung tissue and causes alveolar septal cell loss. RT-PCR analysis of class 1 HDACs in rat lung tissues showed that HDAC2 and HDAC3 expression was moderately decreased in TSA-treated rat lungs compared with control rat lungs (Fig. 1A), and Western blot analysis likewise showed that HDAC2 and HDAC3 protein expression was decreased in TSA-treated rat lungs compared with control rat lungs (Fig. 1B). Whereas HDAC activity was slightly decreased in TSA-treated lung tissues compared with that of control rat lungs (Fig. 1C), the nuclear expression of the acetyl histone 3 was significantly increased in TSA-treated rat lung extracts compared with control rat lungs, indicating an increase in histone acetylation (Fig. 1D). We also performed a time course study where we varied the time of animal death and lung tissue extraction from 3 to 24 h after one single TSA injection. The expression of acetylated histone 3 peaked at 3 h after one single injection, yet sequential daily injection resulted in a nontransient elevation of lung tissue acetylated histone 3 levels (Fig. 4B). To address the question whether TSA-induced HDAC inhibition affected the alveolar septal structures, we examined rat lung sections stained with hematoxylin and eosin and found that chronic TSA treatment caused a significant emphysematous destruction of the lungs, which was uniformly present throughout the TSA-treated rat lungs, and an increase of the MAAA and MLI compared with the control rat lungs (Figs. 2A and 4A). When we performed histological inspections of heart, liver, spleen, and kidney, we did not recognize any organ pathologies. Immunohistochemical analysis of tissues stained with an antibody directed against cleaved caspase-3 showed that TSA treatment significantly increased the index of positively labeled caspase-3-positive alveolar septal cells compared with the sections obtained from the control rat lungs.

![Fig. 5. TSA decreases HIF-1α and VEGF and increases p53, LC3, cleaved caspase-3, and miR34a expression in cultured human microvascular endothelial cells (HPMVEC).](image-url)

- **A**: the bar graph shows the ratio of HIF-1α and VEGF mRNA expression of cultured HPMVEC treated with TSA (0.1–10 \( \mu \)M) for 24 h measured by RT-PCR analysis.
- **B**: the bar graph shows the ratio of microRNA (miR17, miR18, miR19a, miR19b, miR20a, and miR34a) expression in cultured HPMVEC treated with TSA (0.1–10 \( \mu \)M) for 24 h measured by RT-PCR analysis.
- **C**: representative Western blot analysis of HIF-1α, cleaved caspase-3, lamin A/C, p53, LC3, VEGF, and β-actin from cultured HPMVEC protein extracts treated with TSA (0.01–10 \( \mu \)M) for 24 h. The top bar graph shows the ratios of HIF-1α and cleaved caspase-3 protein expression relative to lamin A/C protein. The bottom bar graph shows the ratios of p53, LC3, and VEGF protein expression relative to RPL13A protein. Data are expressed as means ± SE (\( n = 4 \) wells or dishes for each group). *\( P < 0.05 \) vs. control; **\( P < 0.01 \) vs. control.
Thus these experiments established that TSA treatment resulted in lung cell apoptosis and septal cell loss. Inflammation was assessed histologically, i.e., multiple tissue sections were examined, and inflammatory cell infiltrates were not found in the parenchyma. There were no obvious accumulations of alveolar macrophages nor were there peribronchial or perivascular cell infiltrates. This may not be surprising because TSA, in several studies, has been shown to have anti-inflammatory actions (9, 15, 21).

To address the question whether HDAC inhibition was associated with activation of proteases, we performed zymography of lung tissue protein extracts and found that HDAC inhibition due to TSA treatment did not result in activation of matrix metalloproteinase MMP-2 or MMP-9 (Supplemental Fig. S1).

TSA decreases lung tissue expression of HIF-1α and VEGF and increases expression of miR34a. RT-PCR analysis demonstrated that HIF-1α, VEGF, and LOX mRNA expression was significantly decreased in TSA-treated rat lungs compared with control lungs. There was no significant treatment effect on the mRNA expression of elastin, fibulin-1, fibulin-3, and fibrillin-1, all known as important components of the lung tissue matrix. However, COL1A1, COL1A2, and COL3A mRNA expression was significantly suppressed in TSA-treated rat lungs (Fig. 3A). There was a significant increase in the expression of miR34a in TSA-treated rat lungs, whereas miR17, miR18, miR19a, miR19b, and miR20 (all known regulators of HIF-1α expression) were not affected (Fig. 3B).

Western blot analysis showed that HIF-1α, VEGF, and LOX protein expression was suppressed in TSA-treated lungs compared with controls, whereas the protein expression of cleaved caspase-3, microtubule-associated protein 1 light chain 3 (LC3), and p53 was increased. Also, we found that TSA decreased the protein expression of COL1A and COL3A (Fig. 3C). Both p53 and HIF-1α protein expression were already significantly altered at 12 h after one single TSA injection (Fig. 4B).

TSA decreases HIF-1α and VEGF expression while increasing the expression of p53 and LC3 in HPMVECs. RT-PCR analysis of cultured HPMVEC showed that HIF-1α and VEGF mRNA expression was significantly and dose-dependently suppressed by TSA starting with a concentration of 0.1 μM (Fig. 5A). Again, as in the rat lung tissue extract, the miRNA analysis showed a significant increase in the expression of miR34a, whereas miR17, miR18, miR19a, miR19b, and miR20 expression did not change (Fig. 5B). Western blot analysis of cultured HPMVEC showed that HIF-1α and VEGF protein expression was dose-dependently suppressed by TSA. The expression of cleaved caspase-3, LC3, and p53 protein was dose-dependently increased by TSA (Fig. 5C).

To further understand the role of TSA on HIF-1α mRNA and p53 protein expression, we analyzed the stability of HIF-1α mRNA and p53 protein expression using the transcription inhibitor ActD and the protein synthesis inhibitor CHX. The stability of HIF-1α mRNA was assessed by mRNA chase experiments using ActD. HIF-1α mRNA stability of cultured HPMVEC was not changed in the combined presence of TSA and ActD, suggesting that TSA has little effect on HIF-1α mRNA stability (Fig. 6A). Because p53 protein expression was increased in the combined presence of TSA and CHX (Fig. 6B), a longer half-life of p53 protein of TSA-treated cells was suggested.

In addition, HIF-1α mRNA and protein expression were increased in p53 gene-silenced HPMVECs compared with the control siRNA-treated cells. Whereas the expression of HIF-1α mRNA was not changed in p53-silenced HPMVECs, 1 μM TSA decreased HIF-1α mRNA and protein expression in p53-silenced cells (Fig. 7).
Gene silencing of HDAC2 decreases expression of HIF-1α and VEGF in HPMVECs. HDAC2 siRNA and HDAC3 siRNA transfection nearly abolished the expression of both HDAC2 and HDAC3 mRNA (Fig. 8, A and B) and HDAC2 protein (Fig. 8C) in HPMVEC. The expression of HIF-1α and VEGF mRNA was significantly suppressed in HPMVECs after HDAC2 silencing (Fig. 8A). The protein expression of HDAC2 was significantly inhibited in the HPMVECs, and HIF-1α and VEGF protein expression was significantly suppressed and p53 protein expression was induced in HDAC2-silenced HPMVECs (Fig. 8C). Please see Fig. 9, which attempts to connect the results of our studies schematically.

DISCUSSION

Oxidative stress and the uncontrolled attack of proteases on lung structure cell and matrix proteins, both caused and sustained by chronic inflammation, are the cornerstones of our concepts of the pathogenesis of emphysema (23, 47). Here, we provide experimental evidence that independent of inflammation, fundamental dysregulation of chromatin remodeling-controlled gene transcription is sufficient to cause the destruction of the adult gas exchange units of the lung. Our data indicate that appropriately functioning HDACs are required for the maintenance of the adult lung structure. Using TSA, a global HDAC inhibitor known to induce apoptosis of cancer cells (19, 54), we show that HDAC inhibition impairs the production of the trophic factor VEGF and inhibits the expression of LOX, which is required for collagen and elastin cross-linking, and we also show that HDAC inhibition impairs the production of lung collagen proteins. Animals treated with TSA injections for 42 days and examined 4 wk after cessation of TSA treatment demonstrated that the lung tissue destruction in these rats can, to some degree, spontaneously repair itself (Supplemental Fig. S2).

Because it has previously been shown that lung tissue from patients with COPD/emphysema is characterized by a reduction of expression and activity of HDAC2 (25, 52), we first established that chronic treatment of rats with an HDAC inhibitor decreased the expression and activity of HDAC2 in their lung tissues. TSA treatment did indeed inhibit HDAC2 expression in the lung and also reduced the expression of HDAC3 mRNA in the rat lung tissues, as has been shown for the treatment of fetal fibroblasts with another HDAC inhibitor, sodium butyrate (32). Remarkably, the reduction in lung tissue HDAC activity achieved by TSA treatment in our experiments was smaller than that reported for human COPD lung tissue by Ito et al. (25). In contrast, TSA did not affect the tissue expression of HDAC8, which had been reported to be reduced in the lungs from COPD patients (25). Whereas TSA is not presently used clinically, TSA effects have been widely examined in cancer cell lines and in preclinical rodent models (9, 10, 15, 19, 21, 49).

Given this evidence of effective HDAC inhibition as a consequence of TSA treatment, we addressed the question...
whether TSA treatment also affected the adult lung structure, and we found a significant loss of alveolar septal cells and apoptosis of alveolar septal cells. HDAC inhibition increased the expression of cleaved caspase-3 and LC3, key mediators of apoptosis and autophagy both in lung tissue and cultured lung cells. These results suggest that inhibition of HDAC activity is functionally linked to the induction of apoptosis and likely autophagy. Clearly, epithelial cells in the lung express genes and proteins that contribute to the maintenance of the alveolar structures. However, for the purpose of this investigation, we

Fig. 9. The schematic depicts cellular and molecular interactions that control lung structure maintenance. The inhibition of HDAC may increase p53 expression. Both increased p53 and HDAC inhibition could reduce expression of HIF-1α. Insufficient HIF-1α causes impaired expression of the downstream mRNA encoding LOX, which may impair the cross-linking of collagen and elastin. Insufficient HIF-1α also impairs the expression of VEGF, which may result in lung cell apoptosis and emphysema. HDACs may also have a separate direct effect on collagen synthesis, which may contribute to alterations in lung structure and emphysema. Small up and down arrows indicate increased or decreased expression and loss of lung structure cells.

Fig. 8. Gene silencing of HDAC2 decreases HIF-1α and VEGF expression in HPMVECs. A: the bar graph shows the ratio of HDAC2, HIF-1α, and VEGF mRNA expression of cultured HPMVECs transfected with HDAC2 siRNA measured by RT-PCR analysis. B: representative Western blot analysis of HIF-1α, HDAC2, lamin A/C, VEGF, p53, and RPL13A from control or HDAC2 siRNA-transfected HPMVEC protein extracts. The top bar graph shows the ratios of HIF-1α and HDAC2 protein expression relative to lamin A/C protein. The bottom bar graph shows the ratios of p53 and VEGF protein expression relative to RPL13A protein. Data are expressed as means ± SE (n = 4 wells or dishes for each group). *P < 0.05 vs. control siRNA.
restricted our next experimental steps to lung microvascular endothelial cells, which are frequently not considered in discussions pertinent to emphysema genesis. In addition, we measured IL-1β and TNF-α in serum and lung tissue protein extracts and found that the tissue levels of these cytokines were decreased by TSA treatment (Supplemental Fig. S3).

Apoptosis of lung endothelial cells has been described in emphysematous lungs (26) and VEGF receptor inhibition (27) or conditional knockout of the VEGF ligand in mouse lungs (46) results in emphysema. Indeed, we reproduced the antiangiogenic actions of TSA, which have been documented in cancer cell lines and in in vitro experiments, which demonstrated that TSA inhibits VEGF-stimulated angiogenesis of human umbilical vein endothelial cells (14, 41). A number of published reports indicate that HDAC inhibition causes a reduction in the expression of HIF-1α (16, 30, 37). We (52) recently reported that both HDAC2 and HIF-1α protein levels are reduced in lung tissue samples from patients with COPD/ emphysema, and we postulate that the reduction of HIF-1α mRNA, found by us in TSA-treated lung tissues and HPMVECs, caused the inhibition of VEGF expression. Likewise, we propose that the reduction in the expression of LOX mRNA was a consequence of impaired HIF-1α expression. We propose such a causality based on the studies published by Erler et al. (17, 18) and Chinnaiyan et al. (7) that demonstrated a reduction of HIF-1α mRNA expression in three different cancer cell lines. Interestingly, HDAC inhibition affected HIF-1α not only on the protein level, but also on a transcriptional level. Our observations suggest that the activation of HIF-1α gene expression is regulated also by the chromatin structure modification itself. Previous reports show that HIF-1α and its transcription have been implicated in the neovascularization and apoptosis resistance in cancer (12, 53). Possibly, the transcriptional control of HIF-1α is essential to the survival of endothelial cells. Likewise, in the normal lung, which is normoxic and therefore expected to exhibit a continuously high degree of HIF-1α ubiquitination, there is apparently a role for HIF-1α as a controller of cell survival and organ structure maintenance. We considered that TSA treatment would affect HIF-1α expression via changes in the expression of the miRNA cluster miRNA17–92 (45) and probed both lung tissue extracts and cultured HPMVECs for changes of expression of these miRNAs and concluded that the TSA-induced decrease in HIF-1α gene expression was not explained by changed expression of these miRNAs. The changes in lung tissue HIF-1α, VEGF, LOX, COL1A, and COL3A gene expression were confirmed on the protein level. In addition, we found a robust upregulation of p53 protein expression in the lungs from TSA-treated rats and suggest that increased p53 expression is involved in the down-regulation of HIF-1α protein expression, as reported by Sano et al. (42) and Kim et al. (29) in the heart. In the context of TSA-induced apoptosis of lung alveolar septal cells, a recent study by Chang et al. (4) connects p53 with the transactivation of miRNA34a, and we now report the novel finding of upregulated expression of miR34a in the lungs and HPMVECs after HDAC inhibition. Loss of miRNA34a expression has been linked to apoptosis resistance of tumor cells and, conversely, increased expression of miR34a to induction of apoptosis and cellular senescence (24). Our findings may support a role of increased miRNA34a expression in lung cell apoptosis.

It is generally accepted that acetylation of several lysine residues in the COOH-terminal domain of p53 contributes to the stability of p53 (19, 54). This lysine residue acetylation status may account for the increased expression of p53 in the lungs from TSA-treated rats and TSA-treated cells. Thus p53 protein stability emerges as an important controller of lung alveolar structure cell survival. To focus specifically on HDAC2, which is reduced in the lungs from COPD patients (25), we employed a knockdown strategy using a siRNA directed against HDAC2. These experiments show that the targeted knockdown of HDAC2 causes a decreased expression of HIF-1α and a decreased expression of VEGF in HPMVECs.

Taken together (Fig. 9), our results of lung tissue and lung cell analysis support the concept of an impairment of angiogenic maintenance (47) and impaired synthesis of collagens as well as a defect of matrix protein cross-linking due to impaired LOX expression (28, 44), each likely a consequence of impaired HDAC and HIF-1α function. Working with this model may lead us to an enriched view of mechanisms of the adult lung maintenance program that may perhaps also be connected to another set of controls, which have been found in the nuclear regulation of histone acetylation by sphingolipid metabolites. Hait et al. (20) recently showed that sphingosine kinase 2 increased histone lysine acetylation, that both sphingosine 1 phosphate (S1P) and dihydro-S1P inhibited HDAC activity in nuclear extracts, and that S1P inhibited the enzymatic activity of endogenous HDAC. An imbalance of S1P and ceramide, particularly under conditions of oxidative stress, may lead to emphysematous lung tissue destruction (35).

In conclusion, we have introduced a novel animal model of emphysema, which is based on alterations of histone acetylation, and we propose a pathogenetic model of lung cell apoptosis based on interactions between HIF-1α, VEGF, LOX, and p53.

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DISCLOSURES

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