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Organic dust inhibits surfactant protein expression by reducing thyroid transcription factor-1 levels in human lung epithelial cells

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Abstract

Exposure to organic dust is a risk factor for the development of respiratory diseases. Surfactant proteins (SP) reduce alveolar surface tension and modulate innate immune responses to control lung inflammation. Therefore, changes in SP levels could contribute to the development of organic-dust-induced respiratory diseases. Because information on the effects of organic dust on SP levels is lacking, we studied the effects of dust from a poultry farm on SP expression. We found that dust extract reduced SP-A and SP-B mRNA and protein levels in H441 human lung epithelial cells by inhibiting their promoter activities, but did not have any effect on SP-D protein levels. Dust extract also reduced SP-A and SP-C levels in primary human alveolar epithelial cells. The inhibitory effects were not due to LPS or protease activities present in dust extract or mediated via oxidative stress, but were dependent on a heat-labile factor(s). Thyroid transcription factor-1, a key transcriptional activator of SP expression, was reduced in dust-extract-treated cells, indicating that its down-regulation mediates inhibition of SP levels. Our study implies that down-regulation of SP levels by organic dust could contribute to the development of lung inflammation and respiratory diseases in humans.

Keywords

Lung surfactant, lung epithelial cells, organic dust, lung inflammation, respiratory diseases

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Introduction

Workers in animal and agricultural farms are exposed to high concentrations of aerosolized dust containing organic and inorganic constituents and microorganisms,[1,2] and are at risk of developing respiratory symptoms and respiratory diseases.[3] In fact, lung diseases in agricultural workers are one of the earliest recognized occupational hazards. Lung diseases such as organic dust toxic syndrome, allergic pneumonitis, chronic bronchitis, and occupational asthma are common among agricultural workers.[3] Due to high-density animal farming, workers are exposed to higher concentrations of aerosolized dust and its constituents for prolonged periods of time. As such, there is a strong association between exposure to aerosolized agricultural/animal dust and respiratory symptoms and respiratory diseases.[4] In this investigation, we used poultry dust as a model organic dust to study its effects on the regulation of surfactant protein (SP) expression. The United States is the second largest poultry producer in the world, and several hundred thousand workers are employed in the poultry industry.[5,6] Workers in poultry production facilities are exposed to higher concentrations of aerosolized dust compared with other

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animal production workers, and experience a higher prevalence of lower and upper respiratory symptoms and lower baseline lung function.[7,8]

Lung surfactant is primarily a mixture of phospholipids and lung-specific proteins, and has two major functions: firstly, it functions to reduce surface tension at the alveolar air–tissue interphase to prevent collapse of the alveoli to ease the work of breathing,[9] and, secondly, it serves as a component of the lung's innate immune system to maintain sterility and balance immune responses in the lung.[10] To date, four distinct SPs, SP-A, SP-B, SP-C, and SP-D have been isolated and characterized. Human SP-A protein consists of two variants, SP-A1 and SP-A2, that are encoded by the genes *SP-A1* and *SP-A2*.[11] Alveolar type II epithelial cells express SP-A, SP-B, SP-C, and SP-D, whereas bronchiolar or Clara epithelial cells express only SP-A, SP-B, and SP-D. As in the alveoli, surfactant stabilizes small airways and participates in local host defense.[12] SP-A and SP-D are hydrophilic proteins that belong to the collectin family of proteins and serve important functions in the control of innate immune and inflammatory responses.[10] SP-B and SP-C are hydrophobic proteins that are important for the maintenance of the surface tension reducing properties of the surfactant.[13] SP-A and SP-D not only have bactericidal action but also enhance uptake of bacterial and viral pathogens by phagocytes.[10] SP-A and SP-D have also been implicated in the regulation of various cellular responses, such as production of inflammatory mediators and oxygen and nitrogen metabolites.[10] Additionally, SP-A and SP-D promote the clearance of apoptotic cells by alveolar macrophages.[10] SP-B is essential for lung function as its complete absence results in death in newborn human infants,[14] and in newborn mice.[15,16] SP-B deficiency causes lung inflammation, indicating that, apart from maintaining lung function, SP-B also reduces lung inflammation.[17] SP-C binds to LPS and modulates macrophage cytokine activity *in vitro*.[18] SP-C-deficient mice have reduced ability to resolve inflammation due to LPS exposure.[19] Therefore, changes in SP levels could modulate organic-dust-induced lung inflammatory responses and development of disease. However, there is very little or no information on the effects of organic dust on the regulation of SP levels. We hypothesized that exposure of lung epithelial cells to organic dust alters SP expression. In this investigation, we studied the effects of aqueous poultry dust extracts (hereafter referred to as dust extracts) on the regulation of SP-A, SP-B, SP-C, and SP-D mRNA and protein levels in H441 human bronchiolar and in primary human alveolar epithelial cells *in vitro*.

We previously reported that poultry dust extract contains LPS[20] and elastase- and trypsin-like protease activities.[21] We also reported that poultry dust extracts induce inflammatory and immune response gene expression in lung epithelial and THP-1 monocytic cells.[20,22] Furthermore, we reported that aqueous poultry dust extracts were equally effective as poultry dust particles in inducing inflammatory gene expression.[22] The induction of cytokine gene expression was dependent on protease activities and mediated via production of reactive oxygen species and protein kinase activation.[20,21]

In this study, we found that treatment of H441 bronchiolar epithelial cells with dust extract reduced SP-A and SP-B mRNA and protein levels, but had no effect on SP-D protein levels. Dust extract reduced SP-A and SP-C protein levels in human primary alveolar epithelial cells similarly as in H441 cells. In H441 cells, LPS inhibitor, polymyxin B, or serine protease inhibitors such as α1-antitrypsin or soybean trypsin inhibitor did not prevent the inhibitory effects of dust extract on SP-A and SP-B protein levels; however, heat treatment of dust extract at 95°C blocked its inhibitory effects. Dust extract inhibited SP-A and SP-B levels primarily by inhibiting their promoter activities. Treatment of cells with antioxidants such as dimethylthiourea did not prevent inhibition of SP levels by dust extract. Inhibition of SP levels in H441 cells was associated with reduced levels of thyroid transcription factor-1 (TTF-1/Nkx2.1/TITF1).

Materials and methods

Preparation of dust extracts

Settled broiler poultry dust collected previously from a commercial poultry farm in East Texas, USA, when the chickens were approximately 8 wk of age, was stored at −70°C. Dust was mixed with serum-free F12 K medium containing penicillin (100 U/ml), streptomycin (100 μg/ml), and amphotericin B (0.25 μg/ml) at a ratio of 1:10 (mass/volume) and extracted as described previously.[20] The concentration of dust extract thus obtained was arbitrarily considered as 100%. The protein concentration of dust extracts was typically found to be in the range of 0.2–0.4 mg/ml.

Ultrafiltration

Dust extracts were subjected to ultrafiltration using 3, 10, and 30 kDa molecular mass cut off centrifugal filters (Amicon Ultra, Millipore, Bedford, MA) according to the manufacturer's instructions. The retentate and the filtrate were tested for their inhibitory effects on SP levels in H441 cells.

Chemicals and Abs

Cell culture media and cell culture reagents were from Life Technologies (Carlsbad, CA). Polymyxin B, actinomycin D, N^{ω}-nitro-L-arginine methyl ester (L-NAME), α1-antitrypsin and soybean trypsin inhibitor were from Sigma-Aldrich (St. Louis, MO). Dimethylthiourea was from Acros Organics (Morris, NJ). SP-A Abs were from Chemicon International (catalog # AB3030) (Temecula, CA) or kindly provided by Joanna Floros, Penn State College of Medicine (Hershey, PA). SP-B Abs were from Chemicon International (catalog # AB3034) (Temecula, CA) or from Seven Hills Bioreagents (catalog # WRAB-48604) (Cincinnati, OH). SP-B Abs from Chemicon International were reacted with the blot under reducing conditions, whereas SP-B Abs from Seven Hills Bioreagents were reacted under non-reducing conditions. SP-C (catalog # sc-13979) and actin (catalog # sc-1616, sc-47778) Abs were from Santa Cruz Biotechnology (Dallas, TX) and tubulin Abs (catalog # MS-581-P0) were from Thermo Fisher Scientific (Fremont, CA). Mouse monoclonal SP-D Abs (catalog # WMAB-1A10A9) were from Seven Hills Bioreagents (Cincinnati, OH). TTF-1 Abs were purified by Protein A-Sepharose chromatography from rabbit antisera against N-terminal portion of rat T/EBP (TTF-1/Nkx2.1) kindly provided by Shioko Kimura, National Cancer Institute (Bethesda, MD). We have used these Abs for Western blot detection of TTF-1.[23]

Cell culture

NCI-H441 cells (ATCC HTB-174), a human lung adenocarcinoma cell line with characteristics of bronchiolar (Clara) epithelial cells were grown on plastic cell culture dishes in RPMI 1640 medium supplemented with 10% FBS, penicillin (100 U/ml), streptomycin (100 µg/ml), and amphotericin B (0.25 µg/ml) in a humidified atmosphere of 95% room air and 5% CO_2. H441 cells were treated with dust extracts in complete cell culture medium. Human primary alveolar epithelial cells (ScienCell, Carlsbad, CA) that are comprised of alveolar type I and alveolar type II cells were grown on poly-L-lysine coated plastic dishes in alveolar epithelial cell medium (ScienCell, Carlsbad, CA) containing FBS and epithelial cell growth supplements. For treatments, alveolar epithelial cells were maintained in RPMI 1640 medium without serum overnight and treated with dust extract in the same medium.

Cell viability

Cell viability was measured using CellTiter 96 Aqueous Non-Radioactive Cell Proliferation Assay (MTS) kit (Promega, Madison, WI).

RNA isolation, Northern blot analysis, and real-time quantitative RT-PCR

Total RNA was isolated using TRI-Reagent (Molecular Research Center) and treated with TURBO DNAse (Ambion) to remove genomic DNA and cDNA synthesized using random hexamers and reverse transcriptase (Applied Biosystems). Levels of mRNAs and 18S rRNA were determined by TaqMan assays (Invitrogen) and the levels of mRNAs normalized to 18S rRNA levels. Gene expression IDs for Taqman assays are listed in Table 1.

Preparations of cell lysates and nuclear extracts

Cells were lysed in buffer (50 mM Tris-HCl, pH 7.4 containing 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 15% glycerol, and 1X protease and phosphatase inhibitor cocktail) and cleared by centrifugation at 16,000 *g* for 10 min at 4°C. Nuclear extracts from H441 cells were isolated as described previously.[24] Protein concentrations of lysates and nuclear extracts were determined by Bradford assay.

Western immunoblotting

Equal amounts of proteins were separated by SDS-PAGE on 10% Bis-Tris gels with MOPS or MES as the running buffer. Separated proteins were transferred to PVDF membranes by electroblotting, probed with specific Abs, and the proteins were visualized by enhanced chemifluorescence detection method (GE Healthcare). Membranes were stripped and re-probed for actin or tubulin levels for correcting loading errors. Protein bands were quantified using QuantityOne software (Bio-Rad).

Cloning of SP-A1 and SP-A2 promoters and transient transfection analysis

5′-Flanking DNA sequences of human SP-A1 (–1111/+99 bp)[25] and SP-A2 (–1111/+69 bp)[26] genes were amplified by polymerase chain reaction using H441 genomic DNA as the template and gene-specific primers. The forward and reverse primers for amplifying SP-A1 and SP-A2 DNA sequences are shown below. SP-A1 primers contained *Mlu*I and *Xho*I restriction enzyme sites (underlined) and SP-A2 primers contained

Table 1. Taqman assay gene expression IDs.

Gene symbol	Gene name	Human assay ID
SFTPA1	Surfactant protein A1	Hs00831305_s1
SFTPA2	Surfactant protein A2	Hs04195463_g1
SFTB	Surfactant protein B	Hs01090667_m1
18S	18S rRNA	Hs99999901

*Xho*I and *Hin*dIII restriction sites to facilitate cloning of amplified DNA fragments into luciferase reporter vector.

SP-A1 (F): 5'-gatacgcgtaaccacaaggccctgcac-3'
SP-A1 (R): 5'-cgcactcgagctctctgcctgcgatcct-3'
SP-A2 (F): 5'-cgcactcgaggggtcaaaactcaggtgtgc-3'
SP-A2 (R): 5'-gcaaagcttgggatgtggctaaactcacc-3'

Amplified DNA fragments were digested with restriction enzymes, purified by agarose gel electrophoresis, and cloned into promoter-less luciferase vector pGL3lucbasic (Promega, Madison, WI). SP-A1 and SP-A2 clones were sequenced and their sequence verified by alignment with published sequences. Cloned SP-A1 and SP-A2 promoter sequences showed 96% and 98% sequence similarities, respectively, with the published SP-A1 and SP-A2 promoter sequences. Cloning of human SP-B promoter sequence (–911/+41 bp) has been described previously.[27] Promoter plasmids were transiently transfected into H441 cells using Lipofectamine 2000 (Invitrogen, Carlsbad, CA).

Statistical analyses

Data are shown as means ± SD or SE. The levels of mRNA, protein or promoter activity in control or dexamethasone treated samples were arbitrarily considered as 100 and relative levels in treated samples are shown. Statistical significance was evaluated by one sample *t*-test, and one-tailed P values < 0.05 were considered significant.

Results

Dust extract inhibits SP-A and SP-B protein and mRNA levels

H441 cells display the characteristics of bronchiolar epithelial cells and express SP-A, SP-B, and SP-D, but not SP-C, and have been used widely to study the regulation of SP expression.[28] Because SP-B protein levels are rather low in H441 cells, the effect of dust extract treatment on dexamethasone induction of SP-B was determined. Treatment with dust extract at 0.01% or 0.1% for 24 h did not significantly alter SP-A protein levels; however, 0.25% and higher concentrations inhibited SP-A protein levels compared with untreated cells (Figure 1a and b). Treatment with 0.01% dust extract for 24 h increased SP-B protein levels compared with untreated cells; however, treatment with 0.1% or higher concentrations reduced SP-B protein levels (Figure 1c and d), while SP-D protein levels were not affected (Figure 1e and f). To determine whether the inhibitory effects are due to changes at the gene expression level, the effects of dust extract on SP-A1, SP-A2, and SP-B mRNA levels were determined. Treatment with dust extract reduced SP-A1, SP-A2, and SP-B mRNA levels with 0.25% or 1% dust extract causing maximal inhibition (Figure 1g–i). Treatment with 0.1%–1% dust extract for 24 h reduced SP-B mRNA levels in H441 cells in the presence of dexamethasone (data not shown). Viability of H441 cells was not affected by treatment with dust extract (0.1–2%) for 24 h (Supplementary Figure 1). To determine if dust extract has similar effects on SP levels in primary cells, we investigated the effects of dust extract on SP levels in primary human alveolar epithelial cells. Treatment with dust extract (0.25%) for 24 h reduced SP-A and SP-C protein levels (Figure 1j and k); however, SP-B protein expression could not be detected in control or treated cells (data not shown).

Dust extract inhibits SP-A and SP-B promoter activities

To determine the role of transcriptional mechanisms in the inhibition of SP-A and SP-B expression, the effects of dust extract on SP-A and SP-B promoter activities were investigated by transient transfection analysis. Treatment with 1% dust extract for 24 h had no effect on SP-A1 promoter activity, but inhibited SP-A2 and SP-B promoter activities (Figure 2).

Dust extract does not alter the stabilities of SP-A1, SP-A2, and SP-B mRNAs

To determine the role of mRNA stability in the inhibition of SP-A and SP-B mRNA levels, the effects of dust extract treatment on SP-A and SP-B mRNA stabilities were determined. Cells were first treated with or without dust extract for 24 h, and mRNA degradation determined after inhibition of new RNA synthesis with actinomycin D. Data showed that SP-A1, SP-A2, and SP-B mRNA degradation rates were similar in untreated and dust extract treated cells (Figure 3).

Polymyxin B does not block inhibition of SP levels

As dust extracts contain significant amounts of LPS,[20] the effects of polymyxin B—an inhibitor of LPS—on the inhibition of SP levels were determined. Polymyxin B by itself appeared to increase SP-A protein levels in a dose-dependent manner, and did not prevent inhibition of SP-A and SP-B protein levels by dust extract (Figure 4).

Protease inhibitors do not block inhibition of SP levels

Dust extracts contain trypsin- and elastase-like activities;[21] we therefore investigated if these activities control inhibition of SP levels. Results indicated that

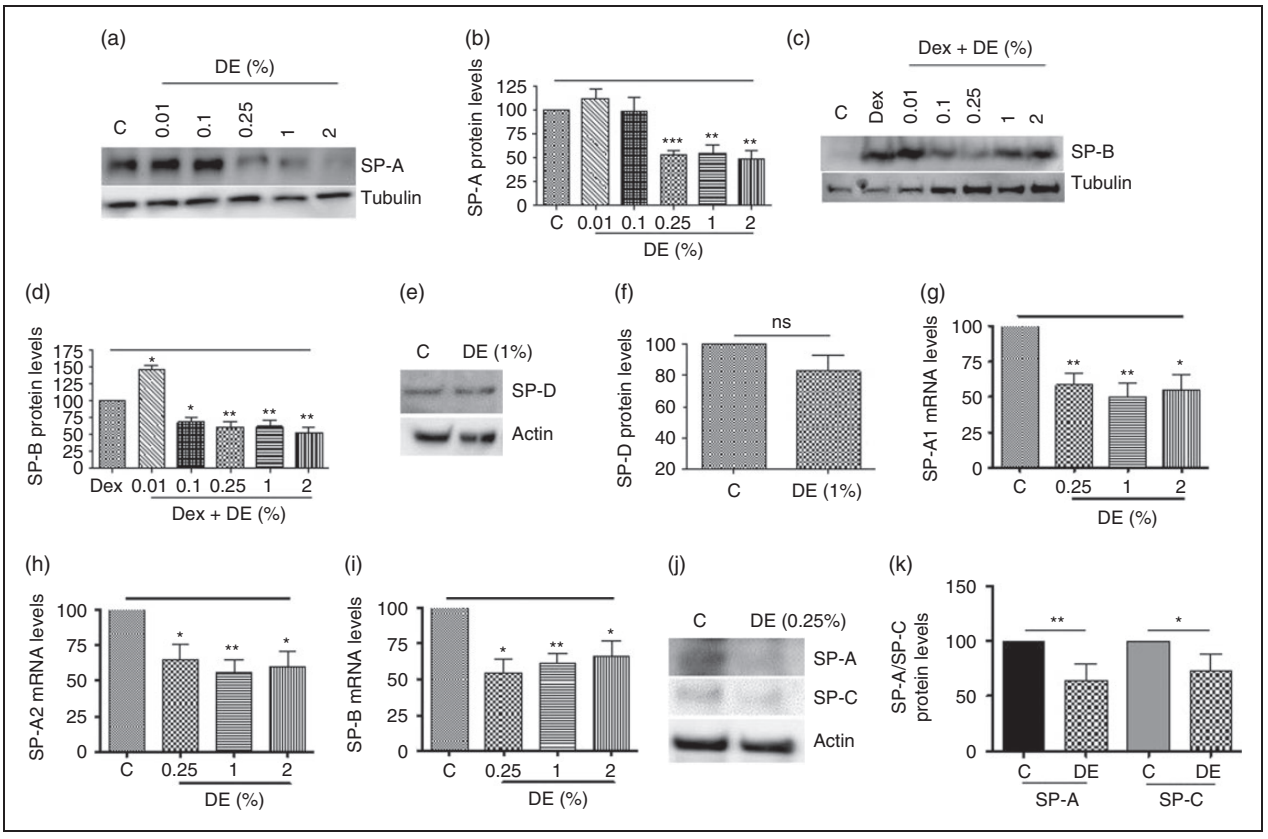

Figure 1. Dust extract inhibits surfactant protein gene expression in H441 and primary human alveolar epithelial cells. (a–f) H441 cells were treated for 24 h with medium, different concentrations of dust extract, dexamethasone (0.1 μM), or a combination of dexamethasone (0.1 μM) and dust extract. SP-A, SP-B, and SP-D protein levels were determined by Western immunoblotting and normalized to tubulin or actin levels. Representative Western blot images are shown. Data are mean ± SE ($n = 3$–6). Because SP-B protein levels are low in untreated cells, SP-B protein levels in dexamethasone treated cells were arbitrarily considered as 100, and relative levels in cells treated with dust extract in the presence of dexamethasone are shown. ns: not significant; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. (g–i) H441 cells were treated with medium or indicated concentrations of dust extract for 24 h, and SP-A1, SP-A2, and SP-B mRNA levels were determined by real-time qRT-PCR and normalized to 18S rRNA levels (n = 4–5). Data are shown as mean ± SE. $^{*}P < 0.05$, $^{**}P < 0.01$. (j, k) Primary human alveolar epithelial cells were treated for 24 h with medium or medium containing dust extract (0.25%) and SP-A and SP-C protein levels were determined by Western immunoblotting and normalized to actin levels. A representative Western blot image is shown. Data are shown as mean ± SD of duplicates ($n = 2$). $^{*}P < 0.05$, $^{**}P < 0.01$. C: control; DE: dust extract; Dex: dexamethasone.

α1-antitrypsin and soybean trypsin inhibitor did not suppress reduction in SP-A and SP-B protein levels in dust extract-treated cells (Figure 5).

Although α1-antitrypsin and soybean trypsin inhibitor failed to block inhibition of SP-A and SP-B protein levels, heating dust extracts at 95°C for 10 min prevented inhibition of SP-A and SP-B protein levels (Figure 6a, b, e, and f). Changes in SP levels were associated with similar changes in SP mRNA levels (Figure 6c, d and g). Fractionation of dust extract using 30 kDa molecular mass cutoff centrifugal filter demonstrated that the retentate, but not the filtrate inhibited SP-A and SP-B protein levels (Figure 6h–k). Similar results were obtained with 3 kDa and 10 kDa molecular mass cutoff filters (data not shown). Similar to unfractionated dust extract, the retentate fraction's ability to inhibit SP levels was sensitive to heating at 95°C for 10 min (Figure 6h–k).

Oxidant and nitrosative stress are not involved in the inhibition of SP levels

We have found that dust extract increases ROS production and 4-hydroxynonenal (4-HNE) staining in lung epithelial cells;[21] therefore, we examined whether the inhibitory effects of dust extract on SP-A and SP-B levels are mediated via production of ROS and nitric oxide. Treatment with antioxidant, dimethylthiourea, or NO synthase (NOS) inhibitor, L-NAME did not prevent the inhibition of SP levels by dust extract (Figure

7a–f). Additionally, treatment with a different antioxidant, *N*-acetylcysteine or 4-HNE scavenger, histidyl hydrazide did not prevent inhibition of SP levels (data not shown).

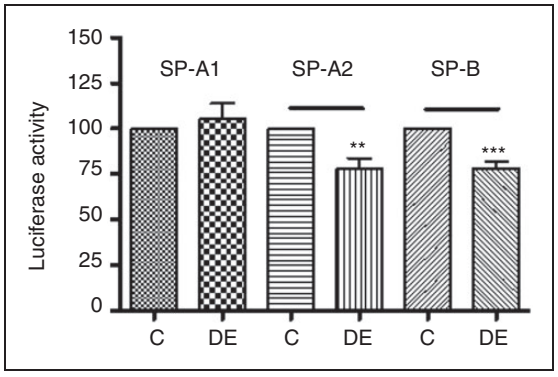

Figure 2. Dust extract inhibits SP-A and SP-B promoter activities. H441 cells were transiently transfected with human SP-A1, SP-A2, or SP-B promoter plasmids containing luciferase reporter gene and then treated with medium or medium containing dust extract (1%) for 24 h. Luciferase activities in cell lysates were determined and normalized to total protein concentration of cell lysates. Data are mean ± SE ($n = 6$–7). $^{**}P < 0.01$, $^{***}P < 0.001$. C: control; DE: dust extract.

Effects of dust extract on TTF-1/Nkx2.1/TITF1) levels

TTF-1 is a key transcriptional activator of SP-A, SP-B, and SP-C genes.[28] We determined the effects of dust extract on TTF-1 levels in H441 cells in an attempt to understand the mechanisms controlling the regulation of SP-A and SP-B gene expression. TTF-1 protein levels in nuclear extracts of H441 cells treated with or without dust extract for 24 h were determined. It was found that treatment of H441 cells with dust extract reduced TTF-1 levels (Figure 8a and b). To determine if lack of inhibition of SP-A and SP-B protein levels in heat-treated dust extracts is associated with similar lack of inhibition of TTF-1 levels, the effects of heat-treated dust extract on TTF-1 protein levels were determined. Results demonstrated that heat treatment prevented inhibition of TTF-1 protein levels (Figure 8c and d).

Discussion

Along with surfactant lipids, SPs maintain the integrity of the alveoli and preserve the patency of the airways during respiration.[9] Additionally, SPs protect against

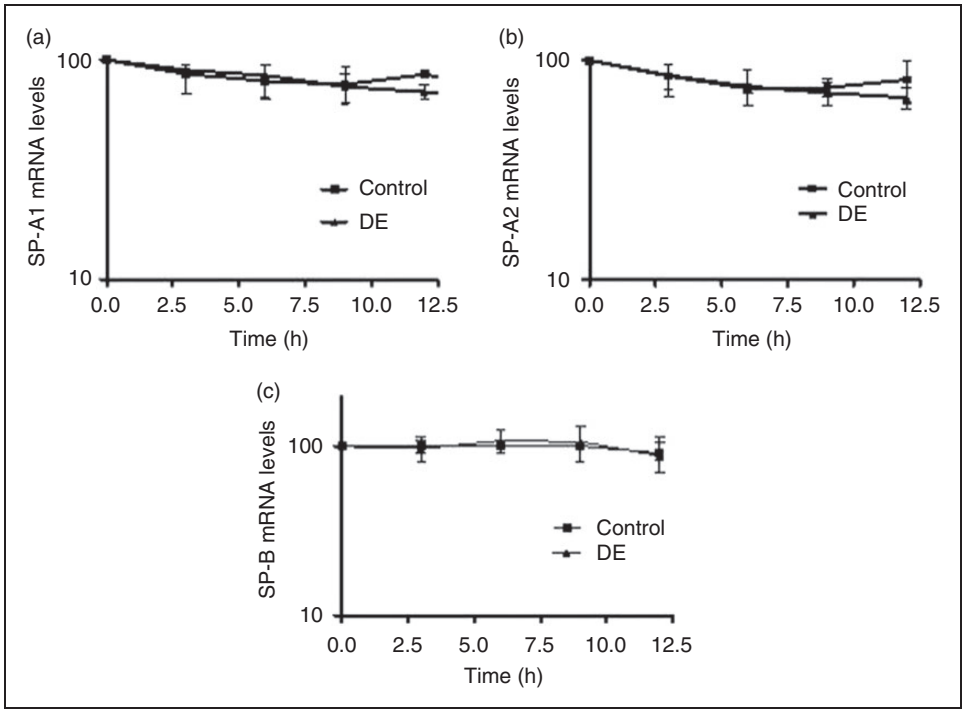

Figure 3. Dust extract does not alter the stabilities of SP-A1, SP-A2, and SP-B mRNAs. H441 cells were first incubated with medium (control) or dust extract (1%) for 24 h, and incubation continued in the presence of actinomycin D (5 μM) for the indicated periods of time. Total RNA was isolated and SP-A1, SP-A2, and SP-B mRNA levels were quantified by real-time qRT-PCR and normalized to 18S rRNA levels. Data are mean ± SE ($n = 3$) for SP-A1 and SP-A2 mRNAs and ± SD ($n = 2$) for SP-B mRNA.
DE: dust extract.

lung injury caused by inhaled particles and infectious agents through their actions to enhance clearance of particulates and infectious agents and reduce inflammation.[10] Surfactant abnormalities such as inactivation of SPs and/or alterations in SP levels could be contributing factors for the development of lung diseases, such as acute respiratory distress syndrome, asthma, bronchiolitis, hypersensitivity pneumonitis, idiopathic pulmonary fibrosis, and others.[29] Agricultural workers experience respiratory symptoms and respiratory diseases due to acute and chronic exposure to organic dusts, and hypersensitivity pneumonitis, organic dust toxic syndrome, asthma-like syndrome, and chronic bronchitis are commonly found among such workers.[3] It is unknown if alterations in SP levels and/or activities are associated with the development of lung diseases in agricultural workers. It is also not known if exposure to organic dust alters the expression of SPs in lung epithelial cells *in vitro*.

Aqueous organic dust extracts have been employed in *in vitro* cell culture,[20,30,31] and *in vivo* mouse studies to investigate the effects on lung inflammation and on lung inflammatory gene expression.[32–34] We found that poultry dust extracts reduced SP-A and SP-B protein and SP-A and SP-B mRNA levels in a dose-dependent manner without affecting the viability of H441 cells. Dust extracts also reduced SP-A and SP-C protein levels in primary human alveolar epithelial cells, indicating that the observed effects are independent of immortalized or primary cell status, and, hence, are biologically relevant. Our previous studies showed that aqueous poultry dust extracts are equally as capable as dust particles in inducing inflammatory genes in lung cells, indicating that aqueous extracts are suitable for studying the effects of dust.[22] The lack of effect of polymyxin B suggested that LPS present in dust extracts might not be solely responsible for the inhibition of SP-A and SP-B protein levels. We previously

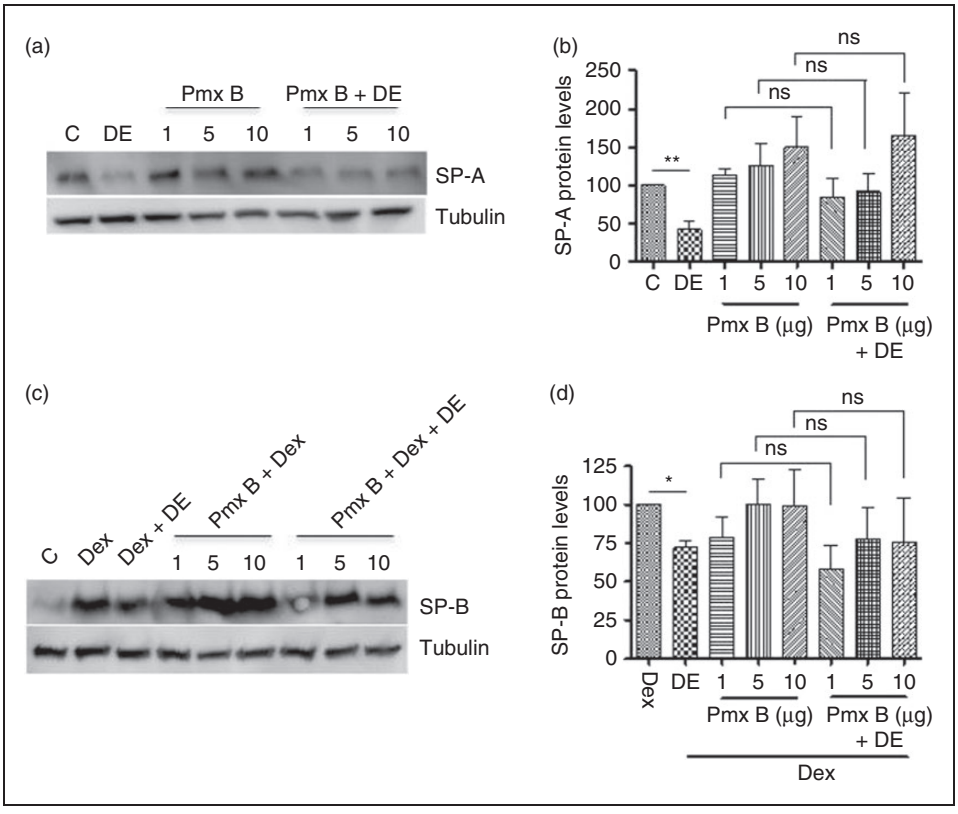

Figure 4. Polymyxin B does not prevent dust extract inhibition of SP-A and SP-B protein levels. (a, b) H441 cells were treated for 24 h with medium, indicated concentrations of polymyxin B (μg/ml), dust extract (1%), or a combination of polymyxin B and dust extract (1%) ($n = 4$). (c, d) H441 cells were treated for 24 h with medium, dexamethasone (0.1 μM), combination of dexamethasone (0.1 μM) and dust extract (1%), combination of polymyxin B (μg/ml) and dexamethasone (0.1 μM), or combination of polymyxin B (μg/ml), dexamethasone (0.1 μM) and dust extract (1%). SP-A and SP-B protein levels were determined by Western immunoblotting and normalized to tubulin levels. Representative Western blot images are shown. SP-B protein levels in dexamethasone treated cells were arbitrarily considered as 100 and relative levels in cells treated with dust extract in the presence of dexamethasone are shown ($n = 3$). All data shown are mean ± SE. ns, not significant, $^*P < 0.05$, $^{**}P < 0.01$.
C: control; DE: dust extract; Pmx B: polymyxin B; Dex: dexamethasone.

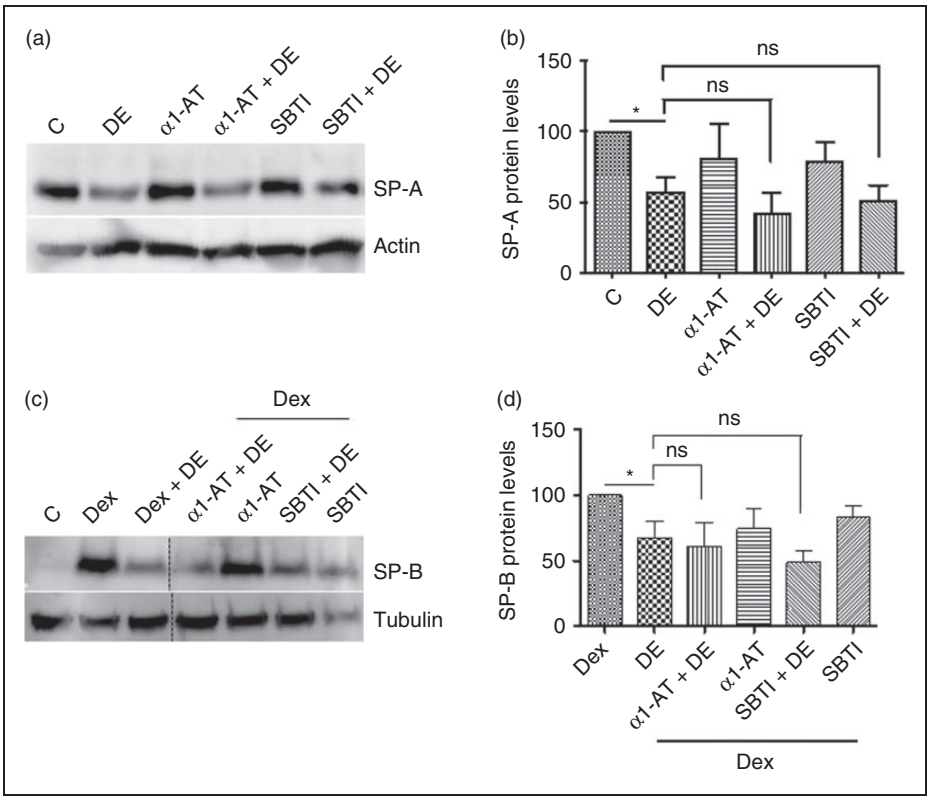

Figure 5. Serine protease inhibitors do not suppress dust extract inhibition of SP-A and SP-B protein levels. (a, b) H441 cells were treated for 24 h with medium, dust extract (1%), α1-AT (50 μg/ml), SBTI (50 μg/ml), or combination of dust extract (1%) with α1-AT (50 μg/ml) or SBTI (50 μg/ml). (c, d) H441 cells were treated as outlined above, except that the medium contained dexamethasone (0.1 μM). SP-A and SP-B protein levels were determined by Western immunoblotting and normalized to actin/tubulin levels. Representative Western blot images are shown, and dotted black lines show reassembly of non-contiguous lanes. SP-B protein levels in dexamethasone treated cells were arbitrarily considered as 100 and relative levels in cells treated with dust extract in the presence of dexamethasone are shown. Data shown are mean ± SE ($n = 4$). *$P < 0.05$, ns, not significant.
C: control; DE: dust extract; α1-AT: alpha1-antitrypsin; SBTI: soybean trypsin inhibitor; Dex: dexamethasone.

found that polymyxin B failed to prevent the inductive effects of organic dust on inflammatory gene expression.[20] The lack of effects of polymyxin B to suppress the inhibitory effects of dust extract on SP-A and SP-B expression could be due to the insensitivity of LPS in dust extract to polymyxin B inhibition. LPS present in poultry dust are likely to be heterogeneous as several Gram-positive and Gram-negative bacteria are found in the dust.[35] It is known that LPS display differential sensitivities to polymyxin B inhibition depending on their origin.[36] It was found that inhalation exposure of mice to corn dust extract or purified *Escherichia coli* LPS increased SP-A mRNA expression in the lung, but decreased SP-A protein levels in the lung lavage.[37] Inhalation exposure of mice to purified *Pseudomonas aeruginosa* LPS decreased SP-B mRNA expression and SP-B protein levels in lung lavage in mice.[38]

Poultry dust extracts contain elastase- and trypsin-like protease activities that are important for the induction of inflammatory gene expression in lung cells.[21] We found that serine-protease inhibitors such as α1-antitrypsin and soybean trypsin inhibitor failed to prevent the inhibitory effects of dust extract on SP-A and SP-B expression indicating that protease activities present in dust extract may not be responsible for the inhibition. On the other hand, heating dust extract for 10 min at 95°C prevented the inhibitory effects suggesting that a heat labile factor(s) that is yet to be characterized may be responsible for the inhibition of SP-A and SP-B expression. Dust extract retentate obtained using 30 kDa molecular mass cutoff filter retained inhibitory effects on SP-A and SP-B protein levels, whereas the filtrate did not, indicating that the factor (s) responsible for inhibition is a high molecular mass macromolecule.

Dust extracts reduced SP-A2 and SP-B promoter activities, but had no effect on SP-A1 promoter activity or the stabilities of SP-A1, SP-A2, and SP-B mRNAs, suggesting that transcriptional mechanisms mediate

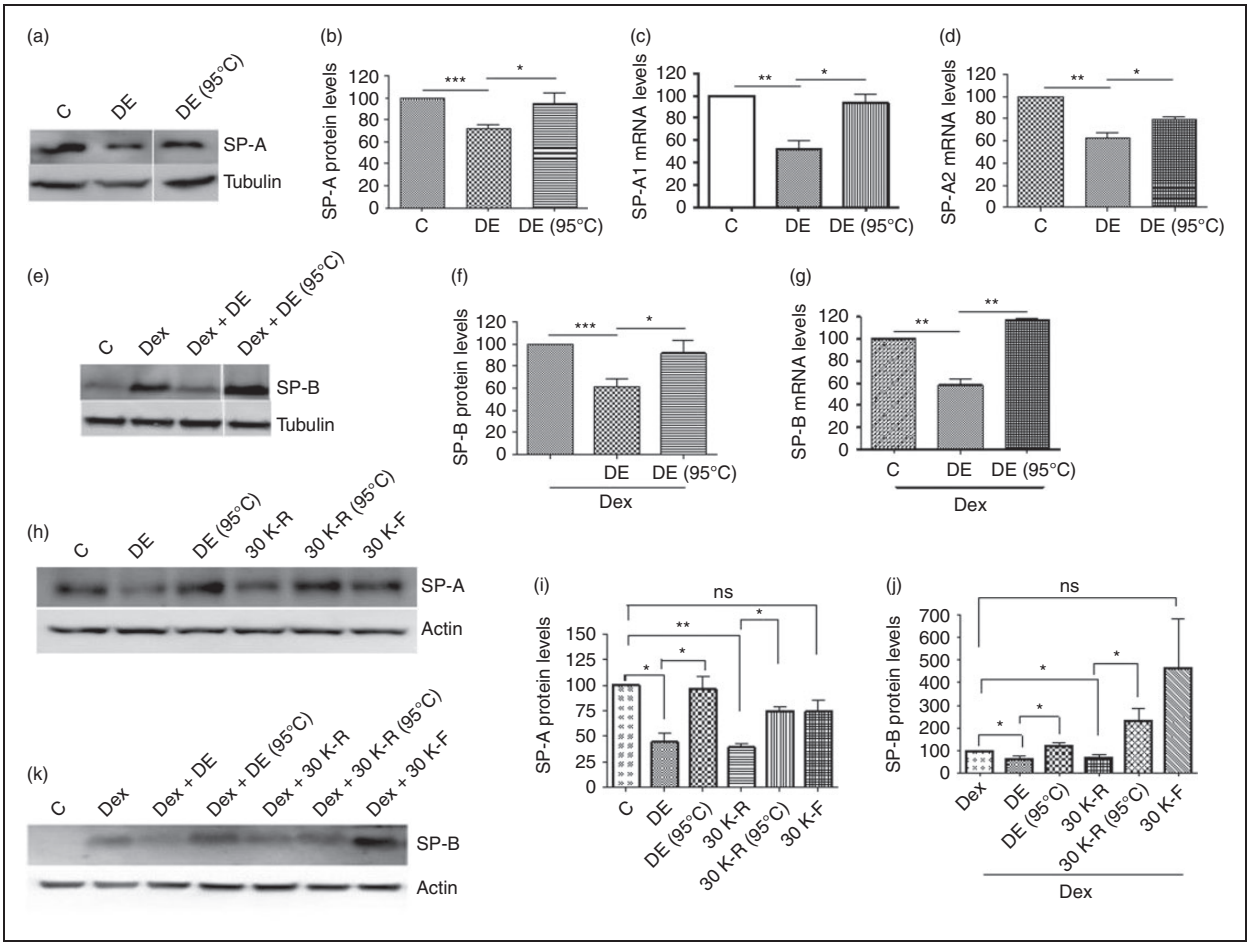

Figure 6. Heat-sensitive factor(s) controls dust extract inhibition of SP-A and SP-B protein and mRNA levels. (a–d) H441 cells were treated for 24 h with medium, dust extract (1%) or dust extract (1%) heated at 95°C for 10 min. (e–g) H441 cells were treated as outlined above, but in the presence of (0.1 μM) dexamethasone. SP-A and SP-B protein levels were determined by Western immunoblotting and normalized to tubulin levels. Representative Western blot images with reassembled noncontiguous lanes highlighted by white lines are shown. SP-A1, SP-A2, and SP-B mRNA levels were quantified by real-time qRT-PCR and normalized to 18S rRNA levels. SP-B protein/mRNA levels in dexamethasone treated cells were arbitrarily considered as 100, and relative levels in cells treated with dust extract in the presence of dexamethasone are shown. Data shown are mean ± SE ($n = 5$–8) for protein levels and ($n = 3$) for mRNA levels. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. C: control; DE: dust extract; Dex: dexamethasone. (h, i) H441 cells were treated for 24 h with medium, dust extract (1%), or dust extract (1%) heated at 95°C for 10 min. Cells were also treated for 24 h with retentate fraction (equivalent to 1% of dust extract), retentate fraction heated at 95°C for 10 min, or flow through fraction (equivalent to 1% of dust extract) that had been obtained by passing dust extract through a 30 kDa molecular mass cutoff centrifugal filter. (j, k) H441 cells were treated as outlined above, but in the presence of (0.1 μM) dexamethasone. SP-A and SP-B protein levels were determined by Western immunoblotting and normalized to actin levels. Representative Western blot images are shown. SP-B protein levels in dexamethasone treated cells were arbitrarily considered as 100, and relative levels in cells treated with dust extract or retentate and flow through fractions in the presence of dexamethasone are shown. Data shown are mean ± SE ($n = 3$). ns, not significant, *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.
C: control; DE: dust extract; Dex: dexamethaonse; 30 K-R: retentate fraction; 30 K-F: flow through fraction.

inhibition of SP-A and SP-B expression. The lack of inhibitory effects on SP-A1 promoter or SP-A1 mRNA stability is intriguing, even though SP-A1 mRNA levels are decreased. The lack of effect on the SP-A1 promoter could be due to the promoter assay not faithfully recapitulating the native chromosomal environment of the gene by lacking additional regulatory sequences such as enhancers and introns and higher order structure. The use of actinomycin D to block de novo RNA synthesis could have stabilized SP-A1 mRNA, if a labile protein(s) is necessary for mRNA degradation. SP-A, SP-B, and SP-C genes are subject to multifactorial regulation with glucocorticoids and cyclic AMP exerting stimulatory effects,[39–45] and TNF-α,[46–48] TGF-β,[49–51] and insulin[52–55] exerting inhibitory effects. Transcription and mRNA stability control regulation

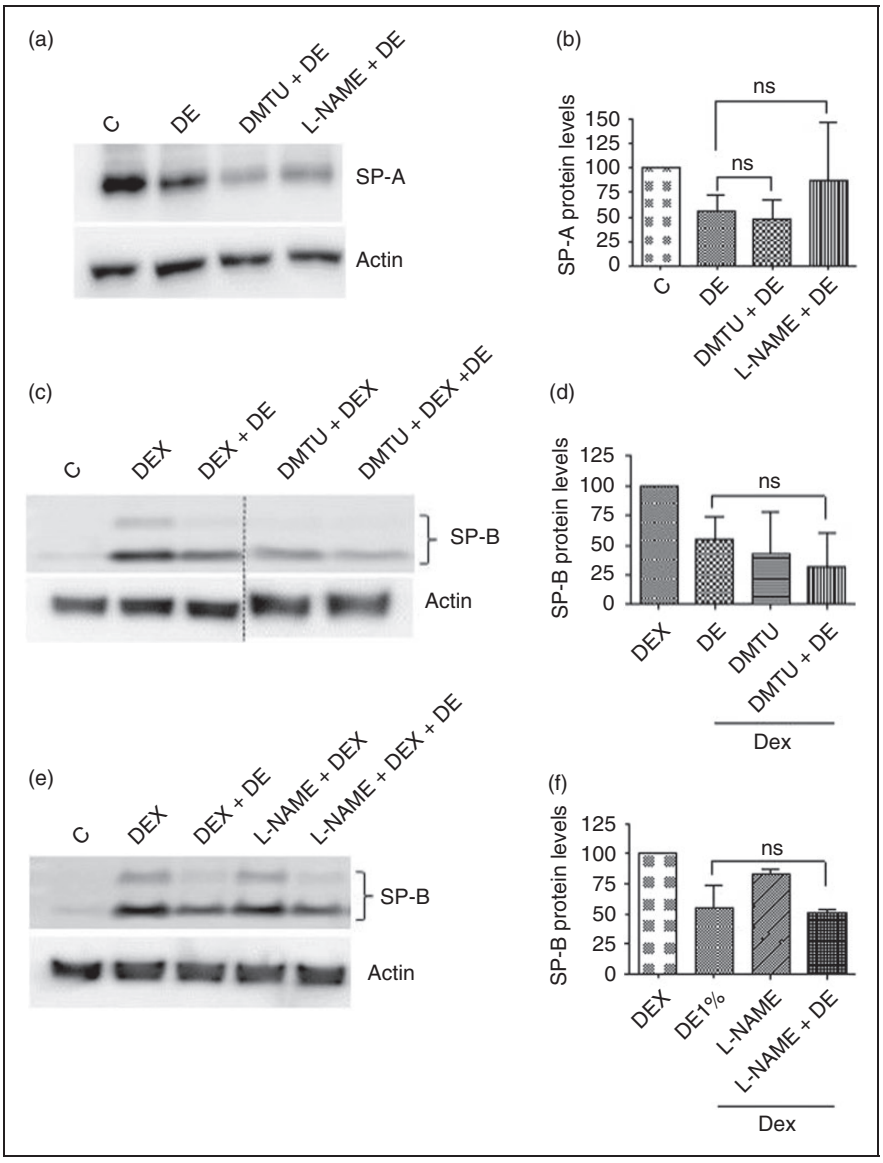

Figure 7. DMTU and L-NAME do not prevent inhibition of SP-A and SP-B protein levels by dust extract in H441 cells. (a, b) H441 cells were first incubated in medium or medium containing DMTU (15 mM) or L-NAME (1 mM) for 1 h and then treated with or without dust extract (1%) for 24 h. (c–f) H441 cells were treated as outlined above, but in the presence of (0.1 μM) dexamethasone. Representative Western blot images are shown, and reassembly of noncontiguous lanes is highlighted by dotted line. Data are shown as mean ± SD ($n = 2$ for SP-A and SP-B). ns: not significant.
C: control; DE: dust extract; Dex: dexamethasone; DMTU: dimethylurea; L-NAME: N^{ω}-nitro-L-arginine methyl ester.

of SP gene expression by glucocorticoids and TNF-α.[56–61] Two SP-A genes that have been named as *SP-A1* and *SP-A2* encode SP-A. SP-A1 and SP-A2 proteins are differentially expressed by different cell types in the lung,[11] and are differentially regulated by glucocorticoids and cAMP.[62,63] SP-A1 content in bronchoalveolar lavage was found to decrease with age in humans and increase in cystic fibrosis patients.[64]

Poultry dust extracts induce inflammatory gene expression in lung epithelial cells via generation of oxidant stress.[21] We found that antioxidant dimethylthiourea and NOS inhibitor, L-NAME did not prevent the inhibition of SPs, indicating that dust extract induced ROS production is not responsible for the inhibition of SP-A and SP-B levels in H441 cells.

Exposure of bronchial epithelial cells *in vitro* and mice *in vivo* to organic dust extracts increases levels of inflammatory cytokines such as, IL-6, IL-8, KC, and TNF-α.[22,29,33,65] Because TNF-α is known to inhibit SP expression,[30,33] we investigated if dust extract treatment increases TNF-α levels in H441 cells. We could not detect TNF-α in medium of control

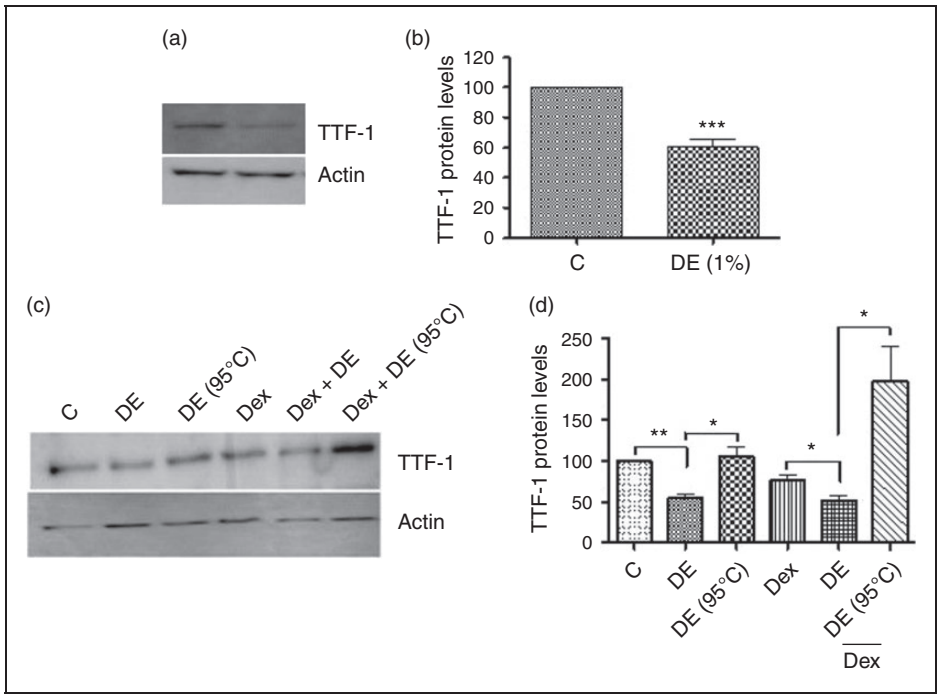

Figure 8. Dust extract reduces TTF-1 levels in H441 cells. (a, b) H441 cells were treated for 24 h with medium or medium containing dust extract (1%). TTF-1 and actin levels in nuclear extracts were determined by Western immunoblotting and TTF-1 levels normalized to actin levels. Data (mean ± SE) shown are from duplicate samples from three independent experiments. ***$P < 0.001$. (c, d) H441 cells were treated for 24 h with medium, dust extract (1%), or dust extract (1%) heated at 95°C for 10 min, dexamethasone (0.1 μM), a combination of dexamethasone (0.1 μM) and dust extract (1%), or a combination of dexamethasone (0.1 μM) and dust extract (1%) heated at 95°C for 10 min. TTF-1 and actin levels in nuclear extracts were determined by Western immunoblotting and TTF-1 levels normalized to actin levels. Data shown are mean ± SE ($n = 3$). *$P < 0.05$, **$P < 0.01$.
C: control; DE: dust extract; Dex: dexamethasone.

or treated H441 cells for 24 h (data not shown). In agreement with these data, pentoxifylline—an inhibitor of TNF-α secretion and TNF-α neutralizing Abs—failed to prevent inhibition of SP levels (data not shown).

SP promoters are regulated by the combinatorial actions of TTF-1, Sp1, HNF-3, and proteins that bind to cyclic AMP regulatory element-like sequences, and TTF-1 is a common transcriptional activator for SP-A, SP-B, and SP-C promoters.[28] We found that dust extract treatment reduced TTF-1 levels in H441 cells, suggesting that the inhibitory effects of dust extract on SP-A and SP-B levels in H441 cells could be due to reduced TTF-1 levels. The lack of effect of heat-treated dust extract on TTF-1 levels concomitant with lack of effect on SP-A and SP-B protein levels further support a role for TTF-1 in the inhibition of SP-A and SP-B protein levels. There is relatively little information available on the regulation of TTF-1 expression by endogenous or exogenous factors. TTF-1 expression is known to be down-regulated by TNF-α in H441 cells,[23] and by TGF-β1 in rat primary alveolar epithelial cells.[66]

Conclusion

Poultry dust extract reduced SP-A and SP-B gene expression by inhibiting their promoter activities in human lung epithelial cells, and the decrease of SP-A and SP-B gene expression was due to reduced TTF-1 levels. Reduction in SP-A and SP-B levels in H441 cells was dependent on high molecular mass heat-labile factor(s) and not due to LPS or proteases present in dust extract or cellular oxidative stress. Inhibition of SP levels in lung epithelial cells could contribute to the development of lung inflammation and lung diseases in humans exposed to organic dust.

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