Surfactant Protein D Protects against Acute Hyperoxic Lung Injury

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***Rationale*: Surfactant protein D (SP-D) is a member of the collectin family of soluble, innate, host defense molecules with demonstrated immunomodulatory properties *in vitro*. Constitutive absence of SP-D in mice is associated with lung inflammation, alteration in surfactant lipid homeostasis, and increased oxidative-nitrative stress.**

***Objectives*: To test the hypothesis that SP-D would protect against acute lung injury from hyperoxia *in vivo*.**

***Methods*: Transgenic mice overexpressing rat SP-D constitutively (SP-D OE) or conditionally via regulation with doxycycline (SP-D Dox-on) were subjected to continuous hyperoxic challenge for up to 14 days.**

***Measurements and Main Results*: Compared with littermate control mice (wild-type [WT]), SP-D OE mice exposed to 80% O_2 demonstrated substantially increased survival accompanied by significant reductions in wet to dry lung ratios and bronchoalveolar lavage (BAL) protein. Although SP-D OE and WT mice exhibited a twofold increase in total BAL cells and neutrophilia in response to hyperoxia, the SP-D OE group had lower levels of BAL proinflammatory cytokines and chemokines, including IL-6, tumor necrosis factor-α, and monocyte chemotactic protein-1; increased mRNA levels of the transcription factor NF-E2 related factor-2 (NRF-2) and phase 2 antioxidants hemoxygenase-1 (HO-1), glutathione peroxidase-2 (GPx-2) and NAD(P)H quinone oxidoreductase-1 (Nqo-1); and decreases in lung tissue thiobarbituric acid–reactive substances. As proof of principle, the protective role of SP-D on hyperoxic injury was confirmed as SP-D Dox-on mice exposed to 85% O_2 demonstrated increased mortality upon withdrawal of doxycycline.**

***Conclusions*: Local expression of SP-D protects against hyperoxic lung injury through modulation of proinflammatory cytokines and antioxidant enzymatic scavenger systems.**

Keywords: innate immunity; inflammation; collectin; antioxidants; oxidative stress

AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

Surfactant protein D (SP-D), a pulmonary collectin with important immunomodulatory properties, is up-regulated in response to various inflammatory and infectious stimuli. The role of SP-D in modulating inflammatory events in acute lung injury is undefined.

What This Study Adds to the Field

These results provide *in vivo* evidence for an antiinflammatory effect of SP-D in response to noninfectious acute lung injury and suggest a potential new therapeutic role for SP-D against hyperoxic lung injury.

The distal pulmonary airspaces perform the coordinated function of gas exchange through a delicate balance of ventilation and perfusion matching requiring regulated blood flow to functional alveolar-capillary units. Alveolar integrity during the respiratory cycle *in vivo* is maintained by a functionally active monolayer of lung surfactant produced by alveolar type 2 cells and deposited at the air–liquid interface (1). The role of surfactant lipid and its hydrophobic surfactant protein (SP) components, SP-B and SP-C, in reducing surface tension and stabilizing alveolar structures at low lung volumes is well known (2). However, in addition to its gas exchange functions, the epithelial surface of the alveoli and small conducting airways must contend with a continuous bombardment with pathogens, particles, and toxins during the course of normal breathing. Nonetheless, the lung surface is maintained in an immunologically quiescent state through a complex interaction of effector immune cells and soluble mediators. Related to this, it is becoming increasingly recognized that two other surfactant protein components, SP-A and SP-D, are a critical part of this local immunologic modulation during lung injury (3–5).

SP-D, a 43-kD member of the collectin superfamily, is a relatively minor component of lung surfactant but is of critical importance to lung homeostasis. From a large volume of *in vitro* and *in vivo* studies, SP-D has been shown to be an immunologically multifunctional, innate immune molecule. In addition to recognizing and binding allergens, particles, bacterial cell wall components, and viral envelope proteins in a pattern-specific manner (4), SP-D can influence recruitment and activation of effector immune cells in the lung. SP-D enhances actin polymerization (6), promotes chemotaxis of macrophages and monocytes (7), and modulates recruitment and function of neutrophils (8, 9) and lymphocytes (10, 11). *In vivo*, the use of transgenic mouse models suggests a protective role for SP-D against damage by inflammatory stimuli. Local up-regulation of SP-D protein levels in wild-type mice occurs after a variety of infectious and inflammatory lung injuries, including bacterial and fungal pneumonia, bleomycin, and hyperoxia (12–14), while mice constitutively deficient in SP-D develop, at baseline, progressive lung inflammation and time-dependent airspace remodeling (15).

Hyperoxic lung injury is mediated by reactive oxygen species (ROS) formed at a rate that overwhelms the endogenous capacity of the lung (cellular and extracellular pathways) to effect their removal. The injury is characterized by molecular and structural modifications to proteins, lipids, and nucleic acids, accompanied by morphologic and physiologic damage to the lung. Breakdown of epithelial–endothelial barrier function, accumulation of edema, recruitment and activation of inflammatory cells, elaboration of cytokines, and development of shunt physiology combine to produce a lesion of diffuse alveolar damage similar to that seen in the acute respiratory distress syndrome. The exact role of pulmonary surfactant components in the pathogenesis or evolution of hyperoxic lung injury is incompletely defined. Perturbations in homeostasis and biophysical activity of lung surfactant lipids after ventilation-induced or hyperoxic injury are well established (16, 17). Although these hydrophobic surfactant

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components are altered, SP-A and SP-D expression are enhanced at the mRNA and/or protein levels after exposure to O_2 concentrations greater than or equal to 80% (5, 18).

Although distinct from hyperoxia, the intratracheal administration of bleomycin to rodents also produces subacute lung injury followed by resolution, fibrosis, and/or repair via a mechanism that involves free radicals and oxidant stress. We have previously shown that SP-D null mice demonstrate enhanced susceptibility to intratracheal administration of bleomycin, whereas SP-D overexpressing mice were protected (14). Based on these results and on data showing increased oxidative stress in SP-D null mice, we hypothesized a protective role for SP-D in hyperoxic lung injury. Using a transgenic model that constitutively overexpresses rat SP-D (SP-D OE) and a doxycycline-regulated, conditional SP-D expressing mouse, we characterized the effects of SP-D on the pulmonary response to hyperoxia. We demonstrate that SP-D is protective against hyperoxia and that the enhanced survival in SP-D OE mice is accompanied by suppression of proinflammatory cytokines and induction of key antioxidant response genes. Collectively, these results demonstrate important novel functions for SP-D in the local modulation of lung inflammation in the distal airways. A preliminary report of some of these results has been previously published in the form of an abstract (19).

METHODS

SP-D OE Mice

Mice overexpressing the rat isoform of SP-D (rSP-D) in a lung-specific fashion using the human −3.7 kb SP-C promoter on the Swiss Black (SWB) background (SP-D OE) were provided by Dr. Frank McCormack and have been described previously (14, 20). Heterozygous transgenic mice carrying a single concatamer of the rSP-D transgene were crossed with wild-type SWB mice to generate SP-D OE mice and littermate control (wild-type [WT]) mice. All mice were maintained under specific pathogen-free conditions in the barrier facilities at the University of Pennsylvania. Experiments were typically performed on 7- to 8-week-old male mice.

Triple Transgenic SP-D Conditionally Expressing Mice

Breeding pairs of conditional SP-D expressing mice were kindly provided by Dr. Jeffery Whitsett and have been described previously (21). Briefly, rat*CCSP-rtTA* transgenic mice were mated with $(tetO)_7$-rSP-D transgenic mice to generate double transgenic mice (rat*CCSP-rtTA* +, $(tetO)_7$-*rSP-D* +). Double transgenic mice were mated with *SP-D*$^{-/-}$ mice to generate heterozygous (rat*CCSP-rtTA* +, $(tetO)_7$-*rSP-D* +, *mSP-D*$^{+/-}$) mice. Heterozygous triple transgenic mice expressing rat SP-D on a SP-D$^{-/-}$ background were then bred to homozygosity in our facility and maintained on a doxycycline-supplemented diet (625 ppm).

Hyperoxic Challenge

Mice were exposed to hyperoxia in the Core Facility at the Institute for Environmental Medicine as described in detail previously (18). SP-D OE and littermate WT mice were exposed to room air (normoxia control group) or to 80% or 85 ± 2% O_2 (hyperoxia group) in sealed Plexiglas chambers (Braintree Scientific, Inc., Braintree, MA). O_2 levels were continuously monitored using an O_2 analyzer (Pacifitech, Temecula, CA). Animals were housed in environment-controlled cages and allowed food and water *ad libitum* during the exposure. Cages were opened periodically for change of water, food, and bedding and as required for removal of dead mice. Conditional SP-D mice were kept on a doxycycline-supplemented diet (~625 ppm) or transferred to normal chow 2 weeks before hyperoxia exposure.

All study protocols, animal care, and procedures had been reviewed before the initiation of work by the Institutional Animal Care and Use Committee of the University of Pennsylvania.

Preparation and Analysis of Bronchoalveolar Lavage

Lungs were lavaged with five 1.0-ml aliquots of sterile saline. Processing and analysis of bronchoalveolar lavage (BAL) has been described previously (18). Briefly, cell pellets obtained by centrifuging BAL samples at 400 × *g* for 10 minutes were re-suspended in 1 ml of PBS, and total cell counts were determined using a Z1 particle counter (Beckman-Coulter, Inc., Miami, FL). Cytospins prepared from an aliquot of each cell suspension were stained with Diff-Quik, and manual differential cell counts were performed.

A 200-μl aliquot of each cell-free BAL was removed from the first collected sample and stored at −80°C for cytokine analysis. An array of nine cytokines was analyzed by SearchLight Technology multiplex cytokine assay by Pierce Biotechnology (Woburn, MA). The remaining BAL was separated into large-aggregate (LA) and small aggregate fractions by centrifugation at 20,000 × *g* for 60 minutes at 4°C as described previously (22).

Total protein content of both fractions was determined by the Bradford method with bovine IgG as a standard (23). Total lipids were extracted from LA and small aggregate surfactant fractions with chloroform-methanol as described previously (24). Total phospholipid content in each fraction was determined by Bartlett's colorimetric estimation of inorganic phosphorus (25).

PAGE and Immunoblotting

BAL samples were separated using NuPAGE NOVEX-10% Bis-Tris gels (Invitrogen, Inc., Carlsbad, CA). Immunoblots were performed with monospecific, polyclonal surfactant protein antisera to SP-A or SP-D. Total SP-D was determined using a rabbit polyclonal antibody that recognizes mouse and rat SP-D (22). Because the SP-D OE mice express a rSP-D transgene, rat SP-D levels were determined using a commercially available monoclonal antibody against rat SP-D (Clone VIF9; HyCult Biotechnology, Uden, The Netherlands). Bands were visualized using horseradish peroxidase–conjugated secondary goat anti-rabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and enhanced chemiluminescence (Amersham, Inc., Arlington Heights, IL). Band intensity was quantitated by densitometric scanning of exposed films or by direct acquisition on a Kodak 440 Imaging System (Eastman Kodak Co., New Haven, CT).

Lung Histology and Estimation of Lipid Peroxides

After lavage, the left lobe of the lung was inflation fixed with 0.5 ml of 10% neutral buffered formalin for histologic analysis. Paraffin sections prepared from the lungs were stained with hematoxylin and eosin for evaluation of airway inflammation and were scored based on the presence or absence of peribronchiolar infiltration, alveolar septal thickening, vascular congestion, alveolar edema, and cellular infiltration into the alveoli as described previously (18).

Total lipid peroxides in lung samples were estimated by the method of Fisher and colleagues (26). Briefly, lungs snap frozen in liquid N_2 were homogenized in ice-cold saline containing 0.01% butylatedhydroxytoluene, deproteinized using 15% trichloroacetic acid, and boiled with thiobarbituric acid at 90°C for 15 minutes. Samples were immediately cooled on ice and centrifuged at 4,000 rpm for 30 minutes, and the absorbance of the resulting supernatant was read at 535 nm. Thiobarbituric acid reactive substances (TBARS) were estimated using an extinction coefficient of 1.56×10^5/M/cm and expressed as pmol/mg protein.

Measurements of Surface Tension

The LA fraction of BAL was diluted to 1 mg/ml phospholipid concentration for assessment of biophysical activity of recovered surfactant in a capillary surfactometer as previously described (27). Samples (0.5 μl) introduced into a glass capillary were compressed for 120 seconds, resulting in cyclic extrusion of the surfactant from the capillary permitting detectable airflow. Biophysical dysfunction in the sample results in loss of capillary "openness." Data were expressed as the percentage of the 120-second study period that the capillary is open to a free airflow.

Quantitative Real-Time Reverse Transcription–Polymerase Chain Reaction

Isolation of total lung RNA was performed using Trizol reagent (Invitrogen, Inc., Carlsbad, CA). Reverse transcription of the DNase-treated total RNA was performed using a RETROscript First Strand Synthesis kit for real-time reverse transcription–polymerase chain reaction (Applied Biosystems, Foster City, CA) and random decamer primers. Quantitative real-time reverse transcription–polymerase chain reaction analyses of transcription factor NRF-2 (Mm00477784_m1) and

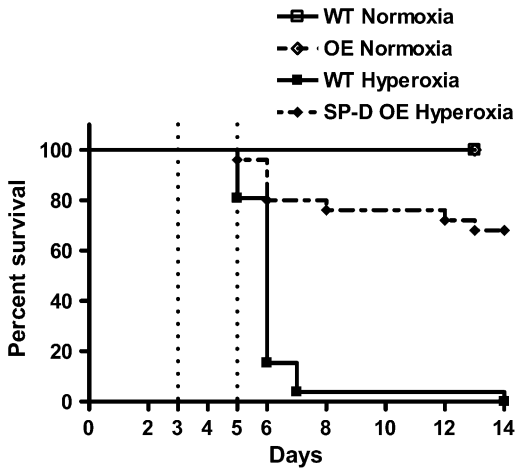

Figure 1. Effect of surfactant protein D (SP-D) overexpression on survival under hyperoxic conditions. SP-D overexpressing mice (OE) and littermate control mice (7–8 wk of age) were exposed to 80% oxygen. Survival for each group was recorded throughout a 14-day observation period and plotted as Kaplan-Meier survival analysis. Comparison of mortality at Days 7 and 14 revealed enhanced tolerance in the SP-D OE mice ($P < 0.0001$ vs. corresponding control groups). Data are representative of two separate experiments. n = 25 for hyperoxia groups; n = 5 for normoxia control mice. WT = wild type.

antioxidant genes GPx-2 (Mm00850074_g1), HO-1 (Mm00516004_m1), and Nqo-1 (Mm00500821_m1) were performed with TaqMan Gene Expression Assays (Applied Biosystems; Foster City, CA) using an ABI 7500 FAST real-time polymerase chain reaction system. 18S RNA (Hs99999901_s1) was used for normalization.

Data Analysis

Parametric data from experimental and control groups were expressed as mean ± SEM, and groups were compared using an unpaired, two-tailed Student's *t* test or analysis of variance (ANOVA) using Instat version 3.0 (GraphPad Software, Inc., La Jolla, CA). Analysis on all parametric data was done using one-way ANOVA followed by Tukey-Kramer multiple comparisons test or unpaired *t* tests. Nonparametric data were expressed as group mean values ± SEM, and statistical comparison was done using Kruskal-Wallis test followed by Dunn's multiple comparison test or Mann Whitney *U* test. In all cases, $P \leq 0.05$ was accepted as significant.

RESULTS

SP-D OE Mice Demonstrate Increased Survival in Hyperoxia

SP-D OE mice survived considerably longer in 80% O_2 than similarly exposed SWB littermates. The median survival for the WT group subjected to hyperoxia was 6 days, with 100% mortality by Day 14 (Figure 1). In contrast, over two-thirds (68.8%) of SP-D OE mice survived at least 14 days under identical conditions. When assessed just before the onset of mortality (Day 5), WT mice were found to have a significantly greater loss of body weight compared with the similarly exposed SP-D OE group (84 ± 2% of initial body weight vs. 90 ± 1% of initial body weight for SP-D OE; n = 11–12; $P < 0.05$).

To gain insight into the underlying mechanisms mediating this survival advantage, we elected to assess the kinetics of hyperoxia-induced injury in this model by evaluating these models at the acute early phase of injury (i.e., Days 3 and 5 after initiation of exposure).

SP-D OE Mice Have Less Hyperoxia-induced Lung Leak

Lung edema was significantly worse in WT mice exposed to hyperoxia. There was a 25% increase in wet to dry lung weight ratio in the WT group after 5 days of exposure (Figure 2A).

Commensurate with this observation, the alveolar protein leak seen during hyperoxic exposure was markedly attenuated in the SP-D OE group (Figure 2B). Under normoxic conditions, no differences were observed between the strains (375.6 ± 49.4 μg in SP-D OE vs. 374.2 ± 47.2 μg in WT). During hyperoxia, BAL protein levels rose in both groups when assessed at 3 days (639.7 ± 77.9 μg in SP-D OE vs. 987.7 ± 108.4 μg in WT; $P < 0.05$) or at 5 days of exposure (2,678.2 ± 267.4 μg in SP-D OE vs. 3,984.3 ± 503.6 μg in WT; $P < 0.05$).

Surfactant Composition and Function Is Altered in SP-D OE Mice and Littermate Control Mice

Surfactant activity can be altered in various forms of lung injury due to protein leak or altered component expression. Measurement of total BAL phospholipids revealed that these were similarly depleted to about half that of the normoxia-exposed mice after 3 days of exposure in the SP-D OE and WT groups (Figure 3A). There was a partial recovery to approximately 60% of baseline values by Day 5. Concomitant with this loss of surfactant, each hyperoxic group had similar degrees of inhibition of surfactant biophysical function as assessed by capillary surfactometer (capillary open time = 47.6 ± 12.6% for WT and 31.6 ± 10.7% for OE; $P > 0.05$). Therefore, the differences in survival observed between the groups cannot be attributed solely to differences in surfactant function.

SP-D levels were differentially regulated by hyperoxia in these models. BAL from SP-D OE mice contained six- to eightfold higher amounts of SP-D than littermate control mice at baseline (Figure 3B). During hyperoxia, WT mice developed an acute SP-D deficiency, with significant depletion of BAL SP-D to about 25% of the baseline after 3 days of exposure and recovery by Day 5. Total BAL SP-D in SP-D OE mice continued to increase throughout hyperoxic exposure, reaching levels four- to fivefold

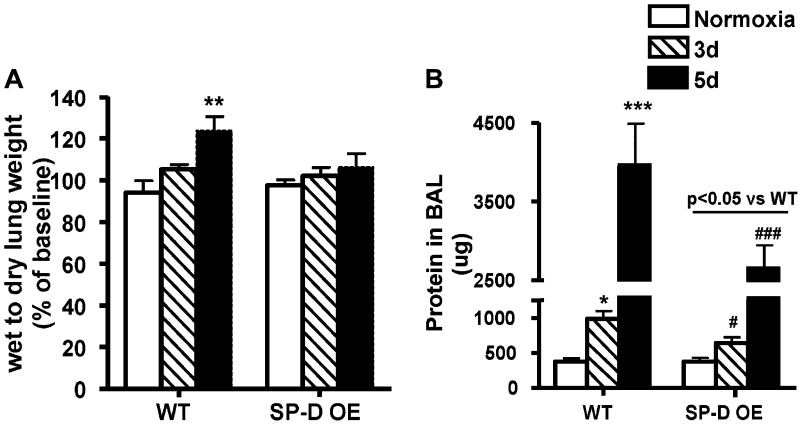

Figure 2. Effect of surfactant protein D (SP-D) overexpression on alveolar–capillary integrity. (*A*) Alveolar capillary leak was determined as the ratio of wet to dry lung weights (n = 6–11) in wild-type (WT) and SP-D overexpressing (SP-D OE) mice. Data for each group are expressed as mean + SEM, expressed as % of WT normoxia. $^{**}P < 0.01$ vs. WT normoxia. (*B*) Bronchoalveolar lavage (BAL) total protein from the same time points (n = 9–12 per group) were determined as described in Methods. Data are mean + SEM, expressed as μg protein in BAL. $^{*}P < 0.05$; $^{***}P < 0.0001$ vs. WT normoxia group; $^{\#}P < 0.05$; $^{\#\#\#}P < 0.0001$ vs. SP-D OE normoxia group.

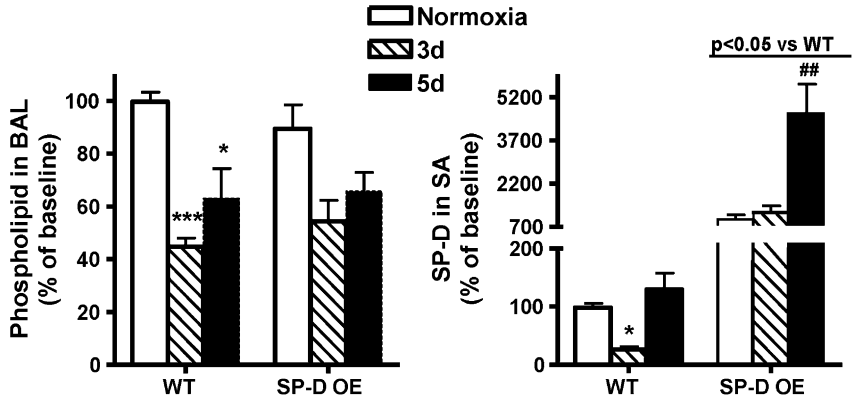

Figure 3. Effect of surfactant protein D (SP-D) overexpression on surfactant components during hyperoxic injury. SP-D overexpressing (SP-D OE) mice and littermate control mice were exposed to 80% O_2, and mice were killed at different time points. Total phospholipid and SP-D in bronchoalveolar lavage (BAL) was determined as described in METHODS. (*Left*) Total phospholipid in BAL was estimated using a modification of the colorimetric Bartlett method as described. Data, normalized to % wild-type (WT) level, are expressed as mean ± SEM. (n = 9–12; $^{*}P < 0.05$; $^{***}P < 0.0001$ vs. WT control group. BAL surfactant phospholipid levels in SP-D OE and WT mice are similarly altered after hyperoxia. (*Right*) Immunoblots against SP-D in small aggregate of BAL were performed as described in METHODS (n = 5–12) and quantified by densitometry on a KODAK imaging system. Data are expressed as mean ± SEM, normalized to % WT level ($^{*}P < 0.05$ vs. WT control group; $^{\#\#}P < 0.01$ vs. SP-D OE control group). Unlike WT mice, SP-D OE mice do not demonstrate an initial depletion of BAL SP-D.

higher than the SP-D OE normoxia group after 5 days. Using a monoclonal antibody specific for rat SP-D, Western blotting revealed that after 5 days of O_2 exposure rSP-D immunoreactivity increased in SP-D OE mice by approximately threefold (vs. the normoxia SP-D OE group), indicating that the increased levels observed were in part due to up-regulation of transgene expression (data not shown). Despite changes in SP-D, there were no major differences in SP-A levels in the LA fraction of BAL at baseline (99.9 ± 19.6% of normoxia control in WT and 72.8 ± 13.7% in SP-D OE) or after exposure (66.1 ± 12.1% of normoxia control in WT and 63.8 ± 7.9% in SP-D OE).

Inflammatory Cell Influx Is Similar in WT and SP-D OE Mice

Total and differential cell counts were performed on BAL cell pellets (Figure 4). WT and SP-D OE mice had similar total cell counts at baseline ($3.5 \pm 0.13 \times 10^5$ vs. $4.1 \pm 0.42 \times 10^5$ cells, respectively). Both groups exhibited approximately a twofold increase after 5 days of exposure to 80% O_2, but there was no significant difference between groups (Figure 4A). Similarly, BAL neutrophils in WT and SP-D OE mice increased to similar levels after hyperoxia ($2.3 \pm 0.4 \times 10^5$ vs. $1.7 \pm 0.5 \times 10^5$ cells, respectively) (Figure 4B). There were no differences in BAL macrophages, lymphocytes, and eosinophils before or after exposure or between groups (not shown).

Histopathologic examination and semiquantitative scoring of hematoxylin-and-eosin-stained lungs of WT mice exposed to 80% O_2 showed marked perivascular cuffing, interstitial thickening, and damage caused by inflammatory cell infiltration, which was significantly attenuated in the SP-D OE mice (Figure 5).

Cytokine Production Is Substantially Repressed in SP-D OE Mice after Hyperoxia

Cytokine profiles in BAL were assessed using a mutiplex assay. We observed a significant repression of cytokine production in SP-D OE group. IL-10, IFN-γ, granulocyte/macrophage colony–stimulating factor, and IL-8 levels in BAL of SP-D OE mice were significantly lower after 5 days of hyperoxia, whereas IL-6 and monocyte chemotactic protein (MCP)-1 levels were reduced after 3 and 5 days of exposure (Table 1).

SP-D OE Mice Respond to Hyperoxia by Up-regulating the Expression of Phase II Antioxidant Genes

Resistance to hyperoxia has been associated with selective up-regulation of antioxidant genes and detoxifying enzymes. We first looked at the transcription factor NRF-2, which acts via the ubiquitous antioxidant response element (ARE) binding site in the promoter region to regulate expression of various stress response genes, such as HO-1, GPx-2, and Nqo1. We observed a 2.5-fold increase in NRF-2 mRNA levels after 5 days of hyperoxia compared with no change in similarly exposed littermate control mice (Figure 6). Concomitant with this increase, we observed a parallel three- to fivefold increase in mRNA levels of stress-responsive genes HO-1, GPx-1, and Nqo1. Oxidant-mediated tissue injury in these mice was assessed by measuring TBARS in postlavage lung homogenates. We observed a twofold increase in the level of TBARS in WT mice exposed to 80% O_2 for 5 days, which was completely abrogated in the SP-D OE group (Figure 7).

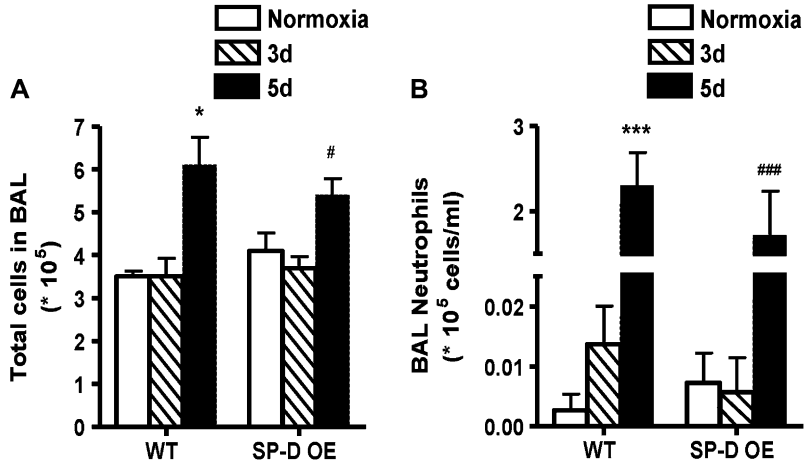

Figure 4. Effect of surfactant protein D (SP-D) on inflammatory cell recruitment. Bronchoalveolar lavage (BAL) (*A*) total and (*B*) neutrophil cell counts were performed as described in METHODS after exposure of SP-D overexpressing (OE) and wild-type (WT) mice to 80% O_2. Data for each group are expressed as mean ± SEM (n = 9–13) ($^{*}P < 0.05$ and $^{***}P < 0.0001$ vs. WT normoxia group; $^{\#}P < 0.05$ and $^{\#\#\#}P < 0.0001$ vs. SP-D OE normoxia group). SP-D OE and littermates demonstrate similar infiltration of proinflammatory cells in the lungs in response to hyperoxia.

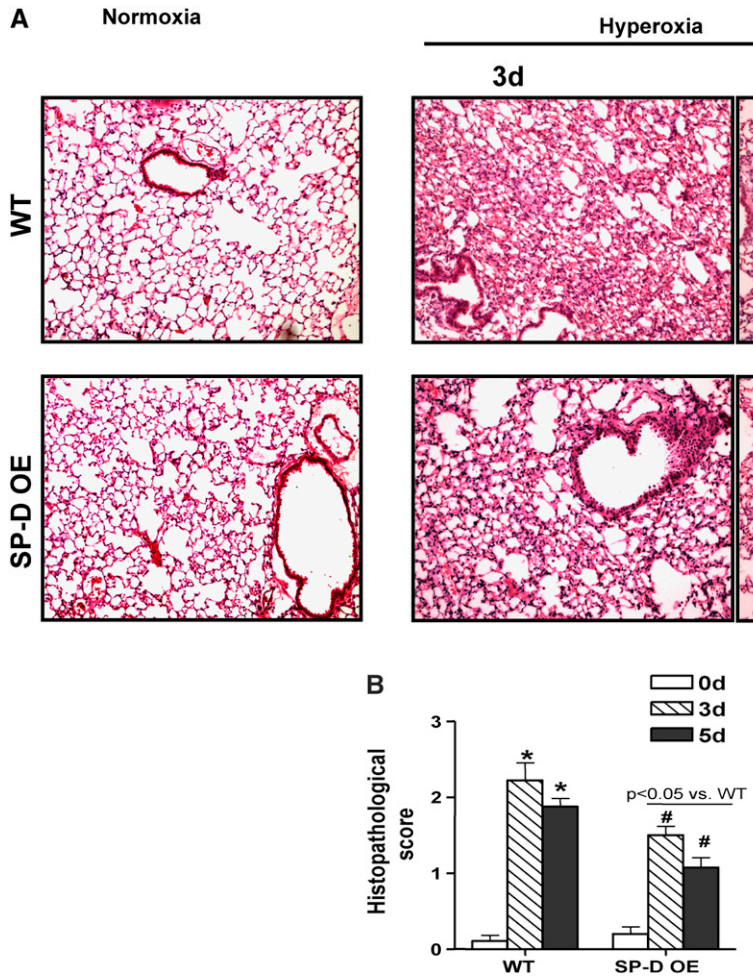

Figure 5. Effect of surfactant protein D (SP-D) on pulmonary parenchymal inflammation. (*A*) Representative hematoxylin-and-eosin–stained lung sections from SP-D overexpressing (OE) and littermate wild-type (WT) mice exposed to 21% O_2 (Day 0) or to hyperoxia for 3 and 5 days. (*B*) Histologic scoring was performed as published previously (18). Data for each group are expressed as mean ± SEM (n = 5–12). Multiple group comparisons were done by nonparametric Kruskal-Wallis analysis of variance, followed by Dunn's multiple group comparisons. *$P < 0.05$ vs. WT normoxia group; $^{\#}P < 0.05$ vs. SP-D OE control (normoxia) group. Similarly exposed WT and SP-D OE hyperoxia groups were compared by Mann-Whitney *U* tests.

Loss of Lung-specific Expression of SP-D Confers Susceptibility to Hyperoxia

To confirm our hypothesis that SP-D plays a protective role in hyperoxic lung injury, we used mice expressing SP-D under the control of a doxycycline-dependent promoter on a SP-D–null background. These mice, on an FVB background, were bred to homozygosity in our laboratory. Western blots on BAL from these mice demonstrate that the animals maintained on a doxycycline-supplemented diet (SP-D Dox-on group) produce about 10- to 15-fold higher basal levels of SP-D than B6 mice. When doxycycline is removed from the diet (Dox-off group), SP-D levels decline precipitously (Figure 8A). Using prolonged exposures, trace amounts of SP-D could be detected at Day 14 on Western blots but account for less than 3% of the levels seen in

TABLE 1. BRONCHOALVEOLAR LAVAGE PROFILE AFTER HYPEROXIA

	WT		SP-D OE		
	Hyperoxia		Normoxia	Hyperoxia	
Cytokine	3 d	5 d	0 d	3 d	5 d
IL-6	**8.7 ± 1.9**†	**5,284.3 ± 1,941.7***	**0.97 ± 0.15**	**1.8 ± 0.1**‡	**423.6 ± 211.1**†‡
IL-10	1.8 ± 0.3	7.1 ± 2.7*	0.99 ± 0.17	1.4 ± 0.2	0.6 ± 0.2†
IL-13	3.8 ± 0.8†	0.2 ± 0.1	1.0 ± 0.15	2.04 ± 0.9	0.1 ± 0.07
IFN-γ	1.4 ± 0.3	7.96 ± 2.9*	0.94 ± 0.18	1.69 ± 0.15	1.1 ± 0.65†
TNF-α	**50.0 ± 19.4**†	**1.7 ± 0.7**	**1.0 ± 0.15**	**2.57 ± 0.9**†	**1.0 ± 0.5**
GM-CSF	3.1 ± 0.4	4.8 ± 1.5†	0.96 ± 0.22	2.0 ± 0.28	1.3 ± 0.57‡
MIP-2	2.1 ± 0.47	5.8 ± 2.2†	0.92 ± 0.15	1.4 ± 0.17	1.7 ± 0.8
KC/IL-8	2.16 ± 0.28	12.6 ± 5.37†	0.98 ± 0.1	1.08 ± 0.3	3.08 ± 1.08‡
JE/MCP-1	**2.16 ± 0.27**	**153.3 ± 60.1**†	**0.95 ± 0.15**	**1.08 ± 0.2**	**43.7 ± 27.0**‡

Definition of abbreviations: GM-CSF = granulocyte/macrophage colony-stimulating factor; KC = keratinocyte chemoattractant; MCP-1 = monocyte chemotactic protein-1; MIP-2 = macrophage inflammatory peptide-2; SP-D OE = surfactant protein D–overexpressing mice; WT = wild-type mice.

Cytokine levels in bronchoalveolar lavage were estimated by Searchlight Multiplex cytokine assay. Values are fold changes as compared with WT normoxia group. Data for each group are expressed as mean ± SEM (n = 9–12). Multiple-group comparisons were done using one-way analysis of variance, followed by *t* tests.

* $P < 0.05$ vs. WT 0 d.

† $P < 0.05$ vs. similarly exposed WT.

‡ $P < 0.05$ vs. SP-D OE 0 d.

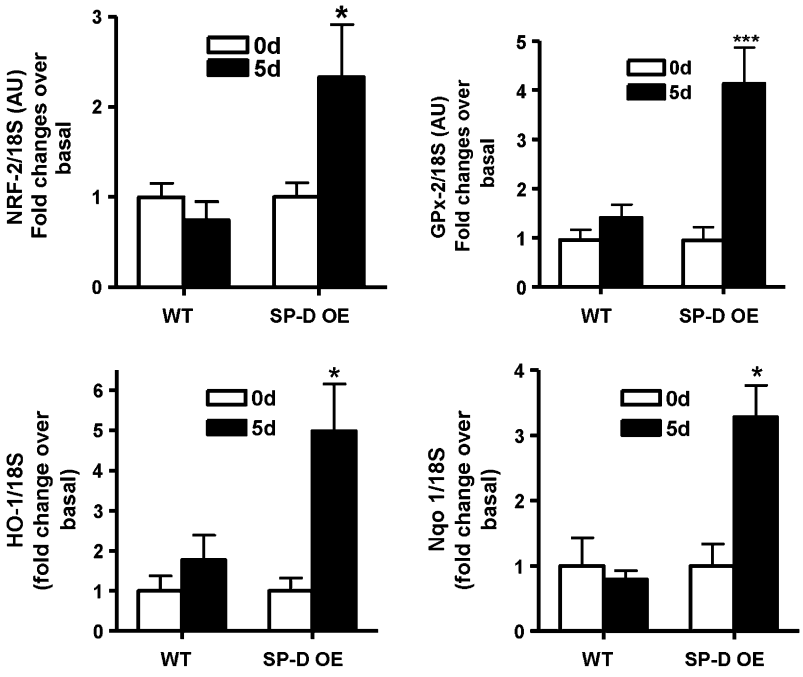

Figure 6. Gene expression of NF-E2 related factor-2 (NRF-2) and downstream antioxidant genes in wild-type (WT) and surfactant protein D overexpressing (SP-D OE) mice. Total lung mRNA was extracted from SP-D OE and littermate WT mice exposed to 21% or 80% O_2 for 5 days. Quantitative real-time reverse transcription–polymerase chain reaction analysis was performed for NRF-2, glutathione peroxidase-2 (Gpx-2), hemoxygenase-1 (HO-1), and NAD(P)H quinone oxidoreductase-1 (Nqo-1) and normalized to 18S RNA as described. Data are expressed as mean ± SEM (n = 5–10), normalized to WT level (*$P <$ 0.05; ***$P <$ 0.0001 vs. SP-D OE normoxia group). Hyperoxia-exposed SP-D OE mice exhibit significantly higher levels of NRF-2, GPx-2, HO-1, and Nqo-1 mRNA in the lung as compared with similarly exposed WT group.

SP-D Dox-on mice. However, acute loss of SP-D did not alter baseline total phospholipid levels (Dox-on group, 220.4 ± 30.9 μg, vs. Dox-off group, 273.2 ± 28.1 μg; $P > 0.05$) (Figure 8C).

Subsequent hyperoxia exposure experiments were done on age-matched SP-D Dox-on and Dox-off mice. Under hyperoxic conditions (85% O_2), the SP-D Dox-on group survived considerably longer than the Dox-off SP-D–deficient group (median survival = 11 days for Dox-on vs. 7 days for Dox-off; $P <$ 0.0001) (Figure 8B). Dox-off mice developed 100% mortality by Day 13, whereas more than 20% of the Dox-on mice survived the 14-day exposure protocol.

DISCUSSION

It is increasingly recognized that factors intrinsic to the lung play a role in the differential susceptibility to O_2 toxicity observed in patients and in animal models exposed to hyperoxia (28). In the present study, we show that local overexpression of SP-D resulted in an enhanced median survival for transgenic mice exposed to high concentrations of O_2 that was accompanied by preservation of alveolar capillary barrier integrity, a decrease in proinflammatory cytokines, and an up-regulation of NRF2 and its dependent phase 2 antioxidant genes. Furthermore, the effect of SP-D occurred in a dose-dependent fashion as mice conditionally expressing SP-D and mice rendered acutely SP-D–deficient through withdrawal of doxycycline were more susceptible to hyperoxia.

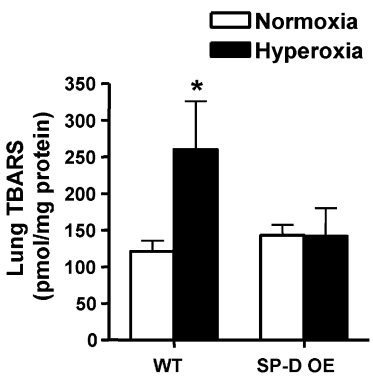

Figure 7. Lung lipid peroxidation in wild-type (WT) and surfactant protein D overexpressing (SP-D OE) mice after hyperoxia. Total lung lipid peroxides were measured in SP-D OE and WT mice before or after exposure to 80% O_2 for 5 days. Data are expressed as mean ± SEM (n = 5–10), normalized to WT level (*$P <$ 0.05 vs. WT normoxia group). Hyperoxia-induced increases in lung thiobarbituric acid reactive substances are abrogated in SP-D OE mice.

Lung surfactant lipid content and *in vitro* biophysical activity were mildly inhibited in the SP-D OE and littermate control groups and could not account for observed changes seen in response to hyperoxia. This is in contrast to our experience with the effects of hyperoxia on a murine model constitutively deficient in SP-D. The SP-D knockout mouse exhibits a variety of baseline phenotypic alterations, including increased surfactant phospholipid pools, enlarged but nonfunctional pulmonary macrophages, and chronic inflammation including elevation of proinflammatory cytokines. Paradoxically, that model had enhanced survival when subjected to hyperoxic exposure (18). After an extensive evaluation, it was demonstrated that this effect could be attributed to secondary enlargement of alveolar surfactant phospholipid pools and SP-B, which develop in this phenotype. In contrast, the triple transgenic SP-D conditional mice used in this study, when maintained on doxycycline from birth, have no discernible phenotype (21). Unlike chronically SP-D–deficient mice, SP-D conditional mice removed from doxycycline for 2 weeks before hyperoxia (Dox-off) did not demonstrate significant changes in BAL phospholipid content (Figure 8B). Similarly, we and others have found that constitutive local SP-D overexpression in the SP-D OE line also does not affect alveolar phospholipid homeostasis (20). Taken together, the use of these two models has allowed us to investigate the protective effects of SP-D independent of any mechanistic contributions from surfactant phospholipids or other phenotypic alterations associated with constitutive knockdown of the SP-D gene.

Despite an accompanying inflammatory cell infiltrate similar to that found in identically exposed littermate control mice (Figure 4), the lungs of SP-D OE mice subjected to hyperoxia had paradoxically less lung damage histologically (Figure 5) and biochemically (Figure 2) in association with a highly immunosuppressive microenvironment. Inflammatory and chemotactic cytokine levels in the BAL of these mice were attenuated (Table 1). Because the measured BAL cytokines represent products of multiple cell types present in the distal airways, including macrophages (tumor necrosis factor-α), epithelial cells (IL-6, IL-8

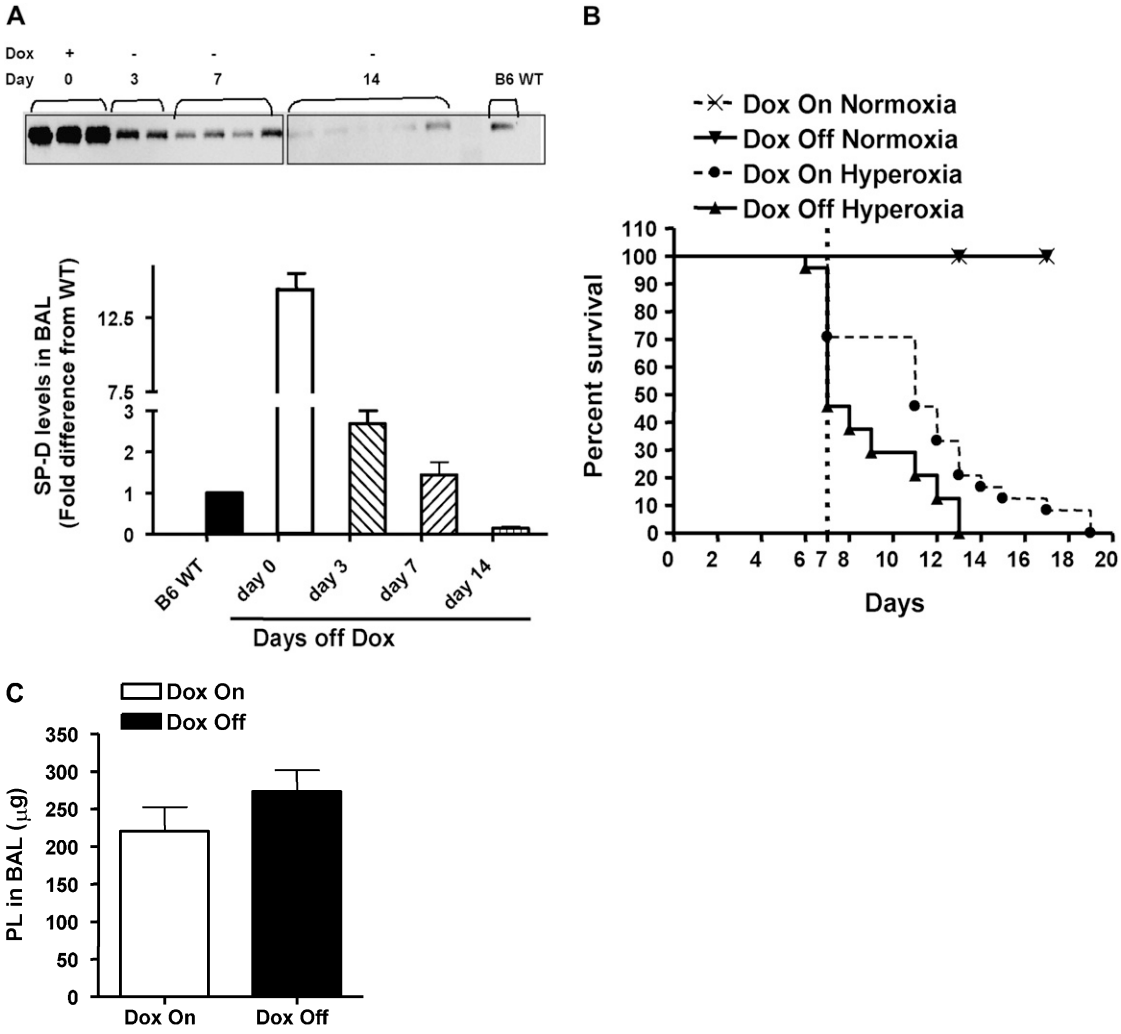

Figure 8. Transgenic mice with doxycline-dependent, lung-specific expression of surfactant protein D (SP-D) show increased survival under hyperoxic conditions. (*A*) SP-D expression in triple transgenic (conditional SP-D expressing) mice fed doxycycline chow diminishes rapidly after removal of doxycycline. Western blots of bronchoalveolar lavage (BAL) fluid obtained from mice bred and maintained from birth on 625 ppm dox feed pellets and then fed normal feed pellets for 0, 3, 7, or 14 days detected using polyclonal SP-D antiserum. Data are expressed as mean ± SEM, normalized to % B6 wild-type (WT) level (n = 1 for WT; n = 2–7 for conditional SP-D–expressing mice). (*B*) Triple transgenic mice at 8 weeks of age maintained on 625 ppm doxycycline feed pellets ("on dox") or taken off doxycycline feed pellets 2 weeks earlier ("off dox") were exposed to 85% O_2 as described in METHODS. Comparison of Kaplan-Meier survival curves for each group revealed enhanced tolerance in the SP-D–expressing mice ($P < 0.0001$ vs. corresponding control groups). Data are representative of two separate experiments (n = 24 for hyperoxia groups; n = 10 for normoxia control mice). All normoxia mice survived at the end of the experiment (data lines are superimposed). (*C*) Total phospholipid (PL) levels in BAL from "Dox on" and "Dox off" (2-week) groups at baseline were determined as described in METHODS. Data are expressed as mean ± SEM (n = 8–9). There were no significant differences between baseline BAL PL levels in "Dox On" and "Dox Off" groups.

[keratinocyte chemoattractant (KC)], MCP-1), neutrophils (MCP-1), and T cells (IL-13), the data suggest widespread inflammatory suppression by SP-D in these mice. From a number of *in vitro* studies, SP-D has previously been shown to be immunosuppressive of many types of these same effector cells in part through alterations in nuclear factor-κB signaling (29), a common mechanism for cytokine elaboration and oxidative burst (11, 30–37). Thus, teleologically, it appears plausible that the rise in SP-D seen in mice exposed to a variety of inflammatory stimuli (18, 38, 39) represents an acute phase response to suppress further inflammatory damage through a direct effect on the local pulmonary microenvironment interacting with these effector cells and/or other cytokine-producing cell types, such as alveolar or airway epithelia.

In addition to alterations in the local cytokine milieu, we have shown that the observed protection against hyperoxia in SP-D OE mice is associated with up-regulation of NRF-2 and increased expression of phase 2 antioxidant genes. Transcription of various antioxidant enzymes, including HO-1 (40) and GPx (41), is regulated by cis-regulatory elements known as antioxidant response elements (AREs). The ARE consensus sequence TGA(G/C)nnnGC is recognized by a number of transcription activator proteins, including a family of cap-n-collar basic leucine zipper proteins and nuclear erythroid factor 2 (NF-E2)–related factors 1 and 2 (NRF-1 and NRF-2). Recent studies have confirmed that activation of NRF-2 and induction of mRNA expression for ARE-bearing antioxidant and detoxifying enzymes is protective against oxidative stress *in vitro* (42–45) and *in vivo* (46–48). A threefold increase in lung NRF-2 mRNA expression occurred in mice after exposure to O_2 (48) at higher doses (>95% O_2) than in the current study where quantitative real-time reverse transcription–polymerase chain reaction analysis demonstrated that 80% O_2 exposure induces a 2.5-fold increase in NRF-2 and a concordant three- to fivefold increase in downstream antioxidant enzyme expression (GPx-2, HO-1, and Nqo-1) that was not observed in the littermate control mice (Figure 6). Thus, because SP-D OE mice at baseline lack an enhanced antioxidant phenotype and because the SWB littermates did not up-regulate a phase 2 antioxidant response at this dose of O_2, it seems that SP-D in concert with hyperoxic challenge can synergistically participate in a coordinated up-regulation of this protective response at inspired concentrations lower than previously reported to enhance NRF-2 gene responses (48).

The current free radical theory of O_2 toxicity implicates a shift in the balance between intracellular oxidants and antioxidants

as responsible for tissue damage by lipid peroxidation, enzyme inactivation, DNA oxidation, or altered gene expression (49). In addition to induction of enzymatic detoxifying proteins for ROS scavenging described above, protection against oxidant-mediated cell death in the lung is also afforded by up-regulation of nonenzymatic detoxifying agents like vitamins E and C (50). Functionally, using total TBARS as an experimental readout, overexpression of SP-D was associated with marked attenuation in generation of ROS by hyperoxia. SP-D has been shown to act as a potential direct free radical scavenger *in vitro*, limiting peroxidation of surfactant lipid mixtures and protecting a macrophage cell line from oxidant-induced cell death by acting as a free radical chain terminator that could then provide rapid quenching of diene formation, thereby limiting lipid peroxidation (51).

Beyond damage by ROS, exposure to inflammatory stimuli results in the production of reactive nitrogen species (RNS). Classically, inflammatory lung injury has been amplified by NO formed during the inflammatory cascade reacting with ROS to form highly reactive molecules such as peroxynitrite (52). In this model of hyperoxia, we have shown complete attenuation in SP-D OE mice of the two- to threefold increase in oxidative stress in WT mice (Figure 7). Although this study did not directly assess the affect of hyperoxia on NO metabolism, it is likely that protection from hyperoxic injury in SP-D overexpressing mice could also be partly related to secondary abrogation of nitrative stress. This is based on the consideration that because RNS are generated by the reaction of NO with O_2 radicals, and even if NO were to increase modestly during hyperoxia (e.g., by ~35% as we have shown previously [18]), the marked SP-D–induced attenuation in ROS alone could account for a reduction in RNS.

In addition to up-regulation of antioxidant enzymes, SP-D expression in SP-D OE mice exposed to hyperoxia increased to a much greater extent than in littermate control mice (Figure 3B). Analysis of the cis-active human SP-C gene promoter that is used to drive rSP-D expression in our SP-D overexpressing mice revealed a potential binding site for NRF-2 at 2,924 bp upstream of the start codon. This promoter region also contains at least three other AREs at position −2763, −1972, and −866, suggesting a potential for the transgene to be subject to regulation by ROS through ARE. In contrast, the regulatory region of the endogenous SP-D promoter is mainly characterized by the presence of TTF-1 and AP-1 binding sites (53) and is not expected to be directly sensitive to oxidant stress. Hence, we speculate that SP-D OE mice exposed to 80% O_2 demonstrate significantly greater increases in BAL SP-D as a result of an oxidant-induced activation of transgene expression. The resultant 30- to 40-fold increase in SP-D achieved in SP-D OE versus similarly exposed littermates would also be possible pharmacologically via intratracheal instillation with or without surfactant. We and others have measured BAL SP-D concentrations in humans (54, 55) and found them to be approximately 1 μg/ml. Levels found in rodents are of similar magnitude (0.25–0.5 μg/ml BAL, which translates to 0.75–2.0 μg/mouse of total alveolar SP-D) (56). Pharmacologic instillation of SP-D has been reported by Clark and colleagues (57), who repetitively instilled recombinant forms of SP-D intranasally at doses of 10 to 50 μg in 50 μl. Such an instillation enriches alveolar SP-D content by 20- to 40-fold over the basal WT levels.

In summary, we have shown in two different animal models that SP-D is an important regulatory molecule in the protection from hyperoxic lung injury. This protection occurs through a variety of pathways resulting in generalized proinflammatory cytokine immunosuppression and effects on phase II antioxidant expression. Our results raise the possibility that exogenous replacement pulmonary surfactants (heretofore deficient in collectins) that are replete in SP-D could offer an advantage through protection from lung damage when administered to patients at risk for hyperoxic pulmonary injury.

Conflict of Interest Statement: None of the authors has a financial relationship with a commercial entity that has an interest in the subject of this manuscript.

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