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Selective Inhibition of Inducible NO Synthase Activity In Vivo Reverses Inflammatory Abnormalities in Surfactant Protein D-Deficient Mice

Elena N. Atochina-Vasserman,* Michael F. Beers,* Helchem Kadire,* Yaniv Tomer,* Adam Inch,* Pamela Scott,† Chang J. Guo,† and Andrew J. Gow2†

Surfactant protein D (SP-D)−/− mice exhibit early development of emphysema. Previously we have shown that SP-D deficiency results in increased production and activity of inducible NO synthase (iNOS). In this study, we examined whether treatment with the iNOS inhibitor 1400W could inhibit the inflammatory phenotype. Mice were treated with 1400W systemically for 7 wk from 3 wk of age. Treatment reduced total lung NO synthase activity to 14.7 ± 6.1% of saline-treated 10-wk-old SP-D−/− littermates. Long-term administration of 1400W reduced lung inflammation and cellular infiltration; and significantly attenuated the increased levels of matrix metalloproteinases 2 and 9, chemokines (KC, TARC), and cytokines (IFN-γ) seen in bronchoalveolar lavage (BAL) of SP-D−/− mice. Abrogation of these levels was associated with decreasing BAL chemotactic activity for RAW cells. Two weeks of treatment with 1400W reduced total lung NO synthase (NOS) activity to 12.7 ± 6.3% of saline-treated SP-D−/− mice. Short-term iNOS inhibition resulted in attenuation of pulmonary inflammation within SP-D−/− mice as shown by decreases in total BAL cell count (63 ± 6% of SP-D−/− control), macrophage size (>25 μm) within the BAL (62 ± 10% of SP-D−/− control), and a percentage of BAL macrophages producing oxidants (76 ± 9% of SP-D−/− control). These studies showed that s.c. delivery of 1400W can be achieved in vivo and can attenuate the inflammatory processes within SP-D deficiency. Our results represent the first report linking defects in the innate immune system in the lung with alterations in NO homeostasis. The Journal of Immunology, 2007, 179: 8090–8097.

Surfactant protein D (SP-D) belongs to a family of innate host defense proteins termed collectins because of the presence of collagenous and lectin-like domains. It is a multimeric Ca2+−binding protein produced primarily by alveolar type II cells and nonciliated bronchiolar cells in the lung (1). In vivo, targeted ablation of the SP-D gene results in an increase in the baseline level of inflammation in the lung (2). The pulmonary phenotype of SP-D-deficient (SP-D−/−) mice includes the early development of emphysema accompanied by alterations in the surfactant phospholipid profile, an increase in the number and size of macrophages within the airways, increases in metalloproteinase activity, and biochemical evidence of enhanced oxidative-nitritative stress (3). In addition to studies showing that macrophages from SP-D−/− mice generate significantly greater levels of hydrogen peroxide and superoxide than those from wild-type (WT) controls (4), we have recently shown that SP-D also appears to operate as a potent regulator of NO metabolism in the lung as evidenced by the marked increases in NO production, up-regulation of expression of iNOS within the cells of the alveolar space, and a shift in the distribution and oxidative state of NO metabolites (5).

To date, the relationship between SP-D, NO metabolism, and inflammation remains incompletely defined. Within the lung, NO possesses a well-recognized role in a number of physiological processes, including regulation of both airway and vessel tone (6). It has been suggested that S-nitrosylation, i.e., the formation of S-nitrosothiols, may represent one mechanism of NO signaling and thus play a role in the control of pulmonary function (7–10). Conversely, the interaction of NO with other oxidants (e.g., superoxide), as evidenced by the formation of higher oxides of nitrogen such as nitrotrrosine, has been associated with a variety of lung pathologies (11).

The increased iNOS expression and altered NO metabolism observed within SP-D−/− mice indicates that iNOS may be a critical mediator of the inflammatory process seen within these animals (5, 12). Furthermore, it has been previously observed that inhibition of iNOS can reduce pulmonary inflammation in response to allergen challenge (13). To better define the relationship of SP-D, NO metabolism, and inflammation, we examined the effects of iNOS inhibition using the specific inhibitor 1400W upon the indices of inflammation seen in the SP-D−/− mice. In this study, we show that mice treated with 1400W had marked attenuation of bronchoalveolar lavage (BAL) inflammatory cell counts, macrophage size, lung parenchymal inflammation scores, matrix metalloproteinase (MMP) protein expression and cytokine/chemokine levels. These results demonstrate a novel link between lung collectins,
inflammation, and NO metabolism and support the potential use of selective iNOS inhibitors in pulmonary inflammatory disease.

Materials and Methods

**SP-D****mice**

SP-D−/− mice were generated by targeted gene inactivation as previously described (2). This was followed by backcrossing 10 generations into C57BL/6 background. The lungs of SP-D−/− mice in this background do not contain detectable full-length SP-D mRNA or protein (2). SP-D−/− mice were bred in the barrier facilities at the Children’s Hospital of Philadelphia (Philadelphia, PA). Through extensive serologic and bacteriologic screenings, the colony has been shown to be free of detectable viral or bacterial infection.

WT C57BL/6 littermates as well as mice purchased from The Jackson Laboratory were used as controls. Experiments were performed between 3 and 12 wk of age and between 8 and 12 wk of age on mixed populations of male and female mice. All experimental animals used in this study were under a protocol approved by the Institutional Animal Care and Use Committee of the University of Pennsylvania and at Children’s Hospital of Philadelphia.

**Preparation and implantation of micro-osmotic pumps**

Before installation, the specific iNOS inhibitor 1400W (Cayman Chemicals), or control saline, was filtered through a 0.22-μm filter to ensure the sterility of the infusate. Alzet micro-osmotic pumps (model 1002) were filled under sterile conditions with 100 μl (10 mg/kg/h) of 1400W of saline. Loaded pumps were subcutaneous and were placed s.c. After the pumps were inserted into the pocket with the flow modulator pointing away from the incision, the skin incision was closed with sutures and the animals were allowed to recover. Perioperative mortality in experimental animals was <1%. In one set of experiments osmotic pumps were implanted for 2 wk at 8 wk of age, at a time when SP-D−/− mice had developed clear inflammatory disease. Mice were sacrificed at 10 wk of age.

Alternatively inhibition of iNOS activity was started as early as 3 wk of age. Upon weighing, SP-D−/− mice and WT mice were p.i. injected daily with 1400W or saline for 3 wk. At the age of 6 wk, osmotic pumps were implanted for 2 wk and then replaced for another 2 wk such that the dose could be matched to animal growth. The effects of iNOS inhibition could be age-dependent and therefore we examined mice at three ages during the development of pulmonary inflammation (ages 6, 8, 10 wk). Inhibitor 1400W administration had no apparent effect on the general well-being of the mice and did not alter weight gain throughout the experiment (mean weight at 10 wk of age was as follows: WT saline, 23.6 ± 1.0 g; WT 1400W, 23.6 ± 0.8 g; SP-D−/− saline 23.7 ± 0.9 g; and SP-D−/− 1400W, 23.1 ± 0.6 g).

**Differential cell counts**

Lungs of WT and SP-D−/− mice were lavaged with 0.5-ml aliquots of sterile saline to a total of 5 ml, and total cell counts were performed as published (12). Differential cell counts were performed manually on stained cytospins. Cells were identified as macrophages, eosinophils, neutrophils, neutrophils, lymphocytes by standard morphology. The size (diameter) of each macrophage identified during differential counting was determined using an eyepiece objective containing a grid of known size and magnification (model WH10X2-H; Olympus). Data were stratified into two groups, greater than and less than 25 μm in diameter (3 times normal size), and recorded as a percentage of total macrophage number (n = 50 macrophages per cytospin sample).

**Measurement of oxidant production within macrophages by flow cytometry**

Cell pellets from BAL samples collected by centrifugation at 400 × g for 10 min at room temperature were gently resuspended in 5 ml of medium and counted in a Coulter counter. Cells were diluted to a concentration of 6 × 105 cell/ml in RPM 1040 with 10% FCS, penicillin/streptomycin, and L-glutamine. A total of 500 μl of cells (3 × 106 cells) were placed per well and incubated overnight in 24-well tissue-culture plates at 37°C with 5% CO2. Wells containing BAL cell from WT or SP-D−/− mice were subdivided into three groups: positive control (incubated with 200 μM of H2O2 for 30 min at 37°C, followed by incubation with 10 μM H2DCF-DA (2’,7’-dichlorodihydrofluorescein diacetate) for 30 min at 37°C); negative control (incubated without H2O2, or H2DCF-DA); and test group (incubated only with H2DCF-DA). Between every step cells were washed with medium. Cells were harvested on ice and gated fluorescence measured by flow cytometry. Data are expressed as the percentage of macrophages producing oxidants. Dichlorodihydrofluorescein (DCF) fluorescence has previously been used as a measure of the production of one electron oxidant (14).

**Histology and immunostaining of lung tissue**

Following lavages, the left lung was frozen in liquid nitrogen for biochemical and NO measurements; the right lung was inflated and fixed in 4% paraformaldehyde with 0.1 M cacodylate (pH 7.3) for histochemical and immunohistochemical analysis. Paraffin-embedded lung sections were stained with H&E to evaluate intensity of airway inflammation. Sections were scored in a blinded fashion to grade the intensity of inflammation by using a scoring system previously described in detail and validated (15). Lung sections of WT and SP-D−/− mice at three different ages (6, 8, and 10 wk) were deparaffinized and rehydrated. To quench background fluorescence, sections were washed in fresh solutions of saturated sodium borohydride. After blocking nonspecific Ag binding, sections were incubated overnight at 4°C with specific primary antibodies (Santa Cruz Biotechnology), anti-iNOS polyclonal antibodies, anti-neuronal NOS (nNOS; BD Transduction), or nonimmune IgG (Sigma-Aldrich) diluted at 1/100 concentration in blocking solution. Anti-rabbit goat secondary Ab labeled with Alexa Fluor 680 (Molecular Probes) at 1/200 dilution was used for visualization. Fluorescence images of air-dried and Mowiol mounted slides were viewed on an Olympus BX-70 inverted fluorescent microscope. Fluorescence and phase images were captured using a Hamamatsu 12-bit, double-charged device camera. Image processing and overlay analysis were performed under identical conditions at the same setting using IMAGE 1.4.1 software (Universal Imaging), with an exposure times of 1500 ms for all stains. Images were taken at a magnification of ×200. To compare the magnitude of Ab binding for iNOS and IgG, we used computer-assisted image quantification through the Metamorph system. Three to four random images from different slides were analyzed under each condition.

**NOS activity measurement**

Lung tissue homogenates were prepared by pulverizing frozen tissue and placing it in a lysis buffer (20 mM HEPES [pH 7.4], 150 mM NaCl, 10% glycerol, 0.1% Triton X-100, 1 mM EGTA, 1.5 mM MgCl2, 0.33 U of calmodulin, 5 mM CaCl2, 1 mM NaN3, 5 mM -arginine) and incubated for 1 h on a shaking platform at 37°C with 5% CO2. Following incubation, the reaction was stopped by the addition of 2 volumes of acetone and placed at 4°C for 30 min. Samples were centrifuged for 30 min at 14,000 × g and the supernatant was analyzed for NO metabolite content using vanadate chloride reduction and the Sievers NO Analyzer as per the manufacturer’s instructions. NO generation by NOS was calculated as the difference between the 0- and 60-min time points and is expressed as micromole per minute per milligram. This assay provides a simpler but less rigorous measure of NOS activity than the citrulline assay and thus allows for the assessment of the effectiveness of iNOS inhibition within the lung (16).

**Quantitation of cytokine content and chemotaxis**

A 200-μl aliquot of cell-free BAL from the first 1 ml of collected samples was stored at −80°C for cytokine analysis by SearchLight Technology multiplex cytokine assay (Pierce Biotechnology). The remaining aliquots were used for differential chemotaxis (chemoattractant concentration was previously described (17). Briefly, 50 μl of RAW 264.7 cells (American Type Culture Collection), suspended at 2 × 106 cells per ml in DMEM, were placed in the upper wells of a 48-well microchemotaxis chamber (NeuroProbe). The lower chambers contained 41 μl of test solution, consisting of DMEM and either nothing (control); BAL from saline- or 1400W-treated WT or SP-D−/− mice. All test solutions were used in triplicate in each assay. A polycarbonate membrane-pore polycarbonate filter, with 5-μm pores (NeuroProbe), was placed between the wells along with the rubber gasket of the assembly. The chamber was incubated for 3 h at 37°C with 5% CO2 and then disassembled. Nonmigrating cells were scraped from the upper surface, and the migrating cells were stained with the Hemacolor differential blood stain. The filter was placed on a glass coverslip and mounted with coverglass in a glass slide. Cells that migrated through the filter were counted in 10 randomly selected oil-immersion fields in each well at 1000-fold magnification. Data were expressed as the average of the three fields in cells per oil-immersion field.
our personal observations). Therefore to better understand how within lung homogenate as determined by Western blot (12 and significant increase in iNOS expression within the cells of the BAL we have studied mice at 10 wk of age and found that there is a representative images are shown for each time point (6, 8 and 10 wk). Three to four random images from different slides from multiple mice were analyzed under each condition. Original magnification was at $\times$200. Immunostains of lung sections reveal age-related increases of iNOS expression within the inflammatory cells and airway epithelia of SP-D$^{-/-}$ mice (top) when compared with WT mice (bottom).

Data analysis

Experimental data were analyzed with the GraphPad InStat software (v.3.0 for Windows). To test differences between groups, parametric data were analyzed with ANOVA or the Student's $t$ test assuming equal variances. Data are expressed as mean $\pm$ SEM. In all cases, a value for $p < 0.05$ was considered as significant.

Results

As iNOS has been shown to play a role in infection based pulmonary inflammation (18), we wished to study its role in the development of inflammatory disease within SP-D$^{-/-}$ mice. Previously we have studied mice at 10 wk of age and found that there is a significant increase in iNOS expression within the cells of the BAL as indicated by immunohistochemistry (5, 12). Furthermore, we have consistently observed a slight increase in iNOS content within lung homogenate as determined by Western blot (12 and our personal observations). Therefore to better understand how NOS expression is altered within SP-D$^{-/-}$ mice we have examined lung tissue at three ages during the development of pulmonary inflammation (6, 8, and 10 wk of age) by immunohistochemistry. There is no significant change in either eNOS or nNOS expression, and the pattern of immunostaining for these proteins is similar to that previously published (19). Within WT mice there is minimal expression of iNOS and what staining is observable is indistinguishable from the nonimmune IgG control. However, within SP-D$^{-/-}$ there is a significant increase in iNOS immunostaining (Fig. 1). This increase is progressive with the most extensive staining observable at 10 wk of age. iNOS is clearly seen within inflammatory cells within the alveoli, however, there is also strong staining within the large airways. Interestingly, iNOS is also discernible within pulmonary epithelial cells of the alveoli.

To gauge the importance of iNOS function in the development of pulmonary inflammation associated with ablation of the SP-D gene, we examined mice 6, 8, and 10 wk of age with and without 1400W administration from 3 wk of age. Weaning routinely occurs in our animal facility at 3 wk of age. Upon weaning, mice were i.p. injected daily with 1400W or saline for 3 wk, followed by implantation of osmotic pumps for another 4 wk as described in Materials and Methods. Total lung NOS activity in SP-D$^{-/-}$ mice increased gradually with age and become statistically different from saline-treated WT mice at 10 wk of age (Fig. 2). In contrast, treatment with 1400W inhibited total lung NOS activity in SP-D$^{-/-}$ mice to comparable levels seen in WT group. Lung NOS activity of saline-treated WT mice was at a baseline level at all age time points and was not statistically significantly different when compared with 1400W-treated WT mice.

One of the hallmarks of the inflammation that occurs within SP-D$^{-/-}$ mice is the presence of peribronchial mononuclear infiltrates, as well as an increase in the total number of macrophages in the alveolar space. Histological sections of 10-wk-old WT mice treated with saline or 1400W were uniformly normal and showed no evidence of lipoproteinosis or other abnormalities in lung morphology (Fig. 3A). Similarly, there were no visible signs of inflammatory disease in SP-D$^{-/-}$ mice at 4 wk of age (data not shown). In contrast, by 6 wk of age in SP-D$^{-/-}$ mice treated with saline, there were some signs of inflammatory injury, as indicated

![FIGURE 1. The iNOS expression is up-regulated in an age-dependent manner within the lung tissue of SP-D$^{-/-}$ mice. Lung sections of mice 6, 8, and 10 wk of age for WT (bottom) and SP-D$^{-/-}$ (top) animals were stained with anti-iNOS Abs as described in Materials and Methods. The specificity of staining was confirmed using parallel sections stained with nonimmune IgG (at right). Representative images are shown for each time point (6, 8 and 10 wk). Three to four random images from different slides from multiple mice were analyzed under each condition. Original magnification was at $\times$200. Immunostains of lung sections reveal age-related increases of iNOS expression within the inflammatory cells and airway epithelia of SP-D$^{-/-}$ mice (top) when compared with WT mice (bottom).](http://www.jimmunol.org/)

![FIGURE 2. Treatment with 1400W from 3 wk of age resulted in significantly reduced total lung NOS activity in SP-D$^{-/-}$ mice. WT and SP-D$^{-/-}$ mice 3 wk of age were i.p. injected daily with saline or 1400W for 3 wk, followed by implantation of osmotic pumps for another 4 wk as described in Materials and Methods. After 7 wk of treatment, all mice were sacrificed, and lung homogenates from WT and SP-D$^{-/-}$ mice were analyzed for NOS activity as described in Materials and Methods. Data are expressed as total picomoles per microgram of protein per hour. Values shown as mean $\pm$ SEM ($n = 3–10$ animals in each group). Animals at 6-wk-old ( ), 8-wk-old ( ), and 10-wk-old ( ) time points are shown. #, $p = 0.044$ for significant difference from the corresponding WT level; and *, $p = 0.022$ from the corresponding saline level. Treatment of WT mice with saline or 1400W had no effect on lung NOS activity.](http://www.jimmunol.org/)
by both parenchymal inflammation and airway neutrophilia. However, these changes become more pronounced with age. SP-D$^{-/-}$ mice at 8 wk of age, had developed perivascular and peribronchial infiltrates consisting of mainly mononuclear cells and they had accumulated large foamy macrophages in the alveolar space (Fig. 3A). In comparison with saline, treatment with 1400W attenuated the establishment of these inflammatory lung disease markers in SP-D$^{-/-}$ mice (Fig. 3A, bottom panels, and B). Scoring of the histological findings (Fig. 3B) confirmed the enhanced inflammatory response in SP-D$^{-/-}$ mice at all three time points (median score of 1 and 2 vs 0 for WT mice, $p < 0.05$). This enhanced inflammatory response in SP-D$^{-/-}$ mice was significantly attenuated by iNOS inhibition (median score of 1 vs 2 for saline-treated SP-D$^{-/-}$ mice, $n = 4–8, p < 0.05$).

The effect of 1400W on inflammation within the tissues was reflected in changes in macrophage morphology. BAL cytospins were examined after staining with Diff-Quik. Quantitative analysis was done by manual identification and counting of macrophages in BAL cytospins followed by stratification according to size (>25 μm in diameter being considered enlarged) as previously described (20). Examination of BAL cell pellet from saline-treated 10-wk-old WT controls showed that the macrophages appeared normal in size and shape. However, macrophages from saline-treated SP-D$^{-/-}$ mice, consistent with the existence of an activated state, had large and foamy cytoplasm at all time points (Fig. 4A). Inhibition of iNOS for 7 wk had no effect on the size of macrophages in WT mice (3.5 ± 1% macrophages >25 μm and 3.43 ± 1.43% macrophages >25 μm for saline- and 1400W-treated mice, respectively, $n = 5$) (Fig. 4B). The percentage of alveolar macrophages with a diameter >25 μm in the BAL of SP-D$^{-/-}$ mice was reduced with 1400W treatment relative to saline over time (Fig. 4A). This reduction was statistically significantly in mice treated for 5 wk with 1400W and approached WT levels in mice treated for 7 wk (Fig. 4B) (10.57 ± 2% and 6.67 ± 0.88% macrophages >25 μm, for 8- or 10-wk-old SP-D$^{-/-}$ mice, $n = 15, p < 0.05$).

Activation and recruitment of inflammatory cells is dependent upon the expression and release of chemokines and cytokines. Using a cytokine multiplex assay (SearchLight Technology), an array of nine different cytokines in the BAL of WT and SP-D$^{-/-}$ mice treated with saline or 1400W was analyzed (Fig. 5). The samples were assayed for key chemokines (KC/IL-8, MCP-1, MIP-2, and TARC) (Fig. 5A), cytokines (GM-CSF, IFN-γ) (Fig. 5B), and metalloproteinases MMP-2, MMP-3, MMP-9 (Fig. 5C). BAL from saline-treated SP-D$^{-/-}$ mice exhibited a significant elevation of KC/IL-8 and TARC when compared with WT mice (210 ± 34% and 390 ± 47% of corresponding saline-treated WT mice, $n = 6$), (Fig. 5A). Inhibition of iNOS for 7 wk significantly attenuated these increases seen in SP-D$^{-/-}$ mice to 35 ± 7% and 60 ± 13% of saline-treated SP-D$^{-/-}$, respectively. Saline-treated SP-D$^{-/-}$ mice demonstrated increased BAL content of the cytokines GM-CSF and IFN-γ when compared with WT (142 ± 25% and 149 ± 19% of corresponding saline-treated WT, $n = 6$) (Fig. 5B). In addition, 1400W administration significantly reduced the level of IFN-γ (93 ± 7% of saline-treated WT), but not GM-CSF in SP-D$^{-/-}$ mice. In contrast, 1400W treatment has no effect on chemokine and cytokine levels in WT mice.

The expression of certain metalloproteinases has been shown to be increased in SP-D$^{-/-}$ mice (3, 21). Fig. 5C confirms that the protein levels of MMP-9 and MMP-2 are significantly elevated in SP-D$^{-/-}$ mice (541 ± 142% and 190 ± 20% of saline-treated WT.

**FIGURE 3.** SP-D$^{-/-}$ mice demonstrated significantly reduced lung inflammation following 1400W treatment. WT and SP-D$^{-/-}$ mice were treated with saline or 1400W for 7 wk as described in Materials and Methods. At different time points (6, 8, and 10 wk) mice were sacrificed and inflation fixation for histopathology was performed as described in Materials and Methods. A, Representative H&E stains of lung sections from WT and SP-D$^{-/-}$ mice receiving either saline (top panels) or 1400W (bottom panels) are shown at equal magnification of $×200$. Saline-treated SP-D$^{-/-}$ mice demonstrated age-related increases of inflammatory injury and accumulation of large alveolar macrophages (top) and reversal of these abnormalities after 1400W treatment (bottom). B, Intensity of inflammation in the lung tissue of WT or SP-D$^{-/-}$ mice treated with saline or 1400W. Histologic sections of lungs from each of the mice obtained at 6, 8, 10 wk of age were scored for inflammatory changes as described in Materials and Methods. Animals at 6-wk-old (III), 8-wk-old (III), and 10-wk-old (■) time points are shown. Data represent median values, with 5–20 samples in each group. #, $p < 0.05$ for significant difference from the corresponding WT level; and *, $p < 0.05$ from the corresponding saline level.
mice, respectively). In addition, iNOS inhibition significantly attenuates this up-regulation (57 ± 7% and 63 ± 10% of saline-treated SP-D mice, respectively, n = 6). In contrast, the level of MMP-3 was not elevated in SP-D mice. Interestingly, 1400W treatment significantly reduced MMP-3 level in both WT and SP-D groups (43 ± 7% and 65 ± 12% of corresponding saline-treated controls, respectively, n = 6).

The chemokines and cytokines analyzed have been shown to be pleiotropic in their inflammatory effects. However, among those raised within SP-D mice, TARC, MCP-1, and IFN-γ have been shown to have stimulatory effects upon macrophages. Therefore, as a functional correlate of these increases, the ability of BAL to induce RAW cell chemotaxis was analyzed using a modified Boyden chamber assay (Fig. 5D). BAL of SP-D mice induced a
Significantly greater level of cell migration than that from WT mice (515 ± 45% of saline-treated WT mice). Inhibition of iNOS significantly reduced the number of migrated cells to 45 ± 5% of saline-treated SP-D−/− mice.

Having established that iNOS inhibition could modulate the development of pulmonary inflammation within SP-D−/− mice, we examined whether 2 wk of selective iNOS inhibition could reverse previously developed pulmonary inflammation in SP-D−/− mice starting at 8 wk of age (a time at which SP-D−/− mice have developed a clear inflammatory disease). For these studies, 1400W or saline was administered via osmotic pump for 2 wk. As with the long-term inhibition study, NOS activity was measured in the lung tissue to gauge the effectiveness of 1400W administration. Fig. 6 shows that lung NOS activity in 10-wk-old SP-D−/− mice was significantly higher at baseline level when compared with WT control littermates and that treatment with 1400W significantly reduced NOS activity in the lungs of SP-D−/− mice. There were no statistically significant differences in NOS activity between 1400W- or saline-treated WT mice (28 ± 2 vs 20 ± 10 pM/mg of protein/hour, respectively, n = 3).

In accordance with previous observations, saline-treated 10-wk-old SP-D−/− mice showed a 3-fold increase in the number of BAL macrophages when compared with littermate WT control mice (Fig. 7A). Inhibition of lung NOS activity for 2 wk partially reversed total BAL cell counts in SP-D−/− mice (63 ± 6% of SP-D−/− saline level, n = 20). Within saline-treated 10-wk-old WT mice 93 ± 2% of BAL macrophages appeared normal in size and shape (Fig. 7B, left column). Consistent with our previous observations (5), saline-treated 10-wk-old SP-D−/− mice demonstrated a significant increase in the percentage of foamy, and often multinucleated, alveolar macrophages whose diameter was >25 μm (Fig. 7B, right column, and C). However, treatment with 1400W significantly reduced this percentage in SP-D−/− mice (Fig. 7C); macrophages were more frequently normal in appearance (29 ± 2% macrophages >25 μm for saline-treated mice, n = 10 vs 18 ± 3% for 1400W-treated mice, n = 15, p < 0.05).

Previous studies have shown that SP-D−/− mice exhibit markers of increased oxygen radical production (3, 5, 21). Intracellular radical production in alveolar macrophages was determined by monitoring fluorescence following DCF diacetate treatment (Fig. 7D). We observed a 2-fold increase in DCF fluorescence within macrophages from saline-treated SP-D−/− mice when compared with macrophages from WT saline-treated controls (30% DCF-positive macrophages for saline WT mice). Two weeks of iNOS inhibition significantly reduced oxidant production by macrophages in SP-D−/− mice (64 ± 3% DCF-positive macrophages for saline-treated SP-D−/− mice vs 49 ± 6% for 1400W-treated SP-D−/− mice, n = 4) (Fig. 7D). These data demonstrate that inhibition of lung NOS activity for 2 wk following the development of pulmonary inflammation is associated with a partial reversal of those endpoints in SP-D−/− mice. iNOS inhibition had a significant effect upon BAL cell count, size of macrophages, and percentage of macrophages producing oxidants.

**Discussion**

The most striking phenotype feature of the SP-D−/− mouse has been enhanced lung inflammation. SP-D−/− mice develop alveolar proteinosis and pulmonary emphysema, characterized by chronic low-grade inflammation (2) and a high number of alveolar macrophages with enhanced oxidant production (21). Hydrogen peroxide formation by alveolar macrophages from SP-D−/− mice is significantly increased and is associated with enhanced expression of the metalloproteinases MMP-2, MMP-9, and MMP-12 (3).
development of inflammatory lung disease within SP-D−/− is surprising in light of the already established role of SP-D in the clearance of infectious agents. The SP-D−/− pulmonary phenotype has been shown to be partially rescued by conditional re-expression of SP-D in adulthood (22). To date no other treatment has been reported to alter the phenotype of these mice. The current study uses a selective iNOS inhibition to show that SP-D−/− mice phenotype can be manipulated pharmacologically.

Recently, we reported that NO metabolism was severely disrupted within the SP-D−/− mouse, specifically there was an increase in the production of higher oxides of nitrogen (5). The combination of increased higher nitrogen oxide production and inflammation suggests that iNOS may be a contributing factor. It has been proposed that SP-D operates under basal conditions to inhibit activation of the inflammatory process, but upon inflammation guides the activated immune cells to their appropriate target. The mechanism for these changes has not been defined; however, it would appear that ablation of SP-D results in progressive activation of alveolar macrophages with age. Such activation would be expected to lead to increased iNOS activity, which may contribute to the changes in NO metabolism. In addition, it is possible that iNOS-derived NO acts as a key signaling molecule in the inflammatory process, which results from SP-D ablation. In the present study, we investigated the effects of reducing iNOS-mediated production of NO by using the specific inhibitor 1400W. This inhibitor binds tightly to iNOS and has minimal effect on eNOS or nNOS (23). The iNOS inhibition was achieved using i.p. injection or osmotic pumps loaded with 1400W (using saline as a control) placed s.c. in WT and SP-D−/− mice either before or following the establishment of inflammation in the lung.

In the absence of 1400W total lung NOS activity in SP-D−/− mice was significantly higher than in WT littermate controls at 10 wk of age (Figs. 2 and 6). Treatment with 1400W significantly reduced NO activity in the lungs of SP-D−/− mice (Figs. 2 and 6). iNOS, although normally present within the lung, is significantly induced by inflammation. Furthermore, once expressed it produces a higher flux of NO than either nNOS or eNOS, which are also present within the lung (19). The ability of 1400W treatment to reduce total NOS activity to the control level indicates that iNOS activity dominates within the inflammatory process induced within SP-D−/−. The ineffectiveness of this inhibitor on NOS activity within WT mice, indicates that iNOS is not significant source of NO for the resting lung. Such an observation is in accordance with the apparently low level of iNOS expression in the lung at baseline (Fig. 1) (19).

Previously we have observed that NO production in total is increased within SP-D−/− mice and that a higher proportion of this NO is metabolized as higher oxides of nitrogen (5). The increased iNOS activity within SP-D deficiency, almost certainly a result of the inflammatory process, as well as being the source of the increased NO production, could play a role in these alterations in NO chemistry. The higher rate of NO flux produced by iNOS will significantly increase the proportion of higher nitrogen oxides formed within the oxidizing environment of the lung. However, the inflammatory response also increases the production of oxidants that may be a factor in the increased proportion of higher oxides formed. Increased iNOS activity within the SP-D−/− mouse may not just be a result of inflammatory signaling, but may also play a role in perpetuating that signal, either through increased NO production or through the formation of altered NO metabolites with signaling properties of their own, such as nitroalkenens (24). If iNOS-derived NO is part of the inflammatory signal, then its inhibition should result in a reversal of the observed inflammatory processes within SP-D deficiency.

In the present study, inhibition of iNOS immediately after weaning attenuated the percentage of enlarged, foamy macrophage in SP-D−/− mice to a level similar to that seen in normal WT mice (Fig. 4). In addition, iNOS inhibition reduced inflammation within the lung as indicated by reduced parenchymal inflammation (Fig. 3) and normalization in cytokine and chemokine production (Fig. 5). Furthermore, iNOS inhibition in adult mice from 8 to 10 wk of age, after inflammation develops, produced a significant reduction in BAL cell count and macrophage activation state (Fig. 7). Therefore, it would appear that iNOS-derived NO plays a role both in the establishment and the maintenance of the pulmonary inflammatory phenotype seen in SP-D−/− mice.

Previously Tino and Wright (25), as well as Bridges et al. (26), demonstrated that SP-D directly protects macrophages and surfactant phospholipids from oxidative damage (25, 26). However, from these studies it remains unclear whether SP-D protection occurs directly via reductions in cellular oxidant production or indirectly through function as an antioxidant or scavenger. The effects of iNOS inhibition upon oxidant generation by alveolar macrophages were assessed using DCF diacetate, a fluorescent target for intracellular oxidants. Increased DCF fluorescence was observed in macrophages from saline-treated SP-D−/− mice when compared with those from WT saline controls (data not shown). Furthermore, 2 wk of iNOS inhibition significantly reduced the level of oxidant generation within macrophages in SP-D−/− mice (Fig. 7D). These data suggest that the increased oxidant production observed within alveolar macrophages from SP-D−/− mice is at least partially mediated through increased iNOS activity. Taken together with the findings demonstrating a decreased number and percentage of large foamy macrophages within 1400W-treated mice (Figs. 4 and 7), these present data support the concept that inhibition of iNOS reduces inflammatory signaling and oxidant production within SP-D deficiency.

Recent data from Clark and coauthors (27) demonstrated that an increased number of alveolar macrophages isolated from SP-D−/− mice appears to be apoptotic and necrotic. Furthermore, intrapulmonary replacement of a human recombinant SP-D reduces the percentage of apoptotic alveolar macrophages and the level of mRNA for the proinflammatory cytokines MCP-1 and MIP-1α in the lungs (27, 28). Recent findings that NF-κB activity was up-regulated within alveolar macrophages from SP-D−/− mice (21) and was inhibited by S-nitrosylation of the p50 subunit within lung epithelial cell lines (29), suggests a signaling mechanism by which SP-D achieves regulation of the inflammatory process. Because NF-κB is a critical mediator of transcriptional responses during inflammation, these findings support the concept that SP-D is required for appropriate regulation of both oxidant production and inflammatory responses by alveolar macrophages.

Our results demonstrate that inhibition of lung NOS activity, either before or at a time point when SP-D−/− mice have developed a clear inflammatory disease, is associated with a reversal of the inflammatory phenotype. Therefore, this study identifies iNOS, and its resultant products, not only as a downstream target of SP-D signaling but also a mediator of the inflammatory response that occurs in its absence. Reversibility of pulmonary abnormalities within SP-D deficiency has been previously reported by two groups (22, 30). However, in these studies reversal was achieved by either the local expression of SP-D or its conditional replacement in the lung. The results presented suggest that pharmacologically iNOS may be an appropriate target in pulmonary inflammation associated with emphysema, although caution should be exercised in extrapolating mouse data to the human disease.
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References