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Role of NOS2 in pulmonary injury and repair in response to bleomycin

Changjiang Guo^a, Elena Atochina-Vasserman^b, Helen Abramova^a, Blessy George^a,
Velepparambil Manoj^c, Pamela Scott^a, and Andrew Gow^{a,*}

^aDepartment of Pharmacology & Toxicology, Rutgers, the State University of New Jersey, Piscataway, NJ, USA

^bDivision of Pulmonary, Allergy, and Critical Care Medicine, Department of Medicine, University of Pennsylvania School of Medicine, Philadelphia, PA, USA

^cDepartment of Molecular Genetics, Cleveland Clinic, Lerner Research Institute, Cleveland, OH, USA

Abstract

Nitric oxide (NO) is derived from multiple isoforms of the Nitric Oxide Synthases (NOSs) within the lung for a variety of functions; however, NOS2-derived nitrogen oxides seem to play an important role in inflammatory regulation. In this study, we investigate the role of NOS2 in pulmonary inflammation/fibrosis in response to intratracheal bleomycin instillation (ITB) and to determine if these effects are related to macrophage phenotype. Systemic NOS2 inhibition was achieved by administration of 1400 W, a specific and potent NOS2 inhibitor, via osmotic pump starting six days prior to ITB. 1400 W administration attenuated lung inflammation, decreased chemotactic activity of the bronchoalveolar lavage (BAL), and reduced BAL cell count and nitrogen oxide production. *S*-nitrosylated SP-D (SNO-SP-D), which has a pro-inflammatory function, was formed in response to ITB; but this formation, as well as structural disruption of SP-D, was inhibited by 1400 W. mRNA levels of IL-1 β , CCL2 and Ptgs2 were decreased by 1400 W treatment. In contrast, expression of genes associated with alternate macrophage activation and fibrosis Fizz1, TGF- β and Ym-1 was not changed by 1400 W. Similar to the effects of 1400 W, NOS2^{-/-} mice displayed an attenuated inflammatory response to ITB (day 3 and day 8 post-instillation). The DNA-binding activity of NF- κ B was attenuated in NOS2^{-/-} mice; in addition, expression of alternate activation genes (Fizz1, Ym-1, Gal3, Arg1) was increased. This shift towards an increase in alternate activation was confirmed by western blot for Fizz-1 and Gal-3 that show persistent up-regulation 15 days after ITB. In contrast arginase, which is increased in expression at 8 days post ITB in NOS2^{-/-}, resolves by day 15. These data suggest that NOS2, while critical to the development of the acute inflammatory response to injury, is also necessary to control the late phase response to ITB.

*Correspondence to: Ernest Mario School of Pharmacy, Rutgers, Room 009A, 160 Frelinghuysen Road, Piscataway, NJ 08854, USA. Fax: +1 848 445 1191. gow@rci.rutgers.edu (A. Gow).

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Keywords

Pulmonary inflammation; NOS2 inhibitor; Bleomycin; Surfactant protein-D; S-nitrosylation

1. Introduction

Nitric oxide (NO) is a pluripotent molecule with a wide range of bioactivity and chemical reactivity [1]. Within the lung it is both an important physiological regulator and a key agent within pulmonary disease [2–6]; it regulates airway and vascular tone as well as pulmonary surface tension. However, under pathologic conditions, excessive expression of iNOS results in high efflux of NO, leading to post-translational modification of proteins, such as surfactant protein-D (SP-D), and mediates lung inflammation [7]. NO, and NO-mediated modifications, have been identified in pulmonary pathology in animal and human studies [2,8–12]. NO is produced by alveolar macrophages and pulmonary epithelial cells upon exposure to pro-inflammatory stimuli such as LPS, TNF- α , IL-1 β and interferon- γ [13–21]. These inflammatory mediators induce NOS2 expression through a series of signal transduction pathways including NF- κ B and interferon regulatory factors (IRFs). Induction of iNOS, the product of NOS2, results in high efflux of NO, which further accelerates pulmonary inflammation. It has been suggested that inhibition or ablation of NOS2 inhibits persistent injury of the lung including pulmonary inflammation and fibrotic processes [22].

Pulmonary inflammation and the subsequent transition to fibrosis is a complicated process involving multiple cellular activators and growth factors including such well known cytokines as TNF- α , IL-1 β and TGF- β . A key step in the transition from inflammation to fibrosis is the development of a re-organized extracellular matrix. Both reactive oxygen and nitrogen species appear to play a role in the inflammation-fibrosis transition. Recently, Pini et al reported that inhibition of cyclooxygenase and NO-donation attenuates pulmonary fibrosis induced by bleomycin [23]. To further address the potential role of iNOS-derived NO in pulmonary inflammation and fibrosis, we have examined the effects of genetic and pharmacological inhibition of iNOS function in the well-established lung injury model of intratracheal instillation of bleomycin (ITB). ITB in rodents has relevant clear phases of pulmonary pathology including sub-acute lung injury, inflammation, and fibrosis [24–27]. The production of free radical species such as nitric oxide is considered as a factor resulting in endothelial and epithelial cell damage, the appearance of DNA-damage inducible proteins, increased micro-vascular permeability, and respiratory distress with surfactant dysfunction. ITB produces an initial inflammatory response marked by peak levels of TNF- α and TGF- β 7–10 days after injury mediated by increased activity of NF- κ B that corresponds with maximal inflammatory cell infiltrate and respiratory distress. From 14 to 21 days, a transition from inflammation to either extracellular matrix production or to tissue healing and repair ensues [24–27].

The increased NOS2 expression and altered NO metabolism observed within bleomycin-induced acute inflammation in mice indicate that NOS2 is a critical mediator of the inflammatory process seen within these animals [24–28]. We have previously demonstrated

that selective inhibition of NOS2 can reverse chronic pulmonary inflammation [10]. In the present study, we investigated the effect of systemic NOS2 inhibition using the specific inhibitor 1400 W or genetic ablation of the NOS2 gene upon the indices of inflammation and fibrosis seen following acute injury with ITB. In this paper we demonstrate that 1400 W treatment attenuated bronchoalveolar lavage (BAL) inflammatory cell counts, macrophage size, SNO-SP-D formation and inflammatory cytokine/chemokine gene expression. However, the fibrotic precursors were not altered by systemic NOS2 inhibition. Similarly, NOS2 ablation while reducing inflammation early in the response to ITB does not improve the fibrotic endpoint, evidence is shown that indicates that macrophage phenotype may play a role in determining the outcome of ITB.

2. Material and methods

2.1. Preparation and implantation of micro-osmotic pumps and 1400 W treatment

Before instillation, the specific NOS2 inhibitor 1400 W (Cayman Chemicals), or control saline, was filtered through a 0.22- μ M filter to ensure the sterility of the infusate [10]. Alzet micro-osmotic pumps (model 1002) were filled under sterile conditions with 100 μ l (10 mg/kg/h) of 1400 W or saline. Loaded pumps were submerged overnight in sterile saline at 37 °C before implantation. C57 BL6/J mice were each anesthetized with 50 mg/kg i.p. injected pentobarbital. Under sterile conditions a small incision was made in the skin between the scapulae, and osmotic pumps were placed s.c. After the pumps were inserted into the pocket with the flow moderator 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%. 1400 W was treated for 6 days at the age of 8 wk. Bleomycin was instilled and the animals were sacrificed at day 8 post bleomycin administration.

2.2. Model of bleomycin injury

All animals for this study were housed in the Animal Care Facility of Rutgers under standard conditions with free access to food and water. Human clinical-grade, sterile, and lipopolysaccharide (LPS)-negative saline or 3.0 U/kg of bleomycin sulfate (Bristol Myers Squibb) in 50 μ l of saline was administered intratracheally to 10-wk-old C57 BL6/J mice (25–35 g) or NOS2^{-/-} mice as previously described. All protocols were reviewed and approved by the Institutional Animal Care and Use Committees of Rutgers University and adhered to the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals.

2.3. Biotin-Switch assay for detection of SNO-SP-D

Detection of SNO-SP-D was performed *via* an adaptation of the biotin switch method [7]. BAL (30 μ g total protein) in HEN buffer (25 mM Hepes, pH 7.7/0.1 mM EDTA/0.01 mM neocuproine), and 20 μ M *N*-ethylmaleimide (NEM) at 37 °C for 30 min to block free thiols. Excess NEM was removed by protein precipitation using cold acetone. Protein pellets were resuspended in HENS buffer (HEN 1% SDS), SNO bonds were decomposed by adding 20 mM sodium ascorbate. The newly formed thiols were then linked with the sulhydryl-specific biotinylating reagent *N*-[6-biotinamido)-hexyl]-1-(2-pyridyldithio) propionamide (Pierce).

Biotinylated proteins were precipitated with Streptavidin-agarose beads and Western blot analysis was performed to detect the amount of SP-D remaining in the samples.

2.4. Histopathology

The lungs were inflated and fixed in 3% paraformaldehyde and processed for paraffin embedding. Sections of lung were stained with hematoxylin and eosin or with a Masson trichrome stain to assess the degree of fibrosis/long term inflammation. The extent of lung fibrosis was graded on a scale of 0 for normal lung to 8 for severe distortion of structure and large tissue areas as previously reported by Ashcroft and colleagues [29]. The major criteria examined included interstitial thickening of alveolar or bronchiolar walls, collagen deposition, and inflammatory cell infiltration.

2.5. Polyacrylamide gel electrophoresis and immunoblotting

Total BAL SP-D was analyzed by denaturing sodium dodecyl sulfate–polyacrylamide gel electrophoresis, SP-D quaternary structure by native gel electrophoresis, and S-nitrosylated SP-D by the biotin-switch method as previously described [7].

2.6. Quantitation of cytokine content and chemotaxis

A 200- μ l 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). Directed migration (chemotaxis) of cells was performed as previously described [7]. Briefly, 100 μ l of cells suspended at 1×10^6 cells/ml in DMEM were placed in the upper wells of a 48-well microchemotaxis chamber (Neuro Probe). The lower chambers contained 31 μ l of test solution, consisting of DMEM and either nothing (control) or BAL. All test solutions were used in triplicate in each assay. A polyvinylpyrrolidone-free polycarbonate filter was placed between the wells along with the rubber gasket of the assembly. The filters used for macrophage chemotaxis had 5- μ m pores (Neuro Probe). The chamber was incubated at 37°C with 5% CO_2 for 3 h, and then disassembled. Non-migrating 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 immersion oil onto a glass slide. Cells that migrated through the filter were counted in ten randomly selected oil-immersion fields in each well at $400 \times$ magnifications. Data were expressed as cells per oil-immersion field for the three wells used for each solution.

2.7. NF- κ B p50 Transcription factor assay

Nuclear proteins from BAL cells were extracted using the Sigma Nuclear Extract Kit (Sigma St. Louis, MO) according to the manufacturer's instructions. Activation of the NF κ B p50 subunit in 5 μ g nuclear extract was determined using the NF- κ B enzyme-linked immunosorbent assay (ELISA)-based transcription factor kit (TransAM assay) according to the manufacturer's protocol.

2.8. RNA isolation of alveolar inflammatory cells and reverse transcription

Total cellular RNA was isolated from alveolar immune cells using Tri-Reagent (Molecular Research Center, Cincinnati, OH, USA) [30]. In brief, total RNA was extracted by a single-step, guanidium thiocyanate–phenol–chloroform extraction. After centrifugation at 13 000g for 15 min at 4 °C, the RNA-containing aqueous phase was precipitated in isopropanol. RNA precipitates were washed once in 75% ethanol and re-suspended in 20 µL Rnase-free water. Total cellular RNA was subjected to reverse transcription using the reverse transcription system from Promega, with haxmer for 1 h at 42 °C. The reaction was terminated by incubating the reaction mixture at 99 °C for 5 min and then kept at 4 °C. The resulting cDNA was ready to serve as a template for PCR amplification.

2.9. Real-time qPCR

Real-time qPCR was performed with ABI Prism 7300 Sequence Detection System (Perkin Elmer). The target genes for IL-1 β , Arginase 1, CCL2, Ptgs2, NOS2, Fizz1, Ym-1 and β -actin were amplified by ABI gene assays. The cycle conditions were set as follows: 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. All controls and samples were run in triplicate in the same plate. β -actin mRNA levels of the samples in the same plate were analyzed by real-time PCR to normalize the mRNA contents among the samples tested.

2.10. Statistical analysis

To test differences between groups, parametric data were analyzed with ANOVA or the Student's *t* test assuming equal variances and using a Bonferroni correction for multiple comparisons. Data are expressed as mean $\pm$ SEM. For non-parametric data, comparisons were made by Wilcoxon-Rank Sum test. Data are expressed as median and 25th to 75 quartile range. In all cases, a value for $p < 0.05$ was considered as significant.

3. Results

3.1. 1400 W attenuates NO metabolite formation in response to ITB

NOS2 expression and increased production of NO have both been observed in response to ITB. In this study we delivered a continuous dose of the selective iNOS inhibitor 1400 W via osmotic pump. Previously, we demonstrated that we could inhibit lung NOS function by this method in a long term inflammatory model [31]. To ensure that this method was effective in an acute injury model, such as ITB, we measured NO metabolites within the BAL. 8 days after ITB the concentration of nitrogen oxides (NO_x) within BAL rose significantly from 1.2 ± 0.44 µM to 4.2 ± 0.57 µM ($p < 0.05$). This increase was significantly attenuated by treatment with 1400 W, where BAL NO_x fell to 2.1 ± 0.41 µM, which was not significantly greater than control.

3.2. 1400 W inhibits inflammatory cell infiltration in the acute lung inflammation

The hallmark pathologic effects of bleomycin administration include pro-inflammatory cell infiltration and alveolar macrophage activation, along with morphologic changes. As one can see in Fig. 1 there is pronounced inflammatory response to ITB, characterized by

distinct perivascular and peribronchial infiltration (Fig. 1A). The cellular content of the BAL was examined both by coulter counter and by cytopsin followed by staining with Diff-Quik. 1400 W treatment significantly attenuated the increase in total cell count that is observed with ITB (Fig. 1B); as well as decreasing the size of individual macrophages. Not coincidentally the chemotactic activity of the BAL was attenuated by 1400 W treatment relative to ITB alone (Fig. 1D). Although the total cell count was attenuated by 1400 W treatment the reduction did not alter the overall cellular profile of the BAL, as defined by cell type (Fig. 1C).

3.3. 1400 W treatment reduces formation of SNO-SP-D and disruption of SP-D multimer

NO modification of SP-D to form SNO-SP-D is a regulator of pulmonary inflammation [7]. Since 1400 W administration inhibited NO production in the lung, we sought to determine whether SNO-SP-D formation was attenuated. Biotin switch shows that SNO-SP-D is increased in response to ITB, which is consistent with our previous findings. However, SNO-SP-D was dramatically reduced by 1400 W treatment (Fig. 2A). As *S*-nitrosylation of SP-D disrupts the protein quaternary structure by forming trimers, we examined SP-D structure by native gel electrophoresis (Fig. 2B). ITB resulted in an increase in SP-D trimer formation that was inhibited by 1400 W administration (Fig. 2B). It should be noted that multimeric SP-D is very large (> 1 MDa) and does not enter a native gel easily, but for the native gel analysis equal amounts of total SP-D, as determined by reducing gel electrophoresis (Fig. 2A), were loaded.

3.4. Inhibition of iNOS alters gene expression profile of pro-inflammatory mediators within BAL

Pro-inflammatory mediators are produced from alveolar macrophages during the acute inflammatory process [24–27]. Macrophages differentiate to two main phenotypes upon activation [32–34]. In order to further understand the activation stage of pulmonary macrophages within ITB, the gene expression of BAL cells was assayed by real-time-qPCR. Classical pro-inflammatory genes such as IL1B, CCL2 and PTGS2 were up-regulated in response to ITB (Table 1). 1400 W administration inhibited ITB-mediated induction of these genes. Retnla, which encodes for Fizz1 (found in the inflammatory zone) a resistin-like secreted protein is up-regulated during allergic, alternative pulmonary inflammation [35]. ITB induced Retnla expression within BAL cells, however, in contrast to IL1B, CCL2 & PTGS2, 1400 W did not affect its expression (Table 1). Chil3 encodes for the chitianse-like molecule Ym-1, a secretory lectin that forms crystals within alveolar spaces and within the cytoplasm of alveolar macrophages and multinucleate giant cells in the lungs of mice exhibiting hyperactivity of alveolar macrophages [35]. Ym-1 is considered as an anti-inflammatory molecule and has been shown to be involved in eosinophilic inflammation [36].

3.5. 1400 W administration exacerbates markers of long term inflammation post ITB

The alteration in gene expression profile observed within the BAL in response to 1400 W indicated that loss of iNOS function may lead to a failure to regulate the late phase/fibrotic response to ITB. Consistent with this proposal we observed that TGF- β staining was increased by 1400 W treatment in the context of ITB (Fig. 3A). Trichrome staining was used

as a marker of chronic inflammation and the initiation of fibrosis (Fig. 3B) ITB resulted in a significant increase in trichrome score (4 vs 0 for ITB vs saline), which was not attenuated by inhibition of iNOS (5 vs 0 for ITB vs saline in the presence of 1400 W) (Fig. 3B). In confirmation of the altered TGF- β staining seen in panel A, TGFB mRNA levels were measured within the lung tissue. As was seen with the staining ITB resulted in a significant increase in TGFB1 mRNA level (Fig. 3C). Treatment with 1400 W actually increased TGFB1 expression in the presence of ITB.

3.6. NOS2 ablation reduces early stage of inflammation but increases long term inflammation/fibrosis

Fibrosis develops later than in the inflammatory response to ITB. Therefore, in order to better understand the importance of iNOS in the time course of inflammation and fibrosis, we examined the response to ITB in NOS2 $^{-/-}$ mice. Similar to 1400 W administration, the absence of NOS2 reduces ITB-mediated injury early in the time course with reduction in cellular invasion 3 days post injury (Fig. 4 A and E). 8 days post ITB, the inflammatory response in C57 Bl6/J and NOS2 $^{-/-}$ mice appear similar (Fig. 4 B and F). However, 21 days post injury, while inflammation in C57 Bl6/J mice appears to have resolved with minimal fibrosis (Fig. 4 C), as shown by trichrome stain (Fig. 4 D), there is extensive inflammatory proliferation and fibrotic staining in NOS2 $^{-/-}$ mice (Fig. 4 G and H).

3.7. NOS2 ablation reduces NF- κ B DNA binding activity and increases MMP-9 and TGF- β 1 production

NF- κ B is a key transcription factor in macrophage activation and in the production of pro-inflammatory mediators. NF- κ B DNA binding activity in BAL cells was measured by TransAM assay 3 days post ITB. As predicted, ITB resulted in a significant increase in NF- κ B DNA binding in C57Bl6/J mice, however, this induction was attenuated in NOS2 $^{-/-}$ animals (Fig. 5A).

MMP9, a matrix metalloproteinase involved in neutrophil migration and pulmonary epithelial repair processes, was found to be up-regulated by ITB (Fig. 5B). In contrast to NF- κ B DNA binding activity, however, MMP9 expression was increased in NOS2 $^{-/-}$ mice. MMP9 can operate in concert with TGF- β 1 to induce fibrotic processes [37]. Notably, there was no increase in TGF- β 1 within C57BL6/J mice 3 days post ITB, however, there was significant induction within NOS2 $^{-/-}$ animals (Fig. 5B) indicative of an increase in proliferative signaling.

3.8. NOS2 ablation increases expression of markers associated with alternative macrophage activation following ITB

Macrophage phenotype is dynamically regulated in the lung and is thought to be a critical component in maintaining the balance between inflammation and resolution [39]. Therefore, we examined the time course of changes in expression of three proteins known to be associated with alternative macrophage activation and fibrosis Gal3, Fizz1, and Arg1 [40–42]. All three proteins were induced in their expression in response to ITB, although the time course and genotype dependency of these changes varied (Fig. 6). Fizz1 was induced as early as 3 days post ITB, however, it remained elevated through to day 15 in NOS2 $^{-/-}$ mice.

Arg1 did not increase expression until 8 days post ITB and returned to control levels in both genotypes by day 15. There is an indication of increased expression within NOS2^{-/-} mice, although the time course is not altered by the lack of iNOS function. Gal3, which is present irrespective of injury at day 3, increased in expression in response to ITB by day 8. It remains elevated at day 15 within NOS2^{-/-} mice. These results are consistent with a pro-fibrotic phenotype in the NOS2^{-/-} mice.

4. Discussion

In the present study we show that loss of iNOS function, either through pharmacologic inhibition or through genetic deletion, attenuates the acute pulmonary inflammatory process in response to ITB, however, it appears to exacerbate the long term inflammation/fibrosis. Evidence from both the use of 1400 W and NOS2^{-/-} mice shows that inflammation at 3 days post ITB is attenuated (Figs. 1,3 and 4). However, these same studies demonstrate that lack of iNOS function exacerbates the late phase inflammatory/fibrotic response. Additional data, suggests that iNOS function may alter macrophage phenotype in response to bleomycin and this provides a potential mechanism for the opposite effects in the early and late stages of the response. The importance of iNOS in murine pulmonary inflammation has previously been seen in both chronic and acute models [10,28] and it has been shown that chronic responses to ovalbumin are also NOS2 dependent [43,44]. The data presented here are consistent with these previous observations; however, they raise the possibility that iNOS function is critical to limiting fibrotic responses, potentially via regulation of macrophage phenotype.

Pre-treatment with 1400 W inhibited lung inflammation 3 days post ITB (Fig. 1A) and in conjunction there was a reduction in the increase in macrophage size and number (Fig. 1B). Alveolar macrophages are known to increase in size upon classical activation, a response that has been previously observed in response to ITB. Activated macrophages are both responders to and mediators of acute inflammation. They secrete pro-inflammatory cytokines, chemokines, MMPs and other growth factors, and reactive oxygen and nitrogen species producing pulmonary epithelial injury and reducing the surface-active function of the lung lining fluid. As well as responding to inflammatory cytokines, such as TNF- α , we have demonstrated that the NO-modified form of surfactant protein-D, SNO-SP-D, can shift pulmonary macrophages to a pro-inflammatory phenotype [7,9,11,12]. In agreement with these earlier studies we found that ITB induced the formation of SNO-SP-D (Fig. 2) and acute inflammatory cytokines such as IL1B, CCL2, and Ptg2 (Table 1). That iNOS plays a role in this acute inflammatory process is shown by the reduction in SNO-SP-D formation and cytokine induction by 1400 W. A further indication of the importance of iNOS in this early response is shown by the lower level of NF- κ B DNA binding activity in NOS2^{-/-} compared to C57 Bl6/J (Fig. 5). At these early time points in the ITB response loss of iNOS may be producing a shift in the inflammatory profile. With both 1400 W treated and NOS2^{-/-} animals, while there was a reduction in the expression of acute inflammatory genes, alternative activation markers such as Retnla, Arg1, and CCL20 were either maintained or elevated (Table 1 and Fig. 6). Also Chi3l3, which encodes for the chitinase YM-1 (a macrophage surface marker of alternative activation [45–47]), was suppressed in its expression by ITB, but this response was abrogated by 1400 W. This bias towards alternate

activation is continued at 15 days post ITB in NOS2^{-/-}, where Fizz1, the Retnla1 product, and Gal3 are still elevated (Fig. 6).

TGF- β signaling is critical in the development of long term inflammation/fibrotic responses. At 8 days post ITB there is an expected increase in TGF- β staining within the lung tissue in response to ITB. However, in contrast to its effects on markers of inflammation and injury 3 days post-ITB, 1400 W treatment does not decrease the production of TGF- β 8 days post injury. This raises the possibility that loss of iNOS function leads to a persistent inflammatory response and potentially aggravated fibrosis. This concept is further supported by a lack of reduction in trichrome staining with 1400 W treatment and exacerbated TGF- β expression (Fig. 3). Inherent within the experimental design of using osmotic pumps, one can only examine iNOS inhibition effects in the early phases of ITB response. Therefore, we examined NOS2^{-/-} mice at later time points and found a significant exacerbation of the cellular consolidation and fibrosis than was observed in C57 BL6/J mice (Fig. 4). Previously, it has been proposed that both TGF- β and NO are required for resolution of the inflammatory response to injury [48]. Therefore, it is reasonable to suppose that the increased TGF- β and lack of NO production may produce an imbalance that leads to a persistent inflammatory activation. In support of this idea of altered inflammatory signaling, we observed that within NOS2^{-/-} mice NF- κ B binding activity was attenuated and both MMP9 and TGF- β levels are increased early in the ITB response (Fig. 5).

These changes indicated that the lack of iNOS function may have produced an altered inflammatory activation profile within the cells of the lung lining. Further confirmation of continued alternate activation was seen when the lung tissue was examined by western blot for Fizz-1 (the product of Retnla, which was recently identified as being critical to the post ITB fibrotic response [49]), Arg1 and Gal3. All three of these proteins were induced at 8 days post ITB within C57 BL6/J mice. However, expression returned to baseline by day 15, however, within NOS2^{-/-} mice this expression profile was either extended in time scale, i.e. there was still significant expression at day 15, as was the case for Gal3 and Fizz1; or intensity at day 8 as for Arg1. The persistent expression of these proteins appears to confirm that loss of iNOS function results in increased alternative inflammatory activation.

Within these studies we have utilized both pharmacologic and genetic reduction in NOS2 function. The use of these two methods does complicate the observations; however, it is necessary as both systems have their limitations. iNOS is only one of three main isoforms of nitric oxide synthase and thus within the knockout experiments one cannot rule out the possibility that other isoforms compensate for the lack of iNOS function. Furthermore, as with any constitutive knockout there is always the possibility that effects seen are not truly as a result of altered ITB response but rather occur due to alterations in lung or immune system development. With the use of an inhibitor, such as 1400 W, there are pharmacokinetic limitations. One can measure that NOS function is attenuated in general but one cannot determine how much NOS function has been lost in a specific cell type. Therefore, that similar results are observed within both models is critical for limiting these individual methodological limitations and can increase one's confidence in the significance of the observations.

In summary, the data presented here demonstrate that iNOS plays a critical role in both the early and late inflammation/fibrosis that occurs consequent to ITB. While limiting iNOS function reduces the acute injury in response to ITB, it exacerbates the later inflammation/fibrosis that occurs 21 days later. In addition, data are presented that indicate that this double role for iNOS may be a result of altered macrophage phenotype, with iNOS being critical to classical M1 activation and in limiting alternative activation. These observations provide a rationale for the difficulty in using iNOS inhibitors within acute lung injury. Further understanding of how iNOS function is critical to these processes, including what molecular targets, such as SP-D, are involved will be critical in identifying novel therapeutic avenues.

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Abbreviations

SP-D	surfactant protein-D
NOS2	inducible nitric oxide synthase
SNO-SP-D	S-nitrosylated surfactant protein-D
IL-1β	Interleukin-1 β
Fizz1	found in the inflammatory zone 1
CCL2	CC chemokine ligand-2
BAL	bronchial alveolar lavage

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Fig. 1.

1400 W inhibits pulmonary inflammation. (A) C57 B16/J mice were treated with 1400 W by Alzet micro-osmotic pumps (model 1002) from 6 days prior to intratracheal instillation of bleomycin, the right lungs were harvested 8 days after instillation and inflation fixed for histology. Treatment groups were saline, bleomycin, 1400 W plus bleomycin or 1400 W plus saline. Representative pictures were taken with Nanozoomer (Olympus) at the magnification of 100 $\times$. (B) The cells recovered from the BAL were counted using a Beckman coulter counter and cell differentiation was assessed following cytopsin and staining of 30,000 cells. (C) Data are presented as the mean $\pm$ SE ($n=5-7$ in each group), *, $p < 0.05$, compared to bleomycin alone. (D) Chemotactic activity of the BAL was assessed for the ability to induce Raw 264.7 macrophage migration using a modified Boyden Chamber. Data are expressed as the number of migrated cells per field. Values are presented as the mean $\pm$ SE ($n=5-9$ in each group). **, $p < 0.01$, compared to saline and 1400 W; #, $p < 0.05$, compared to Bleomycin.

Fig. 2.

1400 W reduces SNO-SP-D formation and multimeric disruption. (A) C57 Bl6/J mice were treated with 1400 W by Alzet micro-osmotic pumps (model 1002) from 6 days prior to intratracheal instillation of bleomycin. Treatment groups were saline, bleomycin, 1400 W plus bleomycin or 1400 W plus saline; BAL was collected at day 8 after instillation. SNO-SP-D content was assessed by the biotin switch method. The total input SP-D was calculated for equal amounts (as determined by reduced SDS PAGE). (B) Equal amounts of total SP-D from the BAL were also analyzed by native PAGE and Western blotting with SP-D antiserum to determine quaternary structure of SP-D. Multimeric SP-D is incapable of entering the gel at the top, while smaller molecular mass forms are seen only in BAL from bleomycin-injured mice. Data are representative of duplicate determinations from two independent experiments.

Fig. 3.

Effect of 1400 W on bleomycin-induced TGF- β 1 expression and late stage inflammation/fibrosis. (A) Lung sections prepared 8 days after bleomycin administration in mice with or without 1400 W treatment were stained with antibodies to TGF- β 1. Binding was visualized using a peroxidase DAB substrate kit. One representative section of the alveolar region is shown (original magnification, $\times 400$). (B) Five-micron-thick sections were stained with Masson Trichrome and examined microscopically. Representative pictures were taken with the magnification of $100\times$. Each successive field was individually assessed for severity of interstitial fibrosis and allotted a score between 0 and 8 using a predetermined scale of severity as described in the MATERIALS AND METHODS. Scores from the fields were averaged to obtain the pathologic score for each animal. Median values and the 25th to 75th quartile ranges are shown in the corner of each image ($n=3-7$ in each group). *, $p < 0.01$, compared to Saline and 1400 W; 1400 W plus bleomycin was not significantly different from bleomycin. (C) Total nucleotides were extracted from Lung tissue prepared 15 days after bleomycin administration in mice with or without 1400 W treatment and subjected to the real-time reverse transcription polymerase chain reaction (RT-qPCR) assay for expression of TGFB1 mRNA. β -actin mRNA was used as internal controls. The data were expressed as mRNA levels for the TGFB1 relative (folds) to the Sham control (which is defined as 1). Values are presented as the mean $\pm$ SE ($n=3-4$ in each group). *, **, $p < 0.01$, compared to saline, **, $p < 0.05$, compared to Bleomycin.

Fig. 4.

Histology of C57 Bl6/J and NOS2^{-/-} mice following ITB. Bleomycin was instilled intratracheally at a dose of 3 U/kg. After 3, 8 and 21 days animals were sacrificed and the left lung was inflation fixed. After fixation, lungs were embedded in paraffin blocks, sectioned and stained with H&E or Mason's Blue trichrome. Images are shown at 200 ×. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article)

Fig. 5.

NOS2 ablation reduces NF- κ B binding activity and increases MMP-9 and TGF- β 1 production. C57 Bl6/J and NOS2^{-/-} mice were intratracheally administrated with bleomycin (3 U/kg) for 3 days. (A) NF- κ B activity was assayed in nuclear extracts from alveolar macrophages by TransAM ELISA. *, $p < 0.05$, compared to C57 Bl6/J Bleomycin ($n=5$ in each group). (B) Cytokine levels in the BAL were determined by Search light multiple-cytokine ELISA. Data are presented as the mean $\pm$ SE ($n=3-5$ in each group). *, $p < 0.05$, compared to C57 Bl6/J Bleomycin. #, $p < 0.05$, compared to C57 Bl6/J Saline.

Fig. 6.

Expression of alternate activation proteins in lung tissues after bleomycin injury. C57 Bl6/J or NOS2^{-/-} mice were administrated with bleomycin for 3, 8, and 15 days. Lung was harvested and lung protein was prepared to determine marker expression by western blot. GAPDH was used as control to ensure the equal loading.

Table 1

Gene expression of pulmonary immune cells in 1400 W inhibition. The pulmonary immune cells were isolated at day 8 post-bleomycin administration and subjected to RNA isolation and reverse transcription and quantified specific gene expression by real-time-qPCR. The changes of genes were calculated by 2^{-Ct} method and normalized to β -actin mRNA as input controls. The fold change was calculated by saline controls. Values are presented as the mean $\pm$ SE ($n=5-7$ in each group), n.d., not detectable;

mRNA induction (fold)	Bleo ($n=6$)	1400 W + Bleo ($n=7$)	1400 W ($n=5$)
IL1B	9.2 ± 0.8	$4.8 \pm 0.4^*$	n.d.
CCL2	7.7 ± 1.15	$3 \pm 0.6^*$	0.8 ± 0.3
Ptgs2	5.4 ± 1.1	$1.4 \pm 0.6^{**}$	3.3 ± 0.9
Arg1	57.6 ± 7.5	44.8 ± 3.5	2.3 ± 0.11
Retnla	18.6 ± 3.3	19.8 ± 1.3	0.7 ± 0.5
Chi3l3	0.25 ± 0.3	0.59 ± 0.5	0.71 ± 0.5

* $p < 0.05$, compared to bleomycin.

** $p < 0.01$, compared to bleomycin.