Free Radical Biology and Medicine 91 (2016) 293–301

Contents lists available at ScienceDirect

Free Radical Biology and Medicine

journal homepage: www.elsevier.com/locate/freeradbiomed

Original Contribution

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

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ARTICLE INFO

Article history:
Received 25 November 2014
Received in revised form
19 October 2015
Accepted 20 October 2015
Available online 23 October 2015

Keywords:
Pulmonary inflammation
NOS2 inhibitor
Bleomycin
Surfactant protein-D
S-nitrosylation

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 broncheoalveolar 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-1β, 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-κB 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.

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-1β and interferon-γ [13–21]. These inflammatory mediators induce NOS2 expression through a series of signal transduction pathways including NF-κB 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 fibrocystic 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-1β 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

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

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http://dx.doi.org/10.1016/j.freeradbiomed.2015.10.417
0891-5849/

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-κB 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- μM filter to ensure the sterility of the infusate [10]. Alzet micro-osmotic pumps (model 1002) were filled under sterile conditions with 100 μl (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 μl 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 μg total protein) in HEN buffer (25 mM Hepes, pH 7.7/0.1 mM EDTA/0.01 mM neocuproine), and 20 μM *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-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). Directed migration (chemotaxis) of cells was performed as previously described [7]. Briefly, 100 μl 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 μl 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-μm 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× magnifications. Data were expressed as cells per oil-immersion field for the three wells used for each solution.