Contribution of Nitric Oxide to the Pathogenesis of Pulmonary Infections (a Review)

Tomas Kačergius,
Arvydas Ambrozaitis

Department of Infectious Diseases and Microbiology, Faculty of Medicine, Vilnius University, LT-2004 Vilnius, Lithuania

Nitric oxide (NO), being a free radical molecule, mediates a variety of physiologic and pathologic effects *in vivo*. An increasing number of investigations indicates that excessive NO generation by inducible nitric oxide synthase (iNOS) in the sites of infection leads to tissue damage. The current review concentrates on the pulmonary inflammation brought about by overproduction of NO within the lungs during viral and bacterial infections. Also, the aspects relevant to the regulation of iNOS gene expression and conditions under which iNOS is activated in mouse and human systems are discussed. Importantly, analysis of data reviewed in this paper provides an absolutely new insight into the pathogenesis of pulmonary infections, suggesting that new therapeutic strategies might be designed to modify inflammatory reaction in the infected tissues.

Key words: nitric oxide, inducible nitric oxide synthase, cytokines, pulmonary infections

INTRODUCTION

The discovery of nitric oxide (NO) by Louis Ignarro in 1987 (1) as a transcellular signaling molecule synthesized by vascular endothelial cells has led to the intensive studies regarding its production and function in various cells over the last 16 years. These studies identified that NO, a gaseous free radical molecule, is produced in mammalian cells from amino acid L-arginine by one of the three isoforms of nitric oxide synthase (NOS): neuronal NO synthase (nNOS), inducible NO synthase (iNOS) and endothelial NO synthase (eNOS) (2). Constitutively expressed nNOS and eNOS are involved in regulation of synaptic transmission and maintaining vascular tone, respectively. Activity of nNOS and eNOS depends on the concentration of intracellular calcium (Ca^{2+}). Both of these enzymes generate NO for the short periods of time in the low-output fashion (at nanomolar levels). Contrarily, iNOS activity is independent of Ca^{2+} concentration variations, and its induction occurs at the transcriptional level by bacterial lipopolysaccharide (LPS) or proinflammatory cytokines in many cell types, including vascular smooth muscle cells, monocytes/macrophages and lung epithelial cells (3). Following stimulation, this enzyme can produce sustained and high levels of NO at micromolar concentrations. The main functions of NO synthesized by iNOS are immunomodulatory and antimicrobial activities during infections with bacteria, viruses, protozoa, fungi. In this respect, iNOS expression is a beneficial for host defense against infectious agents. However, excessive NO production by iNOS can cause tissue damage in the sites of infection. This detrimental effect of NO has been recognized especially well in experimental studies with pneumotropic virus infections (4). Hence, it appears that NO is implicated in a broad spectrum of physiologic and pathophysiologic conditions. The present review is focused on the aspects of iNOS gene expression and NO-induced pulmonary pathology during certain viral and bacterial infections.

REGULATION OF iNOS GENE EXPRESSION

Transcriptional activation of human iNOS gene is complex and tightly depends on the microenvironment surrounding the cell. Reasons for this complexity lie in the length of promoter region of iNOS

Address correspondence to: Tomas Kačergius, Department of Infectious Diseases and Microbiology, Faculty of Medicine, Vilnius University, Birutės 1, LT-2004 Vilnius, Lithuania. Tel.: +370-5 2724657. Fax.: +370-5 2758885. E-mail: infek@auste.elnet.lt

gene. The promoter of human iNOS gene is very large and spans over 16 kilobases (kb), whereas the murine iNOS promoter has a length of approximately 1 kb (3, 5). In addition, human and murine iNOS genes exhibit an 80% homology in nucleotide sequences. The mechanisms of iNOS regulation have been studied well in experimental mouse models, but those are poorly understood in humans. Despite that, similarities exist between murine and human iNOS genes in terms of activating factors and conditions. The viral replication (or viral components), bacterial LPS and cytokines, such as interleukin 1β (IL-1β), tumor necrosis factor α (TNF-α), interferon α/β (IFN-α/β) and IFN-γ, can stimulate expression of iNOS gene in mouse and human cells during infectious processes (3–6). These inducers mediate the activation of cellular transcription factors – nuclear factor κB (NF-κB), signal transducer and activator of transcription (STAT), interferon regulatory factor 1 (IRF-1), which, in turn, bind to their respective binding sites in the promoter region of iNOS gene and initiate transcription of the gene. Noteworthy, the binding sites for transcription factors in murine iNOS promoter have different locations comparing with their counterparts in the promoter of human iNOS gene. Many of the binding elements within the region of murine iNOS promoter are juxtaposed and situated in a close proximity to the transcriptional start site. That is a contrast to the human iNOS promoter where the binding motifs are arranged more distantly from the start point of transcription. Interestingly, it contains multiple NF-κB response elements – the unique feature of human iNOS gene. These underlying discrepancies determine the different characteristics of iNOS gene inducibility between the species. In mouse system, even a single stimulus can readily induce the iNOS gene, whereas two or three activating factors are required for an efficient expression of human iNOS. Plausibly, the latter occurrence indicates a cooperative action of several inducers necessary for the formation of protein–protein–DNA complex between transcription factors (e.g., STAT and NF-κB) and corresponding binding sequences within the human iNOS promoter in order to stimulate the gene effectively (7). For instance, the interacting IL-1β, TNF-α and IFN-γ synergistically induce expression of iNOS and increase production of NO in human alveolar epithelial cells *in vitro* (8). It is important to emphasize that the transforming growth factor β (TGF-β) and glucocorticoids (e.g., dexamethasone) have a capacity to downregulate the expression of iNOS by decreasing its mRNA stability (9, 10).

NITRIC OXIDE-MEDIATED CYTOTOXICITY AND PATHOLOGY IN PULMONARY INFECTIONS

NO is an inert radical because of a relatively stable electronic configuration, and does not exert the cytotoxic effect directly (11). Since NO carries an unpaired electron, thus it can easily combine with oxygen radicals resulting in the formation of highly reactive nitrogen oxides. This radical–radical reaction occurs between NO and superoxide anion (O_2^-), yielding a potent oxidizing and nitrating agent – peroxynitrite anion radical ($OONO^-$) (12). The latter is mostly responsible for the cytotoxic effects attributed to NO. An amount of the formed peroxynitrite strongly depends on the NO and O_2^- production rates by iNOS and NADPH oxidase, respectively. In this regard, one of the major cells containing both of these enzymatic systems are macrophages. They possess the distinctive ability to produce NO and O_2^- abundantly after activation. Moreover, once expressed in macrophages, the iNOS protein can synthesize NO for prolonged periods of time. Peroxynitrite generated under such circumstances readily permeates the membranes of adjacent cells and reacts with the target molecules. Thereby, peroxynitrite can nitrate tyrosine residues of proteins decreasing their activity, inhibit mitochondrial electron transport complexes I and II, oxidize membrane-lipids and cause DNA strand breaks (12). All these alterations irreversibly damage cellular functions leading to cell death. In a broader view, excessive formation of peroxynitrite contributes to tissue injury in the site of infection and, if sustained for a longer time, can result in the organ disorder.

Convincing evidences for pulmonary pathology brought about NO-induced oxidative stress were given by experiments with mice upon pneumotropic virus infections, in particular, influenza virus infection. In 1996, Akaike et al. (13) demonstrated that overproduction of NO in mouse lungs during influenza virus infection leads to the development of pneumonia. Following challenge of mice with the virus, they found a considerable increase of NOS activity in the bronchoalveolar lavage (BAL) fluid, enhanced levels of iNOS mRNA in the lungs and intense infiltration of the pulmonary tissues by macrophages and neutrophils. iNOS expression occurred due to the augmented IFN-γ production, which peaked on day 6 after infection. The excessive generation of NO in the lungs was confirmed using a quantitative and precise method – electron spin resonance (ESR) spectroscopy. Nitrotyrosine formation (a consequence of peroxynitrite caused protein nitration) served as a marker to evaluate the extent of NO-mediated tissue damage. Indeed, the immu-

nohistochemical staining of tissues applying specific anti-nitrotyrosine antibodies showed extensive accumulation of nitrotyrosine in the influenza virus-infected lungs, predominantly in the areas infiltrated by macrophages and neutrophils. Adler et al. (14) revealed the similar pathological findings within the lungs of mice exposed to herpes simplex virus type 1. The latter infection also caused pneumonia via the mechanisms related to increased iNOS expression and nitrotyrosine formation. Most importantly, in both cases, treatment of the virus-infected mice with N^{ω}-monomethyl-L-arginine and N^{G}-monomethyl-1-arginine, the non-selective inhibitors of NOS, significantly improved the survival rate and reduced the lung consolidation score comparing with placebo, despite the presence of high pulmonary viral titers.

The link between elevated NO production and the development of viral pneumonia was investigated in further experiments using genetically deficient $iNOS^{-/-}$ mice (15). The results unraveled that influenza virus infection of the wild-type ($iNOS^{+/+}$) mice rose NO levels in the BAL fluid and led to high mortality because of consolidating pneumonitis characterized by massive inflammatory foci and edema within the lungs. In contrast, the mice lacking iNOS gene did not exhibit any significant signs of pulmonary inflammation or morbidity. Recently, Akaike et al. (16) have identified another footprint of the nitrative stress associated with tissue injury. After exposure of mice to influenza virus or murine paramyxovirus (Sendai virus), they found a considerable formation of 8-nitroguanosine in the lungs of wild-type mice. Since the latter occurrence is the outcome of nucleotide base guanosine nitration, therefore, *in vivo*, it can reflect a degree of nucleic acids' (DNA and RNA) damage caused by reactive nitrogen oxides. In fact, the most intense immunostaining for 8-nitroguanosine was detected in bronchial and bronchiolar epithelial cells at 6–8 days post-infection. The staining was lighter in alveolar macrophages. Other findings included a marked increase of NO synthesis and an extensive 3-nitrotyrosine generation in the virus-infected lungs, which correlated with great mortality rates. Histological analysis showed strong infiltration of lung tissues by inflammatory cells, alveolar exudates and destruction of pulmonary architecture. As distinct from the wild-type mice, infection of iNOS-deficient mice with influenza virus did not induce a detectable formation of 8-nitroguanosine within the lungs. Also, the histopathological changes (typical for pneumonia) were absent or moderate, thereby accounting for a higher survivability of iNOS-deficient mice. Taken together, data of the experiments evidently indicate that in a murine system pneumotropic viruses can damage lung tissues through the excessive iNOS expression, and IFN-γ is a key cytokine mediating this process.

However, it has been difficult to demonstrate such deleterious effects of NO in humans having pulmonary infections. The fact is that investigation of the consequences of iNOS activity directly *in vivo* requires sophisticated methods (e.g., ESR spectroscopy), which have a very limited application for humans. Another difficulty consists in the incomplete understanding of molecular mechanisms regulating expression of human iNOS gene. For example, it is still undefined how exactly iNOS induction occurs in human macrophages. Nevertheless, the progress in this field has been made. The evidence about iNOS expression and activity during infectious process within human lungs came with a study of Nicholson et al. (17). Patients having active and untreated pulmonary tuberculosis were involved in this study. Examination of alveolar macrophages obtained from BAL fluids of tuberculosis patients showed positive immunostaining for iNOS protein in the average of 65%. Importantly, the iNOS protein had enzymatic activity as it was tested by diaphorase cytochemistry. The latter can be a good indication of high-output NO production in human macrophages. Furthermore, expression of iNOS mRNA was also determined in tuberculosis patients' BAL cells by the reverse transcriptase-polymerase chain reaction. Several investigations pointed out iNOS implication in the pathogenesis of acute respiratory distress syndrome (ARDS) (18, 19). This syndrome is a disease process characterized by diffuse inflammation in the lung parenchyma. Kobayashi et al. (18) studied patients with ARDS developed under bacterial sepsis and bacterial pneumonia. In BAL fluids of the patients, they found significantly elevated nitrite and nitrate (stable metabolites of NO) concentrations as well as increased levels of IL-6 and IL-8. Strong immunostaining for iNOS protein, IL-6, IL-8 and weaker for IL-1β was observed within the cytoplasm of alveolar macrophages. A further study performed by Sittipunt et al. (19) revealed nitrotyrosine formation (along with iNOS) in the BAL cells, including macrophages, derived from the lungs of patients at risk for ARDS and with established ARDS. Noteworthy, nitrite and nitrate accumulated in the lungs during the course of ARDS and their levels were greater in the BAL fluid of patients who afterwards died, specifically in the cases with sepsis.

As regards human pneumotropic viruses, known is the capacity of respiratory syncytial virus (RSV) to up regulate the expression of iNOS gene in lung alveolar epithelial cells (20). Relevant to that, research also demonstrated a cooperative action between RSV and cytokines (IL-1β, TNF-α, IFN-γ) in

the stimulation of iNOS gene. Such interaction significantly enhanced NO production in alveolar epithelial cells as compared to the cells exposed to RSV alone. The fact that RSV infection can upregulate the expression of iNOS gene was confirmed *in vivo* conditions by the same study. In this respect, the elevated iNOS mRNA levels were detected in nasopharyngeal exudate cells obtained from infants during the acute phase of RSV bronchiolitis. The mentioned clinical studies provide indirect evidences that the infectious process triggers a high generation of reactive nitrogen oxides within human lungs, consequently leading to a severe pulmonary inflammation. In addition, the formation of NO metabolites and nitrotyrosine was identified in the lungs of patients having such inflammatory disorders as asthma, idiopathic pulmonary fibrosis and cystic fibrosis (21).

Sharara et al. (22) investigated IFN-α ability to induce iNOS in human monocytes/macrophages *in vitro* and *in vivo*. Exposure of purified blood mononuclear cells derived from healthy donors to IFN-α2b or IFN-α2a *in vitro* stimulated the expression of iNOS gene, augmented iNOS enzyme activity and NO synthesis in the dose-dependent manner. Moreover, the blood mononuclear cells of IFN-α2b-treated patients (having hepatitis C infection) exhibited a significantly higher iNOS activity and iNOS mRNA levels in comparison with the cells of untreated patients. These results signify that possibly IFN-α plays the pivotal role in mediating iNOS expression within human monocytes/macrophages.

Infectious process initiates a cascade of host immune responses, which involves production of proinflammatory cytokines and recruitment of macrophages to the infected tissues. Hence, during pulmonary infection, the microenvironment within lungs where macrophages are present contains the elevated levels of cytokines. For instance, influenza virus in human respiratory tract activates production of various cytokines, and predominantly of IL-6, TNF-α, IFN-α, IFN-γ and IL-10 (23). Such conditions inevitably lead to the induction of iNOS in macrophages and high-output synthesis of NO, which, in turn, can cause pulmonary inflammation and tissue injury. A particular importance in this context has influenza virus infection because of the frequent complications (primary viral pneumonia and secondary bacterial pneumonia). On the basis of data from mouse model experiments, it is most likely that influenza virus in humans evokes pneumonia through the same mechanism as described above. Interestingly, macrophages derived from senescent animals are capable to generate higher levels of NO in a long-lasting fashion *versus* the macrophages obtained from young animals (24). The latter finding could provide a possible explanation why during influenza virus infection older people exhibit a higher morbidity and mortality than their younger counterparts. Thus, it is necessary to design alternative treatment approaches (acting via inhibition of iNOS expression) in order to reduce lung damage brought about pulmonary infections.

CONCLUSIONS

Several concluding remarks could be made in accordance with results of the discussed studies. The first is that an infectious agent triggers production of proinflammatory cytokines whose cooperative interaction can efficiently (or at the maximum) activate the iNOS gene. Secondly, macrophages play a key role in the exacerbation of inflammation by expressing iNOS abundantly. However, more investigations need to be performed to attain a complete understanding what conditions and stimuli lead to iNOS induction in human macrophages. Finally, it appears that overproduction of NO in the lungs as a nonspecific immune response to infectious agent is one of the most detrimental factors contributing to the pulmonary injury.

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T. Kačergius, A. Ambrozaitis

AZOTO MONOKSIDO VAIDMUO PLAUČIŲ INFEKCIJŲ PATOGENEZĖJE

S a n t r a u k a

Būdamas laisvuoju radikalu, azoto monoksidas (NO) yra daugelio fiziologinių ir patologinių efektų mediatorius *in vivo*. Didėjantis tyrimų skaičius rodo, kad indukuojamos azoto monoksido sintetazės (iNOS) pagaminti pernelyg dideli NO kiekiai infekcijos vietose pažeidžia audinį. Šios literatūros apžvalgos objektas yra intensyvaus NO gaminimosi sukeltas plaučių uždegimas, esant virusinėms ir bakterinėms plaučių infekcijoms. Be to, aptariami tam tikri aspektai, susiję su iNOS geno reguliacija, ir aplinkybės, kurios aktyvuoja iNOS ekspresiją pelės ir žmogaus organizmuose. Svarbiausia yra tai, kad esamų studijų duomenų analizė pateikia visiškai kitokį supratimą apie plaučių infekcijų patogenezę bei nurodo naujų gydymo strategijų, mažinančių uždegiminę reakciją infekuotose audiniuose, sukūrimo galimybę.

Raktažodžiai: azoto monoksidas, indukuojama azoto monoksido sintetazė, citokinai, plaučių infekcijos