Nitric oxide and virus infection

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SUMMARY
Nitric oxide (NO) has complex and diverse functions in physiological and pathophysiological phenomena. The mechanisms of many events induced by NO are now well defined, so that a fundamental understanding of NO biology is almost established. Accumulated evidence suggests that NO and oxygen radicals such as superoxide are key molecules in the pathogenesis of various infectious diseases. NO biosynthesis, particularly through expression of an inducible NO synthase (iNOS), occurs in a variety of microbial infections. Although antimicrobial activity of NO is appreciated for bacteria and protozoa, NO has opposing effects in virus infections such as influenza virus pneumonia and certain other neurotropic virus infections. iNOS produces an excessive amount of NO for long periods, which allows generation of a highly reactive nitrogen oxide species, peroxynitrite, via a radical coupling reaction of NO with superoxide. Thus, peroxynitrite causes oxidative tissue injury through potent oxidation and nitration reactions of various biomolecules. NO also appears to affect a host’s immune response, with immunopathological consequences. For example, overproduction of NO in virus infections in mice is reported to suppress type 1 helper T-cell-dependent immune responses, leading to type 2 helper T-cell-biased immunological host responses. Thus, NO may be a host response modulator rather than a simple antiviral agent. The unique biological properties of NO are further illustrated by our recent data suggesting that viral mutation and evolution may be accelerated by NO-induced oxidative stress. Here, we discuss these multiple roles of NO in pathogenesis of virus infections as related to both non-specific inflammatory responses and immunological host reactions modulated by NO during infections in vivo.

INTRODUCTION
Free radical species with oxygen- or nitrogen-based unpaired electrons are now considered to play diverse roles in many aspects of physiological and pathological events. In the past decade, particular attention has been paid to the unique biological functions of nitric oxide (NO), a gaseous nitrogen-centred inorganic radical that is produced endogenously in a number of cells and tissues. NO is critically involved in non-specific (innate) and immunological host defense. It has antimicrobial actions against various pathogens via its cytotoxic or cytostatic effects.1–5 Potent host defence against intruding microbes is also mediated by oxygen radicals and active oxygen species, including superoxide anion radical (O2–), hydrogen peroxide (H2O2), and hypochlorite anion (OCl–), produced from phagocytic cells such as neutrophils and activated macrophages.6 It is now well accepted that the chemical and biological reactivities of NO produced in environments such as inflamed tissues are greatly affected by concomitantly formed oxygen radicals, particularly O2–, through formation of reactive nitrogen and oxygen intermediates.7–12 Although the importance of these reactive nitrogen and oxygen intermediates has been documented for host defence reactions against bacteria and fungi,1–5 their role in the pathogenesis of virus infections is only partly understood.

Because pathological consequences of microbial infections are determined by the interaction of the host and the pathogen, a central theme in modern microbiology is overall understanding of the mechanism of host–pathogen interaction rather than gaining insight about a particular microbe. It is thus critical to evaluate the pathogenesis of virus infection as related to the emerging concept of free radicals that are generated as...
host-derived factors during interactions between viruses and hosts. In this review, the biological relevance of NO production is discussed in view of oxidative stress and immunomodulation of the host's responses caused by NO during virus infections.

INDUCTION OF NO BIOSYNTHESIS AND OXYGEN RADICALS IN VIRUS INFECTION

Overproduction of NO, mainly caused by inducible NO synthase (iNOS), which is usually expressed by inflammatory phagocytic cells and other types of cells (e.g. epithelial and neuronal cells), has a defence function against bacteria, fungi, and parasites. iNOS produces a much larger amount of NO for a longer time (i.e. 10–100 times more) than do the other two constitutive enzymes, neuronal NOS and endothelial NOS. Although NO seems to have a limited bactericidal effect, suppression or lack of NO production results in impaired clearance of some types of bacteria by the host.

NOS is induced in a variety of experimental virus infections in rats and mice, including those with neuroviruses, such as Borna disease virus, herpes simplex virus type 1 (HSV-1), rabies virus, and pneumotropic and cardiotropic viruses, such as influenza virus, Sendai virus, and coxsackievirus. iNOS expression is also observed in human diseases caused by human immunodeficiency virus-1 (HIV-1) and hepatitis B virus (HBV). It seems therefore that iNOS is ubiquitously expressed during host responses to viral replication in vivo.

For example, iNOS is expressed by exudate macrophages and bronchial epithelial cells in lung tissues infected with influenza virus in mice; the high output of NO has been clearly identified and quantified by electron spin resonance (ESR) spin trapping with the use of a dithiocarbamate-iron complex. iNOS induction in virus infection is mediated by pro-inflammatory cytokines such as interferon-γ (IFN-γ) (Fig. 1a).

![Image](https://via.placeholder.com/150)

**Figure 1.** (a) Mechanisms of iNOS induction in viral diseases. In many virus infections, iNOS expression appears to be regulated indirectly via interferon-γ (IFN-γ) induction. Direct iNOS induction may occur in some cases, such as with respiratory syncytial virus and HIV-1 (gp41). (b) NO generation detected by ESR spectroscopy with N-dithiocarboxy(sarcosine) (DTCS)-iron complexes in influenza virus-infected lung (7 days after virus infection). Wild-type mice (C57BL/6, B6), iNOS heterozygotes (iNOS+/−), and mice deficient in iNOS (iNOS−/−) were inoculated with 2 × LD50 of influenza virus, and ESR was performed as described previously. 16

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In pneumonia induced by influenza virus or Sendai virus, NO production is greatly attenuated in IFN-γ-deficient mice (Akaike et al., unpublished observation). Furthermore, the iNOS-inducing potential in bronchoalveolar lavage fluid in influenza virus pneumonia is attributable solely to IFN-γ, as revealed by an immunoadsorption study using a specific anti-IFN-γ antibody. These results strongly support the suggestion that IFN-γ is a major cytokine inducing iNOS and NO overproduction in pathogenesis of virus infection.15,16,31

Many previous reports indicate that type 1 helper T cell (Th1) responses are important for viral clearance. However, IFN-γ, a Th1-dependent cytokine, seems to be inefficient in host defence against various viral pathogens including influenza virus, Sendai virus, and vaccinia virus.42–44 In addition, lack of an iNOS-dependent antiviral effect is also noted for the same virus infections, which was recently confirmed by a number of studies using iNOS-deficient (iNOS−/−) mice.44–48

Downregulation of iNOS expression is also reported for some cytokines, e.g. interleukin (IL)-4, IL-10, and transforming growth factor-β. In addition, these suppressor cytokines may reduce NO production indirectly via induction of arginase, which diminishes the supply of the substrate (L-arginine) for iNOS. Because IL-4 and IL-10 are induced by regulatory T cells and both are antiviral cytokines, expression of these cytokines may reduce NO production indirectly via induction of arginase.52–54 Thus, NO produced in response to the intruding virus. In fact, in our influenza model, induction of IL-4 seems to be inversely related to IFN-γ and iNOS induction in virus-infected lungs, suggesting downregulation of IFN-γ and NO production in pathogenesis of various proteins and sulfhydryl targets of pathogens is of great interest in view of the diverse functions of NO.54–69 However, it remains ambiguous whether selective toxicity of NO for virus and virus-infected cells is brought about by NO-dependent S-nitrosylation. In fact, considerable evidence shows redox regulation by S-nitrosylation of sulfhydryl-containing proteins involved in intracellular signaling pathways, including neurotransmission, transcription, and apoptosis, containing proteins involved in inter- and intracellular signal pathways, including neurotransmission, transcription, and apoptosis, containing proteins involved in inter- and intracellular signal pathways, including neurotransmission, transcription, and apoptosis.

Activity of NO against other viruses remains unclear, however. Recent reports suggest that NO has no appreciable antiviral effect on several types of viruses such as ortho- and paramyxoviruses, murine vaccinia virus, coronavirus (mouse hepatitis virus, MHV), lymphocytic choriomeningitis virus (LCMV), murine encephalomyocarditis virus (EMCV), tick-borne encephalitis virus (TBE-V), and others. This lack of antiviral activity of NO has been proven in murine pneumotropic virus infections caused by influenza and Sendai viruses in a series of cell, mouse, and in vivo studies (Akaike et al., unpublished observation). Exposure of these viruses to biologically relevant concentrations of NO produces no appreciable reduction of viral growth in cultured cells in vitro. More important, antiviral host defence is not impaired by pharmacological interventions producing NO inhibition or by genetic iNOS deficiency of in mice infected with either influenza virus or Sendai virus. Such NO inhibition and lack of NO biosynthesis, however, significantly reduce the pathological consequences of various virus infections, including viral pneumonia in mice caused by influenza virus, Sendai virus, and HSV-1; HSV-1-induced encephalitis in rats; EMCV-induced carditis and diabetes; and murine encephalitis induced by flavivirus (Murray Valley encephalitis virus; TBE-V). It is thus conceivable that NO is not entirely an antiviral molecule in various, if not all, virus infections.

**EFFECTS OF NO ON IMMUNOLOGICAL RESPONSES DURING VIRUS INFECTION AND THE PATHOLOGICAL CONSEQUENCES**

It has been suggested that NO affects the polarized Th1–Th2 response, causing a Th2-biased immunoregulatory balance, via a relatively specific suppressive effect on Th1 subpopulations. Such NO-induced immunomodulation occurs during virus infection in mice, as revealed by recent studies of HSV-1 and influenza virus infections. These biased Th2 responses are most clearly demonstrated by using iNOS−/− mice, which show enhanced Th1 immune responses after these infections. It is believed that Th1 cells produce IL-2 and IFN-γ, whereas production of IL-4 and IL-10 depends on Th2 cells. NO thus seems to downregulate the Th1-associated cytokine IFN-γ, which is a major iNOS-inducing cytokine in virus infections as described above, and increases the Th2-associated IL-4 and IL-10 during virus infections in mice.

However, the immunoregulatory effects of NO on Th1–Th2 balance are not commonly observed among the different types of...
Defence is eliminated in coxsackievirus-infected iNOS–/– mice, Similarly, Lowenstein's group reported that the antiviral host defence in iNOS–/– mice is significantly exacerbated, even though the Th1-dependent IFN-γ response is critical for viral clearance, NO may impair antiviral responses by suppressing Th1-dependent IFN-γ induction and tipping the Th1–Th2 balance toward Th2 domination. However, it has now been demonstrated that IFN-γ, a Th1-dependent cytokine, is eventually inefficient in clearance of influenza virus from infectious foci, and even IL-4, induced by Th2 responses, possesses antiviral activity against murine paramyxovirus (Sendai virus). Our recent experiments using iNOS−/− mice indicate that clearance of virus from lungs infected with either influenza virus or Sendai virus is not affected by a lack of iNOS expression (Akaie et al., unpublished observation). In fact, iNOS−/− mice recover from viral pneumonia much better than do wild-type animals, because of reduced levels of oxidative stress in virus-infected tissues. Therefore, not only NO-induced Th1 suppression, but also NO-induced oxidative injury may be attributable to pathogenesis of infection with certain viruses that are resistant to the direct antiviral actions of NO.

In addition, NO seems to have profound immunosuppressive and immunopathological effects, most typically in Mycobacterium avium and Salmonella typhimurium infections, which may be due to NO-induced cytotoxic effects on immune effector cells such as macrophages. Similar immunosuppression by NO is clearly demonstrated with vaccinia virus-infected murine macrophages, which show a loss of antiviral activity because of inhibition of IFN-α/β production by NO.

Thus, NO has complex roles in immunological host responses against viruses. The mechanism of pathogenesis of virus infection is mediated by the following three classes of biological events affected by NO:

1. Direct antiviral effects of NO that may contribute to innate resistance of hosts to viruses: e.g. coxsackievirus and EV appear to be potentially susceptible to NO. Therefore, virus infection in iNOS−/− mice is significantly exacerbated, even though the Th1-dependent IFN-γ response is enhanced by the disrupted iNOS gene. Similarity, Lowenstein's group reported that the antiviral defence is eliminated in coxsackievirus-infected iNOS−/− mice. However, in infections with vaccinia virus and corona virus (MHV), iNOS deficiency affects neither antiviral host defence nor pathology of viral diseases. Also, the antiviral immunological response against LCMV is unimpaired in iNOS−/− mice lacking iNOS, although T-cell-mediated inflammation induced by LCMV is reduced.

2. Effects of NO on antiviral defence mediated by polarized Th1–Th2 immunological reactions of hosts: if the Th1 response is critical for viral clearance, NO may impair antiviral responses by suppressing Th1 functions.

3. Contribution of NO-induced oxidative stress: NO-induced cytotoxicity via oxidative injury may cause not only immunosuppression and immunopathology, but also cellular and organ dysfunctions (detailed molecular mechanisms are described in the following section).

**NO-INDUCED OXIDATIVE STRESS IN PATHOGENESIS OF VIRUS INFECTION**

No itself is an inert radical and much less reactive compared with other naturally occurring oxygen and alkyl radicals. Of the complex chemistry of NO, the most important and biologically relevant reaction is formation of...
ONO\textsuperscript{O} via a very rapid radical coupling with \(O_2\) (NO + \(O_2\) \(\rightarrow\) ONOO\textsuperscript{−} \(k = 6.7 \times 10^7 \ \text{M}^{-1} \ \text{s}^{-1}\)).\textsuperscript{7,9,11,12} Although NO can function as an antioxidant, particularly in lipid peroxidation,\textsuperscript{9} it also has indirect pro-oxidant activity after conversion to a strong oxidant and a potent nitrating agent (ONO\textsuperscript{O}) causing oxidative stress.\textsuperscript{9} In addition, although NO and nitrosothiols show strong anti-apoptotic effects as described above,\textsuperscript{67–71} ONOO\textsuperscript{−} induces apoptosis, possibly via mitochondrial damage leading to cytochrome \(c\) release.\textsuperscript{10,92} NO chemistry in biological systems is shown schematically in Fig. 2. As mentioned above, the reaction between NO and \(O_2\) takes place in virus-infected inflammatory tissues, leading to formation of ONOO\textsuperscript{−}. Immunohistochemical analysis with anti-nitrotyrosine antibody shows positive staining in macrophages and neutrophils infiltrating the alveoli and interstitial tissues, as well as in inflammatory intra-alveolar exudate in virus-infected lung,\textsuperscript{34} which provides indirect indication of ONOO\textsuperscript{−} generation during virus infection.

ONOO\textsuperscript{−} may cause various pathological events in virus infections, such as host cell apoptosis and necrosis. It may be also involved in NO-induced suppressive effects on macrophages, as described in earlier sections. In addition, we recently found that ONOO\textsuperscript{−} activates matrix metalloproteinases (MMPs), which are involved in extracellular tissue damage and remodelling.\textsuperscript{97} Accordingly, oxidative tissue injury in virus-infected lung may be mediated by ONOO\textsuperscript{−}-induced MMP activation. In fact, remarkable improvements in pathological condition in the lung and in survival rate of virus-infected mice were observed with \(l\)-NMMA, and with the \(O_2\) scavenger SOD and the NO inhibitor allopurinol as well.\textsuperscript{34,38} Furthermore, a therapeutic effect on influenza virus-induced pneumonia in mice (Sendai) containing a marker gene (green fluorescent protein, GFP) for genetic mutation and iNOS knockout mice, we clearly showed that oxidative stress induced by NO in wild-type mice \textit{in vivo} remarkably increases and accelerates viral mutation rates compared with the situation in iNOS-deficient mice (Fig. 3). This process of accelerated mutation may occur in other virus infections \textit{in vivo}. For example, NO-induced oxidative stress in virus infection may cause increased survival of heterogeneous mutants, resulting in selection of highly pathogenic variants of coxsackievirus.

In addition, our recent study verifies for the first time that oxidative stress induced by a high output of NO accelerates mutation of RNA virus.\textsuperscript{48} By using a recombinant RNA virus (Sendai) containing a marker gene (green fluorescent protein, GFP) for genetic mutation and iNOS knockout mice, we clearly showed that oxidative stress induced by NO in wild-type mice \textit{in vivo} remarkably increases and accelerates viral mutation rates compared with the situation in iNOS-deficient mice (Fig. 3). This process of accelerated mutation may occur in other virus infections \textit{in vivo}. For example, NO-induced oxidative stress may cause greater heterogeneity of variants of RNA viruses including HIV and influenza virus, leading to rapid viral evolution under selective pressure and to production of drug-resistant and immunologically tolerant and cell tropism-altered mutants (Fig. 4). We now know that NO and \(O_2\), and hence ONOO\textsuperscript{−} and other reactive molecular species in the pathogenesis of influenza virus-induced pneumonia in mice.
species such as NO₂, ClO⁻, and H₂O₂, are generated universally as a result of host responses during infections. Therefore, we may expect such chemical mutagenesis in other DNA viruses, bacteria, and even host cells, although it may not be as effective as that in single-strand RNA viruses.

CONCLUSION

Biological consequences of NO generation and implications for pathogenesis of virus infections are discussed by illustrating NO-modulated non-specific and virus-specific immune responses of the hosts. Free radicals are produced primarily as effector molecules of the host defence response. Their biological effects, however, are not necessarily beneficial to the infected host. Understanding of the pathophysiological functions of NO and oxygen radicals will provide profound insights into many aspects of infectious diseases.

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Figure 3. NO-dependent Sendai virus mutation as revealed by genetic mutation of GFP in Sendai virus during Sendai virus-induced pneumonia in mice. (a) The mutation frequency of the virus isolated from the lung of wild-type B6 and iNOS⁻/⁻ mice was quantified by use of the GFP-based mutation assay. (b) Virus yield in the lung of wild-type B6 and iNOS⁻/⁻ mice. Data are the mean ± SEM (n = 4). *P < 0.05, †P < 0.01, between wild-type B6 and iNOS⁻/⁻ mice (t-test). Adapted from Akaike et al. (FASEB J 2000; 14:1447).

Figure 4. Schematic drawing of the possible involvement of NO-induced oxidative stress and mutagenesis in viral mutation and evolution. NO-derived reactive nitrogen intermediates, via their potent mutagenic activities, may contribute to the molecular evolution of viruses. Alternatively, NO may affect viral evolution by inhibiting a host’s antiviral immune responses, which may impair clearance of viral mutants.
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