Antiviral Activity of Nitric Oxide

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Nitric oxide (NO) is a gaseous free radical that is largely produced by the enzyme NO synthase (NOS) in cells. NO produced by upper epidermal cells contributes to the inactivation of viruses and bacteria contained in air or aerosols. In addition to enzymatic production, NO can be generated by the chemical reduction of inorganic nitrite (NO_2^-), an alternative mechanism for NO production in living organisms. Dietary vitamin C, largely contained in fruits and vegetables, can reduce the nitrite in saliva to produce NO in the oral cavity when chewing foods. In the stomach, salivary nitrite can also be reduced to NO by vitamin C secreted from the epidermal cells of the stomach. The strong acidic pH of gastric juice facilitates the chemical reduction of salivary nitrite to produce NO. It is evident that NO exhibits substantial antiviral activity against many types of viruses, including SARS-CoV-2.

Keywords: antiviral activity ; nitric oxide ; nitrite ; SARS-CoV-2

1. Smokers' Paradox

Asthma and cigarette smoking, as well as obesity, diabetes, and chronic heart disease, are considered high-risk factors for acquiring COVID-19 or poorer outcomes. Early in the pandemic, however, there were few asthma patients with severe cases of COVID-19 [1][2]. A recent meta-analysis study also supported that people with asthma have a lower risk of SARS-CoV-2 infection than those without asthma [3]. The use of inhaled corticosteroids might partly account for a protective effect against SARS-CoV-2 infection, due to decreased ACE2 in asthma patients [4]. It is important to note that NO emission is generally high due to eosinophilic airway inflammation in asthmatic patients. In fact, the fraction exhaled NO (FE_{NO}) has been adopted as a non-invasive indicator of the type 2 airway inflammation of asthma [5]. Although non-allergic asthma (non-type 2) seems to have a greater risk, there were fewer asthma patients with COVID-19 in many countries [2], implying that high NO emission from the airway may protect against SARS-CoV-2 infection.

Cigarette smoking has also been listed as a risk factor for contracting COVID-19. In general, cigarette smoking is associated with relatively poor outcomes in respiratory infectious diseases [6]. In 2020, the WHO and the FDA released statements warning that smoking may increase the risk and severity of COVID-19. However, only a low proportion of smokers suffered from SARS-CoV-2 infection [7]. This is referred to as the "smokers' paradox" [8]. Although smoking cannot be recommended as a protective measure for COVID-19, the underlying mechanism for the smokers' paradox may give a clue for the consideration of preventing SARS-CoV-2 infection. Farsalinos et al. proposed that nicotine intake could be the reason for the low prevalence of smoking among hospitalized patients [6], whereas Hedenstierna et al. hypothesized that the short burst of concentrated NO (approximately 250 to 1350 ppm per puff) contained in cigarette smoke may prevent SARS-CoV-2 infection [9], an explanation similar to that given for why asthmatic patients are less likely to contract COVID-19.

2. RNS Biochemistry

Apart from its potent actions on cardiovascular systems, NO is involved in innate immunological host defense. The innate immune response is also mediated by reactive oxygen species (ROS), including O_2^-, H_2O_2, and hypochlorite anion (OCl^-), which are produced by phagocytic cells such as neutrophils and activated macrophages [10]. Its action is non-specific, and potentially inactivates a broad range of pathogens, including parasites, fungi, bacteria, and viruses [11]. As ROS is the term for a group of reactive molecules derived from O_2, reactive molecules originating from NO are referred to as "reactive nitrogen species (RNS)" [12]. **Figure 1** illustrates the pathophysiological conditions associated with the major RNS.

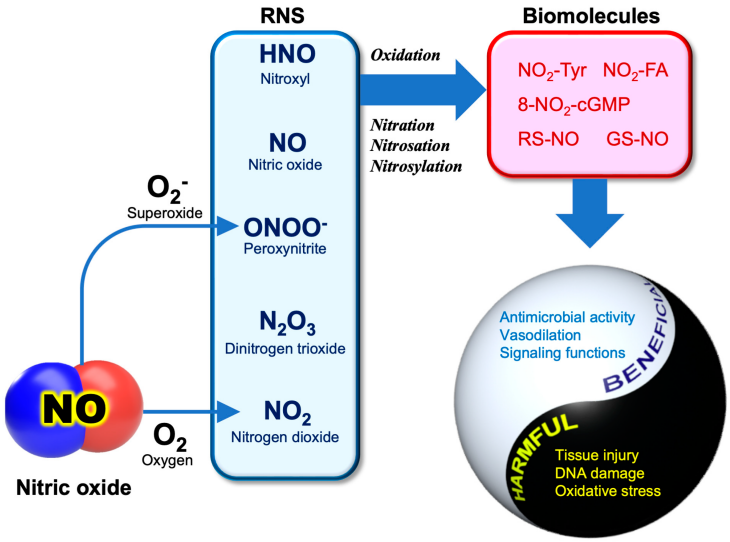

Figure 1. NO and RNS in COVID-19. NO and its derived reactive molecules are frequently referred to as "reactive nitrogen species" (RNS). Peroxynitrite ($ONOO^-$) is a reaction product between NO and superoxide (O_2^-). RNS potentially mediate the oxidation, nitration, nitrosation and nitrosylation of biomolecules. Those reactions exhibit both beneficial and harmful effects. NO_2-Tyr, nitro-tyrosine; 8-NO_2-cGMP, 8-nitroguanosine 3′,5′-cyclic monophosphate; NO_2-FA, nitro-fatty acids; RS-NO, *S*-nitrosothiol; GS-NO, *S*-nitrosoglutathine.

Like ROS, RNS are highly reactive oxidants that are associated with oxidative stress in living organisms. Compared with ROS, the chemistry of RNS-related reactions is much more complicated, especially under in vivo conditions, and most of them are not fully understood. Basically, the reactions of NO are involved in the oxidation, nitration (the addition of NO_2), nitrosation (the addition of NO^+), and nitrosylation (the addition of NO) of biomolecules [13]. An uncontrolled situation of these reactions may cause "nitrative" or "nitrosative stress", leading to cellular damage or cell death.

Most classes of biomolecules, including proteins, nucleic acids, and lipids, can be nitrated, generating products such as nitro-tyrosine (NO_2-Tyr) [14], 8-nitroguanosine 3′,5′-cyclclic monophosphate (8-nitro-cGMP) [15], nitro-fatty acids (NO_2-FA) [16], and nitro-phenolics [17]. NO^+ could directly react with the thiols (RSH) of cysteine residues or the reduced form of glutathione (GSH) to produce *S*-nitrosothiols (RS-NO, GS-NO). Regarding RS-NO chemical generation in biological systems, several possible mechanisms have been proposed, but currently none of them have reached a consensus [18]. The antipathogenic activity of NO relies on these unique RNS reactions (nitration, nitrosation, and nitrosylation) that are capable of inactivating or killing pathogens through the modification of biomolecules, including enzyme proteins. It is important to remember that these reactions are non-specific, thereby also causing cellular damage to the host cells during inflammation [19]. It appears that the use of RNS as a countermeasure against pathogens is a risky business for hosts.

Under oxidative stress conditions where ROS are overproduced, such as during inflammation, peroxynitrite ($ONOO^-$) can be produced as the reaction product between NO and O_2^-, an important interplay between ROS and RNS [20].

The rate constant for the reaction between NO and O_2^- is near diffusion controlled (Equation (1)), which is faster than the superoxide dismutase (SOD) reaction that removes O_2^-. The product $ONOO^-$ is stable at pH 12 in the absence of target molecules. At physiological pH, $ONOO^-$ is in rapid equilibrium with its conjugated acid peroxynitrous acid (ONOOH, pK_a 6.8) (Equation (2)), which is a short-lived molecule that spontaneously decays to nitrate (Equation (3)) [21]. Due to its high reactivity, $ONOO^-$ is considered the major cytotoxic agent in RNS.

$$NO + O_2^- \rightarrow ONOO^-$$

(1)

$$ONOO^- + H^+ \rightarrow ONOOH$$

(2)

$$ONOOH \rightarrow NO_3^- + H^+$$

(3)

$ONOO^-$ and ONOOH are strong oxidants capable of oxidizing various molecules, such as thiols, sulfides, ascorbate, and phenols [22]. In addition to the oxidation of molecules, $ONOO^-$ can chemically nitrate aromatics, with a reaction being

facilitated in the presence of bicarbonate anion (HCO_3^-) [23]. The dysfunction of proteins or enzymes may occur due to the formation of nitro-tyrosine residues (NO_2-Tyr). $ONOO^-$ is also involved in DNA fragmentation [22] and RNA viral mutation [24] through deamination of the bases.

3. Anti-SARS-CoV-2 Activity of NO

The antiviral activity of NO has been reported for many types of viruses, most typically, DNA viruses such as murine poxvirus, herpesviruses, and some RNA viruses [10]. The direct action of NO as an antiviral agent involves the inhibition of viral replication and viral entry into the host [25][26]. In 1999, Saura et al. demonstrated that the in vitro replication of the RNA virus coxsackievirus is suppressed by NO-dependent *S*-nitrosylation that causes the inactivation of viral cysteine protease, an enzyme necessary for replication [27]. The *S*-nitrosylation of the cysteine-containing enzymes of viruses is thought to be a general mechanism for the antiviral activity of NO [28].

SARS-CoV-2 is a positive-sense RNA virus belonging to the family Coronaviridae, which includes severe acute respiratory syndrome coronavirus (SARS-CoV), the pathogen that caused the SARS outbreak. In 2005, Akerstrom et al. reported that the NO chemical donor SNAP inhibits the in vitro replication cycle and the protein and RNA synthesis of SARS-CoV [29]. This inhibitory effect was not observed with SNP (sodium nitroprusside), another chemical NO donor [30]. Likewise, NO released from SNAP was reported to inhibit the replication of SARS-CoV-2 in Vero E6 cells through the inhibition of the SARS-CoV-2 3CL cysteine protease [31].

Macrophages are multifunctional innate immune cells that play an essential role in the clearance of pathogens and control inflammatory responses. Recent studies have suggested that *S*-palmitoylation is a key reaction for control macrophages in the processes of endocytosis [32]. Interestingly, NO was reported to suppress the palmitoylation of the spike (S) proteins that is needed for their binding to ACE2 [33]. The spike (S) proteins of coronaviruses are receptor-binding proteins that are synthesized in the endoplasmic reticulum (ER), followed by complex post-translational modification in the host Golgi apparatus [34][35][36]. *S*-Palmitoylation is one of the post-translational modifications in the Golgi apparatus where palmitoyl acyltransferase (PAT) adds the saturated fatty acid palmitate (C16:0) to the cysteine thiol group (-SH) of proteins [34][36]. Protein modification causes an increase in the hydrophobicity of the proteins, which is essential for cell-cell fusion activity [35][37]. Endothelial NO synthase (eNOS), an isoform of the host's NO-producing enzyme, can be modified by palmitoylation, and its activity is decreased by the modification [38]. *S*-nitrosylation of the SARS-CoV spike (S) protein with NO may reduce cell-cell fusion activity through decreased amounts of spike (S) protein palmitoylation [33]. It is presumable that the disturbance of the cysteine palmitoylation of the spike (S) proteins is also involved in the mechanism for antiviral activity of NO against the coronavirus [39].

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