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BRIEF REPORT

Nitric oxide inhibits the replication cycle of porcine parvovirus in vitro

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Abstract This study investigated the inhibitory effect and mechanism of nitric oxide (NO) on porcine parvovirus (PPV) replication in PK-15 cells. The results showed that two NO-generating compounds, *S*-nitroso-L-acetylpenicillamine (SNAP) and L-arginine (LA), at a noncytotoxic concentration could reduce PPV replication in a dose-dependent manner and that this anti-PPV effect could be reversed by the NO synthase (NOS) inhibitor *N*-nitro-L-arginine methyl ester (L-NAME). By assaying the steps of the PPV life cycle, we also show that NO inhibits viral DNA and protein synthesis. This experiment provides a frame of reference for the study of the anti-viral mechanism of NO.

Porcine parvovirus (PPV), characterized as a member of the autonomous parvoviruses, is a major cause of reproductive failure in swine, resulting in early embryonic death, fetal death, still births, and delayed return to estrus [3, 19]. Presently, PPV is recognized as one of the most serious viral infections of swine worldwide, causing heavy economic losses to the pig industry [21, 22]. PPV has a single-stranded DNA genome encapsidated by a nonenveloped icosahedral particle of 25 nm in diameter that is composed of three structural proteins: VP1, VP2 and VP3, of which VP2 is the major component [27]. In the past decades, knowledge in molecular biology, antigenicity, and virulence of the virus has rapidly grown, but little is known about the pathogenicity of PPV infection.

Nitric oxide (NO), an important molecule for signaling between cells, is synthesized from L-arginine (LA) by a family of complex enzymes know as NO synthases (NOS), which includes at least three different isoforms: neuronal (nNOS), inducible (iNOS), and endothelial constitutive (eNOS) [16]. It has been shown to have antimicrobial activity for several bacteria and protozoa and for some viruses [1, 7, 15, 17, 23, 25]. It also has been demonstrated that NO hinders the productive infection of several animal virus, including vaccinia virus (VV) [4], influenza virus [29], Japanese encephalitis virus [17, 26, 31], Marek's disease virus [31], and murine Friend leukemia retrovirus [2]. NO exerts its inhibitory effect on viral infection by interfering with specific stages in the cycle of viral replication. For example, NO inhibits viral DNA synthesis, late protein translation, and virion assembly, resulting in lower viral yields and more efficient host clearance of the infection [8, 10, 13, 14, 18]. In this study, the inhibitory effect of NO on PPV replication and the effect of NO on different stages of the PPV replication cycle were investigated in vitro.

The PPV-NJ strain (kindly provided by Professor Fu-yan Chen, Nanjing Agricultural University, China), was isolated from breeding sow tissue and detected by polymerase chain reaction (PCR). The virus was propagated in PK-15 cells (purchased from China Institute of Veterinary Drug Control, China). *S*-nitroso-L-acetylpenicillamine (SNAP) and L-arginine (LA) were used as NO-generating

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compounds, and *N*-nitro-L-arginine methyl ester (L-NAME) was used as an NO synthase (NOS) inhibitor. These reagents were purchased from Sigma Chemical Company.

Virus titer was determined using a plaque assay. The culture supernatants were harvested and diluted serially tenfold using RPMI 1640 medium. For passage in host cells, the cells were inoculated in 24-well plates with 100 μl of diluted virus per well. The plate was then incubated for 24 h at 37°C, the medium was removed, and the cells were washed twice with Hank's medium. One milliliter of 1.0% agar (about 45–50°C) was added to each well. The plaques were counted after a 24-h incubation at 37°C. All experiments were done in triplicate.

The concentration of NO in the culture medium was determined by using an NO reagent kit (Jiangcheng Co., China). In brief, 100-μl samples were used for assaying the stable end product, NO_2^-. All of the media were mixed in 96-well microplates, and the plates were incubated at room temperature for 10 min. The reaction produced a pink color, which was measured at 550 nm using a micro-ELISA reader (model UV-2102, UNICO).

Viral DNA was extracted from the culture supernatant using a DNA Extraction Kit (Takara, China) according to the manufacturer's protocol. The PPV VP2 gene was quantified using a real-time PCR that had already been established in our laboratory [9].

A double-antibody (sandwich) ELISA was used to quantify PPV antigen in the culture supernatants. The ELISA procedure was performed as described by Dong et al. [11]. A sample was considered positive if the OD was more than the mean background +3 standard deviations. Optimal dilutions of affinity-purified rabbit antibodies and mouse antibodies were determined for each batch prepared by box titration.

Results are presented as means ± standard error (SE). The SE was multiplied by an index that was determined by the degree of freedom for 95% confidence. Statistical significance at $P < 0.05$ was determined by either *t* test or rank analysis.

To explore the inhibitory effect of NO donors (SNAP or LA) on PPV replication, different concentrations of SNAP (or LA) were added to the cultures, which were infected with PPV at an MOI of 1. As shown in Fig. 1a, SNAP and LA have the ability to inhibit PPV replication in PK-15 cells, and there is a dose-dependent relationship between the inhibitory effect and the SNAP (or LA) concentration. A slight decrease in virus titer was observed with both 50 μM SNAP and LA ($P > 0.05$), while the number of PFUs in PK-15 cells was clearly reduced after treatment with 100 μM of SNAP or LA ($P < 0.05$). However, in samples with 100 μM of SNAP (or LA) plus 50 μM of L-NAME (a competitive inhibitor of NOS), the number of PFUs was higher than with SNAP (or LA) treatment alone, although the virus titer was still lower than that of the control, indicating that L-NAME partially reversed the inhibitory effect of SNAP (or LA) on PPV replication.

To verify that the antiviral effect was caused by NO released from the SNAP or LA in the cultures, NO

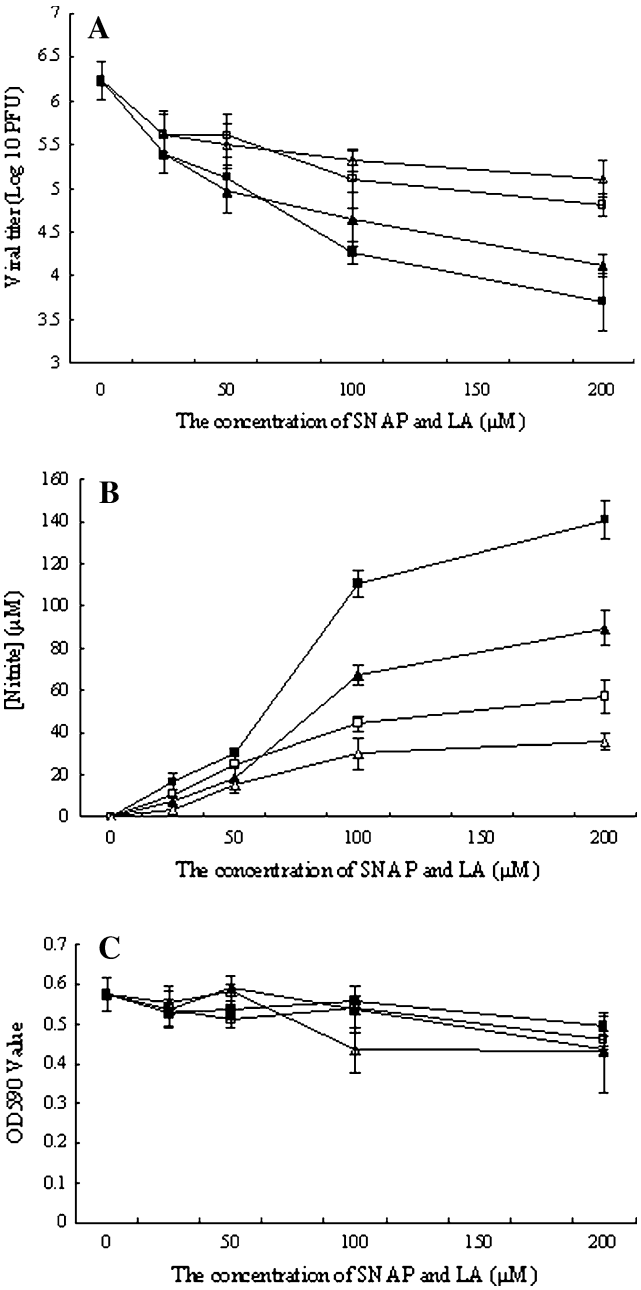

Fig. 1 Antiviral effect of NO on PPV infection. PK-15 cells were infected with PPV at an MOI of 1. At 1 h p.i., the cells were treated with different concentrations of SNAP (*filled square*), SNAP + L-NAME (50 μM, *open square*), LA (*filled triangle*) or LA + L-NAME (50 μM, *open triangle*). **a** Virus was harvested at 24 h p.i., and virus titers were determined. **b** Nitrite concentration produced at 24 h p.i. with addition of different concentrations of SNAP and LA with or without 50 μM of L-NAME. **c** Cell viability was determined using MTT assays. The mean values from three experiments are indicated

concentration in the cultures was next calculated. Figure 1b shows that the antiviral effects of SNAP or LA appears to correlate with the amounts of NO produced in the cultures with increasing SNAP or LA concentration. Treatment with SNAP or LA plus L-NAME resulted in an inhibition of NO production, with a rate of up to 35%.

To exclude the possibility that the detected antiviral effect might have resulted from the toxicity of SNAP or LA for the cells, we performed an MTT cell proliferation test. The results clearly excluded the possibility that the antiviral effect was due to general cytotoxicity of SNAP (or LA) itself (Fig. 1c).

Based on the above results, we selected 100 μM as the optimal SNAP or LA concentration for further experiments. The inhibitory effect of SNAP or LA on PPV infection of PK-15 cells was further investigated by determining the kinetics of PPV reproduction. The infected cells (MOI = 1) were treated with 100 μM SNAP or LA at different time points. As shown in Fig. 2, the PFU value increased with later addition of SNAP (or LA), and adding SNAP (or LA) 6 or 3 h prior to viral infection resulted in the highest level of inhibition.

To investigate whether NO inhibits PPV DNA replication, viral DNA was isolated, and the partial VP2 gene was quantified by real-time PCR. As shown in Fig. 3a, the amounts of viral DNA in SNAP- or LA-treated cells were reduced significantly compared to mock-treated samples ($P < 0.05$). Adding SNAP or LA 6 or 3 h prior to infection resulted in a greater inhibition of the amount of viral DNA produced than adding SNAP or LA at the time of infection. There was less reduction when 50 μM L-NAME was added.

The kinetics of protein synthesis in infected PK-15 cells was analyzed. From the ELISA results, we can see that

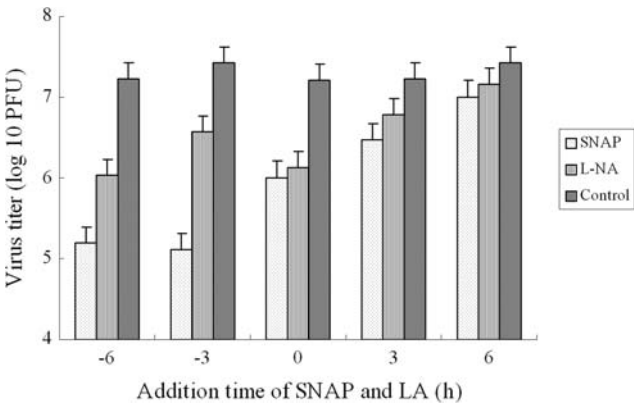

Fig. 2 Inhibition kinetics of virus yields in PK-15 cells treated with NO donors. PK-15 cells were infected with PPV at an MOI of 1. SNAP or LA (100 μM) was added to the cultures 6 and 3 h before (−6, −3), during (0), or 6 and 3 h after (+6, +3) the infection. The virus titer in the culture supernatant was measured by plaque assay 24 h p.i. The virus titer values shown here are the mean and SEM of three independent experiments

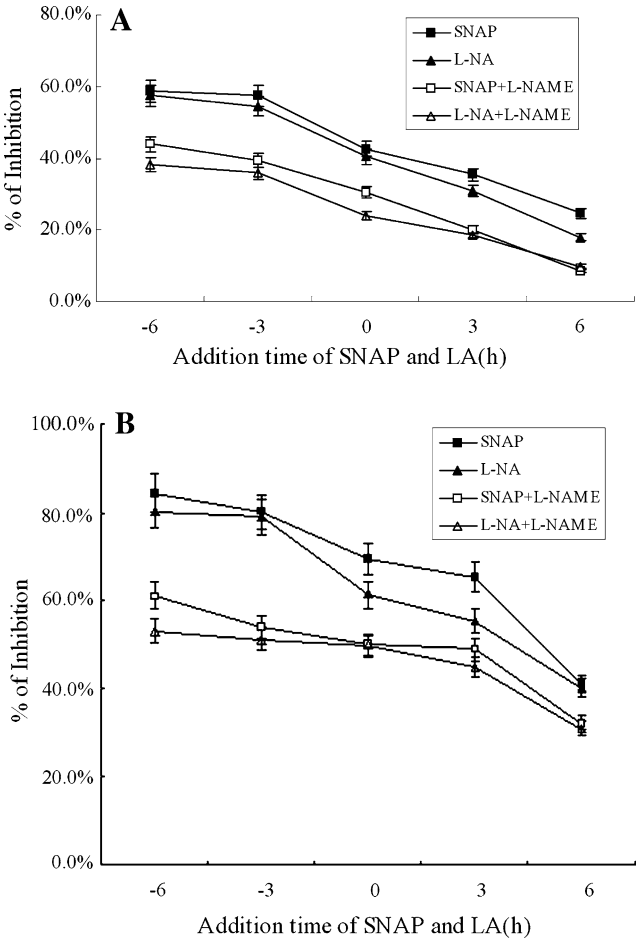

Fig. 3 NO donor blocks viral DNA and protein synthesis of PPV. PK-15 cells were infected with PPV at an MOI of 1 and 100 μM of SNAP (or LA) with or without 50 μM L-NAME was added to the PK-15 cells either before (−6, −3 h), during (0 h) or after (3, 6 h) infection with PPV. After 24 h p.i., culture supernatants were harvested. **a** The PPV VP2 gene was detected at the DNA level using real-time PCR. **b** A double-antibody (sandwich) ELISA was used to quantify PPV antigen in the supernatant

SNAP or LA (100 μM) inhibited viral protein synthesis at each time point. The inhibitory rate was high (up to 80%) when SNAP or LA was added 6 h prior to infection (Fig. 3b), and the inhibitory rate became gradually lower with later addition of the drug. In addition, there was less reduction of protein synthesis when 50 μM L-NAME was added.

This study demonstrates that the antiviral effect of NO was generated by the organic donors SNAP and LA in the PK-15 cell line, reaffirming the antimicrobial capacity of NO against a wide range of intracellular pathogens. Furthermore, we also provide the first direct evidence that the inhibitory effect of NO on the PPV life cycle occurs at the step of viral DNA and protein synthesis. These results are important for the understanding of the pathogenesis of PPV, and the use of PK-15 cells to study the inhibitory

effects of NO on PPV replication provides an important cell model for investigating viral replication in vitro.

Our data demonstrated that the addition of SNAP and LA inhibited PPV replication in PK-15 cells in a dose-dependent manner. This finding is consistent with previous reports in which severe acute respiratory syndrome coronavirus (SARS-CoV) and vesicular stomatitis virus (VSV) were studied [5, 25]. Although this reduction in viral replication is seen with NO itself, it is not certain whether the antiviral effects of SNAP or LA are actually due to some other NO-related species. To address this question, three lines of evidence from our experiments indicate that the inhibition of PPV replication in PK-15 cells is most likely caused by NO and not by other factors. Firstly, SNAP and LA could induce the release of NO and inhibit PPV replication in a dose-dependent manner (Fig. 1b). Secondly, the addition of L-NAME, a NOS inhibitor, could reduce NO production in stimulated cultures, and consequently, the inhibition of PPV replication was reversed (Fig. 1a). Finally, the result of the MTT assay clearly excluded the possibility that the antiviral effect of SNAP or LA was due to the general cytotoxicity of SNAP or LA itself (Fig. 1c). Taken together, these results strongly suggest that NO, generated from SNAP or LA, could inhibit the replication of PPV in PK-15 cells.

By investigating the time-of-addition effects of SNAP and LA on anti-PPV in PK-15 cells, our data confirmed that when PK-15 cells were pretreated with SNAP or LA 6 h prior to infection, there was a maximal inhibitory effect on PPV replication (Fig. 2). These results were similar to those of a previously study by Rimmelzwaan et al. [24], which showed that addition of NO donor 3 h prior to infection significantly reduced the synthesis of both vRNA and mRNA. In contrast, some papers have indicated that pretreatment of N18 and SW480 cells with SNAP did not enhance anti-JEV and anti-Sindbis virus inhibition, respectively [8, 16]. These differences in antiviral responses were probably due to the different natures of the viruses and cell lines in the experiments. More importantly, these phenomena are possible because of the different infection mechanisms of different viruses.

Our experiments demonstrate that NO can inhibit PPV replication by blocking viral DNA synthesis. This finding is consistent with previous studies [8, 12, 25]. Harris et al. [12] found that NO affects the late stages, including viral DNA replication, viral protein synthesis, and virion maturation, of VV in macrophages. Sara et al. also showed, using a real-time PCR assay, that SNAP significantly inhibited SARS-CoV viral RNA production in Vero E6 cell [25]. These inhibitory effects suggest that NO inhibits cellular enzymes necessary for viral DNA or RNA synthesis, such as eIF-4G. NO typically interacts with iron-containing proteins and interferes with the function of ribonucleotide reductase [6, 20].

NO might act upon certain viral targets that are necessary for protein synthesis and processing, either by directly acting on host translation enzymes or by reducing the levels of high-energy phosphate compounds. For example, NO inhibits enzymes implicated in diverse metabolic processes, such as glyceraldehydes-3-phosphate dehydrogenase [20], *cis*-aconitase [30] and NADPH-ubiquinone reductase [28], reducing the production of ATP. Our results demonstrate that NO specially inhibits the PPV replication cycle during the step of viral protein synthesis (Fig. 3b). The rates of inhibition by SNAP and LA on viral protein synthesis were high—up to 80%—when these compounds were added 6 h prior to infection, whereas the inhibition rates of SNAP and LA on viral DNA synthesis were about 60%, showing that the inhibitory effect on DNA replication was less than that on the viral protein synthesis. The reason for this discrepancy is uncertain but may reflect an inhibition by NO of one or more steps of the PPV life cycle. These results are consistent with a previous report by Lin et al., who showed that NO is able to reduce the amount of viral glycoprotein and packaged virion RNA secreted from JEV-infected cells into the medium, implying that NO may interfere with the release and/or maturation of virions [16]. However, since our data are unable to furnish us with information on how NO inhibits PPV at the molecular level, more studies are required to elucidate the potential viral and cellular targets of NO.

In conclusion, we have demonstrated that NO inhibits PPV replication in PK-15 cells by inhibiting synthesis of viral DNA and protein. However, further study is needed to identify the host and viral targets of NO, and the exact mechanism by which NO inhibits viral replication in vitro and in vivo remains to be determined.

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