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Short communication

Nitric oxide is elicited and inhibits viral replication in pigs infected with porcine respiratory coronavirus but not porcine reproductive and respiratory syndrome virus

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ABSTRACT

There is little information on the role of nitric oxide (•NO) in innate immunity to respiratory coronavirus (CoV) infections. We examined •NO levels by Greiss assay in bronchoalveolar lavage (BAL) of pigs infected with either porcine respiratory coronavirus (PRCV) or porcine reproductive and respiratory syndrome virus (PRRSV), a member of *Nidovirales*, like CoV. The antiviral effects of •NO on these two viruses were tested in an *in vitro* system using a •NO donor, S-nitroso-N-acetylpenicillamine (SNAP). We detected a large increase in •NO levels in BAL fluids of PRCV-infected pigs, but not in PRRSV-infected pigs. Pulmonary epithelial cell necrosis induced by PRCV coincided with increased •NO. Moreover, •NO levels in cell culture medium of PRRSV-infected alveolar macrophages (AMs) did not differ from that of mock-infected AMs. Antiviral assays showed that •NO significantly inhibited PRCV replication in swine testicular (ST) cells, whereas PRRSV was not susceptible to •NO based on the conditions tested. Our study suggests that unlike PRRSV which induces apoptosis in AMs, respiratory CoVs such as PRCV that infect pulmonary epithelial cells and cause cytolysis, induce •NO production in the respiratory tract. Thus, •NO may play a role in innate immunity to respiratory CoV infections by inhibiting viral replication.

1. Introduction

Nitric oxide (•NO) mediates innate and adaptive immunity by inhibiting viral replication and modulating T-helper (Th) 1/Th2 responses, respectively (Akaike and Maeda, 2000; Davis and Matalon, 2001; Xu et al., 2006). During immune responses to respiratory viral infections, such as influenza A, •NO is generated by inducible nitric oxide synthase (iNOS) in alveolar macrophages (AMs), pulmonary epithelial cells, and neutrophils. To produce •NO, IFNα/β and the dsRNA-activated protein kinase (PKR) system is required which triggers the iNOS gene at an early stage of viral infection. Subsequently, IFN-γ through the signal transducer and activator of transcription (STAT) pathway induces persistent •NO production at the later stages of infection (Xu et al., 2006). The •NO likely functions as a signalling molecule in initiation and progression of immune responses to viral infections.

The •NO is known to inhibit viral protein and/or RNA synthesis by modification of target molecules essential for viral replication, such as ribonucleotide reductase and viral protease (Davis and Matalon, 2001; Xu et al., 2006). The •NO has antiviral effects on RNA viruses, such as influenza A virus, and DNA viruses, such as human herpes virus (Davis and Matalon, 2001). However, studies exploiting immortalized cell lines may not fully elucidate the roles of •NO in the host. In addition, since •NO is a highly reactive nitrogen/oxygen radical, in cases of its excessive generation during viral infection, it may be detrimental by damaging

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uninfected host cells rather than inhibiting viral replication in infected cells (Davis and Matalon, 2001).

Porcine respiratory coronavirus (PRCV) is a positive-strand RNA virus which belongs to group 1 coronaviruses (CoVs) in the family *Coronaviridae*, order *Nidovirales* (Saif, 2004). In pigs, PRCV induces upper and lower respiratory tract disease. The virus mainly damages pulmonary epithelial cells at the onset of infection and thereafter induces lymphohistiocytic interstitial pneumonia (Cox et al., 1990; Jung et al., 2007). The pulmonary lymphohistiocytic inflammation induced coincided with increased Th1 (IFN-γ) cytokines in serum and lung (bronchoalveolar lavage) and large numbers of IFN-γ secreting T cells infiltrating the lungs and regional lymph nodes (Jung et al., 2007; Zhang et al., 2008).

Porcine reproductive and respiratory syndrome virus (PRRSV) is a positive-strand RNA virus in the family *Arteriviridae*, and like CoVs, it belongs to the order *Nidovirales*. The virus replicates in AMs in lung resulting in apoptotic death of infected cells, and it also increases Th1 (IFN-γ) serum (systemic) and lung (local) cytokine responses (Costers et al., 2008; Mateu and Diaz, 2008). We previously reported that co-infection by PRRSV and PRCV increased Th1 (IFN-γ) serum cytokine responses and enhanced apoptosis of AMs in dual-infected pigs compared to single PRCV- or PRRSV-infected pigs (Jung et al., 2009). The cytokine IFN-γ is also known to promote •NO production, resulting in enhanced cell apoptosis (Akaike and Maeda, 2000; Schroder et al., 2006).

The aims of our study were twofold: first, to examine •NO levels in the lungs of pigs infected with either PRCV or PRRSV, or co-infected with PRRSV and PRCV over the time course of the infection; and second, to analyze the antiviral effects of •NO on these two viruses in an *in vitro* system using a •NO donor, S-nitroso-N-acetylpenicillamine (SNAP). We further investigated if the induced •NO coincides with the increased Th1 (IFN-γ) serum and lung cytokine responses after PRCV, PRRSV, or PRRSV/PRCV co-infections, as reported in an earlier publication (Jung et al., 2009).

2. Materials and methods

2.1. Animal infection and sample collection

Based on the availability of different bronchoalveolar lavage (BAL) fluids, the •NO was estimated in BAL fluids obtained during four of five independent animal trials that were conducted earlier (Jung et al., 2009). Specific-pathogen-free, 20- to 25-day-old, Large White-Duroc crossbred weaned pigs ($n = 92$) were randomly assigned to one of four groups: PRCV single-infection ($n = 26$), PRRSV single-infection ($n = 20$), PRRSV/PRCV dual-infection ($n = 26$), and mock control ($n = 20$). As reported previously (Jung et al., 2009), subsets of pigs were first inoculated intranasally (IN) with 3×10^4 50% tissue culture infectious dose ($TCID_{50}$) and intramuscularly with 2×10^4 $TCID_{50}$ of PRRSV (North American SD23983 strain) or mock and, 10 days later, inoculated IN with 4×10^6 plaque-forming units (PFU) and intratracheally with 6×10^6 PFU of PRCV (ISU-1 strain) or mock. At early [post-inoculation day (PID) 2 and 4], middle (PID 8 and 10), and late (PID 14) stages of PRCV infection, four to six pigs per group were euthanized to collect BAL samples, as described previously (Jung et al., 2007, 2009; Zhang et al., 2008). The BAL (25–30 ml) was centrifuged at $800 \times g$ for 10 min at 4 °C to separate the BAL cells. The •NO concentrations were determined only in BAL fluids, devoid of BAL cells.

2.2. Determination of •NO concentration in BAL fluids

The BAL samples were stored at −70 °C until tested. Since the final products of •NO *in vivo* are nitrite (NO_2^-) and nitrate (NO_3^-), the sum of both nitrite and nitrate were measured by the Griess method using a commercially available kit (Cayman Chemical Co., MI). All assays were performed in duplicate.

2.3. In vitro evaluation of antiviral effects of •NO on PRCV and PRRSV

The antiviral effects of •NO on PRCV and PRRSV replication were tested in viral-replication competent ST and MARC145 cells, respectively. Based on previous similar studies (Akerstrom et al., 2005; Davis and Matalon, 2001), the confluent cell monolayers seeded in six well plates were treated with SNAP at different concentrations (0, 50, 100, 200, 400, and 800 μM; Sigma–Aldrich) to select an optimal concentration that produced maximum •NO without affecting cell viability. Based on cell toxicity observed, 400 and 800 μM SNAP were added to MARC145 and ST cells, respectively. The SNAP-treated, virus-infected and SNAP-untreated, virus-infected cells were infected with PRCV or PRRSV (10^{5-6} $TCID_{50}$/ml), with or without SNAP, respectively. At PTH 24 and 48, the cells were fixed in 95% ethanol and the $TCID_{50}$/ml was determined by evaluating virus infectivity in cells by immunofluorescent staining using virus-specific monoclonal antibodies (SDOW-17 for PRRSV and 25H7/14E3 for PRCV) (Jung et al., 2009). The $TCID_{50}$/ml was calculated according to the Reed and Muench method (Reed and Muench, 1938). All assays were performed independently three times.

2.4. Statistical analysis

For •NO levels in BAL, all data are expressed as the means ± standard deviation of the means (SDM) or standard error of the means (SEM). Data among the four treatment groups were analyzed and compared by the Kruskal–Wallis test (nonparametric) using the Statistical Analysis Systems software (SAS Institute Inc., Cary, NC). For comparison of viral titers between SNAP-treated, virus-infected and SNAP-untreated, virus-infected cells, all data are expressed as the means ± SDM and analyzed by unpaired Student's *t*-test. A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

The Griess assay results revealed a large increase in BAL •NO levels of PRCV-infected pigs, but not in PRRSV single-infected pigs. The PRRSV/PRCV dual- and PRCV

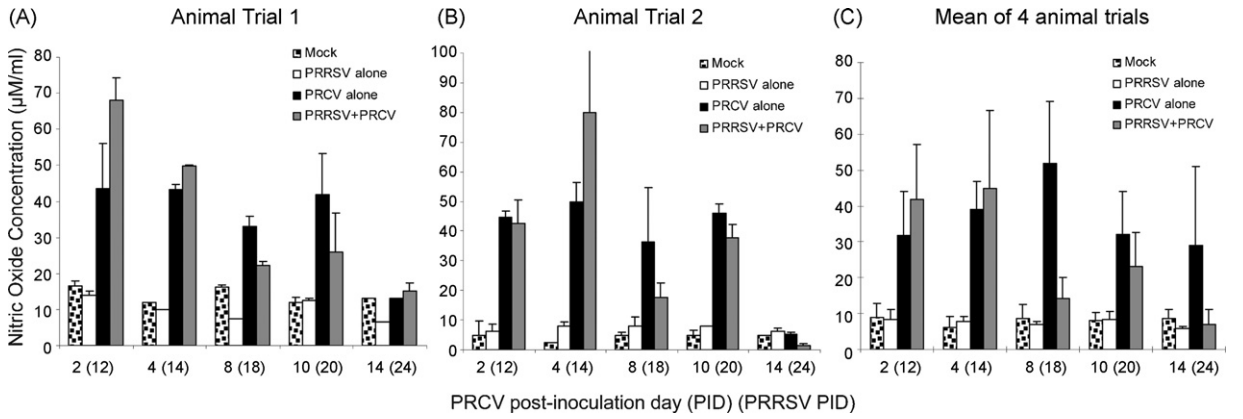

Fig. 1. PRCV, but not PRRSV, induced increased •NO levels in BAL during the early and middle stages of infection (PRCV PID 2–10). (A and B) BAL •NO levels in different independent animal trials. BAL samples were collected from each pig ($n = 1$ at all PIDs for mock and PRRSV single-infected pigs, $n = 1$–2 at each PID for PRRSV/PRCV dual- and PRCV single-infected pigs). All data are expressed as the means ± SDM. (C) Mean BAL •NO levels in four independent animal trials. BAL samples were collected from each pig ($n = 4$ at all PIDs for mock and PRRSV single-infected pigs, $n = 4$–6 at each PID for PRRSV/PRCV dual- and PRCV single-infected pigs). All data are expressed as the means ± SEM. Pigs were first inoculated with PRRSV or mock and, 10 days later, inoculated with PRCV or mock. Total •NO (nitrite and nitrate) in BAL fluids were measured by the Griess method. All assays were performed in duplicate.

single-infected pigs had 2.4–14 times higher BAL •NO levels at PRCV PID 2–10 than the mock control pigs (Fig. 1A–C), although there were no significant differences in mean •NO levels among treatment groups due to high variability among pigs. In our Greiss assays, large variability in BAL •NO levels was observed among the four independent animal trials. In mock control pigs, mean BAL •NO levels showed a wide range from 2.5 to 16.63 μM/ml among the four trials. For this reason, no significant differences were detected in mean BAL •NO levels among the four experimental groups in our study. These results also might be explained by use of outbred pigs and different storage times for the BAL samples after collection, because of large intervals between each animal trial. However, the trend toward increased BAL •NO levels in PRCV-infected pigs at PRCV PID 2–10 was consistent and reproducible (Fig. 1A–C).

As verified by others (Cox et al., 1990) and in our previous studies using the PRCV ISU-1 strain (Jung et al., 2007, 2009), at PRCV PID 2 and 4 (acute stage of infection), PRCV mainly infected upper and lower pulmonary epithelial cells (bronchial/bronchiolar epithelial cells and type 2 pneumocytes) and caused cytolysis, which coincided with the highest BAL •NO levels among all PIDs. Pulmonary epithelial cells are known to contribute to substantial increased levels of •NO in respiratory viral infections (Xu et al., 2006), whereas porcine AMs are likely to be less involved in induction of •NO production in the respiratory tract (Pampusch et al., 1998). Thus, the elevated BAL NO levels following PRCV infection might originate as a result of viral-induced damage to pulmonary epithelial cells; subsequently, the increased •NO might also contribute to further damage to the pulmonary epithelial cells. In addition, we speculate that the persisting, increased BAL •NO levels at PRCV PID 8 and 10 also might be associated with the large numbers of AMs and lymphocytes infiltrating the lungs in parallel with necrosis of respiratory epithelial cells occurring during the same period. However, further studies are needed to identify if iNOS gene expression or if •NO amounts are increased in AMs and lymphocytes following PRCV infection, although unlike murine macrophages, porcine macrophages stimulated by either IFN-γ or lipopolysaccharide (LPS) did not induce •NO production (Pampusch et al., 1998). We suggest that respiratory CoVs such as PRCV that infect pulmonary epithelial cells and cause cytolysis, induce •NO production in the lungs. Based on the results, SARS-CoV that caused massive necrosis of pulmonary epithelial cells such as type 1 and 2 pneumocytes, similar to that observed in lungs of PRCV-infected pigs (Gu and Korteweg, 2007; Jung et al., 2007), might also induce •NO production in the respiratory tract. It is unknown whether SARS-CoV induces •NO production in the lungs and serum of patients (Akerstrom et al., 2005). In humans, concentrations of exhaled •NO are measured in some clinical cases to monitor the progress of pulmonary diseases, such as asthma and viral pneumonia (Xu et al., 2006). Our study demonstrated that as compared with earlier PIDs, BAL •NO levels decreased dramatically on PRCV PID 14 by which time PRCV-induced pneumonia had mostly resolved (Fig. 1A and B) (Jung et al., 2009). Thus, •NO levels might be monitored as an indicator of the progression of bronchopneumonia induced by PRCV, or possibly other respiratory CoVs, and to monitor the effectiveness of treatment.

The IFN-γ contributes to •NO production by inducing iNOS gene expression in AMs and pulmonary epithelial cells during the late stages of viral infection (Xu et al., 2006). In our previous study, we noted elevated IFN-γ serum levels beyond PRCV PID 10 in PRRSV/PRCV dual-infected pigs compared to either group of PRRSV or PRCV single-infected pigs, which might be associated with the more severe pneumonia in dual-infected pigs (Jung et al., 2009). Therefore, we hypothesized that increased IFN-γ serum levels followed by elevated •NO production in lung of the dual-infected pigs might be related to the exacerbated pneumonic lesions. In pigs infected with the influenza H1N2 virus, increased BAL IFN-γ levels at PID 7–10 coincided with increased BAL •NO levels during the same period, possibly promoting viral clearance from the lungs during the middle and late stages of infection (Jung et al.,

2004). In contrast, our study demonstrated that the trend toward increased Th1 (IFN-γ) serum cytokine responses beyond 14 days after PRCV single or PRRSV/PRCV co-infection resulted in the reverse trend of decreased BAL •NO levels in the PRCV single and PRRSV/PRCV dual-infected pigs. Thus, the induced •NO levels in BAL of PRCV singly and PRCV/PRRSV dually infected pigs might not be related to the increased Th1 (IFN-γ) serum and lung cytokine responses.

In PRRSV single-infected pigs, BAL •NO levels were unchanged at PRCV PID 2–14 (PRRSV PID 12–24) compared to mock control pigs (Fig. 1A–C). Nevertheless, during the test period, high PRRSV titers in lungs and severe pneumonia were observed in PRRSV single-infected pigs as reported earlier (Jung et al., 2009). Our results concur with a prior report showing that •NO levels in BAL of experimentally PRRSV (strain VR2332)-infected pigs were not increased at PID 2 and 9 (Pampusch et al., 1998), although the infected pigs had viremia at these PIDs. Our study was expanded to confirm whether PRRSV induces •NO production in AMs, the major target cells for viral replication. Briefly, AMs-rich BAL cells (1×10^6 cells/ml) collected from the lungs of a five-day-old gnotobiotic pig were infected with either North American PRRSV VR2332 (10^5 $TCID_{50}$/ml) or MN184 (10^5 $TCID_{50}$/ml). At PTH 24,

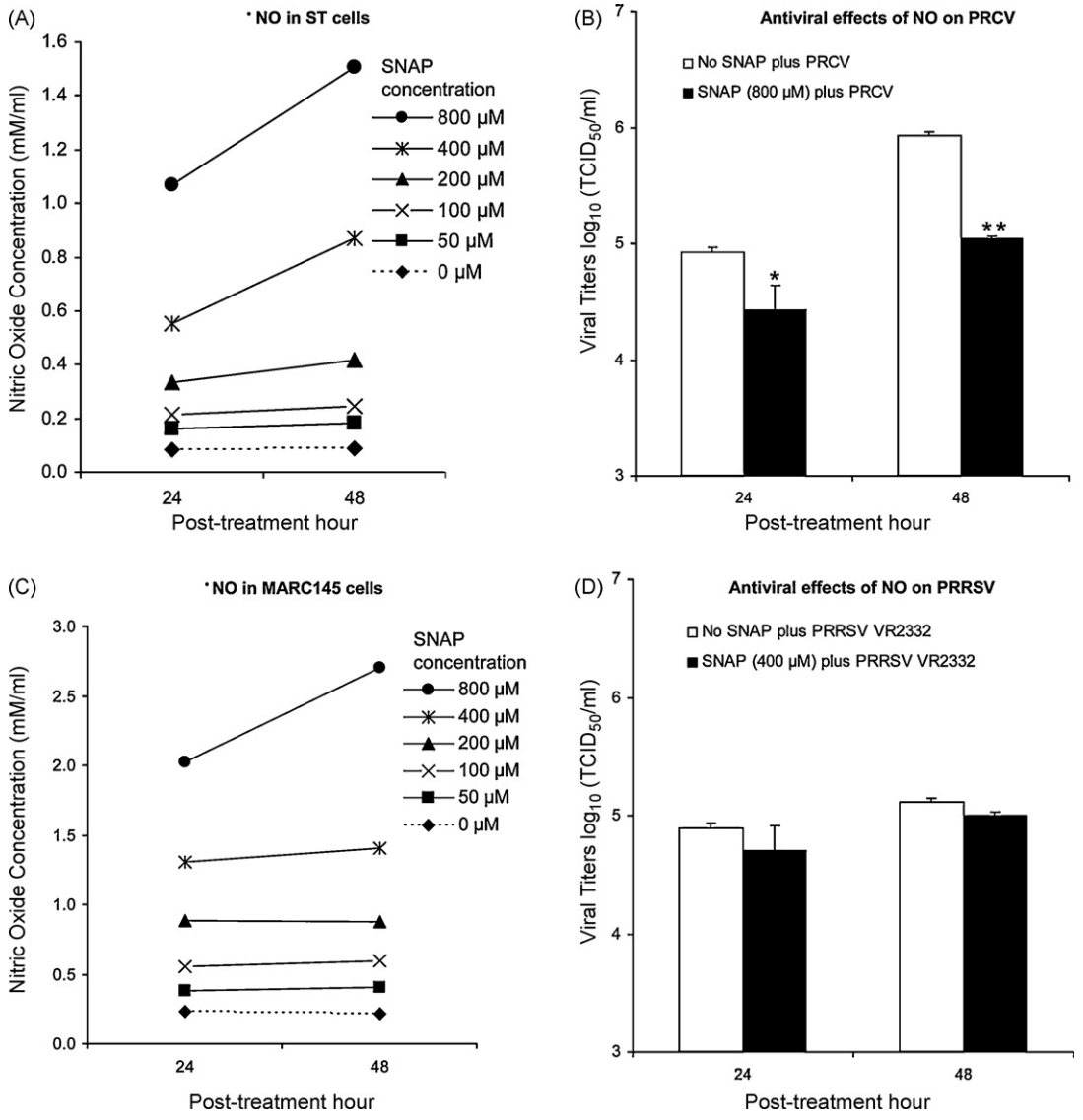

Fig. 2. •NO significantly inhibited PRCV replication in ST cells, whereas PRRSV was not susceptible to •NO. (A and C) •NO production in ST and MARC145 cells, respectively. ST and MARC145 cells were treated with SNAP at different concentrations (0, 50, 100, 200, 400, and 800 μM), and cell supernatants were harvested at PTH 24 and 48 for the Griess assay. (B and D) The antiviral effects of •NO on PRCV replication in ST cells and PRRSV replication in MARC145 cells, respectively. The SNAP-treated, virus-infected and SNAP-untreated, virus-infected cells were infected with PRCV or PRRSV (10^{5-6} $TCID_{50}$/ml), with or without SNAP, respectively. At PTH 24 and 48, the cells were fixed in 95% ethanol and the $TCID_{50}$/ml was titrated by immunofluorescent staining using virus-specific monoclonal antibodies (SDOW-17 for PRRSV and 25H7/14E3 for PRCV). The $TCID_{50}$/ml was calculated according to the Reed and Muench method. All assays were performed independently three times. All data are expressed as the means ± SEM. Asterisks ($^*P < 0.05$; $^{**}P < 0.01$) indicate statistically significant differences between SNAP-treated, virus-infected and SNAP-untreated, virus-infected cells by unpaired Student's *t*-test.

48 and 60, the cell culture medium was harvested for the Greiss assay. There were no statistically significant differences in •NO levels in the cell culture medium between PRRSV- and mock-infected AMs, although large numbers of PRRSV antigen-containing AMs were confirmed by immunofluorescent staining during the test period (data not shown). Therefore, we conclude that although PRRSV induces severe apoptotic death of AMs in lungs, it may not induce •NO in the respiratory tract, and that •NO might not play a critical role in the pathogenesis of PRRSV.

From antiviral assays of •NO induced by PRCV infection, treatment with 800 μM SNAP in ST cells produced maximum •NO levels of 1.07 and 1.51 mM/ml in a dose-dependent manner at PTH 24 and 48, respectively. The •NO significantly inhibited PRCV replication in ST cells at PTH 24 ($P < 0.05$) and 48 ($P < 0.01$) compared to SNAP-free, virus-infected cells (Fig. 2A and B). Similarly, a group 2a CoV, mouse hepatitis virus (MHV) was also susceptible to the antiviral effects of •NO, which reduced viral replication in OBL21a cells. However, •NO production was not a determinant in viral clearance from the lesions (Lane et al., 1997). Also the group 2b CoV, SARS-CoV was inhibited in Vero E6 cells treated with 400 μM SNAP (Akerstrom et al., 2005); however, as noted earlier, there is no information about •NO levels in SARS patients (Akerstrom et al., 2005). On the other hand, •NO did not significantly inhibit PRRSV replication in MARC145 cells at PTH 24 and 48 compared to SNAP-free, virus-infected cells (Fig. 2C and D). Moreover, treatment with 400 μM SNAP in MARC145 cells produced as much •NO (1.31 and 1.41 mM/ml in MARC145 cells at PTH 24 and 48, respectively) as in ST cells treated with 800 μM SNAP (Fig. 2A and C). Therefore, our results imply that PRRSV is not susceptible to •NO under the conditions tested.

Collectively, we suggest that unlike PRRSV, respiratory CoVs such as PRCV that infect pulmonary epithelial cells, cause cytolysis, and stimulate IFN-α secretion, induce •NO production in the lungs. Based on the *in vivo* and *in vitro* data, we conclude that •NO may play a role in innate immunity to respiratory CoV infections by inhibiting viral replication, but not in PRRSV infections, at least based on the conditions tested.

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