Virology 426 (2012) 66–72

Contents lists available at SciVerse ScienceDirect

Virology

journal homepage: www.elsevier.com/locate/yviro

Reactive oxygen species regulate the replication of porcine circovirus type 2 via NF-κB pathway

Xingxiang Chen [a], Fei Ren [a], John Hesketh [b], Xiuli Shi [a], Junxian Li [a], Fang Gan [a], Kehe Huang [a,*]

[a] College of Veterinary Medicine, Nanjing Agricultural University, Nanjing 210095, PR China
[b] Institute for Cell and Molecular Biosciences, University of Newcastle, The Medical School, Framlington Place, Newcastle Upon Tyne NE2 4HH, UK

ARTICLE INFO

Article history:
Received 22 October 2011
Returned to author for revision
29 November 2011
Accepted 10 January 2012
Available online 11 February 2012

Keywords:
Porcine circovirus type 2
PCV2 replication
Reactive oxygen species
NF-κB

ABSTRACT

Intracellular redox state has been suggested to have various effects on the replication of different viruses within host cells. The aim of the present study was to investigate the influence of reactive oxygen species (ROS) on replication of porcine circovirus type 2 (PCV2), in PK15 cells. Following PCV2 infection there was a time-dependent increase in ROS. Antioxidant N-acetyl-L-cysteine treatment of cells resulted in lower ROS levels and lower PCV2 replication. In contrast, treatment by buthionine sulfoximine (BSO), a GSH synthesis inhibitor, resulted in elevation of ROS levels and increased PCV2 replication. Furthermore, inhibiting the activity of NF-κB, a redox-responsive transcription factor, suppressed BSO-mediated increase of PCV2 replication, indicating that increased PCV2 replication likely occurs via ROS activation of NF-κB. Taken together, our results indicate that the generation of ROS during PCV2 infection is involved in its replication and this progression is associated with the alteration in NF-κB activity induced by ROS.

Introduction

Porcine circovirus (PCV) is classified in the genus *Circovirus* of the family *Circoviridae* (Todd et al., 2005). Two genotypes of PCV have been identified. PCV type 1 (PCV1), which was first recognized in 1974 as a contaminant of a continuous porcine kidney cell line (PK15) (Tischer et al., 1982), is known to be non-pathogenic to pigs (Allan et al., 1995). Infection with PCV type 2 (PCV2) has been associated with postweaning multisystemic wasting syndrome (PMWS) in young weaned pigs, a disease first recognized in Canada in 1991 (Clark, 1997). Usually PMWS appears in pigs at the age of 5 to 18 weeks and affected pigs show fever, wasting or unthriftiness, respiratory distress, enlarged lymph nodes and, occasionally, jaundice and diarrhea (Darwich et al., 2004; Segales and Domingo, 2002). Mortality rates may vary from 1% to 2% up to 30% in complicated cases when co-infections with porcine reproductive and respiratory syndrome virus, porcine parvovirus, or *Mycoplasma hyopneumoniae*. Several studies have suggested that severely PMWS affected pigs may develop immunosuppression (Segales et al., 2004). Nowadays, PMWS and other related PCV2-associated diseases are occurring in all swine-producing areas of the world and have become increasingly serious threats to global pig production (Allan and Ellis, 2000; Allan et al., 1998; Choi et al., 2000; Fenaux et al., 2000; Grau-Roma et al., 2011; Mankertz et al., 2000; Patterson and Opriessnig, 2010). However, not all pigs infected with PCV2 will develop PMWS. Even if PMWS occur, severity of the disease is different in different pig farms. Obviously, infection of pigs with PCV2 and other infectious/noninfectious triggers are required for PMWS to occur (Grau-Roma et al., 2011; Patterson and Opriessnig, 2010). Several studies have linked PMWS expression to management measures, presence of concurrent viral infections, stimulation of the immune system, nutrition, male castration and lower piglet weight at weaning (Grau-Roma et al., 2011), but the pathogenic mechanism of PCV2 remains poorly understood.

Oxidative stress results from an imbalance between ROS production and anti-oxidant activity conferred by enzymes such as the thioredoxin, reductases, glutathione peroxidases and glutathione reductase (Schafer and Buettner, 2001). Viral infection is often accompanied by alteration of intracellular redox state (Baruchel and Wainberg, 1992; Casola et al., 2001; Jamaluddin et al., 2009; Korenaga et al., 2005; Seet et al., 2009; Wang et al., 2001; Waris et al., 2005). Infection by the HIV is associated with decreased levels of GSH and increased production of ROS (Ciriolo et al., 1997; Garaci et al., 1997; Palamara et al., 1995). Additionally, the viral TAT protein increases intracellular ROS levels by inhibiting the antioxidant enzyme manganese superoxide dismutase (Flores et al., 1993). Direct interaction of core protein with mitochondria is an important cause of the oxidative stress seen in chronic hepatitis C (Korenaga et al., 2005). ROS have also been shown to affect viral replication, promoting HIV replication (Baruchel and Wainberg, 1992; Staal et al., 1990) but being associated with lower bovine leukemia virus proviral loads (Bouzar et al., 2009). However, there is no information available concerning the production of ROS induced by PCV2 infection and the effects of ROS on PCV2 replication, which may contribute to interpret why there are different incidences of PMWS in different pig farms with PCV2 infection.

* Corresponding author. Fax: +86 25 84398669.
E-mail address: khhuang@njau.edu.cn (K. Huang).

0042-6822/$ – see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.virol.2012.01.023

Intracellular redox state has been suggested to affect the replication of viruses through different mechanism within host cells according to previous studies. Inhibition of influenza infection by GSH appears to occur via GSH-dependent inhibition of apoptosis which may slow virus release (Cai et al., 2003). Increased HIV replication likely occurs via ROS activation of NF-κB (Korenaga et al., 2005). However, a mechanism of action on Sendai virus replication is not known (Ciriolo et al., 1997). It has been reported that NF-κB activation occurs principally in virus infected cells through a direct mechanism because a co-localization of ORF1 protein with nuclear translocation of p65 was detected in the PCV2-infected cells but not in UV-irradiated PCV2-infected cells (Wei et al., 2008). However, it is still unclear whether activation of NF-κB as an oxidant sensitive pathway contributes to PCV2 replication in the host cells.

The present study was performed to investigate whether PCV2 infection is accompanied by alteration of ROS generation, whether PCV2 replication in PK15 cells is affected by intracellular ROS and whether PCV2 replication is affected by the activation of oxidant sensitive NF-κB pathways.

Results

PCV2 infection of PK15 cells induces ROS production

To determine whether PCV2 infection induced ROS production, PK15 cells were infected with PCV2 at MOI of 1 at different time points. At the same end time point DCFH-DA was added to cultures and incubated for 30 min at 37 °C. Fluorescence was measured within 60 min according to the manufacturer's instruction. When compared with controls, a significant production of ROS was observed at different time points after infection, reaching a plateau at around 72 h (Fig. 1B). At 96–120 h post infection, ROS levels had declined from the maximum level but were still elevated above those in untreated cells. As a positive control for DCF-DA oxidation, PK15 cells exposed to 100 μM ROSUP (Beyotime, China) for 20 min also exhibited a significant increase over un-stimulated cells (data not shown). These results demonstrate that ROS are produced following PCV2 infection.

Reduction of ROS levels induced by NAC can decrease PCV2 replication

The finding that ROS are generated during PCV2 infection prompted us to examine whether decreasing the production of ROS would alter PCV2 replication. Firstly, cells were treated with the antioxidant NAC, a precursor in glutathione synthesis which can promote the generation of cysteine and indirectly increase glutathione levels. PK15 cells were seeded at a density of 1×10^4/well in 96-plate and incubated with NAC (5 mM) at different time points. At the same end time point PK15 cells were collected for measuring DCFH-DA oxidation. ROS levels were significantly decreased at 24–120 h after 5 mM NAC treatment compared with control cells (Fig. 1). After incubation with 1, 3 and 5 mM NAC for 72 h, ROS levels were reduced significantly when compared with control cells (Fig. 2A). After PK15 cells were incubated with various concentration of NAC for 24 h before PCV2 infection and for a further 72 h, ROS levels were increased when compared with uninfected cells but were lower when compared to infected control cells (Fig. 2A). These results indicate that ROS levels were lowered by NAC.

To determine whether reduction of ROS levels play any role in the replication of PCV2, the effects of NAC on PCV2 replication were evaluated. PK15 cells were seeded in 24-well cell culture plates and cultured overnight with various concentration of NAC before PCV2 infection at MOI of 1 and incubation with NAC for 72 h. Results

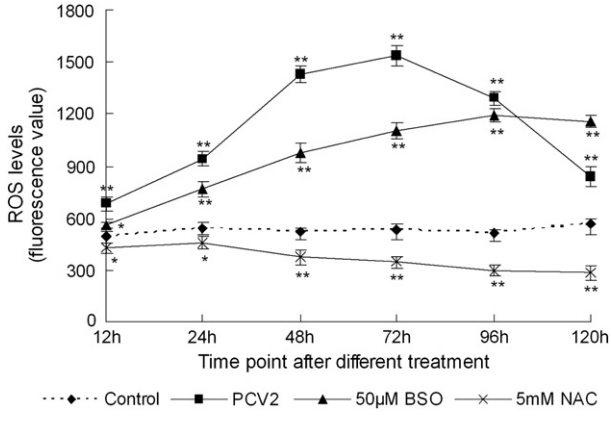

Fig. 1. Time course of ROS production after PCV2 infection, BSO and NAC treatments. (A, B) PK15 cells were infected with PCV2 at MOI of 1 at different time points. At the same end time point DCFH-DA was added to cultures and incubated for 30 min at 37 °C. Fluorescence was measured within 60 min at 485 nm for excitation and 530 nm for emission. (C, D) PK15 cells were treated with 50 μM BSO or 5 mM NAC at different time points. At the same end time point PK15 cells were loaded with DCFH-DA and fluorescence was measured within 60 min. Values shown are means ± SD from four independent experiments. Groups were compared by a 1-way ANOVA followed by least-significant difference test (*P<0.05, **P<0.01).

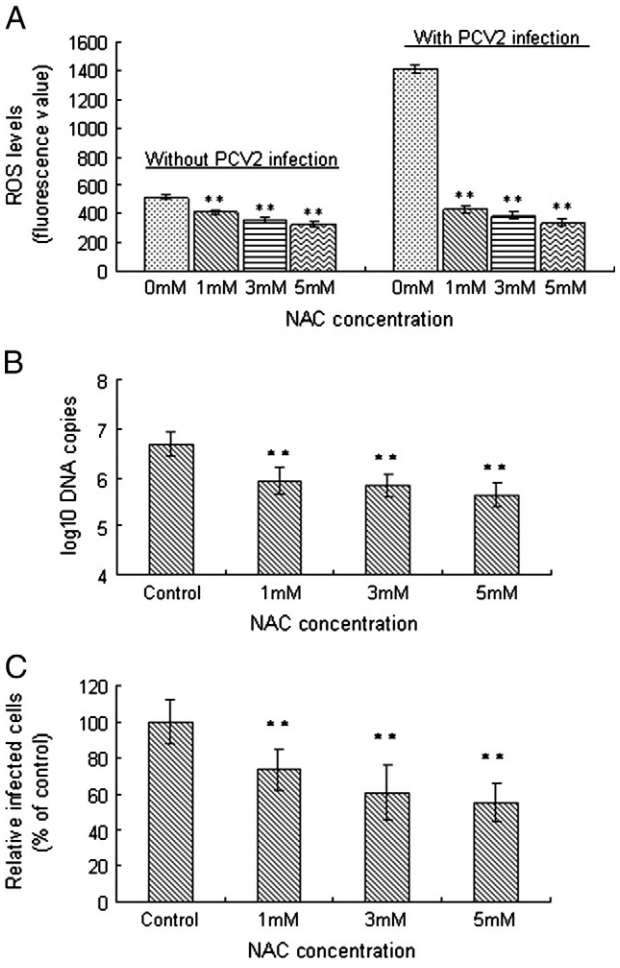

Fig. 2. Reduction of ROS levels induced by NAC decreases PCV2 replication. (A) ROS levels in NAC-treated PK15 cells at 72 h were determined with DCFH-DA. For the PCV2-infected groups, PK15 cells were incubated with various concentration of NAC for 24 h before PCV2 infection and then incubated with NAC for a further 72 h. Fluorescence was measured within 60 min at 485 nm for excitation and 530 nm for emission. Values shown are means ± SD mean from four independent experiments. (B/C) PK15 cells were cultured overnight with various concentration of NAC before PCV2 infection. PCV2-infected PK15 cells at 72 h in the presence of various concentration of NAC were assayed for the viral DNA copies by real-time PCR and the amount of infected cells by IFA. Values shown are means ± SD mean from three independent experiments. Asterisks indicate groups statistically significantly different from control by a 1-way ANOVA followed by least-significant difference test (*P<0.05, **P<0.01).

showed that NAC treatment decreased the progression of the infection as measured by PCV2 DNA copies and the number of infected cells (Figs. 2 and 3). The PCV2 log10 DNA copies significantly decreased (Fig. 2B) and the percentage of PCV2 infected cells dropped by 74%, 61% and 55% after 72 h incubation with 1, 3 and 5 mM NAC respectively when compared with the control cells (Figs. 2C, 3). A high titer viral stock solution was treated with NAC at 5 mM for 1 h at 37 °C to determine whether NAC affected infectivity and replication of virus. No effect of NAC treatment on virus infectivity was observed when these cells were compared with a mock treatment control (data not shown), indicating that NAC is not directly inactivating the virus. Overall, the results suggest reduction of ROS levels induced by NAC decreases viral replication.

Elevation of ROS levels induced by BSO can increase PCV2 replication

Data presented in Figs. 1 and 2 suggest that elevated GSH, resulting from incubation with antioxidant NAC, decreases the level of viral DNA copies and the number of infected cells. Therefore, we tested for the converse effect: does lower antioxidant activity accelerate viral replication? To address this question, intracellular ROS levels were increased by the compound BSO, which can cause irreversible inhibition of GSH biosynthesis and decrease the capacity to detoxify ROS. PK15 cells were seeded at a density of 1×10^4/well in 96-plate, incubated with BSO (50 μM) at different time points, and ROS levels measured by DCFH-DA oxidation. When compared with control cells, a significant increase of ROS levels was observed at different time points after 50 μM BSO treatment (Fig. 1). After incubation with BSO at different concentrations for 72 h, ROS levels were elevated significantly when compared with control cells (Fig. 4A). After PK15 cells were incubated with various concentrations of BSO for 24 h before PCV2 infection and then incubated with BSO for a further 72 h, ROS levels were increased when compared with uninfected and infected control cells (Fig. 4A). These results imply that ROS levels were increased as a result of BSO treatment.

To investigate whether elevation of ROS levels play any role in the replication of PCV2, the effects of BSO exposure on PCV2 replication in PK15 cells were evaluated. PK15 cells were seeded in 24-well cell culture plates and cultured for overnight with various concentrations of BSO before PCV2 infection at MOI of 1 and incubated with BSO for a further 72 h before determination of PCV2 replication and infection. The results presented in Fig. 3 indicate that BSO treatment led to an increased progression of the infection as measured by the appearance of PCV2 DNA copies and infected cells. The PCV2 log10 DNA copies significantly increased and the fold increases in numbers of PCV2 infected cells were 1.15, 1.28 and 1.41 after 72 h incubation with 10, 30 and 50 μM BSO respectively when compared with the control cells (Figs. 4C, 3). A high titer viral stock solution was treated with BSO at 50 μM for 1 h at 37 °C to determine if BSO directly affected virus infectivity and replication; a mock treatment served as a control. No effect of BSO treatment on virus infectivity was observed (data not shown), showing that BSO did not directly activate the virus but may affect some aspect of viral replication. Overall, these results indicate that the elevation of ROS levels induced by BSO increases viral replication.

Effect of BSO and NAC treatment on the activation of NF-κB-dependent luciferase

It has been reported that PCV2 infection can activate NF-κB via IκBα phosphorylation and degradation in PK15 cells, and the activation of NF-κB is required for virus replication and PCV2-induced apoptosis (Wei et al., 2008). Since NF-κB activity is susceptible to be regulated by alterations in the intracellular redox state, it is possible that the effect of NAC and BSO treatment on PCV2 replication is associated with alteration in NF-κB activity induced by ROS. To address this question, we determined NF-κB activation by measuring pNF-κB-luc-reporter gene expression in transiently transfected PK15 cells treated with PCV2 infection, NAC or BSO. As shown in Fig. 5, luciferase activity in PCV2-infected PK15 cells was 244% of that found in uninfected control cells, indicating an activation of NF-κB by PCV2 infection. Luciferase activity in BSO-treated cells was lower, showing a

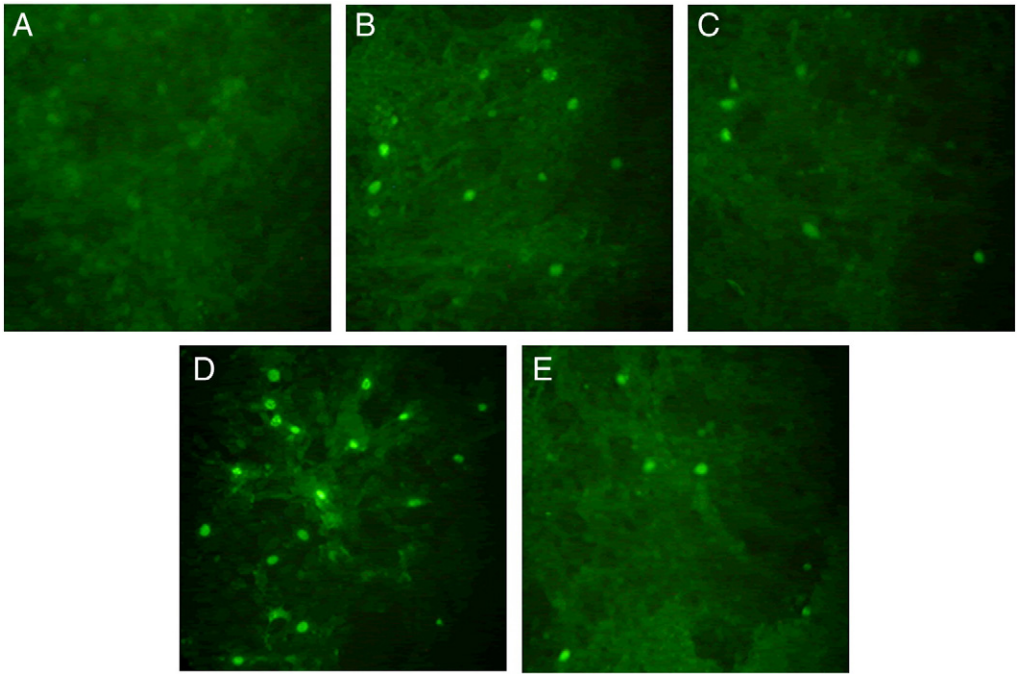

Fig. 3. Assay of infected cells by indirect fluorescence assay (IFA). PCV2-infected cells were detected by immunofluorescence (10×20 magnification). (A) Non-infected control group, (B) control cells stained for PCV2, (C) 5 mM NAC-treated group, (D) 50 μM BSO-treated group and (E) 10 μM BAY 11-7082 treated groups all stained for PCV2. Cells positive for PCV2 viral antigens were counted in six fields of view and number of positive cells calculated as percentages of PCV2-positive cells compared to control cells infected with PCV2 alone (data shown in Fig. 2C, Fig. 4C and Fig. 6B).

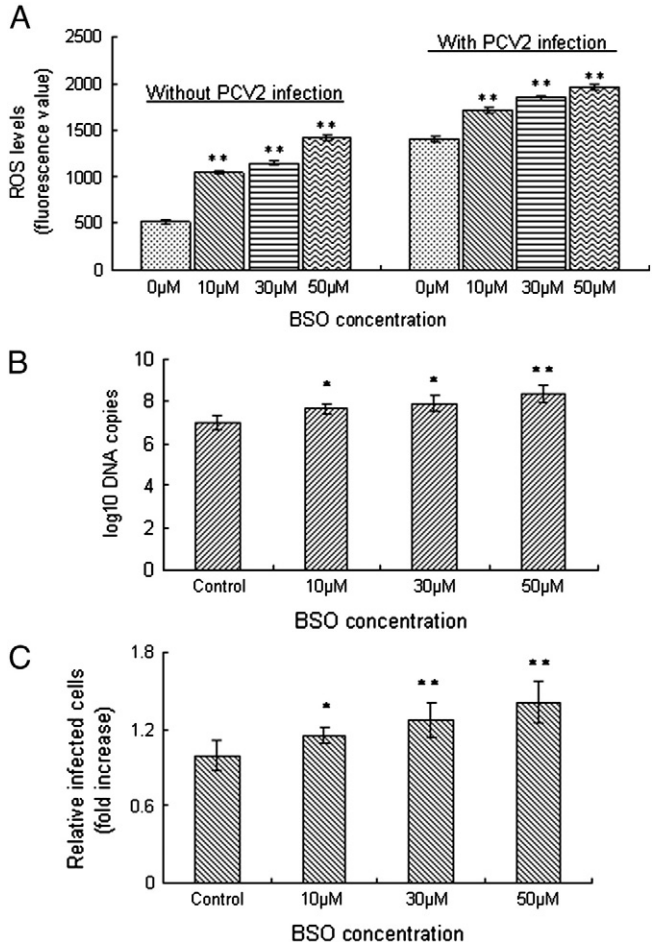

Fig. 4. Elevation of ROS levels induced by BSO increases PCV2 replication. (A) ROS levels in BSO-treated PK15 cells at 72 h were determined with DCFH-DA. Fluorescence was measured within 60 min at 485 nm for excitation and 530 nm for emission. For the PCV2-infected groups, PK15 cells were incubated with various concentration of BSO for 24 h before PCV2 infection and then incubated with NAC for a further 72 h. Values shown are means ± SD from four independent experiments. (B/C) PK15 cells were cultured overnight with various concentration of BSO before PCV2 infection. PCV2-infected PK15 cells at 72 h in the presence of various concentration of BSO were assayed for the viral DNA copies by real-time PCR and the amount of PCV2 viral antigen by IFA. Values shown are means ± SD mean from three independent experiments. Asterisks indicate groups statistically significantly different from control by a 1-way ANOVA followed by least-significant difference test (*$P<0.05$, **$P<0.01$).

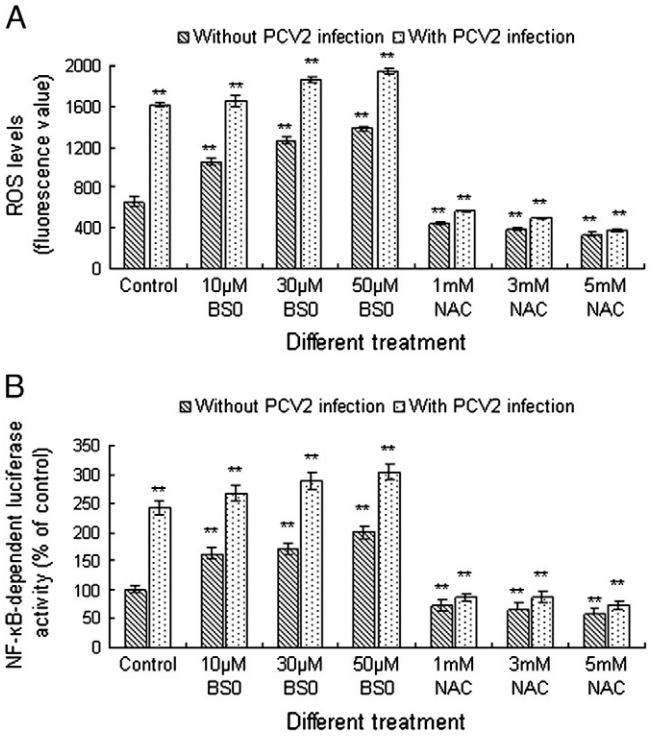

Fig. 5. Effect of BSO and NAC treatment on ROS levels and activation of NF-kB-dependent luciferase. PK15 cells were seeded at a density of 5×10^3/well in 96-well plates one day prior to transfection for 6 h with pNF-κB-luc containing NF-κB binding motifs (GGGAATTTCC). The cells were washed and then cultured in 100 μl DMEM with 5% FBS for 24 h before infection with PCV2 at MOI of 1 and incubated with various concentrations of BSO or NAC for 72 h. (A) Effect of BSO and NAC treatment on ROS levels was determined with DCFH-DA. Fluorescence was measured within 60 min at 485 nm for excitation and 530 nm for emission. (B) Activity of NF-κB-dependent luciferase of cell extracts from each sample was measured using a luciferase assay kit according to the manufacturer's protocol. Activation of NF-κB-dependent luciferase was recorded as a percentage of mock infection. Values shown are means ± SD from three independent experiments. Asterisks indicate groups statistically significantly different from control by a 1-way ANOVA followed by least-significant difference test (*$P<0.05$, **$P<0.01$).

rise of only 164%, 173% and 201%, respectively after 10, 30 and 50 μM BSO treatment, as compared with untreated controls. But luciferase activity in PCV2-infected cells showed an increase of 268%, 290% and 304%, respectively after 10, 30 and 50 μM BSO treatment, as compared with untreated controls. In contrast, after 1, 3 and 5 mM NAC treatment, NF-κB activity in infected or non-infected PK15 cells decreased significantly when compared to untreated PK15 cells. Furthermore, both the patterns and magnitudes of increased or decreased luciferase activity were similar to that of ROS levels in PK15 cells with the same treatments. These results indicate that NF-κB activation could be induced by PCV2 infection and is closely associated with ROS levels in host cells after BSO and NAC treatment.

Inhibition of NF-κB activation suppresses BSO-mediated increase in PCV2 replication

BAY 11-7082 ((E)-3-[4-methylphenylsulfonyl]-2-propenenitrile) is widely used as an inhibitor of cytokine-induced IκBα phosphorylation and consequently as an agent which results in decreased expression of NF-κB (Hernandez-Garcia et al., 2010). To confirm the relationship between NF-κB and ROS-mediated viral replication, PK15 cells were treated with 10 μM BAY 11-7082 for 5 h. After 5 h of BAY 11-7082 treatment, the medium was removed, and fresh basal medium containing fresh inhibitor BAY 11-7082 and BSO was added to the culture. 24 h later, the PK15 cells were then infected with PCV2 and incubated with BAY 11-7082 and BSO for 72 h prior to determination. The results presented in Fig. 6 indicate that BSO-mediated increase in PCV2 replication was suppressed by BAY 11-7082 treatment as measured by the number of PCV2 DNA copies and infected cells. After 72 h incubation with 10, 30 and 50 μM BSO alone, the percentage of PCV2 infected cells increased when compared with the control cells, but after BAY 11-7982 treatment and 72 h incubation with BSO, the percentage of PCV2 infected cells dropped by 62%, 74% and 77% when compared to the control (Figs. 6B, 3). After BAY 11-7982 treatment, the percentage of PCV2 infected cells decreased significantly when compared to cells without BAY 11-7982 treatment at the same concentration of BSO. These results indicate that inhibition of NF-κB activation suppresses BSO-mediated increase in PCV2 replication.

Discussion

The intracellular redox state is the result of a dynamic equilibrium between oxidant and antioxidant molecules. It has been suggested to affect viral replication within a host cell (Ciriolo et al., 1997; Garaci et al., 1997; Michalek et al., 2008; Staal et al., 1990). Results from the

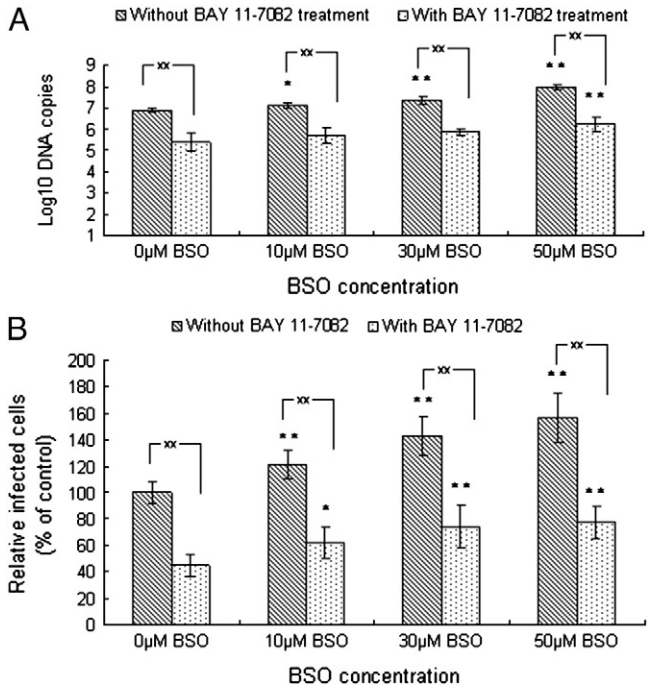

Fig. 6. Inhibition of NF-κB activation suppresses BSO-mediated increase in PCV2 replication. After 5 h of treatment with 10 μM BAY 11-7082, PK15 cells were cultured overnight with various concentration of BSO in the presence of 10 μM BAY 11-7082 before PCV2 infection. PCV2-infected PK15 cells at 72 h in the presence of 10 μM BAY 11-7082 and various concentrations of BSO were assayed for the viral DNA copies by real-time PCR (A) and the amount of infected cells by IFA (B). Values shown are means ± SD from three independent experiments. Within the groups with/without BAY 11-7082 treatment, bars with "*" are statistically significantly different from control (0 μM BSO) by oneway ANOVA followed by least-significant difference test (*$P<0.05$, **$P<0.01$). "x" and "xx" denote significant difference (x$P<0.05$, xx$P<0.01$) between treated cells and untreated cells by BAY 11-7082.

present study provide evidence that PCV2 infection induces ROS production in PK15 cells. First, antioxidant NAC treatment of cells resulted in reduction of ROS levels and an inhibitory effect on virus growth. Second, BSO treatment of cells resulted in elevation of ROS levels and increased number of viral DNA copies and infected cells. Since NF-κB is a factor activated in response to cellular stress, including oxidative stress (Morgan and Liu, 2011), these results raised the possibility that effect of NAC and BSO treatment on PCV2 replication would be associated with alteration in NF-κB pathway induced by ROS. To test this hypothesis we used a reporter gene assay to determine the NF-κB activity after PCV2 infection, NAC and BSO treatment. NF-κB activity increased in PCV2-infected or BSO-treated PK15 cells while it decreased in NAC-treated PK15 cells when compared with control cells. These results indicate that NF-κB activation could be induced by PCV2 infection and is closely associated with ROS levels in host cells after BSO and NAC treatment. The involvement of NF-κB regulation in ROS-mediated PCV2 replication was supported further by the effects of the IκBα phosphorylation inhibitor BAY 11-7982. Overall these data provide the first report of an association between increased ROS production in PK15 cells and PCV2 infection and they demonstrate that intracellular ROS level play a role in PCV2 replication and this progression is associated with the alteration in activation of the NF-κB pathway induced by ROS.

Porcine circovirus type 2 is found worldwide in pigs and has been linked to several pathological conditions collectively named porcine circovirus diseases (PCVD) (Allan and Ellis, 2000; Allan et al., 1998; Gillespie et al., 2009). The most economically important PCVD is post-weaning multisystemic wasting syndrome (PMWS), which results in losses of €900 million per year in the European Union (Armstrong and Bishop, 2004). However, not all pigs infected with PCV-2 will develop PMWS even though PCV2 is recognized as an essential infectious agent of PMWS (Grau-Roma et al., 2011). Infection of pigs with PCV2 and other unknown triggers are required for PMWS to occur (Grau-Roma et al., 2011). Evaluation of the role of these triggers is essential in order to understand the incidence of PMWS. Previous studies have demonstrated that some viruses, such as HIV, influenza, RSV and LCMV, can promote ROS generation during infection and ROS have also been shown to promote viral replication and immuno-suppression (Baruchel and Wainberg, 1992; Casola et al., 2001; Jamaluddin et al., 2009; Korenaga et al., 2005; Seet et al., 2009; Wang et al., 2001; Waris et al., 2005). In contrast, the levels of ROS inversely correlated with bovine leukemia virus proviral loads (Bouzar et al., 2009) and glutathione is required for efficient production of infectious picornavirus virions (Smith and Dawson, 2006). The present observations that elevation of ROS is a direct consequence of PCV2 infection, and that PCV2 replication can be affected by ROS levels after BSO and NAC treatment, suggest that redox factors contribute to the complex interrelationship between PCV2 and porcine circovirus associated diseases.

The results presented here for PCV2 are consistent with those obtained with HIV and Sendai virus where BSO treatment increased virus replication and NAC treatment decreased virus replication (Garaci et al., 1997; Macchia et al., 1999). In addition, it has been suggested that oxidative stress caused by elevated inflammatory cytokines and decreased GSH-dependent antioxidant functions in HIV infection promotes the activation of NF-κB and replication of HIV, resulting in the disease progression associated with the CD4+ T-cell loss, immunodeficiency, and opportunistic infection (Nakamura et al., 2002). Influenza replication is inhibited by GSH and is enhanced when GSH levels are reduced (Cai et al., 2003). The intracellular GSH levels decrease during influenza virus infection and the more oxidative environment established by the influenza virus's depletion of GSH would increase the expression and oxidation of protein disulfide isomerase in the endoplasmic reticulum, accelerating disulfide bonding and enhancing viral glycoprotein maturation, which is supported by the fact that BSO treatment significantly decreased intracellular GSH levels and increased HA expression and viral titers in cell supernatants (Sgarbanti et al., 2011). Our results demonstrate that effect of BSO and NAC on PCV2 replication is associated with the alteration in NF-κB pathway induced by ROS.

In conclusion, our results demonstrate that the PCV2 infection and replication not only generate ROS but also is affected by redox state, and that this progression is associated with the alteration in NF-κB pathway induced by oxidative stress. Our research may contribute to interpret the incidence of PMWS when oxidative stress from other unknown triggers is present. Future studies targeting effect of ROS on PCV2 replication in vivo and in vitro may lead to improving treatment of PCV2-associated diseases and production of PCV2 vaccine.

Materials and methods

Reagents and antibodies

Buthionine sulfoximine (BSO) was purchased from Sigma. N-acetyl-L-cysteine (NAC) and (E)-3-[4-methylphenylsulfonyl]-2-propenenitrile (BAY 11–7082) were purchased from Beyotime Institute of Biotechnology, China. The cytotoxicity of BSO, NAC and inhibitor BAY 11-7082 on PK15 cells was determined by trypan blue exclusion dye staining. It was noted that throughout all doses of BSO, NAC and inhibitor BAY 11-7082 used in the present study, cell viability assay showed no detectable cell death in the PK15 cells. Porcine anti-PCV2 antibody for immunofluorescence was purchased from Univ Biotech Co. Ltd., China. FITC-linked secondary antibody (rabbit anti-pig IgG) was purchased from Sigma.

Virus and cells

PK-15 cells (porcine kidney) were provided by China Institute of Veterinary Drug Control and free of PCV1. PK-15 cells were propagated at 37 °C in an atmosphere of 5% CO2 in DMEM (Invitrogen, USA) supplemented with 5% FBS, penicillin (20 mg/ml), streptomycin (20 mg/ml), referred hereafter as the culture medium. The wild-type PCV2 (PCV2NJ2002) used in the experiment was isolated originally from a kidney tissue sample of a pig with naturally occurring PMWS. The determination of PCV type was performed by sequencing (Invitrogen, USA). PCV2 stocks were generated from PK-15 cells infected with PCV2 according to the following procedure: PK-15 cells were infected with PCV2 at a multiplicity of infection (MOI) of 1, when cells had reached approximately 40%–50% confluence. After 1 h absorption, the inoculum was removed, and the cell monolayer was washed three times with phosphate-buffered saline (PBS). DMEM medium including 5% FBS, penicillin (20 mg/mL) and streptomycin (20 mg/mL), was subsequently added, and incubation was continued at 37 °C with 5% CO2 for 72 h. Then the infected cells were subcultured in DMEM and the PCV2 was serially passaged in PK-15 cells. The virus harvested at each passage was stored at −80 °C.

SYBR green real-time PCR for the quantification of PCV2 DNA copies

Newly synthesized viral DNA in PK15 cell culture was assayed by SYBR green real-time PCR as described elsewhere (Pan et al., 2008). Briefly, the PCV2 infected PK15 cells were harvested at 72 h post-inoculation and DNA extractions were carried out using the TaKaRa DNA Mini kit (TaKaRa, China). The purified DNA was used as templates for real-time PCR amplification. A region of 117 bp was amplified from the PCV2 ORF2 gene with a pair of PCV2 specific primers (Forward primer: 5′-TAGTATTCAAAGGGCACAG-3′, Reverse primer: 5′-AAGGCTACCACAGTCAG-3′). Quantitative real-time PCR was carried out using the ABI PRISM 7300 Detection System (Applied Biosystems, USA). A recombinant pMD19 plasmid vector (TaKaRa, China) containing PCV2 genome insert was used as standard reference. The viral DNA quantity was detected using the TaKaRa SYBR Green qPCR Kit (TaKaRa, China).

Assay of infected cells by indirect fluorescence assay (IFA)

After 72 h post-inoculation, PK15 cells were fixed in methanol and washed with phosphate-buffered saline (PBS). After fixation, the cells were blocked by PBS with 2% BSA at room temperature for 1 h. The porcine anti-PCV2 antibody (Univ Biotech, China), diluted in PBS containing 1% BSA (PBSB) (1:50), was added to the cells and incubated at 37 °C for 1 h. After washing with PBS containing 0.1% Tween-20, an optimum dilution (1:100) of FITC-conjugated rabbit anti-pig antibody (Sigma, USA) was added and incubated for 1 h at 37 °C. After washing, the cells were examined under a fluorescence microscope. Cells positive for PCV2 viral antigens were counted in six fields of view.

Fluorescence assay of ROS

Assay of intracellular ROS were measured with 2′, 7′-dichlorofluorescein diacetate (DCFH-DA; Beyotime, China) (Hernandez-Garcia et al., 2010). DCFH-DA passively diffuses into cells and is deacetylated by esterases to form nonfluorescent 2′, 7′-dichlorofluorescein (DCFH) which in the presence of ROS forms the fluorescent product DCF, which is trapped inside the cells. PK15 cells were seeded at a density of 1×10^4/well in 96-plate (Corning, USA). 5 h after seeding, the culture wells were infected with PCV2 at MOI of 1 and incubated with various concentrations of BSO or NAC for 72 h. To obtain dissociated PK15 cells for the ROS assay, culture medium was first removed and the cells were washed three times with PBS. DCFH-DA, diluted to a final concentration of 10 μM with a serum free DMEM, was added to cultures and incubated for 30 min at 37 °C. The fluorescence was measured at 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (TECAN infinite M200, TECAN).

pNF-κB-luc transfection and luciferase reporter assays

One day prior to transfection, PK15 cells were seeded at a density of 5×10^3/well in 96-well plates (Corning, USA). The pNF-κB-luc reporter gene constructs (Beyotime, China) containing NF-κB binding motifs (GGGAATTTCC) were transfected into PK15 cells using Lipofecter reagents (Beyotime, China) in serum free DMEM. The cells were washed twice with PBS and then cultured in 100 μl DMEM with 5% FBS for 24 h. PK15 cells were infected by PCV2 at MOI of 1 or incubated with various concentrations of BSO or NAC for 72 h before determination. Activity of NF-κB-dependent luciferase of cell extracts from each sample was measured using a luciferase assay kit according to the manufacturer's protocol. Three independent experiments were performed to assess luciferase activity.

Statistical analysis

Statistical analysis of the experimental data was performed using the statistical package (SPSS) V17.0 for windows. Data were analyzed for establishing their significance using one-way analysis of variance (ANOVA) followed by least-significant difference test. Data are expressed as means ± SD. Differences were regarded as significant at $P<0.05$.

Acknowledgments

This work was funded by the National Natural Science Foundation of China (NFSC) (Grant numbers 30871892, 31011130155), an international collaboration grant to JEH and KH from The Royal Society and NSFC, the Research Fund for the Doctoral Program of Higher Education of China (Grant number 20110097110014) and the Priority Academic Program Development of Jiangsu Higher Education Institutions.

References

Allan, G.M., Ellis, J.A., 2000. Porcine circoviruses: a review. J. Vet. Diagn. Investig. 12, 3–14.
Allan, G.M., McNeilly, F., Cassidy, J.P., Reilly, G.A., Adair, B., Ellis, W.A., McNulty, M.S., 1995. Pathogenesis of porcine circovirus; experimental infections of colostrum deprived piglets and examination of pig foetal material. Vet. Microbiol. 44, 49–64.
Allan, G.M., McNeilly, F., Kennedy, S., Daft, B., Clarke, E.G., Ellis, J.A., Haines, D.M., Meehan, B.M., Adair, B.M., 1998. Isolation of porcine circovirus-like viruses from pigs with a wasting disease in the USA and Europe. J. Vet. Diagn. Investig. 10, 3–10.
Armstrong, D., Bishop, S., 2004. Does genetics or litter effect influence mortality in PMWS? Proceedings of the 18th International Pig Veterinary Society Congress, Hamburg, Germany, p. 809.
Baruchel, S., Wainberg, M.A., 1992. The role of oxidative stress in disease progression in individuals infected by the human immunodeficiency virus. J. Leukoc. Biol. 52, 111–114.
Bouzar, A.B., Boxus, M., Florins, A., Francois, C., Reichert, M., Willems, L., 2009. Reduced levels of reactive oxygen species correlate with inhibition of apoptosis, rise in thioredoxin expression and increased bovine leukemia virus proviral loads. Retrovirology 6, 102.
Cai, J., Chen, Y., Seth, S., Furukawa, S., Compans, R.W., Jones, D.P., 2003. Inhibition of influenza infection by glutathione. Free Radic. Biol. Med. 34, 928–936.
Casola, A., Burger, N., Liu, T., Jamaluddin, M., Brasier, A.R., Garofalo, R.P., 2001. Oxidant tone regulates RANTES gene expression in airway epithelial cells infected with respiratory syncytial virus. Role in viral-induced interferon regulatory factor activation. J. Biol. Chem. 276, 19715–19722.
Choi, C., Chae, C., Clark, E.G., 2000. Porcine postweaning multisystemic wasting syndrome in Korean pig: detection of porcine circovirus 2 infection by immunohistochemistry and polymerase chain reaction. J. Vet. Diagn. Investig. 12, 151–153.
Ciriolo, M.R., Palamara, A.T., Incerpi, S., Lafavia, E., Bue, M.C., De Vito, P., Garaci, E., Rotilio, G., 1997. Loss of GSH, oxidative stress, and decrease of intracellular pH as sequential steps in viral infection. J. Biol. Chem. 272, 2700–2708.
Clark, E., 1997. Postweaning multisystemic wasting syndrome. Proceedings of the Annual Meeting of the American Association of Swine Practitioners, Quebec City, Canada, pp. 499–501.

Darwich, L., Segales, J., Mateu, E., 2004. Pathogenesis of postweaning multisystemic wasting syndrome caused by Porcine circovirus 2: an immune riddle. Arch. Virol. 149, 857–874.

Fenaux, M., Halbur, P.G., Gill, M., Toth, T.E., Meng, X.J., 2000. Genetic characterization of type 2 porcine circovirus (PCV-2) from pigs with postweaning multisystemic wasting syndrome in different geographic regions of North America and development of a differential PCR-restriction fragment length polymorphism assay to detect and differentiate between infections with PCV-1 and PCV-2. J. Clin. Microbiol. 38, 2494–2503.

Flores, S.C., Marecki, J.C., Harper, K.P., Bose, S.K., Nelson, S.K., McCord, J.M., 1993. Tat protein of human immunodeficiency virus type 1 represses expression of manganese superoxide dismutase in HeLa cells. Proc. Natl. Acad. Sci. U. S. A. 90, 7632–7636.

Garaci, E., Palamara, A.T., Ciriolo, M.R., D'Agostini, C., Abdel-Latif, M.S., Aquaro, S., Lafavia, E., Rotilio, G., 1997. Intracellular GSH content and HIV replication in human macrophages. J. Leukoc. Biol. 62, 54–59.

Gillespie, J., Opriessnig, T., Meng, X.J., Pelzer, K., Buechner-Maxwell, V., 2009. Porcine circovirus type 2 and porcine circovirus-associated disease. J. Vet. Intern. Med. 23, 1151–1163.

Grau-Roma, L., Fraile, L., Segales, J., 2011. Recent advances in the epidemiology, diagnosis and control of diseases caused by porcine circovirus type 2. Vet. J. 187, 23–32.

Hernandez-Garcia, D., Wood, C.D., Castro-Obregon, S., Covarrubias, L., 2010. Reactive oxygen species: a radical role in development? Free Radic. Biol. Med. 49, 130–143.

Jamaluddin, M., Tian, B., Boldogh, I., Garofalo, R.P., Brasier, A.R., 2009. Respiratory syncytial virus infection induces a reactive oxygen species-MSK1-phospho-Ser-276 RelA pathway required for cytokine expression. J. Virol. 83, 10605–10615.

Korenaga, M., Wang, T., Li, Y., Showalter, L.A., Chan, T., Sun, J., Weinman, S.A., 2005. Hepatitis C virus core protein inhibits mitochondrial electron transport and increases reactive oxygen species (ROS) production. J. Biol. Chem. 280, 37481–37488.

Macchia, I., Palamara, A.T., Bue, C., Savini, P., Ciriolo, M., Gaziano, R., di Francesco, P., 1999. Increased replication of Sendai virus in morphine-treated epithelial cells: evidence for the involvement of the intracellular levels of glutathione. Int. J. Immunopharmacol. 21, 185–193.

Mankertz, A., Domingo, M., Folch, J.M., LeCann, P., Jestin, A., Segales, J., Chmielewicz, B., Plana-Duran, J., Soike, D., 2000. Characterisation of PCV-2 isolates from Spain, Germany and France. Virus Res. 66, 65–77.

Michalek, R.D., Pellom, S.T., Holbrook, B.C., Grayson, J.M., 2008. The requirement of reactive oxygen intermediates for lymphocytic choriomeningitis virus binding and growth. Virology 379, 205–212.

Morgan, M.J., Liu, Z.G., 2011. Crosstalk of reactive oxygen species and NF-kappaB signaling. Cell Res 21, 103–115.

Nakamura, H., Masutani, H., Yodoi, J., 2002. Redox imbalance and its control in HIV infection. Antioxid. Redox Signal. 4, 455–464.

Palamara, A.T., Perno, C.F., Ciriolo, M.R., Dini, L., Balestra, E., D'Agostini, C., Di Francesco, P., Favalli, C., Rotilio, G., Garaci, E., 1995. Evidence for antiviral activity of glutathione: in vitro inhibition of herpes simplex virus type 1 replication. Antiviral Res 27, 237–253.

Pan, Q., Huang, K., He, K., Lu, F., 2008. Effect of different selenium sources and levels on porcine circovirus type 2 replication in vitro. J. Trace Elem. Med. Biol. 22, 143–148.

Patterson, A.R., Opriessnig, T., 2010. Epidemiology and horizontal transmission of porcine circovirus type 2 (PCV2). Anim. Health Res. Rev. 11, 217–234.

Schafer, F.Q., Buettner, G.R., 2001. Redox environment of the cell as viewed through the redox state of the glutathione disulfide/glutathione couple. Free Radic. Biol. Med. 30, 1191–1212.

Seet, R.C., Lee, C.Y., Lim, E.C., Quek, A.M., Yeo, L.L., Huang, S.H., Halliwell, B., 2009. Oxidative damage in dengue fever. Free Radic. Biol. Med. 47, 375–380.

Segales, J., Domingo, M., 2002. Postweaning multisystemic wasting syndrome (PMWS) in pigs. A review. Vet. Q. 24, 109–124.

Segales, J., Domingo, M., Chianini, F., Majo, N., Dominguez, J., Darwich, L., Mateu, E., 2004. Immunosuppression in postweaning multisystemic wasting syndrome affected pigs. Vet. Microbiol. 98, 151–158.

Sgarbanti, R., Nencioni, L., Amatore, D., Coluccio, P., Fraternale, A., Sale, P., Mammola, C.L., Carpino, G., Gaudio, E., Magnani, M., Ciriolo, M.R., Garaci, E., Palamara, A.T., 2011. Redox regulation of the influenza hemagglutinin maturation process: a new cell-mediated strategy for anti-influenza therapy. Antioxid. Redox Signal. 15, 593–606.

Smith, A.D., Dawson, H., 2006. Glutathione is required for efficient production of infectious picornavirus virions. Virology 353, 258–267.

Staal, F.J., Roederer, M., Herzenberg, L.A., 1990. Intracellular thiols regulate activation of nuclear factor kappa B and transcription of human immunodeficiency virus. Proc. Natl. Acad. Sci. U. S. A. 87, 9943–9947.

Tischer, I., Gelderblom, H., Vettermann, W., Koch, M.A., 1982. A very small porcine virus with circular single-stranded DNA. Nature 295, 64–66.

Todd, D., Biagini, P., Bendinelli, M., Hino, S., Mankertz, A., 2005. Circoviridae. In: Fauquet CM, M.M., Maniloff, J., Desselberger, U., Ball, L.A. (Eds.), Virus Taxonomy, VIIIth Report of the International Committee for the Taxonomy of Viruses, London, pp. 327–334.

Wang, Y., Oberley, L.W., Murhammer, D.W., 2001. Evidence of oxidative stress following the viral infection of two lepidopteran insect cell lines. Free Radic. Biol. Med. 31, 1448–1455.

Waris, G., Turkson, J., Hassanein, T., Siddiqui, A., 2005. Hepatitis C virus (HCV) constitutively activates STAT-3 via oxidative stress: role of STAT-3 in HCV replication. J. Virol. 79, 1569–1580.

Wei, L., Kwang, J., Wang, J., Shi, L., Yang, B., Li, Y., Liu, J., 2008. Porcine circovirus type 2 induces the activation of nuclear factor kappa B by IkappaBalpha degradation. Virology 378, 177–184.