The NS1 protein of avian influenza virus H9N2 induces oxidative-stress-mediated chicken oviduct epithelial cells apoptosis

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The pathogenesis of H9N2 subtype avian influenza virus infection (AIV) in hens is often related to oviduct tissue damage. The viral non-structural NS1 protein is thought to play a key role in regulating the pathogenicity of AIV, but its exact function in this process remains elusive. In this study, the pro-apoptosis effect of H9N2 NS1 protein was examined on chicken oviduct epithelial cells (COECs) and our data indicated that NS1-induced oxidative stress was a contributing factor in apoptosis. Our data indicate that NS1 protein level was correlated with reactive oxygen species (ROS) in COECs transfected with NS1 expression plasmids. Interestingly, decreased activities of antioxidant enzymes, superoxide dismutase and catalase, were observed in NS1-transfected COECs. Treatment of COECs with antioxidants, such as pyrrolidine dithiocarbamate (PDTC) or N-acetylcysteine (NAC), significantly inhibited NS1-induced apoptosis. Moreover, although antioxidant treatment has little effect on the activation of caspase-8 in NS1-transfected cells, the activation of caspase-3/9 and Bax/Bcl-2 were significantly downregulated. Taken together, the results of our study demonstrated that expression of H9N2 NS1 alone is sufficient to trigger oxidative stress in COECs. Additionally, NS1 protein can induce cellular apoptosis via activating ROS accumulation and mitochondria-mediated apoptotic signalling in COECs.

INTRODUCTION

H9N2 subtype avian influenza virus (AIV) has been widespread in domestic poultry in Asian countries since the mid-1990s with a mortality rate ranging from 5% to 30%. H9N2 subtype AIV has been classified as low-pathogenicity avian influenza (LPAI) based on molecular characterization and pathotyping (Abolnik et al., 2007). It is known that apoptosis and hyperinduction of inflammatory cytokines caused by AIV in the respiratory and digestive tracts are the major causes of respiratory and digestive failure, respectively (Bi et al., 2010; Kim et al., 2006). Although extensive efforts have been made to understand influenza virus infection in mammalian species, little is known about AIV infection in the oviducts of egg-laying hens. The non-structural protein 1 (NS1) of influenza A virus is considered to be a determining factor for viral virulence and plays key roles in promoting the expression of viral proteins, modulating the growth and metabolism of the host cells, as well as contributing to the pathogenicity and virulence of the virus (Hale et al., 2008; Seo et al., 2002). Moreover, NS1 protein has been shown to regulate host innate/adaptive immune responses along with a variety of cellular signalling pathways, including apoptosis (Han et al., 2012; Yan et al., 2016; Zhang et al., 2015). In our previous study we identified the oviduct as the potential target for the H9N2 subtype AIV virus and showed that H9N2 AIV infection in the oviduct resulted in excessive apoptosis and tissue damage (Wang et al., 2015a, b). However, the mechanism underlying the promotion of apoptosis by H9N2 NS1 during viral infection is still unclear.

Reactive oxygen species (ROS) are constantly produced during normal cell metabolism and are involved in a variety of cellular pathways. Regulation of ROS levels and maintenance of redox homeostasis is necessary to control cell metabolism. ROS readily interact with a large number of molecules, including small inorganic molecules as well as proteins, lipids, carbohydrates and nucleic acids. ROS may irreversibly alter the functions of the target molecule through such interactions. Therefore, oxidative stress caused by ROS could result in damage to organelles. However, various cellular enzymes, such as superoxide dismutases (SODs), catalase and glutathione peroxidase, act as antioxidants against oxidative damage to maintain cellular redox homeostasis. Accumulating evidence indicates that intracellular ROS may lead to various consequences for the cell depending on their concentration. At normal physiological levels, they can be beneficial to the cell and organisms by functioning as the ‘redox messengers’ of cellular signalling pathways. However ROS, including superoxide anion...
and its derivatives, are indiscriminately toxic to cells when excessively produced (Bensaad & Vousden, 2005; Circu & Aw, 2010). It has been reported that viral infections, including hepatitis C virus (HCV), herpes simplex virus (HSV), respiratory syncytial virus (RSV) and influenza virus, induce the generation of excess ROS (Akaike et al., 1996; De Mochel et al., 2010; Gonzalez-Dosal et al., 2011).

In this study, the NS1 protein from chickens obtained from H9N2 AIV (A/Chicken/shaanxi/01/2011) was shown to induce ROS production and lead to oxidative stress in primary chicken oviduct epithelial cells (COECs). Moreover, disruption of the cellular redox balance by NS1 caused apoptosis in COECs via a mitochondria-dependent pathway.

RESULTS

Purification and transfection of COECs

The purification of primary cells was confirmed by the presence of intermediate filaments as well as cell morphology. As shown in Fig. 1(a), after 72 h of in vitro culturing, the primary COECs formed monolayers of polyhedral appearance. Immunostaining indicated that the COECs were stained by the epithelium-specific cytokeratin antibody (Fig. 1a). In addition, the NS1 gene transfection efficiency was analysed by EGFP gene expression. Fluorescence microscopy analysis demonstrated that the transfection efficiency for NS1 in COECs was 75% at 48 h post-transfection (Fig. 1b). To access the expression of NS1 protein in COECs after transfection, Western blot analysis showed that the influenza A NS1 antibody specifically recognized a 55 kDa protein, matching the predicted size of the H9N2 NS1 protein (Fig. 1c). Furthermore, Western blot analysis showed a time-dependent expression level of NS1 protein in homogenates from primary COECs after transfection (Fig. 1c).

NS1-induced time-dependent apoptosis and enhanced viral replication in COECs

To examine whether apoptosis was initiated in COECs transfected with pEGFP-NS1, flow cytometry measuring apoptotic markers for early apoptosis (Annexin-V-PE) or late apoptosis (7-AAD) was carried out. The cells were collected for analysis at the indicated time points post-transfection. As shown in Fig. 2(a, b), the apoptosis rate was significantly increased in NS1-transfected cells compared with pEGFP-C1-transfected cells at all examined time points (P<0.05). Interestingly, a time-dependent increase in the apoptosis rate in COECs transfected with pEGFP-NS1 was detected (Fig. 2b). Additionally, we observed that viral replication increased in a time-dependent manner both in pEGFP-C1- and pEGFP-NS1-transfected COECs infected with H9N2 AIV. In particular, virus yields in pEGFP-NS1-transfected COECs were significantly higher compared with those of cells transfected with pEGFP-C1 at 24, 36 and 48 h post-transfection (Fig. 2c).

NS1-induced time-dependent ROS accumulation in COECs

To investigate the role of NS1 protein in inducing ROS accumulation in COECs, a DHE-based assay was used to examine the superoxide activity in COECs transfected with pEGFP-NS1 or with pEGFP-C1. The results indicated that the intensity of fluorescence was increased in NS1-transfected cells compared with control cells transfected with

![Fig. 1. Immunofluorescence and Western blot analysis of NS1-transfected chicken oviduct epithelial cells (COECs). (a) Phase-contrast microscopy (left panel) or immunocytochemical staining with the use of mouse monoclonal antibodies directed against intermediate proteins (right panel) showing the cobblestone structure and expression of cytokeratin 18 in primary COECs. (b) Fluorescence microscopic images of COECs transfected with pEGFP-NS1. Scale bars, 500 µm. (c) Western blot analysis showing time-dependent NS1 protein expression levels in NS1-transfected COECs. β-actin was used as a loading control.](image)
pEGFP-C1 at all time points examined, especially at 48 h post-transfection (Fig. 3a). To further evaluate the ROS level, quantitative analysis of ROS in NS1-transfected COECs was performed using flow cytometry. As shown in Fig. 3(b, c), ROS was significantly accumulated at all tested time points compared with control cells in a time-dependent manner ($P<0.05$).

**Effect of NS1 on enzymatic antioxidant activities in COECs**

As shown in Fig. 4(a, b), NS1-transfected COECs had a significant decrease in catalase ($P<0.05$) and SOD activities ($P<0.05$) at 24, 36 and 48 h post-transfection compared with the control cells. Interestingly, a modest decrease in enzymatic antioxidant activity was detected in NS1-transfected COECs along with the transfection time course (Fig. 4a, b).

**The role of ROS in NS1-induced apoptosis in COECs**

To explore the effect of ROS on NS1-inducing apoptosis in COECs, we used ROS antioxidants pyrrolidine dithiocarbamate (PDTC, 100 µM, Merck) and N-acetylcysteine (NAC, 100 µM, Sigma-Aldrich) to suppress ROS production in NS1-transfected COECs and to evaluate the numbers of cells undergoing apoptosis. Both PDTC and NAC treatment significantly decreased the percentage of apoptosis in NS1-transfected COECs compared with untreated NS1-transfected cells (Fig. 5a, b). These results indicated that NS1...
protein induced apoptosis in COECs through ROS accumulation.

To further investigate the role of ROS accumulation in apoptosis, we analysed the activities of several key caspases, including caspase-3, -8 and -9, protein level of Bcl-2 and translocation of Bax, as well as mitochondrial membrane potential in NS1-transfected COECs treated with PDTC and NAC at 48 h post-transfection. The results indicated that the caspase-3, -8 and -9 were significantly activated in NS1-transfected COECs compared with the controls. However, the activities of caspase-3 and -9 were significantly decreased in NS1-transfected COECs treated with PDTC or NAC compared with COECs solely transfected with NS1 (P<0.05, Fig. 6a, b). It is notable that treatment of PDTC or NAC has no significant effect on the activity of caspase-8 in NS1-transfected COECs (Fig. 6a, b). Moreover, decreased expression of Bcl-2, increased Bax expression, as well as the increased ratio of Bax:Bcl-2 in the NS1-transfected COECs at 48 h post-transfection, were blocked by PDTC and NAC treatment (P<0.05, Fig. 6c, d).

Assessment of mitochondrial membrane potential showed that both PDTC and NAC significantly reduced Δψm in NS1-transfected COECs at 48 h post-transfection (P<0.05, Fig. 6e).

**DISCUSSION**

It is well known that egg-laying hens infected with H9N2 AIV show symptoms of mild bleeding in the respiratory, digestive and reproductive tracts (Kwon et al., 2008; Pantin-Jackwood et al., 2012; Zhang et al., 2008). Our *in vivo* experiments have verified that H9N2 AIV replicates in the oviducts of egg-laying hens and that this results in tissue damage (Qi et al., 2016; Wang et al., 2015a, b).

NS1 is the only influenza virus protein that has been shown to be both pro- and anti-apoptotic in infected cells (Han et al., 2012; Yan et al., 2016; Zhang et al., 2008). The contrasting data may be a result of differences in the influenza virus subtypes and strains as well as in the host cell system.

![Fig. 3. The levels of ROS in NS1-transfected COECs. (a) Observation of ROS production in NS1-transfected and control COECs. Cells were transfected with H9N2 NS1 and harvested at 12, 24, 36 and 48 h post-transfection. Then the cells were stained with DHE and observed under a fluorescence microscope. (b) Representative flow cytometry histograms showing the relative ROS level of cells transfected with NS1 at 12, 24, 36 and 48 h post-transfection. (c) The levels of ROS were calculated from the fluorescence intensity in NS1-transfected cells by subtracting the fluorescence intensity in control cells. Columns and vertical bars represent the mean±SEM from three independent experiments. *P<0.05, ** P<0.01 versus the control cells at the same time point. Scale bar, 50 µm.](image-url)
being used in the experiments (Ehrhardt et al., 2007; Lam et al., 2008; Shin et al., 2007). It has been reported that the expression of H9N2 NS1 correlated with the induction of Fas-mediated apoptosis in chicken macrophages (Lam et al., 2011; Xing et al., 2009). However, it is still unclear whether apoptosis is due to direct or indirect effects of NS1 and how these changes are mediated by oxidative stress remains to be confirmed in other cell types. In this study, we found that H9N2 NS1 transfection induced apoptosis of COECs via activating a caspase-9-mediated pathway and that NS1-induced intracellular ROS accumulation was involved in promoting apoptosis. Although the results of our previous study confirmed that H9N2 AIV infections in egg-laying hens are often accompanied by a high viral load, excessive cellular apoptosis and tissue damage in the oviduct (Wang et al., 2015b), the effects of NS1 on apoptosis and H9N2 avian influenza virus replication in COECs remain largely unknown. In this study, flow cytometry analysis revealed that transfection of NS1 induced time-dependent apoptosis in COECs. Interestingly, the apoptosis rate was significantly increased in NS1-transfected cells compared with pEGFP-C1-transfected cells at all examined time points. Furthermore, virus load checking confirmed a significant increase in progeny virus yields in H9N2-infected COECs transfected with pEGFP-NS1 compared with those transfected with pEGFP-C1.

Accumulating data have demonstrated that oxidative stress is an important regulatory factor of virus-induced apoptosis and could be triggered by various factors (Kim & Wong, 2013, 2015). Intracellular ROS may lead to various consequences depending on their concentration. Any imbalance in the production and elimination of ROS is often referred to as

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**Fig. 4.** The activities of enzymatic antioxidant in NS1-transfected COECs. The activities of catalase (a) or superoxide dismutase (SOD, b) in NS1-transfected and control COECs were tested using activity assay kits. Columns and vertical bars represent the mean±SEM from three independent experiments. *P<0.05, **P<0.01 versus control cells.

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**Fig. 5.** Involvement of ROS production in NS1-induced apoptosis in COECs. (a) Representative flow cytometry histograms showing the analysis of apoptosis in NS1-transfected or control COECs in the presence or absence of antioxidant at 48 h post-transfection. (b) Analysis of flow cytometry data obtained from three independent experiments. Columns and vertical bars represent the mean±SEM from three independent experiments. *P<0.05, **P<0.01 versus control cells.
oxidative stress. Excessive ROS production in a cell can lead to the oxidation of macromolecules. During influenza virus infection, ROS often emerges due to disturbance of redox balance (Peterhans et al., 1987). In this study, we explored whether expression of H9N2 NS1 alone is sufficient to induce ROS production in COECs. The immunofluorescence and flow cytometry data revealed that intracellular ROS levels in NS1-transfected COECs were increased in a time-dependent manner after transfection and were significantly higher compared with those of controls at the tested time points. Interestingly, flow cytometry analysis indicated significantly higher ROS levels starting from the time NS1 became detectable in transfected COECs by Western blot analysis. Given that a time-dependent increase in NS1 was observed in transfected COECs, these results indicate that H9N2 NS1 protein alone can induce dose-dependent ROS upregulation in COECs.

To prevent oxidative stress, various cellular enzymes, such as superoxide dismutase (SOD), catalase and glutathione peroxidase, act as antioxidants against oxidative damage and maintain cellular redox homeostasis (Dimayuga et al., 2007; Pyo et al., 2014). Analysis of the antioxidant content revealed an overall decrease in the concentration of antioxidants during infection (Pyo et al., 2014). It has been previously shown that H5N1 AIV infection increases the mRNA level of NADPH oxidases but reduces the mRNA and protein levels of SOD in human alveolar epithelial cell line A549 (Lin et al., 2016). A further knockdown of SOD led to initiation of apoptosis in T-cells and resulted in prevention of the activation of adaptive immune response and increased susceptibility of the host to the influenza A virus (Lin et al., 2016). In this study, we found a time-dependent decrease in intracellular antioxidant activity in parallel with a gradual increase in NS1 expression and the protein levels of NS1 in the transfected COECs. These results further support the hypothesis that oxidative damage is related to the amount of H9N2 NS1 expressed in COECs. Results of previous in vitro studies demonstrated that influenza virus-induced oxidative stress appeared to be directly related to viral gene expression. PB1-F2, the key virulence factor of influenza A virus, was found to be responsible, at least in part, for the ROS generation through lowering the SOD level in alveolar epithelial A549 cells (Shin et al., 2015). However, NS1 did not trigger significant changes in SOD levels and ROS production in A549 cells (Shin et al., 2015). The contrasting data may be attributed to the differences between the influenza virus subtypes and strains as well as the host cell systems being used in the experiments. In addition, this does not rule out the possible involvement of other virulence factors via different mechanisms in infected COECs.

Although results from a number of studies have demonstrated that viruses induce the generation of ROS and alter

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**Fig. 6.** The effects of ROS induction on the activity of caspase and Bcl-2/Bax as well as mitochondrial membrane potential (ΔΨm) changes in NS1-transfected COECs. (a) The activities of caspase-3, -8 or -9 in NS1-transfected and control COECs in the presence or absence of antioxidant at 48 h post-transfection were tested using colorimetric assay kits. (b) Western blot analysis of caspase-3, -8 or -9 levels in NS1-transfected COECs in the presence or absence of antioxidant at 48 h post-transfection. (c) The expression of Bcl-2 and Bax levels in NS1-transfected COECs was analysed by Western blot assay in the presence or absence of antioxidant at 48 h post-transfection. Actin was used as a loading control. (d) The ratio of Bax : Bcl-2 in the presence or absence of antioxidant at 48 h post-transfection were tested using colorimetric assay kits. (e) The changes in mitochondrial membrane potential (ΔΨm) in NS1-transfected COECs in the presence or absence of antioxidant at 48 h post transfection were assessed using a JC-1 Mitochondrial Potential Detection Kit. *P<0.05.
antioxidant levels, the exact molecular mechanism of influenza-virus-induced decreased activity of antioxidant enzyme has not been clearly identified. The transcription factor Sp1, which is a major element of the proximal region of the SOD promoter, was slightly downregulated at the transcriptional level during IAV infection, and subsequently modulated by post-translational control (Pyo et al., 2014). Further studies are needed to determine whether N1-mediated downregulation of SP1 results in depletion of the major antioxidant SOD.

ROS are well-established activators of cellular apoptosis pathways and are probably responsible for the influenza-virus-induced apoptosis in a caspase-dependent manner. (Lin et al., 2016; Scherz-Shouval & Elazar, 2007). Consistent with the role of ROS in N1-induced apoptosis in COECs, the antioxidant treatment of PDTC or NAC was able to protect cells from N1-induced apoptosis. However, as the application of antioxidants at the concentrations used in our experiments did not completely protect the cells from apoptosis, it appears that N1-induced apoptosis in infected cells may involve other uncharacterized mechanisms acting in parallel with ROS.

Two major apoptosis signalling pathways involve both upstream initiator caspases (caspase-8 and caspase-9) and downstream effectors (caspase-3 and caspase-7) (Iwai et al., 2013; Wurzer et al., 2004). Caspase-3 is an extensively investigated effector caspase and considered to be a major player in apoptosis regulation (Sanghavi et al., 1998). In this study, caspase activity detection and Western blot analysis revealed significantly increased caspase-3/8/9 activation in N1-transfected COECs compared with controls. It is notable that treatment with PDTC or NAC has no significant effect on the activation of caspase-8 in N1-transfected cells while caspase-9/3 activation was significantly downregulated in response to the antioxidant treatment. Caspase-9 is known to be an indicator of the mitochondrion-mediated apoptosis pathway (Marsden et al., 2002). Moreover, increasing evidence has demonstrated that Bcl-2 family proteins, including Bcl-2 and Bax, regulate the mitochondria-associated pathway (Sharpe et al., 2004). Here, we also observed a significantly decreased ratio of Bax:Bcl-2 and reduced mitochondrial membrane potential in the N1-transfected COECs treated with PDTC and NAC. Taken together, these results clearly indicate that N1-protein-dependent ROS accumulation induced the apoptosis of COECs via the mitochondria-mediated pathway. However, the mechanism of N1-induced ROS accumulation and its effect on replication of avian influenza A virus, as well as the functional domains of N1 involved in this regulation, need further investigation.

Collectively, we provide evidence that expression of H9N2 NS1 alone is sufficient to trigger oxidative stress in COECs. NS1 protein can induce apoptosis of COECs via activating ROS accumulation and mitochondria-mediated apoptotic signalling.

**METHODS**

**Virus.** The virus strain used in this study, H9N2 subtype AIV strain (A/Chicken/shaanxi/01/2011), was isolated from a diseased chicken in Shaanxi, China and propagated in 10-day-old embryonated chicken eggs (ECs) at 37 °C for 72 h as previously described (Qi et al., 2016; Wang et al., 2013).

**Isolation and primary culture of COECs.** Primary COECs were isolated as described previously (Ebers et al., 2009), with slight modifications. The oviduct tissues of 10-week-old layer hens (single comb, white leghorn) were obtained from the poultry farm of Northwest A&F University. The isthmal portion of the oviduct was removed, flushed thoroughly with HBSS (Sigma-Aldrich) containing 200 U penicillin ml⁻¹ (Sigma-Aldrich) and 200 mg streptomycin ml⁻¹ (Sigma-Aldrich). The epithelial cells were gently scrapped off the tissue and treated with 20 ml HBSS containing 1 mg collagenase ml⁻¹ (Sigma-Aldrich) for 30 min at 37 °C. After collagenase treatment, the supernatant was discarded and digestion of tissue fragments was done using 0.25% trypsin and 3 mM EDTA in 20 ml HBSS for 10 min at 37 °C. The cell suspension was stop to 10% in heat-inactivated FBS (Thermo Fisher Scientific) to stop the activity of trypsin. The cell suspension was then passed through a cell filter (100 µM) in order to remove any undigested tissue. The epithelial cells were centrifuged as 50 g for 5 min to separate epithelial cells from erythrocytes, platelets and other immune cells. The supernatant was discarded after centrifugation and the pellet containing epithelial cells was re-suspended in minimal essential medium (MEM, Thermo Fisher Scientific) supplemented with 10% FBS and 2% heat-inactivated chicken serum (HICS; Gibco, Thermo Fisher Scientific), along with insulin (0.12 U ml⁻¹, Sigma-Aldrich) and oestradiol (50 nM, Sigma-Aldrich). The COECs were incubated in a tissue culture flask for 2 h at 39 °C in 5% CO₂ to allow fibroblast attachment. Following incubation, the unattached epithelial cells were collected by gentle pipetting followed by centrifugation at 125 g for 10 min. The pelleted epithelial cells were resuspended in whole medium and allowed to grow until a monolayer was formed. After four passages, the cells were seeded onto 24-well cell culture plates (2×10⁵ cells per well), and grown at 39 °C under 5% CO₂ for 24–36 h. The identification of COECs was confirmed by determining the expression of cytokeratin as previously described (Ebers et al., 2009).

**Vector construction and transfection.** Total RNA was extracted from the cell lysates of ECs infected with avian H9N2 influenza virus strain using Trizol reagent (Thermo Fisher Scientific). Full-length NS1 cDNA was amplified by using a first-strand cDNA synthesis kit (Takara Bio). The positive PCR products were ligated into the pMD18-T vector (Takara Bio), and then sub-cloned into EcoRI/BamHI sites of pEGFP-C1 (Clontech). The insertion of the NS1 coding region was confirmed by DNA sequencing, and the recombinant vector was named pEGFP-NS1.

For transfection experiments, COECs were seeded on six-well plates with 2.5×10⁶ cells per well and cultured until they reached 80% confluence 24 h after seeding. Transfection of plasmids was conducted using TurboFect Transfection Reagent (Thermo Fisher Scientific) according to the manufacturer’s instructions. The COECs transfected with pEGFP-C1 or pEGFP-NS1 were harvested at 12, 24, 36 and 48 h post-transfection. The NS1 gene transfection efficiency was observed using an inverted fluorescence microscope. In specified experiments, the media was changed to DMEM containing the ROS antioxidants pyrrolidine dithiocarbamate (PDTC, 100 µM, Merck) and N-acetylcysteine (NAC, 100 µM, Sigma-Aldrich) before transfection, and cells were harvested at 48 h after transfection. Neither PDTC nor NAC showed cytotoxicity at the concentrations used (data not shown).
Immunofluorescence. Cells subjected to the indicated treatments were fixed with 4% paraformaldehyde and washed with phosphate-buffered saline (PBS, TransGen Biotech, China). Following permeabilization with 0.2% Triton X-100 in PBS, the cells were then blocked with 5% BSA in PBS for 30 min and incubated overnight with mouse anti-cytokeratin 18 (1:1000; Santa Cruz Biotech) or mouse anti-influenza A NS1 (1:500; Santa Cruz Biotech) as primary antibodies in blocking buffer. After washing the sample with PBS three times, the reactions of primary antibodies and target proteins were visualized using FITC-conjugated goat anti-mouse antibody (1:500) diluted in 5% BSA + 5% heat-inactivated goat serum in PBS. The cell nuclei were visualized using 4', 6-diamidino-2-phenylindole (DAPI; Santa Cruz Biotech). The stained cells were monitored under a TCS NT confocal microscope (Leica). The images obtained with the confocal microscope were further processed and merged using ImageJ software.

Western blot. Cells subjected to the indicated treatments were washed once with pre-cooled PBS and lysed in Tris lysis buffer (Cell Signaling Technology) containing 1% EDTA-free protease inhibitor (Roche) in ice bath for 30 min. The cells were then briefly sonicated and cleared via centrifugation at 15 500 g for 10 min at 4°C. The supernatant was subsequently denatured by boiling for 5 min in SDS loading buffer. An equal amount of protein was subjected to SDS-PAGE and then transferred to nitrocellulose membranes (Whatman). After the membranes were blocked in Tris-buffered saline with Tween (TBST) containing 1% BSA, the membranes were incubated with different primary antibodies for 2 h at room temperature (RT). The membranes were then washed with TBST three times and reacted with the corresponding HRP-conjugated secondary antibodies for 1 h at RT. The blots were visualized using an Immobilon Western chemiluminescent HRP substrate kit (Thermo Fisher Scientific) in an ECL detection system (Amersham Biosciences).

TCID50 assay. Total virus yields (intracellular and extracellular viruses) were determined by the microtitration infectivity assay, calculated using the Reed–Muench methods (Reed & Muench, 1938), and recorded as TCID50 ml−1. Briefly, the COECs transfected with pEGFP-C1 or pEGFP-NS1 cultivated in 96-well plates were inoculated with H9N2 AIV dilutions (100 µl well−1) prepared by serial 10-fold dilution. After adsorption for 1 h at 37°C, culture supernatants were removed and DMEM with 2% FBS was added to the wells. Plates were harvested at 12, 24, 36 and 48 h post-infection. Finally, virus titres were determined by calculating the presence of a visible CPE in the wells with different dilutions.

ROS determination. Dihydroethidium (DHE, Molecular Probes, Invitrogen) fluorescent probe was used for detecting generation of ROS. Briefly, COECs were seeded into 24-well plates and transfected with pEGFP-NS1 or empty vector. Then, the cells were stained with 10 µM DHE fluorescent probe in a dark room at 12, 24, 36 and 48 h post-transfection and visualised using a fluorescence microscope (Olympus).

Determination of catalase activity. Differently treated cells were homogenized in cold PBS with 1 mM EDTA, centrifuged at 10 000 g for 15 min at 4°C and the supernatant was collected. The Oxiselect Catalase Activity Assay Kit (Cell Biolabs) was used to determine catalase activity in samples. Briefly, 20 µl of the diluted catalase standard or sample and 50 µl of the H2O2 working solution (12 mM) were added to a 96-well microplate. The samples were then mixed and incubated for 1 min. The reaction was stopped by adding 50 µl of the catalase quencher to each well, 5 µl from each reaction well was transferred to a fresh well, and 250 µl of the chromogenic working solution was added to each well. The samples were then mixed well and incubated for 60 min. The A520 was evaluated by the spectrophotometric microplate reader (Thermo Fisher Scientific).

Determination of SOD activity. Differently treated cells were homogenized in cold 1× lysis buffer (containing 10 mM Tris, pH 7.5, 150 mM NaCl and 0.1 mM EDTA), centrifuged at 12 000 g for 10 min and the supernatant was collected for analysis. The Oxiselect SOD Activity Assay Kit (Cell Biolabs, Cat No.: STA-340) was used for evaluating SOD activity. Basically, 20 µl samples, 5 µl xanthine solution, 5 µl chromagen solution, 5 µl 10× SOD assay buffer and 50 µl deionized water were added (total volume 90 µl) to a 96-well microplate, and then 10 µl pre-diluted 1× xanthine oxidase solution was added to each well. The samples were then mixed well and incubated for 1 h at 37°C. The A405 was measured using the spectrophotometric microplate reader (Thermo Fisher Scientific).

Flow cytometry. Cells transfected with empty vector or pEGFP-NS1 were harvested at the indicated times and washed twice with DMEM without FBS. The apoptosis of COECs transfected with pEGFP-C1 or pEGFP-NS1 at the indicated times post-transfection was determined using the Annexin V-PE/7-AAD kit (BD Biosciences) according to the manufacturer’s instructions. For ROS quantitative analysis, the cells were incubated with 10 µM DHE fluorescent probe at 37°C for 30 min in a light-proof environment and analysed using flow cytometry (EPICS ALTRA; Beckman Coulter). Cell apoptosis was also evaluated by using the PE Annexin V Apoptosis Detection Kit I (BD Biosciences) according to the manufacturer’s instructions. At least 1×10⁶ cells in each sample were analysed.

Caspase activity detection. The caspase activities were measured by colorimetric assay kit (BioVision) following the manufacturer’s manual. Briefly, the protein concentration was measured using BCA Protein Assay Reagent (Pierce). For each sample, 150 µg total protein was incubated with each caspase substrate in a 96-well plate for 4 h at 37°C. The A405 was measured with a spectrophotometer (Thermo Fisher Scientific).

Mitochondrial membrane potential (Δψm) assessment. The cells were harvested and stained with tetrachlorotetrabenzyloxymethylcarbocyanine iodide 1 (JC-1) for 15 min at room temperature in the dark. Then the Δψm was assessed using a JC-1 Mitochondrial Potential Detection Kit (Biotium) following the manufacturer’s protocol. The fluorescence signal was measured at 550 and 485 nm.

Statistical analysis. All data are presented as means ± SEM. Significant differences were analysed using a two-tailed Student’s t test. Values of $P<0.05$ were considered statistically significant. The image data shown in some figures (e.g. photographs of scanning electron microscope analysis) were selected from at least five independent experiments.

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REFERENCES


(TRAIL) and FAS/FASL is crucial for efficient influenza virus propagation.


