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Influenza A virus-induced autophagy contributes to enhancement of virus infectivity by SOD1 downregulation in alveolar epithelial cells

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ABSTRACT

Infection with influenza A virus (IAV) A/WSN/1933 (H1N1) causes oxidative stress and severe lung injury. We have demonstrated that the generation of reactive oxygen species (ROS) during IAV infection is tightly regulated by superoxide dismutase 1 (SOD1) and correlated with viral replication in alveolar epithelial cells. However, the molecular mechanism underlying SOD1 reduction during IAV infection is uncertain. Here we demonstrate that the autophagy pathway is activated by IAV infection and involved in enhanced ROS generation in the early phase of infection. We observed that IAV infection induced autophagic vacuolation, leading to autophagic degradation of cellular proteins, including the protease sensitive antioxidant SOD1. Silencing of the microtubule-associated protein 1A/1B-light chain 3 (LC3) gene in A549 cells supported the critical role of autophagy in the ROS increase. The decrease in viral titer and viral polymerase activity caused by LC3 silencing or the autophagy inhibitor clearly evidenced the involvement of autophagy in the control of ROS generation and viral infectivity. Therefore, we concluded that early stage IAV infection induces autophagic degradation of antioxidant enzyme SOD1, thereby contributing to increased ROS generation and viral infectivity in alveolar epithelial cells.

1. Introduction

Influenza A virus (IAV) is a highly infective and pathogenic virus, and causes severe respiratory illness. IAV has been known to induce the generation of excess reactive oxygen species (ROS) in infected host cells. ROS is normally formed as a natural byproduct of the metabolism with oxygen molecules, and has a variety of roles in cell signaling and homeostasis. However, ROS has often appeared due to the disruption of redox balance during times of environmental stress [1,2]. IAV infection has been identified as one of the major causes of ROS generation, which may lead to the activation of acute or sustained oxidative stress, resulting in significant damages to respiratory tissues [3].

In order to cope with the severe oxidative damage associated with IAV infection, cells utilize various cellular defense mechanisms, one of which could be detoxication using antioxidant enzymes. Superoxide dismutases (SODs), the major cellular antioxidant enzyme group, have been found to interfere with excess ROS generation in various diseases [4]. Although many aspects of ROS generation in response to a variety of environmental stresses remain to be investigated, the impact of oxidative stress on IAV infection is likely to benefit viruses at a certain stage of their life cycle. A previous study showed that IAV infection led to a decrease in SOD1 levels and its key regulator Sp1, resulting in down-regulation of SOD1 gene expression [5]. In addition to regulation at the transcriptional level, the significant shift to cleaved Sp1 and SOD1 indicates the involvement of post-translational modification including protein degradation. Interestingly, the enhancement of ROS levels by IAV-induced SOD1 down-regulation appeared to influence virus replication [5]. Antioxidant treatment or SOD1 gene overexpression in cells infected with IAV resulted in lower virus titration, thereby supporting a pivotal role of SOD1 in virus propagation. Although the mechanism underlying the down-regulation of Sp1 and SOD1 in IAV infection is not fully understood, alteration of SOD1 activity appeared to be strongly connected to virus replication and ROS control.

A possible control mechanism could be activation of the catabolic process [6]. In general, viruses that infect animal cells bring about cell destruction as a result of the high demands of viral replication. IAV is a cytolytic virus, and its infection indeed brings about the significant proteolytic degradation of cellular components and proteins. Proteasomal and lysosomal degradation are the

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two major degradation processes for proteins, and are involved in many important physiological events in eukaryotic cells [7]. Especially lysosomes play a critical role in the cellular defense against invading pathogens through autophagic degradation. Although autophagy is a fundamentally a self-degradative process essential for cellular maintenance [8,9], it may also be utilized by the virus to support its own propagation. In this study, we investigated whether the autophagy pathway activated by IAV could be involved in the enhancement of ROS generation and virus production.

2. Materials and methods

2.1. Cells and reagents

Human alveolar epithelial A549 cells were cultured in RPMI (Invitrogen) supplemented with 10% (v/v) FBS (Corrning) and antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin, Invitrogen) and maintained in a humidified incubator with 5% CO_2 at 37 °C. *N*-acetyl-cysteine (NAC), chloroquine diphosphate salt solid and 3-Methyladenine (3-MA) were purchased from Sigma.

2.2. Virus production

The recombinant influenza virus (A/WSN/1933) was generated by DNA transfection as described [10]. The set of 8 plasmids was generously provided by R.G. Webster (University of Tennessee, TN, USA). Titer of virus was determined on MDCK cells by plaque assay as described previously [11].

2.3. RNA preparation and RT-PCR

Cellular total RNAs were extracted using RNAiso reagent (TAKARA). RT-PCR was performed using reverse transcriptase according to manufacturer's instructions (Invitrogen). The primer set for SOD1 and GAPDH were synthesized by IDT (Coralville, IA, USA). The cDNAs were PCR-amplified by primers specific for the indicated genes. Each primer is as follows. Primers for SOD1 and GAPDH are as fallows. GAPDH (forward), 5′-AGAAGGCTGGGGCTCATTTG-3'; GA PDH (reverse); 5′-AGGGGCCATCCACAGTCTTC-3′, SOD1 (forward); 5′-ACAAAGATGGTGTGGCCGAT-3′, SOD1 (reverse); 5′- TGGGCGAT CCCAATTACACC -3'.

2.4. Western blot analysis

All procedures for western blot were performed as previously described [5]. Anti-bodies against SOD1 and LC3 were purchased from Cell Signaling. Anti-bodies against PB1 and actin were purchased from Santa Cruz Biotech.

2.5. Determination of superoxide anion

The level of superoxide anion was determined using the superoxide detection system (Enzo Life Sci.) following the manufacturer's instructions. 2×10^5 cells were seeded in 6 well plates. After staining cells with superoxide staining solution, samples were analyzed using a flow cytometer (FACS Caliber, Becton and Dickinson).

2.6. Transmission electron microscopy

Collected samples were fixed in 2.5% glutaraldehyde mixed with 2% paraformaldehyde solution (0.1 M cacodylate buffer, pH 7.0) for 1 h and 2% osmium tetroxide for 1 h at 4 °C. The samples were stained with 2% uranyl acetate, followed by dehydration with a graded acetone series and embedding in low-viscosity medium. Samples were sectioned at 60 nm using an ultramicrotome (MTXL, RMC, Tucson, AZ, USA). The sections were double stained with 2% uranyl acetate for 20 min and lead citrate for 10 min, then viewed with the aid of transmission electron microscopy operating at 120 kV (Tecnai 12, FEI, Netherlands; H-7600, Hitachi, Tokyo, Japan).

2.7. Immunofluorescence staining and confocal imaging

For immunofluorescence microscopy, A549 cells were grown on coverslips and infected with the virus. 24 h after infection, immunofluorescence assay was carried out as described elsewhere [12]. Images were analyzed with confocal laser scanning microscope (Carl Zeiss).

2.8. Viral polymerase assay

Polymerase I-based luciferase reporter plasmid (pPolI-Luc) was constructed as described [5]. For the influenza virus polymerase assay, cells were transfected with the pPolI-Luc reporter plasmid, *Renilla* luciferase plasmid (pRL-Luc), Flag-tagged SOD1 (pSOD1) or empty vector, and then subsequently infected with IAV. After incubation for 24 h, cell lysates were prepared, and the luciferase level was measured by dual-luciferase assay method with GLOMAX™ 20/20 luminometer (Promega).

3. Results

3.1. IAV infection induces ROS generation through SOD1 down-regulation involving lysosomal proteolysis

In previous studies, IAV is shown to alter the cellular ROS level by SOD1 reduction [5]. To investigate the mechanism of redox control in response to IAV infection, we carried out western blot and RT-PCR analysis after IAV infection at an MOI of 0.01 with different hours post infection (hpi) in alveolar epithelial A549 cells. As shown in Fig. 1A, both SOD1 protein and mRNA levels were gradually reduced with time post-infection. Interestingly, the decrease of SOD1 protein level occurred earlier than that of mRNA, indicating the involvement of protein degradation after infection (Fig. 1B).

Since the reduction of SOD1 levels might cause impaired redox balance in host cells, we analyzed the generation of superoxide anions in infected cells. As shown in Fig. 1C, the superoxide anion level in the infected cells was markedly elevated compared that in the uninfected cells. The IAV-induced decrease of SOD1 seemed to result in the accumulation of superoxide anions. Therefore, to verify the role of SOD1 in redox balance in response to IAV infection, we attempted to express the exogenous SOD1 gene to see whether it can reduce the level of superoxide anion. SOD1 expression diminished the level of the superoxide anion in infected cells but not as strongly as in the mock cells, 20.4% vs. 34.8%, respectively. Presumably high levels of exogenous SOD1 proteins might also be sequestered as soon as they were expressed in the infected cells. To verify involvement of the proteolytic activity upon IAV infection, we employed a lysosomal inhibitor, chloroquine, which impairs the breakdown of cellular proteins after entering the lysosome system. When chloroquine was treated, IAV-infected cells restored largely the SOD1 protein level (Fig. 1D). Taken together, these results imply that autophagy might be induced during the initiative stage of IAV infection, and its proteolytic activity influences the redox balance in host cells.

3.2. IAV infection induces autophagic vacuolation

Autophagy has been generally seen as a catabolic process for the