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Alteration of copper–zinc superoxide dismutase 1 expression by influenza A virus is correlated with virus replication

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ABSTRACT

Viruses have evolved mechanisms designated to potentiate virus replication by modulating the physiological condition of host cells. The generation of reactive oxygen species (ROS) during infection with influenza virus A (IAV) is a well-established mechanism in animals, but little is known about the generation of ROS in *in vitro* cell culture models and about its role in virus replication. We show here that IAV H1N1 infected human alveolar cells increased superoxide anion level mainly by suppressing the copper–zinc superoxide dismutase 1 (SOD1) gene, and that the SOD1-controlled generation of ROS was tightly correlated with virus replication. The transcription factor Sp1, which is a major element of the proximal region of the *sod1* promoter, was slightly downregulated at the transcriptional level during IAV infection, and subsequently modulated by post-translational control. A gradual reduction of whole Sp1 was largely responsible for the repression of *sod1* transcription with increasing time post-infection, and their rescue by the proteasome inhibitor, MG132, proved the involvement of proteasomal degradation in Sp1 regulation during IAV infection. Furthermore, we observed that expression of viral polymerase PB1 was inversely proportional to SOD1 level. The antioxidant *N*-acetyl-cysteine (NAC) neutralized IAV-mediated oxidative stress, and either NAC treatment or *sod1* transfection considerably diminished viral polymerase activity. These data indicate that IAV-induced SOD1 repression, which may cause impaired redox balance in host cells, can be attributed, at least in part, to enhance viral replication.

1. Introduction

Influenza A virus (IAV) infection is a malicious cause of respiratory diseases. However, the underlying mechanisms are still obscure, although host cell damage of has been intensively studied from the view of disease development by morbidity of influenza virus genes [1]. Increasing evidence suggests that viral diseases are linked to physiological changes directed by the response of host cells [2]. Influenza virus infection often accompanies the generation of excess reactive oxygen species (ROS), which leads to activation of specific oxidative stress-sensitive signaling pathways inducing apoptosis in respiratory epithelial cells [3,4]. This causal relationship has been evidenced by studies in which adding an antioxidant reduces tissue damage in influenza virus-infected mice [5].

ROS can be generated by activated monocytes and leukocytes in virus-infected cells. ROS released from phagocytes may contribute to cell death or to the pathogenesis of influenza virus-induced diseases [1,6]. In addition, induction of intracellular ROS is triggered by a number of external agents including stress from virus infection [7,8]. Excess ROS may cause oxidative damage by participating in the modification of macromolecules, which alters gene expression and modulates cellular signaling pathways. A variety of cellular enzymes are modulated to defend against oxidative stress, to cope with injury from oxidative damage, and maintain redox homeostasis in cells. However, we and others have observed that IAV induces a significant increase in ROS levels, indicating an imbalance between ROS generation and antioxidant defense [9]. A similar report revealed that significant down-regulation of antioxidant genes is induced in mice infected with respiratory syncytial virus which causes oxidative injury [10]. Although ROS have been implicated in activation of gene expression and cellular signals, which play a central pathogenic role, the mechanisms underlying IAV-induced increase in ROS and eventually associated consequences for virus remain unclear.

Superoxide dismutases (SODs) are the major class of enzymes that catalyze ROS detoxification reactions. Regulation of SOD genes at both the expression and activity levels plays a critical role balancing the concentration of ROS [11]. Proximal region of human SOD genes contains several *cis*-acting regulatory elements [12], in which Sp1 is a principal factor for SOD transcription [13]. In

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the present report, we demonstrate that IAV (A/WSN/1933) induces a Sp1-dependent decrease in Cu–Zn SOD (SOD1) protein level, thereby contributing to an increase in intracellular ROS in cultured cells. The ROS generation in the context of Sp1 in IAV-infected cells is implicated in virus replication, and is tightly regulated by the level of antioxidants including SOD1 protein and *N*-acetyl-cysteine.

2. Materials and methods

2.1. Cell culture

Human alveolar epithelial cell line A549 and canine kidney cell line MDCK were purchased from ATCC (Rockville, MD, USA) and cultured as described previously [14].

2.2. Virus production

The recombinant influenza virus (A/WSN/1933) was generated by DNA transfection as described previously [15]. The required set of 8 plasmids was generously provided by R.G. Webster (University of Tennessee, TN, USA).

2.3. Immunoprecipitation and Western blot

All procedures for immunoblotting and immunoprecipitation were followed as previously described [16]. Antibodies for Sp1 and actin were purchased from Santacruz (Dallas, TX, USA). SOD1 antibody was purchased from Sigma Aldrich (St. Louis, MO, USA). PB1 and NP antibodies were purchased from GeneTex (Irvine, CA, USA)

2.4. RNA preparation and qRT-PCR

Total RNA was extracted using TRIzol reagent (Invitrogen). First strand cDNA conversion by reverse transcriptase was proceeded according to manufacturer's instructions (Invitrogen). The primer set for *sp1, thioredoxin, catalase, sod1, gapdh, IAV-pb1* and *IAV-np* were synthesized by IDT (Coralville, IA, USA). The primer set used are listed in supplementary Table 1.

2.5. Chromatin immunoprecipitation assay (ChIP) and electrophoretic mobility shift assay (EMSA)

Briefly, cells were fixed with 1% formaldehyde for 10 min at room temperature, lysed with lysis buffer containing 1% SDS and then sonicated for 5 min using Bioruptor™. The solutions were incubated with Sp1 antibody (each 2 μg) for 18 h at 4 °C. Precipitated DNAs were reverse purified and amplified with primers containing the *sod1* proximal region (−162 to+4) of the promoter. A549 nuclear extracts were prepared for EMSA as described [17]. The primer set used are listed in supplementary Table 1.

2.6. In vitro polymerase assay

Polymerase I-based luciferase reporter plasmid (pPolI-Luc) was constructed by flanking the firefly luciferase gene between non-coding regions of the influenza NP gene. The NP segment of influenza A/WSN/33 used as the backbone was generously provided by R.G. Webster. For the influenza virus *in vitro* polymerase assay, A549 cells were transfected with the pPolI-Luc reporter plasmid, *Renilla* luciferase plasmid (pRL-Luc), Flag-tagged SOD1 (pFlag-SOD1) or empty vector, and then subsequently infected with IAV at an MOI of 0.01. To investigate antioxidant effect, *N*-acetyl-cysteine (10 mM) was additionally treated at the end of infection. After 24 h, cell lysates were prepared, and the luciferase activity was measured by dual-luciferase assay method with GLOMAX™ 20/20 luminometer (Promega).

3. Results

3.1. Influenza virus infection induces oxidative stress in human lung alveolar epithelial cells

Our first objective was to determine whether IAV infection contributes to an increase in intracellular ROS in cultured cells. We therefore measured levels of superoxide anion and hydrogen peroxide in infected A549 cells. Confluent culture A549 cells (5×10^5 cells) were infected with IAV at an MOI of 0.01 for 1 h post infection (hpi), and continuously incubated for 24 hpi after changing media. At the end of the incubation, fluorescence level was measured by fluorescence-activated cell sorting (FACS) using either superoxide anion- or hydrogen peroxide-sensitive dye. As shown in Fig. 1, the superoxide anion level in infected cells increased markedly compared to that in mock infected cells, but the hydrogen peroxide level was slightly increased. These results suggest that complicating factors may be involved in the increase in superoxide anion level in infected cells.

3.2. SOD1 is down-regulated by IAV and tightly correlated with viral gene expression

Next, we assessed expression of key antioxidant genes in response to IAV infection, although it is known that endogenous antioxidant proteins are poorly expressed in the lung. The SOD1 mRNA level in infected A549 cells as an endogenous ROS scavenging enzyme was clearly reduced compared to that in mock infected cells, whereas transcription of other antioxidant genes, including SOD2 and thioredoxin, was relatively stable (Fig. 2A). This observation may explain how IAV infection induces ROS generation, as superoxide anion can accumulate when SOD1 is depleted [18]. Interestingly the catalase mRNA level was much lower than expected with the data shown in Fig. 1, so it could be another candidate regulatory gene during virus infection. However, superoxide anion is the well known starting compound in detoxification pathways leading to the formation of hydrogen peroxide and ultimately water. In addition, the catalase reaction may be mainly dependent upon SOD processes. Therefore, we focused our study on SOD1 repression as one of the causes of IAV-mediated ROS.

We carried out Western blot with a variety of doses and incubation time to determine the minimal virus titer and incubation time sufficient to affect the SOD1 level in the culture system. Fig. 2B shows that SOD1 protein decreased gradually as the MOI increased in a range from 10^{-4} to 10^{-1}, and was significantly lower at an MOI $\geqslant$ 0.01. A time course experiment at an MOI of 0.01 demonstrated that a significant reduction in the SOD1 protein began as incubation progressed past 12 hpi and showed the lowest level after 24 hpi (Fig. 2C). As infection was performed at higher MOIs than 0.1 or for a longer period than 24 hpi, the population of infected cells was severely reduced. Therefore, the effect of IAV infection on SOD1 regulation was illustrated at an MOI of 0.01 and 24 hpi in A549 cells. On the other hands, we found that the SOD1 level was tightly correlated with viral gene expression. Western blot showed an inverse correlation between SOD1 level and expression of viral protein, including PB1 and NP. Interestingly, while viral protein expression was initiated after 6 hpi, a significant decrease in SOD1 was detected after 12 hpi (Fig. 2C). Collectively, these results support that IAV infection may downregulate the SOD1 gene at the transcriptional level, thereby resulting in

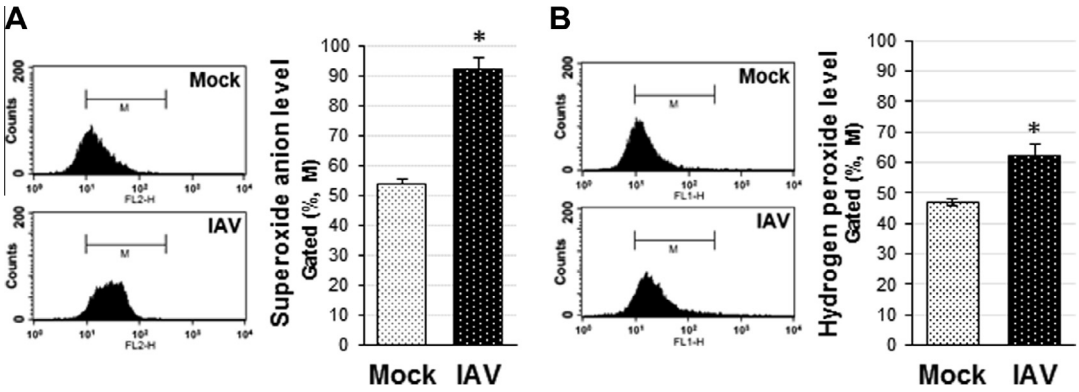

Fig. 1. Influenza virus infection induces oxidative stress in human lung cells. Human lung alveolar epithelial A549 cells were infected with IAV (A/WSN/1933) at an MOI of 0.01 for 1 h. After infection, the media were changed and the cells were incubated for 24 h. After the incubation, the cells were continuously stained with reactive oxygen species (ROS) sensitive dye for superoxide anion or hydrogen peroxide, respectively. The amounts of superoxide anion (A) and hydrogen peroxide (B) in cells were measured by detecting ROS-inducible fluorescence level by flow cytometry. Bar graphs are means ± standard deviation (SD) for three independent experiments. Statistical significance is relative to mock and was determined using the *t*-test ($^{*}p < 0.01$).

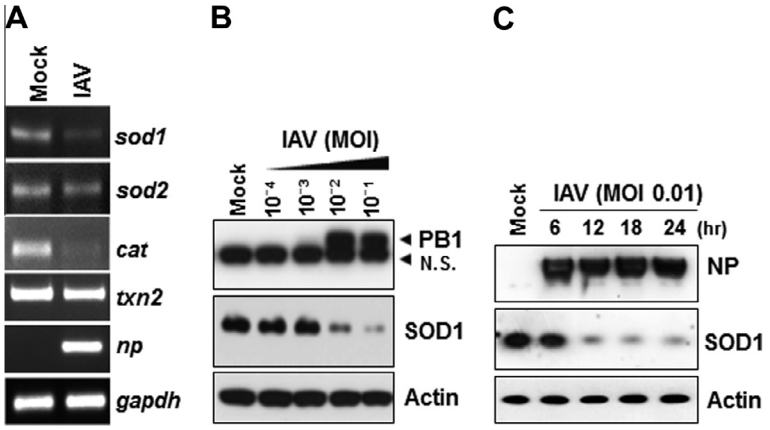

Fig. 2. Superoxide dismutase 1 (SOD1) is downregulated by influenza virus and tightly correlated with viral gene expression. (A) A549 cells were infected with IAV at an MOI of 0.01 for 24 h. Total RNA from mock and infected cells were subjected to quantitative reverse transcription-polymerase chain reaction for the indicated genes. (B) A549 cells were infected with IAV in a dose-dependent manner using various MOIs. After 24 h incubation, total protein was extracted and Western blot analysis was performed to determine the expression levels of PB1, SOD1 and actin. (C) Upon IAV infection at an MOI of 0.01, cells were harvested after incubation for the indicated period, and total proteins were analyzed by Western blot analysis for NP, SOD1 and actin.

ROS generation, which may, in turn, facilitate more efficient expression of viral genes.

3.3. Sp1 is down-regulated at both the transcriptional and post-translational levels and controls the expression of SOD1 and the viral gene in IAV infected cells

According to recent reports, the SOD1 gene has complex promoter elements at its proximal region (−70 to +1) [19–21]. We compared the expression levels of several transcription factors in infected cells and those of basal expression in mock to identify which transcription factor during SOD1 gene expression is influenced by virus infection (data not shown). We found that IAV infection controls expression of Sp1, which is a principal regulatory factor for SOD1 expression (Fig. 3A). Upon virus infection, down-regulation of Sp1 initiates slightly at the transcriptional level, and subsequently a post-translational modification occurs Sp1 protein levels in a time-dependent manner. As hpi increased, a gradual shift from whole Sp1 (95/106 kD) to cleaved Sp1 (about 75 kD) indicated involvement of virus-induced proteolysis in degradation of both pre-existing and *de novo* synthesized Sp1. As expected, the availability of active Sp1 appeared to be reflected accordingly in both SOD1 mRNA and protein levels. It is likely that cleaved 75 kD Sp1 may not be active enough to contribute to SOD1 expression, as SOD1 mRNA was definitely proportional to 95/106 kD Sp1 rather than the cleaved Sp1. Taken together, these results suggest that the decrease in SOD1 protein was due mainly to post-translational degradation of Sp1 as well as transcriptional down-regulation at an early stage of infection. The proteasome inhibitor MG132 was added to infected cells to assess whether activation of Sp1 proteolysis is involved. As shown in Fig. 3B, MG132 treatment restored the levels of Sp1 and SOD1, which were almost depleted by virus infection, suggesting that virus-mediated Sp1 proteasomal degradation played a critical role in the decrease of IAV-mediated SOD1. Furthermore, IAV PB1 expression was inversely proportional to the availability of active Sp1/SOD1 protein in a time course experiment as well as Western blot using MG132. We did not investigate exactly how IAV induced the proteolytic process and post-translational phosphorylation of Sp1. However, we determined how much Sp1 was active in binding to the core promoter of the SOD1 gene. EMSA and ChIP assay showed that IAV infection strongly interfered with binding of Sp1 to the promoter (Fig. 3C). An interesting observation was that the actual amount of bound Sp1 was much less than we expected considering the Sp1 level on the Western blot, indicating the presence of additional activity, including ROS-mediated modification of binding activity.

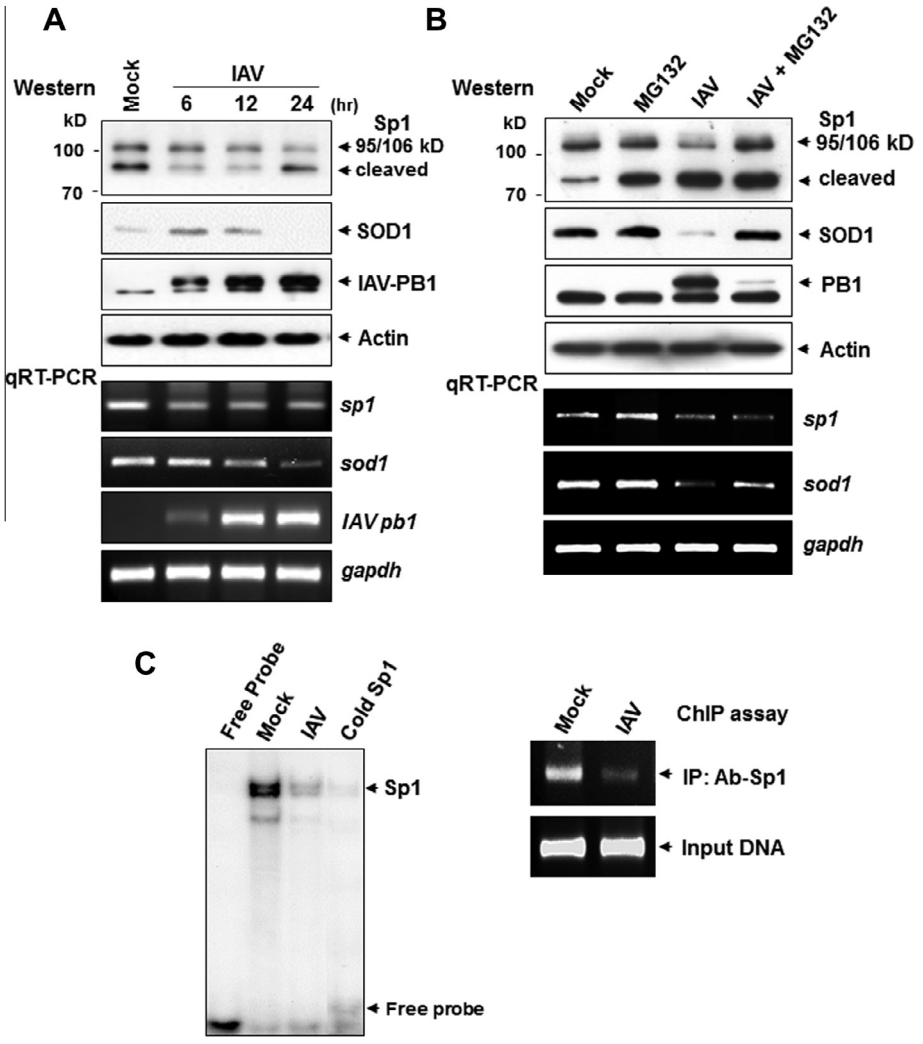

Fig. 3. Sp1 controls the expression of SOD1 and viral gene in influenza virus A (IAV) infected cells. (A) A549 cells infected with IAV at an MOI of 0.01 for various hours post infection (hpi) were subjected to Western blot and quantitative reverse transcription-polymerase chain reaction (qRT-PCR). (B) Mock and IAV-infected cell at MOI of 0.01 were treated with 10 μM MG132 for 1 h after infection. Total protein and RNA were prepared 24 h after MG132 treatment and subjected to Western blot and qRT-PCR analysis. (C) Nuclear extracts from mock and infected A549 cells were subjected to electrophoretic mobility shift assay. The proximal region (−70 to −38) of the SOD1 gene promoter was used as a probe after labeling with [^{32}P]-γATP-labeled DNA probe (left). Unlabeled Sp1 oligonucleotides were used as the competitor. Chromatin immunoprecipitation assay was performed with chromosomal DNA extracted from Mock cell and IAV-infected cells as described in Materials and Methods. Un-immunoprecipitated shared chromosomal DNA was subjected to input PCR amplification (right).

3.4. Antioxidants inhibit IAV-induced ROS generation and attenuate viral gene expression

In order to investigate whether virus-induced oxidative stress is responsible for the inverse relationship between expression of Sp1/SOD1 and PB1, we evaluated the effect of the ROS scavenger NAC on ROS generation and subsequent viral PB1 gene expression. To exclude any difference in infection rate after NAC pretreatment, NAC was added to the media at the end of virus infection. As shown in Fig. 4A, NAC treatment clearly inhibited superoxide anion formation, and also significantly reduced PB1 expression. These data are consistent with our previous results demonstrating that IAV infection induces intracellular ROS generation via Sp1/SOD1 down-regulation and eventually facilitates viral gene expression. To support our finding, we monitored viral gene expression by employing the influenza virus luciferase reporter gene flanked by the non-coding regions of the IAV NP gene and RNA polymerase I promoter (pPolI-Luc), as the *in vitro* polymerase assay can be an indirect assessment for viral gene expression (Fig. 4B). Viral PB1 polymerase activity was reduced in the presence of NAC in the media. Furthermore, transient transfection of exogenous SOD1 gene partially overrode IAV-induced polymerase activity. In order to obtain a better understanding of the roles of SOD1 in viral gene expression, IAV NP protein level was determined. As shown in Fig. 4C, SOD1 expression resulted in a decrease in the viral NP protein level in the infected cells. Therefore, our results strongly suggest that SOD1 depletion in IAV infected cells is a major cause of intracellular ROS generation and oxidative stress-induced stimulation of virus replication.

4. Discussion

Although a number of studies have demonstrated that viruses induce the generation of ROS and alter antioxidants [9,22], the exact molecular mechanism for influenza virus-induced ROS generation has not been clearly identified. In the present study, we show that IAV (A/WSN/1933) infection induced specific downregulation of Sp1 and SOD1 gene expression, thereby resulting in accumulation of intracellular ROS in a lung cell culture system.

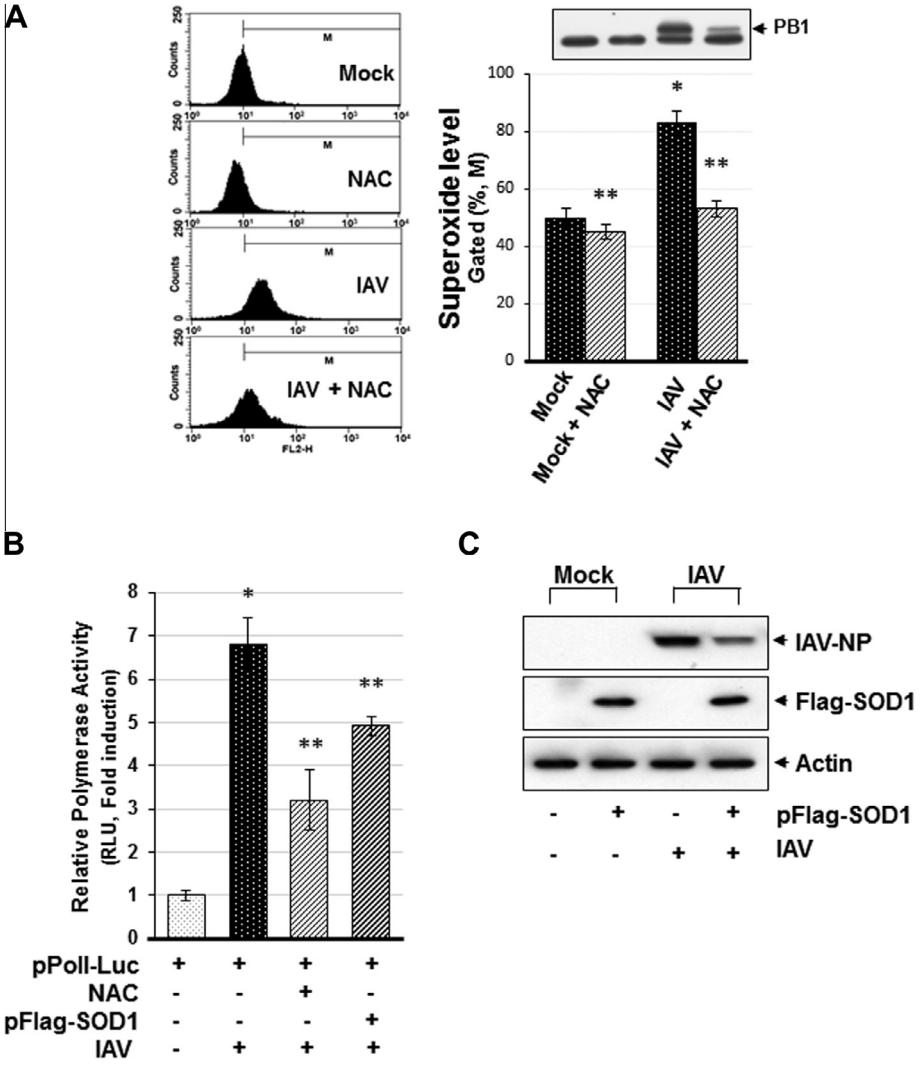

Fig. 4. Blocking IAV-induced ROS accumulation by NAC treatment and *sod1* transfection. (A) A549 cells were infected at an MOI of 0.01. After 1 h incubation, NAC (10 mM) was added to fresh media. After 24 h incubation, superoxide anion level was measured by flow cytometry. Using the same samples, total proteins were subjected to Western blot analysis using antibody against PB1. Bar graphs represent mean ± SD from at least three independent experiments. Asterisks indicate statistical significance. $^{*}p < 0.05$ compared with Mock, $^{**}p < 0.01$ compared with cells untreated with NAC, respectively. (B) Viral polymerase activity was assayed by an *in vitro* polymerase assay using RNA polymerase I-driven luciferase reporter (pPolI-Luc). Cells co-transfected with pPolI-Luc, pRL-Luc, pFlag–SOD1 or empty vector were subsequently infected with IAV at an MOI of 0.01. NAC (10 mM) treatment was performed continuously 24 h following infection. At 24 hpi, luciferase activity was measured with normalization by the activity of *Renilla* luciferase. Results represent mean ± SD from at least three independent transfections. $^{*}p < 0.05$ compared with Mock, $^{**}p < 0.01$ compared with IAV-infected cell, respectively. (C) A549 cells were transfected with indicated combination of expression vectors 10 h prior to infection and subsequently infected with IAV. At 24 hpi, cell lysates were prepared and Western blot analysis were applied to determine IAV NP protein level.

Notably, IAV-induced oxidative stress appeared to be directly related to viral gene expression.

SODs are ubiquitous components of cellular antioxidant systems that guard against oxidant toxicity. Cu–Zn SOD1 is one of three human superoxide dismutases identified in mammals that detoxifies superoxide radicals primarily in the cytoplasm where it is highly expressed. While extracellular ROS released from activated phagocytes cause cellular damage, ROS triggered intracellularly by various mechanisms can severely accumulate within virus-infected cells. A variety of cellular enzymes including SOD1 are modulated to cope with excess oxidative stress and maintain redox homeostasis in cells. According to recent studies, the control mechanism of the generation and elimination of ROS might be of great interest, as altered SOD1 expression appears to be implicated in a variety of human diseases and pathological states [21]. In addition, various SOD1 inducers have been reported, suggesting the presence of a specific regulatory mechanism in SOD1 gene expression. The proximal region (−157 to −25) of the SOD1 gene contains many essential *cis*-acting elements for basal and inducible expression of SOD1 [19]. This region includes several transcription factor binding sites, in which Sp1 is a principal factor for SOD transcription [12,20]. Therefore, our interests were focused on the regulation of Sp1 including alteration in the basal level and DNA binding activity under IAV infection. In this study, we demonstrate that IAV induces a Sp1-dependent decrease in SOD1 protein level, leading to a rise in intracellular ROS in cultured A549 cells. Upon virus infection, downregulation of Sp1 was initiated weakly at the transcriptional level, and subsequent post-translational modification occurred gradually. The time-dependent shift to cleaved Sp1 protein indicates involvement of virus-induced proteolysis in degradation of both pre-existed and *de novo* synthesized Sp1. We do not rule out the possibility of the involvement of proteolysis in lowering SOD1 protein level in infected cells, but its contribution was minor compared to the effect of downregulation by Sp1. To support this result, we showed that infected cells treated with the proteasome inhibitor MG132 restored the levels of Sp1 and

SOD1 almost to their basal levels, and that SOD1 expression depended largely on Sp1 protein level. Therefore, we conclude that IAV induces downregulation of Sp1 at both the transcriptional and post-translational levels, thereby leading to diminishing SOD1 expression. In addition, decreased binding activity of Sp1 on the proximal region of the SOD1 promoter shown in the EMSA and ChIP assays also contributed to SOD1 downregulation in infected cells. However, we do not exclude the possible involvement of Sp1 post-translational modification in its DNA binding activity, as recent studies have reported that sustained Sp1 phosphorylation might be correlated with its proteolytic process [23–25], and DNA binding [20,26].

Furthermore, we observed an inverse relationship between SOD1 level and viral gene expression. We did not investigate the mechanism in which ROS induces expression of viral genes including PB1 and NP. According to recent studies, apoptosis is beneficial for viral propagation and replication [27,28]. Presumably, IAV-mediated ROS generation induces apoptotic cell death after IAV infection. Many reports have demonstrated that IAV-mediated excess ROS are implicated in pathogenesis including inflammation and cell death in respiratory cells and organs [29,30]. The increase in ROS during IAV infection is believed to be a major cause of viral diseases. Thus, synthetic antioxidants have been suggested as a potential therapeutic approach to eliminate ROS or reduce ROS-derived pathological responses. For this purpose, NAC has been intensively studied as the most effective antioxidant in laboratory experiments. In our study, treatment with NAC showed a decrease in levels of superoxide anion and PB1 in infected cells, indicating a critical role of SOD1 in viral PB1 expression. To verify the relationship between SOD1 and viral gene expression, we carried out *in vitro* polymerase assays, and observed that polymerase activity was substantially reduced when exogenous SOD1 was overexpressed. We also observed that the viral NP expression was noticeably suppressed while SOD1 levels were restored to the basal level by the overexpression of the exogenous SOD1 gene. These results clearly suggest a possibility of ROS-dependent viral induction and a pivotal role of SOD1 in viral gene expression. Therefore, while additional experiments are required, it is likely that certain ROS-induced signals may serve as a trigger for virus replication. In conclusion, our results demonstrate a novel mechanism by which IAV-mediated downregulation of Sp1 results in depletion of the major antioxidant SOD1, thereby leading to excess intracellular ROS, which are consequently implicated in virus replication. Overall, our findings contribute to understanding the IAV-mediated pathogenicity and propose a potential target for diminishing viral infectivity.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.06.037.

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