Antiviral Research 119 (2015) 78–83

Contents lists available at ScienceDirect

Antiviral Research

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

Mitochondrial reactive oxygen species modulate innate immune response to influenza A virus in human nasal epithelium

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ARTICLE INFO

Article history:
Received 13 October 2014
Revised 13 April 2015
Accepted 21 April 2015
Available online 28 April 2015

Keywords:
Influenza A virus
Interferon-stimulated genes
Reactive oxygen species
Mitochondria

ABSTRACT

The innate immune system of the nasal epithelium serves as a first line of defense against invading respiratory viruses including influenza A virus (IAV). Recently, it was verified that interferon (IFN)-related immune responses play a critical role in local antiviral innate immunity. Reactive oxygen species (ROS) generation by exogenous pathogens has also been demonstrated in respiratory epithelial cells and modulation of ROS has been reported to be important for respiratory virus-induced innate immune mechanisms. Passage-2 normal human nasal epithelial (NHNE) cells were inoculated with IAV (WS/33, H1N1) to assess the sources of IAV-induced ROS and the relationship between ROS and IFN-related innate immune responses. Both STAT1 and STAT2 phosphorylation and the mRNA levels of IFN-stimulated genes, including *Mx1*, *2,5-OAS1*, *IFIT1*, and *CXCL10*, were induced after IAV infection up to three days post infection. Similarly, we observed that mitochondrial ROS generation increased maximally at 2 days after IAV infection. After suppression of mitochondrial ROS generation, IAV-induced phosphorylation of STAT and mRNA levels of IFN-stimulated genes were attenuated and actually, viral titers of IAV were significantly higher in cases with scavenging ROS. Our findings suggest that mitochondrial ROS might be responsible for controlling IAV infection and may be potential sources of ROS generation, which is required to initiate an innate immune response in NHNE cells.

Recently, it was confirmed that interferon (IFN) is primarily responsible for protection against viral invaders in the respiratory tract and IFN-related immune responses play an important role in local antiviral innate immunity (Hansel et al., 2013). Type I and III IFNs trigger strikingly similar responses in target cells resulting from phosphorylation of transcription factors STAT1 and STAT2, which facilitates intracellular induction of IFN-stimulated genes (ISGs) (Schneider et al., 2014). These ISGs encode proteins such as Mx1, OAS or IFIT and, reportedly, rapid production of ISGs could actually be associated with controlling viral infections and degradation of viral particles (Schneider et al., 2014).

Reactive oxygen species (ROS) are believed to be inevitable toxic by-products that cause cellular damage or stress (Mittal et al., 2014). However, mounting evidence suggests the generation of ROS to be an important component of the host's arsenal against invading microorganisms (Kotsias et al., 2013; Kim et al., 2011). Mitochondria are dynamic double-membrane-bound organelles that are involved in a wide range of cellular processes and. mitochondria are a significant source of ROS that modulate several signaling pathways (Cloonan and Choi, 2012; West et al., 2011). Until now, it has been assumed that ROS are primarily produced by NADPH oxidase systems in non-phagocytic cells, such as respiratory epithelial cells (Kim et al., 2011). However, it has also been suggested that mitochondria are involved in oxidative phosphorylation and ROS generation in respiratory inflammatory diseases (West et al., 2011). Although mitochondrial oxidative metabolism also seems to be a critical source of cellular ROS in many eukaryotic cells, it is not clear whether mitochondrial ROS facilitate immune signaling; additional research is needed to delineate the mechanisms of mitochondrial ROS in the nasal epithelium.

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http://dx.doi.org/10.1016/j.antiviral.2015.04.011
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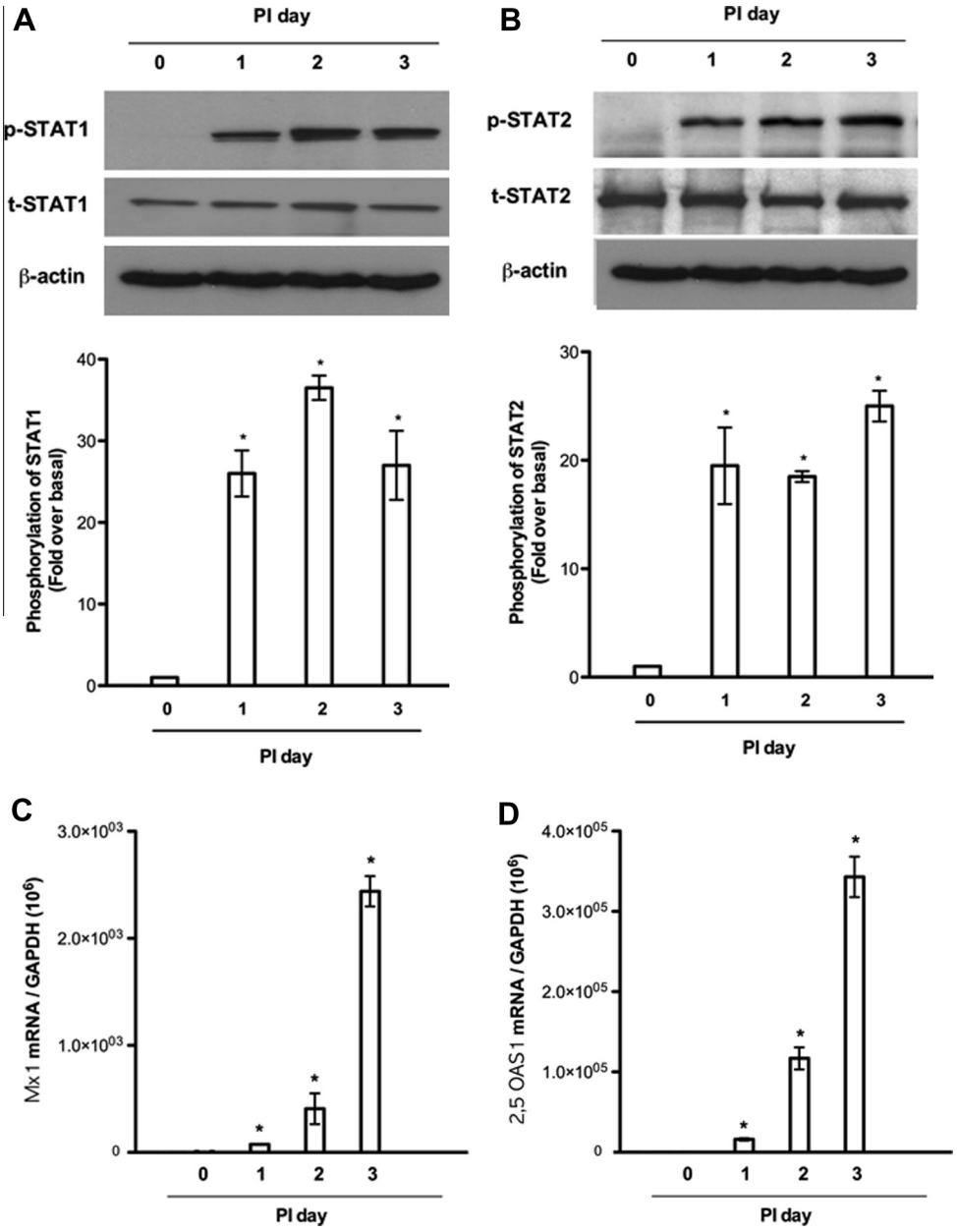

Fig. 1. IFN-related immune response is induced after IAV infection in NHNE cells. NHNE cells from five healthy volunteers were inoculated with WS/33 (H1N1) for 1, 2, and 3 days at MOI 1. Western blot analysis and densitometry revealed that phosphorylation of STAT1 (A) and STAT2 (B) is enhanced from until 3 dpi. Cell lysates were assayed using real-time PCR. The results show that Mx1 (C), 2,5-OAS1 (D), IFIT1(E) and CXCL10 (F) mRNAs are induced after IAV infection from 1 dpi and are significantly higher until 3 dpi. The protein levels of Mx1, 2,5-OAS1, IFIT1 (G) and secreted CXCL10 (H) were induced from 1 day after IAV infection. The Western blot results presented here are representative of five independent experiments. PCR and ELISA results are presented here as the mean ± standard deviation (SD) from five independent experiments ($^*p < 0.05$ when compared to mRNA levels of mock-infected cells).

In this study, we investigated whether mitochondrial ROS induce antiviral immune response in the nasal epithelium and described the specific roles of mitochondria in IFN-related innate immune system, subsequent ROS generation against influenza A virus (IAV) infection.

Previously, we performed gene expression analysis using the Affymetrix GeneChip Human Gene 1.0 ST Array (Santa Clara, CA, USA) with total RNA from NHNE cells after 2 days of IAV infection. The analysis indicated that 11 genes are active in IFN-mediated immune responses, including four ISGs, *MX1*, *OAS1*, *IFIT1* and *CXCL10*, and their transcription factors STAT1 and STAT2 (Kim et al., 2013). To analyze this in greater detail, we obtained NHNE cells from five healthy subjects and infected cells with WS/33 (H1N1) at MOI 1. The cell lysates were harvested one, two, and three days post infection (dpi). We then measured the phosphorylation of STAT1 and STAT2 using Western blot analysis, and the results showed that both STAT1 and STAT2 phosphorylation increased significantly from 1 dpi (STAT1: 26.3-fold over PI 0 day, STAT2: 19.5-fold over PI 0 day), and was maintained for up to 3 dpi (STAT1: 27.6-fold over PI 0 day, STAT2: 25.0-fold over PI 0 day, Fig. 1A and B).

Next, we determined the levels of transcription for ISGs, including *Mx1*, *2,5 OAS1*, *IFIT1*, and *CXCL10* after 1, 2, and 3 days post IAV infection using real-time PCR. Increased Mx1, 2,5 OAS1, IFIT1, and CXCL10 gene expression was observed from 1 dpi and peaked at 3 dpi (Mx1: 2440.1 ± 141.4, 2,5 OAS1: 343024.35 ± 25196.1, IFIT1: 286054.3 ± 27535.9, CXCL10: 1,760,637 ± 280324.5, Fig. 1C–F). We then measured the protein expression of Mx1, 2,5

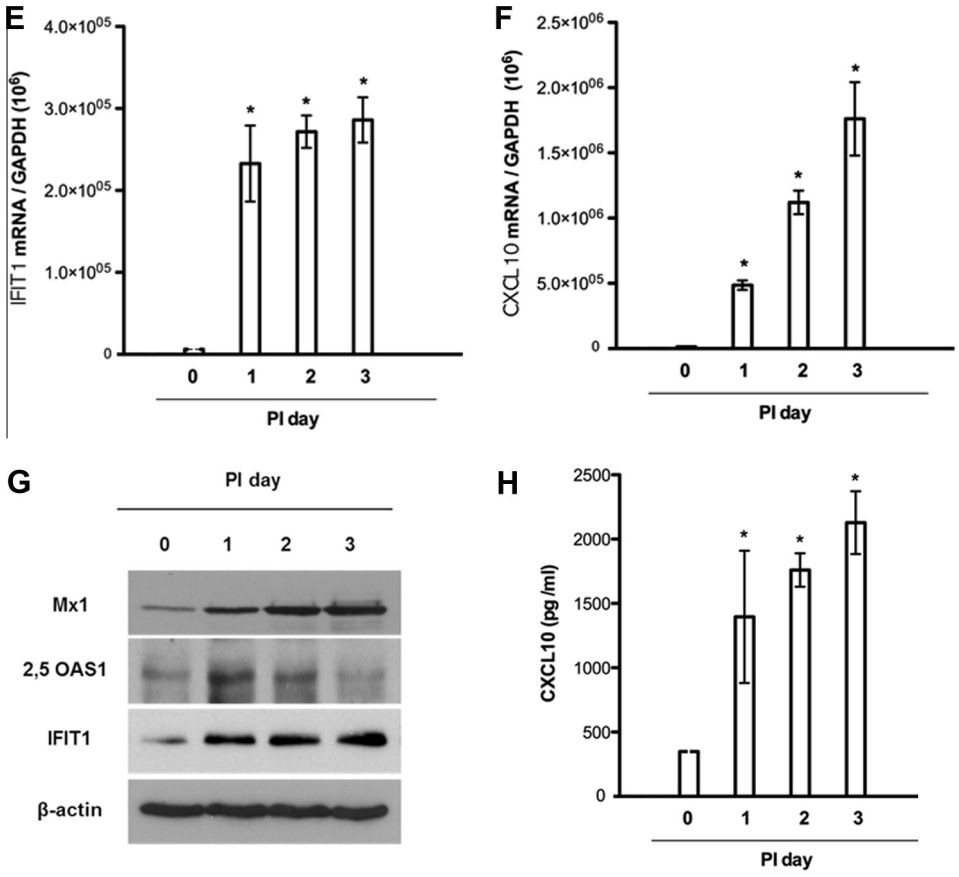

Fig. 1 (*continued*)

OAS1, IFIT1 using Western blot analysis and the amount of secreted CXCL10 protein was measure through ELISA. The results showed that protein levels of Mx1, 2,5 OAS1, IFIT1 increased significantly from 1 dpi, and was maintained for up to 3 dpi. Secreted CXCL10 protein was also significantly induced from 1 day after IAV infection (Fig. 1G and H).

These data suggested significant activation of IFN-related antiviral immune response against IAV, at least at 1 dpi in NHNE cells, which was maintained for up to 3 days after infection.

NHNE cells were inoculated with WS/33 (H1N1) for 10, 30 min; 1, 2, and 8 h; and 1, 2, and 3 days. Mitochondrial ROS was then measured using a fluorescence-based assay with mitoSOX. Infection of cells resulted in a time-dependent increase in mitoSOX fluorescence intensity with a maximum increase at 2 dpi (28.1 ± 1.6, $p < 0.05$), compared to the control intensity (4.3 ± 0.3).

Subsequently, we used mitoTEMPO, which inhibits mitochondrial ROS generation in the nasal epithelium. After pretreating NHNE cells with 500 μM of mitoTEMPO for 1 h, NHNE cells were infected with WS/33 (H1N1) and treated with mitoTEMPO for two days. The IAV-elevated mitoSOX fluorescence intensity was significantly attenuated (Fig. 2B) and the viral titer was considerably elevated in cells where the mitochondria respiratory chain reaction was inhibited (Fig. 2C). At 2 dpi, the viral titer was 1.5×10^6 pfu/ml, but pretreatment with mitoTEMPO elevated the IAV viral titer to 4.2×10^6 pfu/ml ($p < 0.05$).

To determine whether mitochondria promote significant induction of IFN-related innate immune response against IAV infection, cells were treated with mitoTEMPO and inoculated with WS/33 (H1N1) and we then measured the phosphorylation of STAT1 and STAT2. The results indicated that IAV-induced phosphorylation of STAT1 and STAT2 decreased in the cells where mitochondrial ROS were considerably inhibited 2 dpi (STAT1: 24.9 vs 10.2-fold over basal, STAT2: 21.3 vs 3.9-fold over basal, Fig. 3A and B). Lastly, the mRNA levels of ISGs at 2 dpi were analyzed by real-time PCR and the results suggested that IAV-induced *Mx1*, *2,5 OAS1*, *IFIT1*, and *CXCL10* mRNA levels decreased significantly in the cells with diminished mitochondria respiratory chain reaction (Mx1: 5.0×10^3 vs. 2.4×10^3, 2,5-OAS1: 1.8×10^5 vs. 7.4×10^4, IFIT1: 1.5×10^6 vs. 7.1×10^5, CXCL10: 2.6×10^6 vs. 1.2×10^6, $p < 0.05$, Fig. 3C–F). We found that the mitochondria respiratory chain was involved in intracellular ROS generation against IAV infection and IAV-induced ROS generation might mediate the activation of STAT1 and STAT2 and enhancement of ISG transcription in the nasal epithelium.

Initiation of these innate immune responses is achieved through the recognition of invading viruses by pattern recognition receptor (PRR) and virus-derived nucleic acids are considered to activate various PRRs, including members of the membrane-bound Toll-like receptor family such as TLR3, 7, and 9, and the recently identified cytoplasmic retinoic acid-inducible gene 1 (RIG)-like receptors, including RIG-I and melanoma differentiation-associated protein 5. Following the recognition of viral RNAs, the antiviral innate immune response is activated, mainly through the rapid expression of IFNs in nasal epithelium. IFNs from infected cells acts in a positive feedback loop and primes adjacent, uninfected cells for rapid induction of anti-viral immunity via further IFN secretion, JAK/STAT signaling, and ISGs (Schneider et al., 2014). ISGs elicit the anti-viral immune response by degrading viral RNA, preventing virus-associated protein trafficking and virion assembly, and inducing apoptosis of infected cells (Durbin et al., 2013). ISGs inhibit the very early steps in the viral multiplication cycle that affect

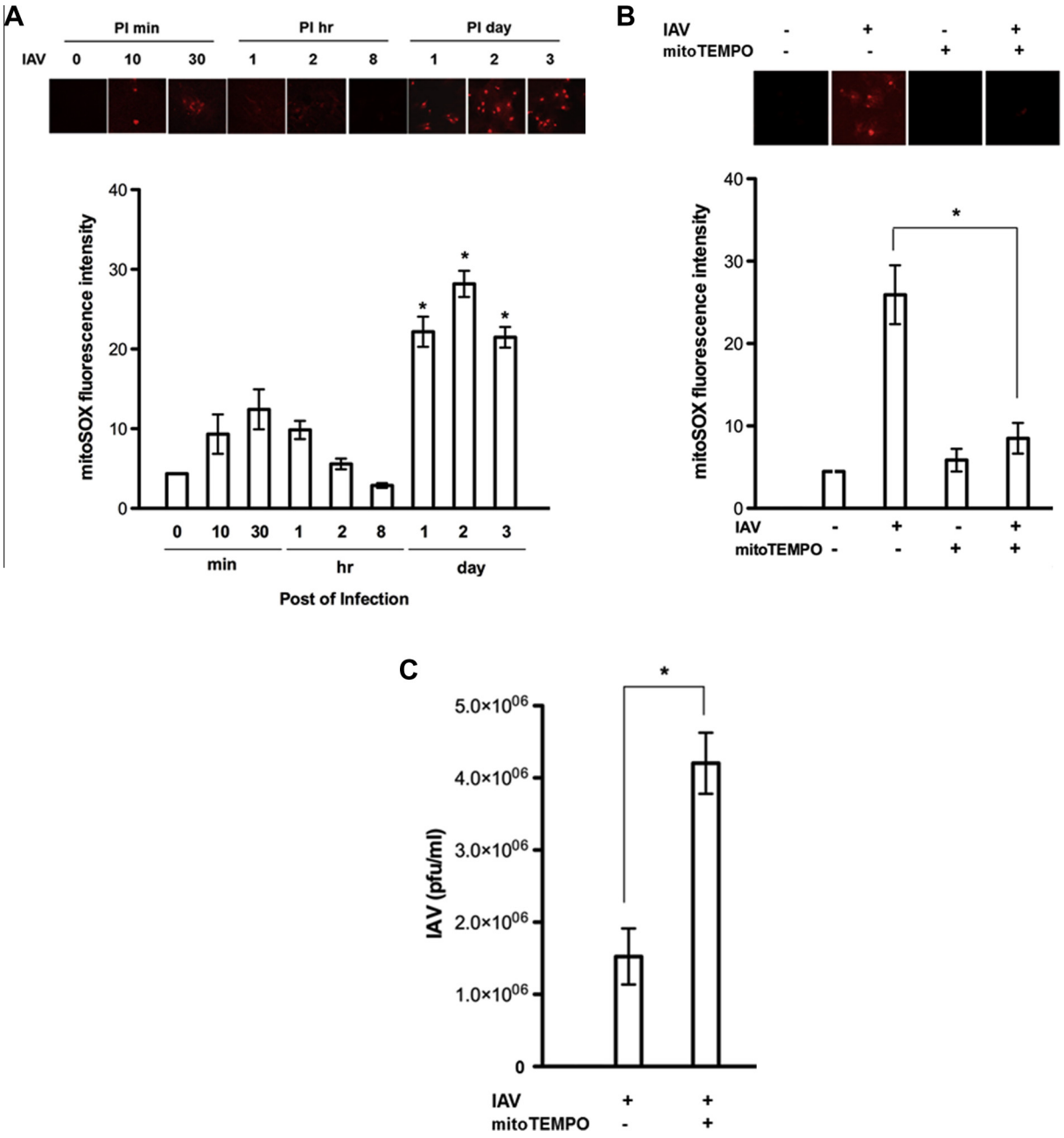

Fig. 2. Mitochondrial ROS are generated after IAV infection and related to antiviral immune response in NHNE cells. NHNE cells were infected with WS/33 (H1N1) for 10 min, 30 min, 1, 2, and 8 h and 1, 2, and 3 days; then, intracellular ROS generation was measured with mitoSOX Red. After IAV infection, MitoSOX Red fluorescence intensity was preferentially elevated after 1 dpi (A). NHNE cells treated with mitoTEMPO (500 μM) 1 h before IAV infection and infected with WS/33 (H1N1) for 2 days at MOI 1. Mitochondrial ROS were measured with mitoSOX Red (B) and plaque assay was performed using cellular supernatant (C). The fluorescence intensity data are representative of five independent experiments, and results are presented here as the mean ± SD from five independent experiments ($^{*}p < 0.05$ when compared with the level of IAV-infected cells or cells treated with mitoTEMPO).

primary transcription of the incoming genome. In the present study, we suggest that ISGs, including *Mx1*, *2,5-OAS1*, *IFIT1* and *CXCL10*, are predominantly induced to resist IAV infection in the nasal epithelium by suppressing the transcription or replication of IAV, thereby contributing to the control of viral infection.

ROS have recently been shown to function as a messenger influencing a variety of host immunologic processes and to enhance host immunity through prevention of pathogen-induced pro-inflammatory cytokines (Kim et al., 2011). ROS generation by exogenous pathogens has also been established in nasal epithelial cells, and modulation of ROS was reported to be important for respiratory virus-induced innate immune mechanisms (Kim et al., 2013).

We found that mitochondria induce expression of ROS-regulated ISGs after IAV infection in NHNE cells. Furthermore, inhibition of the mitochondrial respiratory chain reaction reduced transcription of ISGs and aggravated IAV infection. Together, ISGs such as Mx1, 2,5-OAS1, IFIT1, and CXCL10 play a role in protecting the nasal epithelium from IAV infection, and mitochondrial ROS might be potent mediators of ISG transcription induction against IAV infection. Our previous findings indicate that both IFN-β and IFN-λ are preferentially elevated to resist IAV infection and exogenous IFN-λ treatment might be more potent to control IAV infection in the nasal epithelium (Kim et al., 2013). The cells transfected with IFNAR1 shRNA, IL28Rα and IL10Rβ shRNAs had

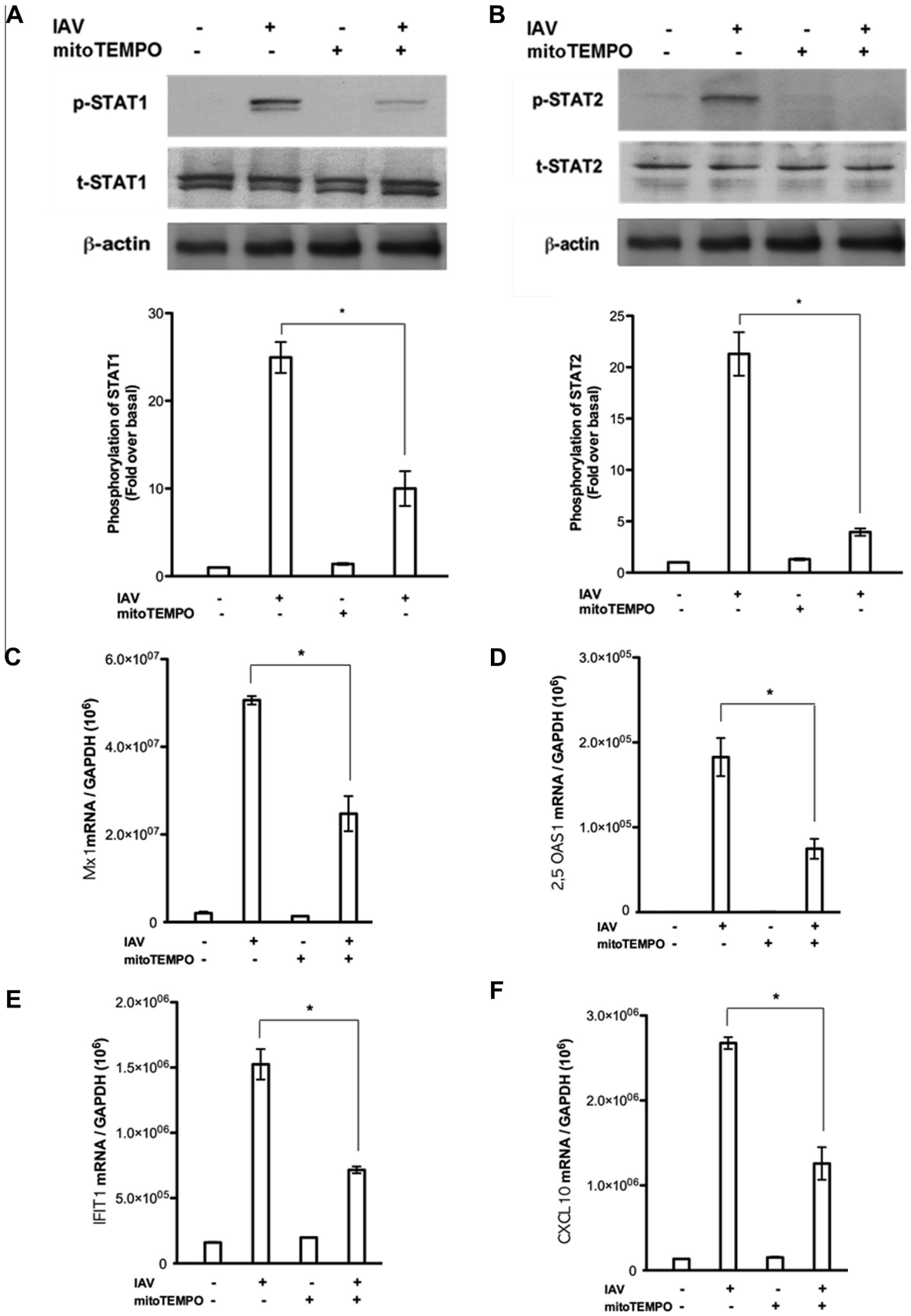

Fig. 3. Mitochondrial ROS are involved in IAV infection-induced STAT phosphorylation and IFN-stimulated genes expression. NHNE cells were treated with mitoTEMPO (500 μM), after which Western blot analysis for STAT1 and STAT2 and real-time PCR for ISGs were performed. The densitometry results show that IAV-induced phosphorylation of STAT1 (A) and STAT2 (B) was reduced in cells treated with mitoTEMPO. IAV-induced Mx1 (C), 2,5-OAS1 (D), IFIT1(E) and CXCL10 (F) gene overexpression were also attenuated in the cells where mitochondrial ROS were not generated. The Western blot results presented here are representative of five independent experiments and PCR results of real-time PCR are presented here as the mean ± SD from five independent experiments ($^*p < 0.05$ when compared with the level of IAV-infected cells or cells treated with mitoTEMPO).

significantly attenuated mitochondrial ROS generation (Supplementary Fig. 1) and showed more completely reduced mRNA levels of Mx1, 2,5-OAS1, IFIT1, and CXCL10 against IAV infection (Supplementary Fig. 2A–D). These results indicate that IAV-caused IFNs' induction might be responsible for mitochondrial ROS generation which was involved in the induction of ISGs' transcription in nasal epithelium.

Herein, we show that mitochondrial ROS contribute to the defense mechanism against IAV infection in the nasal epithelium through IFN-related innate immune responses. Mitochondrial ROS positively regulates transcription of ISGs, and may be critical elements in controlling acute IAV infection in the upper airway.

Acknowledgements

This work was supported by the National Research Foundation of the Korean Government (Ministry of Science, ICT & Future Planning, MSIP), grant 2007-0056092 (J.H.Y.), National Research Foundation of Korea (NRF) funded by the Ministry of Science, ICT & Future Planning (2012M3A9C5048709) (J.H.Y.), National Research Foundation of Korea (NRF) grant funded by the Korean Government (MSIP) (2014R1A2A01003385) (J.H.Y.) and the Basic Science Research Program of the National Research Foundation of Korea (NRF) funded by the Ministry of Education, Science and Technology grant 2013R1A1A2011612 (H.J.K.).

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.antiviral.2015.04.011.

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