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Influenza A virus (H1N1) triggers a hypoxic response by stabilizing hypoxia-inducible factor-1α via inhibition of proteasome

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ABSTRACT

Virus reprogramming of host cellular function is a critical strategy for viral survival and replication. A better understanding of virus-host interaction may provide new potential avenues for the treatment of viral diseases. It has been reported that hypoxia-inducible factor-1 (HIF-1) pathway is activated by a range of pathogens via different mechanisms, but the impact of Influenza A virus on HIF-1 signaling is still unclear. In this study, we observed H1N1 infection stabilized HIF-1α under normoxic conditions. In detail, H1N1 did not increase HIF-1α mRNA transcription, nor impaired posttranslational prolyl hydroxylation or ubiquitination of HIF-1α, but inhibited the function of proteasome, resulting in HIF-1α accumulation. Furthermore, a decreased expression of factor inhibiting HIF-1 (FIH-1), which hydroxylates asparagine 803 within HIF-1α to repress HIF-1α activity, was seen after H1N1 infection. Taken together, these findings reveal a previously unrecognized mechanism of viral activation of the HIF-1 pathway, resembling a hypoxic response in normoxia.

1. Introduction

Viruses depend on host cell machinery to complete their life cycle. Many viral pathogens have evolved sophisticated strategies to alter host system and function for their own benefit (Thai et al., 2014; Mazzon et al., 2018; Fontaine et al., 2015; Ding et al., 2017; Jarret et al., 2016), such as reprogramming of host cellular metabolism and modulation of host immune response. Consequently, virus-host interaction has become a new therapeutic target for viral diseases (Zumla et al., 2016). Influenza A virus, a single-stranded, negative-sense RNA virus with an eight-segmented genome, has become a worldwide threat to public health causing severe morbidity and even mortality (Iuliano et al., 2018). Recent studies have suggested that Influenza A virus also has a lot of interactions with host factors (Wang et al., 2018; Zhang et al., 2016; Zhou et al., 2014; James et al., 2015).

Hypoxia-inducible factor 1 (HIF-1) is a transcriptional activator of various genes related to cellular adaptive responses to hypoxia (Wang and Semenza, 1993). It is composed of a regulatory HIF-1α subunit and a HIF-1β subunit which is constitutively expressing (Wang and Semenza, 1995). Under normoxic conditions, HIF-1 activity is suppressed by two oxygen-dependent dioxygenases which hydroxylate conserved proline or asparagine residues within HIF-1α (Bracken et al., 2003). The hydroxylation of proline 402 (P402) and proline 564 (P564) within HIF-1α by prolyl-hydroxylase domain containing protein (PHD) leads to quick degradation of HIF-1α by the ubiquitin-proteasome system (Ivan et al., 2001; Jaakkola et al., 2001). The hydroxylation of asparagine 803 (N803) within HIF-1α by factor inhibiting HIF-1 (FIH-1) blocks the association between HIF-1α with co-activators P300/CBP, which represses HIF-1's transcriptional activity (Lando et al., 2002; Mahon et al., 2001). In hypoxia, HIF-1α avoids hydroxylation, is stabilized, translocates to the nucleus, and interacts with its partner subunit HIF-1β and co-activators P300/CBP, forming a functional HIF-1 transcription complex. Activated HIF-1 induces a battery of target genes that play important roles in cell metabolism, angiogenesis, and tumor metastasis (Koyasu et al., 2018; Semenza, 2012).

A variety of viral pathogens are able to activate the HIF-1 pathway,

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inducing different downstream effects such as altering host cellular metabolism, promoting inflammation, and facilitating viral replication (Duette et al., 2018; Nicholas and Sumbayev, 2009; Yogev et al., 2014; Moon et al., 2004; Guo et al., 2014; Chen et al., 2017). However, the mechanisms of viral activation of the HIF-1 pathway are diverse. Some viruses such as hepatitis B virus, vaccinia virus, and Epstein-Barr virus can stabilize HIF-1α by interfering in the process of HIF-1α post-translational prolyl hydroxylation or ubiquitination (Mazzon et al., 2013; Kondo et al., 2006; Haeberle et al., 2008; Moon et al., 2004; Guo et al., 2014; Yogev et al., 2014). The parapoxvirus orf virus activates the HIF-1 pathway through the sequestration of FIH-1 by viral ankyrin repeat (ANK) proteins, leading to derepression of HIF-1 activity (Chen et al., 2017). However, the impact of Influenza A virus on the HIF-1 pathway is still unknown. In this study, we observed that the expression of HIF-1α increased after H1N1 infection both in mouse lung tissues and in human lung epithelial cells (A549). Accumulation of HIF-1α following H1N1 infection was not due to increased transcription of HIF-1α mRNA, but due to inhibited degradation of HIF-1α caused by impaired proteasome function. Additionally, the expression of FIH-1 decreased after H1N1 infection, which further boosted HIF-1 activity. Correspondingly, we found increased expression of HIF-1-responsive genes VEGF and GLUT-1. Thus, H1N1 activates the HIF-1 pathway under normoxic conditions mimicking a hypoxic response.

2. Materials and methods

2.1. Reagents

MG132 (catalog number S2619) and dimethyloxalylglycine (DMOG; catalog number S7483) was purchased from Selleck Chemicals (Houston, TX, USA). Cycloheximide (CHX; catalog number HY-12320) was purchased from MedChemExpress (Monmouth Junction, NJ, USA). MG132, DMOG, and CHX were used at a final concentration of 10 μM, 1 mM, and 50 μg/mL, respectively. Ham's F-12K and fetal bovine serum were purchased from Gibco/BRL Life Technologies (Grand Island, NY, USA). Anti-HIF-1α (catalog number SAB2702132) and anti-hydroxy-HIF-1α P402 (catalog number 07–1585) primary antibodies were purchased from Merck (St. Louis, MO, USA). Anti-hydroxy-HIF-1α P564 (catalog number 3434), anti-p53 (catalog number 2524), anti-GFP (catalog number 2955), and anti-β-actin (catalog number 4970s) primary antibodies were purchased from Cell Signaling Technology (Boston, MA, USA). Anti-FIH-1 (catalog number ab92498), anti-H1N1 influenza A virus nucleocapsid (NP; catalog number ab104870), and anti-Tubulin (catalog number ab6160) primary antibodies were purchased from Abcam (Cambridge, MA, USA). Anti-ubiquitin (catalog number sc-8017) was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Their respective horseradish peroxidase (HRP)-conjugated secondary antibodies were from Beyotime (Shanghai, China). Protein lysis buffer Radio Immunoprecipitation Assay (RIPA), Phenylmethanesulfonyl fluoride (PMSF), a Bicinchoninic Acid (BCA) protein concentration assay kit, and an SDS-PAGE gel preparation kit were from Beyotime (Shanghai, China). PVDF membrane and highly sensitive Enhanced chemilumescent (ECL) agent were purchased from Bio-Rad (Richmond, CA, USA) and Thermo Fisher Scientific (Waltham, MA, USA) respectively. TRIzol was purchased from Invitrogen (Grand Island, NY, USA). A PrimeScript™ RT Master Mix Kit and a SYBR Premix Ex Taq™ II Kit were purchased from TAKARA (Dalian, Liaoning, China). A Pierce™ Classic Magnetic IP/Co-IP Kit was purchased from Thermo Fisher Scientific (Waltham, MA, USA).

2.2. Cells and viruses

All cells were cultured in a humidified atmosphere at 37 ℃ with 5% CO_2. Human lung adenocarcinoma epithelial cells (A549) was purchased from ATCC. A549 cells were cultured in Ham's F-12K medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotics (100 U/mL penicillin and 0.1 mg/mL streptomycin). The influenza A/PR/8/34 strain was purchased from ATCC. A549 cells were seeded and after overnight incubation, washed with PBS twice, and then infected with influenza virus at indicated MOI. After 2 h adsorption, the inoculum was removed and the cells were maintained in maintenance media (Ham's F-12K containing 25 mM HEPES, 1% antibiotics, and 0.25 μg/mL TPCK treated trypsin) for the indicated time.

2.3. Animal model

BALB/c mice were purchased from the Chinese Academy of Sciences experiment center in Shanghai. All animal experiments were conducted in compliance with the guidelines from the Institutional Animal Care and Use Committee and approved by the Ethics Committee of Shanghai General Hospital, Shanghai Jiaotong University School of Medicine. Mice were intranasally infected with influenza A virus (A/PR/8/34) 800 PFU/mouse or saline at a volume of 50 μL. After 6 d, the mice were sacrificed. The mouse lung tissues were rapidly collected and cryopreserved in liquid nitrogen.

2.4. Patient serum samples and ethical statement

Serum samples were collected from 12 patients diagnosed with H1N1 infection between 2009 and 2016 at the Shanghai General Hospital and Shanghai Public Health Clinical Center (SHPHCC), Shanghai, China and control serum samples were obtained from 25 healthy volunteers. The diagnoses were according to the diagnostic criteria defined by the National Health and Family Planning commission of the People's Republic of China (http://www.nhfpc.gov.cn/yzygj/s3593g/201306/5fc4b2d158d7475fa0da32e959f9a7ac.shtml). The study was approved by the Ethics Committee of SHPHCC, and conducted in accordance with ethical principles of the World Medical Association Declaration of Helsinki and local legislation. Informed consent was signed by all participants prior to this study.

2.5. ELISA

Serum HIF-1α concentrations of H1N1 patients and healthy controls were measured according to the ELISA kit manufacturer's instructions (R&D Systems, USA). All of the samples were performed in triplicate.

2.6. Western blot

Total protein from the mouse lung samples and the cultured cells was extracted with RIPA. The protein concentrations were detected using a BCA protein assay kit. Then total protein samples were separated on an 10% SDS-PAGE and transferred onto a PVDF membrane. The membranes were blocked with 5% non-fat milk in Tris-buffered saline with Tween-20 (TBST) and incubated overnight at 4 ℃ with primary antibodies against HIF-1α (1:1000), hydroxy-HIF-1α P402 (1:1000), hydroxy-HIF-1α P564 (1:1000), ubiquitin (1:500), p53 (1:1000), GFP (1:1000), FIH-1 (1:7000), NP (1:1000), Tubulin (1:5000), and β-actin (1:1000). After washing in TBST, the bands were incubated with HRP-conjugated goat anti-rabbit secondary antibody (1:1000) or goat anti-mouse secondary antibody (1:1000) at room temperature for 1 h. After washing in TBST again, the bands were visualized with the ECL reagent according to the manufacturer's instructions.

2.7. Immunoprecipitation: HIF-1α polyubiquitination

The immunoprecipitation assay was performed using PierceTM Classic Magnetic IP/Co-IP Kit according to the manufacturer's instructions. Briefly, cell lysate containing 500 μg total protein was incubated with 5 μg HIF-1α antibody for IP overnight at 4 ℃. Then the antigen/antibody complex was incubated with 0.25 mg Protein A/G magnetic

beads for 1 h at room temperature. After washing beads twice with IP Lysis/Wash Buffer and once with purified water, the antigen/antibody complex was eluted from the beads by heating at 100 °C for 10 min. Subsequently, polyubiquitination of HIF-1α was measured by western blotting with anti-ubiquitin antibody.

2.8. RNA quantification

Total RNA was extracted with TRIzol reagent following the manufacturer's instructions. One to two μg total RNA was reverse transcribed using the PrimeScript™ RT Master Mix. Quantitative real-time PCR was performed using SYBR Premix Ex Taq™ II on the Vii7 system (ABI). Relative mRNA level in each individual sample was examined in triplicate, normalized to β-actin, and calculated using the $2^{-\Delta\Delta CT}$ method (Schmittgen and Livak, 2008). Specific primers used for qRT-PCR are listed in Supplementary Table S1.

2.9. GFPu lentivirus

GFP UPS reporter (GFPu), GFP with a degron CL-1 sequence, was subcloned into the lentiviral vector (pTRIP-IRES-puro). The pseudo particle was generated by co-transfected HEK293T cells with the plasmid pTRIP-IRES-puro-GFPu and the plasmids expressing VSVG and Gagpol. Lentiviral stocks harvested were used to infect the A549 cells, followed by cell selection through puromycin (1 μg/mL).

2.10. Statistical analysis

All the data were statistically analyzed using GraphPad prism (version 6.0; San Diego, CA, USA). Measurement data except serum HIF-1α are expressed as mean ± SEM. The data of serum HIF-1α are shown as mean ± SD. An unpaired two-tailed Student's *t*-test was used for comparisons between two indicated groups. A value of $p < 0.05$ was considered significant.

3. Results

3.1. H1N1 infection accumulates HIF-1α in vivo and in vitro

It has been reported that HIF-1α is stabilized by many viral pathogens (Kondo et al., 2006; Mazzon et al., 2013; Haeberle et al., 2008; Yogev et al., 2014; Moon et al., 2004; Guo et al., 2014). To investigate the expression of HIF-1α after H1N1 infection, the mice were intranasally administered H1N1 (A/PR/8/34) 800 PFU/mouse or the same volume of saline as a control (3 mice per group). After 6 d post-infection (p.i.), protein extracted from mouse lungs was subjected to western blotting (the specificity of HIF-1α antibody was validated in Supplementary Fig. S1). As shown in Fig. 1**A**, the expression of HIF-1α apparently increased after H1N1 infection compared with the mock infection group. To validate the influence of H1N1 on the expression of HIF-1α, A549 cells were infected with H1N1 at different multiplicity of infection (MOI) values under normoxic conditions. At 24 h p.i., HIF-1α had accumulated to significant levels at MOI values of 0.1, 0.5, and 1 compared with mock infected cells (Fig. 1**B**). We further examined the expression pattern of HIF-1α at different post-infection time points. A549 cells were infected with H1N1 at an MOI of 1 in normoxia, and HIF-1α protein was detected at different time points by western blotting. To exclude the impact of cell culture conditions, the expression of HIF-1α in mock infected cells at different time points were also measured. The results showed that HIF-1α had accumulated at 16 and 24 h p.i., and was significantly higher than that in mock infected cells (Fig. 1**C**). Consistent with these observations, the serum HIF-1α concentrations in patients with H1N1 infections were significantly higher than those in healthy controls (Fig. 1**D**). Collectively, these data show that HIF-1α accumulates following H1N1 infection both *in vivo* and *in vitro*.

3.2. H1N1 infection stabilizes HIF-1α by blocking its degradation

The expression of HIF-1α is mainly regulated at the transcriptional level and posttranslational level, also called protein stability level. Under normoxic conditions, HIF-1α is hydroxylated by oxygen dependent PHD at P402 and P564 in the oxygen-dependent degradation (ODD) domain. After prolyl hydroxylation, HIF-1α is ubiquitinated by von Hippel-Lindau (VHL) E3-ubiquitin ligase and then degraded by the proteasome (Fig. 2**A**) (Jaakkola et al., 2001; Ivan et al., 2001). Under hypoxic conditions, the activity of PHD is inhibited due to lack of oxygen, which causes HIF-1α's stabilization (Ivan et al., 2001; Jaakkola et al., 2001). As we described previously, H1N1 infection induces HIF-1α overexpression both *in vivo* and *in vitro*. To find the mechanism of this, firstly, HIF-1α transcription level after H1N1 infection was examined by qRT-PCR. As shown in Fig. 2**B** and Fig. 2**C,** HIF-1α mRNA levels were not changed between H1N1 infected groups and mock infected groups either *in vivo* or *in vitro*, indicating that H1N1 infection did not impact HIF-1α mRNA transcription. Then we wondered whether H1N1 infection influenced the stability of HIF-1α. To elucidate this, both H1N1 infected and mock infected A549 cells (22 h p.i.) were treated with DMOG for 2 h and then exposed to CHX to block further HIF-1α mRNA translation. The kinetics of HIF-1α degradation in H1N1 infected and mock infected cells was compared by western blotting. The degradation rate of HIF-1α in H1N1 infected cells was much slower than that in mock infected cells (Fig. 2**D and E**), suggesting that H1N1 infection significantly inhibited HIF-1α's degradation.

3.3. H1N1 infection does not impair HIF-1α prolyl hydroxylation and ubiquitination

As mentioned above, HIF-1α's stability is influenced by the activity of PHD, VHL E3-ubiquitin ligase, and proteasome. Some previous studies have shown that the activity of PHD is inhibited by pathogens by a range of mechanisms (Kondo et al., 2006; Mazzon et al., 2013; Siegert et al., 2015; Yogev et al., 2014). Whether prolyl hydroxylation of HIF-1α was impaired in H1N1 infected cells was examined using two special antibodies targeting P402 or P564 hydroxylated HIF-1α (Siegert et al., 2015). As shown in Fig. 3**A**, we did not detect hydroxylated form of HIF-1α without the use of MG132, a proteasome inhibitor. Hence, we compared the difference of HIF-1α hydroxylation between H1N1 and mock infected cells treated with 10 μM MG132 for 6 h. As expected, both total HIF-1α and hydroxylated HIF-1α were significantly accumulated with the use of MG132. However, an approximately same amount of P402 hydroxylated HIF-1α was accumulated between H1N1 infected and mock infected cells in the presence of MG132, the same as P564 hydroxylated HIF-1α (Fig. 3**A**), clearly indicating that both P402 and P564 hydroxylation of HIF-1α were not impaired in H1N1 infected cells.

It has been reported that some pathogens can perturb the ubiquitination of HIF-1α, causing the accumulation of HIF-1α (Moon et al., 2004; Guo et al., 2014). Next, whether ubiquitination of HIF-1α was impaired in H1N1 infected cells was examined by co-immunoprecipitation. In the absence of MG132, ubiquitinated HIF-1α was nearly undetectable. With 10 μM MG132 treatment for 6 h, ubiquitinated HIF-1α was apparently accumulated. However, compared with mock infected cells, the ubiquitination of HIF-1α in H1N1 infected cells was not impaired (Fig. 3**B**).

3.4. H1N1 infection inhibits the function of proteasome

As demonstrated above, HIF-1α's degradation was inhibited after H1N1 infection but its prolyl hydroxylation and ubiquitination were not impaired, so we turned attention to the function of proteasome, the last step responsible for HIF-1α's degradation. It is known that p53 is also degraded by ubiquitin-proteasome system (UPS) (Maki et al., 1996; Allende-Vega and Saville, 2010), so we measured the expression of p53

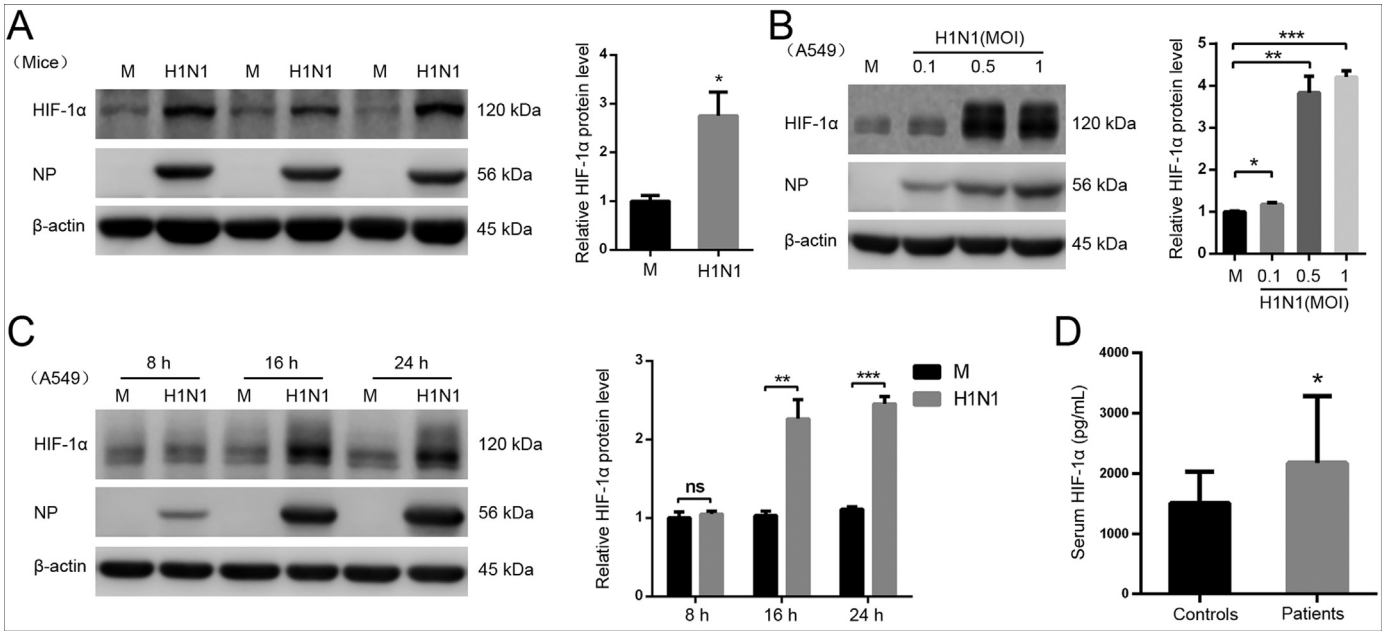

Fig. 1. H1N1 infection causes HIF-1α accumulation *in vivo* and *in vitro*. (A) Mice were intranasally administered H1N1 (A/PR/8/34) 800 PFU/mouse or mock infected as a control (n = 3 mice/group). At 6 d p.i., protein extracted from mouse lungs was subjected to western blotting. (B) A549 cells were infected with H1N1 at different MOI values. At 24 h p.i., cells were harvested and analyzed by western blotting. (C) A549 cells were mock infected or infected with H1N1 at an MOI of 1, cells were harvested at 8, 16, and 24 h p.i., then analyzed by western blotting. (D) Serum HIF-1α concentrations of healthy controls (n = 25) and H1N1-infected patients (n = 12) were measured by ELISA. Data are representative of three independent experiments (A–C). M, mock infection. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$.

after H1N1 infection. As shown in Fig. 4**A,** p53 accumulation was also observed after H1N1 infection, highly indicating that H1N1 might impair the function of proteasome. To confirm this, A549 cells were firstly infected with a lentivirus containing GFP UPS reporter (GFPu), green fluorescence protein (GFP) fused to a short peptide degron CL-1 that facilitates its rapid turnover in conditions of a normal function of proteasome (Bence et al., 2005; Liu et al., 2014). This GFPu stable-expressing A549 cell line was an ideal tool to evaluate proteasome function status. GFP would be accumulated in cells in case of inhibited proteasome function. Then GFPu stable-expressing A549 cells were mock infected or infected with H1N1 for 24 h, and MG132, a proteasome inhibitor, was used as a positive control. As expected, MG132 treated cells significantly accumulated GFP. In the absence of MG132, H1N1 infected cells accumulated obviously more GFP than mock infected cells (Fig. 4**B and C**). Taken together, H1N1 infection impairs the function of proteasome.

3.5. H1N1 infection activates the HIF-1 pathway in normoxia

HIF-1α is accumulated by H1N1 via the inhibition of proteasome. Besides this, we also observed the expression of FIH-1 after H1N1 infection in A549 cells, which suppressed HIF-1α activity by hydroxylating 803 asparagine within HIF-1α then blocking its binding to co-activators p300/CBP (Mahon et al., 2001; Lando et al., 2002). As shown in Fig. 5**A**, FIH-1 decreased at 16 and 24 h p.i., and was significantly lower than that in mock infected cells. Decreased FIH-1 might reduce the hydroxylation of N803 within HIF-1α, which further promoted the transcription of HIF-1 downstream target genes. Then, HIF-1-induced transcription was investigated by quantifying mRNA levels of VEGF and GLUT-1 in H1N1 infected mouse lungs and A549 cells. H1N1 significantly induced transcription of both genes (Fig. 5**B-E**), indicating that the HIF-1 pathway was indeed activated after H1N1 infection.

4. Discussion

HIF-1 is a general transcription factor for cellular adaptation to hypoxia (Wang and Semenza, 1993). It is composed of a regulatory HIF-1α subunit and a constitutive HIF-1β subunit (Wang and Semenza, 1995). When HIF-1α is stabilized, it translocates to the nucleus, and forms the functional HIF-1 transcription factor by binding its partner subunit HIF-1β and recruiting p300/CBP acetyltransferases. This complex induces the transcription of a range of target genes by binding to their hypoxia response elements (HRE), which locate in or around the promoter region of each HIF-1 target gene. Overall, Control of HIF-1 activity is mainly achieved by the expression level of HIF-1α and its binding activity to co-activators p300/CBP.

The expression of HIF-1α is chiefly controlled by HIF-1α mRNA transcription and posttranslational modifications. In our study, H1N1 infection significantly induced HIF-1α overexpression, but did not increase HIF-1α mRNA transcription either in mouse lungs or in human lung epithelial cells, which was in concordance with a published microarray dataset of human bronchial epithelial cells infected with a seasonal H1N1 strain BN/59 (GEO accession number GDS4855) (Gerlach et al., 2013). By comparing the degradation rates of HIF-1α in H1N1 infected cells and mock infected cells, we discovered that H1N1 infection increased the stability of HIF-1α. Hypoxia usually occurs in lung tissues infected with influenza because of inflammation-induced pulmonary capillaries and alveolar epithelium injury (Herold et al., 2015; Traylor et al., 2013), and hypoxia can stabilize HIF-1α via the inhibition of PHD, which gives a potential explanation for HIF-1α accumulation after H1N1 infection *in vivo*. However, HIF-1α was also upregulated even though cells were infected with H1N1 under normal oxygen concentrations, indicating that H1N1-induced HIF-1α accumulation *in vitro* was oxygen-independent.

PHD (including PHD1–3) plays a central role in the posttranslational regulation of HIF-1α (Ivan et al., 2001; Jaakkola et al., 2001; Kaelin and Ratcliffe, 2008). PHD activity requires cosubstrate (oxygen, α-ketoglutarate) and cofactor (iron) (Ivan et al., 2001; Jaakkola et al., 2001). Its activity is also impaired by reactive oxygen species (ROS), reactive nitrogen species (RNS), fumarate, and succinate (Kaelin and Ratcliffe, 2008). Consequently, PHD has been a vulnerable target for pathogens to modulate HIF-1α expression (Yogev et al., 2014; Kondo et al., 2006). The lipopolysaccharide (LPS) of bacteria inhibited PHD activity thereby accumulating HIF-1α through NF-kB mediated

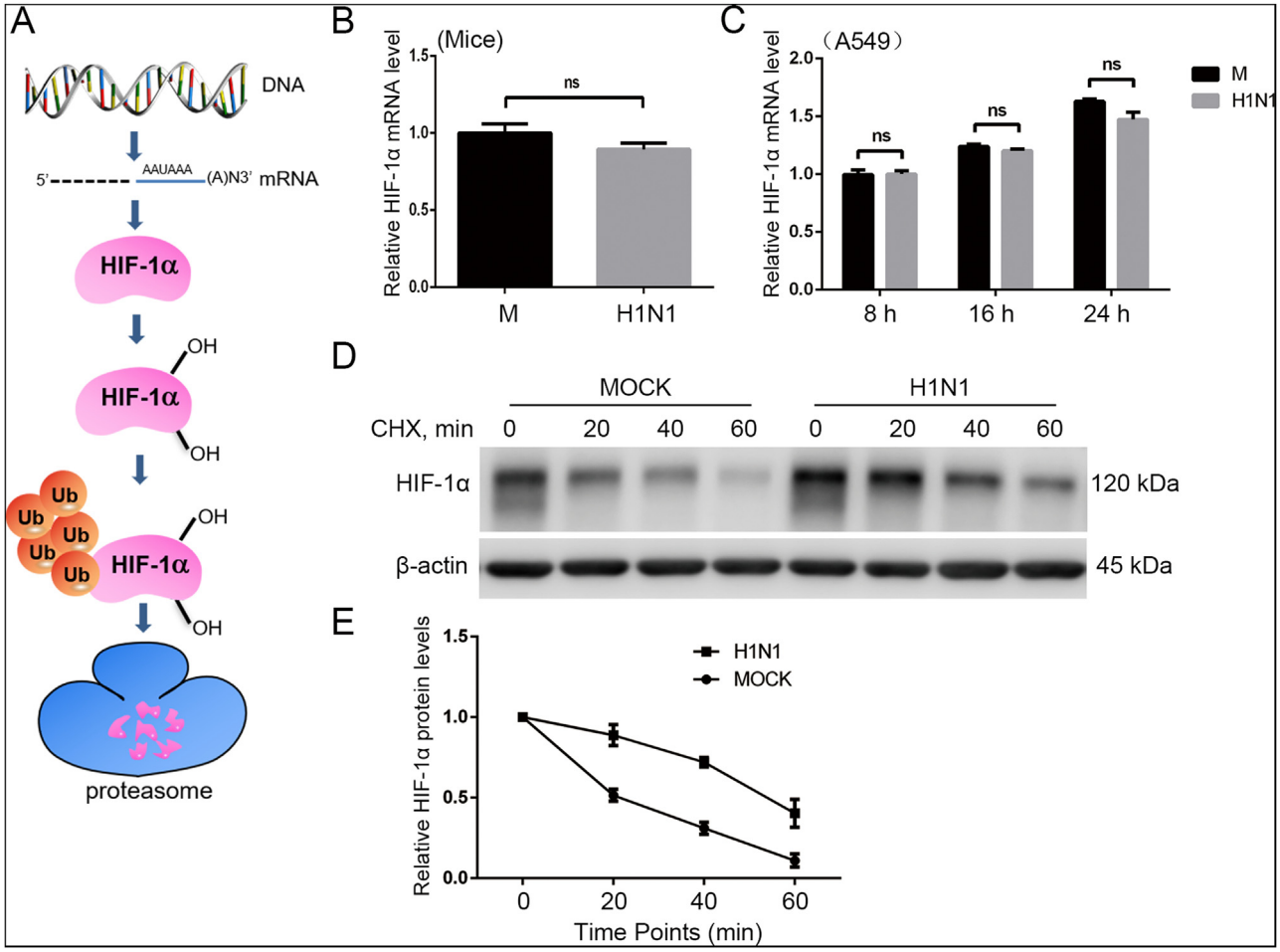

Fig. 2. H1N1 infection does not impact HIF-1α mRNA transcription but inhibits the degradation of HIF-1α. (A) Schematic overview of the synthesis and degradation of HIF-1α. (B) Mice were intranasally administered H1N1 (A/PR/8/34) 800 PFU/mouse or mock infected as a control (n = 3 mice/group). At 6 d p.i., RNA extracted from mouse lungs was subjected to qRT-PCR. β-actin expression was used as an internal control. M, mock infection. (C) A549 cells were mock infected or infected with H1N1 at an MOI of 1, cells were harvested at 8, 16, and 24 h p.i., then total RNA was extracted and HIF-1α mRNA levels were analyzed by qRT-PCR. β-actin expression was used as an internal control. M, mock infection. (D) A549 cells were mock infected or infected with H1N1 at an MOI of 1. At 22 h p.i., cells were treated with DMOG for 2 h and then exposed to 50 μg/mL CHX. Cells were harvested at indicated time points after CHX addition and HIF-1α levels were measured by western blotting. (E) Graphical representation of HIF-1α protein levels from (D) based on densitometry, representing results of three independent experiments. Each experiment was independently performed three times. ns, not significant.

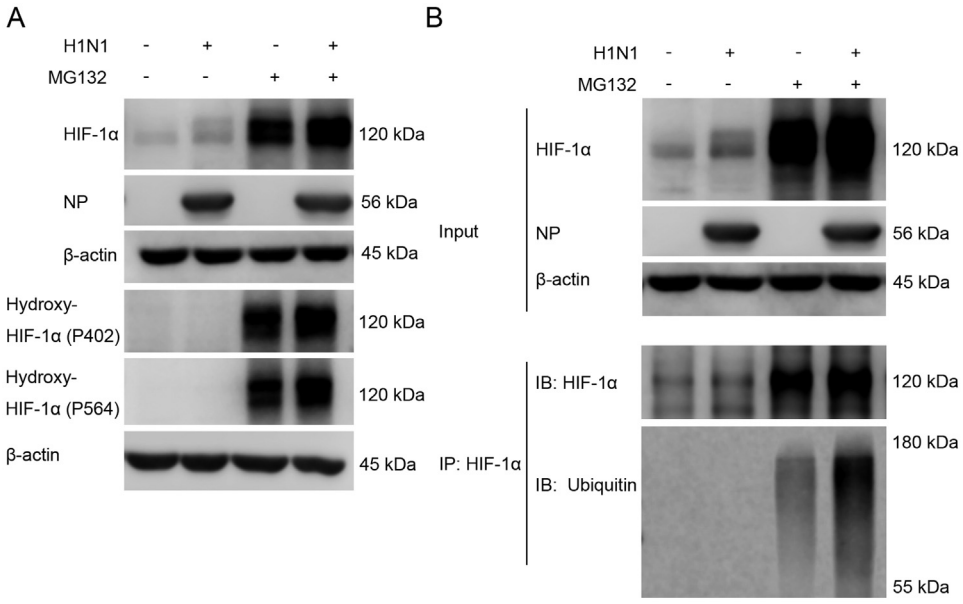

Fig. 3. HIF-1α prolyl hydroxylation and ubiquitination are not impaired after H1N1 infection. A549 cells were mock infected or infected with H1N1 at an MOI of 1 for 24 h, with or without 10 μM MG132 treatment for the final 6 h. (A) total HIF-1α, NP, HIF-1α hydroxylated at P402 and P564 levels are shown. (B) Above, protein levels of HIF-1α, NP, and β-actin in input samples are shown. Below, total protein was immunoprecipitated by HIF-1α antibody, then protein pulled down was analyzed by western blotting. Each experiment was independently performed three times.

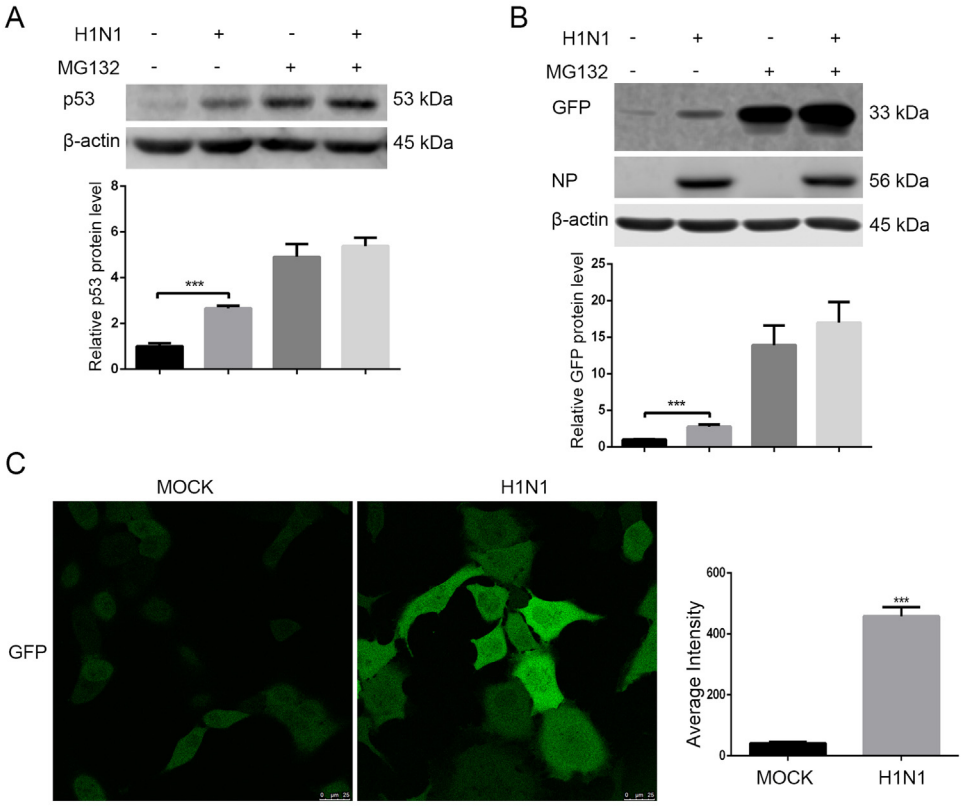

Fig. 4. H1N1 infection impairs the function of proteasome. (A) A549 cells were mock infected or infected with H1N1 at an MOI of 1 for 24 h, with or without 10 μM MG132 treatment for the final 6 h. p53 and β-actin levels are shown. (B) GFPu stable-expressing A549 cells were mock infected or infected with H1N1 at an MOI of 1 for 24 h, with or without 10 μM MG132 treatment for the final 6 h. GFP, NP, and β-actin levels are shown. (C) GFPu stable-expressing A549 cells were mock infected or infected with H1N1 at an MOI of 1 for 24 h, then were observed by fluorescence microscope. Each experiment was independently performed three times. * ** $p < 0.001$.

induction of ferritin and subsequent decrease of intracellular available iron, a critical cofactor of PHD (Siegert et al., 2015). Vaccinia virus protein C16 directly binds to the PHD2 and thereby inhibits PHD2-dependent hydroxylation of HIF-1α, which leads to a rapid stabilization of HIF-1α (Mazzon et al., 2013). HCV triggers stabilization of HIF-1α under normoxic conditions because of impaired PHD activity, caused by increased oxaloacetate and ROS (Nasimuzzaman et al., 2007; Ripoli et al., 2010). Different from these studies, H1N1 did not impair HIF-1α

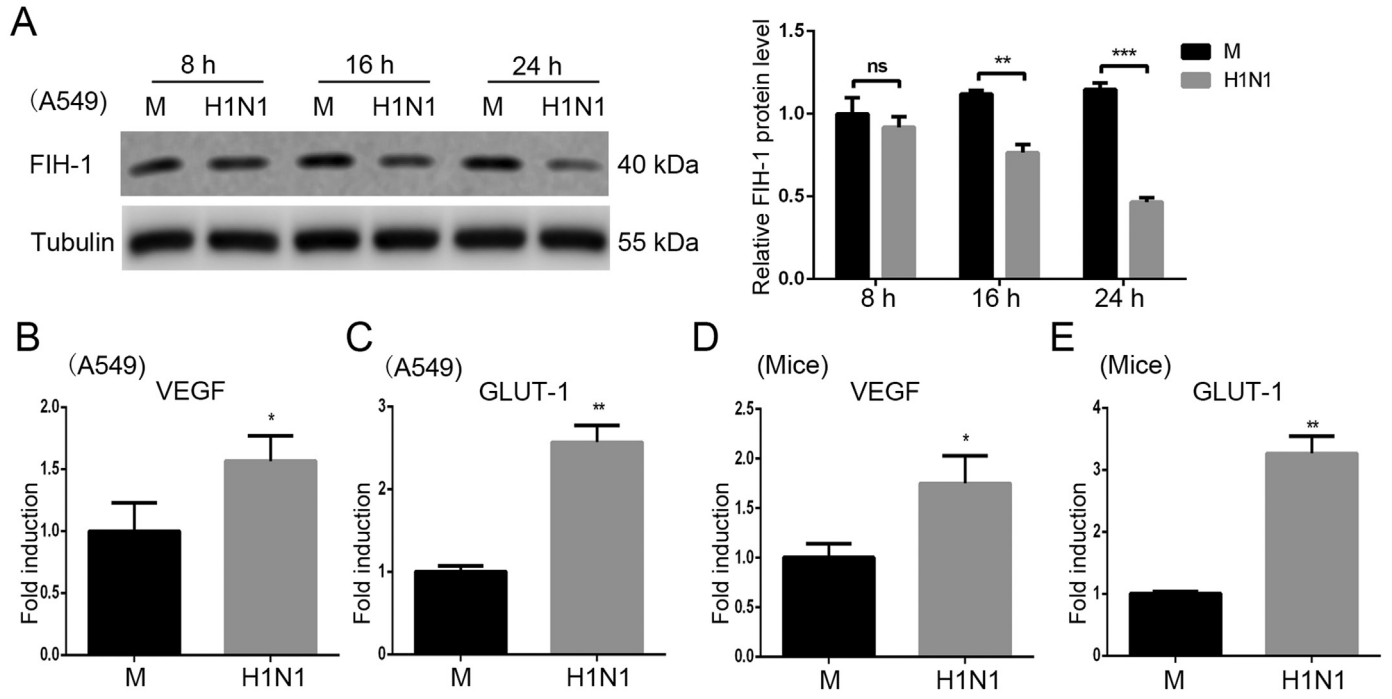

Fig. 5. H1N1 infection activates the HIF-1 pathway. (A) A549 cells were mock infected or infected with H1N1 at an MOI of 1, cells were harvested at 8, 16, and 24 h p.i., then FIH-1 and Tubulin levels were analyzed by western blotting. (B, C) A549 cells were mock infected or infected with H1N1 at an MOI of 1 for 24 h. VEGF and GLUT-1 mRNA levels were measured by qRT-PCR. β-actin expression was used as an internal control. (D, E) Mice were infected with H1N1 800 PFU/mouse or mock infected. At 6 d p.i., RNA extracted from mouse lungs was subjected to qRT-PCR. β-actin expression was used as an internal control. Each experiment was independently performed three times. M, mock infection. * $p < 0.05$, * * $p < 0.01$, * ** $p < 0.001$.

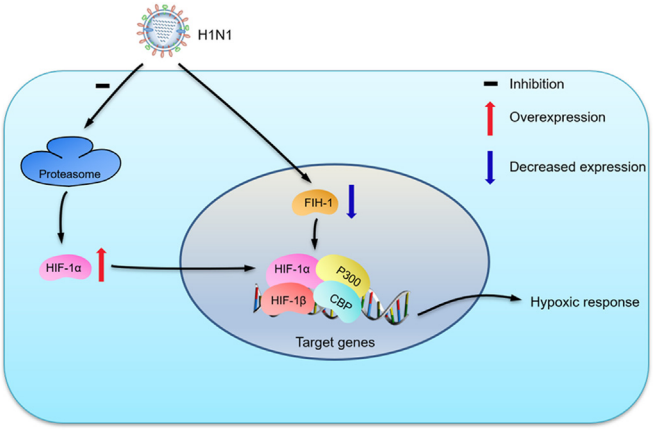

Fig. 6. Schematic of the proposed model for the H1N1 induced hypoxic response in normoxia. H1N1 stabilizes HIF-1α by inhibiting the proteasome function. In addition, a decreased expression of FIH-1 after H1N1 infection leads to derepression of HIF-1 activity.

prolyl hydroxylation, indicating that PHD activity was not inhibited in H1N1 infection. Besides PHD-mediated hydroxylation, the process of HIF-1α ubiquitination is also attacked by some pathogens. For example, Hepatitis B virus and human Papillomavirus 16 stabilize HIF-1α by attenuating the VHL-HIF-1α interaction (Moon et al., 2004; Guo et al., 2014). However, in H1N1 infected A549 cells, HIF-1α ubiquitination was not impaired, revealing that VHL E3-ubiquitin ligase was not affected in H1N1 infection. Instead, H1N1 inhibited the function of proteasome, the last step responsible for HIF-1α degradation, causing HIF-1α accumulation, which showed a previously unrecognized and interesting phenomenon. However, whether the mechanisms for increased HIF-1α accumulation during H1N1 infection *in vivo* consist with that in the *in vitro* system needs to be investigated. Utilizing different inhibitors or genetically modified animals might help to verify this perspective.

The proteasome is a multisubunit enzyme complex responsible for regulated protein degradation in eukaryotic cells (Bard et al., 2018). The ubiquitin-proteasome system (UPS) plays a fundamental role in a wide range of cellular functions by degrading damaged or misfolded proteins and maintaining a critical level of important regulatory proteins (Bedford et al., 2010). The interaction between virus and UPS has been reported in some studies (Nasheri et al., 2015; Minor and Slagle, 2014). The present view holds that UPS serves as a two-edged sword in viral pathogenesis: on the one hand, the UPS is utilized by many viruses to maintain proper level and function of viral proteins; on the other hand, the UPS constitutes a host defense mechanism to eradicate viral components (Luo, 2016). For the first time we showed that H1N1 infection leads to marked reduction of proteasome function, however detailed mechanism of proteasome inhibition still remains unclear. Whether this inhibition is due to direct interaction between viral proteins and proteasome and what roles viral inhibition of proteasome will play in virus life cycle and pathogenesis need further study.

HIF-1 activity is also influenced by FIH-1, which hydroxylates 803 asparagine within HIF-1α then blocks HIF-1α binding to co-activators p300/CBP (Lando et al., 2002; Mahon et al., 2001). In a previous study, FIH-1 protein level was unaffected by parapoxvirus orf virus infection, but sequestration of FIH-1 by parapoxvirus orf virus ANK proteins led to derepression of HIF-1 activity (Chen et al., 2017). In our study, we observed a decreased expression of FIH-1 after H1N1 infection, which further boosted the HIF-1 pathway. However, the detailed mechanism of FIH-1 reduction after H1N1 infection is still unknown.

HIF-1 stimulates the transcription of many downstream target genes, playing important roles in cell metabolism, cell cycle, angiogenesis, immunity, inflammation, and tumor metastasis (Semenza, 2012; Koyasu et al., 2018; Palazon et al., 2014). According to previous studies (Mazzon et al., 2015; Yogev et al., 2014; Xu et al., 2018), we speculate that activated HIF-1 after H1N1 infection may reprogram host cellular glucose metabolism towards enhanced glycolysis to support efficient viral replication. On the other hand, HIF-1 can facilitate host innate and adaptive immune responses (Zinkernagel et al., 2007; Dang et al., 2011; Finlay et al., 2012; Wobben et al., 2013), which promotes virus clearance. The role HIF-1 plays in influenza infection *in vivo* still remains unclear, and further investigations are required to ascertain this issue.

In summary, in this report we observed that H1N1 activated the HIF-1 pathway mimicking a hypoxic response in normoxia by stabilizing HIF-1α via inhibition of proteasome and decreasing FIH-1 expression (Fig. 6).

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Conflict of interest

The authors declare no potential conflicts of interest.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.virol.2019.02.010.

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