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heme oxygenase-1 agonist CoPP suppresses influenza virus replication through IRF3-mediated generation of IFN-α/β

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ABSTRACT

The innate immunity plays an essential role in defending infection of Influenza A virus (IAV). The regulatory effect of heme oxygenase-1 (HO-1), a cytoprotective enzyme, on innate immunity has been revealed. In this study, we aim to confirm the antiviral effect of CoPP (Cobaltic Protoporphyrin IX Chloride), a potent HO-1 inducer on IAV infection and elucidate the possible mechanism of HO-1-mediated host innate immune responses. Our results show that CoPP exhibits broad-spectrum antiviral activities against IAV. Furthermore, CoPP attenuates IAV replication through inducing type I IFNs response, not depending on HO-1 enzymatic activity. We also provide direct evidence that HO-1-mediated type I IFN response activation is largely due to its interaction with IRF3, which then promotes IRF3 phosphorylation and nuclear translocation. These results suggest that HO-1 agonist CoPP suppresses IAV replication through IRF3-mediated generation of IFN-α/β. Thus, therapeutic induction of HO-1 might be a promising strategy to combat IAV epidemics.

1. Introduction

Influenza A virus (IAV) infection remains to be a persistent global health threat with high morbidity and mortality. The antiviral efficacies of available antiviral drugs, including M2 ion-channel inhibitors and neuraminidase inhibitors, were restricted with the emergence of drug-resistant virus strains and recombined pathogenic virus strains (van der Vries et al., 2013; Bright et al., 2005; Shen et al., 2015). This highlights an urgency to develop alternative strategies to overcome pandemic influenza.

The host innate immune response plays a pivotal role in counteracting virus infection (McNab et al., 2015). Type I IFN has been demonstrated to be essential in defending IAV infection by induction of IFN-stimulated genes (ISGs), which works in synergy to inhibit viral replication via multiple distinct mechanisms (Sadler and Williams, 2008; Everitt et al., 2012; Yan and Chen, 2012). However, for colonizing the hosts, IAV evades or inhibits host innate immune responses through making use of IAV NS1 protein or inducing the expression of MAPK phosphatase 5 (MKP5) (Garcia-Sastre, 2001; James et al., 2015). Accordingly, approaches that induce or restore type I IFN production and corresponding antiviral response are likely to inhibit the IAV replication in hosts.

Heme oxygenase-1 (HO-1), a cytoprotective enzyme, catalyzes the rate-limiting step in heme degradation and converts the heme into CO, biliverdin and iron (Motterlini and Foresti, 2014). HO-1 plays a crucial role in the maintenance of cellular homeostasis, which is due to its anti-inflammatory and anti-oxidant effects (Motterlini and Foresti, 2014). In recent years, the relationship between HO-1 and innate immunity has been revealed. For instance, HO-1 regulates innate immunity and autoimmunity by modulating IFN-β production (Tzima et al., 2009). Additionally, the activation of type I IFN response by HO-1 was proved during human respiratory syncytial virus (hRSV) infectionin vivo (Espinoza et al., 2017). Recently, numerous studies have illustrated the role of HO-1 in controlling viral infections, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), Enterovirus 71 (EV 71), Ebola virus (EBOV), hRSV and Dengue (Tzima et al., 2009; Espinoza et al., 2017; Zhu et al., 2008; Protzer et al., 2007; Tung et al., 2011; Hill-Batorski et al., 2013; Tseng

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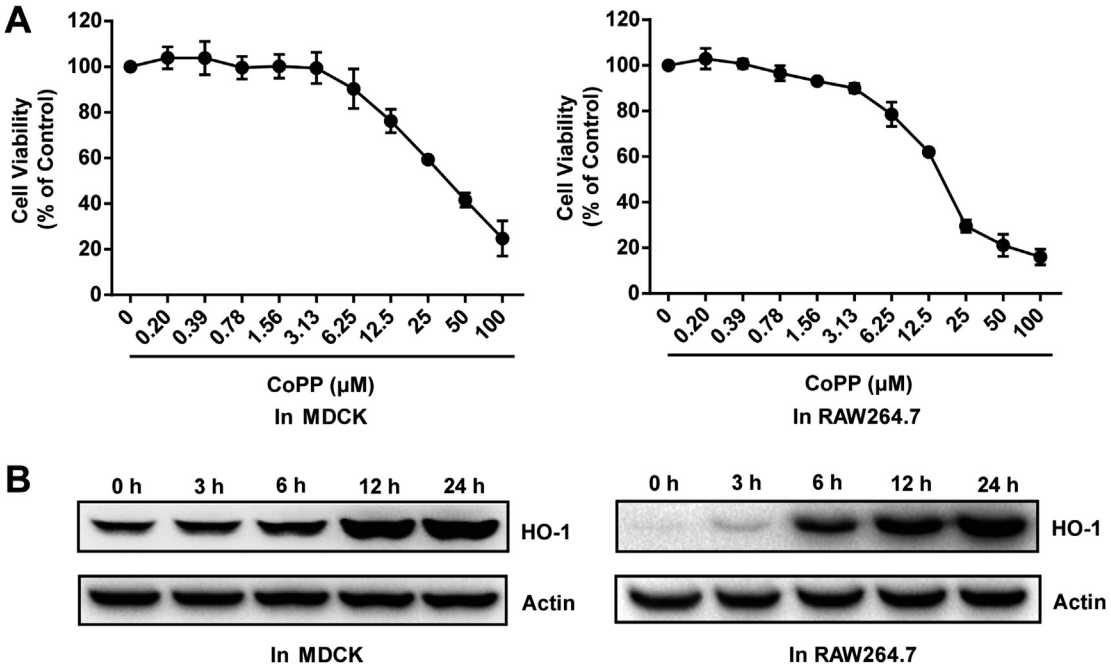

Fig. 1. CoPP enhances the expression of HO-1. (A) The effects of CoPP on cell viabilities of MDCK cells and RAW264.7 cells were determined through MTT assay. (B) The effects of CoPP on protein expression of HO-1 within 24 h in MDCK cells and RAW264.7 cells were analyzed by western blotting assay. The experiments were performed in triplicate, and the data represents mean ± SD.

et al., 2016). Specifically, upregulation of HO-1 mediated by a rupestonic acid derivative was also shown to suppress infection of IAV in our previous study. This compound exhibited potent antiviral activity against multiple influenza A and B viruses through upregulation of HO-1, which implies that inducing HO-1 expression could inhibit IAV replication. Furthermore, the anti-IAV activity of HO-1 was further confirmed by transfection with GFP-HO-1 (Ma et al., 2016). Unlike antiviral mechanism of HO-1 against HCV and EBOV, our previous study provided evidence showing that upregulation of HO-1 inhibited IAV replication via inducing expressions of IFN-α/β as well as ISGs, not depending on its enzymatic activity (Ma et al., 2016). However, the exact mechanism of HO-1 activating type1 IFN response has not been fully illuminated.

In this study, we used CoPP (Cobaltic Protoporphyrin IX Chloride), a potent HO-1 inducer, to confirm the antiviral effect of HO-1 on IAV infection and its role on activating innate immune response. Additionally, we clarified the mechanism of HO-1-mediated host innate immune responses, which might provide clues for us to develop new strategies to overcome pandemic influenza.

2. Materials and methods

2.1. Compounds

CoPP and SnPP (Tin Protoporphyrin IX dichloride) were purchased from Santa Cruz Biotechnology, Dallas, Texas, USA, with oseltamivir carboxylate (OC; Medchem, Princeton, NJ, USA), ribavirin (RBV; Sigma-Aldrich, St Louis, MO, USA) and amantadine hydrochloride (AH; Sigma-Aldrich) were used as reference compounds. 2 mM stock solutions of CoPP or SnPP were firstly dissolved in acidic aqueous solutions, and neutralized with basic aqueous solutions, reaching pH between 8.5 and 9.0. Meanwhile, 2 mM OC and RBV stock solutions were dissolved in culture medium. and 2 mM AH stock solutions were prepared in DMSO. These compounds were diluted to final working solutions with culture medium as indicated in experiments.

2.2. Cells and viruses

Madin-Darby canine kidney (MDCK) (NBL2) cells were obtained from the America Type Culture Collection (ATCC® CCL34™) and cultured in minimum essential medium (MEM; Invitrogen, Carlsbad, CA, USA) containing 10% FBS (Gibco, Grand Island, NY, USA), 1% Penicillin-Streptomycin (10,000 U ml^{-1}, 10,000 μg ml^{-1}) (Invitrogen) and 1% MEM Non-Essential Amino Acids Solution (NEAA, Invitrogen). Mouse macrophage RAW264.7 cells were purchased from Cell Resource Center at Peking Union Medical College, Beijing, China, and grown in Dulbecco's Modified Eagle Medium (DMEM, Invitrogen), supplemented with 10% FBS and 1% Penicillin-Streptomycin.

IAV A/Fort Monmouth/1/1947(H1N1) strain was obtained from ATCC (ATCC® VR-1754™). Clinical isolated influenza virus strains A/TianjinJinnan/15/2009(H1N1, oseltamivir-resistant), A/Wuhan/359/1995(H3N2), A/FujianTongan/196/2009(H3N2, amantadine-resistant) as well as BY/FujianXinluo/54/2006 were kindly donated by Professor Yuelong Shu at the Institute for Viral Disease Control and Prevention, China Centers for Disease Control and Prevention, Beijing, China. All of these viral stocks were propagated in 10-day-old embryonated chicken eggs for 2 or 3 days under BSL-2 condition.

2.3. Cytotoxicity test

The cytotoxicity of compounds was evaluated by a tetrazolium dye (MTT) based assay. Briefly, MDCK cells (2.5×10^4 per well) or RAW264.7 cells (8×10^4 per well) grown in 96-well plates were treated with serial dilutions of CoPP or control drugs for 72 h. Thereafter, 10 μl of 5 mg ml^{-1} MTT (Promega, Madison, WI, USA) dissolved in PBS was added to cells. After 4 h incubation, the medium was aspirated and replaced by 150 μl of DMSO. The plates were shaken for 10 min and then measured by scanning absorbance at 450 nm on Enspire (Perkin Elmer, Waltham, MA, USA). 50% toxicity concentration (TC_{50}) of compounds were determined by Reed and Muench method (Serkedjieva and Ivancheva, 1999).

Table 1
Inhibitory activity of CoPP against five influenza virus strains.

		A/FortMonmouth /1/1947		A/TianjinJinnan /15/2009		A/Wuhan /359/1995		A/FujianTongan /196/2009		BY/FujianXinluo /54/2006	
	TC_{50} (μM)	IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI	IC_{50} (μM)	SI
CoPP	27.07	0.40 ± 0.16	67.68	0.42 ± 0.15	64.45	0.46 ± 0.19	58.85	0.34 ± 0.05	79.62	0.64 ± 0.30	42.30
OC	> 200	0.24 ± 0.05	> 833.33	2.77 ± 0.18	> 72.20	0.11 ± 0.01	> 1818.18	0.26 ± 0.03	> 769.23	0.58 ± 0.03	> 344.83
RBV	> 200	5.98 ± 2.57	> 33.44	6.12 ± 1.78	> 32.68	5.99 ± 0.23	> 33.39	7.33 ± 1.50	> 27.29	2.92 ± 0.46	> 68.49
AH	209.75	0.90 ± 0.28	233.06	16.54 ± 4.16	12.68	15.63 ± 4.84	13.42	80.24 ± 17.89	2.61	> 83.24	–

Note: TC_{50}: 50% toxicity concentration.
IC_{50}: 50% CPE inhibition concentrations.
SI: selectivity index; SI = TC_{50}/IC_{50}.
"–": no antiviral activity at the maximal nontoxic concentration (TC_0).
n = 3, each value represents mean ± SD.

2.4. Cytopathic effect (CPE) assays

MDCK cells seeded in 96-well plates were washed with PBS, then incubated with influenza virus at a concentration of 100 $TCID_{50}$ (50% tissue culture infective dose) in serum-free medium at 37 °C for 2 h. Following the adsorption period, the unbound viruses were removed and replaced by maintenance medium supplemented with 2 μg ml^{-1} TPCK-treated trypsin (Worthington, Lakewood, Colorado, USA) and 0.08% BSA (Beijing Yuan Heng Golden Horse biological technology development Co., Ltd., China) with or without the tested compounds. The reduction of virus-induced CPE caused by compounds were recorded when the CPE of virus control group reached 100%, then the 50% CPE inhibition concentrations (IC_{50}) were calculated using Reed and Muench method (Liu et al., 2008), and the selectivity index (SI) values were determined as the ratio of TC_{50}/IC_{50}.

2.5. Western blot assay

For whole-cell extract preparation, the cells were lysed in M-PER mammalian protein extraction reagent containing halt protease and phosphatase inhibitor cocktail, while nuclear and cytosolic extracts were prepared using nuclear and cytoplasmic extraction kit (Thermo Fisher Scientific, Waltham, MA, USA). The protein concentrations were determined by BCA protein assay kit (Thermo Fisher Scientific) and equal amount of cell proteins were subjected to SDS-PAGE, then transferred to PVDF membrane (Millipore, Billerica, MA, USA). After blocked with 5% milk, the membranes were incubated with antibodies against HO-1 (1:250) (Abcam Cambridge, MA, USA), IAV M2 (1:500), IAV NS1 (1:500), IFITM3 (1:500), PKR (1:500), OAS1 (1:500) (Santa Cruz, Dallas, Texas, USA), total and phospho-specific IRF3 (1:1000), β-actin (1:5000), or histone H3 (1:1000) (Cell Signaling Technology, Beverly, MA, USA), respectively. Lastly, corresponding HRP-conjugated secondary antibodies (1:5000) (Santa Cruz) were applied and the signals were detected using an ECL kit (GE Healthcare Life Sciences, Pittsburgh, PA, USA).

2.6. Quantitative real-time RT-PCR

Total RNAs from cells were isolated using RNeasy Mini Kit (Qiagen, Germantown, MD, USA). The RNAs of IAV M2, IFN-α, IFN-β and GAPDH were amplified by quantitative real-time RT-PCR. It was carried out with an ABI 7500 Fast real-time PCR instrument (Applied Biosystems, Foster City, CA, USA) using SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen) with the following procedures: 50 °C for 3 min, 95 °C for 5 min, followed by 35 cycles of 95 °C for 15 s, 60 °C for 30 s. The relative RNA amount of IAV M2, IFN-α as well as IFN-β were calculated by comparative Ct method after normalizing against the quantity of GAPDH. The primer sequences were used as follows: IAV M2: forward: 5′- GACCRATCCTGTCACCTCTGAC-3′, reverse: 5′-GGGCATTYTGGACAAAKCGTCTACG-3′; IFN-α (Mouse): forward: 5′- CCTGTGTGATGCAACAGGTC-3′, reverse: 5′-TCACTCCTCCTT GCTCAATC-3′. IFN-β (Mouse): forward: 5′-AGCTCCAAGAAAGGACGA ACAT-3′, reverse: 5′-GCCCTGTAGGTGAGGTTGATCT-3′. GAPDH (Mouse): forward: 5′-CTCTGGAAAGCTGTGGCGTGATG-3′, reverse: 5′-ATGCCAGTGAGCTTCCCGTTCAG-3′. GAPDH (Dog): forward: 5′-AGTC AAGGCTGAGAACGGGAAACT-3′, reverse: 5′- TCCACAACATACTCAGC ACCAGCA-3′.

2.7. Over-expression of HO-1 and mutant HO-1

Mouse wild type HO-1 or mouse H25A mutant HO-1 was over-expressed by transfection of pEGFP-C1-HO-1 (GFP-HO-1) or pEGFP-C1-HO-1 H25A (GFP-HO-1 H25A) plasmid, with the pEGFP-C1 (GFP) plasmid as a control. H25A mutant HO-1 cDNA, losing its enzyme activity, was constructed using the PCR based site-directed mutagenesis kit (Tiangen Biotech Co., LTD., Beijing, China). RAW264.7 cells grown

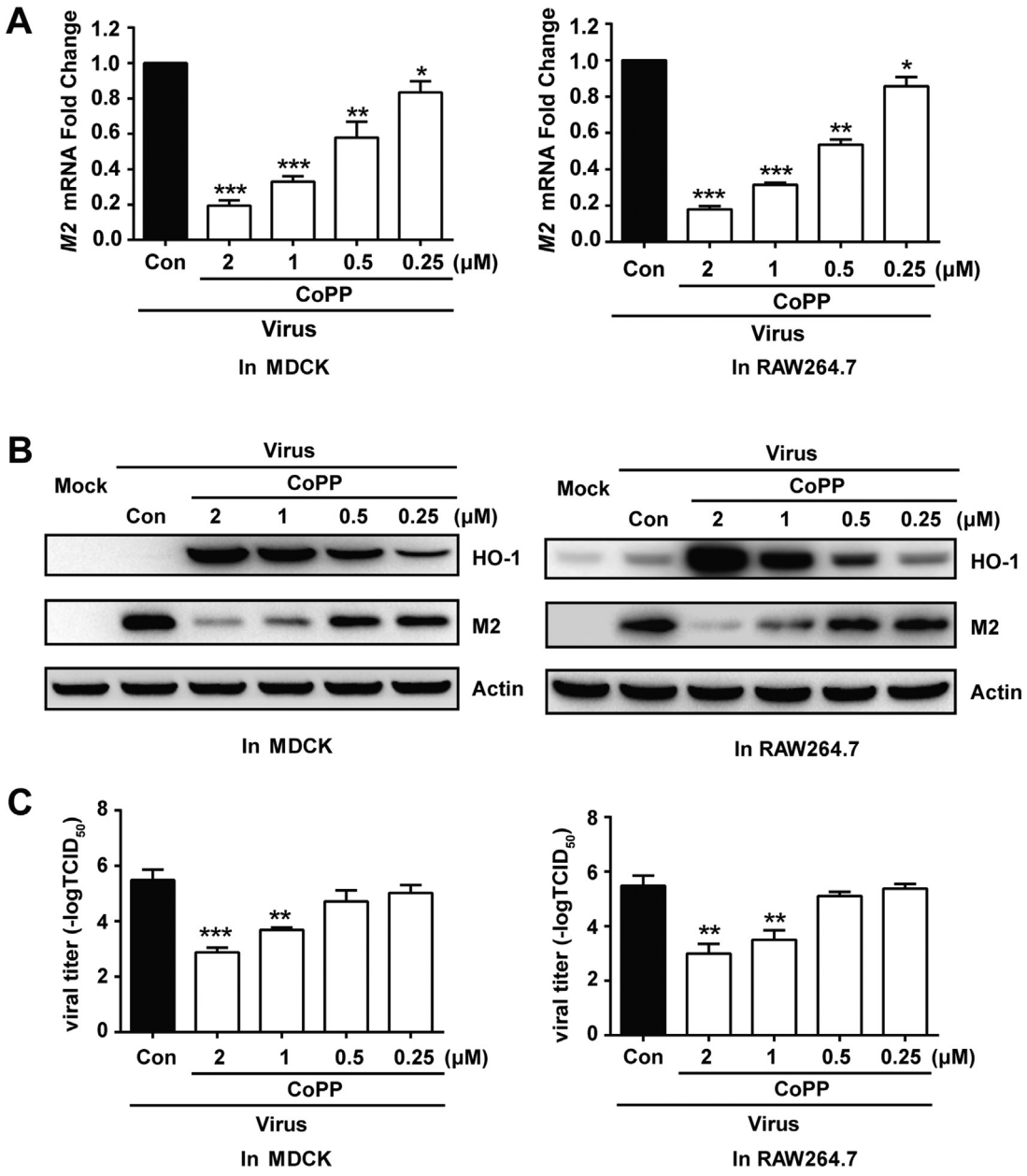

Fig. 2. The effect of CoPP on influenza virus replication. (A-C) The inhibitory effects of CoPP on IAV M2 RNA expressions (A), IAV M2 protein expressions (B), and viral titers (C) in MDCK cells or RAW264.7 cells. MDCK cells or RAW264.7 cells were infected with IAV A/Fort Monmouth/1/1947 (0.005 multiplicity of infection (MOI) or 0.2 MOI) and treated with different concentrations of CoPP simultaneously. The IAV M2 RNA and protein were collected at 12 h and 24 h, and then their expressions were analyzed by qRT-PCR and western blot assay. MDCK cells were infected with a challenge of the diluted cell supernatants at 24 h and viral titers were determined by -Log10$TCID_{50}$. Mock: normal cells without treatment; Virus: cells infected with IAV A/Fort Monmouth/1/1947. Con: treated with equal amounts of solvent as CoPP. The experiments were performed in triplicate, and the data represents mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Con.

in 6-well cell plates (2.5×10^6) were transfected with 1 μg plasmids using Lipofectamine 2000 (Invitrogen). After 6 h, the transfection solution was replaced by complete medium. After further incubation at 37 °C for another 24 h, the amounts of IFITM3, PKR and β-actin in transfected cells were determined by western blotting assay, and the enzyme activity of HO-1 was detected by measuring the amount of bilirubin, the catalytic product, with the QuantiChrom™ bilirubin assay kit (Bioassay Systems, Hayward, CA, USA).

2.8. GST pulldown analysis

Full-length coding sequences of murine HO-1 were amplified and introduced into pGEX-4T-1 vector, the fusion protein and GST protein (control) were expressed in Escherichia coli (Strain BL21), then purified using corresponding affinity chromatography. GST or GST-HO-1 coupled beads (GST·Bind·Resin; Novagen, Darmstadt, German) were used to pull down IRF3. The assay was performed by incubation of GST or HO-1-GST coupled beads with RAW264.7 cell lysates for 3 h at 4 °C, then washed with PBS thoroughly and boiled in SDS-PAGE sample buffer. The interaction between HO-1 and IRF3 was determined by western blotting assay with anti-IRF3 and anti-GST antibodies.

2.9. Coimmunoprecipitation analysis

RAW264.7 cells were co-transfected with pcDNA 3.1-HO-1-HA and pcDNA 3.1-IRF3-His using Lipofectamine 3000 (Invitrogen). At 24 h post-transfection, the cell lysates were incubated with anti-HA antibody or anti-His antibody overnight at 4 °C. and protein A/G-Sepharose

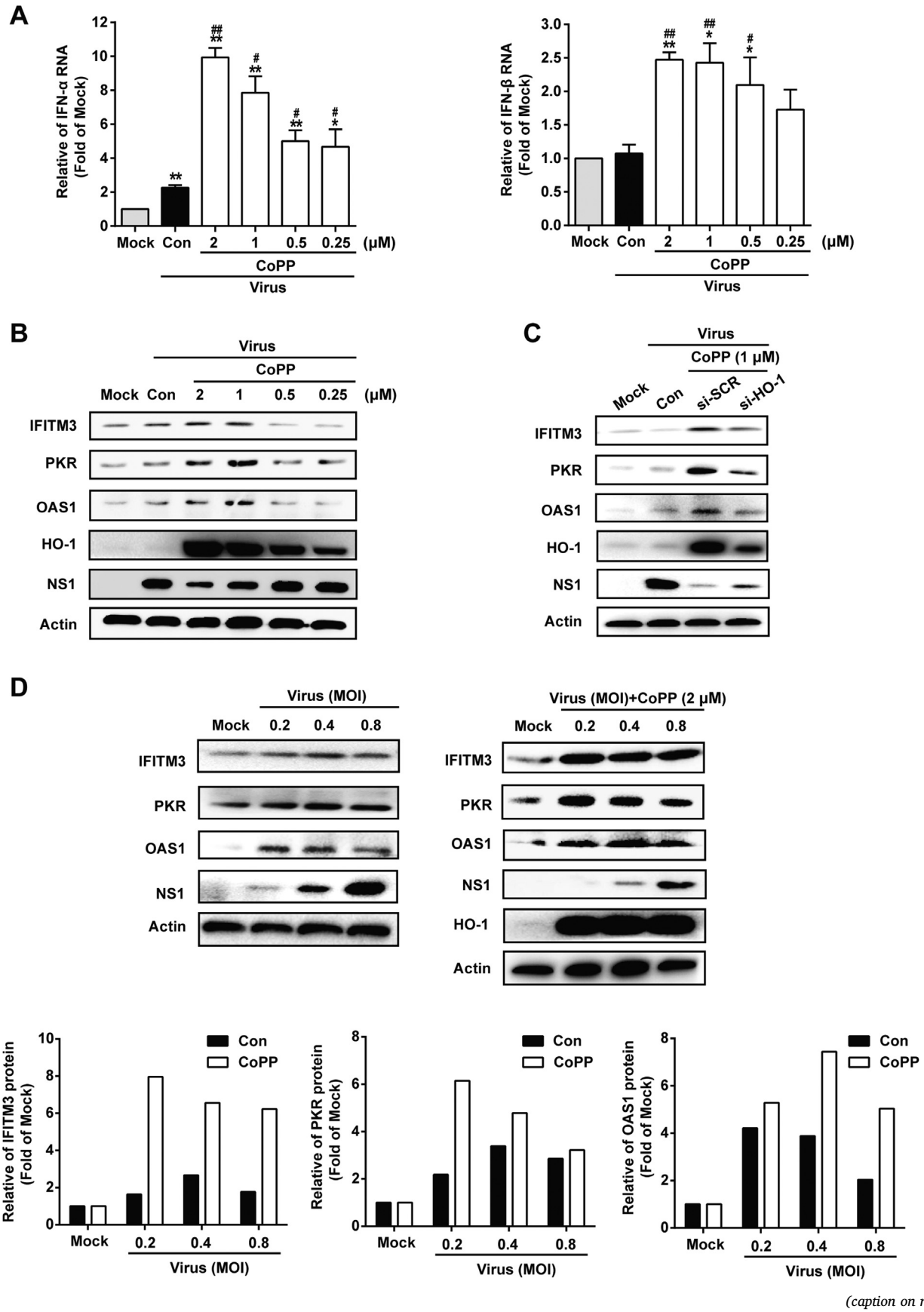

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Fig. 3. HO-1 induction by CoPP augments IFN response. (A-B) The effects of CoPP on mRNA expressions of IFN-α and IFN-β (A) as well as protein expressions of ISGs including IFITM3, PKR and OAS1 (B). RAW264.7 cells were infected with IAV A/Fort Monmouth/1/1947 (0.2 MOI) and treated with CoPP simultaneously, the mRNA expressions of IFN-α/β were determined by qRT-PCR after 18 h. The protein levels of ISGs were measured by Western blotting assay after 24 h. (C) The effect of HO-1 siRNA on CoPP-induced activation of IFN response and anti-IAV effect. RAW264.7 cells were transfected with HO-1 siRNA (si-HO-1) or SCR siRNA (si-SCR) for 24 h and then infected with IAV A/FM1/1947 (0.2MOI) in the presence of 1 μM CoPP for 24 h. (D) The effect of CoPP on type I IFN response and IAV replication at different MOI. RAW264.7 cells were infected IAV at 0.2, 0.4 or 0.8 MOI and treated with 2 μM CoPP simultaneously for 24 h. The protein expression of ISGs, NS1 were determined through western blot assay. The experiments were performed in triplicate, each value represents mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$ versus Mock; $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus Con.

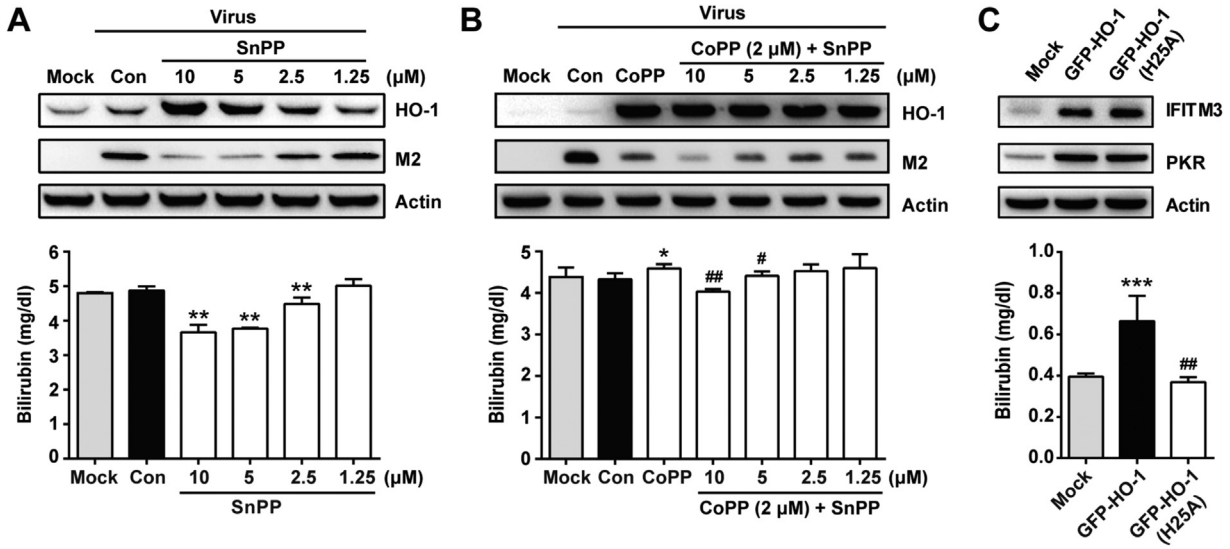

Fig. 4. The HO-1 catalytic function might be not essential for anti-IAV activity of CoPP. (A) The effect of SnPP on IAV replication. RAW264.7 cells were infected with IAV A/Fort Monmouth/1/1947 (0.2 MOI) and treated with SnPP simultaneously for 24 h, the protein expressions of IAV M2 and HO-1 were assayed by Western blotting, the amount of bilirubin was measured by the QuantiChrom™ bilirubin assay kit. (B) The effect of SnPP on anti-IAV activity of CoPP. RAW264.7 cells were infected with IAV and co-incubated with CoPP and SnPP simultaneously for 24 h, the protein expressions of IAV M2 and HO-1 as well as the amount of bilirubin were determined. (C) The effect of mutant construct GFP-HO-1 (H25A) on the expression of ISGs. RAW264.7 cells were transfected with 1 μg GFP-HO-1 or GFP-HO-1 (H25 A). The protein expressions of IFITM3 and PKR were determined by western blot assay, and the amount of bilirubin was detected. The experiments were performed in triplicate, each value represents mean ± SD. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$ versus Con (Figure A-B) or Mock (Figure C). $^{\#}P < 0.05$, $^{\#\#}P < 0.01$ versus CoPP (Figure B) or GFP-HO-1 transfected control (Figure C).

beads (GE Healthcare) was added to the lysate for 3 h at 4 °C. The immunocomplexes were washed three times with lysis buffer. Lastly, the immunoprecipitated proteins were detected by western blotting with a monoclonal anti-HA antibody and anti-His antibody (Santa Cruz).

2.10. Statistical analyses

Statistical analyses were performed using GraphPad Prism 6.0 software (GraphPad, La Jolla, CA, USA). Results are presented as mean ± SD. Data were analyzed by one-way ANOVA with Holm-Sidak multiple comparisons test (three or more data sets in a group). $P < 0.05$ was considered statistically significant.

3. Results

3.1. CoPP enhances the expression of HO-1

To determine the anti-influenza virus activities of CoPP, we firstly determined the effect of CoPP on cell viabilities in MDCK cells and RAW264.7 cells through MTT assay. As shown in Fig. 1A, the maximum nontoxic concentrations (TC_0) of CoPP on MDCK cells and RAW264.7 cells were both 3.13 μM. Therefore, we selected 2 μM as the initial concentration of CoPP in our experiment. Furthermore, as expected, we found that treatment of 2 μM CoPP, the specific HO-1 agonist, significantly increased the intracellular protein levels of HO-1 in a time-dependent manner in MDCK cells and RAW264.7 cells (Fig. 1B).

3.2. Antiviral activity of CoPP against influenza viruses in vitro

Antiviral activities of CoPP and control drugs including OC, RBV and AH were determined based on CPE assay. TC_{50} and IC_{50} values are summarized in Table 1, which shows that CoPP has a broad-spectrum antiviral effect against multiple influenza A and influenza B virus strains tested, including oseltamivir-resistant IAV strain A/Tianjin-Jinnan/15/2009(H1N1) and amantadine-resistant IAV strain A/FujianTongan/196/2009(H3N2). In addition, concerning the antiviral potency, the IC_{50} values of CoPP, ranging from 0.34 to 0.64 μM, is similar to the IC_{50} values of OC.

To further confirm the inhibitory action of CoPP against influenza virus, viral RNA and protein expressions as well as viral titers were evaluated. As presented in Fig. 2A and B, CoPP displayed a potent antiviral activity against IAV A/Fort Monmouth/1/1947 in MDCK and RAW264.7 cells, as judged by dose-dependent decreases of M2 RNA and protein expressions. In addition, CoPP dose-dependently decreased viral titers in the infected MDCK and RAW264.7 cells with more than 1 log drop at 2 μM dose in MDCK cells (Fig. 2C).

3.3. CoPP inhibits IAV replication through augmenting IFN response

HO-1 has been known to be a critical early mediator of the innate immunity through regulating IFN production (Tzima et al., 2009). Also in previous studies, it has been demonstrated to play an important role in suppressing influenza virus replication by activation of HO-1-mediated IFN response (Ma et al., 2016). Hence, to determine whether HO-1 up-regulation by CoPP activates antiviral IFN response in the case of

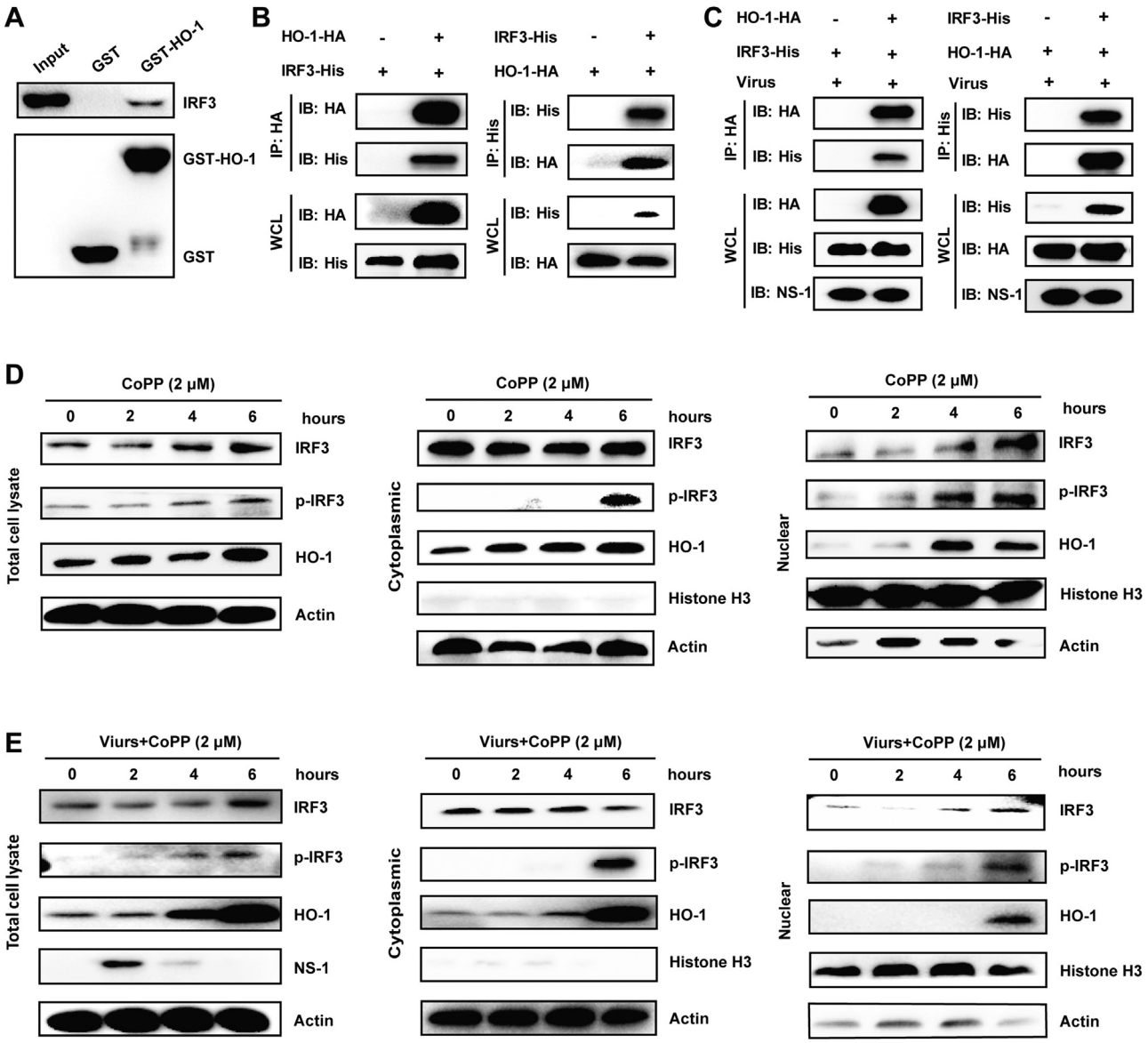

Fig. 5. CoPP promotes IRF3 phosphorylation and nuclear translocation for activating IFN response. (A) The interaction between IRF3 and HO-1 was confirmed by GST pulldown assay. 25 μg GST and GST-HO-1 protein were incubated with RAW264.7 cell lysis respectively. The protein-bound beads were washed after incubation for 3 h at 4 °C. IRF3 and GST fusion proteins were determined by Western blotting. (B-C) The interaction between IRF3 and HO-1 in uninfected (B) or IAV-infected (C) RAW264.7 cells were demonstrated by Co-IP and reverse Co-IP assay. Lysates from RAW264.7 cells transfected with vectors expressing HO-1-HA and IRF3-His and infected with IAV A/Fort Monmouth/1/1947 (0.2 MOI) or not were immunoprecipitated (IP) with anti-HA antibody (left) or anti-His antibody (right), and assayed by protein blot using the indicated antibodies. WCL: whole-cell lysates. (D-E) The effect of CoPP on IRF3 and p-IRF3 proteins in uninfected (D) or IAV-infected (E) total cell lysate, cytoplasm and nucleus. RAW264.7 cells or RAW264.7 cells infected with IAV A/Fort Monmouth/1/1947 (0.2 MOI) were treated with 2 μM CoPP for 0 h, 2 h, 4 h and 6 h. IAV and CoPP were added simultaneously. The total amounts of cellular, cytoplasmic and nuclear IRF3/p-IRF3 proteins were analyzed by Western blotting. The experiments were performed in triplicate.

IAV infection, we studied the effect of CoPP on the expressions of IFN-α/β and ISGs. As indicated in Fig. 3, we found that in RAW264.7 cells infected with IAV, CoPP enhanced mRNA expressions of IFN-α/β (Fig. 3A), as well as protein expressions of some ISGs, such as IFN-inducible transmembrane protein 3 (IFITM3), double-stranded RNA-dependent protein kinase (PKR) and 2′-5′-oligoadenylate synthetase 1 (OAS1), in a dose-dependent manner (Fig. 3B).

To determine whether CoPP-induced type I interferon upregulation and antiviral effect involves in HO-1, RAW264.7 cells were transfected with HO-1 siRNA (si-HO-1) or SCR siRNA (si-SCR) and were then infected with A/Fort Monmouth/1/1947. The results indicated that compared to si-SCR, si-HO-1 treatment partially inhibited increases of ISGs proteins induced by CoPP and reversed its antiviral effect to some extent (Fig. 3C). The result implies that upregulation of HO-1 is at least partly required for the activation of type I IFN response as well as inhibition of influenza viruses induced by CoPP.

Considering the extent of IFN response and ISGs induction depends on specific virus strain and MOI, we tested the effects of CoPP on ISGs expression and IAV replication in RAW264.7 cells infected with different MOI IAV. As shown in Fig. 3D, different MOI infection induced the different increases of ISGs including IFITM3, PKR and OAS1, with 0.4 MOI infection showing the maximal induction of ISGs. However, CoPP treatment further increased ISGs expressions in all the different MOI groups. Of the three tested ISGs, IFITM3, a very important anti-IAV protein (Everitt et al., 2012), showed the maximal increase in the case of CoPP treatment (Fig. 3D). Taken together, these findings suggest that HO-1 induction by CoPP augments IFN response and ISGs expressions, which, at least partly, explains the antiviral mechanism of CoPP against

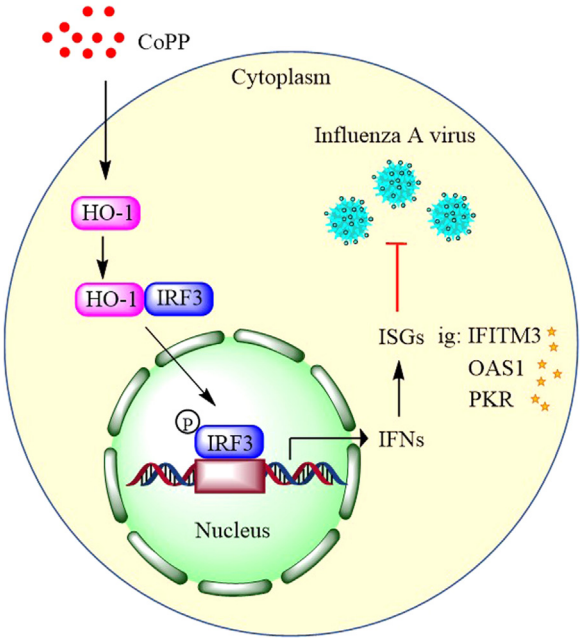

Fig. 6. Schematic showing that CoPP attenuates influenza virus replication. CoPP induces expression of HO-1, which interacts with IRF3, then promotes IRF3 phosphorylation and nuclear translocation. Subsequently, type I IFNs and ISGs including IFITM3, OAS1 and PKR are induced, which finally result in the inhibition of influenza virus replication.

IAV.

3.4. The anti-IAV activity of CoPP is not dependent on the catalytic function of HO-1

To determine whether the HO-1 catalytic function is essential for anti-IAV activity of CoPP, tin protoporphyrin (SnPP), a porphyrin that inhibits HO-1 catalytic activity, was used to treat cells infected with IAV in the presence or absence of CoPP. The results demonstrated that SnPP increased HO-1 expressions, which might be a responsive increase since HO-1 enzymatic activity was inhibited (Fig. 4A). SnPP, interestingly, did not increase IAV replication, but decreased IAV replication. Similarly, we found that SnPP could not reverse the anti-IAV effect of CoPP. Conversely, SnPP and CoPP seems to act in synergy to inhibit IAV (Fig. 4B). Considering no significant difference of HO-1 expression between CoPP-treated group and CoPP/SnPP-treated group, SnPP itself might have other pathway to inhibit IAV replication. Therefore, we confirmed that HO-1 catalytic function is not essential for HO-1-mediated or CoPP-mediated anti-IAV role through the way of pharmacological inhibition. To support this further, genetic mutation was induced in H25A position of HO-1 protein, which results in the loss of HO-1 enzymatic activity as reflected by decreased biliverdin level (Fig. 4C). As expected, this result showed that the mutant construct GFP-HO-1 (H25A) did not attenuate IFITM-3 and PKR protein expression compared to GFP-HO-1 without IAV infection (Fig. 4C). Collectively, these findings indicate that the catalytic function of HO-1 might not be essential for the anti-IAV activity of CoPP.

3.5. HO-1 interacts with IRF3 and promotes IRF3 nuclear translocation

It has been reported that HO-1 in macrophages is required for the activation of IFN regulatory factor 3 (IRF3) after viral infections (Tzima et al., 2009). Therefore, we further determined whether CoPP exhibits anti-IAV activity through regulating IRF3. Firstly, we confirmed the interaction between endogenous HO-1 and IRF3 through GST pulldown assay (Fig. 5A). Also, the interaction between HO-1-HA and IRF3-His was demonstrated by Co-IP and reverse Co-IP assay (Fig. 5B). Of note, their interaction was not affected under IAV infection (Fig. 5C). We next found that CoPP treatment increased protein levels of IRF3 and p-IRF3. In addition, we observed that CoPP promoted the cytoplasmic protein levels of IRF3 and p-IRF3 in RAW264.7 cells following the enhancement of HO-1 protein expression after 6 h. Meanwhile, we demonstrated that CoPP induced nuclear IRF3 and nuclear p-IRF3 accumulation within 6 h of treatment (Fig. 5D). The effect of CoPP on IRF3 phosphorylation and translocation was also demonstrated in IAV-infected cells (Fig. 5E). These findings thus suggest that anti-IAV activity of HO-1 induced by CoPP may depend on IRF3 phosphorylation and translocation, in order to enhance antiviral IFN response.

4. Discussion

The innate immune system plays an important role in protecting against IAV infections. However, the virus has the ability of escaping the innate immune system. Activating or enhancing an innate immune response before or during a viral infection will help increase the host's ability to fight the virus. In our previous study, we showed that HO-1 has the ability to activate IFN-α/β that inhibits replication of influenza virus. However, it is unclear about the mechanism underlying HO-1-mediated activation of IFN-α/β. Hererin, we show that CoPP, a specific activator of HO-1 expression, induces IFN-α/β expression and significantly inhibits the replication of influenza virus. Furthermore, we show that IRF3 involves in HO-1-mediated upregulation of IFN-α/β through the direct binding to HO-1. The proposed mechanism of CoPP anti-IAV effects is illustrated in Fig. 6.

Cobalt protoporphyrin (CoPP) is a type of metalloporphyrins, conjugates of metals binding to porphyrins. CoPP is known as a potent and effective inducer of HO-1 (Shan et al., 2006). Much evidence *in vivo* and *in vitro* models shows CoPP protection from a variety of damages via upregulation of HO-1 (Schmidt, 2007). However, CoPP is not considered as a potential therapeutic agent in view of many side effects at therapeutic doses such as depletion of hepatic cytochrome P450, loss of weight and transient hepatotoxicity (Schmidt, 2007). Hence, CoPP is more suitable as a tool medicine to study the protective effects and mechanisms of HO-1, and alternative HO-1 inducers like hemin or CoPP modifications might be of therpeutic value in clinic when a patient is suffering from infection of drug-resistant or emerging influenza viruses.

HO-1 is a stress-induced and cytoprotective enzyme expressed in most cell types in the organism. HO-1 is known to have anti-inflammatory and antioxidant activities. Recently, HO-1 has been shown to play an important role in modulating immune responses. The immunomodulatory effects of HO-1 have been revealed in several studies, such as HO-1 deficiency-induced chronic inflammation in which HO-1 deficiencies increased counts in blood leukocytes and levels of Th1 cell cytokines including IL-6, IFN-γ and TNF-α (Riquelme et al., 2016). Similar immunomodulatory effects have been observed in HO-1-knockout macrophages in which IFN-β production was impaired after stimulating TLR4 or TLR3. Furthermore, HO-1 deficencies in macrophages and mice result in the deminish of immune responses to bacterial and viral infections (Tzima et al., 2009). CoPP, as a specific activator of HO-1, decreased viral replication and lung inflammation through increasing the production of IFN-α/β in the lung infected with hRSV (Espinoza et al., 2017). Consistent with these findings, in our previous study, we showed that overexpression or pharmacological induction of HO-1 stimulated significant upregulation of IFN-α/β and inhibited replication of influenza virus. In the present study, we again showed that induction of HO-1 expression by CoPP treatment enchanced IFN-α/β expression in the RAW264.7 cells. We obesrved that CoPP dose-dependently inhibit replication of influenza A and B viruses including clinical isolates and drug-resistant strains.

HO-1-mediated inhibition of viral replication is at least partly associated with IFN-α/β induction. The function of IFN-α/β to inhibit IAV replication is mainly attributable to the increased expression of ISG

genes including IFIT, IFITM3, OAS, PKR and MX1 (Diamond and Farzan, 2013; Tecle et al., 2005). The protein products translating from these ISG genes have strong antiviral effects through several mechanisms. For instance, IFIT proteins restrict viral replication by inhibiting the crucial steps in the translation of viral mRNA (Hui et al., 2003). In addition, IFITM3 inhibiting IAV infection by restrecting IAV fuse in late endosomes essential for releasing IAV genetic code (Brass et al., 2009; Huang et al., 2011). In the present study, HO-1 upregulation induced by CoPP siginicantly promotes the production of IFN-α/β and the expression of downstream IFITM3, OAS1 and PKR, which subsequently inhibits replication of the panel of influenza viruses including clincial strains resistant to OC and AH. Thus, the broad-spectrum antiviral activities of CoPP is much likely to be associated with induction of ISGs. These results are consistent with our previous study showing that HO-1 upregualtion induced by another small molecular results in inhibition of a panel of influenza viruses (Ma et al., 2016). It should be noted that one study recently reported HO-1-mediated inhibition of influenza virus replication in MDCK (Hossain et al., 2018), which was considered as the result of HO-1 protective effects against oxidative stress and cell death. Considering that type I intreferon response is weak in MDCK, HO-1-mediated anti-oxidative stress might be also important to inhibit IAV replication. Collectively, therapeutic induction of HO-1 is probably a novel method to prevent the epidemics of influenz viruses. An exceptional study indicated that down-regulation of HO-1 inhibits H9N2 replication in chicken oviduct epithelial cells (COECs) (Qi et al., 2017), this singular inconsistence may be due to the different cells or virus used in their study.

In the previous study concerning HO-1-mediated inhibtion of HCV replication, the mechanism underlying IFN-α/β indcuction by HO-1 involved in HO-1-catalyzed heme metabolic product, biliverdin (Zhu et al., 2010; Lehmann et al., 2010). However, in the present study SnPP, an inhibitor of HO-1 enzymatic activity, did not reverse antiviral activities of CoPP, and mutant construct GFP-HO-1 (H25A) did not inhibit ISGs production compared to GFP-HO-1 treatment. Hence, HO-1 enzymatic activity and metabolic product might be not necessary for HO-1 anti-influenza viruses. Instead, it might work through direct regulation of HO-1 on the signal pathway involved in the production of IFN-α/β.

IRF3 is known to be essential for IFN-α/β production in response to viral infection (Suthar et al., 2013). We therefore speculated that IRF3 was important for HO-1-mediatd IFN-α/β production. As expected, we observed p-IRF3 was accumulated in the nucleus after 6 h of CoPP treatment. Furthermore, we showed that HO-1 interacted directly with IRF3 through GST pulldown and Co-IP assay. These results were consistent with the previous study showing that HO-1 was essential for IFN-β induction in responses to virus infection via interaction with IRF3 (Tzima et al., 2009). Thus, these findings suggest that IFN-α/β generation by HO-1 is associated with interaction between IRF3 and HO-1 which promotes p-IRF3 accumulation in the nucleus.

Taken together, we further confirm using CoPP, a specific HO-1 activator that HO-1 have broad-spectum antiviral activities against influenza viruses through inducing IFN-α/β generation. In addition, we provided direct evidence that HO-1-mediated generation of IFN-α/β is largely attributable to the interaction with IRF3, not to HO-1 enzymatic activity. Combining our previous study, therapeutic induction of HO-1 is probably a novel strategy to combat IAV epidemics.

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Conflict of interest

None.

References

Brass, A.L., et al., 2009. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. Cell 139 (7), 1243–1254.
Bright, R.A., et al., 2005. Incidence of adamantane resistance among influenza A (H3N2) viruses isolated worldwide from 1994 to 2005: a cause for concern. Lancet 366 (9492), 1175–1181.
Diamond, M.S., Farzan, M., 2013. The broad-spectrum antiviral functions of IFIT and IFITM proteins. Nat. Rev. Immunol. 13 (1), 46–57.
Espinoza, J.A., et al., 2017. Heme oxygenase-1 modulates human respiratory syncytial virus replication and lung pathogenesis during infection. J. Immunol. 1601414.
Everitt, A.R., et al., 2012. IFITM3 restricts the morbidity and mortality associated with influenza. Nature 484 (7395), 519–523.
Garcia-Sastre, A., 2001. Inhibition of interferon-mediated antiviral responses by influenza A viruses and other negative-strand RNA viruses. Virology 279 (2), 375–384.
Hill-Batorski, L., et al., 2013. The cytoprotective enzyme heme oxygenase-1 suppresses Ebola virus replication. J. Virol. 87 (24), 13795–13802.
Hossain, M.K., et al., 2018. Bax Inhibitor-1 acts as an anti-influenza factor by inhibiting ROS mediated cell death and augmenting heme-oxygenase 1 expression in influenza virus infected cells. Int. J. Mol. Sci. 19 (3), 712.
Huang, I.C., et al., 2011. Distinct patterns of IFITM-mediated restriction of filoviruses, SARS coronavirus, and influenza A virus. PLoS Pathog. 7 (1), e1001258.
Hui, D.J., et al., 2003. Viral stress inducible protein P56 inhibits translation by blocking the interaction of eIF3 with the ternary complex (eIF2GTPMet-tRNAi). J. Biol. Chem.
James, S.J., et al., 2015. MAPK phosphatase 5 expression induced by influenza and other RNA virus infection negatively regulates IRF3 activation and type I interferon response. Cell Rep.
Lehmann, E., et al., 2010. The heme oxygenase 1 product biliverdin interferes with hepatitis C virus replication by increasing antiviral interferon response. Hepatology 51 (2), 398–404.
Liu, A.L., et al., 2008. Anti-influenza virus activities of flavonoids from the medicinal plant Elsholtzia rugulosa. Planta Med. 74 (8), 847–851.
Ma, L.L., et al., 2016. Rupestonic acid derivative YZH-106 suppresses influenza virus replication by activation of heme oxygenase-1-mediated interferon response. Free Radic. Biol. Med. 96, 347–361.
McNab, F., et al., 2015. Type I interferons in infectious disease. Nat. Rev. Immunol. 15 (2), 87–103.
Motterlini, R., Foresti, R., 2014. Heme oxygenase-1 as a target for drug discovery. Antioxid. Redox Signal. 20 (11), 1810–1826.
Protzer, U., et al., 2007. Antiviral activity and hepatoprotection by heme oxygenase-1 in hepatitis B virus infection. Gastroenterology 133 (4), 1156–1165.
Qi, X., et al., 2017. Down-regulation of cellular protein heme oxygenase-1 inhibits proliferation of avian influenza virus H9N2 in chicken oviduct epithelial cells. J. General. Virol. 99 (1), 36–43.
Riquelme, S.A., et al., 2016. Modulation of antigen processing by haem-oxygenase 1. Implications on inflammation and tolerance. Immunology 149 (1), 1–12.
Sadler, A.J., Williams, B.R., 2008. Interferon-inducible antiviral effectors. Nat. Rev. Immunol. 8 (7), 559–568.
Schmidt, R., 2007. Cobalt protoporphyrin as a potential therapeutic agent? FASEB J. 21 (11) (2639-2639).
Serkedjieva, J., Ivancheva, S., 1999. Antiherpes virus activity of extracts from the medicinal plant Geranium sanguineum L. J. Ethnopharmacol. 64 (1), 59–68.
Shan, Y., et al., 2006. Role of Bach1 and Nrf2 in up-regulation of the heme oxygenase-1 gene by cobalt protoporphyrin. FASEB J. 20 (14), 2651–2653.
Shen, Z., Lou, K., Wang, W., 2015. New small-molecule drug design strategies for fighting resistant influenza A. Acta Pharm. Sin. B 5 (5), 419–430.
Suthar, M.S., Diamond, M.S., Gale Jr, M., 2013. West Nile virus infection and immunity. Nat. Rev. Microbiol. 11 (2), 115.
Tecle, T., White, M.R., Hartshorn, K.L., 2005. Innate immunity to influenza A virus infection. Curr. Respir. Med. Rev. 1 (2), 127–145.
Tseng, C.K., et al., 2016. Human heme oxygenase 1 is a potential host cell factor against dengue virus replication. Sci. Rep. 6, 32176.
Tung, W.H., et al., 2011. Enterovirus 71 induces integrin beta1/EGFR-Rac1-dependent oxidative stress in SK-N-SH cells: role of HO-1/CO in viral replication. J. Cell Physiol. 226 (12), 3316–3329.
Tzima, S., et al., 2009. Myeloid heme oxygenase-1 regulates innate immunity and autoimmunity by modulating IFN-beta production. J. Exp. Med 206 (5), 1167–1179.
van der Vries, E., et al., 2013. Influenza virus resistance to antiviral therapy. Adv. Pharmacol. 67, 217–246.
Yan, N., Chen, Z.J., 2012. Intrinsic antiviral immunity. Nat. Immunol. 13 (3), 214–222.
Zhu, Z., et al., 2008. Heme oxygenase-1 suppresses hepatitis C virus replication and increases resistance of hepatocytes to oxidant injury. Hepatology 48 (5), 1430–1439.
Zhu, Z., et al., 2010. Biliverdin inhibits hepatitis C virus nonstructural 3/4A protease activity: mechanism for the antiviral effects of heme oxygenase? Hepatology 52 (6), 1897–1905.