Fish and Shellfish Immunology 79 (2018) 65–72

Contents lists available at ScienceDirect

Fish and Shellfish Immunology

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

Full length article

Heme Oxygenase-1 inhibits spring viremia of carp virus replication through carbon monoxide mediated cyclic GMP/Protein kinase G signaling pathway

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ARTICLE INFO

Keywords:
Spring viremia of carp virus
Heme oxygenase-1
Carbon monoxide
Biliverdin
Antiviral

ABSTRACT

Spring viremia of carp virus (SVCV) is the etiological agent of spring viremia of carp (SVC) and causes mass mortality in common carp (*Cyprinus carpio*). Currently, no effective treatments or commercial vaccines against SVCV are available. Heme oxygenase-1 (HO-1), an enzyme that catalyzes the degradation of heme to produce carbon monoxide (CO), biliverdin and ferrous iron (Fe^{2+}), exerts anti-oxidant, antiinflammatory and anti-apoptotic properties. Previous studies demonstrated that nuclear factor-erythroid 2 related factor 2 (Nrf2) functions as an important upstream regulator of HO-1 and exhibits robust activity against SVCV infection. In this study, we further examined the antiviral activity of HO-1 against SVCV infection. The elevated expression of HO-1 was induced upon cobalt protoporphyrin (CoPP) treatment in EPC cells without affecting cell viability and thus inhibited SVCV replication in a dose dependent manner. Knocking down of HO-1 rescued SVCV replication. Thereby, the antiviral activity of ROS/Nrf2/HO-1 axis was confirmed in EPC cells. Furthermore, HO-1 enzymatic products CO, but not biliverdin, markedly inhibited SVCV replication via the activation of cyclic GMP/protein kinase G signaling pathway. Collectively, these findings suggest potential drug or therapy that induced the Nrf2/HO-1/CO/cGMP/PKG signaling pathway as a promising strategy for treating SVC.

1. Introduction

Spring viremia of carp (SVC) is a contagious widespread viral disease of fish and causes mass mortality in common carp cultural industry. Spring viremia of carp virus (SVCV), designated as *carp sprivivirus* by ICTV in 2016, was identified as the etiological agent of SVC. SVCV is an enveloped, negative-sense single-stranded RNA virus and belongs to the genus *Sprivivirus* in the family *Rhabdoviridae*. The genome of SVCV is ~11 kb and encodes five proteins: nucleoprotein (N), phosphoprotein (P), matrix protein (M), glycoprotein (G), and viral RNA–dependent RNA polymerase (L) [1]. Currently, there are no effective treatments or vaccines against SVCV infection [2]. It is essential to develop a safe and effective antiviral strategy to against SVCV infection. Our former studies revealed that oxidative injury was the pathogenic mechanism of SVCV infection [3–5]. Thus, the strategy of anti-oxidation might be a promising way against SVCV infection.

Recent studies have showed that heme oxygenase-1 (HO-1) has multiple biological functions including anti-oxidant, anti-inflammatory, and anti-apoptotic properties and also plays an important role in host defense against microbial infection [6]. HO-1 displays important antiviral properties against a growing variety of human viruses, such as human immunodeficiency virus (HIV), hepatitis B virus (HBV), hepatitis C virus (HCV), Ebola virus (EBOV), dengue virus (DENV), enterovirus (EV71), and herpes simplex virus type 2 (HSV-2) [7–11]. Antiviral activity of HO-1 on animal viruses including porcine reproductive and respiratory syndrome virus (PRRSV), bovine viral diarrhoea virus (BVDV) has also been recently reported [12,13]. Small molecular compounds and extracts derived from herbal medicine, including rupestonic acid and celastrols, exert broad-spectrum activity against influenza viruses and hepatitis C virus (HCV) by activating HO-1 [14,15]. Thus, the increasing evidences hold the promise that therapeutic activation of HO-1 represents a potential valuable clinical target to combat infection by a broad-spectrum of viruses. However, the effect of HO-1 on aquatic animal viruses remains unknown. Nuclear factor-erythroid 2 related factor 2 (Nrf2) functions as an important upstream regulator of HO-1 by binding to the anti-oxidant response element (ARE) and shows robust activity against SVCV infection [3]. Our previous study also indicated that SVCV infection down-regulated the expression of HO-1 [4]. These observations implied a link between HO-1 and SVCV infection.

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https://doi.org/10.1016/j.fsi.2018.05.014
Received 5 March 2018; Received in revised form 3 May 2018; Accepted 7 May 2018
Available online 09 May 2018

HO-1 is a highly inducible isoform enzyme that catalyzes the degradation of heme to produce carbon monoxide (CO), biliverdin and ferrous iron (Fe^{2+}) which exert protective effects against oxidative stress [16]. Although the antiviral mechanisms of HO-1 have not been fully elucidated, both of CO and biliverdin have been demonstrated to mediate the antiviral effects during PRRSV infection [17,18]. However, biliverdin inhibits DENV and HCV replication by increasing IFN response, but CO and Fe^{3+} do not [9,19]. While HO-1 activity interferes with the replication cycle of HSV-2 and its antiviral effects can be recapitulated by CO [11]. However, HO-1 had no major effect on the replication of wild-type VSV [8]. These findings indicate that the underlying antiviral mechanism of HO-1 remains elusive referring to different viruses or cell types, which aroused our intense interests to explore the effects of HO-1 on the typical aquatic animal virus of SVCV.

In this study, the effect of HO-1 on SVCV infection was evaluated *in vitro*. The role of HO-1 metabolic products of heme on SVCV infection were further investigated using biliverdin and CO-releasing molecule 2 (CORM-2). We found that HO-1-derived CO significantly inhibited SVCV replication through activating the cellular cGMP/PKG signaling pathway. These findings provide new insights into the molecular mechanism affecting HO-1 inhibition of SVCV replication and promise potential target for drug screening against SVCV infection.

2. Materials and methods

Cell cultures and reagents. Epithelioma Papulosum Cyprini (EPC; ATCC: CRL-2872) cells are SVCV-sensitive and were cultured in Eagle's minimum essential medium (MEM; Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco, Thermo Fisher) at 28 °C in a humidified 5% CO_2 atmosphere. CoPP, CORM-2 [Tricarbonyldichlororuthenium(II) dimer], and the cGMP analog 8-Br-cGMP were purchased from Sigma-Aldrich. Biliverdin was obtained from Frontier Scientific (Logan, USA). The sGC inhibitor 1H- [1,2,4]oxadizolo[4,3-α] quinoxalin-1-one (ODQ), and PKG inhibitor (KT5823) were obtained from Beyotime Biotechnology. With the exception of 8-Br-cGMP, all reagents were diluted in dimethyl sulfoxide (DMSO) to a stock concentration and then further diluted in growth medium to a working concentration before use.

Viral infection. SVCV (ATCC: VR-1390) was a kind gift from Professor Yuanan Lu (University of Hawaii at Manoa) and was used to infect EPC cells for the time points indicated in the figures.

Cell viability assay. Cell viability was evaluated by a tetrazolium dye (MTT) assay. Briefly, EPC cells (1×10^4 per well) grown in 96-well plate were treated with indicated concentrations of CoPP, CORM-2, biliverdin, or vehicle control (DMSO) for 48 h. Then, 20 μl of 5 mg ml^{-1} MTT (Beyotime, China) was added to cells. After 4 h incubation, the medium was aspirated and replaced by 150 μl of DMSO. The plate was shaken for 10 min and measured by scanning absorbance at 570 nm using a microplate reader (Infinite 200 PRO, Switzerland). Results were expressed as relative to the optical density of wells containing untreated control cells defined as 100% viability.

RNA extraction, reverse transcription, and quantitative PCR. Total RNA were extracted by RNAiso Plus (Takara, China) according to the manufacturer's instructions. A 1-μg aliquot of total RNA was used for reverse transcription reaction using the Prime Script™ RT reagent Kit with a gDNA Eraser (Takara, China). Quantitative PCR was performed with Fast SYBR Green PCR Master mix (Bio-Rad, USA) on the LightCycler480 System (Roche, Switzerland). PCR conditions were as follows: 95 °C for 5 min, then 40 cycles of 95 °C for 5 s, 60 °C for 30 s, 72 °C for 30 s. Primers used for RT-qPCR are shown in Table 1, and the β-actin was used as reference gene. The relative fold changes were calculated by comparison to the corresponding controls using the $2^{-\Delta\Delta Ct}$ method.

Western blotting. Cell were harvested and lysed using lysis buffer containing 1 mM PMSF and 65 mM DTT (Beyotime, China). Equivalent amounts of protein samples were separated using 12% SDS-PAGE and then transferred onto a PVDF membrane (Millipore, USA) using the Semi-dry Transfer System (Bio-Rad, USA). To avoid non-specific antibody binding, the membrane was blocked for 1 h at room temperature with 3% bovine serum albumin-TBS solution containing 0.1% Tween 20 (TBS-T). The membrane was incubated overnight at 4 °C with an anti-HO-1 antibody (AB 31163, Chemicon, USA) or an anti-β-actin antibody (SC-1616, Santa Cruz, USA) in TBS-T containing 3% BSA. The membranes were then washed three times with TBS-T for 10 min, incubated with a secondary antibody HRP-conjugated anti-rabbit IgG (Proteintech, China) in 3% BSA solution for 1 h. After three additional washes with TBS-T, the signal detection was performed using enhanced chemiluminescence with the BeyECL Plus reagent (Beyotime, China) on an Amersham Imager 600 system (GE healthcare, USA).

Table 1
Primers used for RT-qPCR.

Primers	Primers(5′-3′)	Purpose
SVCV-G-F	TGCTGTGTTGCTTGCACTTATYT	Real-time PCR
SVCV-G-R	TCAAACKAARGACCGCATTTCG	Real-time PCR
β-actin-F	CACTGTGCCCATCTACGAG	Real-time PCR
β-actin-R	CCATCTCCTGCTCGAAGTC	Real-time PCR
HO-1-F	AGCTGTACAGGAGTCGCATGAA	Real-time PCR
HO-1-R	ACCTGGACGTTGAGCTCGAA	Real-time PCR

HO-1 Knockdown. EPC cells were transfected with HO-1 siRNA or corresponding scrambled siRNA using the VigoFect transfection reagent (Vigorous Biotechnology, China) according to the manufacture's protocol. The efficiency of HO-1 knockdown was determined by Western blotting analysis and RT-qPCR.

Titration. EPC cells (1×10^4 per well) were trypsinized and seeded in 96-well plate 12 h before virus infection. Virus supernatants were 10-fold serially diluted and added to each well with 100 μl with six wells replicates. Seven days after infection, the 50% tissue culture infection dose ($TCID_{50}$) was calculated by the Reed-Muench method [20].

Statistics analysis. The statistical p values were calculated by one-way analysis of variance (ANOVA) with the least significant difference test. P values of less than 0.05 were considered statistically significant.

3. Results

3.1. CoPP upregulates HO-1 expression without affecting cell viability

CoPP is a classical inducer of HO-1 and specially activates the expression of HO-1 in mammalian cells. However, aquatic animal cells were grown at 25–28 °C, a relatively lower temperature than other mammalian cells. We firstly examined whether CoPP treatment can induce the expression of HO-1 in EPC cells. As shown in Fig. 1A, no significant differences on cell viability were observed between CoPP-treated cells and DMSO-treated cells. Then, the transcript and expression of HO-1 were monitored after EPC cells was incubated with CoPP for 24 h. RT-qPCR results showed that CoPP treatment with different concentrations effectively increased the transcriptional level of HO-1 by 20–323 fold compared to negative control (Fig. 1B). Western blot analysis indicated a significant increase in HO-1 expression after treatment with 20 μM or more of CoPP (Fig. 1C). Furthermore, the effect of incubation time of CoPP on the transcription of HO-1 were determined in EPC cells by the treatment of CoPP at 40 μM and the results suggested that HO-1 mRNA was significantly increased 142 fold compared to negative control as early as 3 h post treatment (Fig. 1D). These results indicated that CoPP could upregulate the expression of HO-1 without affecting cell viability in EPC cells.

CoPP inhibits SVCV replication in a dose dependent manner.

In order to determine whether the pharmacological induction can inhibit SVCV replication, EPC cells were treated with CoPP at different concentrations for 6 h prior to infection with SVCV and CoPP was maintained during SVCV infection. As expected, CoPP treatment for 6 h