Antiviral Research 105 (2014) 39–46

Contents lists available at ScienceDirect

Antiviral Research

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

Inhibition of replication of porcine reproductive and respiratory syndrome virus by hemin is highly dependent on heme oxygenase-1, but independent of iron in MARC-145 cells

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ARTICLE INFO

Article history:
Received 3 November 2013
Revised 19 January 2014
Accepted 13 February 2014
Available online 25 February 2014

Keywords:
PRRSV
Hemin
Iron
Heme oxygenase-1
ROS

ABSTRACT

Current vaccines against porcine reproductive and respiratory syndrome virus (PRRSV) have failed to provide sustainable disease control, and development of new antiviral strategies is of great importance. The present study investigated the mechanism of the antiviral effect of hemin during PRRSV infection in MARC-145 cells. Hemin, a commercial preparation of heme, is used as an iron donor or heme oxygenase 1 (HO-1) inducer, and has been shown to provide antiviral activity in many studies. In the current study, the anti-PRRSV activity of hemin was identified through suppressing PRRSV propagation. The 50% inhibitory concentration (IC_{50}) of hemin antiviral activity was estimated to be 32 μM, and the 50% cytotoxic concentration (CC_{50}) of hemin was found to be higher than 125 μM. Further study showed that the antiviral activity of hemin is independent of iron. In addition, after treatment with Protoporphyrin IX zinc (II) (ZnPP) or Sn (IV) Protoporphyrin IX dichloride (SnPP), inhibitors of HO-1, the inhibition of viral replication by hemin was partially reversed. Additionally, it was confirmed that hemin and N-acetyl cysteine were able to significantly reduce reactive oxygen species (ROS) in MARC-145 cells infected with virus. N-acetyl-L-cysteine (NAC), however, did not produce a reduction in viral load or promote expression of HO-1. Taken together, these data indicate that the effect of hemin on the inhibition of PRRSV propagation via HO-1 induction, as well as the antiviral mechanism of HO-1, is not dependent on decreased levels of ROS. In conclusion, these data demonstrate that hemin had antiviral activity against PRRSV and may serve as a useful antiviral agent inhibiting PRRSV replication.

1. Introduction

Porcine reproductive and respiratory syndrome (PRRS) is one of the most important viral diseases in sows, presenting a threat to the swine industry (Xiao et al., 2010a). Annual losses to the U.S. pork industry have been estimated at approximately 664 million U.S. dollars (Board, 2011). The causative agent, PRRS virus (PRRSV) is an enveloped, single-stranded, positive-sense RNA virus belonging to the Arteriviridae family (Meulenberg et al., 1993). PRRSV can be divided into two major genotypes: type 1 (European) and type 2 (North American), represented by Lelystad virus (LV) and VR-2332, respectively (Benfield et al., 1992; Wensvoort et al., 1991). However, incompletely understood mechanisms of immune evasion and immunity have resulted in vaccines against PRRSV failing to provide sustainable disease control, particularly against genetically unrelated strains (Kimman et al., 2009). Therefore, the development of new antiviral strategies with higher efficacy for all viral isolates remains of great importance for a first line of defense against the virus (Liu et al., 2013; Sang et al., 2011; Xiao et al., 2011).

Various types of materials or products have been developed with the objective of producing antiviral activity, among which hemin is a promising compound for inhibition of viruses. Several studies have demonstrated that hemin reduces the infection of different types of viruses, including DNA (Lin and Hu, 2008; Protzer et al., 2007) and RNA viruses (Devadas and Dhawan, 2006; Fillebeen et al., 2005; Hou et al., 2009; Zhu et al., 2010). Nevertheless,

Abbreviations: heme, iron protoporphyrin IX; hemin, chloroprotoporphyrin IX iron (III); DFO, desferrionxamine; CoPP, protoporphyrin IX cobalt chloride; ZnPP, protoporphyrin IX zinc (II); SnPP, Sn (IV) protoporphyrin IX dichloride; HO-1, heme oxygenase-1; FTH1, ferritin heavy polypeptide 1; TFRC, transferrin receptor; NAC, N-acetyl-L-cysteine; ROS, reaction oxygen species.

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http://dx.doi.org/10.1016/j.antiviral.2014.02.010
0166-3542/

the effect of hemin on the antiviral activity of different viruses is different, particularly in the study of hepatitis C virus (HCV). It has been reported both that HCV is inhibited by heme oxygenase 1 (HO-1) induction (Shan et al., 2007; Zhu et al., 2008), and that the inhibition of HCV is due to a limited amount of iron released from hemin (Fillebeen et al., 2005; Yuasa et al., 2006). The antiviral activity of iron against HCV has also been demonstrated in other studies (Fillebeen and Pantopoulos, 2010; Zhu et al., 2010). According to such studies, PRRSV infection altered the concentration of iron in MARC-145 cells (Grebennikova et al., 2006) and slightly up-regulated transferrin (TF) in lung tissue at the protein level (Xiao et al., 2010b). Nevertheless, little information exists regarding the effects of iron during the infection and replication of PRRSV and whether hemin or desferrionxamine (DFO) interferes with iron-dependent viral propagation.

The purpose of the present study was to examine the effect of hemin as an iron donor on the inhibition of PRRSV, and to evaluate whether hemin interferes with viral replication and the expression of viral protein. Results demonstrate that hemin diminishes PRRSV replication in MARC-145 cells and inhibits the synthesis of both viral RNA and protein that is highly dependent on heme oxygenase-1 but independent of exogenous iron. Additionally, HO-1 induction appears to decrease reactive oxygen species (ROS) in MARC-145 cells infected with virus, although the antiviral mechanism of HO-1 is not via ROS reduction.

2. Materials and methods

2.1. Chemicals, cells, and virus

Hemin, desferrionxamine (DFO), Protoporphyrin IX zincs (II) (ZnPP), and Protoporphyrin IX cobalt chloride (CoPP) were purchased from Sigma (St. Louis, MO, USA), Sn (IV) Protoporphyrin IX dichloride (SnPP) was purchased from Frontier Scientific Inc (Logan, UT, USA).

MARC-145 cells were grown in Dulbecco's modified Eagle's medium (DMEM; Sigma, St. Louis, MO, USA) containing 10% FBS (PAA, Pasching, Austria) at 37 °C and 5% CO_2. Classical North American type PRRSV (N-PRRSV) strain CH-1a, was kindly provided by Dr. Guihong Zhang, South China Agricultural University.

2.2. Cell viability and cytotoxicity assays

MARC-145 cells were plated in 96-well plates at a density of 1×10^4 cells per well. After confluent monolayers had formed, cells were treated for 36 h with different concentrations of hemin or DFO. MARC-145 cells were then incubated at 37 °C with fresh DMEM medium containing 10% Alamar Blue (AbD Serotec, Oxford, UK) for 4 h in accordance with the manufacturer's protocol. Fluorescence measurements were taken by using a Synergy 2 Multi-Mode Microplate reader (BioTek, Winooski, VT, USA). The resulting fluorescence intensities were normalized to the control for each sample. The 50% cytotoxic concentration (CC_{50}), defined as the concentration required to cause fluorescence intensity changes in 50% of intact cells, was estimated by comparing hemin-treated and -untreated wells using GraphPad Prism 5.0 software.

2.3. Apoptosis assays

Apoptosis was evaluated using the Apoptosis Kit (Invitrogen, California, USA) containing Alexa Fluor-488 annexin V and propidium iodide (PI). MARC-145 cells were incubated with different chemicals for 36 h, and then apoptosis was measured by annexin/PI staining according to the manufacturer's protocol. Stained cells were then analyzed using a FACSCalibur instrument (Becton Dickinson, Maryland, USA). Cells treated with 5 μM cisplatin for 36 h were used as a positive control.

2.4. Virus titration

Virus titration was processed as described previously (Xiao et al., 2011). For titration, all virus supernatants were diluted 10-fold starting at a dilution of 10. Six days post-infection, the 50% cell culture infection dose (CCID50) was calculated by the Reed-Muench method (Reed and Muench, 1938).

2.5. Reverse-transcription polymerase chain reaction (RT-PCR) and qRT-PCR

Total RNA was isolated from MARC-145 cells using TRIzol reagent (Invitrogen, California, USA) according to the manufacturer's instructions. RNA (2 μg) was reverse-transcribed into first-strand cDNA using a reverse transcription kit (Promega, Madison, WI).

PCR amplification was performed on 1 μl of RT product with ORF7 (N) and specific primers for GAPDH as the endogenous control. Primers were as follows: N-F: 5′-AAAACCAGTCCAGAGGCAAG-3′; N-R: 5′-CGGATCAGACGCACAGTATG-3′ (250 bp); and GAPDH-F: 5′-TGACAACAGCCTCAAGATCG-3′; GAPDH-R: 5′-GTCTTCTGGGTGGCAGTGAT-3′ (141 bp). Real-time PCR was performed using SYBR Premier Dimer Eraser™ (TaKaRa, Osaka, Japan) kit on a Light-Cycler 480 (Roche Basel, Switzerland), and the data was analyzed by relative quantification (△△Ct) method.

For the viral RNA copy assay, the oligonucleotide primers used were as follows: NSP2-F: 5′-GTGGGTCGGCACCAGTT-3′ and NSP2-R: 5′-GACGCAGACAAATCCAGAGG-3′ (172 bp), designed within the gene segment encoding for NSP2. The TaqMan® probe was synthesized as follows: 5′-FAM-CACAGTTCTACGCGGTGCAGG-TAMRA-3′ (Xiao et al., 2010a). A plasmid containing the PRRSV NSP2 sequence was used to generate a standard curve, and RNA copies of PRRSV in all samples were calculated compared to the standard curve.

2.6. Western blot

Whole-cell extracts were prepared by lysing in cell lysis buffer (Beyotime, Jiangsu, China) containing 1 mM phenylmethylsulfonylfluoride. An equal amount of protein (25 μg) was supplemented with SDS loading buffer (Pierce, Rockford, USA) and boiled for 5 min, separated by 12% SDS–PAGE, and then electrotransferred onto polyvinyl difluoride (PVDF) membranes (Millipore, MA, USA). After blocking, the membranes were incubated overnight at 4 °C with one of the following primary antibodies diluted at 1:1000: anti-FTH1 (Cell Signal Technology (CST), MA, USA), anti-HO1 (CST, MA, USA), anti-β-tubulin (CST, MA, USA), anti-TFRC (Lifespan Biosciences, WA, USA), and anti-PRRSV NC protein (Jeno Biotech Inc, Republic of Korea). HRP-conjugated anti-mouse IgG (1:5000; Santa Cruz Biotechnology, Santa Cruz, CA, USA) or anti-rabbit IgG (1:2500, CST, MA, USA) were used as secondary antibodies. The antibodies were visualized using ECL reagent (Pierce, Rockford, USA) according to the manufacturer's instructions.

2.7. Indirect immunofluorescence

The virus-infected cells were grown in the presence of hemin, DFO, or DMSO at 36 hpi and then fixed with 4% paraformaldehyde for 10 min at room temperature (RT). After blocking with PBS containing 1% bovine serum albumin (BSA) for 30 min, the plates were incubated with PRRSV NC MAb (1:400 dilution) at 4 °C overnight and then incubated with goat anti-mouse IgG secondary antibody conjugated with FITC (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at RT for 1 h. Nuclei were stained with Hoechst dye 33258 (Sigma, St. Louis, MO, USA) after the secondary antibody incuba-