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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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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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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-

tion. Cell staining was conducted using the ELYRA P.1 prototype system (Carl Zeiss, Jena, Germany).

2.8. Cytopathic effect reduction assay

The antiviral activity of hemin against PRRSV was measured by CPE inhibition assay, which was processed as previously reported (Li et al., 2013; Ma et al., 2001; Wyde et al., 1993). Briefly, twofold dilutions of hemin were seeded onto cell monolayers cultivated in 96-well culture plates, using 50 μM as the higher concentration. Wells without viruses or hemin were used for the negative control. An equal volume of virus suspension (0.1 MOI) was added to the wells. Cells were incubated at 37 °C for 72 h in a humidified atmosphere containing 5% CO_2 and inhibition of CPE was observed. The concentration reducing CPE by 50% with respect to virus control was estimated from GraphPad Prism 5.0 software, and was defined as the 50% inhibited concentration (IC_{50}) expressed in μM/L. The selectivity index (SI) was calculated from the ratio of CC_{50} to IC_{50}.

2.9. Intracellular reactive oxygen species (ROS) assay

Intracellular ROS levels were measured using the 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Beyotime, Jiangsu, China) according to a protocol provided by the manufacturer. Briefly, the MARC-145 cells were seeded in a 6-well plate. MARC-145 cells were pretreated with or without SnPP for 12 h before PRRSV infection. After 2 h infection, cells were treated with hemin (50 μM) or NAC (10 mM or 20 mM) for 22 h. The cells were then rinsed 3 times with DMEM (serum free) and then incubated for 20 min at 37 °C. At the end of incubation, cells were washed 3 times with DMEM, and fluorescence was measured by a fluorescence microscope (Carl Zeiss, Jena, Germany).

For another comparison of ROS levels, the treatment cells were collected by trypsin treatment and fluorescence was read at 485 nm for excitation and 530 nm for emission with a fluorescence plate reader (BioTek, Winooski, VT, USA).

2.10. Statistical analysis

Data are presented as means ± standard errors and are representative of three independent experiments. Statistical significance was determined by Student's *t* test when only two groups were compared or by one-way analysis of variance (ANOVA) when more than two groups were compared. A P-value < 0.05 was considered statistically significant.

3. Results

3.1. Hemin inhibits PRRSV infection but does not induce apoptosis of cells in vitro

To explore the antiviral activity of iron against PRRSV, PRRSV-infected MARC-145 were exposed for 34 h to increasing concentrations of hemin, an iron donor, or DFO, an iron chelator, and the PRRSV infection was assessed by indirect immunofluorescence staining 36 h post infection. As shown in Fig. 1A, hemin significantly inhibited viral infection in a dose-dependent manner. The number of cells infected with PRRSV was remarkably decreased at a concentration of 50 μM hemin compared with cells treated with DMSO and all doses of DFO.

For assessment of the inhibitory effect of hemin on virus production, cell cultures were harvested at 36 hpi and were then assayed for CCID50. As shown in Fig. 1B, hemin showed greater antiviral activity than DFO. The virus titers were at least 1000-fold lower in MARC-145 cells incubated with 50 μM hemin compared to those infected with PRRSV alone or treated with DMSO.

For investigation of whether concentration-dependent toxicity caused by hemin and DFO affects PRRSV replication, Alamar blue assay results showed that hemin (100 μM) produced a significant reduction in cell viability compared to those treated with 5, 10, or 50 μM. However, no significant difference was found among those cells treated with DFO at various concentrations (Fig. 1C). In addition, the effects of hemin and DFO on apoptosis of MARC-145 cells were also studied using flow cytometry. It was observed that the concentrations of 50 μM hemin and 50 μM DFO do not induce any increased apoptosis of cells compared with control (Fig. 1D).

3.2. Hemin inhibits PRRSV replication in vitro

We further determined whether viral RNA transcription or protein translation was affected by hemin. For this, MARC-145 cells were incubated with strain CH-1a (MOI of 0.1) and cultured for 36 h in fresh medium containing hemin and DFO at the indicated concentration. By using qRT-PCR, it was found that the cells treated with 5, 10, or 50 μM hemin resulted in a significant reduction of viral RNA compared with that obtained in the DMSO-treated or DFO-treated cells (Fig. 2A).

The virus titers in supernatants at 36 hpi after treating with hemin, DFO, or DMSO were also measured using qRT-PCR. The changes of the virus titers in supernatants revealed a similar pattern (Fig. 2B). Meanwhile, the expression of the viral N protein was assessed by western blotting. The MARC-145 cells treated with hemin inhibited the expression of protein N, whereas DFO displayed no response (Fig. 2C). The effectiveness of hemin as an iron donor was demonstrated by the induction of ferritin heavy polypeptide 1 (FTH1) (Fig. 2C), an iron storage protein. Additionally, the levels of the transferrin receptor (TFRC) were decreased when high doses of hemin were added (Fig. 2C). These results are in accordance with iron-dependent regulation of FTH1 and TFRC (Pantopoulos, 2004). In addition, hemin (50 μM) treatment significantly relieved PRRSV-induced cytopathogenic effects (CPE) in MARC-145 cells in comparison to DMSO- or DFO- treated cells, indicating more viable intact MARC-145 cells and fewer dead cells (Supplementary data Fig. S1). To further determine at which point the hemin acted during the infection period, the levels of PRRSV replication were measured indirectly by detecting the cells expressing the N protein through western blotting (Fig. 2D). Treating the cells with hemin between 2 and 12 hpi led to a significant inhibition of viral protein expression. However, partial impairment in virus replication was identified when hemin was added at 24 hpi. These data revealed that hemin was able to efficiently inhibit PRRSV replication. However, the PRRSV replication cycle in macrophages was completed within 12 hpi, so the action of blocking PRRSV propagation is occurs predominantly in the early stage. In addition, to further investigate the antiviral activity of hemin, the IC_{50} and CC_{50} of hemin were evaluated. The results showed that IC_{50} and CC_{50} produced by hemin treatment of MARC-145 cells were 32 ± 0.4 μM and 125 ± 1 μM, respectively, and the SI (CC_{50}/IC_{50}) of hemin was 4.0 (Supplementary data Fig. S2).

3.3. Hemin blocks PRRSV replication independent of iron

Heme can be catalyzed by equimolar concentrations of free iron (Fe^{2+}), biliverdin (BV), and carbon monoxide (CO) by HO-1 (Ryter et al., 2007) (Supplementary data Fig. S3). BV is then rapidly converted to bilirubin (BR) by the biliverdin reductase (BRV). As an

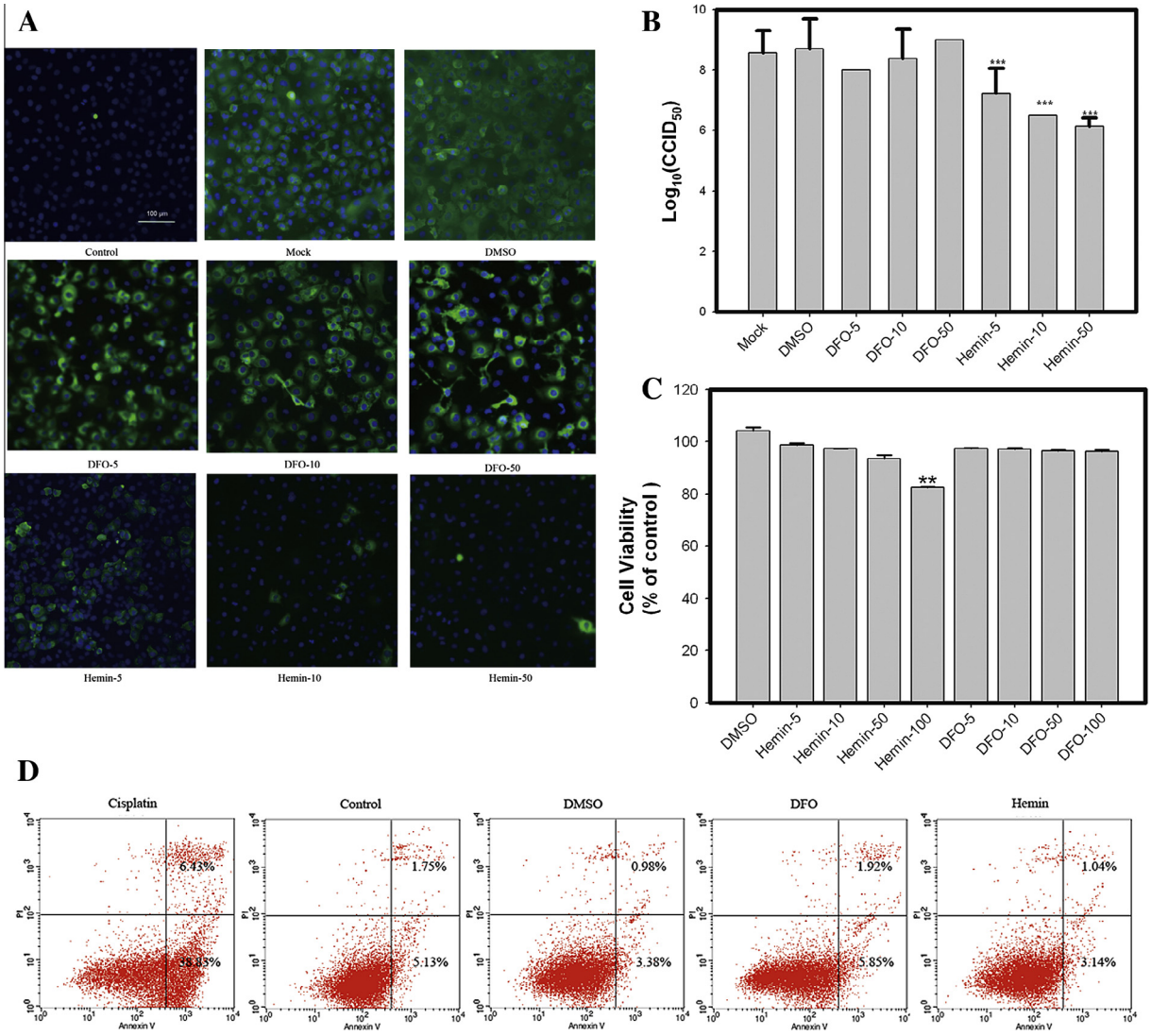

Fig. 1. Anti-PRRSV activity and cellular toxicity of hemin and DFO. (A) For immunostaining, virus-infected cells were fixed at 36 hpi and incubated with PRRSV NC protein antibody followed by FITC-conjugated secondary antibody (green). The cells were then counterstained with Hochest33258 (blue) and examined using a fluorescent microscope at 200× magnification. (B) Upon treatment of infected MARC-145 cells with hemin or DFO for 34 h as described, cell culture supernatants were harvested at 36 hpi and PRRSV titers were determined. (C) The effect of hemin and DFO on MARC-145 cell viability was examined using Alamar blue assay. (D) MARC-145 cells were untreated (control) or treated with hemin (50 μM), DFO (50 μM), or DMSO for 36 h. Apoptosis was then assessed using flow cytometry. Data are representative of three independent experiments (mean ± SE). Significant differences compared with 0.5% DMSO treated group are denoted by $^{**}(P < 0.01)$ and $^{***}(P < 0.001)$. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

iron donor, hemin is also involved in similar reactions in vitro. Thus, whether the antiviral activity of hemin is dependent on iron was next evaluated. In MARC-145 cells, hemin showed significant antiviral activity at a concentration of 50 μM, while the same concentrations of iron ($FeSO_4$) did not show any inhibition of PRRSV replication (Fig. 3A). Interestingly, the increased levels of HO-1 were detected in infected cells treated with hemin (50 μM), while the expression of HO-1 was inhibited in untreated cells or in cells treated with iron. To determine whether the enzymatic activity of iron is impaired by viral RNA transcription in PRRSV, MARC-145 cells were subjected to Mg^{2+} (5 mM) and an indicated concentration of iron manipulations, and then the expression of N protein was analyzed by western blotting (Fig. 3B). Results showed that Mg^{2+} is not required to promote the activity of PRRSV replication. In addition, DFO did not reverse the hemin-reduced viral protein expression even at high concentrations of 100 μM (Fig. 3C). All of these results indicated that iron did not inhibit the expression of viral RNA and protein, even at an increased dosage.

3.4. HO-1 knockdown partially reversed the hemin-reduced viral protein expression

In previous studies (Maines, 1988), hemin was shown to up-regulate heme oxygenase 1 (HO-1) in a variety of cell types. Treatment of MARC-145 cells with hemin also induced HO-1 in a dose- and time-dependent manner (Fig. 4A). The maximum induction was observed when cells were treated with 50 μM hemin for 12 h. To explore the function of HO-1 under PRRSV replication, CoPP, another HO-1 inducer, was evaluated for anti-PRRSV effects of HO-1. The results of western blotting showed that the expression of viral N protein was reduced at a high dose of CoPP, while high levels of HO-1 were induced (Fig. 4B). To further determine the specificity of hemin-induced HO-1 activity in mediating anti-PRRSV activity, zinc protoporphyrin-IX (ZnPP) and Sn(IV) Protoporphyrin IX dichloride (SnPP), both of which are inhibitors of HO-1, were incubated at varying concentrations with viral-infected MARC-145 cells, 12 h before hemin treatment at 50 μM, and then

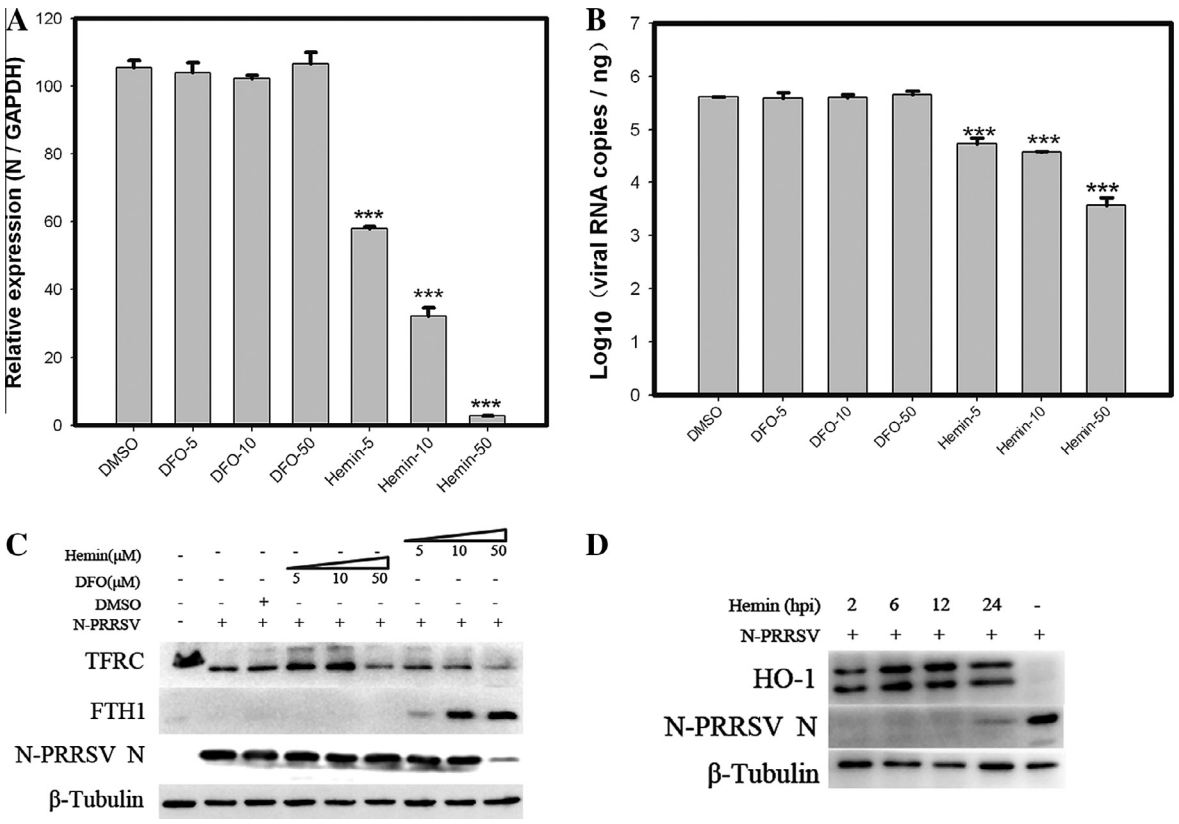

Fig. 2. PRRSV propagation is suppressed by incubating with high doses of hemin. (A) The relative expression of PRRSV ORF7 (N) was determined using quantitative RT-PCR and normalized to that of monkey GAPDH. (B) Virus titers in culture supernatants at 36 h after treatment were measured using Taqman® real-time PCR. (C) The expression of viral N protein, and of cellular FTH1, TFRC1, and β-tubulin in hemin or DFO treated cells was analyzed by western blotting. (D) MARC-145 cells were treated independently with hemin at various time points post infection, 2 hpi, 6 hpi, 12 hpi, and 24 hpi refer to drug on the cells from 2–36 hpi, 6–36 hpi, 12–36 hpi, and 24–36 hpi. At 36 hpi, the levels of PRRSV N protein were measured through western blotting. Data are representative of three independent experiments (mean ± SE). Significant differences compared with 0.5% DMSO treated group are denoted by *** ($P < 0.001$).

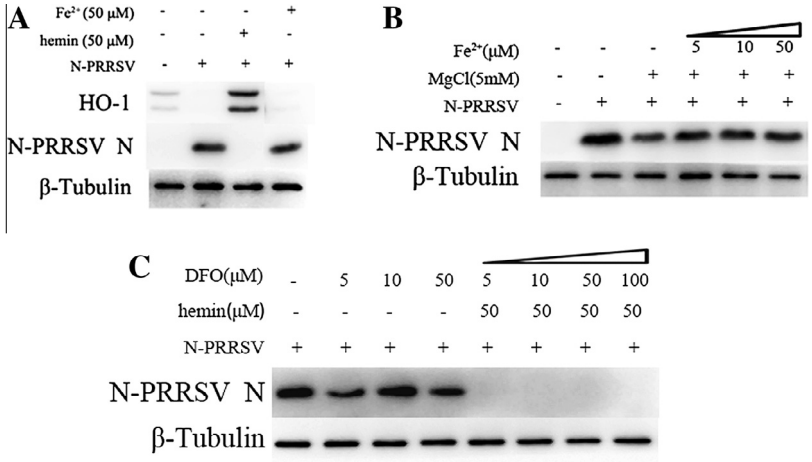

Fig. 3. Hemin inhibits PRRSV expression in the MARC-145 cells independent of iron. (A) The expression of virally encoded N protein, endogenous HO-1 and β-tubulin was analyzed by western blotting. (B) Virus-infected MAR-145 cells were incubated in the absence or presence of 5 mM $MgCl_2$ and the indicated concentration of Fe^{2+}. After 36 hpi, the expression of N protein was detected by western blotting. (C) MARC-145 cells were either untreated or treated with 50 μM hemin, and an indicated dose of DFO was added to the culture medium. After 36 hpi, the expression of viral N protein and β-tubulin was analyzed by western blotting.

evaluated for PRRSV N protein 36 h after infection. Results showed that treatment of cells with ZnPP at a minimum concentration of 200 μM partially reversed hemin-reduced viral protein expression (Fig. 4C). Furthermore, as also shown in Fig. 4C, ZnPP attenuated HO-1 by blocking protein expression, which is in accordance with previous studies (Aizawa et al., 2000; Lee et al., 2003). Additionally, the presence of low-dose SnPP (50 μM) completely reversed the hemin-inhibited N protein expression (Fig. 4D). In contrast, incubation with high-dose SnPP (⩾150 μM) significantly blocked viral protein production by inducing HO-1 expression. As an HO-1 inhibitor, a similar phenomenon was also found in previous studies (Kaizu et al., 2003). Collectively, all of these results demonstrated

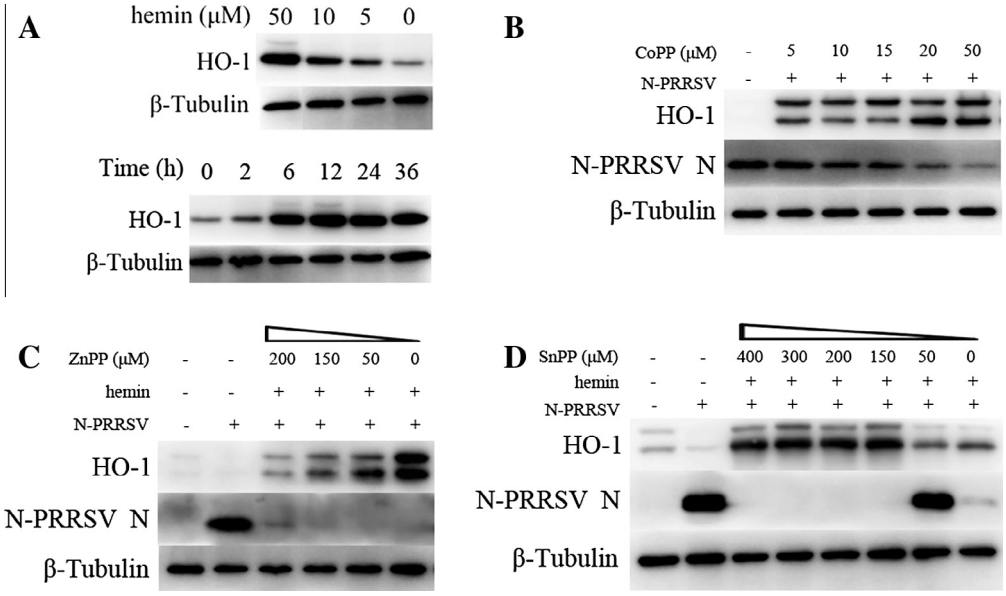

Fig. 4. Hemin mediates the suppression of viral N protein expression in MARC-145 cells by HO-1 induction. (A) The expression of HO-1 in hemin treated cells was analyzed in indicate time and dose by western blotting. (B) The expression of HO-1 and N protein was tested under various dose of CoPP by western blotting. (C and D) PRRSV-infected MARC-145 cells were treated with different concentration of ZnPP or SnPP. The cell lysates were prepared and the levels of viral N protein, of cellular HO-1, and β-tubulin were determined by western blot analysis.

that hemin-mediated inhibition of PRRSV infection was mediated by high HO-1 induction.

3.5. The antiviral mechanism of HO-1 is not via reducing ROS production

Collective evidence has demonstrated the significant role of HO-1 in protection against various diseases (Wu et al., 2011), which results in high levels of ROS. It has also been reported that PRRSV infection increases ROS generation in MARC-145 cells (Yin et al., 2012). To determine if the antiviral effect of HO-1 is due to a reduction in ROS production, the intracellular ROS production was measured using DCFH-DA. The ROS levels were found to be significantly increased by PRRSV infection (Fig. 5A and B). Results showed that pretreatment with hemin (50 μM) attenuated PRRSV-induced ROS production in MARC-145 cells. Furthermore, NAC also decreased the generation of ROS (Fig. 5A and B). However, NAC treatment dose does not inhibit the expression of the viral protein, nor dose it induce the expression of HO-1 (Fig. 5C).

4. Discussion

Iron plays an important role in essential cellular physiology functions. For this reason, iron balance is preserved in most organisms under normal conditions. However, iron balance can be disturbed in pathogenic infection (Drakesmith and Prentice, 2008). It has also been demonstrated that iron is crucial for both host cells and their pathogens. In previous studies, evidence indicates that HIV, Herpes simplex virus (HSV), and Vaccinia virus are iron-dependent (Jordan and Reichard, 1998; McDermid et al., 2007). Thus, several studies have explored methods for controlling virus infection and replication via iron. It was found that patients infected with HIV-1 can survive longer when given higher doses of an iron chelator, deferiprone (Costagliola et al., 1994). Similar results also demonstrated that iron chelators are capable of blocking HIV replication, even though various mechanisms of inhibitory action have been seen in cell culture (Debebe et al., 2007; Sappey et al., 1995). The same inhibitory effects of iron chelators were reported in other viruses such as HSV-1, HBV (Chouteau et al., 2001), and HCMV (Cinatl et al., 1994; Crowe et al., 2004). DFO, an iron chelator, increases HCV replication in hepatic cells while, surprisingly, hemin, which is an iron donor, restricts virus replication in the same cells (Fillebeen et al., 2005). Similar results were found in PRRSV-infected MARC-145 cells (Fig. 1A and B), indicating that synthesis of PRRSV RNA and N protein were both blocked in hemin-treated MARC-145 cells (Fig. 2A and C). The virus titers in supernatants were also reduced in high doses of hemin (Fig. 2B). Furthermore, no significant toxicity and apoptosis were caused by hemin and DFO (Fig. 1C and D). These results indicated that the phenomenon occurs due to hemin, its products, or hemin-induced HO-1. For these reasons, the present study was undertaken.

Hemin was used to demonstrate that iron can block the HCV NS5B RNA-dependent RNA polymerase (RdRP) by high affinity binding to the polymerase (Fillebeen et al., 2005). In addition, excess iron decreases HCV expression by inducing the levels of HO-1 in hepatoma cells (Hou et al., 2009). Thus, iron inhibits HCV replication not only by its high affinity binding to the polymerase, but also by up-regulating HO-1. However, no inhibitory activity and no inducing expression of HO-1 were found in PRRSV-infected MARC-145 cells incubated with iron (Fig. 3B).

For competitive experiments undertaken in the present study, the synthesis of N protein did not increase when adding Mg^{2+} to the culture medium. On the other hand, Fe^{2+} did not inhibit the expression of viral protein even at high dosages (Fig. 3C). However, these findings are not in agreement with previous HCV studies (Behrens et al., 1996; Lohmann et al., 1997) In addition, DFO did not reverse the hemin-reduced viral protein expression, even at a high concentration (Fig. 3D). Based on the above observations, hemin inhibited the replication of PRRSV independent of iron.

HO-1, a heat shock protein (also known as HSP32), can be induced at high levels in response to cellular stressors such as heat shock or physiological or pathological stressors (Immenschuh and Ramadori, 2000). Expression of HO-1 was recently reported to be beneficial to liver cell injury in HCV infected patients (Bonifaz et al., 2009). It is known that HO-1 induction not only offers protection from oxidative injury, but also suppresses HCV replication (Zhu et al., 2008). Similar results were observed in other viruses, such as hepatitis B virus (HBV) (Protzer et al., 2007), human immunodeficiency virus (HIV) (Devadas and Dhawan, 2006), and influ-

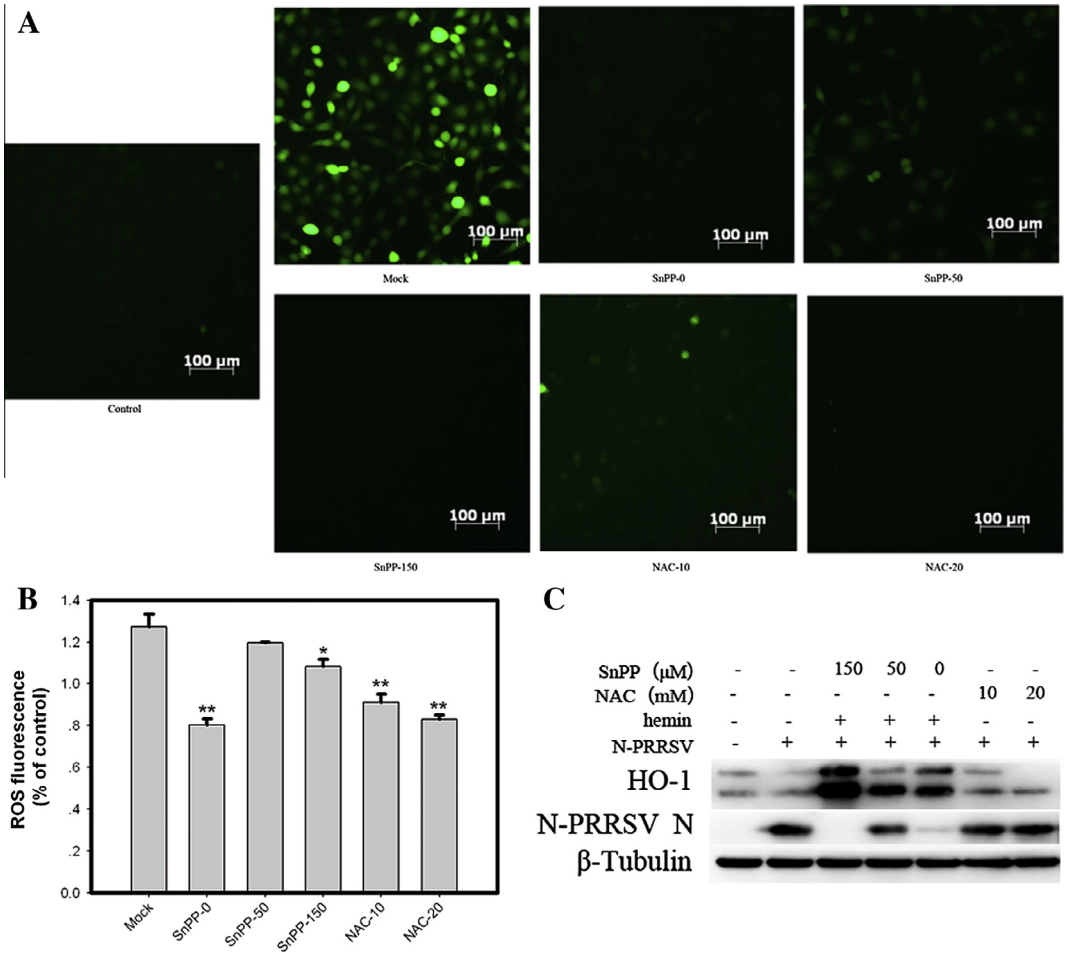

Fig. 5. Hemin treatment reduces ROS production in PRRSV-infected MARC-145 cells. (A) The levels of intracellular ROS in MARC-145 cells were measured by DCFDA as described in the Section 2. The fluorescence intensity was observed using a fluorescence microscope. (B) Experimental procedures were the same as described in (A), and the levels of intracellular ROS in MARC-145 cells were determined with a fluorescence plate reader. Data are the mean ± SEM of three independent experiments. $^{*}p < 0.05$, $^{**}p < 0.01$, compared with PRRSV-infected cells (Mock). (C) Experimental procedures were the same as described in (A), and expression of HO-1 and N protein was determined by western blotting analysis.

enza virus (Hashiba et al., 2001). In the present study, while the levels of HO-1 were significantly inhibited in PRRSV-infected MARC-145 cells (Fig. 3B, Supplementary data Fig. S4), PRRSV N protein was blocked when HO-1 was induced in MARC-145 cells treated with hemin or CoPP (Fig. 3B, Fig. 4B). In addition, as shown in Fig. 4C and D, treatment with HO-1 inhibitors ZnPP or SnPP significantly reversed the inhibitory effect of hemin on viral protein production, suggesting that HO-1 mediates the suppressive effect of PRRSV replication.

HO-1 is considered to have a central role in cardiovascular protection, which protects cells from injury primarily by decreasing oxidative stress (Wu et al., 2011). It is also reported that HO-1 plays a vital role in protecting induced pluripotent stem (iPS) cells from oxidative stress-induced cell death and differentiation (Lin et al., 2012). Surprisingly, the antiviral mechanism of HO-1 is not dependent on decreased levels of ROS (Fig. 5).

Hemin has been approved by the FDA for use in patients with acute porphyria as commercial preparations of heme since the 1970s. In the present study, hemin was shown to be efficacious for use in vitro for inhibition of PRRSV infection and propagation, and is well tolerated (Supplementary data Fig. S2). After demonstrating the anti-PRRSV activity of hemin in vivo, there is reason to believe that pharmacological agents for such diseases are forthcoming.

In summary, this study provides the first evidence, to our knowledge, of an antiviral effect of hemin, independent of iron, by inducing HO-1 expression against PRRSV. Future studies are needed to investigate hemin's antiviral activity in porcine alveolar macrophages (PAM) or in vivo, and which viral proteins contribute to inhibition of the expression of HO-1.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.antiviral.2014.02.010.

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