International Journal of Antimicrobial Agents 49 (2017) 45–52

Contents lists available at ScienceDirect

International Journal of Antimicrobial Agents

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

Hemin ameliorates influenza pneumonia by attenuating lung injury and regulating the immune response

Conghui Wang [a], Yanjing Zhang [a], LianLian Han [a], Li Guo [a], Hui Zhong [a,*], Jianwei Wang [a,b,**]

[a] MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China
[b] Collaborative Innovation Center for Diagnosis and Treatment of Infectious Diseases, Hangzhou 310003, China

ARTICLE INFO

Article history:
Received 23 June 2016
Accepted 17 September 2016

Keywords:
Influenza virus
Hemin
Heme oxygenase-1
Lung injury
Immune regulation

ABSTRACT

The anti-influenza activity of hemin, an inducer, activator and the substrate of heme oxygenase-1 (HO-1), was examined both in vitro and in vivo. The human lung carcinoma cell line A549 was used to evaluate the in vitro effect of hemin on influenza A virus (IAV) replication. A mouse model was used to examine the in vivo activity of hemin. Observation indexes included survival rate and body weight of mice, virus load and pathological examination of the lungs, and characterization of the systemic and local immune responses. The results showed that hemin could induce HO-1 expression in A549 cells and inhibit IAV replication in vitro. The in vivo results showed that injection of hemin could protect mice from death and body weight loss caused by IAV infection. Hemin was administered both at initial and progressive stages of influenza pneumonia (1 day and 4 days after virus infection, respectively) and showed significant anti-influenza activity under both conditions. However, the results showed that although hemin could induce HO-1 expression in vivo, it could not inhibit IAV replication in vivo. Pathological examination showed that hemin significantly attenuated lung tissue injury caused by IAV. Further study showed that hemin could regulate the immune response to IAV infection by reducing lymphocytopenia and local inflammatory cytokine increases caused by IAV infection. This study shows that hemin has the potential for the treatment of IAV infection and its effect may be due to attenuation of lung injury and regulation of the immune response.

1. Introduction

Influenza A virus (IAV) poses an enormous threat to public health. On a global scale, seasonal IAV epidemics cause 3–5 million cases of severe illness and ca. 250,000–500,000 deaths annually [1]. In recent years, avian influenza outbreaks have frequently occurred in Asia, causing extraordinarily high death rates in patients (>30% in H7N9 patients) [2]. Although influenza vaccines are effective in controlling the spread of influenza, their effect is still limited by frequent virus gene drifts and mutations. Two classes of anti-influenza drugs have been licensed: the M2 inhibitors and neuraminidase inhibitors [3]. However, the circulating human IAVs are resistant to M2 inhibitors, and many strains resistant to neuraminidase inhibitors have also emerged [4–6]. Consequently, it is crucial to develop new anti-influenza drugs to defend against a potential influenza pandemic.

There are two different strategies to develop influenza therapies, i.e. targeting IAV directly or targeting the host system. The first strategy is highly efficient but is limited by virus strain sensitivity and resistance mutations [7,8]. The second strategy is not restricted by virus sensitivity or mutations but is limited by relatively low efficacy [9,10]. To date, the second strategy has received increasing attention. For example, the avian IAV can induce an overexuberant immune response known as the 'cytokine storm', which is largely responsible for the lung injury and lethality in avian influenza patients [11–13], and the efficacy of anti-influenza replication drugs is not effective to prevent such lung injury [14]. It is therefore important to develop new agents targeting the host immune response and controlling tissue damage due to influenza infections.

The main activity of the heme oxygenase (HO) enzymes is to catalyze the degradation of heme [15]. HO shows potent anti-apoptosis, anti-inflammatory and immunoregulatory effects [16,17]. There are three isoforms of HO (HO-1, HO-2 and HO-3). HO-2 and HO-3 are constitutively expressed in specific tissues, including the brain and

* Corresponding author. MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China. Fax: +86 10 6782 8516.
E-mail address: zhonghui_bj@126.com (H. Zhong).
** Corresponding author. MOH Key Laboratory of Systems Biology of Pathogens, Institute of Pathogen Biology, Chinese Academy of Medical Sciences and Peking Union Medical College, Beijing 100730, China. Fax: +86 10 6782 8516.
E-mail address: wangjw28@163.com (J. Wang).

http://dx.doi.org/10.1016/j.ijantimicag.2016.09.030
0924-8579/

testes [18]. However, HO-1 is inducible in various cell types under different situations, such as oxidative stress and exposure to heavy metals, heme and its derivatives. The characteristics of HO-1 have been described in multiple inflammatory diseases, including IAV infection [19–21]. Studies in mice showed that overexpression of HO-1 in the lung by an adenovirus vector could attenuate lung pathological changes and increase the survival rate following IAV infection [19]. In addition, HO-1 gene knock-out aged mice showed a decreased survival rate following IAV infection compared with wild-type mice [21]. On the other hand, IAV infection can also increase HO-1 expression in alveolar type I and type II epithelial cells, alveolar macrophages and monocytes [22]. These studies suggest that HO-1 is a potential target for IAV infection.

Hemin is the substrate of HO-1, an inducer of HO-1 expression and an activator of HO-1 activity [15,23]. Hemin for injection (Panhematin®) has been licensed by the US Food and Drug Administration (FDA) to treat porphyria, but its effect on IAV has not been tested so far. In the present study, we investigated the effect of hemin on IAV infection both in vitro and in vivo and ascertained its effects on virus replication, control of tissue damage and immune response regulation.

2. Materials and methods

2.1. Viruses and cells

Influenza A virus (H1N1) strain A/PR/8/34 (PR8) was provided by the Chinese National Influenza Center (CNIC). The human lung carcinoma cell line A549 and Madin–Darby canine kidney (MDCK) cells were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (HyClone, Logan, UT), 100 U/mL penicillin and 100 mg/mL streptomycin at 37 °C with 5% CO_2. PR8 virus was propagated by chicken embryo culture and was titrated in MDCK cells by standard plaque assay.

2.2. Animal experiments

All animal experiments were approved by the Animal Ethics and Experimentation Committee of the Institute of Pathogen Biology of the Chinese Academy of Medical Sciences (CAMS, Beijing, China). BALB/c mice aged 5–6 weeks were purchased from Beijing HFK Bioscience Co. (Beijing, China) and were maintained in an animal biological safety level-2 (BSL-2) facility at the Institute of Laboratory Animal Science, CAMS. Female BALB/c mice were randomly assigned to different groups ($n = 14$ per group) and were intranasally administered 300 PFU/mouse of PR8 virus for infection. The mice received different doses of hemin (Frontier Scientific Inc., Logan, UT) or the same volume of phosphate-buffered saline (PBS) by caudal vein injection. The mice were observed for 12 days, and survival rate [recorded from 0 days post-infection (DPI) to 12 DPI], body weight loss (recorded from 0 DPI to 6 DPI) and other signs of disease were monitored. The mice were sacrificed humanely if their body weight was reduced to less than 70% of the initial weight as permitted by Institutional Animal Care and Use Committees. The mice in each group were euthanized at 5 DPI and 7 DPI. Bronchoalveolar lavage fluid (BALF) and blood with ethylene diamine tera-acetic acid (EDTA) as anticoagulant were harvested. For histopathological analysis, the lungs were harvested and fixed with 4% paraformaldehyde (PFA). Lung samples were embedded in paraffin and were examined by staining with hematoxylin and eosin (H&E) and immunohistochemistry analysis. Fresh lung tissue was also harvested from mice and was frozen at –80 °C to prepare a homogenate to detect virus titre and inflammatory cytokine levels.

2.3. Immunofluorescence assay

A549 cells were seeded in 96-well plates at a density of 10^4 cells/well and were incubated for 18 h. A diluted virus suspension containing 0.5 μg/mL TPCK-modified trypsin was added to each well at a multiplicity of infection of 1, and then different doses of hemin (final concentrations of 0, 0.06, 0.12, 0.25, 0.5 and 1 μM) were added at 24 h post-infection. At 96 h post-infection, A549 cells were fixed in 4% PFA and were permeabilized with PBS containing 0.5% Triton® X-100 (AMRESCO, Houston, TX), followed by blocking with PBS containing 5% bovine serum albumin (Sigma, St Louis, MO). Cells were then incubated with anti-IAV nucleoprotein (NP) monoclonal antibody (1:500) (MAB8251; Millipore, Temecula, CA) in the dark at room temperature for 2 h. After extensive washing, cells were incubated with Alexa Fluor® 488-conjugated anti-mouse IgG antibody (1:400) (Zhongshan Jinqiao Biotech. Co., Beijing, China) for 30 min at room temperature and the cell nuclei were labeled with Hoechst 33342 (Invitrogen). Fluorescence images were obtained using an Operetta® High Content Imaging System (Perkin Elmer, Bridgeville, PA), and the number of infected cells was determined.

2.4. Real-time quantitative PCR (qPCR)

BALF and fresh lungs were harvested from the mice and the lung tissue was homogenized. Total RNA was extracted using TRIzol reagent (Invitrogen). Primers and *Taq*Man probes for real-time qPCR analysis were designed to target the M gene of IAV. The sequences of the forward/reverse primers were 5′-GACCRATCCTGTCACCTCTGAC-3′ and 5′-AGGGCATTYTGGACAAAKCGTCTA-3′. The sequence of the probe was 5′-HEX-TGCAGTCCTCGCTCACTGGGCACG-3′. Each 25 μL reaction mixture consisted of 1.5 μL of RNA plus other components from the Ambion® AgPath-ID™ One-step RT-PCR Kit (Applied Biosystems, Foster City, CA). PCR was conducted for 15 min at 50 °C, 10 min at 95 °C, followed by 45 cycles of 15 s each at 95 °C and 1 min each at 60 °C. The real-time qPCR was performed on a CFX96 Touch™ Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA).

2.5. Western blotting

A549 cells were co-cultured with various concentrations of hemin (final concentrations of 0, 0.06, 0.12, 0.25, 0.5 and 1 μM) and the expression level of HO-1 in A549 cells was determined by western blotting after 24 h. BALB/c mice were treated with PR8 virus and then hemin (0.1 mg/kg/day) or PBS was injected at 4–6 DPI. One day after the last injection, lungs were harvested. HO-1 expression levels in lung tissue homogenates were measured by western blotting. Fresh lung tissue homogenates and A549 cells were lysed in buffer containing 150 mM NaCl, 25 mM Tris (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate and 1 mM EDTA with protease inhibitor cocktail (Roche, Indianapolis, IN). Total protein concentrations were determined using a BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) before separation by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE). Aliquots of tissue or cell lysates were electrophoresed on 12% SDS-PAGE gels and were transferred to a nitrocellulose membrane (Pall, Port Washington, NY). The membranes were blocked with 5% non-fat milk followed by incubation with anti-HO-1 antibody (1:500) (BD Biosciences, San Diego, CA) or anti-actin antibody (1:4000) (Sigma) at 4 °C overnight. This was followed by incubation with the corresponding IRD Fluor 800-labeled IgG or IRD Fluor 680-labeled IgG secondary antibody (Li-Cor, Lincoln, NE). After washing, the membranes were scanned using an Odyssey Infrared Imaging System (Li-Cor). The relative protein expression level was analyzed using the integrated software of the Odyssey system.