Biochimica et Biophysica Acta xxx (2013) xxx–xxx

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

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

Treatment of influenza virus with Beta-propiolactone alters viral membrane fusion

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ARTICLE INFO

Article history:
Received 29 May 2013
Received in revised form 25 September 2013
Accepted 30 September 2013
Available online xxxx

Keywords:
Membrane fusion
Influenza virus
Beta-propiolactone
Cryo electron microscopy
Cryo electron tomography
Vaccine
M2 protonophore
Hemagglutinin

ABSTRACT

Beta-propiolactone (BPL) is commonly used as an inactivating reagent to produce viral vaccines. Although BPL has been described to chemically modify nucleic acids, its effect on viral proteins, potentially affecting viral infectivity, remains poorly studied. Here, a H3N2 strain of influenza virus was submitted to treatment with various BPL concentrations (2–1000 μM). Cell infectivity was progressively reduced and entirely abolished at 1 mM BPL. Virus fusion with endosome being a critical step in virus infection, we analyzed its ability to fuse with lipid membrane after BPL treatment. By monitoring calcein leakage from liposomes fusing with the virus, we measured a decrease of membrane fusion in a BPL dose-dependent manner that correlates with the loss of infectivity. These data were complemented with cryo transmission electron microscopy (cryoTEM) and cryo electron tomography (cryoET) studies of native and modified viruses. In addition, a decrease of leakage irrespective of BPL concentration was measured suggesting that the insertion of HA2 fusion peptide into the target membrane was inhibited even at low BPL concentrations. Interestingly, mass spectrometry revealed that HA2 and M1 matrix proteins had been modified. Furthermore, fusion activity was partially restored by the protonophore monensin as confirmed by cryoTEM and cryoET. Moreover, exposure to amantadine, an inhibitor of M2 channel, did not alter membrane fusion activity of 1 mM BPL treated virus. Taken together these results show that BPL treatment inhibits membrane fusion, likely by altering function of proteins involved in the fusion process, shedding new light on the effect of BPL on influenza virus.

1. Introduction

Infections with influenza viruses cause recurrent epidemics and global pandemics. Seasonal infection is estimated to cause between 250,000 and 500,000 deaths. Moreover, the 2009 pandemic was responsible for more than 250,000 deaths. In addition, these outbreaks have major economic consequences in terms of absenteeism, treatment and hospitalization costs. In the USA alone, this loss is estimated to be about $27 billion per year. The high capability of antigenic shift leads to the emergence of novel viruses, against which acquired immunity by previous influenza infection or vaccination is poorly efficient. Biotechnological improvements relying on genetic, structural, functional and chemical studies are required for the development of better viral treatments and more efficient vaccines to fight against present and future influenza virus variants.

The main targets of the influenza virus are epithelial cells of the respiratory tract. The outer surface of the virus [1] exposes the trimeric transmembrane glycoprotein hemagglutinin (HA) required for viral binding to sialic acid moieties on the surface of host cells [2] which initiates membrane fusion (for reviews, see [3–6]).

Each monomer of HA consists of two disulfide-linked subunits, HA1 and HA2. HA2 contains a transmembrane domain at its C-terminus side and at its N-terminus, the "fusion peptide," an amphiphilic stretch of amino acids which is buried in the HA stem at neutral pH [7]. HA1 monomers contain at the membrane-distal tip of the trimer, a receptor binding pocket composed of highly conserved amino acids (Tyr98, Ser136, Trp153 and His183) that bind sialic acid moieties *via* hydrophobic interactions or hydrogen bonds [2,8]. Once the cell membrane and the virus have been closely juxtaposed by virus–receptor interaction, the complex is internalized by endocytosis through a clathrin dependent pathway. Upon acidification of the interior of the endosome, the viral HA undergoes irreversible conformational rearrangements involving extrusion of the fusion peptide from the HA stem [9] toward the target endosomal membrane [10–13], and transformation of the HA loop region into a large coiled coil domain [9] triggering apposition and fusion of viral and endosomal membranes [12,14,15]. As a consequence of viral interior acidification arising from H^+ transport through the M2 channel, the viral Ribo-Nucleo-Protein complexes (vRNPs) dissociate from M1 matrix

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0005-2736/$ – see front matter © 2013 Published by Elsevier B.V.
http://dx.doi.org/10.1016/j.bbamem.2013.09.021

proteins and escape from endosome after the membrane merger. The vRNPs are then imported into the nucleus for transcription, translation and finally virus amplification [16,17].

Currently, two forms of influenza vaccine are available for widespread distribution: inactivated vaccines and live-attenuated vaccines. Preparation of inactivated vaccines consists of several steps: (i) virus of a chosen subtype is propagated in embryonated chicken eggs, (ii) the virus is purified (iii), inactivated with formaldehyde or beta-propiolactone (BPL), (iv) the inactivating reagents are removed and (v) the virus is split with detergent. To prepare the vaccine, various subtypes of influenza A (H3N2 and H1N1) as well as influenza B are then mixed to provide a broader range of protection against seasonal circulating viruses. Interestingly, the choice of virus inactivation procedure is then of great importance to effectively protect against infection. Indeed a recent report showed that whole inactivated influenza virus with beta-propiolactone (BPL) provided a better protection against different virus subtypes than split or formaldehyde-inactivated virus [18].

BPL is widely used for the inactivation of infectious agents (bacteria, fungi, viruses) in many vaccine preparations as well as in disinfection, plasma sterilization and tissue transplants [19–23]. This very widespread industrial use of BPL contrasts somehow with the limited knowledge available on the molecular consequences of its action. BPL reacts with nucleophilic reagents (including nucleic acids and proteins) leading to alkylated and/or acylated products. BPL is very reactive with different chemical moieties of various biological molecule including proteins (mostly methionine, cysteine and histidine) and nucleic acids (mainly adenosine, cytidine and guanosine moieties of the vRNA) [24–26]. BPL alters the capability of DNA to be used as template by polymerase [22]. From literature, the effect of BPL is dose and specimen -dependent. In the presence of 2 mM BPL infectivity of plasmid pUC18 is reduced while no effect was observed at 250 μM BPL [22]. At 250 μM, BPL drastically inhibits the infectious activity of phage M13 [22]. BPL seems to be more effective in inactivating enveloped viruses than non-enveloped viruses [23]. Interestingly, BPL use appears also suitable to obtain a specific immunity against respiratory syncytial virus and H5N1 virus [18,27]. BPL treatment then mediates a loss of infectivity while maintaining antigenicity. This effect has been obtained for high BPL doses more than several millimolars. These results are promising for the use of BPL in vaccine preparation. However regarding WHO and European Pharmacopoeia recommendations, a better fine-tuning of BPL doses is required for industrial vaccine production processes to assure viral inactivation.

There is a limited understanding of the relation between molecular modification and loss of infectious titer as well as the capacity of BPL to modify proteins. That limits the BPL use for preparing safe vaccines. This lack of knowledge is likely at the origin of "over inactivation" and inappropriate reaction.

In the present work, we aimed at establishing the effects of viral BPL treatment at a molecular level and thus to close this existing gap of knowledge and to study in particular the effect of this inactivating agent on viral proteins. Virus samples of the H3N2 strain were submitted to various doses of BPL. This strain is part of the seasonal influenza virus produced at industrial scale. In addition, the BPL inactivation procedure employed in this study is identical to that used to produce the commercial vaccine. The infectivity of BPL-treated virus was found to be reduced in a BPL dose-dependent manner. By combining fluorescence spectroscopy, mass spectrometry, cryo transmission electron microscopy (cryoTEM), and cryo electron tomography (cryoET), we observed that BPL-treated viruses had lost their capacity to fuse with liposomes in dose dependent manner.

2. Materials and methods

2.1. Chemical products

Egg phosphatidylcholine, *N*-(lissamine rhodamine B sulfonyl)-phosphatidylethanolamine (*N*-Rh-PE) *N*-(7-nitro-2,1,3-benzoxadiazol-4-yl)-phosphatidylethanolamine (*N*-NBD-PE) and GM3 gangliosides were purchased from Avanti Polar Lipids Inc. (Alabaster, Al), monensin from Calbiochem, cholesterol, Porcine trypsin, and amantadine from Sigma-Aldrich (St Louis, MO).

Ultrapure water with a nominal resistance of 18 mΩ cm (Milli-Q, Millipore) was used for all buffers and solutions. All other chemicals were purchased of the highest purity available from Sigma-Aldrich.

2.2. Liposome preparation

Multilamellar vesicles (MLV) were produced by resuspension of dry lipid films of egg phosphatidylcholine, cholesterol, ganglioside GM3 at a molar ration of 8:1:1 in a buffer A (150 mM NaCl, 5 mM HEPES pH 7.4, 1 mM EGTA). The MLV suspension was then quickly frozen in liquid nitrogen and thawed (water at 37 °C) five times. MLV suspension was then forced through 0.1 μm defined pore polycarbonate filters (Whatmann) using a manual extruder (Avanti Polar lipids) to form large unilamellar vesicles. Phospholipid phosphate concentration was determined according to Böttcher et al. [28].

2.3. Influenza virus preparation and BPL inactivation

An egg-grown influenza virus preparation (A/Victoria/210/2009 — H3N2) was obtained from clarified allantoic fluid and purified on a sucrose gradient. All virus work was carried out at 4 °C. Viral preparations were dialyzed over night into phosphate buffer saline (PBS) at pH 7.5 and protein concentrations determined using Bradford protein reagent (BioRad) was adjusted to 2 mg/mL. Four viral preparations were inactivated using different BPL concentrations [22,23,29,30]. In brief, sodium phosphate at a final concentration of 100 mM was added to prevent pH changes that occur with the addition of the desired (2, 20, 250 and, 1000 μM) BPL concentration. Samples were incubated with gentle stirring overnight at 4 °C. Preparations were then again dialyzed 24 hours against PBS at 4 °C. The viral protein concentration was again determined prior to conducting further experiments. Samples were stored at 4 °C with 0.01% (*w*/*v*) sodium azide and used within two months.

2.4. $TCID_{50}$ assay

MDCK cells were seeded in 96 well plates at a density of 3.75×10^4 cells/well in DMEM (Gibco®) supplemented with 1 μg/mL porcine trypsin. Cells were infected with 50 μL of tenfold serial dilutions of virus and incubated for 4 days, at 37 °C. Supernatants from these cultures were then tested in a hemagglutination assay. $TCID_{50}$ titers were calculated according to the method of Spearman–Karber [31].

2.5. Hemagglutination assay

The HA hemagglutination assay was performed by serially diluting 50 μL of culture supernatants 2-fold with PBS in V-bottom plates. Subsequently, 50 μL of 0.5% chicken red blood cells (Sanofi Pasteur, Alba-la-Romaine, France) were added to each well. The plates were incubated for 1 h at 4 °C and the hemagglutination or the absence of hemagglutination was determined visually for each well.

2.6. SDS-PAGE and mass spectrometry analysis

1 mM BPL-treated virus (15 μg proteins) were deglycosylated with PNGase F, denatured in XT sample buffer (Bio-Rad) under reducing conditions, and separated on a Criterion XT 4–12% Bis-Tris gel (BioRad) in MOPS-XT buffer. The gel was fixed in 50% (*v*/*v*) methanol, 7% (*v*/*v*) acetic acid for 30 min and stained using GelCode. The gel was rinsed in distilled water. Excised gel bands of interest were washed alternatively with 50 mM ammonium bicarbonate pH 8.0 and acetonitrile. Proteins were then reduced with 10 mM DTT, alkylated with 55 mM

Please cite this article as: P. Bonnafous, et al., Treatment of influenza virus with Beta-propiolactone alters viral membrane fusion, Biochim. Biophys. Acta (2013), http://dx.doi.org/10.1016/j.bbamem.2013.09.021