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Effect of 25-hydroxycholesterol in viral membrane fusion: Insights on HIV inhibition

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ABSTRACT

Recently, it was demonstrated that 25-hydroxycholesterol (25HC), an oxidized cholesterol derivative, inhibits human immunodeficiency virus type 1 (HIV) entry into its target cells. However, the mechanisms involved in this action have not yet been established. The aim of this work was to study the effects of 25HC in biomembrane model systems and at the level of HIV fusion peptide (HIV-FP). Integration of different biophysical approaches was made in the context of HIV fusion process, to clarify the changes at membrane level due to the presence of 25HC that result in the suppressing of viral infection.

Lipid vesicles mimicking mammalian and HIV membranes were used on spectroscopy assays and lipid monolayers in surface pressure studies. Peptide-induced lipid mixing assays were performed by Förster resonance energy transfer to calculate fusion efficiency. Liposome fusion is reduced by 50% in the presence of 25HC, comparatively to cholesterol. HIV-FP conformation was assessed by infrared assays and it relies on sterol nature. Anisotropy, surface pressure and dipole potential assays indicate that the conversion of cholesterol in 25HC leads to a loss of the cholesterol modulating effect on the membrane.

With different biophysical techniques, we show that 25HC affects the membrane fusion process through the modification of lipid membrane properties, and by direct alterations on HIV-FP structure. The present data support a broad antiviral activity for 25HC.

1. Introduction

Since the early 1980s, there have been several efforts to develop drugs against HIV [1,2]. The fusion between HIV and target cell membranes is a critical moment of the viral infection cycle. The binding of the viral envelope trimeric glycoprotein gp120 with host cell receptors (CD4 and CCR5 or CXCR4) represents the beginning of T $CD4^+$ cells' infection. This interaction promotes a conformational transition in the viral glycoprotein gp41, which leads to an exposure of its hydrophobic N-terminal region, known as the HIV fusion peptide (HIV-FP). The two gp41 helical heptad repeat domains, the C terminal (CHR or HR2) and the N-terminal (NHR or HR1) fold into each other and form a hairpin-like structure. This approximates the cell and the viral membranes, promoting the formation of the fusion pore and the release of the viral content into the cell, where HIV-1 replication occurs [3]. Inhibition of this process could prevent all the subsequent intracellular steps.

Only two HIV entry inhibitors are available in the market: maraviroc, an inhibitor of gp120 binding to the CCR5 co-receptor [4], and enfuvirtide, a fusion inhibitor peptide that targets gp41 [5]. Despite this promising approach, little progress has been reported in developing longer action and orally bioavailable fusion inhibitors [2]. Furthermore, the drawbacks of these approaches include the emergence and selection of drug-resistant viruses [6]. Targeting less variable factors is an attractive concept that is less prone to drug-resistant viruses' selection. A particularly appealing notion is that viral membrane-targeting agents would necessarily limit the development of resistance, as it is not even conceivable how such resistance may develop. Thus, targeting

Abbreviations: 25HC, 25-hydroxycholesterol; HIV, human immunodeficiency virus type 1; HIV-FP, human immunodeficiency virus type 1 fusion peptide; CH25H, cholesterol-25-hydroxylase; IFN, interferons; POPC, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; POPE, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine; POPS, 1-palmitoyl-2-oleyl-*sn*-glycero-3-phospho-L-serine; SM, sphingomyelin; Chol, cholesterol; DPH, 1,6-diphenyl-1,3,5-hexatriene; TMA-DPH, 4′-(trimethylammonio)diphenyl-hexatriene *p*-toluenesulfonate; laurdan, 6-dodecanoyl-2-dimethylaminonaphthalene; MBCD, methyl-β-cyclodextrin; NBD-PE, 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl; Rhodamine-PE, Rhodamine B 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine; di-8-ANEPPS, 4-(2-[6-(dioctylamino)-2-naphthalenyl]ethenyl)-1-(3-sulfopropyl)pyridinium inner salt; SUVs, small unilamellar vesicles; FTIR, Fourier transform infrared spectroscopy; FRET, Förster resonance energy transfer

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viral membranes represents an exciting new paradigm to explore regarding the development of broad-spectrum antivirals [7,8].

Several studies suggest that HIV fusion occurs in specialized plasma membrane microdomains, named lipid rafts. The definition of lipid raft is rather controversial, and there is no general agreement about it, mostly due to concept misinterpretations [9], or even about their existence *in vivo*. These domains are enriched in cholesterol and sphingomyelin, which emphasizes the importance of cholesterol on HIV entry [10–12]. However, the effects in this process of oxysterols such as 25-hydroxycholesterol (25HC, a cholesterol derivative presenting a second hydroxyl group at position 25) have not been totally clarified yet [13]. For several years, 25HC studies were focused on its influence in the development of atherosclerosis, since deregulation at the level of this sterol is associated with disease progression [14,15]. The hypothesis that 25HC could have a potential immune influence was hinted by the discovery that the enzyme responsible for its production, cholesterol-25-hydroxylase (CH25H), is expressed by macrophages and dendritic cells. In fact, CH25H is directly linked to interferons (IFN; agents from the innate immune system) activity, since it results from an IFN-stimulated gene. There are probably other enzymes with the capability to produce 25HC *in vitro*, but their role in generating this sterol *in vivo* is yet to be clarified [16].

In a study by Liu *et al.* [17], it was found that both the enzyme and 25HC present an extensive ability to neutralize the replication of enveloped viruses. 25HC blocked the entry of vesicular stomatitis virus and HIV in host cells. The authors suggested that 25HC suppresses viral growth by blocking the fusion between viral and cell membranes [17]. More recently, its capacity to block Zika virus infection at the same level was also demonstrated [18]. The hypothesis that the hydroxylation of cholesterol at carbon 25 turns it into a powerful antiviral mediator has gained interest in the last years [19,20]. However, to take advantage of this process, its mechanism of action needs to be understood.

The aim of this work was to address the effect of 25HC in the membranes fusion process. For this, we used synthetic HIV-FP, as well as small unilamellar vesicles (SUVs) and lipid monolayers as membrane model systems. Several biophysical studies were performed to understand the differences that occur at the membrane level when cholesterol is replaced by 25HC. The effect of binary lipid mixtures, with 25HC or cholesterol, on HIV-FP structure was evaluated. In our studies, we mimicked the conversion of cholesterol into 25HC, trying to define the changes at the membrane level that result in the possible mechanism of action of 25HC as viral fusion inhibitor.

2. Material and methods

2.1. Materials

POPC (1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphocholine), DPPC (1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine), POPE (1-palmitoyl-2-oleyl-*sn*-glycero-3-phosphoethanolamine), POPS (1-palmitoyl-2-oleyl-*sn*-glycero-3-phospho-L-serine), 25HC (25-hydroxycholesterol) and SM (egg sphingomyelin) were purchased from Avanti Polar Lipids (Alabaster, AL, USA). Cholesterol (Chol), DPH (1,6-diphenyl-1,3,5-hexatriene), TMA-DPH (4′-(trimethylammonio)diphenyl-hexatriene *p*-toluenesulfonate), laurdan (6-dodecanoyl-2-dimethylaminonaphthalene) and methyl-β-cyclodextrin (MBCD) were from Sigma-Aldrich (St. Louis, MO, USA). NBD-PE (1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine-*N*-(7-nitro-2-1,3-benzoxadiazol-4-yl)), Rhodamine-PE (Rhodamine B 1,2-dipalmitoyl-*sn*-glycero-3-phosphoethanolamine), di-8-ANEPPS and di-4-ANEPPDHQ were from Invitrogen (Eugene, OR, USA). The HIV fusion peptide (AVGIGALFLGFLGAAGSTMGAA), corresponding to the N-terminal domain of HIV-1 gp41 (HXB-2 viral clone) was synthesized by JPT (Berlin, Germany). Peptide stock solutions were prepared in dimethyl sulfoxide (DMSO). The working buffer used throughout the studies was HEPES 10 mM pH 7.4 in NaCl 150 mM.

2.2. Sample preparation

Small unilamellar vesicles were prepared by sonication, due to the impossibility to prepare oxysterols-containing vesicles by extrusion processes [21]. Briefly, a desired amount of lipids from chloroform stock solutions were mixed, and the solvent was evaporated under a nitrogen stream. HEPES buffer was added to resuspend the dispersion. SUVs were prepared by sonication in a water bath ultrasonicator for 10–15 min until the suspensions become transparent. Different compositions were prepared to mimic both mammalian cells and HIV membranes.

SUV stability and size distribution were evaluated by dynamic light scattering (DLS), using a Malvern Zetasizer NanoZS (Malvern, UK), guaranteeing a homogeneous vesicle size distribution regardless of lipid composition.

2.3. Lipid mixing assays

Membrane (hemi)fusion was measured by Förster resonance energy transfer (FRET), by incorporating two membrane probes in the SUV membrane: rhodamine B-PE and NBD-PE. This assay is based on the decrease in the efficiency of the resonance energy transfer between the two probes when the vesicles labelled mix with unlabelled vesicles. It is important to bear in mind that lipid mixing assays are not able to distinguish between membrane hemifusion and membrane fusion. This can only be fully established by using considerably more complex content mixing assays [22,23].

The concentration of each of the fluorescent probes within the prefusion liposome membrane was 0.6 mol%. Unlabelled SUVs were mixed with double-labelled SUVs in a 1:4 labelled:unlabelled proportion, at a total final lipid concentration of 100 μM, at 37 °C, under constant stirring. Fluorescence was measured with excitation at 470 nm and the emission scan between 500 and 650 nm. Excitation and emission slits were set to 10 nm. Lipid mixing, resulting from membrane fusion (or hemifusion), was quantified on a percentage basis according to equation [24,25]:

$$\textit{Fusion efficiency} = \frac{R - R_0}{R_{100\%} - R_0} \quad (1)$$

where R is the ratio between the fluorescence intensity with emissions at 530 nm and 588 nm (corresponding to the fluorescence emission maxima of NBD and Rhodamine B, respectively) obtained 10 min after HIV-FP addition, R_0 is the ratio before peptide addition, and $R_{100\%}$ was set with vesicles labelled with 0.12 mol% of each of the fluorophores (one-fifth of the mol% of the previous measurements, corresponding therefore to a full lipid mixing between labelled and unlabelled vesicles).

2.4. FTIR-ATR spectroscopy

Infrared spectra were recorded on a Bruker Tensor 27 infrared spectrophotometer (Bruker Optik GmbH, Ettlingen, Germany) equipped with a Bio-ATR II accessory (ZnSe/silicon crystal). The spectrophotometer was continuously purged with dried air. Secondary structure of the HIV-FP was studied in the absence and presence of membranes with three lipid compositions: POPC, POPC:Chol (70:30) and POPC:25HC (70:30). SUVs 100 μM were incubated with HIV-FP 20 μM, in HEPES buffer, during 1 h, at 37 °C, under constant stirring.

A film with the samples was prepared by spreading the peptide/lipid solution on the plate under a stream of air to evaporate the solvent. Spectra were recorded at a spectral resolution of 4 cm^{-1} and 120 accumulations were performed per measurement. FTIR spectra were recorded at a wavelength range from 900 to 3500 cm^{-1}. Background of the internal reflection element was collected and subtracted to the samples. The obtained spectra were rescaled in the amide I area, between approximately 1600 and 1700 cm^{-1}.