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Research Paper

Inhibition of herpes simplex-1 virus replication by 25-hydroxycholesterol and 27-hydroxycholesterol

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ABSTRACT

Oxysterols are known pleiotropic molecules whose antiviral action has been recently discovered. Here reported is the activity of a panel of oxysterols against HSV-1 with the identification of a new mechanism of action. A marked antiviral activity not only of 25HC but also of 27HC against HSV-1 was observed either if the oxysterols were added before or after infection, suggesting an activity unrelated to the viral entry inhibition as proposed by previous literature. Therefore, the relation between the pro-inflammatory activity of oxysterols and the activation of NF-kB and IL-6 induced by HSV-1 in the host cell was investigated. Indeed, cell pre-incubation with oxysterols further potentiated IL-6 production as induced by HSV-1 infection with a consequent boost of the interleukin's total cell secretion. Further, a direct antiviral effect of IL-6 administration to HSV-1 infected cells was demonstrated, disclosing an additional mechanism of antiviral action by both 25HC and 27HC.

1. Introduction

Oxysterols are a family of cholesterol oxidation derivatives containing an additional hydroxyl, epoxide or ketone group as to the parental compound. Due to their chemical structure, oxysterols have been consistently demonstrated as more reactive than unoxidized cholesterol, being easily diffusible through cell membranes, and possessing remarkable pro-inflammatory and cytotoxic effects in a number of cells and tissues [1].

The biological role of oxysterols has been reconsidered for their fundamental activity as physiological ligands of liver X receptors (LXRs), a feedback regulating system for cholesterol homeostasis, immune and inflammatory reactions. The oxysterols showing a high affinity for LXRs are essentially those originated enzymatically, mainly 25-hydroxycholesterol (25HC), 27-hydroxycholesterol (27HC) and 24-hydroxycholesterol (24HC) [2]. These oxysterols have been shown to activate either LXR-dependent or LXR-independent cell signaling pathways, suggesting their dual effects as anti- or pro-inflammatory molecules [3].

Of interest, oxysterols have recently been reported to exert antiviral activity against different enveloped and non-enveloped viruses [4–7]. Notably, the large majority of these studies were focused on 25HC, because the antiviral molecule interferon (IFN) proved to induce the transcription of cholesterol-25-hydroxylase (CH25H), the enzyme responsible for 25HC synthesis [4]. Indeed, also other oxysterols are endowed with antiviral activity, in particular side chain oxysterols. 27HC was shown to be active against murine cytomegalovirus [5], human papillomavirus, rotavirus and rhinovirus [6]. 24HC and 25-epoxycholesterol inhibited the growth of MCMV [5], while 22(S)-hydroxycholesterol and 20α-hydroxycholesterol were active against HBV [8].

If, on one hand, the broad antiviral activity of at least certain oxysterols is nowadays well demonstrated and supported by a still growing bulk of experimental evidence, on the other hand, the mechanisms underlying such an important property of different cholesterol oxidation products mostly remain to be elucidated (for an updated review see 7).

A suitable approach for understanding the antiviral effects of oxysterols, in our opinion, should be guided by the awareness that most viral infections induce in the host the activation of innate and acquired immune systems and trigger an inflammatory response. Of note, most of the oxysterols of pathophysiological interest are provided with a marked and wide pro-inflammatory effect [9–11]. In addition, at least with regard to 25HC, solid data are available that support its

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significant involvement in the regulation of immunologic response [12]. Hence, an oxysterol-mediated modulation of immune and flogistic reactions of the infected host appears very likely.

Out of the several viruses whose growth resulted to be inhibited by 25HC, herpes simplex-1 (HSV-1) appeared the most affected one, at least under the experimental conditions adopted [5]. Moreover, a number of reports have outlined an important role played by inflammation against HSV-1 infection. Indeed, an induction of pro-inflammatory cytokines, in particular IL-6, in response to HSV-1 infection was observed in different types of cells including leukocytes [13], EMT-6 epithelial cells, HaCat cells [14,15], cornea epithelial cells, fibroblasts [16], astrocytes [4]. HSV-1-induced up-regulation of IL-6 expression and synthesis was confirmed to be operated through the increase of Toll Like Receptor 3 (TLR3) and Nuclear Factor kappaB (NF-kB) protein levels [4]. The pro-inflammatory burst activated by HSV-1 infection was proved to have an antiviral effect, by the net enhancement of viral growth achieved every time the overproduction of IL-6 and other pro-inflammatory cytokines was quenched or inhibited [17,18]. In this regard, it is noteworthy the very strong enhancement of morbidity, symptoms severity and mortality following HSV-1 respiratory infection in knockout mice deficient for IL-6 [19].

On these bases, we deemed important to investigate the possible anti-HSV-1 effect of a panel of oxysterols, some of them already shown as provided with broad antiviral activity [6] and all being recognized to induce inflammatory cytokines, including IL-6 [20,21]. The results obtained, demonstrate that mainly 25HC and 27HC markedly inhibit HSV-1 replication and point to the oxysterol-dependent further induction of NF-kB nuclear translocation and IL-6 production in the infected cells as a contributing mechanism to their antiviral properties.

2. Materials and methods

2.1. Cell lines and virus

African green monkey kidney cells (Vero) (ATCC CCL-81) were cultured in Eagle's minimal essential medium (MEM) (Gibco/BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% foetal calf serum (FCS) (Gibco/BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany), at 37 °C in an atmosphere of 5% of CO_2. Human epithelial adenocarcinoma HeLa cells (ATCC® CCL-2™) were propagated in Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL, Gaithersburg, MD) supplemented with heat-inactivated 10% fetal bovine serum (FBS) (Gibco-BRL) and 1% antibiotic-antimycotic solution (Zell Shield, Minerva Biolabs GmbH, Berlin, Germany), at 37 °C in an atmosphere of 5% of CO_2. Clinical isolates of HSV-1 were kindly provided by Prof. M. Pistello, University of Pisa, Italy. Viral strains were propagated and titrated by plaque assay on Vero cells.

2.2. Cell viability assay

Vero and HeLa cells were seeded into 96-well plates at a density of 10^4 cells/well, and incubated at 37 °C in a 5% CO_2 atmosphere for 24 h. Oxysterols dissolved in ethanol (25 hydroxycholesterol 25HC, 27 hydroxycholesterol 27-HC, 7α hydroxycholesterol 7αHC, 7β hydroxycholesterol 7βHC and 7Ketocholesterol 7KC) were added to the cells at different concentrations ranging between 150 μM and 0.1 μM, with a replicate number of three wells per concentration. 25-HC was purchased from Sigma Aldrich (Saint Louis, Missouri, USA); 7αHC, 7βHC, 7KC and 27-HC were purchased from Avanti Polar Lipid (Alabaster, Alabama). After 24 or 48 h incubation period, cell viability was measured by the MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] assay by the Cell Titer 96 Proliferation Assay Kit (Promega, Madison, WI,USA) according to the manufacturer's instructions. Absorbance was measured using a Microplate Reader (Model 680, BIORAD) at 490 nm. The percent of viability was calculated in comparison with cells treated with equal volumes of ethanol. The 50% cytotoxic concentrations (CC_{50}) and 95% confidence intervals (CI) were determined using GraphPad PRISM software (Graph-Pad Software, San Diego, CA).

2.3. HSV-1 antiviral assays

Inhibition of HSV-1 replication was evaluated with plaque reduction assay and virus yield reduction assay. Vero cells were seeded in 24-well plates at a density of 9×10^4 cells. The plaque reduction assay was performed pre-treating cells for 16 h with each oxysterol and subsequently infecting cell monolayers with HSV-1 at a multiplicity of infection (MOI) of 0.0004 pfu/cell for 2 h at 37 °C. The inocula were subsequently removed from the wells, and the cells were washed with medium twice and overlaid with a medium containing 1.2% methylcellulose (Sigma). Treatment of control samples with equal volumes of ethanol was performed in order to rule out the possibility of any cytotoxic or antiviral effect ascribable to the solvent. After further incubation for 48 h at 37 °C in 5% CO_2, the supernatant was removed, and the cells were fixed and stained with 0.1% crystal violet in 20% ethanol and viral plaques were counted. The plaques were visualized using a Leica inverted Microscope equipped with a Bresser MikroCam microscope camera and MikroCamLab software (Rhede, Germany). ImageJ software was used to quantify plaque sizes. Ethanol treated HSV-infected monolayers were used as internal control.

For the virus yield reduction assay, cells were pre-treated for 16 h with oxysterols and infected in duplicate with HSV-1 at a MOI of 0.01 pfu/cell. Following virus adsorption (2 h at 37 °C), the virus inoculum was removed and cells were incubated until control cultures displayed extensive cytopathology. Alternatively, cells were infected with HSV-1 at a MOI of 0.01 pfu/cell for 2 h and subsequently incubated with medium containing serial dilutions of oxysterols until control cultures displayed extensive cytopathology. Supernatants were pooled as appropriate 48 h after infection and cell-free virus infectivity titers were determined in duplicate by the plaque assay in Vero cell monolayers.

2.4. Oxysterols intracellular quantification

To a screw-capped vial sealed with a Teflon septum, cellular pellets resuspended in NaCl 0.9% were added together with 2500 ng of D6-25-hydroxycholesterol and 2500 ng of 27-hydroxycholesterol as internal standards, 50 μl of butylated hydroxytoluene (5 g/l) and 50 μl of K3-EDTA (10 g/l) to prevent auto-oxidation. Each vial was flushed with argon for 20 min to remove air.

Alkaline hydrolysis was allowed to proceed at room temperature (22 °C) with magnetic stirring for 30 min in the presence of ethanolic 1 M potassium hydroxide solution. After hydrolysis, the sterols were extracted twice with 5 ml cyclohexane and oxysterols were eluted on SPE cartridge by isopropanol: hexane 30:70 v/v. The organic solvents were evaporated under a gentle stream of argon and converted into trimethylsilyl ethers with BSTFA.

Gas chromatography mass spectrometry (GC-MS) analysis was performed on a GC equipped with an Elite column (30 m × 0.32 mmid × 0.25 mm film; Perkin Elmer, USA) and injection was performed in splitless mode and using helium (1 ml/min) as a carrier gas. The temperature program was as follows: initial temperature of 180 °C was held for 1 min, followed by a linear ramp of 20 °C/min to 270 °C, and then a linear ramp of 5 °C/min to 290 °C, which was held for 10 min.

The mass spectrometer operated in the selected ion-monitoring mode. Peak integration was performed manually, and sterols were quantified from selected-ion monitoring analysis against internal standards using standard curves for the listed sterols. Additional qualifier (characteristic fragment ions) ions were used for structural identification. Interassay CV was 4.6% for of 25-hydroxycholesterol and 3.8% for 27-hydroxycholesterol. Recovery ranged from 98 up to 103%.

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