Antiviral Research 145 (2017) 146–159

Contents lists available at ScienceDirect

Antiviral Research

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

Interferon-independent antiviral activity of 25-hydroxycholesterol in a teleost fish

Patricia Pereiro [a], Gabriel Forn-Cuní [a], Sonia Dios [a], Julio Coll [b], Antonio Figueras [a], Beatriz Novoa [a, *]

[a] Instituto de Investigaciones Marinas (IIM-CSIC), Vigo, Spain
[b] Department of Biotechnology, Instituto Nacional Investigaciones Agrarias (INIA), Madrid, Spain

ARTICLE INFO

Article history:
Received 13 June 2017
Received in revised form
21 July 2017
Accepted 4 August 2017
Available online 5 August 2017

Keywords:
Zebrafish
Cholesterol 25-hydroxylase
25-Hydroxycholesterol
Antiviral
SVCV
Interferon

ABSTRACT

Oxysterols are a family of cholesterol oxygenated derivatives with diverse roles in many biological activities and have recently been linked with the induction of a cellular antiviral state. The antiviral effects of 25-hydroxycholesterol (25HC) extend to several mammalian enveloped and non-enveloped viruses. It has been reported that the expression of the gene encoding cholesterol 25-hydroxylase (CH25H) is induced by interferons (IFNs). In this work, five *ch25h* genes were identified in the zebrafish (*Danio rerio*) genome. The *ch25h* genes showed different tissue expression patterns and differed in their expression after immune stimulation with lipopolysaccharide (LPS), polyinosinic:polycytidylic acid (PolyI:C) and Spring Viremia Carp Virus (SVCV). Only one of the 5 genes, *ch25hb*, was overexpressed after the administration of the treatments. Synteny and phylogenetic analyses revealed that *ch25hb* is the putative homolog of mammalian *Ch25h* in zebrafish, while the remaining zebrafish *ch25h* genes are products of duplications within the teleost lineage. Interestingly, its modulation was not mediated by type I IFNs, contrasting previous reports on mammalian orthologs. Nevertheless, *in vivo* overexpression of *ch25hb* in zebrafish larvae significantly reduced mortality after SVCV challenge. Viral replication was also negatively affected by 25HC administration to the zebrafish cell line ZF4. In conclusion, the interferon-independent antiviral role of 25HC was extended to a non-mammalian species for the first time, and dual activity that both protects the cells and interacts with the virus cannot be discarded.

1. Introduction

Viruses are known to alter lipid metabolism to favor their own replication by promoting the synthesis of cholesterol and fatty acids (Clark et al., 2012; Greseth and Traktman, 2014; Munger et al., 2008; Sanchez and Dong, 2010; Taylor et al., 2011; Waris et al., 2007; Yu et al., 2011). As a consequence, drugs that affect cellular lipid composition have shown effectiveness in reducing viral replication (Bocchetta et al., 2014; Munger et al., 2008; Rodgers et al., 2012; Ye et al., 2003). Alterations in cholesterol metabolism can also be induced by the host as a defense mechanism against viral infections (Moser et al., 2012; Schoggins and Randall, 2013; Seo et al., 2011).

The membrane-associated enzyme cholesterol 25-hydroxylase (CH25H) is involved in cholesterol and lipid metabolism by catalyzing the formation of 25-hydroxycholesterol (25HC, oxysterol) from cholesterol. Cellular cholesterol levels are regulated through the activity of two transcription factors: the sterol regulatory element binding proteins (SREBPs) and the liver X receptors (LXRs), which control multiple genes implicated in cholesterol biosynthesis and uptake (Horton et al., 2002; Joseph et al., 2002). Soluble oxysterols, such as 25HC, can regulate the activity of the LXR/SREBP signaling pathway by reducing cholesterol synthesis and increasing its efflux and elimination (Accad and Farese, 1998; Janowski et al., 1996; Radhakrishnan et al., 2007). In addition to the well-known metabolic role of oxysterols, some recent publications have reported an antiviral state induced by 25HC against numerous viruses affecting mammals (Anggakusuma et al., 2015; Blanc et al., 2013; Cagno et al., 2017; Civra et al., 2014; Iwamoto et al., 2014; Liu et al., 2013; Shrivastava-Ranjan et al., 2016; Xiang et al., 2015). Interestingly, it was recently observed that herpes simplex virus type 1 is able to reduce CH25H levels by degrading its mRNA due to the endonuclease activity of the viral protein UL41

* Corresponding author.
E-mail address: beatriznovoa@iim.csic.es (B. Novoa).

http://dx.doi.org/10.1016/j.antiviral.2017.08.003
0166-3542/

(You et al., 2017).

Type I interferons (IFNs) are the main cytokines regulating the antiviral innate immune response in vertebrates (Fensterl and Sen, 2009). IFNs induce the expression of a broad array of IFN-stimulated genes (ISG), which encode for proteins with direct antiviral activity, including inhibition of viral transcription, degradation of viral RNA, inhibition of translation or modification of protein function (Sadler and Williams, 2008). In addition, IFNs can alter cellular metabolism at different levels in response to viral infection (Fritsch and Weichhart, 2016). The murine *Ch25h* gene belongs to the group of ISG (Park and Scott, 2010); however, concerning the induction of human *CH25H* by IFNs, two recent publications showed conflicting results (Anggakusuma et al., 2015; Xiang et al., 2015). Independently of *CH25H* induction by IFNs, the expression of this gene is mediated, at least partially, by Toll-like receptor (TLR) activation (Bauman et al., 2009; Diczfalusy et al., 2009; Park and Scott, 2010).

There remains controversy about the immune mechanisms induced by the oxysterol 25HC. Liu et al. (2013) concluded that 25HC inhibits many mammalian enveloped viruses, but not a few non-enveloped viruses, by affecting viral entry through inhibition of virus-cell membrane fusion. Other authors have suggested that 25HC blocks viral growth at the post-entry stage using diverse mechanisms (Anggakusuma et al., 2015; Blanc et al., 2013; Cagno et al., 2017; Shrivastava-Ranjan et al., 2016; Tani et al., 2016; Xiang et al., 2015), and the antiviral effect of 25HC was also extended to certain non-enveloped viruses (Civra et al., 2014). Therefore, 25HC can be effective against specific viruses, but not against others, and it is unclear if a multiplicity of functions could be attributed to 25HC depending on the virus and the cellular state. It has also been reported that 25HC can modulate the immune state of the host at different levels (Cyster et al., 2014; Fessler, 2016). These effects include the suppression of immunoglobulin A (IgA) production by B lymphocytes (Bauman et al., 2009), the differentiation of monocytes into macrophages and activation of phagocytosis (Ecker et al., 2010), the modulation of inflammation (Cagno et al., 2017; Gold et al., 2014; Reboldi et al., 2014) and immune cell migration (Hannedouche et al., 2011; Liu et al., 2011; Yi et al., 2012).

In this work, the complete repertoire of *ch25h* genes was analyzed in the teleost model species zebrafish (*Danio rerio*). Constitutive expression of the *ch25h* genes in different tissues, as well as their induction after treatment with LPS or PolyI:C and infection with SVCV, were analyzed. Only *ch25hb* (*ch25h* localized to chromosome 12), which appears to be the homolog to mammalian CH25H gene, was significantly overexpressed by the tested immune stimuli, and this induction was IFN-independent. *In vivo* overexpression of *ch25hb* reduced the mortality of zebrafish larvae after SVCV infection. Finally, *in vitro* experiments in the zebrafish cell line ZF4 showed that 25HC induces an antiviral state that reduces SVCV replication. This work shows for the first time that 25HC possesses antiviral activity against a non-mammalian virus in a non-mammalian species.

2. Materials and methods

2.1. Gene organization and phylogenetic analysis of zebrafish ch25h genes

The *ch25h* genes were searched in the *Danio rerio* full genome (GRCz10 assembly) (http://www.ensembl.org/Danio_rerio/Info/Index?db=core). Synteny conservation of *ch25h* genes between zebrafish, several teleost species and *Homo sapiens* was analyzed using Genomicus v83.01 (http://www.genomicus.biologie.ens.fr/genomicus-83.01/cgi-bin/search.pl).

The presence of specific domains in the encoded proteins was determined with SMART 4.0 (http://smart.embl.de/) (Letunic et al., 2015) to search sequences for SMART and Pfam (Sonnhammer et al., 1998) domain sets using the HMMER package (Eddy et al., 1995).

For phylogenetic analysis, CH25H protein sequences from representative vertebrate species were retrieved from GenBank and ENSEMBL (www.ensembl.org). The MAFFT online server was used to generate a starting alignment following the E-INS-i strategy (Katoh et al., 2005), which was pruned using Gblocks server 0.91b (Talavera and Castresana, 2007). The best-fitting amino acid replacement model (JTT in this case) was determined using ProtTest 3.2 (Darriba et al., 2011) based on the Akaike Information Criterion (AIC) (Akaike, 1974). Finally, the maximum likelihood gene tree was estimated with PhyML 3.0 (Guindon et al., 2010) and represented in FigTree v1.3.1 (http://tree.bio.ed.ac.uk/software/figtree/). Nodal confidence was calculated and represented using the aLRT method (Anisimova and Gascuel, 2006).

Sequence identity and similarity scores were calculated with the software MatGAT (Campanella et al., 2003) using the BLOSUM62 matrix.

2.2. Fish, virus, cell lines

Adults, embryos and larvae from wild-type zebrafish were obtained from our experimental facility, where zebrafish are maintained following established protocols (Nusslein-Volhard and Dahm, 2002; Westerfield, 2000) (see http://zfin.org/zf_info/zfbook/zfbk.html). Zebrafish were euthanized using a Tricaine methanesulfonate (MS-222) overdose (500 mg/l^{-1}). For microinjection experiments, larvae were anesthetized by adding two drops of a 0.05% MS-222 solution to a Petri plate with a volume of 10 ml of water. Fish care and challenge experiments were reviewed and approved by the CSIC National Committee on Bioethics under approval number ES360570202001/16/FUN01/PAT.05/tipoE/BNG.

The rhabdovirus Spring Viremia of Carp virus (SVCV isolate 56/70) was propagated on Epithelioma Papulosum of Cyprinid (EPC) cells (ATCC CRL-2872) and titrated in 96-well plates. The $TCID_{50}$/ml was calculated according to the Reed and Muench method (Reed and Muench, 1938).

The fibroblastic-like cell line, ZF4, derived from 1-day-old zebrafish embryos (ATCC CRL-2050) (Driever and Rangini, 1993) was cultured in Dulbecco's modified Eagle's medium (DMEM/F12, Gibco) supplemented with 100 μg/ml of primocin (InvivoGen) and 10% fetal bovine serum (FBS) at 26 °C. Human HEK-293 cells (ATCC CRL-1573) (Anisimova and Gascuel, 2006) were grown in Eagle's Minimum Essential Medium (Gibco) supplemented with 100 μg/ml primocin (InvivoGen), 1× non-essential amino acids (Gibco), 1 mM sodium pyruvate (Gibco) and 10% FBS. The cells were incubated in a 5% CO2 atmosphere at 37 °C.

2.3. Plasmid constructions

Zebrafish type I interferon (IFNΦ) *ifnphi1*, *ifnphi2* and *ifnphi3* (GenBank accession numbers: NM207640, NC007114 and NC007114, respectively) expression constructs in the pcDNA3.1 backbone were kindly provided by Dr. Mulero (University of Murcia, Spain).

The *ch25hb* gene was amplified using touchdown PCR (primers in Supplementary Table 1), and the PCR product was cloned using the pcDNA 3.1/V5-His TOPO TA Expression Kit (Invitrogen), but the epitope V5 and the polyhistidine (6xHis) tag were not included. One Shot TOP10F competent cells (Invitrogen) were transformed to generate the plasmid construct (pcDNA 3.1-*ch25hb*). Plasmid purifications were conducted using the PureLink HiPure Plasmid Midiprep Kit (Invitrogen).