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The crucial role of bile acids in the entry of porcine enteric calicivirus

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ABSTRACT

Replication of porcine enteric calicivirus (PEC) in LLC-PK cells is dependent on the presence of bile acids in the medium. However, the mechanism of bile acid-dependent PEC replication is unknown. Understanding of bile acid-mediated PEC replication may provide insight into cultivating related human noroviruses, currently uncultivable, which are the major cause of viral gastroenteritis outbreaks in humans. Our results demonstrated that while uptake of PEC into the endosomes does not require bile acids, the presence of bile acids is critical for viral escape from the endosomes into cell cytoplasm to initiate viral replication. We also demonstrated that bile acid transporters including the sodium-taurocholate co-transporting polypeptide and the apical sodium-dependent bile acid transporter are important in exerting the effects of bile acids in PEC replication in cells. In summary, our results suggest that bile acids play a critical role in virus entry for successful replication.

Introduction

Calicivirus is a non-enveloped virus with a diameter of 27–35 nm and possess a single-stranded, positive sense RNA genome of 7–8 kb. The family *Caliciviridae* consists of five genera: norovirus, sapovirus, lagovirus, vesivirus and nebovirus (Carstens, 2010). Noroviruses and sapoviruses cause gastroenteritis in humans and animals, whereas lagoviruses and vesiviruses mostly infect animals causing a variety of diseases (Green et al., 2001). Norovirus infection usually occurs as epidemic gastroenteritis outbreaks and affects 10–21 million people in all age groups in the U.S. each year (CDC, http://www.cdc.gov/norovirus/trends-outbreaks.html). Therefore, norovirus is recognized as the major etiological agent of foodborne and waterborne infections in humans. Human noroviruses have remained uncultivable to date, and it has been a major hindrance to research on viral pathogenesis and development of vaccines and antivirals for norovirus infection. Cultivable caliciviruses include murine norovirus (MNV) (Wobus et al., 2004), porcine enteric calicivirus (PEC) (Flynn and Saif, 1988), feline calicivirus (FCV) and Tulane virus (primate calicivirus) (Farkas et al., 2008). PEC was first isolated in primary porcine kidney cells (Flynn and Saif, 1988) and subsequently in continuous porcine kidney cell line (LLC-PK cells) in the presence of the intestinal content (IC) (Parwani et al., 1991). Later it was found that bile acids in IC were responsible for PEC replication (Chang et al., 2004). The requirement of IC or bile acids in virus replication in cell culture is a unique phenomenon for PEC and implies that biologically important interactions may occur between bile acids and PEC in the intestines (Flynn et al., 1988). Bile acids were also shown to play important roles in the replication of some viruses propagating in the bile rich organs, such as the liver and the intestines. Bile acids were reported to promote hepatitis B and C virus replication (Chang and George, 2007; Chhatwal et al., 2012; Kim et al., 2010; Scholtes et al., 2008) but to inhibit rotavirus replication (Kim and Chang, 2011). Unlike these viruses which are cultivable without addition of bile acids, PEC replication is completely dependent on the presence of bile acids in the medium. While it was previously reported that protein kinase A (PKA) pathway and/or innate immunity elicited by IC or bile acids is involved in supporting PEC replication in LLC-PK cells (Chang et al., 2002, 2004), the detailed mechanism of bile acids in supporting PEC replication is yet to be determined.

Bile acids are amphipathic molecules which are synthesized from cholesterol in the liver. Bile acids are the active constituents of bile and essential for solubilization and absorption of dietary lipids in the digestive tract (Johnson, 1998). In addition to their role in lipid absorption, bile acids are also involved in various metabolic processes, such as cholesterol and lipid homeostasis, and inflammatory process by acting as signaling molecules (Schaap et al., 2014). Primary bile acids, such as cholic acid (CA) and chenodeoxycholic acid (CDCA) and their glycine and taurine conjugates, are synthesized in the liver and excreted into the intestinal tract (Johnson, 1998). Subsequently, secondary bile acids, such as deoxycholic acid (DCA) and lithocholic acid (LCA) and their glycine and taurine conjugates, are produced by intestinal bacteria (Johnson, 1998). The total bile acid concentrations range from

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2 to 30 mM in the small intestines (Dowling, 1973; Northfield and McColl, 1973), and majority of bile acids are reabsorbed in the small intestines and returned to the liver (enterohepatic circulation) (Johnson, 1998). PEC replicates primarily in the proximal intestinal tract (duodenum and jejunum) (Flynn and Saif, 1988) where bile acid concentrations are high. In LLC-PK cells, any bile acid, with the exception of hydrophilic ursodeoxycholic acid (UDCA) and its conjugates, support PEC replication (Chang et al., 2004). Among them, glycochenodeoxycholic acid (GCDCA) and taurochenodeoxycholic acid (TCDCA) support PEC growth at concentrations as low as 50 μM (Chang et al., 2004).

The enterohepatic circulation of bile acids involves various bile acid transporters that include the sodium-taurocholate cotransporting polypeptide (NTCP) and the apical sodium-dependent bile acid transporter (ASBT) expressed in the liver and the intestines (Dawson et al., 2009; Trauner and Boyer, 2003). These bile acid transport proteins are important in maintaining bile acid pool in the enterohepatic circulation and extra-hepatic tissues. Bile acids also bind to specific bile acid receptors to exert various metabolic regulation and bile acid homeostasis (Schaap et al., 2014). These bile acids receptors include a farnesoid X receptor (FXR) and a G-protein coupled receptor (G protein-coupled bile acid receptor, TGR5), which are involved in glucose and lipid metabolism as well as in inflammation (Schaap et al., 2014). In the present study, we investigated the effects of bile acid on PEC replication cycle using various methods including time-of-addition study, gene knockout and activation study, and a confocal microscopy.

Results

Growth kinetics of PEC in the presence or absence of bile acids

To determine bile acid-dependent steps in PEC entry and replication, one step replication studies were conducted by infecting LLC-PK cells with PEC at a high MOI. At up to 4 h PI, viral genome copy numbers were comparable between virus-infected cells with or without GCDCA, indicating that viral attachment and uptake is not influenced by GCDCA (Fig. 1A). The viral RNA levels in the cells incubated with GCDCA increased steadily from 4 h to 12 h PI (Fig. 1A). However, there was no indication of virus replication over time in the cells without GCDCA.

Viral titers determined by the $TCID_{50}$ method at each time point were in line with the viral RNA levels in all samples (Fig. 1A): viral titers were comparable in the cells incubated with or without of GCDCA up to 4 h PI, but viral titers increased from 4.8 to 6.04 $\log_{10}$ $TCID_{50}$/ml at 8 and 12 h PI, respectively, only in the cells incubated with GCDCA (Fig. 1A). In line with these results, extensive CPE started to appear at 12 h PI, and progressed to $>90\%$ of cells at 16 h PI only in the virus-infected cells incubated with GCDCA. At 16 h PI, virus titers of PEC-infected cells incubated with GCDCA further increased to 7.5 $\log_{10}$ $TCID_{50}$/ml, while those of virus-infected cells lacking GCDCA remained low at 3.2 $\log_{10}$ $TCID_{50}$/ml (Fig. 1B). The expression of viral proteins 2AB, POL and VPg was evident only in the virus-infected cells incubated with GCDCA, as monitored by IFA at 12 h PI (Fig. 1C). Similar results were observed with CDCA (100 μM) (data not shown).

Bile acid is required in the early stage of PEC infection

Addition of GCDCA at 0 h (treatment b), 1 h (treatment c) or 2 h PI (treatment d) resulted in marked viral replication, as determined at 12 h PI (Fig. 2A and B), compared to 0 h PI or control (without GCDCA). However, there was no evidence of viral replication when GCDCA was added at 4 h PI (treatment e) (Fig. 2B). Notably, GCDCA was most efficient in inducing viral replication when it was present during 1 h of viral inoculation (treatment b) (Fig. 2B). We also examined if bile acids and/or low pH have deleterious effects on viral particles. Incubation of concentrated PEC with GCDCA (200 μM) at pH 7.0 and 5.0 did not affect virus replication (data not shown). In addition, pre-incubation of cells with GCDCA (treatment a) for 30 min did not lead to virus replication (Fig. 2A and B).

Transfection of viral RNA does not require bile acids to produce infectious viruses

To investigate if bile acids are involved in the virus replication steps following the release of virus genome in the cytoplasm, we transfected LLC-PK cells (permissible) with high concentrations of PEC RNAs and incubated the cells in the presence and absence of GCDCA. In addition, MARC-145 cells (non-permissible) were transfected with PEC RNA in the absence of GCDCA. MARC-145 cells are not susceptible to PEC infection by a usual route of virus infection (incubation of virus in cell culture) regardless of the presence of bile acids, thus described as non-permissible cells for PEC. At 12 h post-transfection, viral protein expression (2AB, POL and VPg) was evident in the LLC-PK cells incubated with or without GCDCA, and also in MARC-145 cells (only LLC-PK cells incubated without GCDCA are shown in Fig. 3B). Virus titration conducted at 16 h post-transfection demonstrated the production of infectious viruses following transfection in both cell lines (Fig. 3A). These results indicate that GCDCA affects the early steps of PEC replication that occur prior to viral replication in the cytoplasm.

Bile acid is required for PEC escape from the late endosomes into the cytoplasm

We examined the entry event of PEC in LLC-PK cells in the presence and absence of GCDCA by studying the subcellular localization of virus with a confocal microscope. At 1 h PI, little immunofluorescence staining for PEC was observed in the cells incubated with GCDCA under confocal microscopy (Fig. 4A). However, positive staining for PEC was observed in the cytoplasm in the cells incubated without GCDCA, or cells incubated with GCDCA and chloroquine at 1 h post-transfection (Fig. 4A). In these cells, confocal microscopy analyses demonstrated colocalization of virus with rab7, a late endosomal marker (Fig. 4A, panels h and p). At 4 and 6 h PI, viruses colocalized with rab7 were still observed by confocal microscopy (Fig. 4B). In the next experiments focusing on the first one hour following virus infection, we observed that fluorescent virus particles gradually decreased over time during 5–60 min PI in the cells incubated with GCDCA (Fig. 4C). ImageJ-colocalization (MBF) analysis and JACoP plugin was used for colocalization analysis. The thresholds were set by the Costes' Auto threshold method and the thresholded Mander's split colocalization coefficients were determined. The split coefficient for colocalization of virus particles (green) with rab7 (red) in the confocal images were found to be >0.90 for all experiments indicating a high degree of colocalization of viral particles with rab7. These results demonstrated that PEC is able to reach the late endosomes without the help of bile acids, but viral escape from the late endosomes requires the presence of bile acids. Our finding on the negating effects of chloroquine in bile acid-supported PEC replication was confirmed by virus titration of the cells treated with chloroquine (data not shown).