HMG-CoA reductase is negatively associated with PCV2 infection and PCV2-induced apoptotic cell death

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We examined the role of HMG-CoA reductase (HMGCR) during porcine circovirus 2 (PCV2) infection. The results demonstrated that levels of endogenous HMGCR were not significantly different in PCV2-infected cells and mock-infected cells. However, the level of phosphorylated HMGCR, an inactivated form of HMGCR, was increased in PCV2-infected cells. Furthermore, HMGCR was upregulated by overexpression, silenced by siRNA or inactivated using its dominant-negative form in PK-15 cells. The results showed that PCV2 infection was inhibited by HMGCR overexpression, whereas it was significantly increased in HMGCR-silenced cells and HMGCR inhibitor-treated cells. Moreover, there was a robust apoptotic response at 48 h post-infection (p.i.) in HMGCR-inactivated cells, and this response was significantly greater than that observed in PK-15 cells. A modest apoptotic response was also observed in HMGCR-silenced cells. Caspase-3 activity was also analysed in PCV2-infected cells at 48 h p.i. As expected, caspase-3 activity was significantly increased in HMGCR-inactivated and -silenced cells compared with PK-15 cells. PCV2 replication was dose-dependently increased in HMGCR-inactivated cells when treated with increasing amounts of caspase-3 inhibitor. Altogether, HMGCR was negatively associated with PCV2 infection and PCV2-induced apoptotic cell death. These data demonstrated that HMGCR can be used as a candidate target for PCV2 disease control and antivirus research. Furthermore, the cells generated in this study can be used to evaluate the potential effects of HMGCR on PCV2 replication.

INTRODUCTION

Porcine circovirus type 2 (PCV2) is the aetiological agent of PCV-associated disease, which is present in every major swine-producing country in the world (Finsterbusch & Mankertz, 2009; Gillespie et al., 2009). PCV2 is a member of the genus Circovirus, family Circoviridae, the members of which are the smallest non-enveloped, single-stranded, circular DNA viruses (Misinzo et al., 2006). Due to its small genome size (1.7 kb) and highly limited coding capacity, the life cycle of PCV2 relies predominantly on host cell factors (Finsterbusch & Mankertz, 2009). However, the cellular processes involved in virus infection are still poorly understood.

The enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase (HMGCR) catalyses the conversion of HMG-CoA to mevalonate, a four-electron oxidation reaction that is the rate-limiting step in the synthesis of cholesterol and other isoprenoids (Friesen & Rodwell, 2004). It was suggested that HMGCR can affect virus infection (Potena et al., 2004; Chang, 2009; Yeo et al., 2009; Soto-Acosta et al., 2013). For example, respiratory syncytial virus (RSV) filament formation coincided with elevated HMGCR expression (Yeo et al., 2009), and HMGCR-mediated changes in F-actin structure play an important role in the intercellular transmission of mature RSV particles (Ravi et al., 2013). Dengue virus infection increased the enzymic activity of HMGCR (Soto-Acosta et al., 2013). The inhibition of HMGCR significantly increased the levels of Norovirus proteins and RNA (Chang, 2009). However, to date, the possibility that the activity of HMGCR may interfere with the replication of PCV2 has never been reported. Here, we explored the relevance of HMGCR during PCV2 infection for the first time. The results demonstrated that HMGCR is negatively associated with PCV2 infection and PCV2-induced apoptotic cell death, indicating that it can be used as a candidate target for PCV2 disease control and antivirus research. Furthermore, the cells generated in this study can be
used to evaluate the potential effects of HMGCR on PCV2 replication.

RESULTS

HMGCR was inactivated during PCV2 infection

Several publications have suggested an association between HMGCR and virus infection (Potena et al., 2004; Chang, 2009; Yeo et al., 2009; Soto-Acosta et al., 2013). In humans, HMGCR was inactivated by phosphorylation at serine 872 by AMP-activated protein kinase (Goldstein & Brown, 1990; Istvan et al., 2000). Thus, we determined whether HMGCR was activated during PCV2 infection. PK-15 cells were infected with PCV2 for 96 h. Whole-cell lysates were prepared at 24, 48, 72 and 96 h p.i. Then, Western blotting was performed to analyse the effects of PCV2 infection on the levels of endogenous HMGCR. The results showed that the levels of endogenous HMGCR were not significantly different in PCV2-infected cells and mock-infected cells (Fig. 1). However, the level of phosphorylated HMGCR, an inactivated form of HMGCR (Goldstein & Brown, 1990; Istvan et al., 2000), was increased in PCV2-infected cells at 48, 72 and 96 h p.i. At the same time, PCV2 capsid protein levels were increased at 24, 48, 72 and 96 h p.i. Together, these results indicate that HMGCR was inactivated during PCV2 infection.

Identification and characterization of positive stable cell lines

We compared the amino acid sequences of human and porcine HMGCR genes, and found that the phosphorylation site of HMGCR was serine 872 in human and serine 869 in pig (Fig. S1 available in the online Supplementary Material). To determine the relevance of HMGCR during PCV2 infection, different lines of cells derived from PK-15 cells in which HMGCR was upregulated by overexpression (PK-HMG), silenced by short interfering RNA (siRNA) (PK-shHMG) or inactivated by its dominant-negative form (PK-S869A mutant) were generated (Fig. 2). Real-time PCR and Western blotting were performed to analyse the characteristics of the cells. As expected, the HMGCR expression levels were significantly increased in PK-HMG cells and PK-S869A mutant cells compared with PK-control cells (PK-15 cells), whereas the amount of HMGCR was significantly decreased in PK-shHMG cells (Fig. 2a–d). However, no significant differences in HMGCR levels were observed among PK-15 cells, PK-C1-control cells and PK-shNC-control cells. A significant amount of phosphorylated HMGCR was detected in PK-15 cells and PK-C1-control cells. In contrast, no phosphorylated protein was detected in PK-S869A mutant cells, indicating that the wild-type HMGCR was inactivated by its dominant-negative form in the PK-S869A mutant cells (Fig. 2e).

We further investigated whether the overexpression or depletion of HMGCR had any substantial effect on total cholesterol levels. As shown in Fig. 2(f), cholesterol levels were significantly increased in PK-HMG cells and decreased in PK-shHMG cells and PK-S869A mutant cells compared with PK-15 cells. Moreover, an MTS assay was performed to evaluate cell viability. The results showed that even though the cells generated in this study exhibited a lower growth rate than did PK-15 cells, there was no significant difference among PK-HMG, PK-S869A mutant, PK-shHMG, PK-shNC-control and PK-C1-control cells (Fig. 2g). These results indicated that the expression levels of the HMGCR gene had no notable toxicity or effect on cell viability.

Overall, the cell lines engineered in this study were well characterized and will undoubtedly be useful to help us understand the impact of HMGCR on PCV2 replication.

PCV2 infection was negatively correlated with HMGCR level

Cells were infected with PCV2 for 72 h and the effect of HMGCR on PCV2 replication and yield was determined by real-time PCR and indirect immunofluorescence assay (IFA), as shown in Fig. 3(a) and Fig. 3(b), respectively. The results showed that overexpression of the HMGCR gene significantly inhibited PCV2 replication and yield, whereas PCV2 replication and yield were significantly increased in the HMGCR-downregulated PK-shHMG cells.

Lovastatin, an inhibitor of HMGCR, was used to validate these results further. PK-HMG cells in 25 cm² flasks were treated with concentrations of lovastatin ranging from 5 to 20 µM, as described in Methods. The effect of lovastatin on PCV2 replication was determined by real-time PCR (Fig. 3c) and IFA (Fig. 3d) at 72 h p.i. PCV2 replicated normally in untreated PK-HMG cells, whereas it was significantly increased in lovastatin-treated cells, indicating that PCV2 replication was affected by HMGCR. Because lovastatin has
a broad effect on cells, its effect on cell viability was determined by MTS assay in PK-HMG cells. PK-HMG cells treated with concentrations of lovastatin ranging from 5 to 20 μM for 72 h exhibited no significant cytotoxicity under our experimental conditions (Fig. 3e).

Taken together, PCV2 replication and yield were negatively correlated with HMGCR levels. Thus HMGCR can be used as a candidate target for PCV2 disease control and antivirus research.

**HMGCR was negatively associated with PCV2-induced apoptosis**

In the present study, we observed that the PK-S869A mutant cells began to detach from the plate at 48 h p.i., and almost no cells remained alive at 72 h p.i., whereas no significant cell death was observed in Z-VAD-FMK treated and untreated PK-15 cells (data not shown).

To determine whether HMGCR is associated with apoptosis in PCV2-infected cells, an apoptosis assay was performed. As demonstrated in Fig. 4(a), there was a robust apoptotic response at 48 h p.i. in PK-S869A mutant cells, and this response was significantly higher than that observed in PK-15 cells. A modest apoptotic response was also observed in PK-shHMG cells. Furthermore, caspase-3 activity was analysed in the PCV2-infected cells at 48 h p.i. As expected, caspase-3 activity was significantly increased in PK-S869A mutant cells and PK-shHMG cells compared with PK-15 cells (Fig. 4b). Moreover, caspase-3 activity was reduced when the PK-S869A mutant cells were treated with the inhibitor Z-VAD-FMK. PCV2 replication was dose-dependently increased in PK-S869A mutant cells treated with...
increasing amounts of Z-VAD-FMK (Fig. 4c). The cytotoxicity of Z-VAD-FMK against PK-S869A mutant cells was determined by MTS assay. For all doses of the inhibitor used in the present study, the cell viability assay showed no detectable cell death (Fig. 4d). Therefore, it is reasonable to conclude that HMGCR is negatively associated with PCV2-induced apoptosis.

**DISCUSSION**

In the present study, we found that the level of phosphorylated HMGCR, as an inactivated form of HMGCR, was increased during PCV2 infection. As is known, the HMGCR pathway is an important metabolic route that is present in nearly all organisms, and HMGCR itself is the rate-limiting enzyme of cholesterol synthesis (Tamasawa et al., 1997; Svoboda, 2007; Zipp et al., 2007; DeBose-Boyd, 2008). It was reported that HMGCR can affect virus infection (Potena et al., 2004; Chang, 2009; Yeo et al., 2009; Soto-Acosta et al., 2013). Cholesterol, fatty acids and lipid rafts are critical for the replication and infection of RNA and DNA viruses (Wang et al., 2009; Pollock et al., 2010). However, unexpectedly, cholesterol does not appear to be involved in infectious PCV2 entry into epithelial cells (Misinzo et al., 2009). Furthermore, there is no significant difference in PCV2 yield between methyl-β-cyclodextrin-treated cells, in which cholesterol has been depleted from...
the plasma membrane, and untreated PK-15 cells (Yang et al., 2013). Therefore, we hypothesized that there might be a direct or indirect relationship between HMGCR and PCV2 infection. The results in this study demonstrated a negative correlation between HMGCR and PCV2 infection (Fig. 3). Although we do not have a clear explanation for this observation, it is possible that HMGCR plays an important direct role, such as participating in viral replicase complexes as an essential cofactor, or an indirect role in PCV2 replication. It is obvious that HMGCR can be considered a candidate target for PCV2 disease control and antivirus research. In future work, the potential interactions between HMGCR and viral proteins will be evaluated by co-immunoprecipitation and mammalian two-hybrid assays.

Furthermore, we will focus on this protein to find the most cost-effective method of controlling the virus.

PCV2 infection induces the activation of the nuclear factor kappa B pathway, the extracellular signal-regulated kinase pathway and the mitogen-activated protein kinase (MAPK) pathway. These pathways play important roles in PCV2 replication and contribute to virus-mediated changes in host cells, including apoptosis (Wei et al., 2008, 2009; Wei & Liu, 2009). It was reported that the ORF3 protein of PCV2 caused apoptosis in virus-infected cells and interacted with the porcine homologue of Pirh2 (Liu et al., 2007; Karupp kannan et al., 2010). PCV2-induced apoptosis may be related to Ca^{2+} concentration (Lv et al., 2012). Apoptosis signal-regulating kinase 1, an upstream enzyme
that activates the c-Jun N-terminal kinase and p38 MAPK pathways, also regulates PCV2-induced apoptotic responses (Wei et al., 2013). However, PCV2 infection can also induce the anti-apoptotic function of the PI3K/Akt pathway to suppress premature apoptosis, ensuring improved virus growth after infection (Wei et al., 2012). The ORF4 protein of PCV2 can inhibit viral replication in the early stage of infection, suppress caspase activity by restricting ORF3 transcription, and regulate CD4+ and CD8+ T lymphocytes during PCV2 infection (He et al., 2013; Gao et al., 2014). In this study, we demonstrated that HMGCR was negatively associated with PCV2-induced apoptosis. Interestingly, a robust apoptotic response was observed in PCV2-infected PK-S869A mutant cells at 48 h p.i., and this response was significantly greater than that observed in PK-15 (normal) cells and Z-VAD-FMK-treated PK-S869A mutant cells. PCV2 replication was dose-dependently increased in PK-S869A mutant cells treated with increasing amounts of Z-VAD-FMK (Fig. 4c). These results indicate that the mutation of the phosphorylation site in HMGCR may promote apoptosis in PCV2-infected cells. However, further studies are required to determine the mechanism involved.

PCV2 is an extremely slow-growing virus; PCV2 infection and replication in cell culture yield very low viral titres (Allan et al., 1998). Meanwhile, PK-15 cells, which are widely used for PCV2 propagation, do not undergo efficient viral infection (Zhu et al., 2007). The results of previous studies have shown that PCV2 propagation in cell culture is not efficient, and the virus isolated from the samples cannot be passaged more than three times (Yang et al., 2013). In this study, different cell lines in which HMGCR was upregulated by overexpression, silenced by siRNA or inactivated using its dominant-negative form were generated. The results indicated that the expression level of the HMGCR gene had no notable effect on cell viability, but did have a significant effect on PCV2 replication and yield. Clearly, these cells will be useful for future studies of PCV2. Furthermore, PCV2 yields were significantly increased in PK-shHMG cells, indicating that this cell line can be used as a sensitive cell line for PCV2 propagation or vaccine development.

In conclusion, HMGCR was negatively associated with PCV2 infection and PCV2-induced apoptotic cell death, suggesting that HMGCR can be used as a candidate target for disease control and antiviral research related to PCV2. We will focus our future efforts on defining the precise mechanisms by which HMGCR affects PCV2 replication. Furthermore, the cell lines generated in this study can be used to evaluate the potential effects of HMGCR on PCV2 replication. In particular, PCV2 yields were significantly increased in the PK-shHMG cells, indicating that they can be used as sensitive cells for PCV2 propagation or vaccine development.

METHODS

Virus and cells. PCV-free PK-15 cells were purchased from the China Veterinary Culture Collection Center and cultured in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 2% FBS and incubated at 37 °C in a 5% CO2 atmosphere.

Cells were infected with PCV2b strain CC1 at 6 × TCID50 (GenBank accession no. JQ955679) (Yang et al., 2012).

Reagents. Lovastatin, an inhibitor of HMGCR, was purchased from Cayman Chemical (Ann Arbor). Lovastatin was dissolved in DMSO at 10 mg ml−1 and then diluted to various concentrations (5–20 μM) in fresh medium. The caspase-3 activity inhibitor Z-VAD-FMK was purchased from Beyotime (Shanghai).

Cells were treated with either 20 μl DMSO, lovastatin (5–20 μM) or Z-VAD-FMK (10–40 μM) for 1 h prior to PCV2 infection. After 1 h of virus adsorption, the virus was removed and fresh medium containing fresh inhibitor was added to the culture.

Plasmid construction. Plasmids encoding the wt and dominant-negative (S869A) forms of HMGCR were constructed. pEGFP-C1 (Clontech) was digested with NdeI and AgeI and subsequently self-ligated to delete the CMV promoter (pEGFP-C1-CMV−). HMGCR cDNA was amplified by reverse transcriptase-PCR from porcine liver. The PCR product was cloned into pEF-GFP (Addgene plasmid 11154) by replacing the EGFP gene using EcoRI and NotI; the plasmid was designated pEF-HMG1. The HMGCR expression cassette was removed from pEF-HMG1 using SalI and HindIII and ligated into pEGFP-C1-CMV− to generate pEF-HMG2 for wt HMGCR or pHMG-mutant for the dominant-negative (S869A) form of HMGCR. The primers were designed based on the cDNA sequence of the porcine HMGCR gene (GenBank accession no. DUQ32054) using vector NTI 10 (Invitrogen). Primers HMG1 and HMG2 were used to amplify the wt HMGCR, and HMG1 and HMG1-R2M were used to amplify the dominant-negative (S869A) form of HMGCR. The primers and oligonucleotides used in this study are listed in Table S1.

siRNAs targeting the HMGCR gene were synthesized and ligated into the pGPH1/GFP/Neo siRNA expression vector (GenePharma) to generate pGPH1/GFP/Neo. The target sequence was 5′-GCAGAAAAC-TGACACCTCAAGC-3′, corresponding to position 1212–1232 of the HMGCR ORF. Scrambled siRNA (a non-targeting siRNA, designated shNC) was used as the control.

Generation of stable PK-15 cells expressing wt HMGCR, HMGCR-S869A and HMGCR-siRNA. To establish a stable cell line, PK-15 cells were seeded in a 60 mm dish at a density of 7 × 104 cells per dish. On the following day, the cells were transfected with 5 μg of linearized plasmid using Fugene HD Transfection Reagent (Roche) according to the manufacturer’s instructions. At 18 h after transfection, the cells were diluted 1:38, plated in six-well plates, and maintained in the presence of 1200 ng G418 ml−1 (Gibco) for 2 weeks. Stable cell lines were harvested and cultured in separate six-well dishes for further study. The cells stably transfected with pEF-HMG2, pGPH1/GFP/Neo and pHMG-Mutant were designated PK-HMG, PK-shHMG and PK-S869A mutant, respectively. Control cells produced by stable transfection with pEGFP-C1 and shNC were designated PK-C1-control and PK-shNC-control, respectively.

Quantitative real-time PCR. Total RNA was extracted from the cells using TRIzol®-A® Reagent (Tiangen) and reverse transcribed into cDNA using the BioRT cDNA First Strand Synthesis kit (Bioer) according to the manufacturer’s protocol. SYBR Green quantitative real-time PCR was used to analyse the expression of the HMGCR gene using the primers HMG3 and HMG4. The experiments were repeated at least three times.

PCV2 DNA was extracted from infected transfected cell lines and purified with the TIANamp Virus DNA/RNA kit (Tiangen) according to the manufacturer’s protocol. The genomic DNA was dissolved in

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50 μl of DNase-free twice-distilled H2O and stored at −20 °C. PCR products amplified using the primers PCV2-2A and PCV2-2B (Grierson et al., 2004) were cloned into pGM-T (Tiangen) to generate PT-PCV2 as the standard plasmid. The viral genome copy number was determined using real-time PCR by comparison to a standard curve, which was generated by PT-PCV2 dilution. SYBR Green quantitative real-time PCR was performed according to the protocol described by Yang et al. (2013) using the Bio-Rad iQ5 Multicolor Real Time PCR detection system and the BioEasy SYBR Green I Real Time PCR kit (Bioer). The experiments were repeated at least three times.

**Virus titration and IFA.** Viruses were collected and serially diluted 10-fold from 10−1 to 10−11. PCV-free PK-15 cells at 50 % confluence were infected and cultured in 96-well plates at 37 °C in a 5 % CO2 atmosphere. Each dilution was replicated eight times and examined for viral proliferation by IFA at 72 h p.i. The TCID50 values were calculated according to the Reed–Muench method.

The IFA assay was performed as described by Yang et al. (2013). Briefly, cells in 96-well plates were infected with PCV2 for 72 h and fixed with 80 % ice-cold acetone for 30 min at −20 °C. The cells were then washed five times with PBS-T (PBS-T) and incubated for 1.5 h with positive anti-PCV2 serum at 37 °C. After an additional five washes with PBS-T, the cells were incubated with goat polyclonal secondary antibody against pig IgG-H&L (TRITC) (Abcam; 1:500) for 1.5 h. After an additional five PBS-T washes and the addition of 10 % glycerol, the cells were examined using an Eclipse TE2000 V microscope (Nikon). The experiments were repeated at least three times.

**Cholesterol assay.** Cells were seeded in 60 cm2 dishes for the cholesterol assay. Total protein extracts from the cells were prepared in cell lysis buffer, and the protein concentration was quantified using the Enhanced BCA Protein Assay kit (Beyotime). Cholesterol assays were performed for each cell line using a Tissue Total Cholesterol Assay kit (Applygen) according to the manufacturer’s instructions.

**MTS assay.** An MTS assay of the cells was performed according to the protocol described by Zhao et al. (2011). Briefly, cells were seeded in 96-well dishes at a density of 1 × 104 cells per dish. On each day for the next five days, MTS assay was performed using the Cell Titre 96 AQueous One Solution Cell Proliferation Assay (Promega). The OD490 values were measured using an ELx800 microplate reader (Bio-TEK) 2 h later. The experiments were repeated at least three times.

**Western blotting.** Whole-cell lysates were prepared with cell lysis buffer for Western blotting and immunoprecipitation (Beyotime) according to the manufacturer’s instructions. The lysates were diluted in 5 × sample buffer and boiled for 5 min. Equivalent amounts of cell lysates (30 μg) were resolved on 12 % SDS-PAGE and subsequently transferred to nitrocellulose membranes using a PowerPac Universal power supply (Bio-Rad). After blocking with 5 % skimmed milk in TBS-T buffer (20 mM Tris/HCl [pH 7.4], 150 mM NaCl and 0.1 % Tween-20) for 2 h at room temperature, the membranes were incubated with rabbit anti-HMGCR antibody (Santa Cruz Biotechnology), rabbit anti-phospho-HMGCR antibody (Beijing Biosynthesis Biotechnology), mouse anti-PCV2 Cap antibody (produced in our laboratory), or mouse anti-β-actin antibody (Proteintech). The membranes were washed three times with TBS-T buffer and incubated for 1.5 h with HRP-conjugated goat anti-rabbit IgG or HRP-conjugated goat anti-mouse IgG (Beyotime). The immunoreactive bands were visualized using the SuperSignal West Pico Chemiluminescent Substrate kit (Thermo Fisher Scientific) according to the manufacturer’s instructions. The experiments were repeated at least three times.

**Apoptosis assay.** Cell apoptosis during PCV2 infection was assayed using the Annexin V-FITC detection kit (Beyotime) according to the manufacturer’s protocol. Briefly, cells were cultured in 24-well plates and inoculated with PCV2. After 48 h, the cells were washed once with PBS and incubated in 195 μl of Annexin V-FITC binding buffer, which was then added to 5 μl of Annexin V-FITC and incubated at room temperature in the dark for 10 min. A 10 μl aliquot of propidium iodide (PI) was added to the mixture, which was then incubated at 4 °C in the dark for 5 min. The cells were examined using an inverted Eclipse TE2000 V microscope (Nikon). The experiments were repeated at least three times.

**Caspase-3 activity assay.** Caspase-3 activity was evaluated in the cell lines after infection with PCV2. In addition, PK-HMG cells infected with PCV2 were treated with the caspase-3 inhibitor Z-VAD-FMK. Whole-cell lysates harvested from PCV2-infected cells at 48 h.p.i. were assayed using the Caspase-3 Activity kit (Beyotime) according to the manufacturer’s instructions. Samples were measured using an ELx800 microplate reader (Bio-TEK) at an absorbance of 405 nm. The experiments were repeated at least three times.

**Statistical analysis.** For each separate set of assays, at least three independent experiments were evaluated. The results are expressed as the mean ± s.d. Statistical significance was calculated using one-way analysis of variance (ANOVA), followed by the Bonferroni multiple comparison test. Statistical analysis was performed using GraphPad Prism software, version 5. The results were considered to be statistically significant at P<0.05.

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HMGCR affects PCV2 infection


