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The Role of Lipid Rafts in the Early Stage of Enterovirus 71 Infection

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Key Words

Enterovirus 71 • Cell entry • Lipid rafts • Signaling pathway

Abstract

Background/Aims: Although it has been widely accepted that Enterovirus 71 (EV71) enters permissive cells via receptor-mediated endocytosis, the details of entry mechanism for EV71 still need more exploration. This study aimed to investigate the role of lipid rafts in the early stage of EV71 Infection. **Methods:** The effect of cholesterol depletion or addition of exogenous cholesterol was detected by immunofluorescence assays and quantitative real-time PCR. Effects of cholesterol depletion on the association of EV71 with lipid rafts were determined by flow cytometry and co-immunoprecipitation assays. Localization and internalization of EV71 and its receptor were assayed by confocal microscopy and sucrose gradient analysis. The impact of cholesterol on the activation of phosphoinositide 3'-kinase/Akt signaling pathway during initial virus infection was analyzed by Western-blotting. **Results:** Disruption of membrane cholesterol by a pharmacological agent resulted in a significant reduction in the infectivity of EV71. The inhibitory effect could be reversed by the addition of exogenous cholesterol. Cholesterol depletion post-infection did not affect EV71 infection. While virus bound equally to cholesterol-depleted cells, EV71 particles failed to be internalized by cholesterol-depleted cells. EV71 capsid protein co-localized with cholera toxin B, a lipid-raft-dependent internalization marker. **Conclusion:** Lipid rafts play a critical role in virus endocytosis and in the activation of PI3K/Akt signaling pathway in the early stage of EV71 infection.

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Introduction

Enterovirus 71 (EV71) is a small, non-enveloped, positive-stranded RNA virus which belongs to human enterovirus species A of the *Enterovirus* genus of the *Picornaviridae* family. EV71 and coxsackievirus A16 are known as major causative agents of hand-foot-and-mouth disease (HFMD) in young children. EV71 infection can progress to severe neurological disease, including fatal encephalitis, aseptic meningitis and acute flaccid paralysis. Following the initial identification of EV71 in USA in 1969, outbreaks have been reported throughout the world, especially in Asia-Pacific region, including Australia, Malaysia, Japan, Vietnam and China [1]. Until now, no antiviral treatment has been developed to treat EV71 infections and effective vaccines are unavailable. Due to the serious consequences of its infection, much effort is being made to understand the basic mechanisms of EV71 life cycle.

The 7.4-kb EV71 RNA genome encodes a single polypeptide that is cleaved by the host and viral protease into four capsid proteins (VP1, VP2, VP3 and VP4) and seven nonstructural proteins (2A, 2B and 2C, 3A, 3B, 3C and 3D). The capsid protein VP1 is variable and confers distinct antigenic properties. Based on VP1 gene sequence, EV71 can be divided into genotype A, B, and C [2]. The capsid proteins mediate the initiation of infection by binding to a receptor on host membrane. To date, several cell type-specific candidate receptors have been identified, including Annexin in HepG2 cells [3], sialylated glycans in DLD-1 intestinal cells [4], human P-selectin glycoprotein ligand-1 (PSGL-1) in Jurkat T cells [5] and scavenger receptor class B, member 2 (SCARB2) in rhabdomyosarcoma (RD) cells [6]. Host endocytic pathways, such as clathrin-mediated endocytosis, caveolae-dependent uptake, macropinocytosis/phagocytosis and cholesterol-dependent endocytosis, are commonly employed to mediate the infectious entry of virus particles into host cells [7]. Although it has been widely accepted that EV71 enters permissive cells via receptor-mediated endocytosis, the detail of virus-host interaction still needs more exploration.

Lipid rafts are membrane microdomains enriched in cholesterol and sphingolipids that accumulate in liquid-ordered, detergent-resistant membrane (DRM) domains [8]. They perform crucial roles in the establishment of cell polarity [9, 10] and act as platforms mediating signal transduction and protein trafficking [11-13]. A number of raft-associated proteins have been identified, including tyrosine kinase cascades, G protein-coupled receptors, Src family kinases, and cytoskeletal and intermediate filament proteins [14]. The association of proteins with lipid rafts is determined by posttranslational modifications, such as glycosylphosphatidylinositol (GPI) anchor, cysteine acylation and N-terminal myristic acid tail [15].

Membrane cholesterol is critical for infection by many viruses. Recent studies have demonstrated the important functional roles of lipid rafts in the entry process of human immunodeficiency virus type 1, Ebola virus, hepatitis C virus, Japanese encephalitis virus, simian virus 40, and severe acute respiratory syndrome coronavirus [16-19]. There were also some evidences that influenza virus, Measles virus and rotavirus utilize lipid rafts for assembly and egress [20, 21]. Members of the picornaviruses have been reported to enter cells in a lipid raft-dependent pathway. Internalization of echovirus 1 depends on caveolae and lipid rafts [22], while poliovirus RNA delivery requires cholesterol after endocytosis [23]. However, the exact step at which lipid rafts play a role in EV71 infection and the mechanism involved remain uncharacterized. In this study, we demonstrated the critical role of lipid rafts in virus endocytosis and in the activation of PI3K/Akt signaling pathway. Our results provided new insights into the endocytic pathway involved in EV71 internalization, which will further improve our understanding of the underlying mechanism of viral entry.

Materials and Methods

Viruses and cells

The EV71 strain FJ08089 was isolated from a confirmed 8-year old boy with HFMD in Fujian Province, China. Human rhabdomyosarcoma (RD) cells were cultured in Dulbecco's modified Eagle's medium

supplemented with 10% fetal bovine serum (FBS, Invitrogen). The virus was propagated in RD cells and viral titers were determined via viral infectious plaque assays using RD cells.

Immunofluorescence assays

RD cells were fixed with 4% paraformaldehyde for 20 min at room temperature and permeabilized with 0.1% Triton X-100 (Sigma). Then cells were stained with anti-EV71 VP1 monoclonal antibody 10F0 (Abcam) at room temperature for 1 h. After being washed with phosphate-buffered saline (PBS) for three times, the cells were reacted with Alexa 488 conjugated anti-mouse antibody (Invitrogen). Nuclei were counterstained with DAPI (Roche).

Quantitative real-time RT-PCR

RNA was isolated from harvested cells using TRIzol reagent (Invitrogen), and RNA was prepared according to the instructions of the manufacturer. RNA from 10⁵ cell equivalents was analyzed by RT-PCR using a one-step reverse transcription-PCR (RT-PCR) system (Applied Biosystems). Genome copy numbers were normalized to GAPDH (glyceraldehyde-3-phosphate dehydrogenase) or β -actin gene copy numbers by using the comparative cycle threshold values determined in parallel [24]. The mRNA levels of 3-hydroxy-methylglutaryl coenzyme A reductase (HMGCR) in RD cells infected with EV71 was determined by one-step RT-PCR. The reagent used was from Applied Biosystems inventoried assay (assay identification no. Hs00168352_m1).

Treatment with the cholesterol disrupting agent

RD cells were washed with PBS, and treated for 1 h at 37 °C with varying concentrations of methyl- β -cyclodextrin (M β CD) (Sigma). To replenish cellular cholesterol level, M β CD-treated cells were washed with PBS and then incubated with 150 μ g/ml cholesterol (Sigma) for 1 h at 37 °C. After treatment, cells were washed three times with PBS and infected with EV71 for 1 h at 37 °C in serum-free medium. At 24 h post infection, cells were fixed and analyzed by immunofluorescence assays and quantitative real-time RT-PCR as described above.

Cell cholesterol quantification

RD cells treated with 10 mM M β CD were washed with PBS for three times and then lysed in lysis buffer (1% Triton X-100, 25 mM HEPES (PH 7.4), 150 mM NaCl, 5 mM MgCl₂, 20 μ g/ml aprotinin, 10 μ g/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride). Lysates were spun at 10,000 g for 5 min to remove debris, and cholesterol levels were quantitated using an Amplex Red cholesterol assay kit (Molecular Probes) according to the manufacturer's instructions. A standard curve using purified cholesterol was generated for each experiment and normalized to the number of cells.

Antibody staining and flow cytometry

Expression of SCARB2 on the surface of RD cells and the binding of EV71 to RD cells were evaluated by flow cytometry. Untreated or M β CD-treated cells were collected and incubated with or without EV71 for 1 h at 4 °C. After washing three times with PBS, the cells were incubated with anti-SCARB2 antibody (R&D Systems) or anti-EV71 VP1 monoclonal antibody (10F0, Abcam) for 1 h at 4 °C. The cells were washed three times with PBS and incubated with Alexa 488-conjugated anti-goat antibody or Alexa 488-conjugated anti-mouse antibody for 30 min at 4 °C. The stained cells were analyzed by flow cytometry.

Cholera toxin B and EV71 endocytosis

RD cells seeded on cover slips were washed twice with PBS and incubated for 45 min at 4 °C with DMEM containing 0.5% bovine serum albumin with or without of Alexa 555-conjugated Cholera toxin B (CTB) (Invitrogen) at 10 μ g/ml and with or without purified EV71. After attachment at 4 °C, cells were transferred to 37 °C for 10 min to allow the endocytosis of CTB and EV71. Cells were washed twice with PBS and then subjected to immunofluorescence analysis as described above.

Virus endocytosis experiments were performed as previously described [25]. Briefly, virus attachment was permitted as described above and virus internalization was allowed at 37°C for indicated time points. Cells were washed, fixed and stained as described above with anti-VP1 antibody, followed by Alexa 488-conjugated secondary antibodies. Nuclei were counterstained with DAPI. Cells were observed using a confocal fluorescence microscope (Zeiss).

Immunoprecipitation and Western blotting

RD cells were suspended and incubated with EV71 for 2 h at 4 °C. Cells were washed with PBS for three times and then lysed in lysis buffer as described above. After 1 h at 4 °C, insoluble material was pelleted at 16,000 g (25 min, 4 °C) and supernatants were precleared by incubation with protein G-Sepharose (Roche Molecular Biochemicals). The supernatants were then incubated with anti-SCARB2 antibody (R&D Systems) for 1 h at 4 °C, followed by overnight incubation with protein G-Sepharose. Immune complexes were collected by centrifugation, washed three times in lysis buffer, and then analyzed by SDS-PAGE (12% acrylamide) under non-reducing conditions. The proteins were transferred to PVDF membranes and probed with primary antibody, followed by horseradish peroxidase (HRP)-conjugated antibodies (Santa Cruz Biotechnology). Bound antibodies were detected with the ECL Plus Western blotting detection reagents (PerkinElmer Life Sciences).

Akt phosphorylation in EV71-infected cells was detected as previously described [26]. Briefly, RD cells were infected with EV71 at 37 °C. After 20, 40 and 60 min of viral infection, the cell lysates were analyzed by Western blotting with specific antibodies for phospho-Akt or Akt (Cell Signaling Technology).

siRNA experiments

Specific siRNAs targeting PIK3CG (M-005274-02) were obtained from Dharmacon. As a control, we used the non-targeting siRNA sequence (D001810-10) (Dharmacon). RD cells were transfected with 30 nM siRNA using Lipofectamine RNAiMAX according to the manufacturer's instructions. Subsequent experiments were carried out 72 h after siRNA transfection. Knockdown efficiencies were analysed by Western blotting.

Lipid raft isolation

Cells (5×10^7) were washed twice with ice-cold PBS and lysed on ice for 30 min in 1 ml TNE-buffer [25 mM Tris (pH 7.5), 150 mM NaCl, 5 mM EDTA, and complete protease inhibitor cocktail (Roche)] with 1% Triton X-100. The cell lysates were homogenized and then centrifuged at 4 °C for 5 min at 1000 g to remove insoluble materials. The supernatant was mixed with 1 ml 80% sucrose in TNE buffer, placed at the bottoms of ultracentrifuge tubes and overlaid with 7 ml 30% and 3 ml 5% sucrose in TNE buffer. The cell lysates were ultracentrifuged at 4 °C for 18 h at 36,000 rpm in a SW41 rotor (Beckman). After centrifugation, twelve 1-ml fractions were collected from the top to the bottom and analyzed immediately by Western blotting.

About 20 μ l of each sucrose gradient fractions was resolved by SDS-PAGE (12% acrylamide). Immunoblotting was performed using anti-caveolin-1 (Sigma), anti-CD71 (Santa Cruz Biotechnology), anti-SCARB2, anti-EV71 VP1, anti-phospho-Akt and anti-Akt antibodies separately and HRP-conjugated secondary antibodies. Blots were visualized by chemiluminescence.

Statistical analyses

All statistical analyses were performed using Microsoft Excel software. All graphs represent means $\pm$ standard deviations (SDs). P values for all data were determined using the paired t test.

Results

Depletion of cholesterol by M β CD inhibits EV71 infection

To study the role of cholesterol in EV71 infection, we used M β CD to deplete cell membrane cholesterol. M β CD is a derivative of cyclic oligosaccharides and has a lipophilic property that extracts cholesterol from membranes, resulting in lipid raft disruption [27]. RD cells were treated with the increasing concentrations of M β CD (from 2.5 to 10 mM) for 1 h at 37 °C, washed and then incubated with EV71. After this treatment, the expression of viral protein detected by the anti-VP1 antibody decreased markedly in a dose-dependent manner (Fig. 1A). The pretreatment of increasing concentrations of M β CD decreased more than 85% intracellular EV71 RNA levels (Fig. 1B). To further investigate that the inhibitory effect was due to cholesterol depletion and not due to a pleiotropic effect of M β CD on other membrane components, we supplemented M β CD-depleted cells with exogenous cholesterol in the same experiment. As shown in Fig. 1C, the decrease in VP1 expression was reversed when M β CD-treated RD cells were incubated with exogenous cholesterol prior to infection.

Fig. 1. Effect of cholesterol depletion by MβCD on EV71 infection. (A and B) RD cells were pre-treated with MβCD (0–10 mM) for 1 h and then infected with EV71 at 37 °C. After adsorption for 1 h, the inoculated virus was washed away and the cells were cultured in fresh medium. At 24 h p.i., the cells were stained with anti-EV71 mAb to visualize viral protein expression (A). Intracellular RNA was isolated and EV71 genome copy numbers were determined by qRT-PCR (B). (C, D, and E) RD cells were treated with MβCD as described above. For cholesterol (Chol) reconstitution, untreated and MβCD-treated cells were incubated with 150 µg/ml cholesterol for 1 h at 37 °C. After intensive wash, cells were infected with EV71 for 1 h at 37 °C. The immunofluorescence assays (C) and quantitative real-time RT-PCR assays (D) were performed at 24 h p.i. Cholesterol levels were measured from equal numbers of control cells, MβCD-treated cells, cholesterol-treated cells, or MβCD-treated cells which were subsequently incubated with MβCD-cholesterol (MβCD+Chol) complexes (E). Data in (B, D and E) represents means±SD in three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

Adding cholesterol to MβCD-treated RD cells also restored intracellular viral RNA levels (Fig. 1D). In order to determine whether MβCD treatment depletes cellular cholesterol levels, we quantitated the amount of cellular cholesterol before and after MβCD treatment. MβCD-treated RD cells reduced the total intracellular pools of cholesterol by 65% compared to mock-treated cells (Fig. 1E). These data demonstrate that EV71 infection in RD cells is dependent on lipid rafts.

Fig. 2. Lipid rafts are required for the initiation of EV71 infection. (A and B) RD cells were pre-treated with 10 mM MβCD for 1 h and infected with EV71, or adsorbed with EV71 for 1 h at 37 °C and cultured in medium supplemented with 10 mM MβCD at the indicated time points. The immunofluorescence assays (A) and quantitative real-time RT-PCR assays (B) were performed at 24 h p.i. (C) RD cells were infected with EV71 at the indicated time points, and cells were harvested for RNA isolation. Copy numbers of HMGCR mRNA were estimated by TaqMan gene expression analysis primer-probe sets as described in Materials and Methods. β-actin cycle threshold values were used for normalization. Data in (C) represents means±SD in three independent experiments. *, $P < 0.05$; **, $P < 0.01$.

EV71 entry is dependent on lipid rafts

To specifically determine which stage of EV71 infection that was affected by MβCD treatment, RD cells were incubated with MβCD for 1 h prior to the start of infection or at the indicated times after infection. As shown in Fig. 2, EV71 infection was maximally inhibited when MβCD was added before viral entry. Interestingly, addition of MβCD at 30 min or later time points after the incubation of EV71 had little to no effect on VP1 expression (Fig. 2A) or intracellular viral RNA levels (Fig. 2B). Since MβCD only had an antiviral effect when added prior to or at the start of infection, these data suggest that MβCD act at a very early stage in EV71 entry.

To determine if cholesterol biosynthesis is influenced during EV71 infection, RD cells were infected with EV71 and then collected at 1, 12 and 24 h post infection for RNA isolation. The levels of mRNA for HMGCR, an enzyme involved in cholesterol biosynthesis, were measured by qRT-PCR. As shown in Fig. 2C, a significant induction of HMGCR was observed in EV71-infected cells at early time points. At 24 h post infection, HMGCR levels were similar to those in uninfected controls (Fig. 2C), indicating that de novo synthesis of cholesterol may not be required for EV71 RNA replication. Collectively, these data demonstrate the requirement of lipid rafts for EV71 entry.

Association of EV71 with lipid rafts in the early stage of infection

Lipid rafts are cholesterol-enriched microdomains, where many cellular proteins, including viral receptors, are preferentially localized. Since membrane cholesterol played an important role in early infection stages, the interaction of EV71 with its receptor SCARB2

Fig. 3. Localization of EV71 and SCARB2 in lipid rafts in mock-infected or EV71-infected RD cells. RD cells were mock infected (A) or infected with purified EV71 (B) for 1 h at 37 °C, and then lysed with 1% Triton X-100 and separated using a discontinuous sucrose gradient. The samples in each fraction were analysed by Western blotting using anti-SCARB2, anti-caveolin-1, anti-CD71 or anti-EV71 VP1 antibody.

may occur in membrane microdomains. To test this hypothesis, RD cells were incubated with EV71 for 1 h at 37 °C and then lipid rafts were isolated from RD cells. Total membranes from mock- and virus-infected RD cells were treated with 1% Triton X-100 at 4 °C, fractionated using a sucrose flotation gradient, and the individual fractions assayed for the presence of EV71 VP1 and SCARB2 by immunoblotting. In mock-treated cells, the majority of SCARB2 was found in detergent-insoluble fractions (Fig. 3A). In EV71-infected cells, we found that VP1 protein is located into the raft fractions, the same fractions as its cellular receptor (Fig. 3B). In control experiments, the raft marker caveolin-1 appeared in low density fractions and the non-raft marker transferrin receptor (CD71) marked in the dense fractions (Fig. 3). Thus, the biochemical fractionation analysis suggests the association of EV71 with lipid rafts during viral entry.

Depletion of cholesterol by M β CD does not affect virus attachment, but shifts EV71 to non-raft microdomains

Since EV71 VP1 and SCARB2 partitioned in lipid rafts, we assessed if the effect of cholesterol depletion of M β CD on viral entry is due to the inefficient virus binding. Equal amounts of EV71 were added to untreated or M β CD-treated RD cells at 4 °C for 1 h. Unbound virus were removed by extensive wash and the amount of cell-associated viruses were determined by flow cytometry using anti-EV71 VP1 antibody. No obvious shifting of peaks was observed, indicating that viral protein bound comparatively to M β CD-treated or untreated RD cells (Fig. 4A). These results suggest that depletion of cholesterol does not affect virus attachment.

RD cells were pre-incubated with M β CD and infected with EV71, after which flow cytometry analysis, biochemical fractionation and co-immunoprecipitation were performed. As shown in Fig. 4B, cell surface expression of SCARB2 was not affected by cholesterol depletion with M β CD. In M β CD-treated cells, high levels of SCARB2 and EV71 VP1 protein were detected, but these were largely confined to the non-raft fractions as well as caveolin-1 (Fig. 4C). The anti-VP1 antibody recognized the 34-kDa protein in the immunoprecipitated complexes of both untreated and M β CD-treated cells (Fig. 4D, lane 3), while no protein bands were observed using a normal rabbit IgG (Fig. 4D, lane 2) or in the lysates without EV71 infection (Fig. 4D, lane 1). As expected, the presence of SCARB2 in total extract from RD cells was also observed (Fig. 4D, lane 1). Taken together, these results demonstrate that M β CD shifts EV71 and its receptor SCARB2 to non-raft microdomains, but the interaction of EV71 VP1 protein with SCARB2 was not affected by M β CD treatment.

Fig. 4. Effects of cholesterol depletion on the association of EV71 with lipid rafts. (A) Flow cytometry analysis of the effect of MβCD on EV71 VP1 binding. RD cells were incubated with or without 10 mM MβCD for 1 h at 37 °C and then labeled with anti- EV71 VP1 mAb and detected by flow cytometry. A peak shift was observed in RD cells incubated with purified EV71 compared with the negative control (RD cells labelled with primary and secondary antibody, without EV71 incubation). (B) Analysis of the effect of MβCD on SCARB2 cell-surface expression. RD cells were incubated with or without 10 mM MβCD for 1 h at 37 °C and then labeled with anti-SCARB2 mAb and detected by flow cytometry. (b) Sucrose gradient analysis. RD cells were pre-incubated with 10 mM MβCD for 1 h at 37 °C and then infected with EV71 for 1 h. Cells were lysed with 1% Triton X-100 and subjected to discontinuous sucrose gradient centrifugation. The samples in each fraction were analysed by Western blotting using the antibodies indicated on the right. (D) Co-immunoprecipitation assays. A coimmunoprecipitation experiment was performed to demonstrate the interaction between SCARB2 and EV71. Untreated or MβCD-treated RD cells were incubated with EV71 and then immunoprecipitated with an anti- SCARB2 antibody. The immunoprecipitated complex was separated by SDS-PAGE (12% acrylamide), transferred to PVDF membranes and probed with specific antibodies. The results were developed by chemiluminescence.

Cellular membrane cholesterol is required for EV71 internalization

To further demonstrate that EV71 entry is mediated by lipid rafts, we examined the internalization of EV71 VP1 and Alexa 555-conjugated cholera toxin B (CTB) by RD cells. CTB has been shown to enter cells by binding to its receptor gangliosides (GM1) in a lipid raft-dependent manner [28]. We permitted EV71 (MOI=0.1) and 10 µg/ml fluorescent CTB to bind to cells at 4 °C for 10 min. Cells were then shifted to 37 °C for 30 min to allow internalization, washed extensively, fixed and analyzed by immunofluorescence microscopy using anti-VP1 antibodies. As shown in Fig. 5A, colocalization of virus and CTB were observed at the plasma membrane of RD cells. This strongly suggests the involvement of lipid rafts for internalization of EV71 virions.

Fig. 5. EV71 internalization is dependent on lipid rafts. (A) EV71 VP1 localizes with CTB at the plasma membrane. RD cells grown on coverslips were infected with EV71 and incubated with 10 μ g of Alexa 555-conjugated CTB/ml for 45 min on ice. Cells were shifted to 37°C for 10 min, after which they were fixed and stained with EV71 VP1 antibodies, followed by Alexa 488-conjugated secondary antibodies. The nuclei were stained by DAPI. Scale bars, 10 μ m. (B) Effect of M β CD on EV71 endocytosis. Virus attachment was permitted as described above and virus internalization was allowed at 37°C for indicated time points. Cells were washed, fixed and stained as described above with anti-VP1 antibody, followed by Alexa 488-conjugated secondary antibodies. The nuclei were stained by DAPI. Scale bars, 10 μ m. (C) Virus attachment was permitted as described in (A) and virus internalization was allowed at 37°C for 60 min. Cells were washed, fixed and stained as described in (B) with anti-VP1 antibody or anti-Na⁺-K⁺-ATPase antibody (Santa Cruz), followed by Alexa 488 conjugated anti-mouse or Alexa 555 conjugated anti-rabbit secondary antibodies. The nuclei were stained by DAPI. Scale bars, 5 μ m.

We next examined the effect of M β CD on EV71 internalization. Cells were infected with EV71 (MOI=0.1) at 4 °C for 30 min for virus attachment and then shifted to 37 °C for 30 min to allow internalization. Immunofluorescence assays were performed to track the entry process and cellular localization of EV71 within infected cells at indicated time points. In control cells, EV71 particles were observed predominantly attached to the plasma membrane of the cells at time 0 (Fig. 5B). At time 30 min, virus particles were clearly observed in an intracellular location (Fig. 5B). No intracellular EV71 particles could be detected in M β CD-treated cells at 30 min p.i., and a few of the viral particles were present at the cell peripheries in the cholesterol-depleted RD cells (Fig. 5B). Na⁺-K⁺-ATPase, a cell membrane marker, was used to demonstrate the retaining or release of viral VP1 protein from the plasma membrane (Fig. 5C). These data demonstrate that cholesterol depletion by M β CD affects EV71 internalization.

Activation of PI3K/Akt signaling in early EV71 infection is dependent on lipid rafts

Previous reports have shown that EV71 infection at early steps stimulates phosphorylation of PI3K/Akt signaling pathways [26]. To detect the role of PI3K/AKT signaling in viral entry, the effect of PI3K depletion by small interfering RNA (siRNA) treatment on EV71 entry was investigated. The efficiency of siRNA-mediated knockdown

Fig. 6. Dependence of the activation of PI3K/Akt signaling on intact lipid rafts. (A) RD cells were transfected with siRNA as indicated for 72 h and then lysed. To verify PI3K knockdown, protein samples from cells expressing non-targeting siRNA or siPIK3CG were analyzed by Western blotting for anti-PI3K antibody (Cell Signaling Technology). (B) RD cells were transfected with siRNA as indicated for 72 h and EV71 infection was then performed. Immunofluorescence assays of the effect of PI3K on EV71 entry. (C) RD cells were infected with EV71 for 0, 20, 40 and 60 min, or mock treated with an isotype-matched goat IgG (10 mg ml⁻¹; Ctrl) for 30 min. Cell lysates were analysed by Western blotting with antibodies specific for phospho-Akt (p-Akt) and Akt. (D) RD cells that had been pre-incubated with MβCD (10 mM) were infected with JEV for the indicated times. The cell lysates were harvested for Western blotting as described in (C).

of PIK3CG (PI3K gamma complex) was analysed by Western blotting. As shown in Fig. 6A, siRNA specific for PIK3CG reduced PI3K expression. Depletion of PI3K resulted in a strong inhibitory effect on EV71 VP1 expression (Fig. 6B), suggesting PI3K/Akt signaling was involved in EV71 infection. Since lipid rafts act as signaling platforms and initiate a variety of signal transduction processes critical for virus entry and propagation, we investigated whether membrane cholesterol is required for triggering PI3K/Akt signaling during EV71 entry. Indeed, cell lysates from RD cells showed increased phosphorylation levels of Akt as early as 20 min post infection (Fig. 6C). As shown in Fig. 6D, a dramatic decrease in Akt phosphorylation was observed after viral infection in MβCD-treated cells. These results clearly suggest that disruption of lipid rafts immediately after EV71 infection impairs the activation of PI3K/Akt signaling pathways.

Discussion

Lipid rafts often serve as an entry site for many viruses. Numerous studies have shown that extraction of cholesterol by MβCD inhibits viral infection in the early stages [19, 29-32]. Our results support the notion that lipid rafts on the plasma membrane facilitate entry of EV71. We observed a reduction in the number of EV71-positive cells and the viral titers in cholesterol-depleted cells. This inhibition probably occurs at initial steps of infection, since no significant inhibitory effect was observed when rafts were disrupted following EV71 adsorption. A dramatic induction of cholesterol biosynthesis during early infection lends further support to the critical role of lipid rafts in EV71 entry. Although no significant upregulation of cholesterol biosynthesis was observed after viral entry in our experiments, we cannot rule out the possibility of the presence of intracellular cholesterol sufficient to participate in EV71 replication. It is possible that cellular cholesterol levels were replenished

by de novo synthesis upon the removal of the drug, allowing for cholesterol engagement in viral replication, assembly or egress.

To infect host cells, the virus initially binds to receptor molecules present on the cell surface. Cholesterol-enriched membrane microdomains may provide a convenient platform to concentrate the receptors on host cell membrane [18]. Our data indicated that the interaction of viral capsid protein with its receptor occurs preferentially at lipid rafts. A number of non-enveloped viruses recognize their receptors in membrane microdomains to access endocytic pathways, thereby facilitating viral entry. Decay-accelerating factor (DAF or CD55), a GPI-anchored membrane glycoprotein, functions as a receptor by many enteroviruses including enterovirus 11 [33], enterovirus 70 [34], hemagglutinating echoviruses [35], and coxsackievirus A21 [36]. Components of the receptor complex for group A rotavirus all localize to cholesterol-rich microdomains prior to or as a result of virus attachment, thus favoring binding and internalization of rotavirus particles [30, 37]. A decrease in productive virus entry may reflect a reduction in virus binding to cells. We then investigated the effect of cholesterol extraction on virus attachment. No significant decrease in the cell surface expression of SCARB2 and the amount of bound virus were observed between M β CD-treated and control untreated RD cells, indicating that cholesterol depletion of cell membrane does not perturb the attachment of virus, but alter viral entry at post-binding steps. We also showed that extraction of cholesterol by M β CD excluded SCARB2 from raft domains. Cholesterol levels are important for maintaining membrane fluidity, and its removal can reduce lateral diffusion within the cell membrane. This reduction in fluidity could perhaps affect migration of SCARB2 within the membranes, which may impair a chain of events that could participate in EV71 pathogenesis triggered by the interaction of viral capsid protein with SCARB2 located in cholesterol-enriched microdomains.

Using confocal microscopy, we found that EV71 particles failed to be internalized in cholesterol-depleted cells. Our results do not dispute the role of clathrin in EV71 endocytosis [38, 39], but suggest that lipid rafts are also required for EV71 internalization. Many viruses are known to enter cells via multiple pathways, such as simian virus 40 (caveolar and cholesterol-dependent pathways) [40], adenovirus (clathrin-mediated endocytosis and macropinocytosis) [41], coxsackievirus A9 (β 2-microglobulin, dynamin and Arf6-mediated internalization) [42], and echovirus 1 (caveolar and dynamin-2 dependent pathways) [22]. Further experiments directed to analyze the endocytic pathways involved in EV71 internalization as well as the role of other proteins in this process are being performed in our laboratory.

Lipid rafts act as functional platforms for multiple signaling and trafficking processes. A number of viruses, including enveloped and non-enveloped viruses, hijack lipid raft machinery to gain access to various signaling elements in the membrane cholesterol for viral entry or the later stages of infection [43]. In picornaviruses infection, the importance of PI3K/Akt pathways in maintaining host cell survival and stimulating anti-apoptotic pathways during early infection has been established [26, 44, 45]. Indeed, our results showed that EV71 activated PI3K/Akt at the early stages of infection. Recently, it has been demonstrated that the silencing of the PIK3CG and PIK3C2G, genes involved in PI3K/Akt signaling pathways, caused a reduction in viral infection [38]. It seemed that the activation of PI3K/Akt signaling pathway was lipid raft-dependent, as disruption of lipid rafts prior to EV71 infection resulted in significant decline in Akt phosphorylation. Therefore, we suggest the critical role of lipid rafts in initiation of signaling cascades during early EV71 infection.

In conclusion, our data demonstrate the importance of lipid rafts for EV71 endocytosis as well as in triggering signaling pathways at the early steps of life cycle. These results further our understanding of the mechanisms of EV71 entry and provide a new target for antiviral potential.

Disclosure Statement

There are no conflicts of interest to disclose for all authors.

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