Sterol Carrier Protein 2, a Critical Host Factor for Dengue Virus Infection, Alters the Cholesterol Distribution in Mosquito Aag2 Cells

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Sterol Carrier Protein 2, a Critical Host Factor for Dengue Virus Infection, Alters the Cholesterol Distribution in Mosquito Aag2 Cells

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ABSTRACT Host factors that enable dengue virus (DENV) to propagate in the mosquito host cells are unclear. It is known that cellular cholesterol plays an important role in the life cycle of DENV in human host cells but unknown if the lipid requirements differ for mosquito versus mammalian. In mosquito Aedes aegypti, sterol carrier protein 2 (SCP-2) is critical for cellular cholesterol homeostasis. In this study, we identified SCP-2 as a critical host factor for DENV production in mosquito Aag2 cells. Treatment with a small molecule commonly referred to as SCPI-1, (N-(4-[[4-(3,4-dichlorophenyl)-1,3-thiazol-2-yl]amino]phenyl)acetamide hydrobromide, a known inhibitor of SCP-2, or knockdown of SCP-2 dramatically repressed the virus production in mosquito but not mammalian cells. We showed that the intracellular cholesterol distribution in mosquito cells was altered by SCP-2 inhibitor treatment, suggesting that SCP-2-mediated cholesterol trafficking pathway is important for DENV viral production. A comparison of the effect of SCP-2 on mosquito and human cells suggests that SCPI-1 treatment decreases cholesterol in both cell lines, but this decrease in cholesterol only leads to a decline in viral titer in mosquito host cells, perhaps, owing to a more drastic effect on perinuclear cholesterol storages in mosquito cells that was absent in human cells. SCP-2 had no inhibitory effect on another enveloped RNA virus grown in mosquito cells, suggesting that SCP-2 does not have a generalized anti-cellular or antiviral effect. Our cell culture results imply that SCP-2 may play a limiting role in mosquito–dengue vector competence.

KEY WORDS Aedes aegypti, Dengue, SCP-2, Cholesterol, host factor

Dengue fever and dengue hemorrhagic fever has re-surged during the past decades. Tens of million cases of dengue fever and 500,000 cases of dengue hemorrhagic fever occur every year. It is estimated that 2.5 billion people, almost half of the world population, are at risk of infection (Kyle and Harris 2008). Currently there is no effective vaccine or specific treatment for dengue disease. Dengue virus (DENV), a positive-stranded RNA virus in Flaviviridae family, is transmitted mainly by the mosquito species Aedes aegypti and Aedes albopictus. Human host cell factors that are required for DENV propagation and pathogenesis have been extensively studied (reviewed in Pastorino et al. 2010, Lindenbach and Rice 2003, Rodenhuis-Zybert 2010). Although DENV infection requires an extensive number of host factors, only a limited number of them have been identified in mosquito (Sessions et al. 2009, Tchantchou-Nguetcheu 2010). New strategies to control the spread of DENV may require a more complete understanding of mosquito vector competence and targeting of mosquito factors that allow robust viral propagation in Aedes mosquitoes.

In human cells, the life cycle of Flavivirus is tightly related to cellular membranes and cholesterol (Fernandez-Garcia et al. 2009). The enveloped DENV (Kuhn et al. 2002) enters cells via receptor-mediated endocytosis (van der Schaar et al. 2008, Acosta et al. 2008, Chu and Ng 2004), followed by fusion of the viral envelope proteins with late endosomal membrane to release the virus RNA in the cytoplasm. However, in insect cells, entry occurs at the plasma membrane (Hase et al. 1987, 1989; Vancini et al. 2013). Flaviviral infections induce dramatic intracellular membrane rearrangement and proliferation (Pena and Harris 2012, Perera et al. 2012, Welsch et al. 2009). Virus RNA replication and assembly of the virus takes place in the membrane-bound complexes derived from the endoplasmic reticulum (ER) (Welsch et al. 2009, Gillespie et al. 2010). Virus particles are assembled and the mature viruses are released via cell secretory machinery (Mackenzie and Westaway 2001). In mammalian cell studies, cholesterol-rich membranes have been shown to mediate Flavivirus viral entry (Valle et al. 2005, Lee et al. 2008, Medigeshi et al. 2008) as well as viral replication (Aizaki et al. 2007, Mackenzie et al. 2007).
DENV replication in human host cells depends on both de novo cholesterol biosynthesis and intracellular cholesterol transport. The key cholesterol synthesis enzymes, such as mevalonate diphospho decarboxylase and 3-hydroxy-methylglutaryl-CoA (HMGCoA) reductase (HMGCR), are necessary for the life cycle of Flaviviruses (Mackenzie et al. 2007, Rothwell et al. 2009). The cholesterol biosynthesis pathway has been suggested as a target for antiviral drugs. For example HMGCR inhibitors (statins, such as lovastatin) lower cholesterol levels (Endo 1992) and reduce DENV titers in epithelial vero cells and endothelial HMEC-1 cells (Martinez-Gutierrez et al. 2011) as does Hymeglusin, an HMGCoA synthesis inhibitor (Rothwell et al. 2009). Inhibitors of squalene synthase, the enzyme that catalyzes the committed step of sterol synthesis, significantly reduces DENV production in human K562 cells (Rothwell et al. 2009). In human cells, the Niemann–Pick disease Type C (NPC)-mediated intracellular cholesterol trafficking pathway is likely involved in DENV replication (Poh et al. 2012). DENV entry and replication in mammalian host cells are suppressed by U18666A (Poh et al. 2012), which is a chemical inhibitor of intracellular transport pathways of low-density lipoprotein-derivived cholesterol from lysosomes to plasma membranes (Liscum et al. 1989) and from plasma membranes to intracellular membranes (Härmäli et al. 1994).

DENV has both invertebrate (mosquito) and vertebrate (human) hosts, but mosquitoes are cholesterol auxotrophs and cannot synthesize cholesterol de novo (Clayton 1964, Rawson 2003). The importance of cellular cholesterol trafficking and distribution in mosquito–virus interactions has not been addressed. DENV infection in mosquito C6/C36 cells has been previously characterized as cholesterol-independent (Unashankar et al. 2008), similar to another enveloped RNA virus, vesicular stomatitis virus (VSV), which can also infect mosquito cells (Phalen and Kielland 1991). Sterol carrier protein 2 (SCP-2) is a cytosolic protein involved in cholesterol binding and transport (Krebs and Lan 2003, Lan and Massey 2004), and is especially important for mosquito to acquire dietary cholesterol (Blitzer et al. 2005, Dyer et al. 2008). Knocking down of SCP-2 expression leads to reduced growth rate in larvae and lowered fertility in adults (Blitzer et al. 2005, Peng et al. 2011, 2012). When SCP-2 has been mutated such that the protein lacks cholesterol-binding capacity, cells expressing these SCP-2 mutants no longer facilitate cholesterol uptake in Ae. aegypti embryonic Aag2 cells (Radek et al. 2010). In addition, SCP-2 protein inhibitors (SCPIs) have been identified and act as insecticides to mosquito larvae and adults (Kim et al. 2005, Larson et al. 2008). We demonstrated that over-expression of SCP-2 protein in mosquito Aag2 cells facilitated uptake of $[^{3}H]$ cholesterol (Lan and Massey 2004, Radek et al. 2010). Given the role of SCP-2 in cholesterol uptake and metabolism in mosquito, we hypothesized that SCP-2 is a potential host factor that facilitates DENV infection in mosquito host cells. In this study, the role of SCP-2 protein in virus–host interaction was investigated in Aag2 cells. In addition, we compared SCP-2’s function in DENV infection between insect and mammalian cells. Our findings suggest that there are differences in the requirements for cholesterol trafficking between dengue replication in human versus mosquito host cells.

Materials and Methods

Chemicals. Chemicals and reagents were purchased from Sigma (St. Louis, MO), Fisher Scientific (Pittsburgh, PA), and Life Technologies (Carlsbad, CA), except when their origins are mentioned in the text. N-((4-[[4-(3,4-dichlorophenyl)-1,3-thiazol-2-yl]amino]phenyl)acetamide hydrobromide (SCPI-1) was purchased from Chembridge Chemical (San Diego, CA).

Virus and Cell Culture. Ae. aegypti Aag2 cells were maintained in Schneider’s Drosophila Medium and Ae. albopictus C6/36 cells were maintained in Leibovitz L-15 medium at 28°C (without CO2). The mosquito cell culture medium was supplemented with 10% fetal bovine serum (FBS), 50 unit/ml penicillin, and 50µg/ml streptomycin. Monkey kidney vero cells and Human Huh7.5 cells were maintained in Dulbecco’s Modified Eagle Medium with 10% FBS, 1% non-essential amino acids, 50 unit/ml penicillin, and 50µg/ml streptomycin at 37°C with 5% CO2. Passages of cells were conducted every 7–10 d with a 1:10 dilution of cells.

To prepare the virus stock, the DENV serotype 2 New Guinea C was used to infect Ae. albopictus C6/36 cell in a T75 flask at multiplicity of infection (MOI) of 0.01 and incubated at 28°C. The medium with virus was harvested 7–10 d post-infection and was concentrated using 100-kDa filters (Millipore, Billerica, MA).

For VSV stock preparation, the VSV-DsRed-Express virus was used to infect BHK-21 cells in a T75 flask at an MOI of 0.001. The infection was carried out for 15-18 hours at 37°C and 5% CO2. The supernatant containing virus was then collected and filtered using 0.22-µm filters (Millipore, Billerica, MA).

Cytotoxicity Assays. Cytotoxicity of sterol carrier protein inhibitor 1 (SCPI-1) was determined using the CellTiter 96 AQueous Cell Proliferation Assay Kit (Promega, Madison, WI) following the manufacturer’s direction. Briefly, cells (1×10^4/well) were seeded in 96-well plates overnight and treated with serial dilutions of SCPI-1 for 72 h. Reaction solution (20µl) provided in the kit was then added to medium and incubated at 37°C for 1 h, and the absorbance was subsequently read at 490 nm. The absorbance was normalized to control and plotted against the log transformation of the concentration of SCPI-1. The 50% cytostatic concentration (CC50) value was determined using GraphPad Prism v4.0 software (San Diego, CA) using nonlinear regression.

DENV Infection and Titration. Aag2 or HuH7.5 cells (5×10^5/well) were seeded in 24-well plates overnight and treated with 0.1µM, 1µM, or 10µM SCPI-1 for 24 h before infection. The control group was treated with the corresponding amount of DMSO to dissolve SCPI-1. DENV was then added (MOI = 0.5–1) in 0.5 ml serum-free medium and incubated at 37°C for
1.5 h. The virus-containing medium was removed after infection. The cells were washed with serum-free medium and cultured in the regular growth medium with SCPI-1. For the time-of-addition experiment, there were three different treatments: cells were only treated for 48 h with 10 μM SCPI-1 before infection (pre-incubation), only treated during the 1.5 h infection period (co-infection), or only treated for 48 h after infection (post-infection), respectively. DENV was harvested from the medium 48 h post-infection.

The virus was tittered using TCID\textsubscript{50} assay described before (Che et al. 2009). Briefly, Vero cells were grown to 50–60% confluent monolayers in 96-well plates, infected with 10-fold serial dilutions (each dilution in triplicates) of virus, and incubated at 37°C with 5% CO\textsubscript{2}. Cytopathic effects were monitored after 5–7 d, and the 50% tissue culture infective dose (TCID\textsubscript{50}) was calculated using the Reed and Muench method (1938). Virus titers were expressed as TCID\textsubscript{50} units per ml.

**VSV Infection and Titration.** The infections were carried out using a fluorescent VSV strain incorporating a DsRed-Express-DR gene (VSV-DsRed-Express); VSV-DsRed-Express has similar infectivity as the wild-type VSV (Swick et al. 2014). Aag2 cells were seeded in 12-well plates of 2.5 × 10\textsuperscript{3} cells/well and cultured for 24 h until the cells formed 70–90% confluent monolayers. The cells were treated 24 h before infection with 1 μM SCPI-1, 10 μM SCPI-1, or DMSO only (mock control). Cells were infected at a multiplicity of 10 in 0.5 ml serum-free medium and incubated at 37°C for 1.5 h to allow for adsorption. The inoculum was then removed, cells were washed twice with serum-free medium, and 0.5 ml/well of medium with 10% FBS was added to the host cells in a well of a 24-well plate containing 0.5 ml growth medium. The treated cells were used for virus infection 48 h post transfection.

**Western Blot.** Western blotting analysis was performed as described, using SDS 15% PAGE. The protein blots were incubated with 1:1000 dilution of rabbit polyclonal anti-AeSCP-2 antibody (Lan and Massey 2004). The goat anti-rabbit horseradish peroxidase conjugated secondary antibody (Jackson ImmunoResearch Laboratory, West Grove, PA) was used at 1:2000 dilution. The ECL Western blot substrate (Pierce) was used to visualize the bound antibodies. The blots were placed in a cassette and pressed against the film for 30 min.

**Cellular Cholesterol Assay.** Aag2 or Huh7.5 cells were seeded in 24-well plates overnight and treated with DMSO or SCPI-1 for 48 h as described above. The cells were washed three times with 1 ml PBS in the well, then were dislodged and transferred to a 1.5-ml centrifuge tube and briefly spun at 800 × g to decant the extra PBS. Cellular cholesterol was extracted with 0.5 ml hexane/isopropanol (hexane/isopropanol = 3:2). The lipid extract and protein pellets were separated by centrifugation at 10,000 × g. The liquid phase was transferred to a new tube, air-dried overnight in the fume hood, and re-dissolved in 0.5 ml reaction buffer (0.1 M potassium phosphate, 0.05 sodium chloride pH = 7.4, 5 mM cholic acid, 0.1% Triton X-100). The total cholesterol concentration was determined using Amplex red cholesterol assay kit (Life technologies) following the manufacturer’s instruction. A standard curve was calculated each time based on the cholesterol standards on the same 96-well plate with samples. The protein pellets were dissolved in 0.2 M KOH at 65°C for 2 h and the soluble protein concentration was determined using BCA assay (Pierce, Rockford, IL).

**Staining for Cellular free Cholesterol and Lipid Droplets.** Cells were grown on sterile coverslips in six-well plates overnight, followed by 10 μM SCPI-1 treatment and/or virus infection (MOI = 10). To detect the cellular free cholesterol, the coverslips were washed three times with 2 ml PBS and fixed in 4% paraformaldehyde for 30 min. The coverslips were washed again with PBS and incubated in 1.5 mg/ml glycine solution for 10 min, and then stained with 50 μg/ml Filipin complex in PBS for 1 h. To visualize the lipid
droplets, the cells with the same treatments were fixed in 10% formalin in PBS for 30 min. The coverslips were washed briefly in distilled water and 60% isopropanol, and then stained with filtered 1.8 mg/ml Oil Red O (ORO) in 60% isopropanol for 30 min. The excess ORO was removed by washing several times with distilled water. The nuclei were stained using 300 nM DAPI dihydrochloride (Sigma) in PBS for 10 min. After staining, the coverslips were washed with distilled water and mounted to a glass slide using fluoromount (Sigma). Photos were taken using Leica (Solms, Germany) DMRB fluorescent microscope with N2.1 and I3 filter.

**Statistical Analyses.** GraphPad Prism v4.0 software was used for statistical analyses. P values were obtained from a two-tailed unpaired Student t-test.

**Results**

**SCPI-1 Suppresses DENV Viral Production in Mosquito Aag2 Cells.** SCPI-1 was one of the Aedes SCP-2 (AeSCP-2) protein-specific inhibitors identified previously, and the affinity of SCPI-1 to AeSCP-2 protein is comparable with that of cholesterol (Kim et al. 2005). The Aag2 cells were pretreated by SCPI-1 and infected by DENV. The result showed that SCPI-1 at 1 μM was able to cause a 10-fold decrease (P = 0.005) in infectious viral particles produced 48 h post-infection (Fig. 1A). More importantly, in the presence of 10 μM of SCPI-1, the infectious viral particle in the medium was below the detection limit of TCID₅₀ assay (Fig. 1A), dropping by more than 6 logs. The suppressed virus production in SCPI-1-treated cells is not due to cytotoxicity of SCPI-1, as the viability of Aag2 cells was not affected by SCPI-1 at 10 μM (Fig. 1B). The 50% cytostatic concentration (CC₅₀) was 22.2 μM, which is similar to its LC₅₀ to mosquito fourth-instar larvae (Larson et al. 2008). Order-of-addition experiments with SCPI-1 demonstrated that exposure of Aag2 cells to SCPI-1 only prior to viral infection was not sufficient to inhibit viral replication and production, but suggests that SCPI-1 does act on an early step in the viral life cycle (Supp Fig. 1 [online only]).

**AeSCP-2 Protein is Critical for DENV Production in Aag2 Cells.** Although SCPI-1 was able to suppress viral production (Fig. 1A), it was still uncertain whether its antiviral effect was owing to inhibiting AeSCP-2 protein or other non-target effects. Aag2 cells express SCP-2 endogenously (Lan and Massay 2004), but DENV infection did not up-regulate SCP-2 mRNA level in Aag2 cells (data not shown). To validate the role of AeSCP-2 protein in viral infection, we transiently over-expressed or knocked down the AeSCP-2 expression in Aag2 cells, as described (Lan and Massay 2004). The over-expression/knock down was verified by examination at the protein level 48 h post transfection (Fig. 2A). Then we examined its impact on infectious viral particle production. There was no significant increase (P = 0.62) in the viral particles produced in AeSCP-2 over-expressing Aag2 cells compared with the siGFP control (Fig. 2B), indicating that the amount of endogenous AeSCP-2 was sufficient to support DENV propagation in Aag2 cells and additional heterogeneous AeSCP-2 did not enhance DENV propagation. In the Aag2 cells transfected with siRNA against AeSCP-2 gene, there was a significant decrease (P < 0.001) of viral particle production by approximately 100-fold (Fig. 2B, siGFP vs siSCP2). Indeed, the virus production was affected (Fig. 2B) when the SCP-2 protein level was lowered (Fig. 2A). Therefore, the results confirmed that AeSCP-2 is an important host factor in the DENV production in Aag2 cells, although over-expression of AeSCP-2 itself did not enhance the viral propagation.

**SCPI-1 has no Effect on VSV Production in Aag2 Cells.** To further investigate whether functional SCP-2 is required for production in mosquito host cells
by other arboviruses, VSV, an enveloped virus in the Rhabdoviridae family, was used to infect SCPI-1-treated Aag2 cells. VSV replicates in mosquito cells (Phalen and Kielian 1991, Marquardt et al. 1993). VSV-DsRed-Express that has similar infectivity as the wild-type VSV (Swick et al. 2014) readily replicates in Aag2 cells, producing high levels of infectious virus particles 24 hours post-infection (Fig. 3, DMSO control). Further, SCPI-1 treatments had minimal effects on VSV production in Aag2 cells, with merely a 3% reduction in virus titer at 10 \( \mu \text{M} \) SCPI-1 concentration (Fig. 3). These results suggest that VSV production in Aag2 cells does not require functional AeSCP-2, which is in sharp contrast to DENV-2 (Fig. 1A) and provides further evidence that 10 \( \mu \text{M} \) SCPI-1 is not toxic to mosquito cells generally but rather specifically blocks DENV-2 production. These results are also consistent with previous studies that show that manipulating cellular cholesterol had little effect on VSV production in mosquito cells (Phalen and Kielian, 1991, Marquardt et al. 1993).

**Human SCP-2 is not Critical for DENV Infection in Human Huh7.5 Cells.** SCP-2 is a ubiquitous protein found in nearly all mammalian cells examined (Gallegos et al. 2001). Mammalian SCP-2 plays a major role in cholesterol homeostasis through uptake and transhepatocyte transfer (Moncecchi et al. 1996, Atshaves et al. 2009). As Human SCP-2 protein (HsSCP-2) shared the similar function as an intracellular cholesterol transporter (Krista et al. 2010) as that of mosquito SCP-2 (Lan and Massey 2004), it is of interest to investigate if it is also an important human host factor in DENV production. The HsSCP-2 was expressed endogenously in the hepatocytes’ Huh7.5 cells, and predesigned siRNA was able to effectively knock down the HsSCP-2 expression (Fig. 4A). In vitro studies showed that SCPI-1 was an effective inhibitor of HsSCP-2 protein (Kriska et al. 2010). However, neither siRNA nor 10 \( \mu \text{M} \) SCPI-1 was able to suppress DENV production in Huh7.5 cells (Fig 4B). The CC\textsubscript{50} of SCPI-1 was 14.6 \( \mu \text{M} \) in Huh7.5 cells (lower than in insect cells), and the cell viability was not affected at 10 \( \mu \text{M} \) (Fig. 4C). It seemed that HsSCP-2 did not play the same role in DENV production in Huh7.5 cells as that of the AeSCP-2 in mosquito Aag2 cells (Fig. 2B), despite having cellular toxicity at a lower drug concentration.

**SCPI-1 Treatment Reduced Cellular Cholesterol Levels in Both Human and Mosquito Cells.** Under normal growth conditions, cells can acquire cholesterol in the cholesterol-rich medium (\( \sim 300 \mu\text{g/ml} \) in FBS according to the manufacturer). SCP-2 protein facilitates the cholesterol uptake (Moncecchi et al. 1996, Lan and Massey 2004). SCPI-1 treatment reduced \(^{3}H\) cholesterol incorporation in Aag2 cells cultured in sterol-free serum medium (Kim et al. 2005). Here we performed the experiment under normal growth conditions and measured the total...
cellular cholesterol levels. When the SCP-2 was inhibited by 10 μM SCPI-1 treatment, there is a 24% decrease ($P < 0.001$) of the total cholesterol content in Aag2 cells and a 15% decrease ($P < 0.001$) in Huh7.5 cells (Fig. 5). Total cellular cholesterol levels were similar in both cell lines (Fig. 5, DMSO). Although SCPI-1 was able to significantly deplete cellular cholesterol levels in both cell lines, it only affected viral production in Aag2 cells (Fig. 2B vs Fig. 4B). It was also noticed that 1 μM SCPI-1 was able to suppress DENV propagation in Aag2 cells (Fig. 1A), whereas the total cellular cholesterol content was not affected at the same concentration of treatment (Fig. 5). The results suggest that lowering total cellular cholesterol alone may not be the underlying mechanism of SCP-2-dependent DENV propagation in Aag2 cells.

**SCPI-1 Treatment Altered the Intracellular Cholesterol Distribution Only in Aag2 Cells.** To investigate the mechanism in which SCPI-1 affects DENV production in mosquito host cells, we closely examined the distribution of cellular cholesterol in Aag2 cells. Filipin stain has been widely used to localize free cholesterol in various cells and tissues, as it does not recognize esterified cholesterol (Rudolf and Curcio 2009, Gimpl 2010). The infected or non-infected Aag2 cells were treated with either DMSO or 10 μM SCPI-1 for 48 h before fixing and staining. In the DMSO-treated control Aag2 cells, the plasma membrane and numerous perinuclear free cholesterol-rich compartments were stained with Filipin (Fig. 6A). When the cells were infected with DENV, the perinuclear free cholesterol-rich compartments were still visualized with bright Filipin staining (Fig. 6C), similar to non-infected cells (Fig. 6A vs C). The result indicates that DENV infection in Aag2 cells did not significantly alter the intercellular free cholesterol distribution. Interestingly, when treated with 10 μM SCPI-1, the intensity of Filipin staining in the perinuclear free cholesterol-rich compartments was significantly reduced, no matter whether the cells were infected with DENV or not (Fig. 6A vs D). The result suggests that SCP-2 may be involved in cellular cholesterol homeostasis via its...
cholesterol transport function. As siRNA against SCP-2 treatment suppressed DENV production in Aag2 cells (Fig. 2B), we speculate that SCP-2-mediated intracellular cholesterol trafficking is critical for DENV propagation in mosquito host cells.

The perinuclear free cholesterol-rich compartments have been reported in *Ae. albopictus* C6/C36 cells (Rolls et al. 1997). The nature of those free cholesterol-rich compartments is unknown, although they resembled the lipid droplet structures reported previously (Samsa et al. 2009). To investigate whether the perinuclear free cholesterol-rich compartments are lipid droplets, we stained the cells using ORO to localize the lipid droplets (Rudolf and Curcio 2009). The results showed that lipid droplets in Aag2 cells were perinuclear but were not as numerous as the perinuclear free cholesterol-rich compartments (Fig. 6E vs A). On the other hand, SCPI-1 treatment did not significantly affect the intensity of ORO staining of the lipid droplets (Fig. 6E vs F). The result is consistent
with the previous finding that the composition of lipid droplets are mainly neutral lipids including sterol esters (Walther and Farese 2012), which would not be stained by Filipin (Rudolf and Curcio 2009, Gimpl 2010). Our results indicated that the perinuclear free cholesterol-rich structures did not have the same composition as that of the lipid droplets. Moreover, inhibition of SCP-2 function had a more pronounced effect on free cholesterol content in perinuclear free cholesterol-rich compartments than that of the esterified lipids in the lipid droplet. Our study suggested that SCPI-1 treatment somehow disrupted the storage of free cholesterol in the perinuclear compartments or the homeostasis of cellular cholesterol distribution.

We also examined the free cholesterol distribution and lipid droplets in non-infected human Huh7.5 cells. Interestingly, perinuclear free cholesterol-rich compartments were not present in Huh7.5 cells (Fig. 7A vs Fig. 6A). SCPI-1 treatment did not significantly affect the free cholesterol distribution (Fig. 7B vs A). There were substantial amounts of lipid droplets in Huh7.5 cells, but their lipid contents were not significantly affected by SCPI-1 treatment, as the ORO staining intensities were similar under both conditions (Fig. 7C vs D).

Results from our study suggested that intracellular cholesterol distribution was not altered by SCPI-1 in Huh7.5 cells, although high concentration of SCPI-1 did reduce cellular cholesterol levels in Huh7.5 cells (Fig. 5B).

Discussion

Production of enveloped viruses including DENV in mammalian cells involves both de novo cholesterol synthesis (Mackenzie et al. 2007, Rothwell et al. 2009) and intracellular transport of cholesterol (Poh et al. 2012). Previously though, DENV production in mosquito cells has been characterized as cholesterol-independent relative to alphaviruses (Umashankar 2008). Here we show that disruption of the cholesterol trafficking pathway mediated by SCP-2 in mosquito, but not human, cells lowers DENV production. Mosquito host cells are auxotrophic for cholesterol, which provide us an optimal model to study the role of intracellular cholesterol trafficking for DENV production. This highlights an interesting and unanswered question of whether males and pre-bloodmeal females are less competent for DENV production in part because cholesterol is limited, or
perhaps a plant sterol can suffice (Martins et al. 2012, Buckner et al. 2013). We focused on the function of mosquito SCP-2, given that the cholesterol transport function of SCP-2 has been demonstrated in Aag2 cells (Lan and Massey 2004, Radek et al. 2010) and in Aedes aegypti mosquito (Blitzer et al. 2005, Dyer et al. 2008). Our study is the first report of a sterol transport protein, AeSCP-2, as a mosquito host factor for DENV propagation in mosquitoes. When the SCP-2 function was inhibited, production of DENV viral particles was impaired, while that of a different enveloped RNA virus was not.

AeSCP-2 protein does not appear to be an essential host factor for DENV propagation in human cells. To understand the difference between Aag2 cells and Huh7.5, we examined the total cellular cholesterol level after SCPI-1 treatment in both. The total cholesterol was significantly reduced by 10 mM SCPI-1 in both types of cells. SCPI-1 was less effective in reducing mammalian Huh7.5 cellular cholesterol level, possibly owing to its ability to synthesize cholesterol de novo. However, cellular cholesterol depletion as the result of SCPI-1 treatment could not fully explain the antiviral effect in mosquito host cells. First of all, 1 mM SCPI-1 did not change the total cellular cholesterol level, but it did affect DENV production. Second, DENV propagation was not hampered in Huh7.5 cells, despite the total cholesterol being reduced by 15%. Third, pre-infection treatment with SCPI-1 did not affect DENV production as much as that of post-infection treatment in mosquito host cells, which is different from the cholesterol depletion drug methyl-beta-cycloedextrin that is most effective in suppressing DENV infection by pre-treating mammalian cells (Lee et al. 2008, Medigeshi et al. 2008). Therefore, the antiviral effect of SCPI-1 may not be solely due to cellular cholesterol depletion. SCPI-1 treatment altered the cellular free cholesterol distribution in either infected or non-infected Aag2 cells but not in Huh7.5 cells. The results suggest that in mosquito host cells, the SCP-2-mediated cholesterol trafficking pathway is critical for DENV production. There are many differences in viral replication in mosquito cells versus human cells (Vasilakis et al. 2009), but to our knowledge, ours is the first report showing that disrupting cholesterol pathways in mosquito cells limits DENV. While both siRNA and small molecule inhibitors can have cascading or secondary effects, here both approaches support that cholesterol pathways are important for DENV replication in mosquito cells.

We speculate that DENV utilizes different cholesterol trafficking pathways in two different host cells. In mammalian cells, inhibition of NPC-mediated intracellular transport pathway leads to suppression of DENV propagation during initial infection as well as post-infection (Poh et al. 2012). Moreover, studies found that the cholesterol containing lipid droplets were colocalized with DENV viral protein in BHK cells (Samsa et al. 2009). A more recent study using Huh7.5 cells revealed that the amount of lipid droplets remained the same post-DENV infection, while the total lipid droplet volume decreased (Heaton and Randall 2010), indicating lipid droplets were consumed during viral infection (Heaton and Randall 2010, Pena and Harris 2012). Over-expression of SCP-2 in mouse intestine L. cells alters the lipids and cholesterol composition of lipid droplets (Atshaves et al. 2001). The subcellular localization of human SCP-2 and mosquito SCP-2 is quite different, even though AeSCP-2 and HsSCP-2 share 31% identity in amino acid sequences (Krebs and Lan 2003). In Aag2 cells, over 90% of the SCP-2 protein was localized to the cytosol (Lan and Massey 2004), whereas the mammalian SCP-2 localizes largely (>50%) to the peroxisome, ER, and mitochondria (Gallegos et al. 2001). Therefore, the HsSCP-2 trafficking pathway might not be critical for the viral infection in human host cells. Rather, the release of free cholesterol from late endosomes mediated via the NPC transport pathways may play a more important role in human cells (Poh et al. 2012).

Stored cellular cholesterol and/or cholesterol trafficking may be more important in DENV production in mosquito than human cells, as mosquitoes lack de novo cholesterol synthesis. Interestingly, about 50–90% of cellular cholesterol in Aag2 cells is unesterified cholesterol (data not shown) and there are not many lipid droplets/cell (Fig. 6E). The majority of cellular free cholesterol was stored in the perinuclear compartment, as reported in mosquito C6/36 cells (Rolls et al. 1997). Possibly, the mosquito SCP-2 protein is involved in loading the cells with free cholesterol to the perinuclear compartment, which is essential for DENV production in mosquito host cells. Whether DENV replication complex localizes to the perinuclear free cholesterol-rich compartments needs further investigation. Although SCP-2 has moderate affinity toward fatty acid (Gallegos et al. 2001, Vyazumova et al. 2007), SCPI-1 specifically impacted cellular free cholesterol distribution in Aag2 cells, because lipid contents of lipid droplets were not significantly affected by SCPI-1 treatment. Therefore, our studies support other data that DENV replication in mosquito and mammalian cells have different requirements.

Supplementary Data
Supplementary data are available at Journal of Medical Entomology online.

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