The Composition of West Nile virus Lipid Envelope Unveils a Role of Sphingolipid Metabolism on Flavivirus Biogenesis

Running title: Sphingolipids and flavivirus biogenesis

Miguel A. Martín-Acebes,a Teresa Merino-Ramos,b Ana-Belén Blázquez,b Josefina Casas,c Estela Escribano-Romero,b Francisco Sobrino,a,# and Juan-Carlos Saizb,#

aDepartment of Virology and Microbiology, Centro de Biología Molecular “Severo Ochoa” (CSIC-UAM), Madrid, Spain; bDepartment of Biotechnology, Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), Madrid, Spain; cDepartment of Biomedicinal Chemistry, Institute for Advanced Chemistry of Catalonia (IQAC-CSIC), Barcelona, Spain

Abstract word count: 235

Text word count: 6,958

#Address correspondence to Francisco Sobrino, fsobrino@cbm.csic.es; Juan-Carlos Saiz, jcsaiz@inia.es

M.A.M.-A. and T.M.-R. contributed equally to this article.

F.S. and J.-C.S. are joint senior authors on this work.
West Nile virus (WNV) is an emerging zoonotic mosquito-borne flavivirus responsible for outbreaks of febrile illness and meningoencephalitis. The replication of WNV takes place on virus-modified membranes from the endoplasmic reticulum of the host cell and virions acquire their envelope by budding into this organelle. Consistent with this view, the cellular biology of this pathogen is intimately ligated to modifications of the intracellular membranes, and the requirement of specific lipids such as cholesterol and fatty acids has been documented. In this study, we evaluated the impact of WNV infection on two important components of cellular membranes, glycerophospholipids and sphingolipids, by mass spectrometry of infected cells. A significant increase in the content of several glycerophospholipids (phosphatidylcholine, plasmalogens and lysophospholipids) and sphingolipids (ceramide, dihydroceramide and sphingomyelin) was noticed in WNV-infected cells, suggesting functional roles of these lipids during WNV infection. Furthermore, the analysis of the lipid envelope of WNV virions and recombinant virus-like particles revealed a unique composition of their envelopes that were enriched in sphingolipids (sphingomyelin) and showed reduced levels of phosphatidylcholine, in a manner similar to that of sphingolipid enriched lipid microdomains. Inhibition of neutral sphingomyelinase (which catalyzes the hydrolysis of sphingomyelin into ceramide), either by pharmacological approaches or siRNA mediated silencing, reduced the release of flavivirus virions as well as virus-like particles, suggesting a role of sphingomyelin to ceramide conversion in flavivirus budding and confirming the importance of sphingolipids in the biogenesis of WNV.
West Nile virus (WNV) is a neurotropic flavivirus spread by mosquitoes that can infect multiple vertebrate hosts including humans. There is no specific vaccine or therapy licensed for human use against this pathogen. Since the multiplication of this virus is associated to rearrangements of host cell membranes, we analyzed the effect of WNV infection on different cellular lipids that constitute important membrane components. Multiple lipid species were increased in infected cells, pointing to major alterations of cellular lipid metabolism induced by WNV infection. Interestingly, certain sphingolipids, which were increased in infected cells, were also enriched in the lipid envelope of the virus, thus suggesting a potential role during virus assembly. We further verified the role of sphingolipids in the production of WNV by means of functional analyses. This study provides new insight in the formation of flavivirus infectious particles and the involvement of sphingolipids in the WNV life cycle.
INTRODUCTION

West Nile virus (WNV) is a worldwide distributed mosquito-borne flavivirus responsible for recurrent outbreaks of febrile illness and encephalitis. The virus maintains in nature into an enzootic infectious cycle between birds and mosquitoes that act as its vectors, although it can also infect multiple vertebrate hosts, including horses and humans (1, 2). The continuing spread of WNV due to a variety of ecological factors, combined with the lack of specific therapeutics or vaccines for human use, make the identification of viral and host processes that control the biology of this pathogen important to improve the design of specific antiviral strategies (3).

As a flavivirus, WNV is an enveloped plus-strand RNA virus (1, 2). A common feature shared with other plus-strand RNA viruses is that WNV virus replication takes place in the cytoplasm of infected cells tightly associated with intracellular membrane rearrangements (4). In the case of flaviviruses, and hence WNV, the membranes associated with virus replication are from the endoplasmic reticulum (ER) (5). Even more, the assembly of flavivirus particles is produced by invagination and budding of the ER membrane into the lumen of this organelle, so the ER also provides the membrane source for WNV envelopment (6). Although the lipid composition of the viral envelopes of a few distinct viruses has been characterized (7-10), to our knowledge, the molecular composition of the flavivirus envelope has not been analyzed. Only the presence of certain lipids in the flavivirus envelope (cholesterol and phosphatidylyserine) has been identified by functional studies rather than by analytical approaches (11, 12).

Lipids are the main components of cellular membranes, playing key roles in viral infections by acting as signalling molecules, as well as by determining physical
properties of the membranes such as fluidity, thickness or shape (13, 14). In fact, a strong manipulation of cellular lipid metabolism by different viruses has recently been documented (15-17). Accordingly, enrichment in specific lipids contributes to the generation of the adequate membrane curvature for the correct replication complex assembly or virus budding, being of remarkable interest the cone shaped or inverted cone shaped lipids, which can govern membrane bending, budding processes, or fusion events (18-20). Whereas differences on lipid requirements between viruses of the same family have been noticed (21), the dependence on certain lipids such as fatty acids (15, 22, 23) or phosphatidylinositol 4-phosphate (PI4P) (21, 24), can be shared by unrelated enveloped and non-enveloped viruses. These observations suggest that each virus creates its own characteristic cellular microenvironment for replication, by developing specialized virus-induced organelle-like structures within infected cells (4, 24, 25).

Regarding flaviviruses, major rearrangements of cellular lipid metabolism have been observed in Dengue virus (DENV) infected cells (26, 27), and several studies have highlighted the importance of both fatty acids and cholesterol for WNV and DENV infection (22, 28-31), as well as a lack of requirement for PI4P (21, 29).

In this study we have analyzed the effect of WNV infection on the cellular content of 11 lipid classes. Our results revealed an increase in the cellular content of multiple lipid species upon WNV infection, which is consistent with major lipid metabolic changes in WNV-infected cells. The proportions of these metabolites in the envelope of WNV virions and virus-like particles were also determined, unveiling a unique composition of the viral envelope and pointing to specific roles of some of these lipids for virus assembly. Supporting this view, a direct link between specific lipids (sphingolipids) and WNV biogenesis was found by means of functional analyses.
MATERIALS AND METHODS

Cells, viruses, infections and virus titrations. All infectious virus manipulations were performed in our biosafety level 3 (BSL-3) facilities. The origin of WNV strain NY99, USUV strain SAAR-1776, and SINV has been previously described (32). Vero and HeLa cells were grown at 37°C in a 5% CO2 atmosphere in Dulbecco’s modified minimum essential medium (DMEM) supplemented with 2 mM glutamine, penicillin-streptomycin and 5 or 10% fetal bovine serum, respectively. A HeLa cell line stably transfected with plasmid pcDNA 3.1 (+) (Invitrogen, Carlsbad, CA) encoding the 25 last amino acids of the WNV-NY99 C protein followed by prM-E tandem (HeLa3-WNV) was obtained by limiting dilution and grown in complete culture medium supplemented with 500 µg/ml G-418 (Merino-Ramos et al. submitted). This line constitutively secreted WNV recombinant subviral particles (RSPs). C6/36 mosquito cells were cultured in M3 medium supplemented with 4 mM glutamine, gentamicin, penicillin-streptomycin, non-essential amino acids, amphotericin B, and 10% fetal bovine serum at 28°C without supplying CO2. For infections in liquid medium, the viral inoculum was incubated with cell monolayers for 1 h at the cell growth appropriated temperature and, then, the inoculum was removed and fresh medium, containing or not 1% fetal bovine serum, was added. Viral titer was determined 24 h post-infection (p.i.) for WNV or USUV, and 8 h p.i. for SINV, by plaque assay on Vero cells (29, 32, 33). Cell associated virus was extracted from infected cells by three cycles of freeze and thaw and titrated as described above. The multiplicity of infection (MOI) used in each experiment was expressed as plaque forming units (PFU)/cell and is indicated in the corresponding figure legend.
Antibodies. Mouse monoclonal antibodies against WNV E protein (Millipore; Temecula, CA), β-actin (Sigma; St. Louis, MO) and GM130 (ECM Biosciences; Versailles, KY), and rabbit sera against WNV M protein (Imgenex; San Diego, CA), calnexin (StressMarq Biosciences Inc.; Victoria, Canada), mannosidase II (Millipore), and nSMase2 (ECM Biosciences) were used as primary antibodies. Anti-mouse or anti-rabbit IgG coupled to Alexa Fluor 594 or 488 (Invitrogen; Carlsbad, CA), and goat anti-mouse or anti-rabbit IgG coupled to horseradish peroxidase (Pierce Biotechnology; Rockford, IL), were used as secondary antibodies.

Drug treatments. Brefeldin A (BFA), Golgicide A (GCA), GW4869 and glutathione were from Sigma. Spiroepoxide was from Santa Cruz Biotechnology (Santa Cruz, CA). BFA, GCA, GW4869, and spiroepoxide stock solutions were prepared in DMSO, while glutathione was directly dissolved in culture medium. Working concentrations of GW4869 were prepared as described (34). Control cells were treated in a parallel manner with the same amount of drug vehicle. For estimation of RSP release, the medium from HeLa3-WNV cells was replaced by serum free medium containing the drugs. Cells were incubated 4 h and the amount of RSPs released to the culture medium was determined by enzyme linked immunodot assay using a monoclonal antibody directed against WNV E glycoprotein as described below. In the case of virus infections, drugs were added after the first hour of infection, when viral inoculum was replaced by medium containing 1% fetal bovine serum. The lack of toxicity of drug concentrations during the assays was evaluated by determination of cellular ATP content with CellTiter-Glo Luminiscent Cell Viability Assay (Promega, Madison, WI).

siRNA experiments. HeLa, or HeLa3-WNV, cells were transfected in serum free culture medium with 100 nM endoribonuclease-prepared siRNAs (esiRNAs) against
human SMPD3 (MISSION, Sigma) or an equivalent amount of MISSION siRNA Universal Negative control #1 (MISSION, Sigma), using siRNA Transfection Reagent (MISSION, Sigma) as indicated by the manufacturer. To estimate the release of WNV RSPs, the culture medium of HeLa3-WNV cells was replaced by fresh serum free medium, and the amount of RSPs in the culture medium was determined after 4 h of incubation by an enzyme-linked immunodot assay. For viral infections, transfected cells were infected (MOI of 1 PFU/cell) as described for infections in liquid medium and subsequent experiments were performed at 48 h post-transfection. The extent of siRNA silencing was analyzed by Western blot (29, 35) using specific antibodies against nSMase2.

Purification of RSPs and virions. HeLa3-WNV cells were incubated 48 h in serum free medium (to avoid possible interference of serum with subsequent lipid determinations) and RSPs were purified by sucrose gradient centrifugation, essentially as described (36). Briefly, culture medium from HeLa3-WNV cells was clarified at 15000 × g for 30 min at 4 °C and then centrifuged through a 20% sucrose cushion for 3.5 h at 112000 × g at 4º C. The pellet containing RSPs was resuspended in PBS, loaded onto a 12 ml 20-60% lineal sucrose gradient and centrifuged at 256000 × g for 18 h at 4º C. For purification of WNV particles, HeLa cells were infected with WNV (MOI of 5 PFU/cell) in serum free medium and incubated for 48 h. Culture medium was cleared from cell debris by centrifugation at 850 × g for 15 min at 4º C, and centrifuged through a 20% sucrose cushion at 112000 × g for 3.5 h at 4º C. The pellet containing virions was loaded onto a six-step discontinuous 20-60% sucrose gradient and centrifuged at 55500 × g for 18 h at 4º C. Gradients were fractioned from the top and the E protein content of each fraction was determined by enzyme-linked immunodot assay as described below. Only the fraction with the highest E protein content was selected for lipid analyses.
Immunofluorescence and confocal microscopy. Cells grown on glass coverslips were fixed with 4% paraformaldehyde in PBS for 15 min, and processed for immunofluorescence as described (29, 35). Cells were observed using a Leica TCS SPE confocal laser scanning microscope using an HCX PL Apo 63×/1.4 oil immersion objective. Images were acquired using Leica Advanced Fluorescence Software and processed with Adobe Photoshop CS2 (Adobe Inc; San Jose, CA). Optical slice thickness for all confocal images displayed was of 1 airy unit.

Transmission electron microscopy. Negative staining of RSPs was performed in dialyzed samples containing the peak of E protein within the gradient as described (36). For immunolabelling and negative staining, samples were adsorbed to ionized collodion-carbon coated grids, washed with PBS, and blocked with 10% fetal bovine serum in PBS for 5 min. Grids were incubated with primary antibodies diluted in 5% fetal bovine serum in PBS for 30 min, washed five times with PBS, and incubated with protein A coupled to 5 nm colloidal gold diluted in 5% fetal bovine serum in PBS for 30 min. Samples were then fixed with 1% glutaraldehyde in PBS for 5 min, washed three times with bidistilled water and negatively stained with 1% uranyl acetate for 2 min. HeLa or Vero cells infected with WNV were fixed in 4% paraformaldehyde-2% glutaraldehyde in 0.1 M phosphate buffer pH 7.4 at 24 h p.i. and processed for electron microscopy as described (29, 35). Samples were examined using a Jeol JEM-1010 electron microscope (Jeol, Japan) operated at 80 kV and images were acquired using a digital camera 4K64K TemCam-F416 (Tietz Video and Image Processing Systems GmbH; Gauting, Germany).

Enzyme-linked immunodot assay. Cell culture medium (usually 5-10 µl) was adsorbed to a nitrocellulose membrane by vacuum using a Bio-Dot apparatus (Bio-Rad;
Hercules, CA). For quantification of the amount of RSPs in the culture medium, and in
order to verify that samples were within the lineal range, a standard curve using
different dilutions was performed. Membrane was blocked with 3% skimmed milk in
PBS and incubated with monoclonal anti-E diluted in 1% skimmed milk in PBS. After
three washes with PBS, membranes were incubated with secondary antibodies coupled
to horseradish peroxidase, washed and proteins were detected by chemiluminescence
using an ImageQuant LAS 4000 mini equipment (GE Healthcare; Buckinghamshire,
United Kingdom).

**Quantitative RT-PCR.** Viral RNA was extracted with NucleoSpin viral RNA isolation
kit (Macherey-Nagel; Düren, Germany). The amount of viral RNA copies was
determined by quantitative RT-PCR (37) as genomic equivalents to PFU/ml by
comparison with RNA extracted from previously titrated samples (38).

**Lipid analysis.** HeLa cells were infected, or not (mock), with WNV (MOI of 50
PFU/cell) in serum free medium, as described for infections in liquid medium, detached
from the flasks, and resuspended in PBS at 24 h p.i. The number of cells in each sample
was determined, and aliquots containing 2.7 × 10⁶ cells were subjected to lipid
extractions. The amount of proteins in each sample was also determined by Bradford
assay. Lipids were extracted using a modified Bligh and Dyer protocol. Cell pellets
were resuspended in 100 µl of PBS and 750 µL of a methanol: chloroform (1:2, v/v)
solution containing 0.01% of BHT and internal standards (1,2-diheptadecanoyl-sn-
glycero-3-phosphocholine, 1,2-diheptadecanoyl-sn-glycero-3-phosphoethanolamine,
1,2-diheptadecanoyl-sn-glycero-3-phosphoserine, 1-heptadecanoyl-2-hydroxy-sn-
glycero-3-phosphocholine, 1-heptadecanoyl-2-hydroxy-sn-glycero-3-
phosphoethanolamine and 1-heptadecanoyl-2-hydroxy-sn-glycero-3-phosphoserine, 200
pmol each, from Avanti Polar Lipids). Samples were extracted at 48 °C overnight, centrifuged at 13000 g for 5 min. Then, the supernatant was transferred to a new vial, evaporated to dryness, stored at -20 °C in an argon atmosphere until the analysis of glycerophospholipids (39). On the other hand, 100 µl of PBS and 750 µL of a methanol: chloroform (2:1, v/v) solution containing 0.01% of BHT and internal standards (N-lauroyl-D-erythro-sphingosine, N-lauroyl-d-erythro-sphingosylphosphorylcholine, D-glucosyl-β-1,1’ N-lauroyl-D-erythro-sphingosine, 200 pmol each, from Avanti Polar Lipids) were added to the cell pellets. Samples were extracted at 48 °C overnight, cooled, 75 µl of 1M KOH in methanol were added and incubated 2h at 37 °C. Following addition of 75 µl of 1M acetic acid, samples were centrifuged at 13000 g for 5 min and the supernatant was transferred to a new vial, evaporated to dryness and stored at -20 °C in an argon atmosphere until the analysis of sphingolipids (40).

Lipids were measured with an Acquity UPLC system (Waters, USA) connected to a Time of Flight (LCT Premier XE) Detector controlled with Waters/Micromass MassLynx 4.1 software. An Acquity UPLC BEH C8 column (1.7 µm particle size, 100 mm × 2.1 mm, Waters, Ireland) at a flow rate of 0.3 ml/min and column temperature of 30 °C was used. The mobile phase was methanol with 1 mM ammonium formiate and 0.2% formic acid (A)/water with 2 mM ammonium formiate and 0.2% formic acid (B). Gradient elution started at 80% of A, increased to 90% A in 3 min, held for 3 min, increased to 99% A in 9 min and held for 3 min. Initial conditions were attained in 2 min and the system was stabilized for 3 min. The acquisition range of the TOF detector was m/z 50 to 1500, the capillary voltage was set to 3.0 kV, the desolvatation temperature was 350 °C and the desolvatation gas flow 600 L/hr (41).
Lipid identification was confirmed by the analysis of one sample from each group with an UHPLC system (Accela) coupled to a Thermo Fischer Scientific LTQ Orbitrap Velos controlled with Thermo Fischer Scientific/Xcalibur software using the same column eluted with the following conditions: Gradient elution started at 85% of A, increased to 90% A in 9 min, held for 2 min, increased to 99% A in 6 min and held for 2 min. Initial conditions were attained in 2 min and the system was stabilized for 3 min. The acquisition range of the Orbitrap detector was m/z 200 to 100, the source voltage was set to 3.5 kV, the capillary temperature was 350 ºC, the sheath gas flow was 50 l/hr, the auxiliary gas flow 20 l/hr and the sweep gas flow 2 l/hr (42).

Individual chromatographic peaks of distinct lipid species were isolated from full scan MS spectra when selecting their theoretical exact masses, extracted from the database previously generated using the spectrum simulation tool of Micromass MassLynx software which was based on our previous results (39). Then, a list of possible candidates fitting the specific exact mass was generated using formula determination tools (Elemental composition search) of Micromass MassLynx software. The elemental number was restricted to include C, H, O, N and P. The formula constraints were C, H, O ≥ 1, P ≥ 0 and N ≥ 1, following the nitrogen rule. The number of double-bond equivalents (DBEs) was set between -0.5 and 15.0. The search was based on single mass analysis and only considered the m/z value of the monoisotopic peak. Positive identification of the lipids was based on the accurate mass measurement with an error < 5 ppm, a low i-Fit parameter in the spectrum window and its LC relative retention time, compared to that of the standard (±2%) (39). The following lipids from Avanti Polar Lipids were used: 1,2-diheaxadecanoyl-sn-glycero-3-phosphocholine, 1,2-dioctadecanoyl-sn-glycero-3-phosphocholine, 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phosphocholine, 1-hexadecanoyl-2-(4Z,7Z,10Z,13Z,16Z,19Z-docosahexaen...
oyl)-sn-glycero-3-phosphocholine, 1-(9Z-octadecenoyl)-2-hydroxy-sn-glycero-3-
phosphocholine, 1-(1Z-octadecenyl)-2-(9Z-octadecenoyl)-sn-glycero-3-
phosphocholine, 1-octadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-
phosphoethanolamine, 1-hexadecanoyl-2-(9Z-octadecenoyl)-sn-glycero-3-phospho-L-
serine, N-hexadecanoyl-D-erythro-sphingosine, N-hexadecanoyl-D-erythro-
sphingosylphosphorylcholine, D-glucosyl-ß-1,1’N-hexadecanoyl-D-erythro-sphingosine
In addition, identifications of lipid were made by searching their molecular weights
against entries in the Lipid MAPS database. As commented above, this identification
procedure has been confirmed by the analysis of selected samples using Thermo Fischer
Scientific Orbitrap (resolution 30000 FWHM) analyzers (42).

Data presented are product of 4 to 5 independent lipid extractions and
determinations. Annotation of lipid species: Glycerophospholipids were annotated as
<l lipid subclass> <total fatty acyl chain length>:<total number of unsaturated bonds>.
Plasmalogen were annotated as above, except that <p> was added. Sphingolipids were
annotated as <lipid subclass> <total fatty acyl chain length>:<total number of
unsaturated bonds>. If the sphingoid base residue was dihydrosphingosine the lipid
class contained a <dh> prefix.

Data analysis. Analysis of the variance (ANOVA) was performed with statistical
package SPSS 15 (SPSS Inc, Chicago IL) applying Bonferroni’s correction for multiple
comparisons. Nonparametric comparisons were performed using the statistical package
GraphPad PRISM v.2.01 (GraphPad Software Inc.; La Jolla, CA). Data are presented as
mean ± standard deviation (SD). Statistically significant differences were considered at
P<0.05. One asterisk (*) or two asterisks (**) in the figures denote statistically
significant differences with P<0.05 or P<0.005, respectively.
RESULTS

Alteration of lipid content in WNV-infected cells. To analyze the effect of WNV infection on the lipid content of the host cell, HeLa cells were infected, or not, at a high multiplicity of infection (MOI) and processed for lipid analysis 24 h p.i. At this time point, infectious virus release to the culture medium was already detected (Fig. 1A) and, when observed by transmission electron microscopy, cells exhibited intracellular membrane rearrangements associated with flavivirus replication and assembly, such as membrane sacs containing small virus-induced vesicles referred to as vesicle packets (VPs) (5, 29, 43), as well as electron dense virions (Fig. 1B). As expected, these rearrangements were not observed in control uninfected cells. Eleven lipid classes [seven glycerophospholipids (GPLs) and four sphingolipids (SLs)] were included in the analysis. The GPLs analyzed were phosphatidylcholine (PC), 1-alkenyl,2-acylglycerophosphocholine [referred to as plasmalogen-PC (p-PC, plasmenylcholine)], 2-acylglycerol-3-phosphocholine (lyso-PC), phosphatidylethanolamine (PE), p-PE, lyso-PE and phosphatidylserine (PS). The four SLs analyzed were ceramide (CER), dihydro-CER (dhCER), sphingomyelin (SM) and dhSM. A tendency for an increase in the content per cell of most of the lipid classes analyzed (except for lyso-PE, a minor lipid class present in the extracts) was noticed in WNV-infected cells (Fig. 1C). In fact, statistically significant increases in the amount of PC (1.7 fold), p-PC (1.9 fold), lyso-PC (1.9 fold), p-PE (1.6 fold), CER (1.8 fold), dhCER (1.3 fold) and SM (1.4 fold) were noticed when infected cells were compared to uninfected cells. Among the eleven lipid classes analyzed, a total of 162 different molecular species were identified in HeLa cells (the complete list of molecular species identified in this and subsequent analyses is included in DATASET S1), being 54 (33.3%) significantly increased upon WNV infection (Fig. 1D). Interestingly, although a tendency for a reduction of some lipid
species was found, at the level of resolution of this analysis, no statistically significant
reduction of individual lipid species could be determined, so we assumed that no major
modifications in the level of the rest of lipid species occurred in infected cells. The
molecular species significantly increased in infected cells included 44 GPLs (13 PCs, 9
p-PCs, 3 lyso-PCs, 5 PEs and 14 p-PEs) and 10 SLs (5 CERs, 1 dhCERs and 4 SMs).
Therefore, WNV infection markedly alters the lipid metabolism of host cells.

**GPL and SL content of the envelope of flavivirus virions and recombinant subviral
particles (RSPs).** Lipid composition of viral envelopes can provide novel clues to
understand the biology of these pathogens (7, 9, 10). Therefore, we studied the GPL and
SL content of the viral envelope of WNV. Since the co-expression of flavivirus
structural glycoproteins [premembrane/membrane (prM/M) and envelope (E)] results in
the assembly and secretion of a sort of virus-like particles termed recombinant subviral
particles (RSPs) that provide a non-infectious a model system to study the assembly and
secretion of flavivirus particles (44, 45), the analysis of the lipid composition of RSPs
was included in this study. A stable HeLa clone (HeLa3-WNV) expressing WNV
structural glycoproteins (Fig. 2A) that constitutively secretes WNV RSPs of about 30
nm in diameter to the culture medium (Fig. 2B) was used as a source of RSPs. The
identity of these structures was confirmed by immunogold electron microscopy using an
antibody against the E glycoprotein (Fig. 2B inset). In this cell line, immunofluorescence and confocal analysis revealed that the WNV envelope
glycoprotein colocalized with markers of the ER (calnexin) and the Golgi complex
(manosidase II) (Fig. 2C and 2D). The molecular weights of the proteins were analyzed
by western blot using anti-E and anti-M antibodies (Fig. 2E). As expected, using anti-E
antibody, a single band [about 53 KDa (46)] was observed in samples containing
purified RSPs. In the case of anti-M antibody, two bands were observed, one
compatible with prM protein and other compatible with mature M protein [about 20 and 6 kDa, respectively (46)]. These results confirmed the correct expression of WNV glycoproteins by HeLa3-WNV cell line. Enzyme-linked immunodot assay is faster than Western blot and allows analyzing up to 96 samples at once. This kind of assay has been successfully applied to quantify the amount of RSPs released to culture medium by other flaviviruses (47). Thus, a dot blot assay was used to detect the WNV RSPs released to the culture medium from HeLa3-WNV cells (Fig. 2F). To further validate this methodology, the amount of RSPs released to the culture medium upon treatment with the Golgi disturbing agents Brefeldin A (BFA) or Golgicide A (GCA) was studied. Treatment of HeLa3-WNV cells with BFA or GCA impaired the release of RSPs to the culture medium (Fig. 3A). The quantification of the blots revealed that concentrations ≥5 µM BFA or GCA, significantly inhibited the release of RSPs to the culture medium without exerting noticeable toxic effects on the cells (Fig. 3B). Immunofluorescence analysis of BFA or GCA treated cells confirmed the disruption of the Golgi complex architecture that these drugs induced (Fig. 3C). Golgi disassembly was concomitant with the accumulation of E glycoprotein inside the cells (Fig. 3C), an observation consistent with a reduction in the release of RSPs into the culture medium observed in Fig. 3A and B. Overall, these observations are consistent with previous data showing that flavivirus RSPs, as well as infectious virions, are assembled by budding into the ER and then traffic through the secretory pathway (44, 45). Therefore, in the HeLa3-WNV model, the lipid envelope of RSPs is acquired from the ER in a parallel manner to that observed for infectious virus (6).

Hence, the lipid composition of the envelope from sucrose gradient purified WNV and RSPs was analyzed by mass spectrometry and compared to that of total cellular membranes of HeLa cells (Fig. 4A), following an approach similar to that
described for other viruses (7-10). The most abundant GPL in virions, RSPs and total cellular membranes was PC, although both RSPs and virions displayed significantly reduced contents of PC relative to the total cellular membranes (about 0.57 fold for both RSPs and virions). Among the SLs, a significant enrichment in SM was noticed in the envelope of RSPs and virions when compared to total cellular membranes (about 3.9 and 2.5 fold, respectively). These analyses also revealed that the lipid composition of both virions and RSPs was very similar for all lipid classes analyzed, except for p-PC that appeared enriched in virions when compared with both RSPs and total cellular membranes. This could be related to the differences in the size between virions (50 nm) and RSPs (30 nm), since vesicles formed with p-PC are bigger than those formed with PC (48).

From the 162 lipid species identified in total cellular membranes, about 95% and 93% were also detected in RSPs (155) and virions (152), respectively (DATASET S1). Significant differences between RSPs and virions were restricted to the proportion of 3 lipid species (lyso-PC 18:2, CER 18:0, and dhSM 24:0; representing only about 2% of the total species identified) (DATASET S1), supporting that the lipid envelope of RSPs and virions shared multiple common features. When compared to total cellular membranes, virions and RSPs displayed significantly altered proportions of 30 (19%) and 36 (24%) lipid species, respectively; being 15 of them significantly altered in both virions and RSPs (Fig. 4B). Among these common species altered, 5 corresponded to PE or its derivative p-PE [4 enriched (PE 32:1, p-PE 32:2, p-PE 34:3 and p-PE 36:3) and 1 reduced (PE 36:4)] and 6 to PC [34:1, 34:3, 36:2, 38:5, 38:6, and 44:3; all reduced]. Regarding SLs, enrichment in dhCER (24:0) and three SM species (16:0, 24:0 and 24:2) was noticed. When lipids enriched in both RSPs and virions (Fig. 4B) were compared with those increased in infected cells (Fig. 1D) only 5 matches were found (p-
Interestingly, three of these species were SLs, suggesting that the enrichment in infected cells of these molecular species could be related to an increasing demand of these SLs for the assembly of the viral envelope.

**Involvement of SM and CER in flavivirus biogenesis.** The SLs are derived from sphingosine, a long chain amino alcohol, which is acylated with a long chain fatty acid to give CER, which in turn is the central core of SM. These lipids are important components for membrane organization and shape, playing key roles in multiple cellular processes (49). Our analysis showed that different SLs (CER, dhCER, and SM) were increased in infected cells (Fig. 1C), particularly SM that was found enriched in the lipid envelope of both RSPs and virions (Fig. 4A). The hydrolytic removal of the phosphocholine moiety of SM by sphingomyelinases (SMases) to render CER has been associated with the induction of membrane curvature in SL enriched membrane domains (50, 51). In fact, compounds that inhibit the activity of the neutral SMase 2 [nSMase2, sphingomyelin phosphodiesterase 3 (SMPD3)] can inhibit budding processes (18). Since these observations are compatible with potential roles of SLs during WNV infection, *i.e.* in virion budding and/or assembly, we first addressed the effect of nSMase inhibition in HeLa3-WNV cells, used as a model system to study flavivirus biogenesis in the absence of virus replication. To this end, we selected three structurally unrelated nSMase inhibitors (18): GW4869, a non-competitive inhibitor of nSMase (34), spiroepoxide a selective irreversible inhibitor of neutral sphingomyelinase (52), and glutathione a cellular regulator of nSMase activity (53). Treatment of these cells with either of these inhibitors significantly reduced the amount of RSPs released to the culture medium without exerting noticeable toxic effects on the cells (Fig. 5A, B and C). GW4869 was the drug that produced significant inhibitions at a lower concentration,
so this compound was selected for further experiments involving virus infections (see below). As a complementary approach, the effect of nSMase2 depletion using RNA interference was also analyzed. Silencing of the expression of nSMase2 by transfection with siRNA, which was verified by western blot (Fig. 5D), significantly inhibited the release of RSPs to the culture medium (Fig. 5D and E). When the E protein content was analyzed in siRNA transfected cells by Western blot (Fig. 5D) or immunofluorescence (Fig. 5F), no reduction was observed in cells transfected with siRNA against nSMase2. These results pointed to an accumulation of viral proteins inside cells depleted from nSMase2, which is consistent with the reduction observed in the release of RSPs from cells depleted from nSMase2. The extent of the inhibition of RSPs release in cells depleted from nSMase2 by siRNA (Fig. 5E) was comparable to that exerted by treatment with nSMase inhibitors (Fig. 5A-C), further supporting the requirement for nSMase function during the biogenesis of flaviviruses.

**Interference on nSMase function reduces flavivirus release from infected cells.** The effect of the nSMase inhibitor GW4869 on the infection of WNV was assayed in two mammalian cell lines (HeLa and Vero) and one mosquito cell line (C6/36) (Fig. 6A-C). Treatment with GW4869 significantly inhibited the release of infectious WNV at concentrations ≥5 µM in these three cell lines; confirming the requirement of nSMase for the production of WNV in both mammalian and insect cells. The effect of this inhibitor on the infection of Usutu virus (USUV), a related emerging flavivirus, was also tested in HeLa cells (Fig. 6D). In these experiments, an inhibition similar to that obtained for WNV at drug concentrations ≥5 µM was observed. To analyze whether the requirement of nSMase function for virus production was shared by other unrelated arboviruses, the effect of GW4869 on the infection of the alphavirus Sindbis virus (SINV) was analyzed (Fig. 6E). Interestingly, the release of SINV infectious particles...
from HeLa cells was not inhibited, but significantly increased by treatment with GW4869. This enhancing effect of virus titer was similar to that previously reported when SINV infection was performed in cells exhibiting an altered SL content by depletion of acid SMase, which was associated with an increase in virion infectivity (54). We next addressed the effect of depletion of nSMase2 on the infection of these three viruses by transfection with specific siRNA (Fig. 6F). Silencing of nSMase2 expression significantly inhibited the production of both WNV and USUV to an extent comparable to that produced by treatment with GW4869. Conversely, a significant increase in SINV titer was found in cells treated with siRNA against nSMase2. Therefore, these observations confirm the results obtained with GW4869, and highlight the involvement of nSMase function during flavivirus infection.

Inhibition of nSMase activity reduces WNV-induced membrane budding. The mechanism behind the reduction of WNV growth upon nSMase inhibition using GW4869 was further studied. The amount of viral particles released to the culture medium, determined by an enzyme-linked immunodot assay, showed a significant reduction in the levels of secreted E glycoprotein in cells treated with GW4869 (Fig. 7A), which was consistent with the decrease observed in RSPs and infectious virus release (Fig. 5A and 6A). The analysis by immunofluorescence and confocal microscopy of infected cells treated with GW4869 showed an accumulation of the E glycoprotein that colocalized with the ER marker calnexin (Fig. 7B). To rule out that the effect of GW4869 on the biogenesis of WNV occurred in a step posterior to virus assembly or maturation, the amount of cell-associated, or medium-released, infectious virus was determined in cells treated with GW4869 (Fig. 7C). Whereas treatment with GW4869 significantly reduced the amount of infectious WNV released, it did not induce an accumulation of cell-associated infectious virus. When the amount of cell-
associated and medium-released viral RNA were analyzed (Fig. 7D), a significant reduction of viral RNA levels in the culture medium of cells treated with GW4869 was observed. Conversely, a significant accumulation of cell associated viral RNA was noticed. Overall, these results were compatible with a reduction in infectious virus release with a concomitant accumulation of viral RNA, but not of infectious particles, inside GW4869 treated cells, which suggests that inhibition of nSMase function alters a process on flavivirus biogenesis previous to the assembly and/or maturation of infectious particles. Interestingly, the extent of PFU release inhibition (about 60%, Fig. 7C) was higher than the inhibition of total particle or viral RNA release observed (about 40%; Figs. 7A and D). This could suggest that GW4869 not only decreases the amount of released viral particles, but also elevates the proportion of secreted noninfectious particles.

As the inhibition of nSMase function can interfere with intracellular budding processes, the effect of GW4869 on virus-mediated budding was analyzed. To this end, infected cells were treated with GW4869 and analyzed by transmission electron microscopy (Fig. 7E). Cells treated with the nSMase inhibitor displayed ultrastructural features associated with flavivirus infection i.e. formation of VPs and the presence of electron dense virions. VPs are produced by budding into the ER, and the abundance of these structures can be quantified by transmission electron microscopy in flavivirus infected cells (55). According to this view, these structures were analyzed to directly evaluate the effect of nSMase inhibition in viral budding. The mean diameter of virus induced vesicles detected inside the VPs was indistinguishable (about 80 nm) in both control and GW4869 treated cells (Fig. 7F). However, when the amount of vesicles inside VPs was scored, a significant reduction in the number of vesicles inside each VP was noticed in GW4869 treated cells relative to control infected cells (mean number of
vesicles/VP of $1.9 \pm 0.2$ and $5.8 \pm 0.6$, respectively; Figure 7G), supporting that viral budding into the ER was reduced upon inhibition of nSMase function.

**DISCUSSION**

Our results illustrate the alteration of the content of a wide variety of lipid metabolites in WNV-infected cells. The increase in the cellular content of PC, lysophospholipids (lyso-PC), SM, and CER in HeLa cells infected with WNV is consistent with previous results with mosquito cells infected with DENV (26). Our analysis also revealed the enrichment in p-PC, p-PE and dhCER in WNV-infected cells. Apart from PC, which is a cylindrical lipid, the rest of lipids found increased in WNV-infected cells were conical. Increase in these conical lipids could be related to their roles on the establishment and maintenance of cellular membrane curvature necessary for proper viral replication complex assembly, as well as for virion envelopment. The enrichment of unsaturated PC species in DENV-infected cells has been suggested to be associated with the development of more fluidic membranes (26). Supporting this idea, all PC species increased in WNV-infected cells were unsaturated. Likewise, the observed enrichment of SLs (CER, dhCER and SM) in WNV-infected cells is also compatible with the requirement of these molecules in the membranes where virus replication and assembly takes place. According to this hypothesis, it has been documented that DENV and hepatitis C virus replication complexes are enriched in SLs (17, 26).

To identify the potential roles of the lipid classes analyzed in the biogenesis of WNV particles, the proportion of each lipid was analyzed in both purified virions and RSPs. All lipid classes analyzed were detected in the envelope of WNV and RSPs including PS that has been shown to play a functional role during flavivirus entry (11). The high degree of similarity between the lipid composition of RSPs and virions, along
with their parallel mechanisms of formation, reinforce the view of RSPs as a valuable tool for the study of lipid involvement during the biogenesis of flaviviruses.

We next addressed the question of whether the lipid composition of the WNV envelope was similar to that of total host cell membranes or if it reflected that of a particular membrane domain, following an approach similar to those performed for characterization of other viral envelopes (7, 10). This analysis indicated that the envelopes of RSPs and virions were both enriched in SM and exhibited a reduction in the content of PC when compared to that of total cellular membranes. Similar features, increase in SM (or other SLs) and depletion of PC, have been also observed in the lipid envelope of human immunodeficiency virus type 1 (HIV-1) and influenza virus (7, 9).

On the contrary, the rhabdovirus vesicular stomatitis virus and the alphavirus Semliki Forest virus acquire their envelope exerting little lipid selection (8). The characteristics of WNV envelope also differed from those of hepatitis C virus, which resembles more that of very low- and low- density-lipoproteins (10). Enrichment in SLs and reduction of PC levels are characteristics of liquid ordered membrane microdomains or membrane rafts (7, 9, 18, 56). Since membrane raft-like microdomains are usually cholesterol enriched (7, 9, 18, 56), including those located in the ER (57), our findings could be connected with the role(s) of membrane microdomains enriched in cholesterol in flavivirus infections (28, 31, 58-60). SLs enriched membrane microdomains have functional properties directly linked to their lipid composition, such as coalescence and promotion of budding processes during virus assembly (7, 9) or exosome biogenesis (18). Indeed, flavivirus assembly by invagination of ER membrane shares topological features with the formation of exosomes, since both processes mainly consist on budding outward from the cell cytoplasm. Supporting this hypothesis, the parallelism between viral budding processes and exosome biogenesis has already been documented.
An important point that has to be also considered in these analyses is that in the RSPs system, HeLa3-WNV cells express viral prM and E proteins in the absence of any other viral protein. Since viral non-structural proteins (such as NS4A) induce membrane proliferation (63, 64), their expression is expected to modify cellular lipids in infected cells. In fact, modification of cellular lipids upon infection with WNV was confirmed by the analysis of lipids in WNV-infected cells. Therefore, it is foreseeable that the lipid components from WNV-infected cells can differ from those of RSPs-producing cells, which may explain some of the minor differences between RSPs and virions found in the analysis, such as the amount of p-PC or the variations between specific lipid species. However, the major features observed (reduction in PC and enrichment in SM) were common between the two systems, supporting the use of RSPs as a model to study flavivirus biogenesis.

The hydrolysis of SM by nSMases converts SM into CER, a compound with a smaller head size (49). CER induces an asymmetric membrane tension and segregates into highly ordered domains triggering modifications of membrane shape (65). These properties have been associated with promotion of membrane bending and budding processes, both on model membranes (50) and in living cells (18, 51, 66, 67). In this scenario, pharmacological inhibition of the activity of nSMase function, which catalyzes SM to CER hydrolysis, impaired release of RSPs to the culture medium and reduced the production of infectious virus particles of WNV and the related flavivirus USUV, in a manner parallel to the effect documented in the production of certain types of exosomes (18, 66, 67). Supporting this observation, depletion of nSMase2 by RNAi inhibited the release of RSPs and the production of WNV and USUV. On the contrary, an increase in the titer of other arbovirus (SINV) was observed upon inhibition of nSMase function, therefore indicating that the requirement for SM to CER conversion...
during the biogenesis of flavivirus was not shared by this alphavirus. Consistent with these results, lipid recruitment during alphavirus envelopment has been proposed to be a low selective process (8) in contrast to the enrichment in SM and reduction in PC described here for flavivirus envelope. Alphavirus biogenesis is dependent on the functionality of the secretory pathway, since viral envelope glycoproteins must traffic from the ER through the Golgi complex to the plasma membrane for viral budding (68). Thus, the lack of inhibition of SINV release upon pharmacological inhibition of nSMase2, or siRNA mediated depletion of this enzyme, evidences the functionality of the secretory pathway in cells treated with GW4869 or siRNA against nSMase2. This observation further supports the specific involvement of nSMase2 in the biogenesis of flavivirus. Although other nSMases are expected to be functional in cells depleted from nSMase2 by siRNA, the degree of inhibition (RSPs and virion release) achieved with specific siRNA against nSMase2 was similar to that observed using the nSMase inhibitor GW4869, thus reinforcing the idea that nSMase2 is the main nSMase involved in flavivirus biogenesis.

When the effect of nSMase inhibition on virus biogenesis was analyzed by several approaches, a reduction of the amount of virus-induced vesicles inside VPs in cells treated with an inhibitor of nSMase function was noticed. Since these vesicles are associated with flavivirus replication and virion envelopment (5, 43, 55), this observation supports the role of SM conversion to CER in virus-induced budding. The diameter of the vesicles found in cells treated with nSMase inhibitor was similar to that observed in untreated cells. A possible explanation for this phenomenon could be that vesicle components others than lipids (presumably viral proteins) also contribute to vesicle formation, although the inhibition of nSMase activity makes this assembly less efficient. Accordingly, an important role for viral glycoproteins on the induction and
maintenance of membrane curvature in flavivirus virions has been proposed (69). Since budding mechanisms are usually driven by a regulated combination of proteins and lipids (70), our results indicate that, indeed, the lipid composition of the flavivirus envelope also contributes to this process.

Interestingly, the dhCER content was found increased in WNV-infected cells, being one of the increased molecular species (dhCER 24:0) enriched in the viral envelope. This metabolite is an intermediate of the SL biosynthetic pathway (49), which points to an upregulation of the de novo synthesis of SLs within WNV-infected cells, as reported in other viral models (17, 26). On the other hand, the involvement of nSMase in WNV and USUV biogenesis indicates that the production of CER by hydrolysis of SM is also required during the infection with these pathogens, evidencing the functionality of these two pathways for CER production in flavivirus infected cells. However, one must be cautious with the interpretation of CER increase in WNV-infected cells, since this can be due to different factors, such as the induction of ER stress (71) mediated by the activation of the unfolded protein response in flavivirus-infected cells (72).

In summary, our results provide additional evidences on the complex degree of manipulation of the host cell lipid metabolism by WNV. These results also unveil a connection between SL metabolism and flavivirus biogenesis. The findings presented could contribute to further development of lipid-based antiviral strategies to combat these pathogens.

ACKNOWLEDGMENTS

We thank G. Fabrias and E. Dalmau for help with lipid analyses, M. Guerra and M. T. Rejas for help with electron microscopy, and M. Calvo for technical assistance.
This work was supported by grants RTA 00036-2011 (J.-C.S.), BIO2011-24351 (F.S.), RTA2013-0013 (J.-C. S. and F.S) and by the Fundació la Marató de TV3 (grant 112130) (J.C.). M.A.M.-A. is a recipient of a “Junta de Ampliación de Estudios (JAE)” post-doctoral fellowship from the Spanish Research Council (CSIC). T.M.-R. is a recipient of a “Formación de Personal Investigador (FPI)” pre-doctoral fellowship from INIA.

REFERENCES


52. Arenz, C., and A. Giannis. 2000. Synthesis of the First Selective Irreversible Inhibitor of Neutral Sphingomyelinase This work was supported by grants from the Fonds der Chemischen Industrie. C.A. is grateful to the Land of Baden-Wurttemberg for a scholarship from the Landesgraduiertenforderung. Angew Chem Int Ed Engl 39:1440-1442.


FIGURE LEGENDS

FIG 1 WNV infection alters lipid metabolism. (A) Time-course analysis of WNV infection. HeLa cells were infected with WNV (MOI of 50 PFU/cell) and the infectious virus released to the culture medium was determined by plaque assay at different time p.i. (B) Intracellular membrane rearrangements in WNV-infected HeLa cells observed by transmission electron microscopy. A micrograph showing the ER from uninfected cells is included for comparison. Characteristics features of flavivirus infected cells, vesicle packets (VP) and virions (Vi), were observed at 24 h p.i. (MOI of 50 PFU/cell). Scale bar: 200 nm. (C) Relative amount of different classes of GPLs and SLs in HeLa cells infected, or not, with WNV (MOI of 50 PFU/cell) determined by mass spectrometry at 24 h p.i. Statistically significant differences are indicated by * for P<0.05, and ** for P<0.005. (D) Fold differences of individual lipid species significantly increased in infected cells analyzed as in (C). Dashed line shows the mean value for each lipid in uninfected cells. Data are presented as mean ± SD.

FIG 2 WNV RSP as a noninfectious system to study flavivirus biogenesis. (A) Immunofluorescence analysis of the expression of the WNV E glycoprotein in HeLa3-WNV cells. HeLa or HeLa3-WNV cells were subjected to immunofluorescence analysis using a monoclonal antibody directed against the E glycoprotein (green) and observed by confocal microscopy. Nuclei were stained with ToPro3 (blue). Scale bar: 10 µm. (B) RSPs produced by HeLa3-WNV cells. The RSP released to the culture medium were purified by sucrose gradient centrifugation and observed by negative staining and transmission electron microscopy. Immunogold staining of RSPs using a monoclonal antibody against the E protein and detected using protein A coupled to 5 nm colloidal gold is displayed in the inset. Scale bar: 100 nm. (C and D) Localization of the WNV E
glycoprotein at the ER (C), and the Golgi complex (D) of HeLa3-WNV cells. Immunofluorescence was performed using a monoclonal antibody directed against the E glycoprotein (green) in combination with rabbit polyclonal antibodies against calnexin (red) to stain the ER, or to mannosidase II (red) to stain the Golgi complex. Nuclei were stained with ToPro3 (blue). HeLa cells not expressing viral proteins were included as negative control. Scale bar: 10 µm. (E) Western blot analysis of RSPs purified through a sucrose cushion from the culture medium of Hela3-WNV. Cell culture medium from control HeLa cells was processed and analyzed in parallel as a negative control. Western blot was performed using anti-E or anti-M antibodies. (F) Enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein of culture supernatants from Hela3-WNV cells. Culture medium from HeLa cells not expressing viral glycoproteins was included as a negative control.

FIG 3 Treatment with BFA or GCA impairs the secretion of RSP to the culture medium. (A) The amount of RSPs released to the culture medium by HeLa3-WNV cells treated (4 h) with the drugs was analyzed by enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein. (B) Quantification of the amount of RSPs released to the culture medium by treated HeLa3-WNV cells shown in panel (A). The cellular ATP content is indicated for each drug concentration as an indicative of cell viability upon drug treatment. (C) Localization of the WNV E glycoprotein in infected HeLa cells treated with Golgi disrupting agents. HeLa3-WNV cells were treated with 10 µM BFA, or GCA, for 4 h. Immunofluorescence was performed using a monoclonal antibody directed against the WNV E glycoprotein (red) in combination with a rabbit polyclonal antibody against mannosidase II (green) to stain Golgi complex. Nuclei were stained with ToPro3 (blue). Left panels display E glycoprotein staining
false colored with the ‘fire’ look-up table (LUT) scheme from dark purple to bright yellow to highlight differences in the intensity of the signal. Scale bar: 10 µm.

FIG 4 Lipid composition of WNV RSP and virions. (A) GPL and SL composition of WNV RSP and virions. The content of individual lipid classes of total HeLa cells and WNV RSPs or virions was determined by summing up absolute abundances of all identified species. Values are standardized to mole percentage of all membrane lipids detected within the sample. Values multiplied by ten for visibility are indicated. (B) Individual lipid species significantly altered in virions or RSPs in comparison to total cellular membranes. Data are presented as mean ± SD. Statistically significant differences are indicated by * for P<0.05, and ** for P<0.005.

FIG 5 Impairment of nSMase function inhibits the release of RSPs. (A, B, C) Treatment with the nSMase inhibitors GW4869 (A), spiroepoxide (B) or glutathione (C) impairs the release of RSPs by HeLa3-WNV cells. The content of RSPs in the culture medium of cells treated (4 h) with the drugs was determined by enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein. The quantification of the amount of RSPs determined by enzyme-linked immunodot assay and the cellular ATP content for each drug concentration is shown in the graphs. (D) Depletion of nSMase2 by RNA interference. HeLa3-WNV cells were transfected with universal negative control siRNA, or with siRNA against nSMase2, for 48 h, and the amount of nSMase2, E glycoprotein and β-actin in cell lysates was analyzed by western blot using specific antibodies. The amount of RSPs released to the culture medium after 4 h of incubation in cells transfected for 48 h with siRNA was analyzed by enzyme–linked immunodot assay using an anti-E antibody. (E) Quantification of the amount of RSPs released to the culture medium by HeLa3-WNV cells transfected with siRNA control, or against
nSMase2, is shown in panel (D). The cellular ATP content is indicated for each drug concentration as an indicative of cell viability upon drug treatment. (F) Immunofluorescence analysis of the expression of the WNV E glycoprotein in HeLa3-WNV cells transfected with siRNAs. HeLa, or HeLa3-WNV cells transfected as described for panel (D), were subjected to immunofluorescence analysis using an antibody directed against E glycoprotein (green) and observed by confocal microscopy. Nuclei were stained with ToPro3 (blue). Left panels display the E glycoprotein staining false colored with the ‘fire’ look-up table (LUT) scheme from dark purple to bright yellow to highlight differences in the intensity of the signal. Scale bar: 10 µm. Data are presented as mean ± SD. Statistically significant differences are indicated by * for P<0.05, and ** for P<0.005.

FIG 6 Impairment of nSMase function inhibits the release of WNV. (A, B, C) Treatment with the nSMase inhibitor GW4869 reduces WNV production in mammalian and insect cells. HeLa (A), Vero (B) or C6/36 (C) cells were infected with WNV (MOI of 1 PFU/cell), treated with the drug and the amount of infectious virus released to the culture medium was determined by plaque assay (24 h p.i.). The cellular ATP content of uninfected cells treated with the different concentrations of the drug is indicated in all graphs as an estimation of the cell viability upon drug treatment. (D) Treatment with GW4869 reduces USUV production. HeLa cells were infected with USUV (MOI of 1 PFU/cell) and the amount of infectious virus released was determined as above. (E) Treatment with GW4869 increases SINV production. HeLa cells were infected with SINV (MOI of 1 PFU/cell) and the amount of infectious virus released to the culture medium was determined by plaque assay (8 h p.i.). (F) Effect of nSMase2 depletion by siRNA on WNV, USUV and SINV infection. HeLa cells were transfected with a universal negative siRNA control, or with siRNA against nSMase2, for 48 h, infected
with WNV, USUV, or SINV, and the virus released to the culture medium was analyzed as described above. Data are presented as mean ± SD. Statistically significant differences are indicated by * for P<0.05, and ** for P<0.005.

FIG 7 Inhibition of nSMase function reduced WNV-induced budding. (A) Treatment with the nSMase inhibitor GW4869 reduces WNV release. HeLa cells were infected with WNV (MOI of 1 PFU/cell), treated with the drug and the amount of virus particles released to the culture medium was estimated by an enzyme-linked immunosorbent assay using a monoclonal antibody against the E glycoprotein (24 h p.i.). (B) Localization of the WNV E glycoprotein in HeLa cells infected with WNV (MOI of 1 PFU/cell) and treated with 10 µM GW4869. Immunofluorescence was performed using a monoclonal antibody directed against the E glycoprotein (green) in combination with a rabbit polyclonal antibody against calnexin (red) to stain the ER (24 h p.i.). Nuclei were stained with ToPro3 (blue). Left panels display the E glycoprotein staining false colored with the ‘fire’ look-up table (LUT) scheme from dark purple to bright yellow to highlight differences in the intensity of the signal. Scale bar: 10 µm. (C) Impairment of nSMase function with GW4869 does not increase the amount of cell associated WNV. HeLa cells were infected with WNV (MOI of 1 PFU/cell) and the amount of virus either released or cell associated was determined by plaque assay at 24 h p.i. (D) The amount of viral RNA released to the culture medium, or cell associated, was determined by quantitative RT-PCR in cells infected and treated as described for panel (C). (E) Representative electron micrographs (24 h p.i.) of cells infected with WNV (MOI of 10 PFU/cell) and treated, or not (control), with 10 µM GW4869 showing the formation of vesicle packets (VP) containing virus-induced vesicles (Ve) and electron dense virions (Vi). Scale bar: 200 nm. (F) Diameter of Ve detected in the VPs of cells infected and treated, or not, with 10 µM GW4869. Each point represents an individual Ve. Solid...
lines denote the mean vesicle diameter. (G) Number of Ve per VP detected in the VPs of cells treated, or not, with 10 μM GW4869. Each point represents the number of Ve detected in an individual VP. Solid lines denote the mean number of Ve per VP. Unless specified, data are presented as mean ± SD. Statistically significant differences (P<0.005) are indicated by **.
FIG 1 WNV infection alters lipid metabolism. (A) Time-course analysis of WNV infection. HeLa cells were infected with WNV (MOI of 50 PFU/cell) and the infectious virus released to the culture medium was determined by plaque assay at different time p.i. (B) Intracellular membrane rearrangements in WNV-infected HeLa cells observed by transmission electron microscopy. A micrograph showing the ER from uninfected cells is included for comparison. Characteristics features of flavivirus infected cells, vesicle packets (VP) and virions (Vi), were observed at 24 h p.i. (MOI of 50 PFU/cell). Scale bar: 200 nm. (C) Relative amount of different classes of GPLs and SLs in HeLa cells infected, or not, with WNV (MOI of 50 PFU/cell) determined by mass spectrometry at 24 h p.i. Statistically significant differences are indicated by * for P<0.05, and ** for P<0.005. (D) Fold differences of individual lipid species significantly increased in infected cells analyzed as in (C). Dashed line shows the mean value for each lipid in uninfected cells. Data are presented as mean ± SD.
FIG 2 WNV RSP as a noninfectious system to study flavivirus biogenesis. (A) Immunofluorescence analysis of the expression of the WNV E glycoprotein in HeLa3-WNV cells. HeLa or HeLa3-WNV cells were subjected to immunofluorescence analysis using a monoclonal antibody directed against the E glycoprotein (green) and observed by confocal microscopy. Nuclei were stained with ToPro3 (blue). Scale bar: 10 µm. (B) RSPs produced by HeLa3-WNV cells. The RSP released to the culture medium were purified by sucrose gradient centrifugation and observed by negative staining and transmission electron microscopy. Immunogold staining of RSPs using a monoclonal antibody against the E protein is displayed in the inset. Scale bar: 100 nm. (C and D) Localization of the WNV E glycoprotein at the ER (C), and the Golgi complex (D) of HeLa3-WNV cells. Immunofluorescence was performed using a monoclonal antibody directed against the E glycoprotein (green) in combination with rabbit polyclonal antibodies against calnexin (red) to stain the ER, or to mannosidase II (red) to stain the Golgi complex. Nuclei were stained with ToPro3 (blue). HeLa cells not expressing viral proteins were included as negative control. Scale bar: 10 µm. (E) Western blot analysis of RSPs purified through a sucrose cushion from the culture medium of Hela3-WNV. Cell culture medium from control HeLa cells was processed and analyzed in parallel as a negative control. Western blot was performed using anti-E or anti-M antibodies. (F) Enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein of culture supernatants from Hela3-WNV cells. Culture medium from HeLa cells not expressing viral glycoproteins was included as a negative control.
FIG 3 Treatment with BFA or GCA impairs the secretion of RSP to the culture medium. (A) The amount of RSPs released to the culture medium by HeLa3-WNV cells treated (4 h) with the drugs was analyzed by enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein. (B) Quantification of the amount of RSPs released to the culture medium by treated HeLa3-WNV cells shown in panel (A). The cellular ATP content is indicated for each drug concentration as an indicative of cell viability upon drug treatment. (C) Localization of the WNV E glycoprotein in infected HeLa cells treated with Golgi disrupting agents. HeLa3-WNV cells were treated with 10 µM BFA, or GCA, for 4 h. Immunofluorescence was performed using a monoclonal antibody directed against the WNV E glycoprotein (red) in combination with a rabbit polyclonal antibody against mannosidase II (green) to stain Golgi complex. Nuclei were stained with ToPro3 (blue). Left panels display E glycoprotein staining false colored with the ‘fire’ look-up table (LUT) scheme from dark purple to bright yellow to highlight differences in the intensity of the signal. Scale bar: 10 µm.
FIG 4 Lipid composition of WNV RSP and virions. (A) GPL and SL composition of WNV RSP and virions. The content of individual lipid classes of total HeLa cells and WNV RSPs or virions was determined by summing up absolute abundances of all identified species. Values are standardized to mole percentage of all membrane lipids detected within the sample. Values multiplied by ten for visibility are indicated. (B) Individual lipid species significantly altered in virions or RSPs in comparison to total cellular membranes. Data are presented as mean ± SD. Statistically significant differences are indicated by * for P<0.05, and ** for P<0.005.
FIG 5 Impairment of nSMase function inhibits the release of RSPs. (A, B, C) Treatment with the nSMase inhibitors GW4869 (A), spiroepoxide (B) or glutathione (C) impairs the release of RSPs by HeLa3-WNV cells. The content of RSPs in the culture medium of cells treated (4 h) with the drugs was determined by enzyme-linked immunodot assay using a monoclonal antibody against the E glycoprotein. The quantification of the amount of RSPs determined by enzyme-linked immunodot assay and the cellular ATP content for each drug concentration is shown in the graphs. (D) Depletion of nSMase2 by RNA interference. HeLa3-WNV cells were transfected with universal negative control siRNA, or with siRNA against nSMase2, for 48 h, and the amount of nSMase2, E glycoprotein and β-actin in cell lysates was analyzed by western blot using specific antibodies. The amount of RSPs released to the culture medium after 4 h of incubation in cells transfected for 48 h with siRNA was analyzed by enzyme-linked immunodot assay using an anti-E antibody. (E) Quantification of the amount of RSPs released to the culture medium by HeLa3-WNV cells transfected with siRNA control, or against nSMase2, is shown in panel (D). The cellular ATP content is indicated for each drug concentration as an indicative of cell viability upon drug treatment. (F) Immunofluorescence analysis of the expression of the WNV E glycoprotein in HeLa3-WNV cells transfected with siRNAs. HeLa, or HeLa3-WNV cells transfected as described for panel (D), were subjected to immunofluorescence analysis using an antibody directed against E glycoprotein (green) and observed by confocal microscopy. Nuclei were stained with ToPro3 (blue). Left panels display the E glycoprotein staining false colored with the ‘fire’ look-up table (LUT) scheme from dark purple to bright yellow to highlight differences in the intensity of the signal. Scale bar: 10 µm. Data are presented as mean ± SD. Statistically significant differences are indicated by * for P<0.05, and ** for P<0.005.
FIG 6 Impairment of nSMase function inhibits the release of WNV. (A, B, C) Treatment with the nSMase inhibitor GW4869 reduces WNV production in mammalian and insect cells. HeLa (A), Vero (B) or C6/36 (C) cells were infected with WNV (MOI of 1 PFU/cell), treated with the drug and the amount of infectious virus released to the culture medium was determined by plaque assay (24 h.p.i.). The cellular ATP content of uninfected cells treated with the different concentrations of the drug is indicated in all graphs as an estimation of the cell viability upon drug treatment. (D) Treatment with GW4869 reduces USUV production. HeLa cells were infected with USUV (MOI of 1 PFU/cell) and the amount of infectious virus released was determined as above. (E) Treatment with GW4869 increases SINV production. HeLa cells were infected with SINV (MOI of 1 PFU/cell) and the amount of infectious virus released to the culture medium was determined by plaque assay (8 h.p.i.). (F) Effect of nSMase2 depletion by siRNA on WNV, USUV and SINV infection. HeLa cells were transfected with a universal negative siRNA control, or with siRNA against nSMase2, for 48 h, infected with WNV, USUV, or SINV, and the virus released to the culture medium was analyzed as described above. Data are presented as mean ± SD. Statistically significant differences are indicated by * for P<0.05, and ** for P<0.005.
FIG 7 Inhibition of nSMase function reduced WNV-induced budding. (A) Treatment with the nSMase inhibitor GW4869 reduces WNV release. HeLa cells were infected with WNV (MOI of 1 PFU/cell), treated with the drug and the amount of virus particles released to the culture medium was estimated by an enzyme-linked immunosorbent assay using a monoclonal antibody against the E glycoprotein (24 h p.i.). (B) Localization of the WNV E glycoprotein in HeLa cells infected with WNV (MOI of 1 PFU/cell) and treated with 10 µM GW4869. Immunofluorescence was performed using a monoclonal antibody directed against the E glycoprotein (green) in combination with a rabbit polyclonal antibody against calnexin (red) to stain the ER (24 h p.i.). Nuclei were stained with ToPro3 (blue). Left panels display the E glycoprotein staining false colored with the 'fire' look-up table (LUT) scheme from dark purple to bright yellow to highlight differences in the intensity of the signal. Scale bar: 10 µm. (C) Impairment of nSMase function with GW4869 does not increase the amount of cell associated WNV. HeLa cells were infected with WNV (MOI of 1 PFU/cell) and the amount of virus either released or cell associated was determined by plaque assay at 24 h p.i. (D) The amount of viral RNA released to the culture medium, or cell associated, was determined by quantitative RT-PCR in cells infected and treated as described for panel (C). (E) Representative electron micrographs (24 h p.i.) of cells infected with WNV (MOI of 10 PFU/cell) and treated, or not (control), with 10 µM GW4869 showing the formation of vesicle packets (VP) containing virus-induced vesicles (Ve) and electron dense virions (Vi). Scale bar: 200 nm. (F) Diameter of Ve detected in the VPs of cells infected and treated, or not, with 10 µM GW4869. Each point represents an individual Ve. Solid lines denote the mean vesicle diameter. (G) Number of Ve per VP detected in the VPs of cells treated, or not, with 10 µM GW4869. Each point represents the number of Ve detected in an individual VP. Solid lines denote the mean number of Ve per VP. Unless specified, data are presented as mean ± SD. Statistically significant differences (P<0.005) are indicated by **.