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Nordihydroguaiaretic acid (NDGA) inhibits replication and viral morphogenesis of dengue virus

Rubén Soto-Acosta [a], Patricia Bautista-Carbajal [a], Gulam H. Syed [b], Aleem Siddiqui [b], Rosa M. Del Angel [a,*]

[a] Departmento de Infectómica y Patogénesis molecular, CINVESTAV-IPN, México, D.F., Mexico
[b] Department of Medicine, Division of Infectious Disease, University of California, San Diego, La Jolla, CA, United States

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ABSTRACT

Dengue is the most common mosquito borne viral disease in humans. The infection with any of the 4 dengue virus serotypes (DENV) can either be asymptomatic or manifest in two clinical forms, the mild dengue fever or the more severe dengue hemorrhagic fever that may progress into dengue shock syndrome. A DENV replicative cycle relies on host lipid metabolism; specifically, DENV infection modulates cholesterol and fatty acid synthesis, generating a lipid-enriched cellular environment necessary for viral replication. Thus, the aim of this work was to evaluate the anti-DENV effect of the Nordihydroguaiaretic acid (NDGA), a hypolipidemic agent with antioxidant and anti-inflammatory properties. A dose-dependent inhibition in viral yield and NS1 secretion was observed in supernatants of infected cells treated for 24 and 48 h with different concentrations of NDGA. To evaluate the effect of NDGA in DENV replication, a DENV4 replicon transfected Vero cells were treated with different concentrations of NDGA. NDGA treatment significantly reduced DENV replication, reiterating the importance of lipids in viral replication. NDGA treatment also led to reduction in number of lipid droplets (LDs), the neutral lipid storage organelles involved in DENV morphogenesis that are known to increase in number during DENV infection. Furthermore, NDGA treatment resulted in dissociation of the C protein from LDs. Overall our results suggest that NDGA inhibits DENV infection by targeting genome replication and viral assembly.

1. Introduction

Dengue virus (DENV), the causative agent of dengue fever, is the most prevalent mosquito borne viral pathogen worldwide. Infection with any of the four DENV serotypes can either be asymptomatic or manifest into mild dengue fever or the more severe dengue hemorrhagic fever and shock syndrome. DENV, a member of *Flavivirus* genera, infects more than 50 million people each year and causes approximately 25,000 deaths (Gubler, 2002). The virion is a spherical particle of approximately 50 nm in diameter and contains a lipid envelope harboring two structural proteins, the envelope (E) and membrane proteins (prM in immature virions and M in mature virions). The envelope surrounds the nucleocapsid, containing the capsid protein (C) associated with the single stranded RNA genome of positive polarity (Kuhn et al., 2002; Mukhopadhyay et al., 2005; Yu et al., 2008). The first step in DENV infection is the viral binding to the cellular receptor molecules on the surface of the target cell, followed by receptor-mediated endocytosis and subsequent fusion of the viral and endosomal membranes leading to genome release into the cytoplasm (Hidari and Suzuki, 2011). The viral RNA acts initially as mRNA for the translation of viral proteins, later, genome replication occurs in two steps. First, the positive polarity RNA is copied to an RNA of negative polarity, which then serves as a template for positive polarity RNA synthesis. The newly synthesized positive polarity RNA associates with C, E and prM to form the immature virions, which travel in vesicles to the Golgi apparatus, where prM is cleaved by furin to generate mature virions which are released outside the cell through the secretory pathway (Mukhopadhyay et al., 2005; Stiasny and Heinz, 2006).

DENV and others positive-strand RNA viruses modify endoplasmic reticulum membranes to allow viral genome replication and encapsidation (Welsch et al., 2009). Perera et al. (2012) demonstrated, in mosquito-derived cells, significant differences in the intracellular lipid profile of DENV infected cells, compared with uninfected cells. The type of lipids enriched in DENV infected cells includes lipids that are capable of altering the membrane both structurally and functionally (Perera et al., 2012); supporting the

* Corresponding author. Address: Departmento de Infectómica y Patogénesis Molecular, Centro de Investigación y de Estudios Avanzados del IPN (CINVESTAV-IPN), Av. IPN 2508, Colonia San Pedro Zacatenco, México, D.F. 07360, Mexico. Fax: +52 5557477107.
E-mail address: rmangel@cinvestav.mx (R.M. Del Angel).

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notion that DENV infection promotes intracellular membrane rearrangements through alterations in lipid metabolism.

During DENV replication and morphogenesis, the participation of several lipids, fatty acid and cholesterol has been reported. For example, Heaton et al. (2010) observed that NS3 protein of DENV was able to recruit the fatty acid synthase (FASN) to the replicative complexes, increasing its activity (Heaton et al., 2010). Moreover, it has been described that the C protein of DENV is recruited to the lipid droplets (LDs) (Carvalho et al., 2012; Samsa et al., 2009). These findings are congruent with the fact that the pharmacological inhibition of FASN using C75, causes a significant inhibition of DENV genome replication and viral morphogenesis (Poh et al., 2012; Samsa et al., 2009).

The importance of cholesterol in DENV replication has been demonstrated in different cell types by the use of drugs that disrupt lipid rafts (Mβcd and filipin III complex), inhibit cholesterol synthesis (lovastatin, fluvastatin and pravastatin) or siRNAs that block the cholesterol biosynthetic pathway (Lee et al., 2008; Martinez-Gutierrez et al., 2011; Rothwell et al., 2009). Since DENV replication requires cholesterol, at early times after viral infection an increase in cholesterol levels is required. This increase is a consequence of an increase in the exogenous cholesterol uptake through the low density lipoprotein receptor (LDLr) and an increase in the 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR) activity (Soto-Acosta et al., 2013). Considering the important role of lipid metabolism in DENV replication, the use of drugs or molecules capable of interfering the cellular lipid metabolic pathways are expected to perturb the viral replicative cycle.

Nordihydroguaiaretic acid (NDGA) is the main metabolite of creosote bush, also known as chaparral; a plant abundant in the desert areas of the United States and Mexico. This arachidonic acid 5-lipoxygenase inhibitor has antioxidant, anti-inflammatory and hypolipidemic properties. NDGA decreases the amount of mature SREBP-1 and its target genes, acetyl-CoA carboxylase and fatty acid synthase, and increases the expression of genes involved in free fatty acid catabolism, promoting a strong hypolipodemic effect *in vitro* and *in vivo*. Interestingly, NDGA is able to decrease the plasmatic cholesterol in high-fat diet (HFD)-fed C57BL/6J mice (Arteaga et al., 2005; Chen, 2009; Lee et al., 2010; Zhang et al., 2013).

The antiviral effect of NDGA and its derivatives has been reported in several studies involving RNA viruses (HCV, HIV, SIV and Influenza virus) (Gnabre et al., 1995; Huang et al., 2003; Syed and Siddiqui, 2011; Uchide et al., 2005; Uchide and Toyoda, 2008) and DNA viruses (poxvirus, HSV and HPV) (Craigo et al., 2000; Park et al., 2003; Pollara et al., 2010). Currently, neither antiviral treatments nor vaccines against DENV are available. Thus, efforts directed to search new therapeutic targets and molecules that inhibit viral infection are required. In this work, we reported the antiviral effect of the potent lipid lowering drug NDGA on DENV infection using the hepatocarcinoma derived Huh-7 cells. Our results show that the hypolipidemic effect of NDGA is able to inhibit DENV infection at least by two mechanisms; through the reduction of viral genome replication and the inhibition of virion assembly.

2. Materials and methods

2.1. Cell culture and viral strain

Huh-7 cells, a differentiated hepatocyte derived cellular carcinoma cell line, (a gift from Dr. Ana Maria Rivas, Autonomous University of Nuevo León) and Vero cells (Green monkey kidney cells) were grown in advanced DMEM supplemented with 2 mM glutamine, penicillin (5×10^4 U/mL)-streptomycin (50 μg/mL), 5% fetal calf serum (FCS) and 1 mL/L of amphotericin B (Fungizone) at 37 °C and a 5% CO_2 atmosphere. Vero-derived cell line, stably transfected with DENV4 encoding luciferase reporter replicon, was grown in advanced DMEM supplemented with G418 (Geneticin) as selective agent. The human monocytic cell line, constitutively expressing DC-SIGN (U937-DC SIGN) receptor was a kind gift from Dr. Aravinda M. de Silva from the University of North Carolina School of Medicine, was grown in RPMI Advanced medium supplemented with 5% FBS, 2 mM glutamine, 2X penicillin/streptomycin and 5 mL/L fungizone at 37 °C and a 5% CO_2 atmosphere.

Propagation of DENV serotype 4, H241 strain and DENV serotype 2, New guinea strain was carried out in CD1 suckling mice and viral titers were determined by plaque assays in BHK-21 cells as was previously described (Mosso et al., 2008). Brains extracts from mock infected CD1 suckling mice were used as control.

2.2. DENV infection and NDGA treatment

The protocol of infection and treatment was performed in the same manner for all experiments. Briefly, the cells were washed three times with Hanks medium, and infected with DENV (serotype 2 or 4) at the MOI indicated in serum free medium. The infection was allowed to proceed for 2 h at 37 °C; then, cells were washed with acid glycine (pH 3) to inactivate non-internalized virus, washed 3 times with PBS and cultured in NDGA containing advance DMEM supplemented with serum for 24 or 48 h at 37 °C. The treatment with NDGA was performed at 35, 50 and 100 μM in complete medium from a stock of 50 mM diluted in DMSO, the maximum concentration of DMSO was 0.5%, the 0 μM of NDGA was only the vehicle (medium with DMSO at 0.5%).

2.3. Determination of total cholesterol and cell viability

Huh-7 cells grown in 6 well plates were infected and treated as described above. Then, cells were washed 3 times with PBS and lysed with 250 μL of lysis buffer RSB-NP40 (1.5 mM $MgCl_2$, Tris–HCl pH 7.5 10 mM, 10 mM NaCl, 1% IGEPAL) at 24 and 48 h of treatment. 50 μL of the lysate plus 50 μL of solution cholesterol oxidase–peroxidase (Biosystems, Barcelona, Spain) were placed in a 96 well-plate. Reactions were permitted for 10–15 min at 37 °C and the absorbance at 490 nm was measured in an ELx808 BioTek plate reader.

Cell viability of Huh-7 cells and Vero cells was evaluated by the MTS reduction according with the manufacturer's protocol (cell titer 96 Aqueous One Solution Cell Proliferation Assay Promega). The cell viability of U937-DC SIGN cells was performed by flow cytometry through the Propidium Iodide (PI) uptake.

2.4. Viral yield and NS1 secretion analysis

Supernatants of infected and treated cells were analyzed for viral yield by plaque assay as previously described and for NS1 secretion by ELISA (Platelia, Biorad) (Ludert et al., 2008).

2.5. Luciferase activity and real time PCR analysis

Luciferase activity of NDGA treated Vero cells expressing a DENV 4 replicon was measured as indicated in the kit protocol (Renilla luciferase assay system, Promega). To determine viral genome, total RNA was obtained using the kit Zymo Research Quick-RNA (MicroPrep). The *q*RT-PCR was conducted by the SYBR green method using the primers of NS5 reported by Chien et al. (2006) in the ECO ILLUMINA system. The amount of viral RNA transcripts was calculated by generating a standard curve from 10-fold dilutions of RNA isolated from a DENV-4 and DENV-2 preparation titrated in BHK-21 cells and expressed as plaque forming units equivalents per milliliter (PFU equivalents/mL). (Callahan et al., 2001; Chien et al., 2006; Johnson et al., 2005)

2.6. Western blot analysis

Huh-7 cells grown in p100 plates were mock or DENV infected and treated with NDGA. At 24 or 48 h post infection, cells were lysed with the lysis buffer described for total cholesterol determination, containing protease inhibitors. The lysate was clarified by centrifugation (10,000 RPM) and 50 μg of protein were assayed by SDS–PAGE and immunoblotting using rabbit polyclonal antibody directed to NS3 and C protein (GENETEX). Densitometry of bands corresponding to the viral proteins was performed using the ImageJ software (National Institutes of Health) and adjusted with its load control (β actin) densitometry. The adjusted densitometry of each condition was normalized with 0 μM NDGA-infected condition and expressed as fold change.

2.7. Immunofluorescence and flow cytometry analysis

Huh-7 cells grown in slides and in 6-wells plates, for confocal microscopy and FACS assays, respectively, were infected and treated as indicated before. At 24 or 48 h of infection, slides or harvested cells were fixed with 1% formaldehyde, incubated for 20 min with permeabilized solution (PBS 1X, saponin 0,1% and FBS 1%), and incubated for 2 h at room temperature with anti-NS3, anti-Capsid or anti-E monoclonal antibodies and detected with donkey anti mouse-Alexa 488 or goat anti rabbit-alexa 555. Lipid droplets were stained with the fluorescent dye bodipy 493/503 (Molecular probes). Slides were observed in a Zeiss LSM700 laser confocal microscopy. Flow cytometry was performed in a BD LSR Fortessa quantifying 10,000 events.

3. Results

3.1. NDGA inhibits DENV infection

Since NDGA has an hypolipidemic effect in *in vitro* and in *in vivo* systems, the influence of NDGA in DENV infection was evaluated in the hepatoma cell line Huh-7 cells. Initially, the amount of total cholesterol was quantified in untreated (DMSO 0.5%) and NDGA treated cells (35, 50 and 100 μM). A reduction up to 33% of total cholesterol levels was achieved in cells treated for 48 h with the highest concentration of NDGA (100 μM) (Fig. 1B). This reduction in cholesterol levels, was not associated with a reduction in cell viability, since none of the concentrations were deleterious for the cells (Fig. 1A).

The next step was to analyze the effect of NDGA in DENV infection. Two different parameters of DENV replication were evaluated; levels of NS1 protein secreted and viral yield. Both parameters were quantified in supernatants of infected Huh-7 cells untreated or treated with NDGA. The levels of secreted NS1, determined by ELISA, were reduced up to 92% with 100 μM of NDGA at 24 h of treatment (Fig. 1C). A statistically significant reduction in viral yield, (up to one log) was achieved with 100 μM of NDGA at 24 h post-treatment (Fig. 1C). Although the inhibitory effect in NS1 secretion and viral yield was also observed after 48 h of treatment, this effect was less dramatic (Fig. 1D) probably due to NDGA getting metabolized during prolonged incubation.

Since NDGA was added after DENV entry, these results suggest that the inhibitory effect of NDGA in DENV infection may be during replication and or morphogenesis.

3.2. NDGA inhibits DENV replication

The first step in DENV replicative cycle after viral entry is viral translation/replication. To evaluate the effect of NDGA in viral translation/replication two different approaches were used. First, a Vero-derived cell line, stably transfected with DENV4 encoding luciferase reporter replicon (kindly donated by Dr. Padmanabhan, Georgetown University Washington DC), was treated with 35, 50 and 100 μM of NDGA. At 24 and 48 h post treatment, the luciferase activity was measured. A concentration dependent decrease, from a half a log to one log, in the luciferase activity was observed in the NDGA-treated cells compared with untreated cells (Fig. 2B). The effect appears to be concentration dependent. A similar, but less dramatic, inhibitory effect in luciferase activity was observed after 48 h of treatment with NDGA, respect to control cells (Fig. 2B). None of the concentrations of NDGA cause a reduction in the Vero-derived cell viability (Fig. 2A), suggesting that NDGA induces an inhibitory effect in translation/replication of DENV. The second approach used to evaluate the effect of NDGA in replication was to quantify the amount of viral proteins by Western blot and viral RNA by qRT-PCR in NDGA treated DENV infected Huh-7 cells. A statistically significant reduction in the amount of NS3 protein was observed after treatment with NDGA for 24 (95%) or 48 h (60%) (Fig. 2C). Moreover, up to 3-log decrease in PFU equivalent/mL of viral RNA from DENV2 and DENV4 was detected after treatment with 50 and 100 μM of NDGA (Fig. 2D). These results confirm that NDGA induces an inhibitory effect on DENV genome replication.

It has been reported that DENV E and NS3 proteins colocalize with dsRNA in the RC (Anwar et al., 2011). Thus, the integrity of RC was evaluated in infected Huh-7 cells treated with NDGA by analyzing the distribution and the colocalization rate of both DENV proteins. In infected and untreated Huh-7 cells, a compact and perinuclear distribution for both E and NS3 proteins was observed (Fig. 3A) with a high colocalization coefficient (0,80) (Fig. 3B and C). However, in the infected and NDGA-treated cells a diffuse perinuclear distribution of DENV proteins (Fig. 3A) and a reduction in the colocalization coefficient (0,45) of NS3 with E was observed (Fig. 3B and C), suggesting that the hypolipidemic effect of NDGA altered the RC structure. The modification in the distribution of both viral proteins may be responsible, at least in part, for the inhibition in replication-of DENV genome observed in NDGA treated cells.

3.3. NDGA inhibits DENV assembly

Since it has been described that NDGA reduces HCV assembly through a reduction of LDs (Syed and Siddiqui, 2011), and DENV also uses these structures for viral assembly (Samsa et al., 2009), the effect of NDGA in DENV assembly was analyzed. The infection level as well as the amount of LDs was evaluated by flow cytometry in uninfected and infected Huh-7 cells untreated or treated for 48 h with 0 (vehicle), 50 and 100 μM of NDGA. In cells treated with the vehicle alone, approximately 30% of the cells infected with DENV4 were positive for E protein and a similar percentage were positive for LDs (Fig. 4A and B). The percentage of E protein positive cells as well as the LD positive cells decreased in the presence of NDGA. In order to determine if infected cells and cells positive for LDs were the same population, a double staining was performed. A population of approximately 30% of the infected and untreated cells was positive for both, E protein and LDs (Fig. 4C). This double positive population was decreased in a dose dependent manner in the presence of 50 and 100 μM of NDGA, The results suggest that DENV infection increases the amount of lipid droplets and NDGA reduces the amount of infected cells through a reduction in the levels of LDs.

It is well known that for DENV assembly the binding of C protein to LD periphery is required. To analyze whether DENV assembly is altered by the LD reduction promoted by NDGA treatment, the distribution of C protein and LD was analyzed. In untreated and infected cells an increase in LDs was observed compared with uninfected cells, correlating with the results obtained by flow cytometry. The localization of LDs matches with the perinuclear

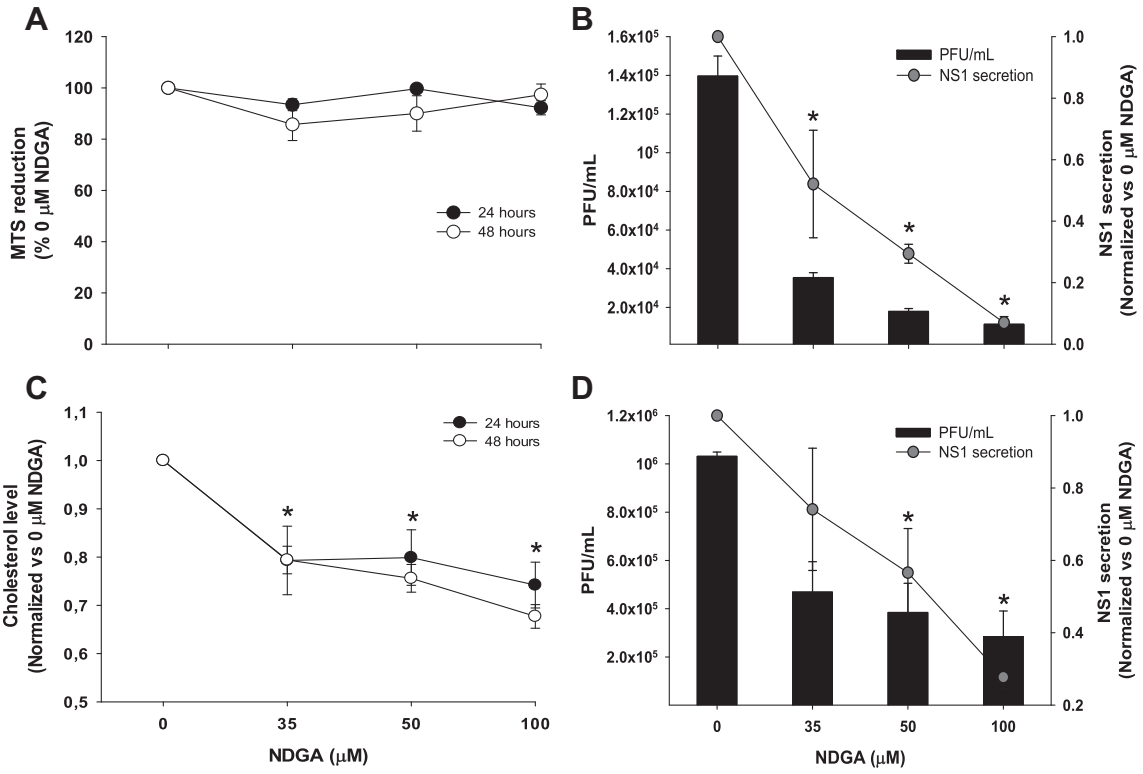

Fig. 1. Validation of NDGA treatment and it effect on viral yield and NS1 secretion in infected and treated Huh-7 cells. The cells treated with NDGA (35, 50 and 100 μM) or vehicle (0 μM), were assayed for viability by MTT reduction (A) and quantification of cholesterol levels by colorimetric assay (B) at 24 and 48 h of treatment. Supernatants of Huh-7 cells infected with DENV 4 and treated with NDGA as described before for 24 (C) and 48 h (D) were analyzed for viral yield (black bars) by plaque assay and NS1 secretion (line) by ELISA. The cell viability, cholesterol levels and NS1 secretion are expressed as mean of normalized absorbance ± SEM. Viral yield is expressed as mean ± SEM of PFU/mL of three independent experiments. $^{*}P < 0.05$.

distribution of the C protein, as can be seen in Fig. 4D panel 0 μM of NDGA. In contrast, in infected and treated cells the distribution pattern of C protein is altered and a significant decrease in the amount of LDs and C protein is observed. This observation supports the idea that NDGA treatment disrupts the binding of C protein with lipid droplet through an effective decrease in the amount of LD and this effect may be translated to an inhibition of viral assembly. The lower amount of C protein observed in treated cells compared with untreated cells was confirmed by WB assays (Fig. 4D).

In order to study whether the antiviral effect of NDGA against DENV infection in hepatic cell line Huh-7 is reproducible in other cell lines, Vero cells and U937-DC SIGN cells were infected and treated with NDGA. Vero cells, infected with DENV2 and 4 were treated with NDGA as described before, at 48 hpi the cells were harvested, fixed, stained for E protein and the amount of infected cells were analyzed by flow cytometry. A 40 and up to 50% reduction in the amount of infected Vero cells was observed with both, DENV 2 and DENV 4 with a treatment of 100 μM of NDGA (Fig. 5A). Similarly, U937 DC-SIGN cells showed a 50% reduction in DENV4 infection when treated with NDGA at 50 μM for 24 h. None of the concentrations of NDGA reduced cell viability in Vero and U937-DC SIGN cells (data not shown).

4. Discussion

The central role of lipids and cholesterol in DENV infection has been demonstrated in part by the fact that several compounds that decrease intracellular levels of cholesterol (statins) and/or free fatty acid (C75 compound) inhibit DENV infection (Lee et al., 2008; Martinez-Gutierrez et al., 2011; Rothwell et al., 2009; Soto-Acosta et al., 2013). Under this perspective, in the present study the antiviral action of the broad-spectrum lipid-lowering drug NDGA on DENV infection using the hepatic cell line Huh-7 was evaluated.

The hypocholesterolemic effect of the NDGA in the Huh-7 cells was corroborated since treatment with 35 μM for 24 and 48 h decreased cholesterol levels as it has been described in other systems (Kelley et al., 2004; Zhang et al., 2013). The mechanism underlying the NDGA-lipid lowering action is based in two main effector molecules. The NDGA treatment performed in *in vivo* and in *in vitro* systems, increases AMPKα activity and the PPARα at mRNA and protein levels. AMPK is a cellular energy regulator that during energy depletion inhibits lipogenesis. It has been reported that NDGA and other polyphenols induce an increase in the AMPK activity through its phosphorylation. The activated AMPK interacts and directly phosphorylates SREBP-1c and -2 (sterol regulatory element binding proteins). SREBPs are lipogenic transcription factors that activate genes involved in fatty acid (SREBP-1c) and cholesterol synthesis (SREBP-2). Both transcription factors are synthesized as precursors proteins inserted into the endoplasmic reticulum membrane. Under sterol depleted conditions, the precursor proteins are transported to the Golgi compartments where they are processed by Golgi-resident S1 and S2 proteases to produce active/mature factors that migrate to the nucleus and promote transcription of target genes harboring sterol regulatory element (SRE). The AMPK interaction and resultant phosphorylation of SREBP-1c and 2 promoted by NDGA inhibits the cleavage of mature SREBP and their nucleus translocation preventing the transcription of fatty acid synthase (FAS) and 3-hydroxy-3-methyl-glutaryl-CoA reductase (HMGCR), limiting enzymes in fatty acid and cholesterol synthesis, respectively. (Lee et al., 2010; Li and Chiang, 2009; Li et al., 2011; Zhang et al., 2013). On the other hand,

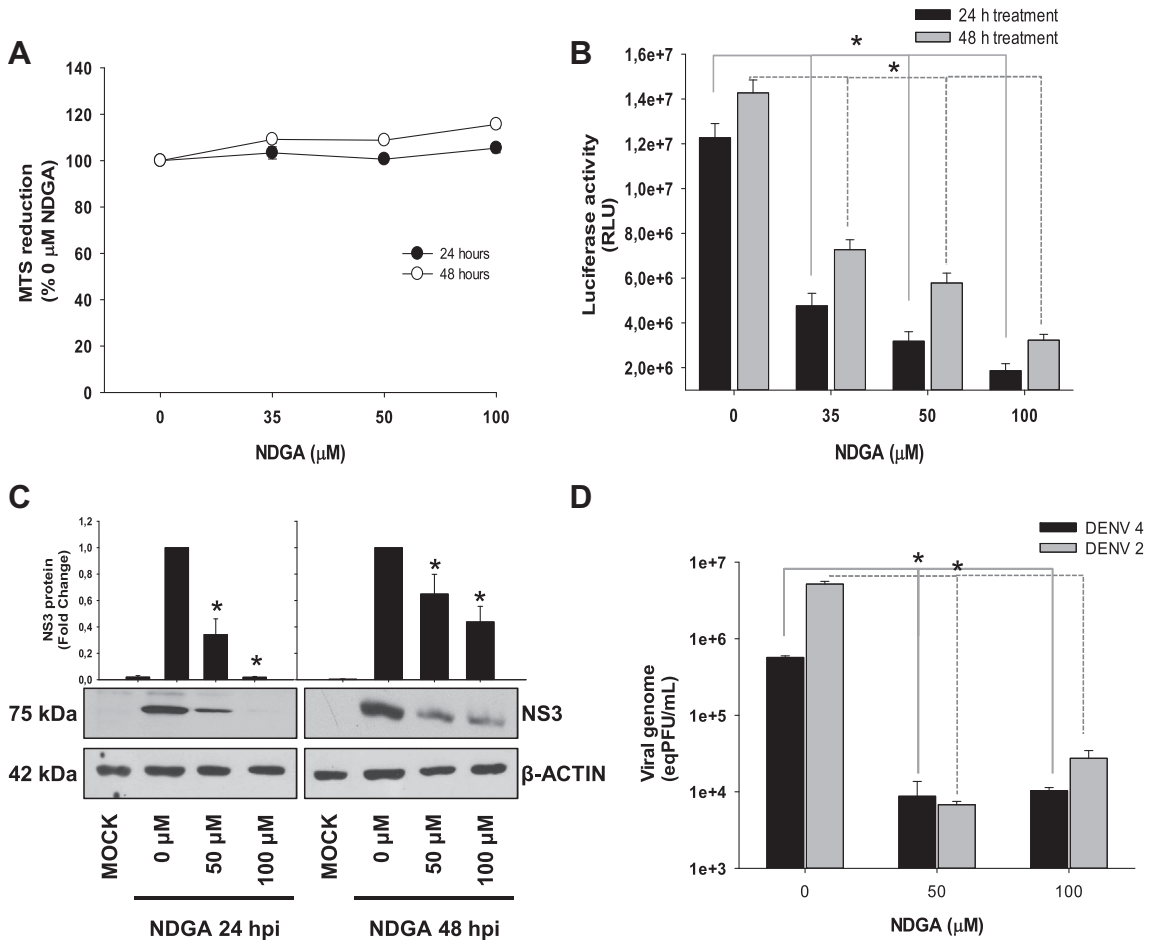

Fig. 2. Effect of NDGA in replication of viral genome. Vero cells, expressing DENV 4 replicon which encodes luciferase gene, were treated with NDGA (35, 50 and 100 μM) or vehicle (0 μM) for 24 and 48 h and analyzed for cell viability by MTT reduction (A) and luciferase activity assay (B). The cell viability is expressed as mean of normalized absorbance ± SEM. Luciferase activity is expressed as relative luciferase units (RLU) and the data is mean ± SEM of three independent experiments. Additionally, infected Huh-7 cells were treated with NDGA or vehicle and the amount of viral protein NS3 (C) and viral genome (D) was assayed by Western blot (24 and 48 h of treatment) and qRT-PCR (24 h of treatment), respectively as described in Section 2. The amount of NS3 protein is expressed as fold change value ± SEM obtained of densitometric analysis of three independent assays. Viral genome is expressed as mean ± SEM of eqPFU/mL of three independent experiments. $^{*}P < 0.05$.

it has been described that NDGA increases PPARα at mRNA and protein levels in livers of rodent models and in the hepatocyte cell line HepG2. PPARα induces free fatty acid oxidation. In the liver, fatty acid β oxidation occurs in mitochondria and peroxisomes meanwhile ω oxidation is carried out in microsomes. It is believed that the NDGA lipid-lowering effect through PPARα is mediated by an increase in genes and proteins involved mainly in mitochondrial β oxidation (Lee et al., 2010; Li et al., 2011). Thus, the hypocholesterolemic effect induced by NDGA in the hepatic cell line is concerted with a reduction of free fatty acid and triglycerides in the intracellular environmental and these effects have a common molecular mechanism.

4.1. NDGA reduces viral yield and NS1 secretion in Huh-7 dengue virus infected cells

A decrease of viral yield and NS1 secretion was observed in cells treated from 35 and up to 100 μM of NDGA for 24 and 48 h. This result correlates with the reduction in cholesterol levels, suggesting that the lipid lowering effect of NDGA is related with the decrease in DENV infection. However, the precise step in DENV replicative cycle blocked after treatment was not identified. Lipid dependence has been described in different steps along the DENV replicative cycle (entry, replication and assembly) (Lee et al., 2008; Puerta-Guardo et al., 2010; Reyes-Del Valle et al., 2005). Although NDGA could promote cholesterol reduction in the plasma membrane of treated cells, as it has been described for other compounds such as statins (del Toro et al., 2010; Zhuang et al., 2005), our observations do not take into account this property of NDGA as the treatment was commenced after infection of the Huh-7 cells with DENV.

4.2. NDGA inhibits DENV genome replication process

The dose-dependent reduction in luciferase activity of the DENV replicon observed in NDGA treated Vero cells suggests that NDGA inhibited viral replication. This conclusion was supported by a reduction in viral RNA and protein synthesis levels in NDGA treated cells. These results suggest that NDGA is able to decrease DENV replication. Syed and Siddiqui (2011), observed that NDGA decreases specifically the HCV genome replication, without affecting translation, however in this study we did not discriminate between these two processes. The upregulation of AMPK activity by NDGA, which in turn, inhibits the synthesis of FAS and HMGCR mainly, through the inhibition of nuclear translocation of SREBP-1c and -2 may be the primary mechanism behind NDGA-mediated reduction in DENV replication. These results are in agreement with previous studies reporting the susceptibility of DENV replication to the treatment with lipid lowering drugs (Martinez-Gutierrez et al., 2011; Martinez-Gutierrez et al., 2014; Rothwell et al., 2009). To study the possibility that NDGA alters the replicative complex

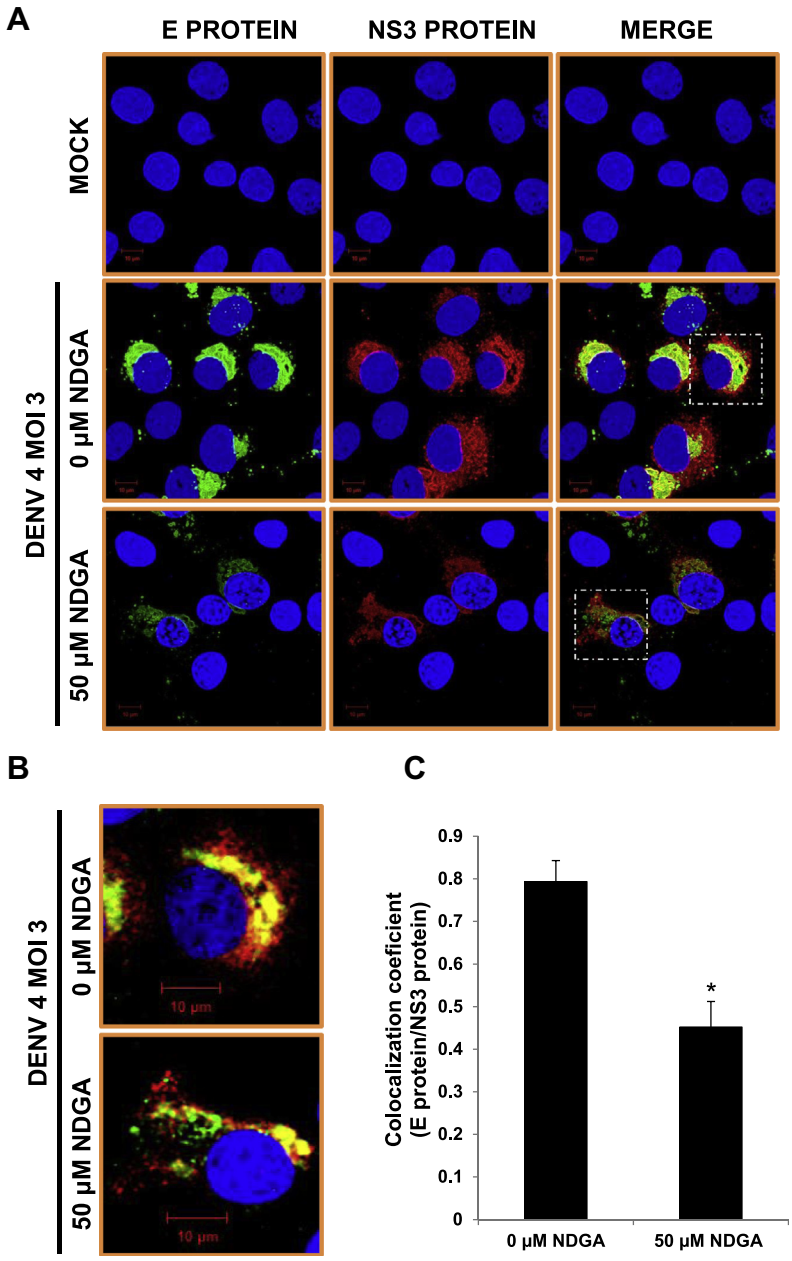

Fig. 3. Effect of NDGA on the distribution pattern of viral proteins. Huh-7 cells were infected with DENV 4 and treated with vehicle or 50 μM of NDGA. After 24 h of treatment the cells were fixed and stained for E (green) and NS3 protein (red). Nuclei were counterstained with Hoescht (Blue). (A) Distribution pattern of viral proteins was analyzed by confocal microscopy, (B) selected area (white box), amplified 2.5X showing the delocalization of viral proteins. The images are representatives of three independent experiments. (C) The column graph compares the colocalization coefficient ± SEM between both viral proteins (E protein/NS3 protein) of infected cells untreated and treated with NDGA. $^{*}P < 0.05$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

structure, confocal analysis were performed in Huh-7 cells infected and treated with NDGA. The results indicate that NDGA promotes a change in the distribution pattern and a decrease in the colocalization levels of E and NS3 viral proteins in the replicative complexes. Considering the possible role of fatty acid and sphingolipids in the formation of replication complex and the direct effect of NDGA in lipid metabolism, these results suggest that NDGA alters the structure of replication complex. The role of the SREBP-2 pathway in the membrane rearrangement is less clear as the inhibition of HMGCR using lovastatin did not inhibit this process induced by DENV in the Human fibrosarcoma 2fTGH cell line (Pena and Harris, 2012). However, the activation of AMPK induced by NDGA and the concerted down regulation of lipogenesis pathways in the hepatic cell line may have an effect in the replicative complex structure. Further studies with AMPK activators would help to determine the role of AMPK in DENV replication. In addition, the potent antioxidant property of NDGA may also be involved in the antiviral effect observed for DENV infection (Chen, 2009; Czapski et al., 2012). DENV infection promotes oxidative stress and increases the generation of reactive oxygen species (ROS) in *in vitro* and *in vivo* systems. When this condition is reverted using antioxidant molecules such exogenous GSH (glutathione) and N-acetylcysteine (NAC) the infective process is reduced (Chen et al., 2011; Tian et al., 2010; Wang et al., 2013).

4.3. NDGA inhibits DENV assembly through the reduction of LD

DENV genome encapsidation is a crucial step in the viral particle assembly and lipid structures, including lipid droplets, are involved in the process (Carvalho et al., 2012; Samsa et al.,

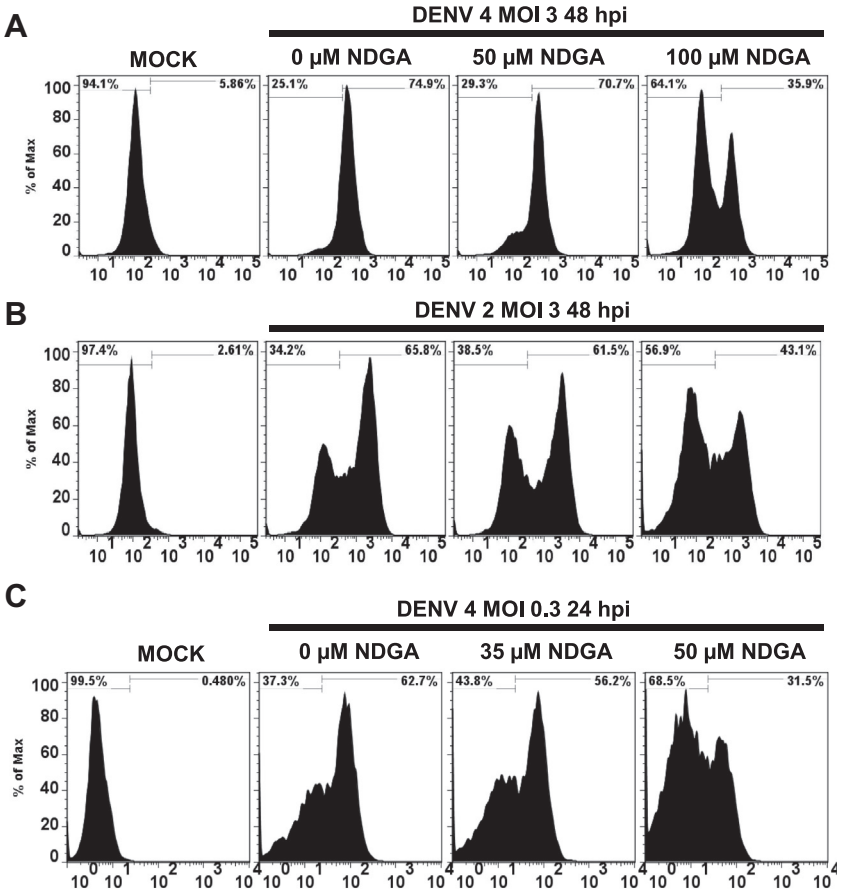

Fig. 5. Antiviral property of NDGA against DENV in Vero and DC SIGN-U937 cells. Vero (A) and DC-SIGN cells (B) were infected with DENV at a MOI of 3 (A) or 0.3 (B) and treated with NDGA. The histograms indicate the mean fluorescence intensity and the amount of infected cells and are representatives of three independent experiments.

2009). In this work the inhibitory effect of NDGA on viral assembly was evaluated using two parameters: first, the amount of LDs positive cells by flow cytometry and second, the colocalization between LD and C protein by confocal microscopy. Our first observation was that as expected, during DENV infection the amount of LD increases, however, NDGA treatment dramatically reduced the abundance of LDs in the infected cells. These results are consistent with previous observations (Samsa et al., 2009). Our second observation was that after NDGA treatment, the amount of C protein decreased in the infected cells, but even more relevant was the fact that the distribution of C protein was significantly alter in treated cells. Although C protein was observed mainly in the cytoplasm, the amount of this protein was increased significantly in the nucleus of treated and infected cells compared with untreated cells. The modification in abundance and distribution of C protein may be related with the reduction of LDs formation induced by NDGA treatment (Fig. 4). Both observations are congruent with the reduction in viral yield observed in treated and infected cells, suggesting that NDGA also alter viral assembly.

In this work the modification of DENV secretion pathway was not evaluated; however, this process is also altered in NDGA treated cells. Virions and NS1 traffic occurs through the host cell secretory pathway where the cellular protease furin cleaves prM protein. Since lipid metabolism and specifically assembly of lipid microdomains is required for the biogenesis of secretory vesicles from the trans-Golgi-network, it would be expected that the cholesterol lowering effect of NDGA inhibit DENV and NS1 secretion through the inhibition of secretory pathway (Fujiwara et al., 1998; Stadler et al., 1997; Yu et al., 2008). Additionally, it has been reported that NDGA inhibits the VLDL biosynthesis and secretion by inhibition of essential genes for VLDL secretion pathway. (Syed and Siddiqui, 2011). Recently, it has been reported that DENV C protein is able to interact with Very Low Density Lipoprotein (VLDL), specifically with Apo E, which is the main surface apolipoprotein in VLDL (Faustino et al., 2014; Samsa et al., 2009). Moreover, it has been proposed that the interaction between HCV C protein and VLDL ApoE may allow the formation of lipoviroparticles (LVP) (Andre et al., 2002). The inhibition of VLDL synthesis could be other mechanism operating in the antiviral effect of NDGA against DENV as has been reported for HCV by Syed and Siddiqui (2011).

4.4. Anti-DENV effects of NDGA are reproducible in other cell lines

The broad-spectrum lipid lowering and antioxidant properties of NDGA described before, was also observed in Vero (infected with DENV 2 and DENV4) and U937 cells (infected with DENV 4) even though DENV infectivity in these cell lines was higher than the infection observed in Huh-7 cells. These results support the possible use of drugs such as NDGA for DENV treatment.

In conclusion, the results of this work clearly indicate that the hypolipidemic and antioxidant drug NDGA had an antiviral effect against DENV inhibiting at least on two important viral processes, the genome replication and viral assembly. Further studies directed to determine the role of AMPK in the antiviral effect induced by NDGA are been performed in our laboratory.

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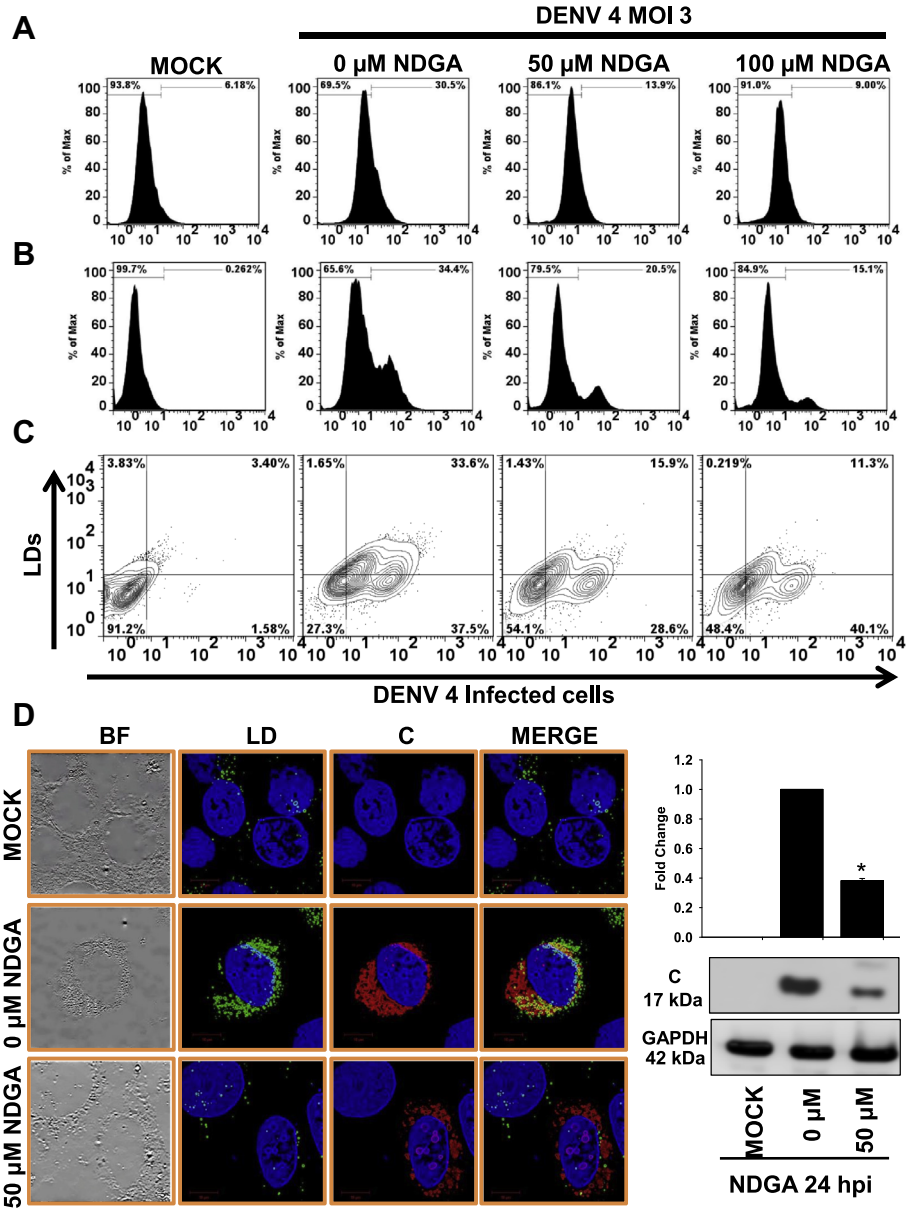

Fig. 4. Effect of NDGA on LDs and capsid protein levels and distribution patterns. Huh-7 cells were infected with DENV and treated with vehicle, 50 and 100 μM of NDGA. The amount of LDs and infected cells were assayed by flow cytometry. The histograms indicate the mean fluorescence intensity and the amount of positive cells for LDs (A) and infection (B). The density plots indicate the population positive for both LDs and infection (C, right upper quadrant). The histograms and plots are representative of three independent experiments. The effect of NDGA in the distribution pattern of LDs and capsid protein observed by confocal microscopy was confirmed by Western blot assay (D). $^{*}P < 0.05$.

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