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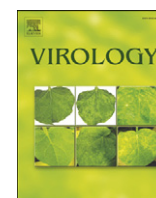
JNK phosphorylation, induced during dengue virus infection, is important for viral infection and requires the presence of cholesterol

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ABSTRACT

Infection with a broad diversity of viruses often activates host cell signaling pathways including the mitogen-activated protein kinase pathway. The present study established that dengue virus infection of human macrophages activates Jun NH(2)-terminal kinase (JNK) and the p38 MAPKs pathways. The activation was observed at early times after infection and occurs when either infectious or UV-inactivated dengue virus was used. The role of these activated kinases in dengue virus infection was evaluated using specific inhibitors. Inhibition of JNK and p38 kinases did result in a significant reduction in viral protein synthesis and in viral yield. Additionally, lipid rafts disruption induced a strong inhibition of JNK activation. These results suggest that, at early stages after dengue virus infection, MAPKs are activated and that activation of JNK and p38 is required for dengue virus infection.

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Introduction

Dengue fever is the viral infection transmitted by mosquitoes most widespread in the world. It is estimated that 2500 million people live in areas at risk of disease transmission (Guzman and Kouri, 2002). The causative agent of dengue fever and its complications is dengue virus (DEN), a member of the family *Flaviviridae* and the genus Flavivirus (Vaughn et al., 2000). Four virus serotypes can be recognized (DEN-1, DEN-2, DEN-3, and DEN-4) and multiple genotypes within each serotype have been identified (Guzman and Kouri, 2002). Morphologically, DEN is a roughly spherical particle of 50 nm in diameter, which contains a capsid of 30 nm surrounded by a lipid envelope (Lindenbach and Rice, 2001). The virus contains two surface proteins, the protein of the envelope (E) and the membrane protein (M). The E glycoprotein contains most of the antigenic determinants of the virus and is essential for viral entry (Modis et al., 2003; Roehring, 2003; Crill and Roehring, 2001; Mandl et al., 2000; Heinz, 1986; Henchal et al., 1985).

The first step in DEN infection is the binding of the mature virion to the receptor present on the cell surface of the host cell. This interaction induces its internalization by receptor-mediated endocytosis and subsequent fusion of the viral with the endosomal membranes, releasing the viral genome into the cytoplasm (Hase et al., 1989; Lim and Ng, 1999). Several molecules have been described as DEN receptors including heparan sulfate (Chen et al., 1997) and unidentified proteins in mosquito (Salas-Benito and del Angel, 1997; Muñoz et al., 1998; Mercado-Curiel et al., 2006; Mercado-Curiel et al., 2008) and mammalian cells (Martínez-Barragán and del Angel, 2001;

Bielefeldt-Ohmann et al., 2001; Ramos et al., 1997; Moreno-Altamirano et al., 2002). Additionally, other molecules have also been involved in DEN attachment and entry, of importance are C-type lectins receptors. Two of these receptors are DC-SIGN in dendritic cells and L-SIGN in hepatocytes (Navarro et al., 2003; Tassaneetrithep et al., 2003; Lozach et al., 2005) and the mannose receptor (Miller et al., 2008). These lectins bind DEN through the recognition of the mannose residues present on DEN E glycoprotein. The DC-SIGN expression is clearly capable of rendering cells permissive to DEN infection (Lozach et al., 2005), indicating that this molecule is important in viral attachment and/or entry. An additional C-type lectin involved in DEN infection is CLEC5A, which is present on the surface of human macrophages. The blockage of CLEC5A-mediated signaling attenuates TNF-α and proinflammatory cytokines production and inhibits plasma leakage, indicating that signaling through the CLEC5A pathway is required for viral pathogenesis (Chen et al., 2008).

Some other molecules have also been involved in DEN infection such as GRP78 and 37/67 kDa high-affinity laminin receptor, which have been described as the HepG2 cell receptors (Jindadamrongwech et al., 2004; Thepparit and Smith, 2004), and the heat shock proteins HSP70 and HSP90 that have been proposed as part of DEN receptor in neuroblastoma cells, human U937 macrophages, and peripheral blood monocytes (Reyes del Valle et al., 2005).

It is remarkable that the proteins so far identified as DEN receptors play important roles in innate immune response and in pathogen recognition, as well as in cellular signaling. Since DEN virus uses different molecules, organelles, and structures to enter into the cells, it is likely that viral infection will activate several cellular signaling pathways. One of the pathways activated during DEN infection is the mitogen-activated protein kinases (MAPK) pathways (Huerta-Zepeda et al., 2008). MAPKs are signaling components that are important in

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converting extracellular stimuli into a wide range of cellular responses (Raman et al., 2007). The extracellular signal-regulated kinases (ERK 1/2) are activated by mitogens and are found to be upregulated in human tumours. Two other major MAPK pathways, the Jun N-terminal kinase (JNK) and p38 MAPK pathways, which are also called stress-activated protein kinase pathways, are activated by environmental and genotoxic stresses as well as viral infection and have key roles in inflammation and in tissue homeostasis (Holloway and Coulson, 2006; Wolf et al., 2008; Monick et al., 2005). In endothelial vascular cells, DEN virus infection up-regulates protease-activated receptor type-1 and tissue factor receptor via the phosphorylation of two of the MAPKs, p38 and ERK 1/2 (Huerta-Zepeda et al., 2008), suggesting that for DEN, these kinases could be involved in viral pathogenesis. However, it is unknown if these MAPKs pathways could also be activated by DEN in other cell types and if their activation is relevant for viral replication. In this article, the activation of MAPKs pathways in macrophages, one of the main target cells for DEN virus, was analyzed, as well as the importance of the activation of these important signaling pathways in viral infection.

Results

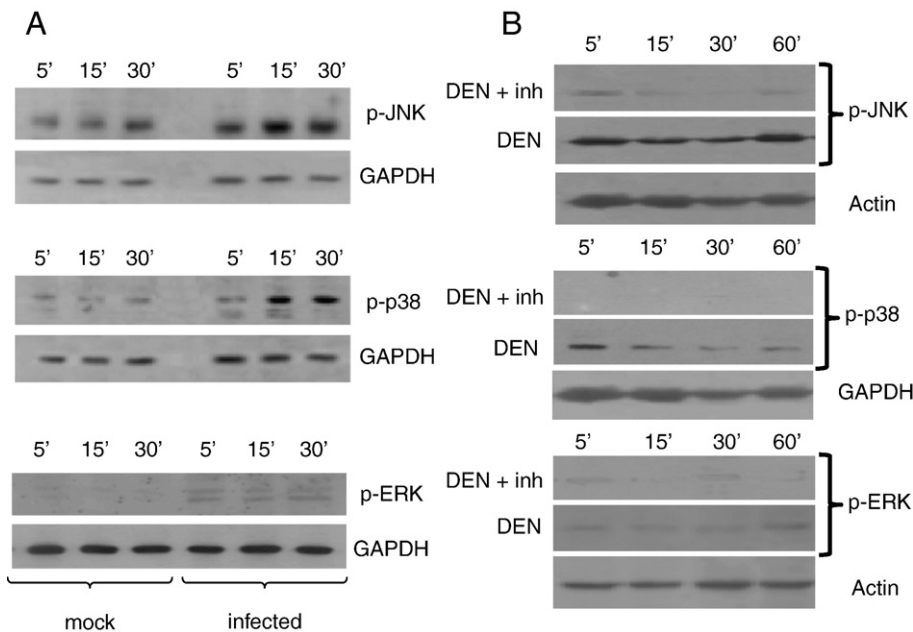### DEN infection induces activation of the MAPKs: JNK, ERK, and p38

For other viral systems, it has been observed that the MAPKs are activated early during viral infection (Holloway and Coulson, 2006; Si et al., 2005). Thus, the presence of the phosphorylated forms of JNK, ERK, and p38 was analyzed from 5 to 30 min post-infection by Western blot. All three kinases were found activated after DEN infection. However, the time course and level of activation among them differed: JNK and p38 were clearly activated at 5 and 15 min after infection and remained activated after 30 min (Fig. 1A), while ERK was only weakly activated 5 min post-infection and no further activation was observed (Fig. 1A).

To confirm that the observed MAPK phosphorylation was indeed induced by DEN infection, macrophages were incubated before infection for 30 min with specific inhibitors for each kinase (to avoid background) and afterward cells were infected with DEN in the presence or in the absence of the inhibitors. Again, as soon as 5 min post-infection, activation of JNK and p38 was observed, verifying our previous finding, while ERK was again weakly activated (Fig. 1B).

To further confirm the activation of JNK and p38 in a quantitative form, the level of the phosphorylated form of JNK and p38 as well as the amount of total JNK and p38 proteins were evaluated using a commercial ELISA kit. In addition, this kit allows correcting the level of phosphorylated and total proteins according to the number of cells analyzed. The phosphorylated form of JNK and p38 evaluated by duplicate in macrophages from five different donors was calculated as percent of total amount of JNK or p38. It can be observed that, 5 min after DEN infection, an increase in phosphorylated form of JNK was detected, and this activation became higher after 30 min (Fig. 2A). For p38, the activation was detected after 5 min and decreased slowly thereafter (Fig. 2B). It is important to notice that in uninfected human macrophages, the basal levels of phosphorylated forms of JNK and p38 varied depending on the blood donor. However, in all cases, a clear increase in the phosphorylation of JNK and p38 was detected in DEN-infected cells.

Since the activation of MAPKs occurs at early times post-infection, it may be expected that the simple interaction between the DEN virion and the host cell surface molecules may trigger cellular signaling. To analyze this possibility, cells were incubated for 15 min with viral particles inactivated with UV light for 0, 15, 30, 45, and 60 min and the phosphorylation of one of the MAPK (JNK) was determined. The level of virion UV light inactivation was evaluated by plaque assay. A proportional reduction in viral titer was observed after 15 min of UV inactivation while the exposure to UV light for 60 min completely abolished plaque formation (Fig. 3A). UV-inactivated viral particles were analyzed by electron microscopy and showed morphology compatible with intact virions (data not shown). Since equal levels of activation of JNK occurred in the presence of viral particles exposed to UV light for 0 and up to 60 min, we can conclude that viral replication is not required to activate the JNK pathway and that viral structural protein present on the virion is sufficient to induce the activation of JNK (Fig. 3B).

Fig. 1. Dengue virus infection induces activation of MAPK. (A) Human macrophages were either mock-infected or infected with DEN-2 at a MOI of 3 and at the indicated times lysed and the activation of MAPK evaluated by Western blot using antibodies specific to the phosphorylated form of the proteins. (B) Human macrophages were pretreated with specific inhibitors for each kinase before infected with DEN in the presence (DEN-inh) or absence of the inhibitors (DEN). Macrophages were lysed at the indicated times and the activation of MAPK evaluated by Western blot. The detection of either GAPDH or actin was used as a load control for the Western blots.
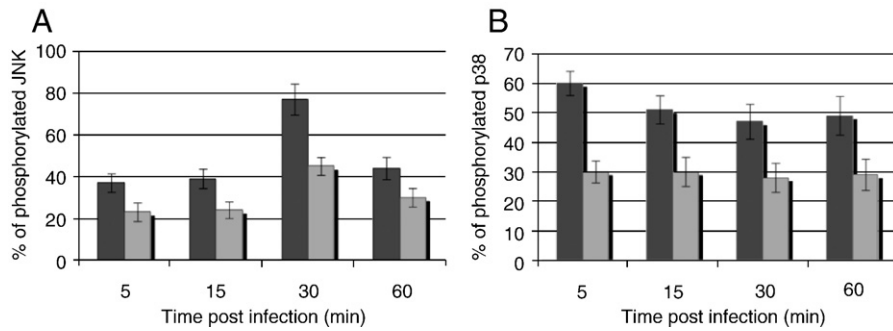
Fig. 2. Activation of JNK and p38 in dengue virus-infected macrophages quantified by ELISA. Macrophages seeded in 96-well plates were infected with dengue virus (dark grey) or mock-infected (light grey) and at the indicated times the level of phosphorylated and total JNK (A) and p38 (B) was quantified by ELISA using a commercial kit. Phosphorylated protein was expressed as percent of total protein, taken as 100% ($n = 5$).

## Inhibitors of JNK and p38 inhibit DEN infection

Given that the activation of MAPKs take place during early times after infection, its activation could either be exploited for DEN to replicate in the host cell or alternatively, it could function as a host cell antiviral response. To determine if the activation of the MAPKs, specifically p38 and JNK, was required for viral infection, macrophages were infected in the absence or in the presence of specific inhibitors of p38 and JNK. The effect of the inhibitors on the viral replicative cycle was monitored determining two parameters; the amount of secreted NS1, evaluated by PLATELIA NS1 Ag (Bio-Rad) and by the viral yield measured by plaque assay. It has been described that the amount of NS1 in the supernatants of DEN-infected cells correlates with the level of viral replication (Ludert et al., 2008). Additionally, the viral titer obtained from supernatants of infected cells is a helpful parameter to evaluate if complete replication cycle is being carried out. Even though the inhibition of the p38 and JNK phosphorylation induced approximately a 50% reduction in the amount of NS1 produced by infected cells (Figs. 4A and B), the effect of both inhibitors in viral yield was distinctive. The inhibitor of p38 reduced viral yield in less than a half of a log, while the JNK pathway inhibitor diminished virus yield in more than two logs (Fig. 4C). These results suggest that the activation of p38 is important during DEN infection; however, the activation of JNK is essential for viral infection.

## DEN infection induces nuclear translocation of AP-1

It is well known that the JNK activation induces c-Jun phosphorylation and translocation into the nucleus. C-Jun and c-Fos form the AP-1, transcriptional factor that promotes the transcription of multiple genes. To determine if JNK activation in DEN-infected macrophages causes nuclear translocation of c-Jun, the intracellular location of c-Jun in infected and uninfected macrophages was evaluated by confocal microscopy. A higher percentage of colocalization of c-Jun with DAPI staining was observed in DEN-infected macrophages ($22 \pm 3\%$) compared to uninfected macrophages ($9 \pm 1.5\%$) (Fig. 5), suggesting that the JNK activation leads to the movement of c-Jun from cytoplasm to nucleus.

Activation of JNK pathway requires the presence of cholesterol. Since JNK activation occurs early during DEN infection, it is likely that this pathway is activated by the initial interaction between DEN and attachment or receptors molecules present on the macrophages surface. It is remarkable that the molecules so far identified as dengue attachment and receptors such as DC-SIGN, heparan sulphate, GRP78, HSP90, and HSP70 are all associated to lipid rafts (Chu and Ng, 2004; Cambi et al., 2004; Philippova et al., 2008; Reyes del Valle et al., 2005). Thus, it is logical to expect that the first contact between DEN and macrophages involve the presence or formation of lipid rafts. To this respect, the relocation of HSP70 and HSP90 to lipid rafts in response to DEN infection has been documented as well as the inhibition of DEN infection after treatment of macrophages with methyl-β-cyclodextrin (Reyes del Valle et al., 2005). Considering that the first steps in DEN infection are dependent of lipid rafts formation and that JNK activation occurs early in DEN infection, we tested if JNK activation is also dependent of lipid rafts formation. To analyze this aspect, human macrophages untreated or treated with methyl-β-cyclodextrin (a drug that sequester cholesterol disrupting lipid rafts) were infected and JNK activation evaluated 30 min post-infection by Western blot. Cells were treated with a drug concentration of 10 mM since a high viability (higher than 95%) with an important reduction in total cholesterol (50% reduction) was observed under this condition (data not shown). Treatment with methyl-β-cyclodextrin totally inhibited the JNK activation induced by DEN infection (Fig. 6), suggesting that activation of this MAPK requires the presence of cholesterol and intact lipid rafts.
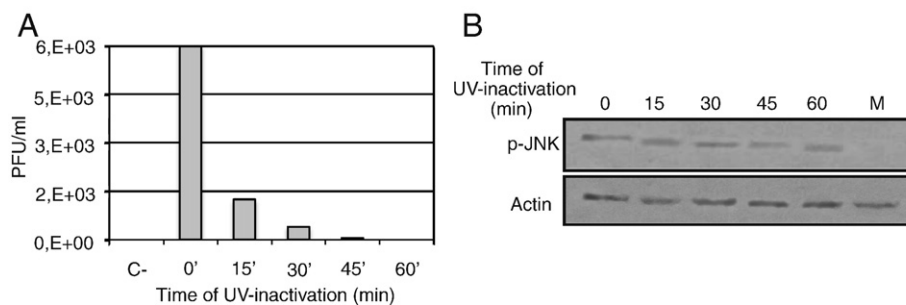

Fig. 3. UV light-inactivated DEN activates JNK pathway. DEN was UV light-inactivated from 5 to 60 min on ice. (A) Inactivation was monitored by reduction in viral titer expressed as plaque-forming units per milliliter (PFU/ml). Sixty minutes of inactivation abolished completely virus plaque formation. (B) UV light-inactivated virus from 0 to 60 min was used to infect human macrophages. Extracts from mock- infected (M) and infected cells were prepared and the activation of JNK was determined by Western blot using anti-phospho-JNK antibodies. As a control, the presence of actin was detected using an anti-actin antibody by Western blot.
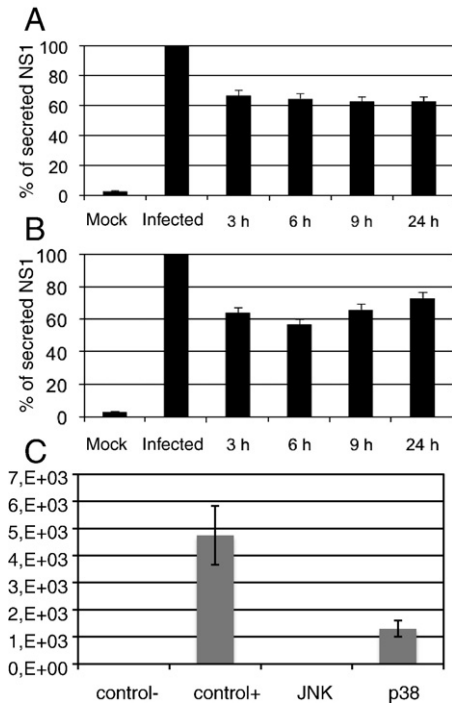
Fig. 4. Inhibitors of JNK and p38 inhibit dengue virus replication. Macrophages were pretreated for 1 h and treated during infection with specific inhibitors for JNK (A) or p38 (B). At the indicated times, supernatants were collected and the amount of secreted NS1 was measured by Platelia™ Dengue NS1 Ag (Bio-Rad). At each time after infection, the OD obtained from infected and untreated cells was considered as 100%. The OD obtained from treated cells was referred to untreated cells and expressed as percent of the control. Supernatants from mock-infected cells were included as negative controls ($n=3$). (C) Macrophages were pretreated for 1 h and treated during infection with specific inhibitors for JNK or p38. At 24 h.p.i., cells supernatant were collected and titrated for virus yield by plaque assay in BHK-21 cells. Supernatants from mock-infected macrophages (control−) and macrophages infected in the absence of inhibitors (control+) were titrated in parallel ($n=3$).

Discussion

DEN infection starts when the E protein, present on the surface of the viral particle, interacts with the cellular receptor on the surface of the host cell. This interaction initiates a chain of dynamic actions that consent to viral internalization and also activate cellular signaling. Viral infections are known to activate various cellular signaling
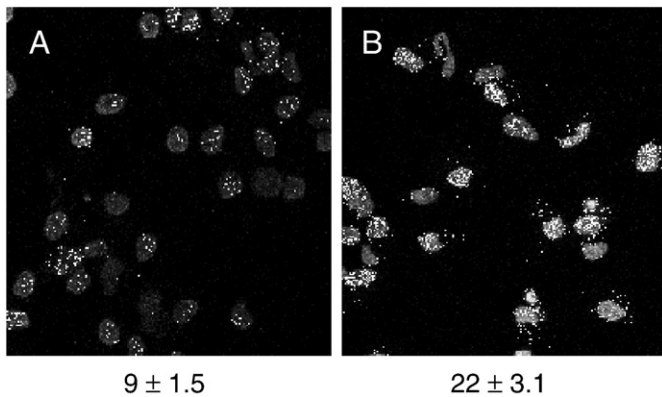
Fig. 5. c-Jun localizes to the nucleus in dengue virus macrophages. Mock-infected (A) or dengue virus-infected (B) human macrophages were fixed 30 min after infection, permeabilized, and stained with anti c-Jun antibody followed by and anti-rabbit antibody coupled with Alexa 647. Cells' nuclei were counterstained with DAPI. The c-Jun and DAPI colocalization is shown as white dots. Numbers indicate percent of colocalization ± SD, measured by confocal microscopy software LAS AS lite ($n=10$).
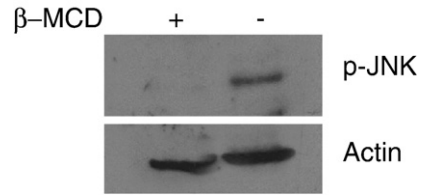
Fig. 6. Activation of JNK requires the presence of cholesterol. Macrophages were pretreated (+) or not (−) with methyl-β-cyclodextrin for 30 min and infected with DEN-2 at a MOI = 3. Fifteen minutes after infection, cells were lysed and the activation of JNK was evaluated by Western blot.

pathways, which can affect cellular function and virus replication. MAPKs are a family of proteins that serve as components of signaling pathways within cells in order to process and respond to extracellular stimuli (Raman et al., 2007). Typically, receptors on the cell surface initiate signaling cascades, which lead to phosphorylation and translocation of MAPKs to the nucleus where they regulate transcriptional activators (Whitmarsh, 2007). In recent years, it has become clear that MAPKs also regulate processes outside of the nucleus such as mRNA translation and cytoskeletal remodeling (Frevel et al., 2003; Huang et al., 2004). Three major MAPK pathways, conserved in all eukaryotic cells, are ERK 1/2, JNK, and p38 (Pearson et al., 2001). In general, the ERK 1/2 pathway is activated by proliferative stimuli, while the JNK and p38 pathways are activated by extracellular stresses such as ultraviolet light, heat, and osmotic shock (Pearson et al., 2001). Subsequent studies have shown that the p38 MAPK pathway is responsible for the phosphorylation of a large group of transcriptional and translational response elements which directly regulate the expression of a wide variety of proinflammatory cytokines (Kumar et al., 2003). In this report, we show using two different types of assays that JNK and p38 pathways were activated during DEN infection in macrophages. Although the activation of the three kinases was observed, the activation of ERK 1/2 was very weak, while the activation of JNK and p38 was strong enough to be clearly detected in Western blot assays. Interestingly, it was observed that the MAPKs were activated with UV-inactivated particles as efficiently as with infectious virions, suggesting that the initial contact between viral particle and host cells could be the step required to trigger signaling.

One important aspect to analyze was if the activation of MAPKs favors or inhibits DEN infection. To analyze this matter, macrophages were treated with specific inhibitors for JNK and p38 and the ability of DEN to replicate was determined using two main parameters: the amount of NS1 secreted to the supernatant of infected cells and viral yield. Our results clearly indicate that the inhibitors for JNK and p38 pathway lead to a reduction of viral activity, as determined by a decrease in viral yield and viral protein secretion. Although the inhibitors of both pathways induced a reduction in viral yield, the JNK inhibitors avoid viral production, supporting the idea that while the activation of p38 is important for viral infection, JNK pathway is crucial. This aspect raises the possibility that DEN has acquired the ability to activate these two MAPK to aid its replication; however, further studies have to be performed to support this hypothesis. Additionally, it has been documented that in endothelial vascular cells, DEN virus infection up-regulates protease-activated receptor type-1 and tissue factor receptor via the phosphorylation of p38 and ERK 1/2 (Huerta-Zepeda et al. 2008), involving MAPKs in viral pathogenesis.

MAPK activation has been shown to be required for the optimal replication of several other viruses. For example, rotavirus replication was inhibited after JNK and p38 inhibition (Holloway and Coulson, 2006). Coxsackievirus B3 activates JNK and p38 MAPK, which appears to facilitate virus replication and virion release (Si et al., 2005).

Additionally, while the blockage of JNK translocation to the nucleus resulted in decreased in HSV virus production (Diao et al., 2005; Hargett et al., 2006; McLean and Bachenheimer, 1999), the production of human immunodeficiency virus type 1 (HIV-1) was increased in p38 MAPK-activated cells (Shapiro et al., 1998). Furthermore, PCV2 infection induces the activation of JNK and p38 and their activation is required for efficient PCV2 replication, as well as PCV2-induced apoptotic cell death (Wei et al., 2009). Thus, MAPKs regulation of viral replication may act at specific steps of the replication cycle, including entry, gene transcription, protein expression, and assembly or in viral pathogenesis. The specific step in DEN replicative cycle in which the activation of JNK and p38 pathways participates is under study in our laboratory.

Since several molecules involved in early stages of DEN infection are associated to lipid rafts (Chu and Ng, 2004; Cambi et al., 2004; Philippova et al., 2008; Reyes del Valle et al., 2005), the importance of these platforms for JNK activation was evaluated treating cells with a drug that sequester cholesterol. The treatment induced a strong inhibition of JNK activation, suggesting that either lipid rafts are the platforms required for the activation of JNK in DEN-infected macrophages or viral attachment or entry, mediated by lipid rafts, are required for JNK activation. In either case, the characterization of the molecules relocated to or present on lipid rafts platforms during DEN infection is important to understand signaling pathways activated at early stages of DEN infection.

Materials and methods

Reagents and antibodies

All fine chemicals were purchased from Aldrich-Sigma. Primary antibodies anti-p-JNK MAPK (rabbit mAb, 81E11), anti-p-p38 MAPK (rabbit mAb, 12F8), and anti-p-ERK MAPK (rabbit polyclonal) used in the Western blot analysis were purchased from Cell Signaling Technology (Beverley, MA). Peroxidase-conjugated anti-rabbit IgGs used as secondary antibodies were obtained from Pierce. Anti-actin monoclonal antibody was a kind donation of Dr. Manuel Hernández (CINVESTAV-México). MAPK-specific inhibitors were acquired from Calbiochem.

Cells and viruses

Monocyte-derived macrophages were obtained from human peripheral blood buffy coats from healthy donors without previous known flavivirus infections. Leukocyte-enriched plasma was centrifuged over Ficoll-Hypaque (Pharmacia). Mononuclear cells were washed, resuspended in RPMI medium supplemented with 10% fetal calf serum, antibiotics (penicillin and streptomycin), 5.95 mg of HEPES/ml, and 2 mM L-glutamine, and plated onto plastic Petri dishes (Costar). After 1 h of incubation at 37 °C in a 5% CO_2 atmosphere, nonadherents cells were removed. Adherent cells were further incubated for 7 days to allow monocytes to differentiate into macrophages. Differentiation was followed by flow cytometry using an anti-CD14 antibody (Zymed). Adherent cell monolayers (2×10^5 cells/well) were subcultured in 24-well plates in RPMI medium.

BHK-21 cells were cultured in MEM supplemented with 10% fetal calf serum. DEN-2 strain 16681 was generously provided by Richard Kinney from the Centers for Disease Control and Prevention (Fort Collins, CO). Virus was propagated in suckling mice brains as described (Reyes del Valle et al., 2005).

For some experiments, DEN virus preparation from suckling mice brains were exposed to short-wave UV light from a germicidal lamp (ULTRA-LUM model UVC-28) for 0, 15, 30, 45, and 60 min. To avoid overheating, virus preparations were kept in ice during UV irradiation. Inactivation of virus infectivity was verified by plaque assay on BKH-21 cells.

DEN virus titration

DEN virus titers were determined by standard plaque assays on confluent monolayers of BHK-21 cells as previously described (Reyes del Valle et al., 2005). Briefly, 10-fold viral serial dilutions were inoculated on BHK-21 confluent monolayers grown on 24-well plates. After 1 h of viral adsorption, the BHK-21 cell monolayers were washed with PBS and overlaid with MEM containing 3% carboxymethyl-cellulose, 0.5% fetal calf serum, and 2 mM L-glutamine. The cultures were incubated at 37 °C for 5 days and then counted for plaque formation after fixation with 10% formalin and staining 0.5% naphthol-blue-black.

Preparation of total cell protein extract and Western blot assay

Human macrophages total protein extracts were prepared as described by Reyes del Valle et al. (2005). In brief, cells were pellet at $840 \times g$ for 10 min and washed three times with PBS. The cell pellet was then resuspended in RSB-NP40 (1.5 mM $MgCl_2$, 10 mM Tris–HCl, 10 mM NaCl, and 1% Nonidet P-40) in the presence of protease inhibitor cocktail (2 mM EDTA, 0.5 mM phenylmethylsulfonyl fluoride, 2 mM benzonidine, 5 μg of aprotinin/ml, 5 μg of pepstatin/ml, 5 μg of leupeptin/ml, and 5 μg of chymostatin/ml) (MiniComplete; Roche Applied Science, Indianapolis, IN). Nuclei and debris were removed by centrifugation at $12,000 \times g$ for 15 min at 4 °C. Cell extracts were separated in a SDS–PAGE and transferred by semidry transfer onto nitrocellulose membrane (Bio-Rad Hercules, CA). Membranes were blocked for 1 h at 37 °C and probed with the appropriate dilution of primary antibody for 1 h at 37 °C, followed by washing with phosphate-buffered saline with 0.05% (vol./vol.) Tween 20 (PBS–Tw). Membranes were incubated with horseradish peroxidase conjugated to either goat anti-mouse IgG or rabbit anti-goat IgG for 1 h at 37 °C. After extensive washing with PBS–Tw, the antigen–antibody complexes were visualized by chemiluminescence using a Femto-Mol (Pierce) detection system according to the manufacturer's instructions.

MAPK phosphorylation analysis

Human monocyte-derived macrophages seeded at 1×10^6 cultivated in serum-free medium for 1 h were mock-infected or infected with DEN-2 at a MOI of 3. At different times after infection (5, 15, and 30 min), the cells were washed twice with cold PBS and lysed in 200 μl of lysis buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 μg/ml leupeptin, 1 mM phenyl-methylsulfonyl fluoride (PMSF), and protease cocktail inhibitor (MiniComplete; Roche Applied Science, Indianapolis, IN)]. Samples were resolved by 10% SDS–PAGE and transferred by semidry transfer onto nitrocellulose membranes (Bio-Rad, Hercules, CA). Membranes were blocked with 5% milk in PBS–Tw and probed overnight with specific primary rabbit anti-phospho-antibodies anti-p-ERK, anti-p-p38, and anti-p-JNK. Finally, membranes were incubated for 1 h at room temperature with goat HRP anti-rabbit (Pierce). Signals were developed by chemiluminescence with Femto-Mol detection system (Pierce) and detected by autoradiography. The films were digitized by densitometry and quantified by image analysis software EDAS (Eastman Kodak).

The amounts of activated p38 and JNK relative to total protein in dengue virus-infected macrophages were also quantified by ELISA using commercial kits (CASE™; SA Biosciences) used following the manufacturer's instructions.

Inhibition of MAPK phosphorylation

Human macrophages, cultivated for 24 h in RPMI supplemented with antibiotics but without serum, were pretreated for 1 h with the

inhibitors against JNK (SP60025, 10 μM), p38 (SB203580, 10 μM), and ERK (FR180204, 10 μM). Under the conditions used, the inhibitors are highly specific as described by the manufacturer (Calbiochem). In addition, under these conditions, none of the MAPK inhibitors showed cytotoxicity for either infected or mock-infected macrophages as assayed by a metabolic colorimetric method (CellTiter 96®; Promega). Pretreated cells were then infected with DEN virus at a MOI = 3 and kept either in the presence or absence of the inhibitors and the infection was allowed for 5, 15, 30, and 60 min. The expression of phosphorylated MAPKs, ERK 1/2, p38, and JNK was evaluated by Western blot.

To analyze the effect of the MAPK inhibitors on DEN replication, cells were pretreated with JNK or p38 inhibitors for 1 h and inoculated, in the presence of the inhibitors, with DEN-2 virus at a MOI = 3. After 1 h, the cells were washed, incubated with RPMI and supernatants collected at 3, 6, 9, and 24 h.p.i. DEN replication was evaluated by measuring secreted NS1 levels using The Platelia™ Dengue NS1 Ag (Bio-Rad), and virus yield was determined by plaque assay (Ludert et al., 2008; Chavez-Salinas et al., 2008).

Indirect immunofluorescence assays

Human macrophages cultured at 60% confluence on coverglasses were infected with DEN at a MOI of 3 for 1 h at 37 °C. After infection, the virus was removed by washing 3 times with PBS and cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Afterward, cells were permeabilized with 4 % paraformaldehyde and 0.5% Triton X-100, blocked for 1 h with blocking buffer (1× PBS, 10% FCS, 3% BSA) and incubated with a rabbit anti-c-Jun (no. sc-45; Santa Cruz) as primary antibody and with an anti-rabbit antibody coupled to Alexa 647, as secondary antibody (Invitrogen). Cell nuclei were counterstained with DAPI. The cells were analyzed under a Leica LSM-SPC-5 Mo inverted confocal microscope fitted with HCXPLapo lambda blue 63× 1.4 oil immersion lens. Images were captured and processed using the LAS AF software confocal (Leica).

Methyl-β-cyclodextrin treatment

Macrophages derived from human peripheral blood monocytes were treated or not with 7.5 mM of methyl-β-cyclodextrin for 30 min at 37 °C prior to DEN-2 infection at a MOI = 3, as described previously (Reyes del Valle et al., 2005). Drug treatment conditions were found to be non-cytotoxic for virus or mock-infected macrophages as assayed by a metabolic colorimetric method (CellTiter 96®; Promega). Fifteen minutes after infection, total cell protein extract was collected and analyzed for Western blot assays to determine MAPK activation. Levels of cell cholesterol after drug treatment or viral infection were measured using a colorimetric commercial assay (Cholesterol, BioSystems) with modifications.

Acknowledgments

References

Bielefeldt-Ohmann, H., Meyer, M., Fitzpatrick, D.R., Mackenzie, J.S., 2001. Dengue virus binding to human leukocytic cell lines: receptor usage differs between cell types and virus strains. Virus Res. 73, 81–89.

Cambi, A., de Lange, F., van Maarseveen, N.M., Nijhuis, M., Joosten, B., van Dijk, E.M., de Bakker, B.I., Fransen, J.A., Bovee-Geurts, P.H., van Leeuwen, F.N., Van Hulst, N.F., Figdor, C.G., 2004. Microdomains of the C-type lectin DC-SIGN are portals for virus entry into dendritic cells. J. Cell Biol. 164, 145–155.

Chavez-Salinas, S., Ceballos-Olvera, I., Reyes del Valle, J., Medina, F., del Angel, R.M., 2008. Heat shock effect upon dengue virus replication into U937 cells. Virus Res. 138, 111–118.

Chen, Y., Maguire, T., Hileman, R.E., Fromm, J.R., Esko, J.D., Linhardt, R.J., Marks, R.M., 1997. Dengue infectivity depends on envelope protein binding to target cell heparan sulfate. Nat. Med. 3, 866–871.

Chen, S.T., Lin, Y.L., Huang, M.T., Wu, M.F., Cheng, S.C., Lei, H.Y., Lee, C.K., Chiou, T.W., Wong, C.H., Hsieh, S.L., 2008. CLEC5A is critical for dengue-virus-induced lethal disease. Nature 453 (7195), 672–676.

Chu, J.J.H., Ng, M.L., 2004. Infectious entry of West Nile virus occurs through a clathrin-mediated endocytic pathway. J. Virol. 78, 10543–10555.

Crill, W.D., Roehring, J.T., 2001. Monoclonal antibodies that bind to domain III of DEN E glycoprotein are the most efficient blockers of virus adsorption to Vero cells. J. Virol. 75, 4002–4007.

Diao, L., Zhang, B., Xuan, C., Sun, S., Yang, K., Tang, Y., Qiao, W., Chen, Q., Geng, Y., Wang, C., 2005. Activation of c-Jun N-terminal kinase (JNK) pathway by HSV-1 immediate early protein ICP0. Exp. Cell Res. 308, 196–210.

Frevel, M.A., Bakheet, T., Silva, A.M., Hissong, J.G., Khabar, K.S., Williams, B.R., 2003. p38 Mitogen-activated protein kinase-dependent and -independent signaling of mRNA stability of AU-rich element-containing transcripts. Mol. Cell. Biol. 23, 425–436.

Guzman, M.G., Kouri, G., 2002. Dengue: an update. Lancet Infect. Dis. 2, 33–42.

Hargett, D., Rice, S., Bachenheimer, S.L., 2006. Herpes simplex virus type 1 ICP27-dependent activation of NF{kappa}B. J. Virol. 80, 10565–10578.

Hase, T., Summers, P.L., Eckels, K.H., 1989. Flavivirus entry into cultured mosquito cells and human peripheral blood monocytes. Arch. Virol. 104, 129–143.

Heinz, F.X., 1986. Epitope mapping of flavivirus glycoprotein. Adv. Virus Res. 31, 103–168.

Henchal, E.A., McCown, J.M., Burke, D.S., Seguin, M.C., Brandt, W.E., 1985. Epitopic analysis of antigenic determinants on the surface of dengue-2 virions using monoclonal antibodies. Am. J. Trop. Med. Hyg. 34, 162–169.

Holloway, G., Coulson, B.S., 2006. Rotavirus activates JNK and p38 signaling pathways in intestinal cells leading to AP-1-driven transcriptional responses and enhanced virus replication. J. Virol. 80, 10624–10633.

Huang, H., Kamm, R.D., Lee, R.T., 2004. Cell mechanics and mechanotransduction: pathways, probes, and physiology. Am. J. Physiol., Cell Physiol. 287, C1–C11.

Huerta-Zepeda, A., Cabello-Gutiérrez, C., Cime-Castillo, J., Monroy-Martínez, V., Manjarrez-Zavala, M.E., Gutiérrez-Rodríguez, M., Izaguirre, R., Ruiz-Ordaz, B.H., 2008. Crosstalk between coagulation and inflammation during dengue virus infection. Thromb. Haemost. 99, 936–943.

Jindadamrongwech, S., Thepparit, C., Smith, D., 2004. Identification of GRP78 (BiP) as a liver cell expressed receptor element for dengue virus serotype 2. Arch. Virol. 149, 915–927.

Kumar, A., Knox, A.J., Boriek, A.M., 2003. CCAAT/enhancer-binding protein and activator protein-1 transcription factors regulate the expression of interleukin-8 through the mitogen-activated protein kinase pathways in response to mechanical stretch of human airway smooth muscle cells. J. Biol. Chem. 278, 18868–18876.

Lim, H.Y., Ng, M.L., 1999. A different mode of entry by dengue-2 neutralisation escape mutant virus. Arch. Virol. 144, 989–995.

Lindenbach, B.D., Rice, C.M., 2001. *Flaviviridae*: the virus and their replication, In: Knipe, D.M., Howley, P.M., Griffin, D.E., Lamb, R.A., Martin, M.A., Roizman, B., Straus, S.E. (Eds.), Fields Virology, 4th ed. Lippincott Williams & Wilkins, Philadelphia, Pa, pp. 991–1042. 2009.

Lozach, P.-Y., Burleigh, L., Staropoli, I., Navarro-Sanchez, E., Harriague, J., Virelizier, J.L., Rey, F.A., Despres, P., Arenzana-Seisdedos, F., Amara, A., 2005. Dendritic cell-specific intercellular adhesion molecule 3-grabbing non-integrin (DC-SIGN) mediated enhancement of dengue virus infection is independent of DC-SIGN internalization signals. J. Biol. Chem. 280, 23698–23708.

Ludert, J.E., Mosso, C., Ceballos-Olvera, I., del Angel, R.M., 2008. Use of a commercial enzyme immunoassay to monitor dengue virus replication in cultured cells. Virol. J. 5, 51.

Mandl, C.W., Allison, S.L., Holzmann, H., Meixner, T., Heinz, F.X., 2000. Attenuation of tick-borne encephalitis virus by structure-based site-specific mutagenesis of a putative flavivirus receptor binding site. J. Virol. 74, 9601–9609.

Martínez-Barragán, J.J., del Angel, R.M., 2001. Identification of a putative coreceptor on Vero cells that participates in dengue 4 virus infection. J. Virol. 75, 7818–7827.

McLean, T.I., Bachenheimer, S.L., 1999. Activation of cJUN N-terminal kinase by herpes simplex virus type 1 enhances viral replication. J. Virol. 73, 8415–8426.

Mercado-Curiel, R.F., Black IV, W.C., Munoz de, L., 2008. A dengue receptor as possible genetic marker of vector competence in *Aedes aegypti*. BMC Microbiol. 8, 118.

Mercado-Curiel, R.F., Esquinca-Aviles, H.A., Tovar, R., Diaz-Badillo, A., Camacho-Nuez, M., Munoz M de, L., 2006. The four serotypes of dengue recognize the same putative receptors in *Aedes aegypti* midgut and *Ae. albopictus* cells. BMC Microbiol. 6, 85.

Miller, J.L., Dewet, B.J., Martinez-Pomares, L., Radcliffe, C.M., Dwek, R.A., Rudd, P.M., 2008. The mannose receptor mediates dengue virus infection of macrophages. PLoS Pathog. 4 (2), e17.

Modis, Y.S., Ogata, S., Clements, D., Harrison, S.C., 2003. A ligand-binding pocket in the dengue virus envelope glycoprotein. Proc. Natl. Acad. Sci. U. S. A. 100, 6986–6991.

Monick, M.M., Cameron, K., Staber, J., Powers, L.S., Yarovinsky, T.O., Koland, J.G., Hunninghake, G.W., 2005. Activation of the epidermal growth factor receptor by respiratory syncytial virus results in increased inflammation and delayed apoptosis. J. Biol. Chem. 280, 2147–2158.

Moreno-Altamirano, M.M.B., Sánchez-García, F.J., Muñoz, M.L., 2002. Non Fc receptor-mediated infection of human macrophages by dengue virus serotype 2. J. Gen. Virol. 83, 1123–1130.

Muñoz, M.L., Cisneros, A., Cruz, J., Das, P., Tovar, R., Ortega, A., 1998. Putative dengue virus receptors from mosquito cells. FEMS Microbiol. Lett. 168, 251–258.

Navarro, S.E., Altmeyer, R., Amara, A., Schwartz, O., Fieschi, F., Virelizier, J.L., Arenzana-Seisdedos, F., Despres, P., 2003. Dendritic-cell-specific ICAM3-grabbing non-integrin is essential for the productive infection of human dendritic cells by mosquito-cell-derived dengue viruses. EMBO Rep. 4, 723–728.

Pearson, G., Robinson, F., Gibson, T.B., Xu, B.E., Karandikar, M., Berman, K., Cobb, M.H., 2001. Mitogen-activated protein (MAP) kinase pathways: regulation and physiological functions. Endocr. Rev. 22, 153–183.

Philippova, M., Ivanov, D., Joshi, M.B., Kyriakakis, E., Rupp, K., Afonyushkin, T., Bochkov, V., Erne, P., Therese, J.R., 2008. Identification of proteins associating with glycosylphosphatidylinositol- anchored T-cadherin on the surface of vascular endothelial cells: role for Grp78/BiP in T-cadherin-dependent cell survival. Mol. Cell. Biol. 28, 4004–4017.

Raman, M., Chen, W., Cobb, M.H., 2007. Differential regulation and properties of MAPKs. Oncogene 26, 3100–3112.

Ramos, C.J., Imbert, J.L., Barron, B.L., Ramos, C., 1997. A 65-kDa trypsin-sensible membrane cell protein as a possible receptor for dengue virus in cultured neuroblastoma cells. J. Neurovirol. 3, 435–440.

Reyes del Valle, J., Chavez, S.S., Medina, F., del Ángel, R.M., 2005. Heat shock protein 90 and heat shock protein 70 are components of dengue virus receptor complex in human cells. J. Virol. 79, 4557–4567.

Roehring, J.T., 2003. Antigenic structure of flavivirus proteins. Adv. Virus Res. 59, 141–175.

Salas-Benito, J.S., del Angel, R.M., 1997. Identification of two surface proteins from C6/36 cells that bind dengue type 4 virus. J. Virol. 71, 7246–7252.

Shapiro, L., Heidenreich, K.A., Meintzer, M.K., Dinarello, C.A., 1998. Role of p38 mitogen-activated protein kinase in HIV type 1 production in vitro. Proc. Natl. Acad. Sci. 95, 7422–7426.

Si, X., Luo, H., Morgan, A., Zhang, J., Wong, J., Yuan, J., Esfandiarei, M., Gao, G., Cheung, C., McManus, B.M., 2005. Stress-activated protein kinases are involved in coxsackievirus B3 viral progeny release. J. Virol. 79, 13875–13881.

Tassaneetrithep, B., Burguess, T.H., Granelli-Piperno, A., Trumpfheller, C., 2003. DC-SIGN (CD209) mediates DEN infection of human dendritic cells. J. Exp. Med. 197, 823–829.

Thepparit, C., Smith, D.R., 2004. Serotype-specific entry of dengue virus into liver cells: identification of the 37-kilodalton/67-kilodalton high-affinity laminin receptor as a dengue virus serotype 1 receptor. J. Virol. 78, 12647–12656.

Vaughn, D.W., Green, S., Kalayanarooj, S., Innis, B.L., Nimmannitya, S., 2000. Dengue viremia titer, antibody response pattern, and virus serotype correlate with disease severity. J. Infect. Dis. 181, 2–9.

Wei, L., Zhu, Z., Wang, J., Liu, J., 2009. JNK and p38 mitogen-activated protein kinase pathways contribute to porcine circovirus type 2 infection. J. Virol. 83, 6039–6047.

Whitmarsh, A.J., 2007. Regulation of gene transcription by mitogen-activated protein kinase signaling pathways. Biochim. Biophys. Acta 1773, 1285–1298.

Wolf, D., Witte, V., Clark, P., Blume, K., Lichtenheld, M.G., Baur, A.S., 2008. HIV Nef enhances Tat-mediated viral transcription through a hnRNP-K-nucleated signaling complex. Cell Host Microbe 4, 398–408.