Antiviral action of nitric oxide on dengue virus type 2 replication

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Recently, nitric oxide (NO) has been shown to suppress dengue virus (DENV) RNA and protein accumulation in infected cells. In this report, the potential target of the inhibitory effect of NO was studied at the molecular level. The NO donor, S-nitroso-N-acetylpenicillamine (SNAP), showed an inhibitory effect on RNA accumulation at around 8–14 h post-infection, which corresponded to the step of viral RNA synthesis in the DENV life cycle. The activity of the viral replicase isolated from SNAP-treated DENV-2-infected cells was suppressed significantly compared with that of the negative-control N-acetyl-DL-penicillamine (NAP)-treated cells. Further investigations on the molecular target of NO action showed that the activity of recombinant DENV-2 NS5 in negative-strand RNA synthesis was affected in the presence of 5 mM SNAP in vitro. RNA-dependent RNA polymerase (RdRp) assays, whereas the RNA helicase activity of DENV-2 NS3 was not inhibited up to a concentration of 15 mM SNAP. These results suggest that the inhibitory effect of NO on DENV infection is partly via inhibition of the RdRp activity, which then downregulates viral RNA synthesis.

INTRODUCTION

Dengue viruses (DENVs) (serotypes 1–4) are members of the genus Flavivirus in the family Flaviviridae. The flavivirus genome is a single-stranded RNA (~11 kb) of positive polarity, which has a type I cap structure at the 5′ end, but no poly(A) tail at the 3′ end. The genome encodes a single polyprotein, which is processed co- and post-translationally into at least ten mature proteins consisting of three structural proteins (C, prM and E) that form the virion and seven non-structural proteins (NS1, NS2A, NS2B, NS3, NS4A, NS4B and NS5) that are important for virus replication (Lindenbach & Rice, 2003). NS5 is an RNA-dependent RNA polymerase (RdRp) required for viral RNA replication (O’Reilly & Kao, 1998; Poch et al., 1989). NS5 also has a 2′-O-methyltransferase activity thought to be important for 5′ capping (Egloff et al., 2002; Rozanov et al., 1992). NS3 is a multifunctional protein; it has the active site of a trypsin-like serine protease and, together with the NS2B co-factor, forms an active protease that is required for polyprotein processing. NS3 also has an RNA-stimulated NTPase and RNA helicase, thought to be important for viral RNA replication, as well as a 5′ RNA triphosphatase activity, which is required in the first step of 5′ capping. In flavivirus-infected cells, NS3 and NS5 exist in a complex and are thought to be components of the viral RNA replicase complex (Kapoor et al., 1995; Westaway et al., 2002, 2003).

The endoplasmic reticulum-associated replicase complex is composed of the consensus components NS1, NS2A, NS3, NS4A and NS5 (Westaway et al., 1997), as well as putative host proteins such as La protein (García-Montalvo et al., 2004; Yocupicio-Monroy et al., 2003). Although the binding of several host proteins to the 3′ and 5′ untranslated regions (UTRs) has been shown, their definitive role in virus replication is still obscure. The enzymic activities of mosquito-borne flaviviral NS3 (Benarroch et al., 2004; Li et al., 1999; Wengler & Wengler, 1991, 1993; Yon et al., 2005) and NS5 (Ackermann & Padmanabhan, 2001; Guyatt et al., 2001; Tan et al., 1996) suggest a role in virus replication and 5′ capping. However, the functions of the other non-structural proteins are not clear.

Flavivirus replication is semi-conservative and asymmetrical, and begins with the synthesis of the genomelength, negative-strand RNA. This negative-strand RNA product then serves as a template for the synthesis of additional, positive-strand RNAs. The study of flavivirus replication has identified three forms of viral RNA species in flavivirus-infected vertebrate cells: a 44S genome-length, single-stranded viral RNA (vRNA), a 20S double-stranded replicative form (RF) and the 20–28S partially nuclease-resistant replicative intermediate (RI) (Chu & Westaway, 1985, 1987; Cleaves et al., 1981). The RI species consist of various lengths of positive-strand RNA intermediates.
synthesized on the negative-strand RNA template with concomitant displacement of the parental positive strand, which is released as vRNA. According to the proposed model for flavivirus replication, the sequential steps are RF→RI→vRNA, where RF acts as the recycling template for positive-strand synthesis (Westaway et al., 2003). Recently, an in vitro RdRp assay has been developed using an exogenous subgenomic RNA template incubated with either DENV-2-infected mosquito cells (C6/36) or purified viral RdRp (Ackermann & Padmanabhan, 2001; You & Padmanabhan, 1999; You et al., 2001). The subgenomic RNA template of positive-strand polarity, unlike the three species of membrane-bound endogenous viral RNA RIs, contains only the 5′ UTR, the 5′ cyclization motif from the 5′ end and the complete 3′ UTR, including the 3′ cyclization motif. Using this system, two forms of RNA have been identified. The first has the same size as the input RNA template (1 ×) and is formed by de novo initiation of RNA synthesis on a subgenomic RNA template, whilst the second is a hairpin product that is twice the size of the RNA template (2 ×) and is formed by 3′-end elongation of a fold-back structure of the RNA template (Ackermann & Padmanabhan, 2001).

Nitric oxide (NO) is a ubiquitous molecule that exerts a diverse range of functions such as smooth muscle relaxation, inhibition of platelet activation, neurotransmission and immune response (Marletta, 1994; Murad, 1996). This diffusible innate defence molecule exhibits a potent antibacterial activity through modification of thiol (cysteine residue) and metal centres via an S-nitrosylation reaction of microbial protein involved in critical catalytic and regulatory functions (Mannick et al., 1994; Persichini et al., 1998; Saura et al., 1999; Venturini et al., 2000). NO is produced by monocytes, macrophages and dendritic cells, which are the hosts for several viruses, including dengue viruses (Lin et al., 2002; Wu et al., 2000). Endogenously produced NO in monocytes and macrophages limits replication of DENV (Neves-Souza et al., 2005) and Japanese encephalitis virus (Lin et al., 1997), but not Tick-borne encephalitis virus (Kreil & Eibl, 1996). In other studies, NO levels were not significantly higher in DENV-infected human monocyte cultures than in uninfected cultures (Chen & Wang, 2002; Espina et al., 2003), which may be related to the sensitivity of the method used for NO detection. DENV infection of human primary Kupffer cells did not produce viral progeny, but the released soluble mediators, which may have included NO, caused apoptosis of these cells (Marienneau et al., 1999). In contrast to the induction of antiviral NO production in response to viral infection in vivo or in vitro in some primary cell types, the direct effect of NO on virus replication has also been studied in other cell types infected with different viruses. For example, NO produced by the addition of a NO donor to DENV-infected mosquito C6/36 cells also inhibited DENV replication (Neves-Souza et al., 2005). The antiviral effect of NO has been demonstrated in various viral systems, such as Japanese encephalitis virus (Saxena et al., 2000), vesicular stomatitis virus (Bi et al., 1995), coxsackievirus (Zaragoza et al., 1999), respiratory syncytial virus (Ali-Ahmad et al., 2003) and Vaccinia virus (Harris et al., 1995). Recently, our group demonstrated that NO inhibits dengue virus replication in N18 neuroblastoma cells via suppression of viral RNA and protein accumulation (Charnsilpa et al., 2005). Cell culture-adapted African green monkey (Vero) and Rhesus monkey (LLC-MK2) kidney cells are used routinely for studies of flaviviruses. In the present study, we sought to identify the molecular target of the inhibitory effect of NO in DENV-2 virus replication in LLC-MK2 cells. We examined the inhibitory effect of NO by quantifying the intracellular viral RNA levels using quantitative real-time RT-PCR (qRT-PCR) after treatment of DENV-2-infected cells with various concentrations of S-nitroso-N-acetylpenicillamine (SNAP), a NO donor. Moreover, the target of NO action in viral RNA synthesis was investigated by utilizing several techniques: an in vitro replicase assay with endogenous membrane-bound replicative viral RNA templates, exogenous viral subgenomic RNA templates and purified NS5, an RNA helicase assay with purified NS3 and an electrophoretic mobility-shift assay (EMSA) to assess the RNA-binding activity of NS5. Our results indicated that NO treatment of DENV-2-infected cells affected virus replication by downregulating the activity of endogenous viral replicase or by inhibition of purified RdRp, but had no effect on the RNA helicase activity of NS3 or on the RNA-binding activity of NS5.

**METHODS**

**Materials, cell cultures, media and virus.** LLC-MK2 monkey kidney cells were maintained in Eagle’s minimal essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 2 mM L-glutamine. DENV type 2 (DENV-2; New Guinea C strain) was grown in C6/36 mosquito cells maintained in RPMI 1640 medium containing 2 mM L-glutamine, 1% non-essential amino acids, 1 mM sodium pyruvate and 10% FBS in tightly capped tissue-culture flasks and incubated at 30°C without CO2. Virus production was titrated by using plaque assays as described previously (Charnsilpa et al., 2005). Infection of LLC-MK2 cells with DENV-2 was carried out at the indicated m.o.i. in 1% FBS/Eagle’s MEM at 37°C in 5% CO2 for 72 h unless indicated otherwise.

SNAP was obtained from Molecular Probes. N-Acetyl-l- penicillamine (NAP) and dithiothreitol (DTT) were obtained from Sigma.

**In vitro replicase assay.** The DENV-2 viral replicase complex was isolated from LLC-MK2 cells infected with DENV-2 (m.o.i. = 25) in T-150 flasks for 48 h at 37°C and 5% CO2. Cells were harvested by centrifugation at 800 g to obtain the cytoplasmic fraction as a source of the replicase complex for in vitro replicase assays as described previously (Chu & Westaway, 1985; Uchil & Satchidanandam, 2003). The in vitro replicase assay and RNA extraction and analysis using a partially denaturing 7 M urea/3% polyacrylamide gel followed by autoradiography were carried out as described previously (Uchil & Satchidanandam, 2003).

**Effect of NO on the status of intracellular replicative viral RNA species.** LLC-MK2 cells were cultured in T-25 flasks at 37°C and 5% CO2. Confluent cultures were infected with DENV-2 at an m.o.i. of 25. SNAP was added to culture media immediately after infection at the indicated concentrations as an exogenous NO donor. To maintain the level of NO in infected cultures, SNAP was
replenished every 4 h up to 8 h after infection. NAP (150 μM) or medium alone was used as a negative control. Cultures were incubated for a further 40 h and cytoplasmic extract was prepared as described above. Total protein (60 μg) from each cytoplasmic extract was added to the in vitro replicate reaction.

In vitro RdRp assay. His-tagged, recombinant DENV-2 NS5 protein was expressed and purified as described previously (Ackermann & Padmanabhan, 2001). Preparation of the DENV-2 subgenomic RNA template was carried out as described previously (You & Padmanabhan, 1999), except that AmpliScribe T7 and SP6 High Yield Transcription kits were used for in vitro transcription (Epitect Biotechnologies) following the manufacturer’s protocol. The in vitro RdRp assay and RNA extraction and analysis using formaldehyde/agarose gel electrophoresis and visualization by autoradiography were carried out as described previously (You & Padmanabhan, 1999; You et al., 2001). Band intensity was measured by densitometry (ImageMaster; Amersham Biosciences).

EMSA. The RNA substrate for EMSA was a DENV-2 subgenomic RNA template as described in the in vitro RdRp assay. It was labelled at the 3′-end with polynucleotide kinase and [γ-32P]ATP by using standard methods. Purified NS5 protein (3 μg) was incubated with 5′-radiolabelled subgenomic RNA template (20–50 nM), RNase inhibitor (20 U) and tRNA (4 μg) in 10 μl binding buffer [14 mM HEPES (pH 7.5), 6 mM Tris/HCl (pH 8.0), 1 mM EDTA, 60 mM KCl] for 30 min at room temperature. RNA–protein complexes were fractionated by electrophoresis at 4 °C on a pre-run, non-denaturing 3% polyacrylamide gel at 100 V for 1 h. The gel was dried and exposed to X-ray film for 10–20 min.

RNA helicase assay. The RNA helicase assay using the recombinant full-length NS3 protein (NS3FL) was as described by Yon et al. (2005). NS3FL protein expression and purification were as described previously (Bartelma & Padmanabhan, 2002). The reaction mixture for the RNA helicase assay (20 μl) contained 20 mM HEPES/KOH (pH 7.0), 1.5 mM MgCl2, 2.5 mM ATP, 500 nM purified NS3 protein and 5 nM helicase substrate. The reaction was carried out at 37 °C for 30 min and terminated by the addition of 5 μl 5× RNA loading dye (100 mM EDTA, 0.7% SDS). The double-stranded RNA substrate and the unwound fraction were fractionated by gel electrophoresis on a 12% native polyacrylamide gel at 100 V for 1 h, followed by autoradiography.

qRT-PCR. The method used was as described by Houng et al. (2001). PCR amplification and real-time data collection and analysis were performed according to the ABI 7900 real-time PCR gene detection system. Real-time detection of the DENV-2 PCR product was correlated with input cDNA copy number and the results were plotted. The DENV genomic copy standard was supplied by WRAIR and ten serial 1:3 dilutions were used from a virus stock containing 4·20 × 106 copies ml−1.

Kinetics of DENV-2 RNA synthesis by RT-PCR. LLC-MK2 cells in six-well plates were infected with DENV-2 at an m.o.i. of 10. Infected cells were harvested at various times after infection and subjected to RNA extraction. The DENV prM gene was detected by standard RT-PCR, as described by Lanciotti et al. (1994).

RESULTS

Kinetics of DENV-2 RNA synthesis in LLC-MK2 cells
To determine the kinetics of DENV-2 RNA synthesis in LLC-MK2 cells, a standard RT-PCR was used to detect the DENV prM gene in infected cells harvested at various times post-infection (p.i.) as described in Methods. As shown in Fig. 1(a), viral RNA was detected as early as 8 h p.i. and levels increased up to 16 h p.i. No signal was observed at earlier time points (0–6 h p.i.) or in mock-infected cells.

![Fig. 1.](http://vir.sgmjournals.org)

(a) Kinetics of DENV-2 RNA synthesis analysed by RT-PCR. LLC-MK2 cells were infected at an m.o.i. of 10. Infected cells were harvested at the indicated times p.i. (lanes 1–8). An equal amount of total RNA from each sample was subjected to RT-PCR for detection of the viral RNA as described in Methods. The results are representative of two independent experiments. Lane 9, mock-infected cells; lane M, molecular size marker (100 bp ladder). (b) Time course of NO inhibition of DENV-2 replication. LLC-MK2 cells were infected with DENV-2 (New Guinea C strain) at an m.o.i. of 10. SNAP (150 μM) was added once at the following time points: 2 h prior to infection, at the time of infection or 2–16 h p.i., as indicated. The amount of intracellular vRNA (copies ml−1) was measured by qRT-PCR. The results are from two independent experiments and data represent means ± SD. (c) Cytotoxicity of SNAP. The cytotoxicity of various concentrations of SNAP towards LLC-MK2 cells was determined in triplicate by using an MTT-based kit. Data represent means ± SD.
Time course of inhibition of DENV-2 replication by NO, analysed by qRT-PCR

Next, we followed the time course of inhibition of DENV-2 replication by SNAP (150 µM) treatment. LLC-MK2 cells in six-well plates were infected with DENV-2 (m.o.i. = 10) and treated once with 150 µM SNAP at the following time points: 12 h prior to infection (−2 h p.i.), at the time of infection (0 h p.i.) and at 2, 4, 6, 8, 10, 12, 14 and 16 h p.i. Viral RNA was extracted from infected cells at 72 h p.i. and the level of intracellular viral RNA synthesis was measured by qRT-PCR. As shown in Fig. 1(b), treatment of infected cells with SNAP 2 h prior to infection and up to 6 h p.i. had no effect on viral RNA synthesis. The inhibitory effect of SNAP treatment was clearly detected at 8 h p.i. at the stage of viral RNA synthesis and exerted its maximum inhibitory effect around 10–14 h p.i., resulting in a 1.5–2 log decrease in RNA levels compared with the control (untreated sample). However, the inhibitory effect of SNAP treatment on RNA synthesis at 16 h p.i. was reduced slightly compared with that at the 14 h time point, for reasons unknown at present. The fact that treatment of cells with SNAP 2 h prior to DENV-2 infection had no effect on viral RNA synthesis suggests that the inhibitory effect of NO may not involve pre-existing cellular factors. However, the effect of NO on viral-induced cellular factors cannot be excluded.

The effect of SNAP on the viability of LLC-MK2 cell cultures was assessed by using an assay based on the activity of mitochondrial dehydrogenases of living cells (TOX-1 MTT-based assay; Sigma). In this assay, the chromogenic substrate 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) is converted into purple formazan and quantified at 570 nm using a spectrophotometer. SNAP did not affect the viability of cells up to a concentration of 200 µM in this assay (Fig. 1c).

Effect of NO on the status of intracellular replicative viral RNA species

As the results shown in Fig. 1(b) suggested that NO exerts its inhibitory effect at the level of viral RNA synthesis, we sought to examine the status of the three intracellular replicative species of DENV-2 RNA – RF, RI and vRNA (Chu & Westaway, 1985; Uchil & Satchidanandam, 2003) – following treatment with SNAP. In vitro viral replicase assays were performed using cytoplasmic extracts containing the endogenous, membrane-bound, replicative RNA templates from DENV-2-infected SNAP-treated and untreated (control) cells, NTPs (with [α-32P]GTP) and an ATP-regenerating system as described in Methods. As shown in Fig. 2, the levels of all three RNA species were clearly reduced when treated with 150 µM SNAP compared with those of the untreated samples. In particular, vRNA was undetectable following 150 µM SNAP treatment, whereas following treatment with 75 µM SNAP, all three RNA species could be detected. Treatment with SNAP, a non-donor of NO used as a negative control for SNAP, did not have any effect on the levels of the three RNA species. The reduced levels of intracellular replicative RNA species following treatment with SNAP were consistent with the results obtained by qRT-PCR analysis of viral RNA from treated cells.

The results shown thus far indicated that NO inhibited virus replication and that the levels of intracellular replicative RNA were reduced, but did not reveal the direct target of NO action. To examine whether a protein(s) in the membrane-bound viral replicase complex might be a direct target for NO action, we performed in vitro replicase assays containing cytoplasmic extracts from DENV-2-infected cells in the presence of SNAP added exogenously to the assay mixtures (or NAP as a control). The three forms of viral RNA species were detected by autoradiography as shown in Fig. 3(a) and the band intensities were plotted (Fig. 3b). The results indicated that the levels of vRNA and RF were clearly reduced (53±7±25·1 and 49·7±23·9% of control levels for vRNA and RF, respectively; Fig. 3a, lane 5) following addition of SNAP at a concentration of 15 mM to the assay mixture, whereas the level of RI was not affected. There was no non-specific signal in the reaction containing mock-infected cytoplasmic extract (Fig. 3a, lane 8). NAP had very little effect on the levels of the three intracellular RNA species (Fig. 3a, lane 7). Moreover, the addition of DTT, a reducing agent for the sulphydryl moiety, reversed the inhibitory effect of SNAP completely when added to the

![Fig. 2. In vitro replicate assay of SNAP-treated, DENV-2-infected LLC-MK2 cells. LLC-MK2 cells were infected with DENV-2 and either left untreated or treated with SNAP or NAP as described in Methods. Cytoplasmic extract was prepared and 60 µg of each extract was added to the in vitro replicate assay. After incubation of the reaction at 37°C for 1 h, RNA products were extracted and separated on a partially denaturing gel (7 M urea/3% polyacrylamide) and analysed by autoradiography. Lanes: 1, control (untreated) extract; 2, extract treated with 75 µM SNAP; 3, extract treated with 150 µM SNAP; 4, extract treated with 150 µM NAP; 5, mock-infected cytoplasmic extract. The results are representative of two independent experiments.](image-url)
reaction containing 15 mM SNAP (Fig. 3a, lane 6). The results from the in vitro viral replicase assays in which SNAP was added exogenously to the reaction mixtures indicated that high concentrations of NO donor were required to achieve the inhibitory effect, whereas 100-fold lower concentrations were sufficient when added to DENV-2-infected cell cultures. However, the reduced synthesis of vRNA and RF forms in vitro was consistent with the effects of NO in both cases.

NS5 and NS3 are multifunctional proteins that form the main components of the replicase complex, which also includes other viral and perhaps cellular factors and plays an important role in RNA synthesis. In vitro assays have already been established for analysing the enzymic activities of NS3 RNA helicase and NS5 RdRp encoded by the DENV-2 RNA (Ackermann & Padmanabhan, 2001; Yon et al., 2005). Therefore, we sought to examine whether any of the enzymic activities of NS3 or NS5 might be a target for NO. To this end, the RdRp activity of NS5 and the RNA helicase activity of NS3 were assayed in vitro by using purified Escherichia coli-expressed proteins.

**Effect of NO on the RdRp activity of purified NS5 protein**

To examine the effect of NO on the RdRp activity of purified NS5, subgenomic RNA containing the 5’ and 3’ UTR and the cyclization motifs was used in the in vitro assay as an exogenous template in the presence of SNAP or in its absence, as described in Methods. RNA products were detected by autoradiography as shown in Fig. 4(a) and band intensities were plotted (Fig. 4b). The two RNA products – the de novo (1×) and the hairpin (2×) products – were clearly detected in the positive control (Fig. 4a, lane 1), but not in the negative control (Fig. 4a, lane 10). The RNA products synthesized in the presence of SNAP indicated that the de novo product was affected much more than the 3’-end elongation product produced from the RNA template by a 3’ copy-back mechanism. The levels of de novo product were 59.4 ± 18.2, 32.5 ± 4.3 and 11.1 ± 4.4% of those of the control at 5, 10 and 15 mM SNAP, respectively (Fig. 4b, lanes 3–5). Moreover, the inhibitory effect of NO following treatment with 5 mM SNAP could be reversed only up to 76.2 ± 6.9% of the level of the control, untreated sample by the addition of DTT to a concentration as high as 15 mM (Fig. 4, lane 7). In contrast, synthesis of the 3’-end elongation product (2× hairpin product) was not suppressed by the addition of 1–10 mM SNAP in the RdRp assays. Suppression was only detected when 15 mM SNAP was used (54.4 ± 17.7% of control levels). NAP had no effect on the synthesis of the two forms of RNA (Fig. 4, lanes 8 and 9).

**Effect of NO on NS5 binding to viral RNA**

To examine whether NO treatment interfered with the binding of NS5 to viral RNA template in the in vitro RdRp assays, EMSAs were performed as described in Methods. Purified NS5 was incubated with 5’-radiolabelled viral subgenomic RNA template in the absence or presence of SNAP and in the presence of tRNA to eliminate non-specific binding of NS5 protein. The results shown in Fig. 5 indicated that the addition of SNAP at the indicated concentrations (5–15 mM) had no significant effect on the
formation of RNA–protein complexes (Fig. 5, lanes 3–5) compared with the control (Fig. 5, lane 2). Under these conditions, there was a strong reduction in the level of de novo RNA product (see Fig. 4a). Another control to ensure the specificity of NS5 binding to the template RNA was included (Fig. 5, lane 6), which showed that the unlabelled template RNA strongly reduced the formation of the labelled RNA–protein complexes. From these results, the possibility that NO interferes with the binding of NS5 protein to viral RNA template can be ruled out.

RNA helicase activity of NS3 protein is unaffected by the presence of NO

The inhibitory effect of NO on NS3 helicase activity was tested as described in Methods. RNA substrate was prepared by annealing a 30 nt oligomer, labelled at its 5′ end with 32P, to a complementary 15 nt oligomer such that the 3′-terminal sequences of the 30 nt oligomer were single-stranded.

Purified NS3 was incubated with various concentrations of SNAP for 5 min at room temperature prior to adding it to the reaction mixture. Fig. 6 lanes 1 and 2) shows the duplex RNA substrate used in this experiment and the single-stranded RNA resulting from unwinding of the substrate following heating. The addition of SNAP at concentrations

![Diagram](image-url)
DISCUSSION

The key finding of this study was that NO inhibited DENV replication partly by inhibiting NS5 polymerase activity. Our previous study (Charnsilpa et al., 2005) showed that NO inhibited DENV replication and that NO reduced both viral RNA and protein accumulation in infected N-18 cells. The results presented in this study indicate the inhibition of NS5 polymerase activity by the action of NO, resulting in suppression of viral RNA synthesis, could explain the reduction of viral RNA and protein levels in infected cells and the reduced numbers of infectious particles in the culture medium, as reported previously (Charnsilpa et al., 2005). However, this study of the inhibitory effect of NO was performed in an established monkey kidney cell line, LLC-MK2. In previous studies, human embryonic kidney (293) cells were used to study the effect of NO (produced by the addition of a NO donor) on vaccinia virus-infected cells, as well as in murine macrophage-like RAW 264.7 cells in which NO was induced by gamma interferon (Harris et al., 1995; Karupiah & Harris, 1995). Thus, it would be of interest to study the effect of NO induced in primary target cells of DENV infection (monocytes, Langerhans cells, macrophages) on levels of membrane-bound viral replicase and the three endogenous viral RNA species.

As the inhibitory effect of NO on DENV infectivity was observed from 8 to 14 h.p.i., this suggested that NO suppressed DENV infection by acting at the viral RNA-synthesis step, where a high activity of replicase complex is observed in the DENV life cycle. Our results showed that the addition of SNAP 2 h prior to infection did not have an inhibitory effect, suggesting that NO does not affect viral entry or uncoating events. Any of the viral or host proteins necessary for viral RNA synthesis are potential targets of NO action. However, our results suggested that NO had no effect on the early step of translation (0–6 h.p.i.) before RNA replication occurred (at ~8 h.p.i.). Initially, only a few viral RNA templates released from the input virions need to be translated before replication can begin. As a result of the inhibitory action of NO, fewer RNA templates would be available for translation and assembly during the replication phase of the virus life cycle. The effect of NO on translation of newly replicated RNA templates may depend on the simple law of mass action and availability of cellular and/or viral factors required for these processes that might be targets of NO.

Several studies have previously reported the action of NO on viral proteins. In one such study, the inhibition of coxsackievirus infection by NO was assigned either to nitration of the VP1 capsid protein (Padalko et al., 2004) or to S-nitrosylation of the cysteine protease 3C (Saura et al., 1999). Moreover, inhibition of human immunodeficiency virus type 1 infection has been shown to be due to S-nitrosylation of the viral protease by NO (Persichini et al., 1998). However, modification of cellular proteins also inhibits virus infection; for example, tyrosine nitration of microtubules attenuates respiratory syncytial virus infection (Huang et al., 2005) and S-nitrosylation of NF-kB downregulates expression of the transactivator Zta required for reactivation of Epstein–Barr virus (Mannick et al., 1994). In the present study, our results obtained by using an in vitro replicate assay showed that NO inhibited vRNA and RF synthesis. The precursor–product relationship of all three RNA species generated from the in vitro replicate assay showed that RI acted as a precursor for both vRNA and RF (Uchil & Satchidanandam, 2003). Therefore, our data suggest that NO might inhibit the step where RI → vRNA and vRNA → RF species in vitro. This conclusion is also consistent with our observation that NO inhibits de novo synthesis of negative-strand RNA by the viral polymerase in the exogenous subgenomic RNA template-dependent RdRp assay. However, a 100-fold higher concentration of SNAP was required for inhibition, as seen in the in vitro viral replicate assays, compared with the concentration required for an inhibitory effect in a cell-culture system (15 mM versus 150 μM SNAP). Several possibilities could explain this phenomenon. Firstly, inhibition of replicase activity may be one of several effects of NO that contribute to the inhibition of DENV infection. Secondly, intracellular conditions might be more favourable for NO activity than in vitro conditions. This notion is consistent with reports that the concentration of NO necessary to elicit maximal activity in vitro was ≥ 1000 times higher than that required in whole cells (Lipton et al., 1993; Stamler et al., 1992). In our study, NO was present continuously during translation of the viral RNA, polyprotein processing and assembly of the viral replicase complex on the host membranes when SNAP was added to the infected cell cultures. However, NO was present only during RNA synthesis in vitro by the pre-assembled replication complex isolated from infected cells or by the purified replication component (NS3 or NS5).

Nevertheless, our study focused on two vital proteins, NS3 and NS5, required for viral RNA synthesis. NO had no effect on the RNA helicase activity of NS3, even with the high concentrations of SNAP (15 mM) used in this assay. However, NO did show a selective inhibitory effect on the de novo synthesis of RNA rather than on the synthesis of RNA by elongation. NO might modify the RdRp enzyme covalently by S-nitrosylation in a way that changes its conformation into a form that loses its ability to initiate de novo synthesis, without affecting its activity in elongation synthesis.

NO modifies proteins by formation of S–NO (S-nitrosylation) or S–S (disulfide) bonds that could be reduced to the free thiol by DTT, a reducing agent, thereby restoring the enzymic activity. However, in our in vitro RdRp assays, DTT only partially restored the enzymic activity lost by treatment
with SNAP. This result suggests that the effect of NO on NS5 protein may not be exclusively via S-nitrosylation of cysteine residues. NO-mediated damage of nucleotide precursors, especially GTP (Rohn et al., 1999), required for RNA synthesis by the polymerase, thereby causing inhibition, cannot be ruled out. Further studies are thus required to identify the target(s) of NO in viral RNA synthesis both in vitro and in vivo in infected cells.

In conclusion, NO inhibits DENV-2 replication at the stage of de novo synthesis of viral RNA by the viral polymerase.

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