Inhibition of Vesicular Stomatitis Virus Replication by Prostaglandin A_1 in *Aedes albopictus* Cells

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Cyclopentenone prostaglandins (PGs) exhibit antiviral activity against RNA and DNA viruses in mammalian cell lines, and this effect has been associated with the induction of a heat shock protein (hsp70). We investigated the effect of prostaglandin A_1 (PGA_1) on the replication of vesicular stomatitis virus (VSV) in *Aedes albopictus* (mosquito) cells. PGA_1 was found to inhibit VSV replication dose dependently. Virus yield was reduced to 50% (3 μg PGA_1/ml) and to 95% with 8 μg PGA_1/ml. Even with the dramatic reduction of virus production observed in cells treated with PGA_1, VSV-specific protein synthesis was unaltered. Treatment of cells with PGA_1 (5 μg/ml) stimulated the synthesis of a polypeptide identified as a heat-shock protein (hsp) by immunoblot analysis. PGA_1 induced hsp70 synthesis in uninfected cells. However, in VSV-infected cells the induction of hsp70 by PGA_1 was reduced. This is the first report of antiviral effects of PGs affecting the replication of VSV in a mosquito cell line.

Key words: Prostaglandin, *Aedes albopictus* Cells, Vesicular Stomatitis Virus

Introduction

Prostaglandins (PGs) are potent eicosanoid lipid mediators, derived from phospholipase-released arachidonic acid, which are involved in numerous homeostatic biological functions and in inflammation. These compounds have been shown to function as microenvironmental hormones and intracellular signal mediators and also to participate in the regulation of a large variety of physiological and pathological processes (Funk, 2001).

During the last decade many new findings of PGs actions have been found and it has now been widely accepted that PGs of the A and J types (cyclopentenone prostaglandins) play a role in the regulation of the cell cycle and in cellular defense mechanisms against viral infection. The mecanisms by which PGs can interfere with viral replication have been intensively investigated and different results have been reported for several virus-cell systems (Parker *et al.*, 1995; Conti *et al.*, 1996). Cyclopentenone prostaglandins inhibit virus replication by acting on multiple cellular and viral targets, and can alter the synthesis, maturation and intracellular translocation of virus proteins. A relationship between heat-shock protein (hsp) synthesis induced by PGs (types A and J) and virus replication has also been described (Santoro, 1997). Recently, it was reported that cyclopentenone PGs are potent inhibitors of nuclear transcription factor NF-$\varkappa$B and of NF-$\varkappa$B-dependent HIV-1 transcription in human cells (Rossi *et al.*, 2000).

While eicosanoids are very well known in mammalian systems, there is increasing recognition of the importance of these compounds in insects and other invertebrates. Eicosanoids have physiological roles in insect reproduction, barnacle hatching, snail egg production, bivalve spawning and sea star oocyte maturation (Stanley-Samuelson, 1994). Additionally, results presented by Stanley-Samuelson *et al.* (1991) strongly support the hypothesis that eicosanoids are mediators in the invertebrate immune response.

Prostaglandins of the A and J types are potent inhibitors of vesicular stomatitis virus (VSV) replication in mouse cells and in monkey cells (Parker *et al.*, 1995; Pica *et al.*, 1993). VSV is a member of the Rhabdoviridae family that has been well characterized at the molecular level. The bullet-shaped virus particles are enveloped by a lipid bilayer. After virus entry and decapsidation, the minus-strand RNA is first transcribed by the virus polymerases, which form part of the nucleocapsid, to produce five distinct mRNA molecules, each

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encoding a single viral polypeptide. Translation of the VSV RNAs produces five proteins: a membrane glycoprotein (G), a nonglycosylated membrane glycoprotein (M), a nucleocapsid protein (N), the viral transcriptase (L) and a phosphoprotein (P), originally designated NS (Rose and Whitt, 2001).

The isolation of *A. albopictus* cell clones capable of producing high yields of infectious VSV has facilitated the study of this virus in mosquito cells (Gillies and Stollar, 1980).

To extend our studies of the effects of prostaglandins in mosquito cells, we decided to examine the effect of PGA_1 on the replication of VSV in *A. albopictus* cells.

Materials and Methods

Cell culture

Aedes albopictus cells, clone C6/36, were used in this study. This cell line was a gift from the Arbovirus Research Unit, Yale University, USA. The cells were maintained at 28 °C in Leibovitz's (L-15) growth medium, supplemented with 0.2 mM non-essential amino acids, 0.3% tryptose phosphate broth, 0.02% L-glutamine, 10% fetal bovine serum, penicillin (500 U/ml), streptomycin (100 μg/ml) and amphotericin B (fungizone, 2.5 μg/ml). Vesicular stomatitis virus (VSV), Indiana strain (obtained from Dr. J. L. Silva, Departamento de Bioquímica Médica. UFRJ, Brasil), was propagated in monolayers of Vero cells. PGA_1 (Sigma Chemical Co, St. Louis, MO) was stored as a stock solution (1 mg/ml) in absolute ethanol and was diluted to the indicated concentrations in growth medium. The control medium contained the same concentration of ethanol diluent (0.02%) and did not affect cell growth or virus replication.

Virus infection

Aedes albopictus cells were infected with 10 plaque-forming units (PFU) per cell in medium without serum. After 60 min at 28 °C, unadsorbed virus was removed by aspiration, fresh medium was added and the culture was incubated at 28 °C. To determine antiviral activity, growth medium containing various concentrations of PGs was added to the cell monolayers after the virus adsorption period. Virus production in untreated and PGs-treated cells was determined by plaque assay, 24 h after infection. Briefly, virus dilutions (0.3 ml) were added to Vero-cell monolayers that had just reached confluency. After 60 min. at 37 °C, unadsorbed virus was removed by aspiration and the monolayers were overlaid with growth medium, supplemented with 10% fetal bovine serum and 50% Karaya gum (Sigma Chemical Co, St. Louis, MO), and were further incubated in an atmosphere of 5% CO_2 at 37 °C for 3 days. The monolayers were then stained with crystal violet (1%) and the virus plaques were counted.

Analysis of [^{35}S]-methionine labeled proteins by polyacrylamide gel electrophoresis

Confluent cell monolayers were labeled with [^{35}S]-methionine (0.74 MBq/ml) in methionine-free medium for the protein synthesis studies. After labeling, cells were washed with PBS and lysed in lysis buffer (62.5 mM Tris-HCl, pH 6.8; 2% SDS; 10% glycerol; 5% 2-mercaptoethanol and 0.001% bromophenol blue). Samples were then heated for 5 min at 100 °C and subjected to electrophoresis on one-dimensional 12.5% polyacrylamide gels. The dried gels were exposed to Kodak X-Omat film and analysed with the Kodak BIOMAX Transcreen (LE) intensifying screen system. Protein synthesis was quantified by densitometric analysis of autoradiographic patterns with a laser-beam densitometer (Ultroscan 2202, LKB Instruments, Bromma, Sweden). VSV proteins were designated by their relative molecular weight estimated by comparison to standard proteins (Pharmacia).

Immunoblot analysis

For immunoblot analysis an equal amount of protein from each sample was separated by SDS-polyacrylamide gel electrophoresis (PAGE), as described above, and blotted onto nitrocellulose. The blots were developed with monoclonal anti-hsp70 mouse antibody, mouse ascites fluid, clone BRM-22 (Sigma Chemical Co) and horseradish peroxidase-linked whole antibody from mice (ECL, Amersham, UK).

Results and Discussion

Prostaglandins have been found to inhibit effectively the replication of VSV in several mammalian cell lines (Parker *et al.*, 1995; Pica *et al.*, 1993; Santoro *et al.*, 1983). We determined the antiviral effect of PGA_1 in a mosquito (*Aedes albopictus*) cell line. PGA_1 inhibited VSV production in a dose dependent manner. Virus yield could be re-

duced to 50% by 3 μg PGA_1/ml and to 95% with 8 μg PGA_1/ml (Fig. 1). No toxic effects on the cells were observed in these antiviral assays, determined by microscopic examination and by trypan blue dye exclusion.

To study the effect of PGA_1 treatment on VSV-protein synthesis, *A. albopictus* cell monolayers infected with VSV (10 PFU/cell) were treated with PGA_1 (5 μg/ml) after the adsorption period. After 24 h post infection proteins were labeled with ^{35}S-methionine (20 μCi/ml) and cellular extracts were processed for electrophoresis and autoradiography. The pattern of VSV protein synthesis in PGA_1 treated cells appeared to be similar to that found for untreated cells. The positions of structural virus proteins designated G, N, NS and M, whose molecular weights are 64, 48, 43 and 29 kDa respectively, are indicated (Fig. 2). This result was confirmed by densitometric analysis of the autoradiogram (data not shown). Therefore, the impairment of VSV production by PGA_1 in *A. albopictus* cells occurs without an apparent modification of VSV-protein synthesis, suggesting that PGA_1 interferes in a late event in the virus replication cycle.

In contrast, in human cells (HeLa), in L cells (mouse fibroblasts) and in monkey cells (MA-104) PGA_1 treatment inhibits VSV protein synthesis (Conti *et al.*, 1996; Pica *et al.*, 1993; Santoro *et al.*, 1983).

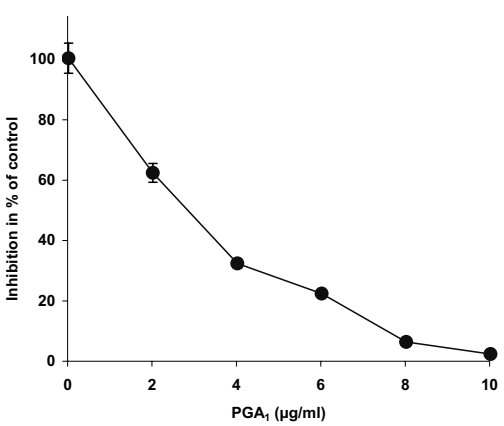

Fig. 1. Effect of PGA_1 on VSV production. *Aedes albopictus* cells were infected with VSV (10 PFU/cell), and after 60 min at 28 °C unadsorbed virus was removed by aspiration. Different concentrations of PGA_1 in growth medium were added to the cells (●). One set of cells was used as an untreated control. Supernatants from treated and untreated cells (triplicate samples) were then tested for their ability to form plaques in Vero cells.

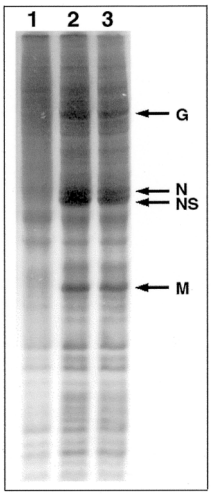

Fig. 2. Effect of PGA_1 on the synthesis of cellular and virus proteins. Cells were infected (lanes 2 and 3) with VSV, 10 PFU/ml or mock infected (lane 1) and maintained in growth medium for 24 h, in the presence of PGA_1 5 μg/ml (lane 3). After this period, the cells were labeled with [^{35}S]-methionine (0.74 MBq/ml) for 3 h and cellular extracts were subjected to PAGE. The positions of structural virus proteins (G, N, NS and M) are indicated. Densitometric analysis of the autoradiographic patterns revealed no alterations of VSV proteins in PGA_1-treated cultures compared with that of the control.

Replication of VSV occurs in several mammalian cell lines with a drastic inhibition in cellular protein synthesis (Rose and Whitt, 2001). We found that cellular protein synthesis was not inhibited in VSV-infected *A. albopictus* cells (Fig. 2). No cytopathic effect was observed, even at later stages of infection (72 h) and the incorporation of ^{35}S-methionine (trichloroacetic acid insoluble material) in VSV-infected *A. albopictus* cells was unaltered when compared with control cells (data not shown). It is interesting to note that Gillies and Stollar (1982), working with *A. albopictus* cells (clone LT-C7), found that the inclusion of serum in the growth medium and incubation at 34 °C are

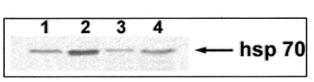

Fig. 3. Immunoblot analysis. Cells were infected with VSV or treated with PGA_1 as described in Fig. 2. After 24 h cells were processed for immunoblot analysis using anti-hsp70 monoclonal antibodies. Control (lane 1); PGA_1 (lane 2); VSV (lane 3); VSV + PGA_1 (lane 4).

conditions required for VSV to induce inhibition of host cell protein synthesis.

PGA_1 treatment induces heat-shock proteins (hsps) in *A. albopictus* cells (Meneses and Rebello, 2001). hsps are a set of proteins synthesized by prokaryotic and eukaryotic cells in response to heat treatment and other environmental stress conditions. The structure of the major hsps (the 70 kDa family) has been widely conserved through evolution, from bacteria to man, indicating an important role for the survival of the organism (Schlesinger, 1990).

The antiviral activity of cyclopentenone PGs has been associated with the induction of heat-shock protein synthesis (Mastromarino *et al.*, 1993). Additional evidence for this association is the fact that only prostaglandins with antiviral activity induce hsp70 synthesis and inhibition of virus replication has always been associated with hsp70 induction. In addition, hsps have been found to interact with viral and cellular proteins, which signifies that stress proteins could be involved in the control of virus replication (Santoro, 1994).

To determine whether PGA_1 treatment could induce hsp70 synthesis in VSV-infected cells, monolayers were treated with PGA_1 (5 μg/ml) during 24 h infection period. After this period, proteins were extracted and an equal amount of protein from each sample was separated by SDS-PAGE and processed for immunoblot analysis using anti-hsp70 monoclonal antibody. As shown in Fig. 3, PGA_1 induced hsp70 synthesis in *A. albopictus* cells and high levels of constitutive hsp70 accumulated in uninfected cells. However, infection with VSV inhibited hsp70 synthesis in PGA_1 treated and untreated cells.

The interference of virus infection with the induction of hsp70 synthesis seems to vary according to the virus-cell system analyzed (Santoro, 1994). Infection of monkey cells with VSV and Sendai virus did not interfere with the induction of hsp70 synthesis by PG. However, Conti *et al.* (1996) observed that in Hela cells infection with poliovirus inhibits the induction of hsp70 synthesis by PGA_1 and PGJ_2. They suggest that the lack of capacity of cyclopentenone PGs to block poliovirus protein synthesis could be due to an impairment of the heat-shock response in poliovirus-infected cells.

Prostaglandins and other derivatives of polyunsaturated fatty acids have been detected in a large number of invertebrate species (Stanley-Samuelson, 1994). Inhibition of eicosanoid formation in larvae of the tobacco hornworm *Manduca sexta*, using specific inhibitors of phospholipase A_2, cyclooxygenase and lipoxygenase, severely weakened the capacity of the larvae to clear the bacterium *Serratia marcescens* from their hemolymph (Stanley-Samuelson *et al.*, 1991). These authors suggested that eicosanoids mediate transduction of the bacterial infection signals into the complex of cellular and humoral responses that comprise invertebrate immunity. Recent studies have addressed the effect of inhibitors of eicosanoid biosynthesis on *Bacillus thuringiensis* δ-endotoxin response in cultured insect cells (Johnson and Howard, 1996). Eicosanoids are also an important component in the overall regulation of basal fluid secretion rates in the Malphigian tubules of the yellow fever mosquito *Aedes aegypti* (Petzel and Stanley-Samuelson, 1992). In our laboratory we found (Barbosa and Rebello, 1995) that in *A. albopictus* cells, PGA_1 inhibits replication of Mayaro virus and induce the synthesis of stress proteins.

Based on what we observed here, we now think it would be useful to determine whether the spread of arboviruses by wild mosquitoes is affected by eicosanoids.

Acknowledgements

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