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INFLUENCE OF THROMBOXANE SYNTHETASE INHIBITORS ON VIRUS REPLICATION IN HUMAN LUNG FIBROBLASTS IN VITRO

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Selective modulation of cellular arachidonic acid metabolism with thromboxane synthetase inhibitors temporarily reduced the yield of viruses hosted by human lung fibroblasts in vitro. The results were similar for several viruses including type I herpes simplex virus, vaccinia, vesicular stomatitis virus, chikungunya virus, and Newcastle disease virus. Thromboxane synthetase inhibitors of different structural classes were effective and their effects were confined to cells that contain the thromboxane synthetase. Virus yields were unaltered by total inhibition of arachidonic acid oxidative metabolism or exogenous addition of prostaglandins. In contrast to most cytopathic agents, viruses destroyed host cells without stimulating prostaglandin synthesis unless interferon induction accompanied the infection in vitro. The results suggest that cellular arachidonic acid metabolism may contribute to the host defense response during virus infections.

Prostaglandins (PG)[1] or other arachidonic acid metabolites exert paradoxical effects on the pathogenesis of viral infection in vivo or in vitro. For certain virus-host combinations exogenously added prostaglandins increase plaque formation, inhibit cell mediated immunity, and antagonize interferon activity (1-4); for others they suppress virus replication, activate cell mediated immunity and supplement interferon activity (6-13). Agents that inhibit prostaglandin biosynthesis can be either proviral or antiviral in vivo (14,15); however, in vivo models are mechanistically ambiguous because of the different components of the host defense response.

To isolate effects derived solely from the host cell we studied the relationship between virus infection and cellular arachidonic acid

[1]Abbreviations used: PG, prostaglandins; HVS-I, type I herpes simplex virus; NDV, Newcastle disease virus; VSV, vesicular stomatitis virus; VACC, vaccinia virus; CV, chikungunya virus; TXB_2, thromboxane B_2; PFU, plaque forming units; MEM, modified Eagle's medium.

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metabolism of WI-38 and MRC-5 human lung fibroblasts *in vitro*. These cells produce both thromboxane A_2 and prostaglandin E_2 from a common substrate (16,17). Selective modulation of endogenous cellular arachidonic acid metabolism with thromboxane synthetase inhibitors altered the replication of several viruses hosted by these cells. Virus infection, itself, did not alter cellular arachidonic acid metabolism except when the virus induced interferon.

EXPERIMENTAL

Materials

Prostaglandin E_2, thromboxane B_2, and selective thromboxane synthetase inhibitors (18-21) were supplied by the Experimental Chemistry Laboratories of the Upjohn Company. [^{3}H] Prostaglandin E_2, 117 Ci-mmole^{-1}; and [^{3}H] thymidine, 14 Ci-mmole^{-1} were purchased from New England Nuclear, Boston, Mass. Antisera against human β interferon was provided by W.E. Stewart II. WI-38, human lung fibroblasts, were purchased from Flow Laboratories, Rockville, MD. MRC-5 human lung fibroblasts and African Green Monkey kidney VERO cells were purchased from Microbiological Associates, Bethesda, MD.

Methods

WI-38, MRC-5, and VERO cells were grown to confluency in 35 mm plastic Petri dishes in modified Eagle's medium, 2 mL per plate, with 5% v/v heat inactivated fetal calf serum; 100 units-mL^{-1} penicillin and 50 μg-mL^{-1} streptomycin. Types I herpes simplex virus, strain 42-D; Newcastle disease virus, Herts strain; vesicular stomatitis virus, Indiana strain; vaccinia virus and chikungunya virus were propagated and titrated as previously described (11). Confluent monolayers of WI-38, MRC-5 and VERO cells were infected by incubating the cells with virus for 1 hour. After rinsing infected cells twice with 2 mL of isotonic phosphate buffer, 0.1 M, pH 7.4 to remove unattached virus particles, the cells were replenished with 1 mL of MEM. In certain experiments, the MEM contained the thromboxane A_2 synthetase inhibitors: 9,11-diazo-prosta-5,13-dienoic acid, 1 μg-mL^{-1} (18); 9,11-iminoepoxy-prosta-5,13-dienoic acid, 5 μg-mL^{-1} (19) n-butylimidazole, 5 μg/mL (20), or the thromboxane A_2 receptor level antagonist, 9,11-epoxyimino-prosta-5,13-dienoic acid, 1 μg/mL^{-1} (21). Medium was collected from the plates at the times indicated in the figures. Samples were analyzed for prostaglandin E_2, thromboxane B_2, virus yield, and interferon yield. The results are representative of several independent experiments each run in duplicate.

Prostaglandin E_2 and thromboxane B_2 were determined by radioimmunoassay (21). Interferon in serially or logarithmically diluted samples (1.0 mL) was measured by VSV plaque reduction assays on confluent monolayers of human foreskin fibroblasts (22). The international reference standard of human interferon, G-023-901-527, provided by the NIH was used for calibration. Virus replication was measured with a modified Dulbecco plaque assay (23). Herpes simplex type I, vaccinia, and vesicular stomatitis were assayed on confluent monolayers of primary rabbit kidney cells. Newcastle disease virus and chikungunya virus were assayed on confluent monolayers of chick kidney cells. The virus cytopathic effect was estimated visually, according to the amount of monolayer destruction.

RESULTS

The addition of 9,11-diazo-prosta-5,13-dienoic acid, a thromboxane synthetase inhibitor, to the culture media reduced the virus yield during the eclipse phase of a productive infection with type I herpes simplex virus in both WI-38 and MRC-5 human lung fibroblasts (Figure 1). The temporary reduction in virus yield coincided with selective inhibition of thromboxane B_2 levels. PGE_2 levels were unaltered or slightly increased due to diversion of unmetabolised substrate from thromboxane synthetase toward prostaglandin isomerase.

Indomethacin, (5×10^{-6}), suppressed the biosynthesis of both metabolites, TXB_2 and PGE_2; but, in contrast to selective modulation, their mutual, simultaneous inhibition had no effect on HSV I virus yield or cytopathic effect during a productive infection in WI-38 and MRC-5 fibroblasts. Alteration of the TXB_2/PGE_2 ratio by the addition of exogenous PGE_2 (0.1-5 μg/mL) to the cell culture media did not influence the HSV I virus yield. PGA_2 (0.1-5 μg/mL) did not affect the HSV I virus yield.

Other thromboxane synthetase inhibitors gave comparable results. In MRC-5 cells, at 4, 8, 12 and 24 hours 9,11-iminoepoxy-prosta-5,13-dienoic acid (5 μg/mL) reduced HSVI virus yields from 2.3×10^2 and 5.3×10^2, 3.5×10^2 and 1.5×10^4 PFU/ml to <50, <50, 1.3×10^2 and 5.5×10^3 PFU/ml respectively. In WI-38 cells 9,11-iminoepoxy-prosta-5,13-dienoic acid, 5 μg/ml reduced HSV I virus yield from 6.3×10^2, 2.8×10^2 and 2.5×10^2 PFU/ml to < 50, <50, and 10^2 at 4, 8 and 12 hours after infection. By 24 hours, HSV I virus yields had returned to control values, 2×10^4 PFU/ml, in WI-38 cells. Radioimmunoassay determinations confirmed that the thromboxane B_2/prostaglandin E_2 ratio was modified. N-butylimidazole, a thromboxane synthetase inhibitor that is structurally unrelated to prostaglandins was equally effective. In MRC-5 cells N-butylimidazole reduced HSV I virus yields from 1.5×10^2, 1.2×10^2 and 5.7×10^5 PFU/mL, to <50, <50, and $3.5 \times$

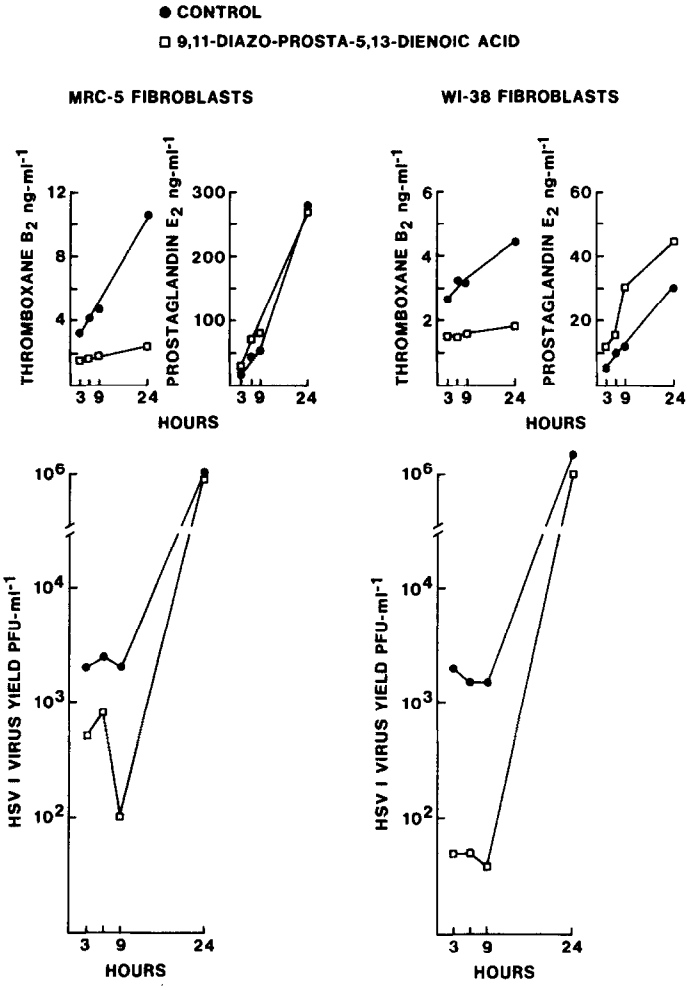

Figure 1. Effect of Thromboxane A_2 Synthetase Inhibition on Herpes Simplex I Virus Replication in MRC-5 and WI-38 Fibroblasts. Cells were infected with 7 x 10^4 PFU of Herpes simplex type I. After a one hour absorption period the unattached virus particles were removed by washing and the cells were replenished with growth medium containing the thromboxane A_2 synthetase inhibitor 9,11-diazo-prosta-5,13-dienoic acid, 1 μg-ml-1. Results for MRC-5 fibroblasts are depicted on the left hand side and results for WI-38 fibroblasts on the right hand side. For both cell types, 9,11-diazo-prosta-5,13-dienoic acid suppressed thromboxane B_2 formation. This suppression was selective, and prostaglandin E_2 levels increased due to the availability of increased prostaglandin endoperoxide H_2 substrate. Treatment with the thromboxane synthetase inhibitor temporarily reduced the herpes simplex type I virus yield during the eclipse phase of the infection.

10^5 PFU/mL at 4, 8, and 24 hours respectively. Thromboxane B_2 and PGE_2 levels were 3.7 ng/mL and 30 ng/mL, respectively, in the media of HSV I infected control cells versus 2.8 ng/mL and 88 ng/mL, respectively, in the media of n-butyl-imidazole treated cells 24 hours after infection.

Results were similar for other viruses. For instance, thromboxane synthetase inhibitors temporarily reduced the vesicular stomatitis virus yield in both MRC-5 and WI-38 fibroblasts (Figure 2). Virus yields returned to control values by 24 hours. A thromboxane A_2 receptor level antagonist, 9,11-epoxyimino-prosta-5,13-dienoic acid consistently suppressed virus yields in MRC-5 cells only.

Neither the inhibitors nor the antagonist affected [^{3}H] thymidine incorporation into WI-38 or MRC-5 cells, or cell proliferation, during their logarithmic growth phase. The compounds did not influence virus infection in human foreskin fibroblasts, a cell line that does not produce thromboxane A_2.

Cellular biosynthesis of thromboxane and prostaglandin E_2 was unaltered by virus infections regardless of their cytopathic effect;

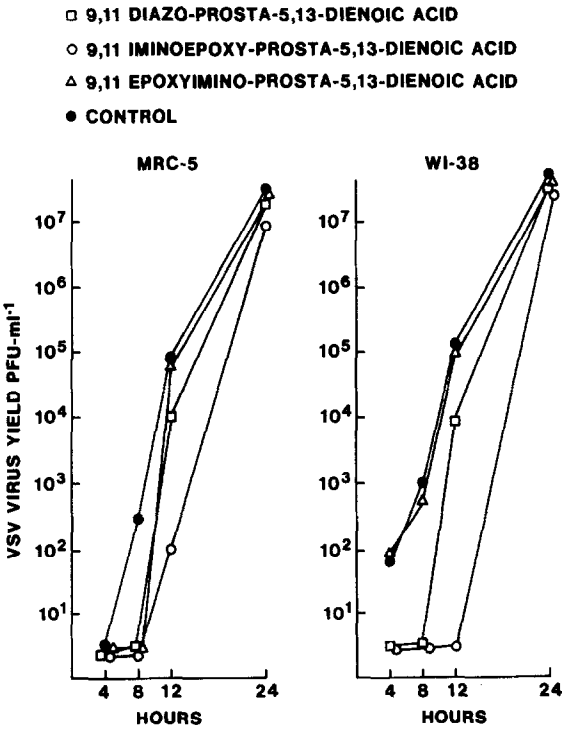

Figure 2. Effects of Thromboxane A_2 Inhibition and Antagonism on Vesicular Stomatitis Virus Replication in MRC-5 and WI-38 Fibroblasts. Cells were infected with 1 x 10^4 PFU of VSV and replenished with growth medium as in Figure 1. Both selective inhibitors temporarily reduced VSV yields in MRC-5 and WI-38 cells. The thromboxane A_2 antagonist was effective only in MRC-5 cells.

however an increased biosynthesis corresponded with interferon induction. Table I summarizes data on the virus yield, cytopathic effect, interferon levels, and TXB_2 and PGE_2 levels 24 hours after infection with representative viruses. TXB_2 and PGE_2 levels were equivalent in infected or uninfected cells prior to detectable interferon induction. VERO cells afford a control to distinguish interferon derived effects from virus derived effects because these cells host inducing viruses, but they lack the capacity to produce interferon (24). In VERO cells, Newcastle disease virus did not induce interferon and it did not stimulate prostaglandin formation, in contrast to its effects on other cells. In separate experiments, treatment of NDV infected MRC-5 cells with human interferon β antibody reduced the interferon titer from 4000 units/mL to 100 units/mL and the corresponding PGE_2 levels declined from 286 ng/mL to 128 ng/mL. Treatment of NDV infected MRC-5 cells with cycloheximide, 50 μg/mL, reduced the interferon titer from 500 units/mL to <10 units/ml and the corresponding PGE_2 levels declined from 529 ng/mL to 84 ng/mL. Treatment with thromboxane synthetase inhibitors did not affect the interferon levels. These results concur with previous reports indicating that, for a battery of viruses and host cells, infection alone does not necessarily activate arachidonic acid metabolism unless accompanied by interferon induction (25). Under certain circumstances, even interferon induction does not activate cellular arachidonic acid metabolism (8).

Our results suggest that cellular thromboxane A_2 biosynthesis may influence virus infection, since its selective modulation temporarily reduced the yield of viruses hosted by human lung fibroblasts *in vitro*. Linkage of this effect to thromboxane synthetase was evident because virus yields were unaltered in control experiments with cells lacking this enzyme, and in control experiments with indomethacin or exogenously added PGE_2. The influence of thromboxane A_2 may be

Table 1

Influence of Virus Infection on Cellular Arachidonic Acid Metabolism

	Virus Titer (PFU/ml)			Cytopathic Effect			Interferon Levels (U/ml)		
	WI-38	MRC-5	VERO	WI-38	MRC-5	VERO	WI-38	MRC-5	VERO
Newcastle Disease	2×10^6	5×10^5	1×10^6	50%	100%	90%	390	160	<10
Chikungunya	5×10^3	2.5×10^5	6×10^5	20%	20%	80%	<50	<50	<10
Herpes Simplex I	1.5×10^6	1×10^6	2.5×10^5	80%	30%	75%	<50	<50	<10
Media Control	0	0	0	0	0	0	0	0	0

	Thromboxane B_2 (ng/ml)			Prostaglandin E_2 (ng/ml)		
	WI-38	MRC-5	VERO	WI-38	MRC-5	VERO
Newcastle Disease	12.3 ± 0.6	37.6 ± 2.3	<0.1	37.1 ± 3.5	495 ± 33	<0.1
Chikungunya	3.1 ± 0.5	11.4 ± 0.8	<0.1	22.2 ± 2.0	245 ± 3	<0.1
Herpes Simplex I	3.4 ± 0.1	10.6 ± 1.5	<0.1	29.6 ± 4.7	280 ± 36	<0.1
Media Control	3.5 ± 0.5	10.2 ± 0.9	<0.1	24.7 ± 2.5	219 ± 20	<0.1

Only interferon induction correlated with increased thromboxane B_2 and prostaglandin E_2 levels, 24 hours after infection. Prior to interferon induction, there was no increase in thromboxane B_2 or prostaglandin E_2 levels. Treatment with cycloheximide abolished the interferon responses and prostaglandin levels reverted to control values.

limited to the early stages of infection, such as attachment and penetration of the cell membrane, since the virus yields returned to control values, eventually, even though thromboxane biosynthesis remained inhibited. The selective inhibitors did not affect cellular proliferation or thymidine uptake by WI-38 or MRC-5 cells but it is notable that these agents, at 5-25 μg/ml, can inhibit DNA synthesis and cell proliferation in tumor cells *in vitro* (26). It is uncertain if the latter effects were mediated via cellular thromboxane A_2 biosynthesis. It is also notable that certain prostaglandins, such as PGA_1 and PGA_2, have anti-viral activity (6-8). Although addition of exogenous PGE_2 or PGA_2 did not alter virus replication in our experiments we cannot exclude their involvement as one component of the mechanism of inhibition. For instance, PGE_2 levels are increased concurrent with thromboxane synthetase inhibition and *in vitro* transformation of E to A series prostaglandins is expected under the experimental conditions reported (27,28). Such *in vitro* transforma-

tions have the demonstrated potential to alter cellular physiology *in vitro* (29).

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