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Inhibition of herpesvirus-induced thymidine kinase and DNA polymerase by β-hydroxynorvaline

Michael J. Massare and Herbert A. Blough

Division of Biochemical Virology and Membrane Research, Department of Ophthalmology, University of Pennsylvania School of Medicine and Scheie Eye Institute, Philadelphia, PA 19104, USA

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Treatment of HSV-infected cells with 5–10 mM β-hydroxynorvaline (Hnv), a threonine analog, specifically affects herpesvirus DNA replication: both the rate of and total DNA synthesis are reduced, the former ~15-fold by Hnv (6 h post-infection) and the latter by 12-fold (between 3 and 12 h post-infection). The effect on DNA replication was due to inhibition of HSV-1 thymidine kinase (TK) and DNA polymerase (DP) activities; the former is reduced by 75% and whereas DP returns to baseline levels (when compared to untreated and/or uninfected cells). Host cell TK and DP activities are unaffected. It is suggested that β-hydroxynorvaline is incorporated into these enzyme(s), either close to or at the active site thus perturbing viral DNA synthesis. β-Hydroxynorvaline should have unique utility as a targeted antiviral compound, acting on both membrane-mediated phenomena (fusion, penetration and attachment) and DNA replication.

DNA polymerase; Thymidine kinase; β-Hydroxynorvaline; DNA replication; (Herpesvirus)

1. INTRODUCTION

The incorporation of the threonine analog, β-hydroxynorvaline (Hnv), into some secretory proteins and some viral glycoproteins has been shown to inhibit the attachment of mannose-rich oligosaccharides to the polypeptide backbone [1–4]. We previously reported that Hnv inhibits endogenous fusion and lowers the yield of progeny herpesvirus in infected cells ~ 1–2 $\log_{10}$. Not only was the synthesis of all viral glycoproteins and the major capsid protein inhibited [3], but viral DNA synthesis also appeared to be affected [4].

Infection by HSV-1 results in the synthesis of ~ 50 different viral proteins of which about one-half are structural [5]. Among these are a viral encoded thymidine (dThd) kinase (TK) and a DNA-dependent DNA polymerase (DP). The primary sequence, kinetic and antigenic properties of these viral-induced enzymes are different from those of the host cell [6–9]. Because of their unique properties, both viral TK and DP have been used as selective targets for several different antiherpes chemotherapeutic agents viz, 9-(2-hydroxyethoxymethyl)guanine (acyclovir, ACV), (*E*)-5-(2-bromovinyl)-2′-deoxyuridine (BVDU), 9-(1,3-dihydroxy-2-propoxymethyl)guanine (DHPG) and phosphonoformate (PFA). With the exception of PFA, these agents depend upon the specific phosphorylation of dThd [10–13]. We now report that the amino acid analog, β-hydroxynorvaline, selectively inhibits both TK and DP in either infected or transfected cells.

Correspondence address: H.A. Blough, Division of Biochemical Virology and Membrane Research, Department of Ophthalmology, University of Pennsylvania School of Medicine and Scheie Eye Institute, Philadelphia, PA 19104, USA

2. MATERIALS AND METHODS

Green monkey kidney cells (GMK) and HSV-1 (HF strain) were propagated as described [14]. In

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some experiments primary rabbit kidney cells were used. Biochemically transformed LM(TK$^-$)/TFpMH110E2 cells, expressing only HSV-1 TK, were kindly supplied by Dr Fred Rapp; these were propagated [14,15] and treated with 10 mM Hnv as described [14]. TK activity was measured by the method of Cohen [16] with minor modifications: the reaction mixture contained 0.01 mM dThd and 2 μCi [^{3}H]dThd (46 Ci/mmol, Amersham). The reaction products were absorbed to DE32 gel (Whatman). DP activity was measured by the method of Purifoy and Benyesh-Melnick [17] with the following modifications: the reaction mixture contained 0.3 mM TTP and 5 μCi [^{3}H]dATP (25.4 μCi/mmol, Amersham). Background activity for both assays was measured using either a heat-activated (100°C for 2 min) extract from infected cells (obtained 9 h post-infection) or buffer alone; both gave comparable results. The rate of DNA synthesis was determined by measuring the incorporation of [^{3}H]thymidine (10 μCi/ml, 40 μCi/mmol, Amersham), following a 30 min pulse at various times post-infection, into a trichloroacetic acid-insoluble fraction(s).

3. RESULTS

Since the reduction of infectious particles ~2 $\log_{10}$ in Hnv-treated cells could not be entirely attributed to its effect on glycosylation or on protein synthesis [4], we examined other macromolecular events, viz., DNA synthesis. The rate of DNA synthesis was measured, in uninfected and infected cells, either treated or not treated with 10 mM Hnv, as described above. The results for infected cells are shown in fig.1. At 6 h post-infection, when the rate of DNA synthesis is maximal in HSV-infected cells, there was a 15-fold reduction of DNA in Hnv-treated cells. Uninfected cells, treated with 10 mM Hnv (not shown), at comparable times, revealed a 20% decrease in the rate of DNA synthesis. Total DNA synthesis at 3–12 h post-infection (measured as the area under the curves in fig.1) in Hnv-treated cells was reduced ~12-fold. In comparison to untreated control cells, we observed a reduction of total DNA synthesis of only 1.7 times in Hnv-treated cells. Thus, it appears that in addition to inhibition of glycosylation, the block in synthesis of HSV

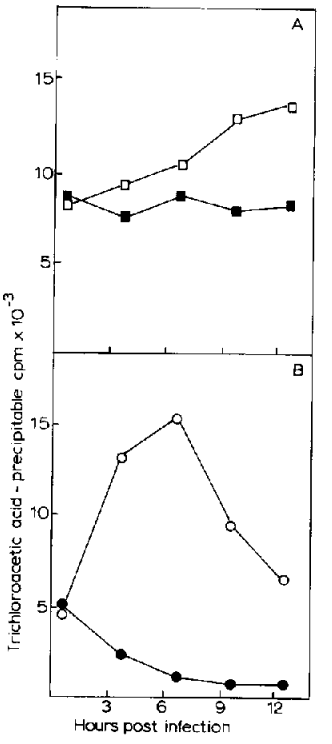

Fig.1. Effect of Hnv on the rate of DNA synthesis in uninfected (A) and HSV-infected (B) GMK cells. GMK cells were infected with HSV as described in section 2. At the indicated times the cells were pulsed for 30 min, at 37°C, with growth medium containing 10 μCi/ml [^{3}H]dThd (Amersham, 46 Ci/mmol), washed with cold HBS and solubilized with 1% SDS in 50 mM NaOH. The trichloroacetic acid-precipitable radioactivity in 10 μl aliquot is shown. Zero time is when the inoculum was removed from each plate. (–○–) Untreated cells; (–●–) cells treated with 10 mM Hnv. Hnv treatment was begun at time zero.

virions by β-hydroxynorvaline was due to inhibition of DNA synthesis.

In HSV-infected cells the bulk of the DNA produced is viral. We examined the two major viral enzymes which are required for DNA replication: viral thymidine kinase and DNA polymerase. TK activity in Hnv-treated cells was reduced ~ 75% (fig.2); uninfected cells had a TK activity of less than 10 cpm per μg protein. To rule out direct effects on TK or substrate, 10 mM Hnv was added to the reaction mixture (of infected cells). This failed to inhibit TK activity (not shown). In HSV-infected cells, DP activity was reduced to levels

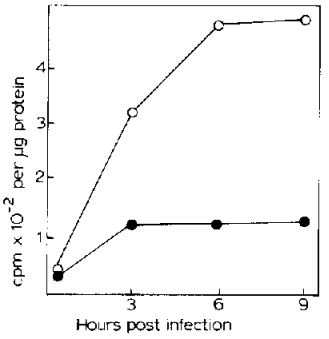

Fig.2. Effect of Hnv on TK in HSV-infected GMK cells. GMK cells were infected with HSV as described in section 2. At the times indicated the cells were rinsed with TBS (10 mM Tris-HCl, 150 mM NaCl, pH 7.8), suspended in 80 mM Tris-HCl, pH 7.8, 2 mM DTT; sonicated for 60 s and centrifuged for 2 min in a Beckman microfuge. The supernatant was used in the enzyme assay. All assays were done in duplicate at each time interval. (−○−) Untreated, HSV-infected cells; (−●−) HSV-infected cells treated with 10 mM Hnv.

found in uninfected cells and in cells at 0 h post-infection (fig.3). From these studies we conclude that Hnv treatment of infected cells reduced DP activity to almost nil. The conditions of the reaction mixture we used were optimal for viral DP and inhibitory for host DP(s). The presence of 100 mM $(NH_4)_2SO_4$, in the reaction mixture inhibited DP activity in uninfected cells ~ 8-fold; whereas that of HSV-infected cells, was enhanced 2.4 times. As little as 2.0 mM Hnv blocked TK activity in HSV-infected cells by ~ 20% (fig.4). The viral origin of TK was confirmed in two ways: Firstly, antibody raised in rabbits against the viral TK [using an infected heterologous cell extract as antigen(s) (gift of Dr Graham Darby, Wellcome Laboratories, England], totally blocked the TK activity. The reaction mixture was incubated with the antiserum for 2–3 h at 20°C and then at 0°C for 1 h; normal rabbit serum had no effect. Secondly, when cells transfected with the plasmid containing the TK gene, LM(TK$^-$)TFpMH110E2, were treated with 5 mM Hnv, for 10 h and the cell extracts examined, as above, TK activity was reduced by ~ 55% when compared to untreated, transfected cells (Massare and Blough, unpublished).

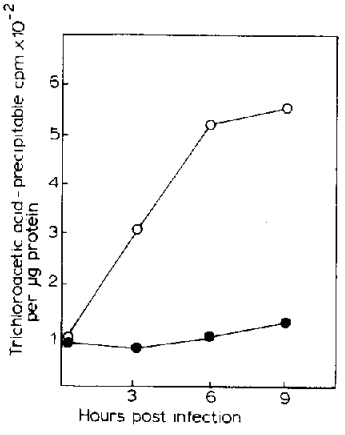

Fig.3. Effect of Hnv on DP in HSV-infected GMK cells. GMK cells were infected with HSV and treated with Hnv as described in fig.1. At the times indicated the cells were washed with TBS, harvested using a rubber policeman, resuspended in 10 mM Tris-HCl, 10 mM NaCl, pH 7.4, and sonicated for 5 min. The cell extract was assayed for DP using heat denatured DNA (salmon sperm, Sigma). (−○−) Untreated, HSV-infected cells; (−●−) HSV-infected cells treated with 10 mM Hnv.

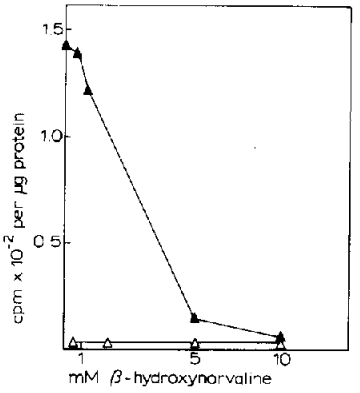

Fig.4. Dose response curve of TK activity in HSV-1-infected (−▲−) and uninfected (−Δ−) primary rabbit kindey cells treated with Hnv. Cells were infected with HSV-1 and treated with various concentrations of Hnv as described in fig.1. TK activity (y-axis) was assayed at 7 h post-infection.

4. DISCUSSION

From the results of this study, it was apparent that β-hydroxynorvaline markedly inhibited viral DNA synthesis by altering both thymidine kinase and DNA polymerase activities of HSV-1; the latter was diminished to almost undetectable levels

(by our assay system). There are several plausible explanations for this: firstly, the incorporation of Hnv into the enzyme(s) produced an aberrant TK and/or DP [as isoenzyme(s)]; secondly, Hnv led to the production of less and/or aberrant regulatory viral polypeptides; thirdly, Hnv caused the production of altered viral polypeptides which were more labile to proteolysis; fourthly, Hnv produced alterations in the regulating genes of the DNA polymerase which modified the TK; finally, any combination of the above leading to decreased synthesis and/or activity.

Recent studies of HSV-1 have shown the presence of at least one Thr residue (at position 63) within the ATP-binding site of the TK molecule [18,19]. In addition, a triplet of Thr residues are present at positions 64–66; the latter is directly adjacent to the ATP-binding site. The subsitution of Hnv into the polypeptide chain(s) does alter the recognition site for the attachment of *N*-linked oligosaccharides [3]. The replacement of Thr with Hnv, at (or proximal to) the active site of these enzyme(s) or regulatory peptide(s), would be expected to alter their tertiary and quarternary structure thus, inhibiting enzymatic function. Similarly, the incorporation of Hnv into α-polypeptides [Translation in HSV-1-infected cells occurs in a regulated cascading fashion. The polypeptides are grouped into α-, β- and γ-polypeptides: the α-polypeptides are synthesized first and induce the synthesis of β-polypeptides. The latter induce the synthesis of γ-polypeptides and shut-off the translation of α-polypeptides. The γ-polypeptides are made last and inhibit β-polypeptide translation. Viral DNA replication occurs after the onset of β-polypeptide synthesis; the latter is required for DNA synthesis.] of HSV-1 may modify the induction of β-peptides (TK and DP) resulting in diminished quantities of TK and DP being synthesized. We have been able to demonstrate, using the cycloheximide block method [20], that the synthesis of β- and γ-polypeptides in HSV-1-infected cells was inhibited by 10 mM Hnv (Massare and Blough, unpublished). However, at this time we are unable to ascertain if these peptides are absent or nonfunctional (if synthesized) by SDS-PAGE and fluorography. There are several known examples where the substitution of a single amino acid in a peptide alters its function and physiochemical properties. These include sickle-cell hemoglobin (valine β-6 substitution), influenza virus hemagglutinin and SV40 T antigen as well as the HSV-1 *syn* polypeptide [21–23]. Thus, β-hydroxynorvaline appears to be targeted at HSV-1 enzymes and regulatory polypeptides, with little or no effect on uninfected cells, suggesting that the de novo pathway (thymidylate synthetase) [24] was used to synthesize DNA. Hnv was not toxic to the cell types used in this experiment. However, certain cells of lymphocytic origin and cells persistently infected with HIV show signs of cytotoxicity at concentrations of Hnv above 0.8 mM [25]. This selectivity, as well as its marked effects on *N*-linked oligosaccharides, enhances the potential of Hnv, as a useful antiviral agent against herpesvirus infections.

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