Suitability of Enalapril as a Probe of the Dipeptide Transporter System: In Vitro and In Vivo Studies

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Purpose. Previous in situ and in vitro studies indicated that the intestinal absorption of enalapril is a saturable carrier-mediated process via the dipeptide transporter system (DTS); however, the oral absorption of enalapril has not been reported to be a saturable process in vivo. Our objectives were to: 1) evaluate the suitability of enalapril as a probe of the DTS, and 2) compare various experimental models as they pertain to studying the DTS.

Methods. The in vitro uptake of enalapril by rat intestinal rings and permeability across Caco-2 cells were studied as a function of concentration and in the presence of compounds that are known substrates of the DTS. The effect of enalapril on the uptake of [3H]-glycyl-L-proline (gly-L-pro) by Caco-2 cells was also examined. In vivo studies were conducted in rats (1 to 50 mg/kg) and dogs (0.06 to 6 mg/kg) to evaluate the oral absorption of enalapril over a wide dose range.

Results. In vitro intestinal uptake/permeability of enalapril was not saturable nor inhibited by β-lactam antibiotics, gly-L-pro, or SQ-29852. Moreover, a 20,000-fold molar excess of enalapril did not inhibit the uptake of [3H]-gly-L-pro by Caco-2 cells. The in vivo studies in rats and dogs did not demonstrate saturable absorption.

Conclusions. The present in vitro and in vivo results indicated that enalapril is primarily absorbed by a non-saturable, passive diffusion process and it is not a suitable model compound for studying the DTS.

KEY WORDS: enalapril; absorption; Caco-2; rats; dogs.

INTRODUCTION

Enalapril maleate (hereafter referred to as enalapril) is an ester prodrug that is converted in vivo to enalaprilat, which is the active angiotensin converting enzyme (ACE) inhibitor. A previous report, based on in situ intestinal perfusion in rats, concluded that the intestinal absorption of enalapril was a saturable carrier-mediated process via the dipeptide transporter system (DTS) (1). Additional studies in vitro (2,3) with rabbit brush border membrane vesicles and Caco-2 cells (4) also concluded that enalapril was absorbed via the DTS.

Our objective was to use a variety of experimental systems to evaluate the suitability of enalapril as a model compound for studying the DTS. This involved determination of: 1) the in vitro uptake (everted rat intestinal rings) and permeability (Caco-2) of enalapril over a wide concentration range and 2) the uptake/permeability of enalapril in the presence of compounds that are known substrates of the DTS. When these in vitro studies failed to demonstrate concentration-dependent uptake/permeability or involvement of the DTS, in vivo studies were then conducted in rats (1 to 50 mg/kg) and dogs (0.06 to 6 mg/kg) over a wide dose range. The current results with enalapril are compared to those reported previously for SQ-29852, an ACE inhibitor that is a stable and specific probe of the DTS (5); this comparison addresses the suitability of the various in vitro, in situ, and in vivo model systems that are currently being used to characterize the DTS.

MATERIAL AND METHODS

Materials

[14C]-Enalapril (5.3 mCi/mmol, radiochemical purity of 97%) and SQ-29852 were synthesized at Bristol-Myers Squibb. [3H]-glycyl-L-proline (proline-3,4-3H; 50 Ci/mmole) and [14C]- Methoxyxynil (5.6 mCi/g) were purchased from NEN Research Products (Boston, MA). Enalapril, β-lactams (Table I), glycyl-L-proline (gly-L-pro), sodium azide, triton X-100 (TX-100), Hank’s balanced salt solution (HBSS), 2-N-morpholinoethanesulfonic acid (MES) and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) were purchased from Sigma Chemical Co. (St. Louis, MO). TX-114 (National Diagnostics, NJ) or Ecolite® (ICN, CA) scintillation cocktail was used for counting radioactive samples. Fetal bovine serum was obtained from Hyclone Lab. Inc. (Logan, Utah). Rat tail collagen-type I was purchased from Collaborative Research Inc. (Bedford, MA). Caco-2 cells (passage #17) were obtained from ATCC (Rockville, MD). Dulbecco’s modified Eagle’s medium, nonessential amino acids, L-glutamine, penicillin-G, trypsin/EDTA, and streptomycin were purchased from JH Biosciences (Lenexa, KS). Transwell® inserts (surface area: 4.7 cm²) with a polycarbonate membrane (3 μm pores) were purchased from Costar (Cambridge, MA). All other reagents were at least analytical grade.

Uptake of [14C]-Enalapril by Rat Everted Intestinal Rings

The present research adhered to the “Principles of Laboratory Animal Care” (NIH publication #85-23, revised 1985). The everted intestinal rings were prepared as follows. Male rats (Sprague-Dawley; 250 to 350 g, Harlan MD) were anesthetized with ether, and 5- to 10-cm segments of jejunum were removed, rinsed with Krebs-Henseleit (KH) buffer and everted with a glass rod. The rings were prepared by cutting several transverse slices (ca. 2 mm, 20 to 30 mg). The time-course of uptake of enalapril (90 cycles/min; 37°C) was determined at selected times up to 3 min and a 45-sec incubation time was selected for subsequent studies. Three rings were transferred into a tube (13 × 100 mm) containing [14C]-enalapril (0.01 to 0.08 mM) and one of the β-lactam antibiotics (6 mM, Table I) in pH 6.0 phosphate buffer. Control experiments were conducted with [14C]-enalapril alone at three bath concentrations: 0.01, 0.08, and 6 mM.
Table I. Uptake of Radioactivity by Everted Rat Intestinal Rings (Mean ± SD; n = 3) 45 Seconds After Incubation with [14C]-enalapril in the Presence of Various β-lactam Antibiotics (6 mM)

<table>
<thead>
<tr>
<th>β-Lactam Antibiotic (6 mM)</th>
<th>Uptake of Radioactivity* (nmol·Eq. mg/min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None (Control)</td>
<td>0.01 mM</td>
</tr>
<tr>
<td>Cefaclor</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Cefazolin</td>
<td>1.2 ± 0.1</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>Cephalosporin-C</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>Cephalexin</td>
<td>1.2 ± 0.4</td>
</tr>
<tr>
<td>Cephradine</td>
<td>1.6 ± 0.3</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>1.2 ± 0.4</td>
</tr>
</tbody>
</table>

* ANOVA indicated lack of significant inhibition by the β-lactam antibiotics (p > 0.05).
§ Expressed as equivalents of enalapril.
\( \text{Bath concentration of [14C]-enalapril.} \)

Prior to analysis of drug uptake, the rings were removed from the bath, rinsed with ice-cold KH buffer, and blotted dry. Each ring was weighed, digested with Solvene-350 (Packard Instruments, Downers Grove, IL), neutralized, and mixed with 15 mL of scintillation cocktail and counted for 14C (Packard LSC Model 1600).

Effect of Enalapril on the Uptake of [3H]-Gly-L-Pro by Caco-2 Cells

Validation of the model used to assess the inhibition of [3H]-gly-L-pro uptake by enalapril is described elsewhere (6). Caco-2 cells (passage number of 25 to 35) were seeded (160,000 cells/well) and grown for 7 to 9 days on 24-well tissue culture plates (2 cm²). Just prior to conducting the uptake study, the culture medium was aspirated and the monolayers were washed with buffer at 37°C. The incubation medium was HBSS with 25 mM MESS, [3H]-gly-L-pro (50 nM), 10 mM proline, 2% DMSO and enalapril (1 or 10 nM). Excess non-radio-labeled proline was added to maximize specific binding because it blocks uptake of the small amount of [3H]-proline that is formed from hydrolysis during the 3 min incubation. Control incubations were done without addition of enalapril. The pH was adjusted to 6 with NaOH (1M). This solution (250 μL) was added to the well and incubated for 3 min at 37°C on an orbital shaker (50 cycles / min). The solution was removed after 3 min and the monolayers were washed with PBS sodium azide 0.05% (w/v) at 4°C. The monolayers were solubilized with 1% TX-100 at 37°C. The cell suspension was mixed with 15 mL of scintillation cocktail and counted for tritium. The data are expressed as the percentage inhibition of [3H]-gly-L-pro uptake by enalapril vs control (no enalapril).

Permeability of Enalapril Across Caco-2 Cells

Caco-2 cells (passage number 33) were seeded (80,000 cells/cm²) onto a collagen coated polycarbonate filter. Growth media was Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 1% nonessential amino acids, 1% L-glutamine, 100 U/ml penicillin-G, and 100 μg/ml streptomycin. The culture medium was replaced every two days for a total of 24 days, and the cells were maintained at 37°C, 90% relative humidity, and 5% CO₂.

The permeability medium was modified Hank’s balanced salt solution (HBSS) containing either 10 mM HEPES, pH 7.4 (basolateral side) or 25 mM MES, pH 6.0 (apical side). The permeability studies were initiated by adding 1.5 mL of HBSS (pH 6.0) containing enalapril (the initial concentration ranged from 0.1 to 10 mM) and [14C]-methoxylin (0.4 μCI) to the apical side of the monolayer. The permeability of enalapril (0.3 mM) was also examined in the presence of several DTS substrates (e.g., SQ-29852, cephradine, or gly-L-pro, 3 mM). The monolayers were placed on an orbital shaker (50 cycles/ min) and incubated up to 3 h at 37°C. At hourly intervals, the Transwell® insert was moved to a new receiver well containing fresh HBSS to maintain sink conditions. Samples were taken from each receiver well and the apical compartment was sampled at the end of the 3-h period.

Enalapril concentrations were analyzed by a specific HPLC-UV assay. A C₁₈ Bondapak column (3.9 mm × 30 cm; Waters, Millipore Corp., Milford, MA) was used. The mobile phase, which consisted of solvent A (water:acetoni-trile:trifluoroacetic acid, 95:5:0.1 v/v/v) and solvent B (water:acetoni-trile:trifluoroacetic acid, 20:80:0.1 v/v/v), was programmed as a linear gradient (flow rate was 1.2 mL/min; 220 nm). The concentrations of [14C]-methoxylin were determined by liquid scintillation counting. Permeability coefficient (Pc) values (expressed as cm/sec × 10⁻⁶) were calculated as follows: \( Pc = dA/dt \cdot S \cdot C_o \), where dA/dt is the flux of drug across the monolayer, S is the surface area of the cell monolayer (4.71 cm²), and C₀ is the initial concentration in the apical fluid.

In Vivo Studies on the Oral Absorption of Enalapril over a Wide Range of Doses

Rats

After an overnight fast, each male rat (about 300 g) received an oral aqueous dose of [14C]-enalapril by gavage at the following doses: 1, 5, and 50 mg/kg (n = 4). In addition, an iv dose of [14C]-enalapril (5 mg/kg) was administered to a separate group of rats (n = 4). Urine was quantitatively collected for 72 h and 0.2-ml aliquots were mixed with TX-114 scintillation cocktail prior to counting. The estimate of absolute absorption was calculated by dividing the amount of radioactivity recovered in 0 to 72-h urine for each rat after oral administration by the average recovery of radioactivity after iv administration.

Dogs

After an overnight fast, each male beagle dog (10 to 15 kg; n = 6) received an oral aqueous dose of [14C]-enalapril by gavage at the following doses: 0.06, 0.6 and 6 mg/kg; at least five days elapsed between doses. Urine was quantitatively collected for 72 h and radioactivity was analyzed as described above for rat urine. The minimum estimate of oral absorption was based on the amount of radioactivity recovered in 0 to 72-h urine. Statistical analysis was performed with a paired student’s t-test with a significance level of p < 0.05.