Oral Absorption of Peptides Through the Cobalamin (Vitamin B12) Pathway in the Rat Intestine

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INTRODUCTION

The increasing availability of therapeutic peptides and proteins would be well served with the development of convenient methods of administering these molecules to the patient. Polypeptides are susceptible to proteolytic degradation and do not diffuse across biological membrane barriers, and are therefore commonly administered by injection. To avoid the encumbrance of parenteral application, most epithelial surfaces are being evaluated as alternative routes of peptide administration (1–3). Oral administration of drugs is the most convenient, economical, and acceptable route and also has the possibility to achieve sustained plasma levels of the peptide. Up until now, approaches for oral administration of peptides have involved the use of complex emulsion systems (4), enzyme inhibitors (5), detergent-like absorption enhancers (6), colonic delivery (7), or via the intestinal peptide and bile acid transporters (8,9) but only limited success has been achieved to date.

Only a few oligopeptides per se meet the physicochemical and structural characteristics that allow for passive or active absorption from the intestine (10,11). For the vast majority of larger polypeptides, oral absorption is limited by their susceptibility to proteolytic degradation and, often more significantly, by their inefficient transport across cellular barriers (4,11). To some extent, both of these barriers can be circumvented by using peptidase inhibitors and detergent-like compounds that facilitate absorption across membranes, although there may be significant safety concerns with their routine use (6).

To avoid the use of enhancers and achieve selective peptide absorption we have attempted to utilize the endogenous intestinal uptake pathway for cobalamin (Cbl) absorption. Cobalamin is actively taken up in the small intestine by receptor-mediated recognition by IF-Cbl receptors (IFCR) on Caco-2 monolayers and oral absorption of the Cbl-conjugates in the rat.

KEY WORDS: cobalamin (vitamin B12); in vitro-in vivo study; Cbl-peptide conjugate; oral absorption.
MATERIALS AND METHODS

Reagents

All cell culture media were from Gibco BRL. [57Co]-Cbl was purchased from Amersham. Cobalamin, a monoclonal anti-VB12 antibody CD-29 and IF and NIF from porcine gastric mucosa were purchased from Sigma. The polyclonal rabbit anti-LHRH antibody was from Biotech Australia, Roseville, Australia. Cbl-LHRH was prepared as described previously (19). DP3 (Glu-Ala-Ser-Ala-Tyr-Ser-Ala) was synthesized from D amino acids (Hoffmann-La Roche Ltd., Basle) according to Pappenheimer et al. (15). Conjugates of DP3 and the “e” isomer of the monocarboxylic acid of Cbl (20) were prepared either by directly coupling to the N-terminus (Cbl-DP3; MW 2123) or via a hexyl spacer (Cbl-Hex-DP3; MW 2236) using EDAC (1-ethyl-3-((3-dimethylaminopropyl)carbodiimide) (19). All other reagents were from Sigma and Fluka.

Cell Culture

Human colon carcinoma Caco-2 cells (passage numbers 100-113, ECACC, England) were seeded at a density of 70,000 cells/cm² on Costar collagen-coated 12 mm Transwells (0.45 µm) and cultured at 37°C in a humidified 5% CO₂ atmosphere. After the first 48h, media (DMEM (Dulbecco’s Modified Eagle Medium) with 10% Fetal Calf Serum (Gibco), 1% nonessential amino acids, 100 U/ml penicillin and 100 µg streptomycin) were changed every two days in both the apical (0.5 ml) and the basolateral (1.5 ml) chambers. Experiments were performed with cells grown for 21–25 days after functionally tight monolayers were formed as assessed by transepithelial electrical resistance and [¹⁴C]-mannitol permeability. DMEM containing 1% nonessential amino acids, 100 U/ml penicillin and 100 µg streptomycin and 0.1% BSA was used for binding and transcytosis studies (transport buffer).

Binding of Cbl-DP3, Cbl-Hex-DP3 and of Cbl-LHRH to Cbl-Binding Proteins

MaxiSorp-break apart microtiter plates (Nunc) were coated overnight at 4°C with 100 µg microtiter plates (Nunc) were coated overnight at 4°C with 100 µg avidin (Fluka, 5 µg/ml in 0.1 M bicarbonate buffer, pH 9.6). Remaining binding sites were blocked for 2 h at room temperature with 150 µl of 1% bovine serum albumin in 0.2 M Tris buffer, pH 7.3 and 100 µl of biotinylated intrinsic (IF) or non-intrinsic factor (NIF) (0.15 U/ml) were added for 2 h at room temperature (Alsenz et al., manuscript in preparation). Mixtures of a constant concentration of 0.5 ng/ml [⁵⁷Co]-Cbl (3.7 × 10⁻¹⁰ M) (Amershams) and serial dilutions of cobamidine, cobalamin (Cbl)(Sigma), Cbl-LHRH, Cbl-DP3 or Cbl-Hex-DP3 were added. After an overnight incubation at 4°C, wells were washed and counted for [⁵⁷Co]-radioactivity.

Labeling of Cbl-Peptides with Iodine

Cbl-conjugates were labeled with [¹²⁵I]iodine (Amersham) using chloramine T as oxidant. Reaction was stopped with ascorbic acid and the peptide was separated from free [¹²⁵I] by using a Supelco RP-18 minicolumn. Any remaining free [¹²⁵I] was further removed by incubation of the Cbl-peptides with an excess of CD-29 (the antibody binds to the Cbl moiety) for 1 h at RT followed by centrifugation through a Centricon-10 concentrator (Amicon). The retentate, containing [¹²⁵I]-peptide bound to CD-29 was washed three times with PBS to remove free [¹²⁵I]. Cbl-peptides were subsequently eluted from CD-29 by washing of the retentate four times with 100 µl 1N HCl. After neutralization of the filtrate with an equal volume of 1N NaOH and addition of bovine serum albumin to a final concentration of 0.1%, samples were stored frozen in aliquots until use.

Association of IF-[⁵⁷Co]-Cbl, IF-Cbl-[¹²⁵I]-DP3, IF-Cbl-Hex-[¹²⁵I]-DP3 or IF-Cbl-[¹²⁵I]-LHRH with Caco-2 Monolayers and Transcytosis of Complexes

Confluent Caco-2 monolayers were cultured as described under ‘cell culture.’ Cells were placed on ice and washed three times with prechilled transport buffer (see above). Prechilled solutions of the various labeled IF- or NIF-complexes (100 fmol/filter) were added to the apical side of the cells in the presence or absence of a 20-fold excess of unlabelled IF-Cbl or NIF-Cbl complexes. After incubation for 2 h at 4°C, samples were removed, the cells were washed three times with DMEM/0.1% BSA and measured for radioactivity. Complexes were prepared by incubating IF or NIF with a 20% molar excess of Cbl, [⁵⁷Co]-Cbl, Cbl-[¹²⁵I]-DP3, Cbl-Hex-[¹²⁵I]-DP3 and Cbl-[¹²⁵I]-LHRH in DMEM/0.1% BSA for 1 h at RT. Samples were loaded onto Centricon-10 concentrators and complexes (concentrate) were separated from unbound ligands (filtrate) by centrifugation and subsequent washing the concentrate three times with 0.3 ml DMEM (5000 g streptomycin and 0.1% BSA was used for binding and measured for radioactivity. Complexes were prepared by incubating IF or NIF with a 20% molar excess of Cbl, [⁵⁷Co]-Cbl, Cbl-[¹²⁵I]-DP3, Cbl-Hex-[¹²⁵I]-DP3 and Cbl-[¹²⁵I]-LHRH in DMEM/0.1% BSA for 1 h at RT. Samples were loaded onto Centricon-10 concentrators and complexes (concentrate) were separated from unbound ligands (filtrate) by centrifugation and subsequent washing the concentrate three times with 0.3 ml DMEM (5000 g streptomycin and 0.1% BSA for 1 h at RT. Samples were loaded onto Centricon-10 concentrators and complexes (concentrate) were separated from unbound ligands (filtrate) by centrifugation and subsequent washing the concentrate three times with 0.3 ml DMEM (5000 g streptomycin and 0.1% BSA for 1 h at RT. Samples were loaded onto Centricon-10 concentrators and complexes (concentrate) were separated from unbound ligands (filtrate) by centrifugation and subsequent washing the concentrate three times with 0.3 ml DMEM (5000 g streptomycin and 0.1% BSA for 1 h at RT.

In Vivo Studies with [⁵⁷Co]-Cbl, Cbl-[¹²⁵I]-LHRH, Cbl-Hex-[¹²⁵I]-DP3, and Cbl-[¹²⁵I]-DP3 in the Rat

The oral absorption of [⁵⁷Co]-Cbl, Cbl-[¹²⁵I]-LHRH, [¹²⁵I]-DP3, Cbl-[¹²⁵I]-Hex-DP3 and Cbl-[¹²⁵I]-DP3 was studied in conscious male Wistar rats after an overnight fast. Samples (1 pMol) were dissolved in 1 ml buffer (PBS/0.1% BSA) with or without a >10⁻⁵-fold excess of Cbl and administered into the stomach of rats by gavage. At the end of the experiment, rats were sacrificed, the intestine was washed thoroughly (>500 ml) with PBS/0.1% BSA to remove unabsorbed ligands and radioactivity in the washing fluid, in urine and in tissue (liver, kidney, spleen, heart, lung, stomach, skin, muscle, testis, brain, plasma, colon, cæcum and washed small intestine) was measured. In case of muscle and skin, two to three 3–4 g samples were washed in PBS and counted together with the filtrate.