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

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Purpose. This study was aimed at examining the extent and mechanism of uptake of cobalamin (Cbl)-conjugated peptides in vitro and in vivo.

Methods. To enable acquisition of quantitative absorption data of Cbl-peptides, metabolically stable octapeptides (DP3), with (Cbl-Hex-DP3) or without a hexyl spacer (Cbl-DP3), were coupled to Cbl and radiolabeled. For comparison, LHRH coupled to Cbl was used as metabolically susceptible peptide. Biological recognition of Cbl-peptides was studied in the physiological order: binding to Intrinsic Factor (IF), recognition and transport of the IF-complexes by IF-Cbl receptors (IFCR) on Caco-2 monolayers and oral absorption of the Cbl-conjugates in the rat.

Results. All Cbl-peptides bound to IF and the IF-complexes were recognized by IFCR receptors on Caco-2 monolayers. Binding was saturable and could be inhibited by a 20-fold excess of IF-Cbl, but not of Non-intrinsic Factor (NIF)-Cbl. Oral administration of these ligands to rats resulted in absorption of 53%, 45%, 42%, and 23% of the applied radioactivity for Cbl, Cbl-LHRH, Cbl-Hex-DP3, and Cbl-DP3, respectively. Simultaneous administration of a >105-fold excess of unlabeled Cbl reduced uptake of all compounds to <4%. Tissue distribution and elimination of the metabolically stable Cbl-conjugates were comparable to Cbl.

Conclusions. The endogenous Cbl uptake pathway can be exploited for oral peptide delivery as indicated by the specific and high (40–45%) uptake of metabolically stable Cbl-coupled octapeptides.

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

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 transcytosis. After binding to intrinsic factor (IF), a 48K gastric protein, the IF-Cbl complex is recognized and internalized by IF receptors (IFCR) on the surface of the ileal epithelium. Cobalamin is subsequently transported to the basolateral membrane and, two to three hours after internalization, it appears in the circulation complexed with Transcobalamin II (TCII) (12–14).

Radiolabelling of peptides facilitates mechanistic and quantitative studies on peptide absorption but their proteolytic degradation may result in absorbable iodinated degradation products that obscure data interpretation considerably. In the present investigation, the mechanism and extent of absorption of Cbl-coupled peptides has been studied both in vitro and in vivo. To enable a clear interpretation of the data and to avoid any misinterpretation by metabolism or pharmacological effects, a pharmacologically inactive octapeptide was selected for the current “proof of concept” study that is reported to possess excellent resistance against proteolytic degradation (15).

Given the fact that the GI tract is designed to break down peptides, the concept of oral protein delivery seems ill-defined; and even the early phases of the cobalamin delivery pathway such as recognition by IF are not without controversy. Early studies suggested that, with the exception of substitutions at the Co-β position (cyanide in native Cbl), substitutions of the Cbl molecule compromise its recognition by IF (16,17), although some other groups have reported substantially better IF binding by VB12 conjugates (18).

Therefore, a consecutive series of experiments was designed to study molecular interactions and recognition of the Cbl-peptides with its natural ligands in the physiological order: (i) binding of the Cbl-peptides to IF; (ii) binding of IF-Cbl-peptides to specific cellular receptors (IFCR) on mature Caco-2 monolayers, (iii) transcytosis of the Cbl-peptide across an endothelial barrier (Caco-2 cells) and (iv), confirmation of the in vitro results in vivo using the rat as a model.

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ABBREVIATIONS: Cbl, cobalamin (Vitamin B12, VB12); CD-29, monoclonal anti-Cbl antibody; IF, intrinsic factor; IFCR, IF-Cbl receptor; NIF, non-intrinsic factor; TCII, transcobalamin II.
MATERIALS AND METHODS

Reagents

All cell culture media were from Gibco BRL. \(^{57}\)Co]-Cbl was purchased from Amersham. Cobalamin, a monoclonal anti-VB12 antibody CD-29 and IF and NIF from porcine gastric mucosa was 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-Ser-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 5 days in both the apical (0.1 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 \(^{[\text{3}]\text{H}}\)-mannitol permeability. DMEM containing 10% FCS was added to each well for 20 h at 37°C. Cell layers were formed as assessed by transepithelial electrical alone or complexed with IF or NIF in DMEM/MEM/Pen-Strep/

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

MaxiSorp-breakapart microwell plates (Nunc) were coated overnight at 4°C with 100 μl aminid (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 \(^{57}\)Co]-Cbl (3.7 \(\times\) 10⁻¹⁰ M) (Amersham) and serial dilutions of cobamide, 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 \(^{57}\)Co-radioactivity.

Labeling of Cbl-Peptides with Iodine

Cbl-conjugates were labeled with \(^{125}\)Iodine (Amersham) using chloramine T as oxidant. Reaction was stopped with ascorbic acid and the peptide was separated from free \(^{125}\)I by using a Supelco RP-18 minicolumn. Any remaining free \(^{125}\)I 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 \(^{125}\)I-peptide bound to CD-29 was washed three times with PBS to remove free \(^{125}\)I. 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-[\(^{57}\)Co]-Cbl, IF-Cbl-[\(^{125}\)I]-DP3, IF-Cbl-Hex-[\(^{125}\)I]-DP3 or IF-Cbl-[\(^{125}\)I]-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, \(^{57}\)Co]-Cbl, Cbl-[\(^{125}\)I]-DP3, Cbl-Hex-[\(^{125}\)I]-DP3 or Cbl-[\(^{125}\)I]-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 3 times with 0.3 ml DMEM (5000 g, 10 min, 4°C). For transcytosis studies, 100 fmol of \(^{57}\)Co]-Cbl or Cbl-[\(^{125}\)I]-peptide alone or complexed with IF or NIF in DMEM/MEM/Pen-Strep/10%FCS was added to each well for 20 h at 37°C. Medium was removed and radioactivity in the basolateral medium was determined. The cells were washed twice with 0.5 ml 50 mM Tris/2.5 mM Ca²⁺/0.1% BSA, pH 7.4 and surface-bound radioactivity was separated from internalized by (i) washing the cells twice rapidly with 0.5 ml glycine-buffer, pH 3 at 4°C as suggested by Dix et al. (19) and (ii) once with 0.5 ml of trypsin/EDTA (Gibco) for 10 min at 37°C; the latter was found to remove surface-bound radioactivity much more efficiently than glycine buffer alone. Cells detached by the washing procedure were collected by centrifugation and counted together with the other cells (internalized radioactivity). Glycine and Trypsin/EDTA washes were combined and radioactivity was determined.

In Vivo Studies with \(^{57}\)Co]-Cbl, Cbl-[\(^{125}\)I]-LHRH, Cbl-Hex-[\(^{125}\)I]-DP3, and Cbl-[\(^{125}\)I]-DP3 in the Rat

The oral absorption of \(^{57}\)Co]-Cbl, Cbl-[\(^{125}\)I]-LHRH, \(^{125}\)I-DP3, Cbl-[\(^{125}\)I]-Hex-DP3 and Cbl-[\(^{125}\)I]-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 >10³-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, caecum and washed small intestine) was measured. In case of muscle and skin, two to three 3-4 g samples