Doctoral Thesis

Kinetics and pathways of intestinal absorption and enzymatic cleavage of therapeutic proteins upon peroral delivery

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Kinetics and Pathways of Intestinal Absorption and Enzymatic Cleavage of Therapeutic Proteins upon Peroral Delivery

A dissertation submitted to the

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for the degree of
Doctor of Natural Sciences

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Meinen Eltern und Fernando
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In all honesty I do feel remorse for all the animals that I killed during my PhD. In my opinion, evaluation of alternative models should always be a great challenge for a scientist. For my part, I hope that I was able to contribute a little by establishing the pig model in my lab. I do hope that financial and scientific interests will help to replace unnecessary animal trials in the future.
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Background and Purpose

There is an increasing use of natural peptides and proteins as therapeutics. For example, eight different protein or peptide drugs have been registered in Switzerland in the last two years\(^1\). In several cases such proteinacious\(^2\) therapeutics are to be applied for long-term treatments. Despite the growing interest in alternative routes of delivery the most popular and desirable method of drug administration remains the oral route. This, however, implies major challenges for the pharmaceutical development and necessitates the invention of efficient and safe delivery systems for peptides and proteins. In addition to the problems which are encountered by all drugs administered perorally, e.g., hepatic first pass metabolism, proteinacious drugs are subjected to the proteolytic activity in the intestine, low intrinsic permeability in the intestinal epithelium and to a certain extent the mucus layer on the intestinal epithelium and the involvement of a secretion pumps, i.e., P-glycoprotein. To overcome these barriers various approaches have been proposed:

- targeting to a particular segment of the gastrointestinal tract in which there is lower enzymatic activity (e.g., the colon)
- using specialised drug carrier systems which shuttle the peptide or protein to its absorption site (e.g., mucoadhesive chitosan-coated liposomes, azo-polymercoated drugs)
- lowering the proteolytic activity (e.g., use of enzyme specific inhibitors)
- improving the resistance to breakdown by structural modification (e.g., cyclisation of peptides)
- inhibition of P-glycoprotein (e.g., verapamil, Tween 80)

To assess the feasibility of peroral delivery of a peptide and/or protein dosing the compound \textit{in vivo} in an animal model or in humans certainly is the method of choice for determination of bioavailability. However, the mechanistic interpretation of the results may be difficult due to the complexity of this model which is mostly due to the overlay of

\(^{1}\text{IKS 1999, personal communications}\)

\(^{2}\text{Hereafter the term "proteinacious" is used as a synonym for "peptide and protein"}\)
various absorption-controlling factors. Therefore, in order to study the influence of each factor involved in the overall absorption process, the use of *in vitro* and *in situ* methods is essential. Various *in vitro* and *in situ* models are applied, each encompassing different phases of the intestinal absorption process. Thus, the choice of a suitable model has to be in accordance with the specific questions of each investigation. For example, the influence of both luminal and brush border membrane bound enzymes on the stability of a proteinacious compound may be determined by a combination of various *in vitro* models, e.g., incubation studies in the presence of pancreatic extract, purified enzymes, perfusates collected from the intestine, brush border membrane vesicles or incubation studies using excised intestinal mucosa.

Well characterised *in vitro* and *in situ* intestinal models for intestinal transport studies are e.g., the Caco-2 cell culture model, excised intestinal mucosa and single-pass perfusion techniques. The excised mucosa represents a more complex model than the homogeneous Caco-2 cell monolayer with respect to the variety of epithelial cells, the mucus layer, the brush border associated enzymes and membrane associated luminal enzymes. In addition, for the assessment of the effective permeability in different intestinal segments, the excised mucosa model may be the first choice. However, in order to investigate the influence of a specific transport carrier or secretion process such as P-glycoprotein on the membrane permeability the Caco-2 cell model might be more adequate since it is easier to characterise and, thus, specific mechanisms can be defined more easily. In contrast to both, excised mucosa and Caco-2 cell model, the *in situ* single-pass perfusion with concurrent blood sampling from the portal vein allows to investigate solute uptake from the intestine into the blood circulation. Thus, this model is suitable when the assessment of the overall absorption of a compound is the primary focus of interest. In this work, the feasibility of peroral delivery was assessed for IGF-I and, with respect to P-gp, also metkephamid as therapeutically relevant peptide. The influence of the main absorption barriers - enzymatic cleavage, mucus and epithelial transport - on the uptake of proteins was studied by means of
stability and transport studies. This work is divided into 4 chapters as shown below:

- An extended introduction about the assessment of barriers for proteinacious drug uptake, such as enzymatic cleavage, mucus and epithelial transport as related to the feasibility of IGF-I absorption.
- The characterisation of P-glycoprotein-mediated efflux in Caco-2 cell monolayers focusing on the influence of culturing conditions and drug exposure on P-gp expression levels.
- Stability of IGF-I in the gastrointestinal tract of various species in relation to typical substrates of pancreatic proteases.
- Transport of IGF-I across the intestinal epithelium: permeation rates and mechanisms relative to permeability markers.

Each of these aspects will be dealt with in separate chapters.
Abstract

The kinetics and pathways of intestinal absorption and enzymatic cleavage of therapeutic proteins upon peroral delivery are the subject of this work. More specifically, Insulin-like growth factor I (IGF-I) was particularly focused on. There is a rapidly growing interest in the therapeutic use of natural peptides in medicine, since several endogenous peptides play an important role in the regulatory processes of body functions as enzymes, hormones, neuropeptides or neurotransmitters and cytokines. In various cases these proteinaceous drugs are applied for long-term treatments. For these chronic therapies the most convenient method of drug administration is via the oral route. This route, however, presents big challenges to the development of effective delivery systems for peptides and proteins, mainly due to enzymatic degradation in the lumen, the mucous layer on the intestinal epithelium and the low permeability. Chapter I gives an extensive overview of the influence of these three main barriers with special focus on IGF-I.

Low absorption of peptides may result from their high hydrophilicity and molecular weight, respectively. Secretion processes may contribute to the limited absorption of various drugs. An important example for an efflux-pump is P-glycoprotein (P-gp). P-gp is known to secrete various peptide drugs. Caco-2 cells are a relevant in vitro model to study the influence of such a secretion process on the permeability of drugs. Therefore, the influence of cell culture conditions and previous drug exposure on P-gp expression levels in Caco-2 cells was determined (Chapter II). In this study, the expression of P-gp is demonstrated (i) visually by confocal laser scanning microscopy (CLSM), (ii) functionally by transport studies with substrates of the efflux pump and (iii) quantitatively by flow cytometry (FCM) analysis using specific monoclonal antibodies (anti P-gp MRK 16 as an external antibody and C219 P-GlycoCheck as an internal antibody). Trypsinisation of the cells post confluency led to a decrease of P-gp expression levels, while prior trypsinsisation led to an increase after long term cultivation. Culturing the cells on polycarbonate filters did not elicit a significant change of P-gp
expression over time in culture, whereas in plastic flasks (polystyrene) a
decrease was detected. Using CLSM a strong fluorescence on the apical
side of Caco-2 cell monolayers was observed, as a result of incubation with
MRK 16 as primary and IgG Cy5 as secondary antibody. Previous drug
exposure of the cells showed that verapamil, celiprolol and vinblastine
induced the P-gp expression, while metkephamid (MKA) decreased the P-
gp expression level as compared to the control. Permeation studies
consolidated the immunohistochemical results that P-gp is expressed in the
Caco-2 cells, albeit at variable concentrations which requires careful
control when this model is to be used in quantitative structure/secretion
studies (Chapter II). For talinolol and MKA a higher transport from
basolateral to apical than in the reverse direction was found. Incubation
of the cell monolayer with MRK 16 reduced the secretory process to the
apical side, but did not influence the $^3$H-mannitol flux.

IGF-I is a 7648 Da protein consisting of 70 amino acids and is
considered among others as a long-term treatment for type II diabetes.

In order to investigate the peroral route of administration as an
alternative to the parenteral injection treatment, the metabolism of IGF-I in
the gut was investigated in the presence of crude porcine pancreatic
enzymes (CPPE) from pig and flushings of the small and large intestine
from pig, rat and dog. Moreover, incubation studies with purified
pancreatic enzymes which are present in the intestine, such as
aminopeptidase M, carboxypeptidase A, $\alpha$-chymotrypsin and trypsin, were
performed in order to determine the most active enzymes responsible for
the metabolism of IGF-I (Chapter III).

IGF-I was degraded in the jejunum, ileum and colon. Significant
intra- as well as inter-species differences of IGF-I stability were observed.
IGF-I was less stable in the ileum as compared to jejunum and colon of all
species examined. It could be shown that IGF-I was mainly degraded by
chymotrypsin ($t_{1/2} = 2.7$ min) and trypsin ($t_{1/2} = 34.6$), whereas in the
presence of aminopeptidase M and carboxypeptidase A it was stable within
90 min. Instability could be improved by adding serineprotease inhibitors
such as aprotinin, soybean trypsin inhibitor and Na-$\alpha$-p-tosyl-L-lysine
chloromethyl ketone (TLCK) or casein. While casein strongly increased the stability of IGF-I in all segments of the three species, the stability of N-acetyl-L-tyrosin ethyl ester and N-benzoyl-L-tyrosin ethyl ester, both typical substrates for chymotrypsin, was only partially increased. Interaction studies with casein and IGF-I elucidated that under the conditions used ~ 70 % of IGF-I was bound to casein leading to the conclusion that the specific protection of IGF-I occurred due to interaction with casein.

It could be shown that IGF-I was mainly degraded by chymotrypsin- and trypsin-like enzymes and that the stability in the intestine could be clearly increased by adding a mixture of chymotrypsin and trypsin inhibitors or casein. We assessed the stability of IGF-I in relation to substrates of the pancreatic enzymes. A degradation rate of 35 nmol mL$^{-1}$ min$^{-1}$ in human jejunal fluid was predicted. Additionally, the stability of IGF-I showed a significant inter-species difference.

The influence of the permeability barrier in the intestine for IGF-I bioavailability was investigated. The transport of IGF-I across intestinal epithelium was characterised with respect to its permeation mechanism and also its magnitude in relation to permeability markers. Two systems were applied in order to study IGF-I absorption: permeation studies across rat, human and porcine excised mucosa in Ussing chambers and single-pass perfusion studies in rats (Chapter IV).

Across porcine, human and rat mucosa of the colon and jejunum, only a low permeability of IGF-I was observed. Addition of casein increased IGF-I permeability significantly, yet permeabilities were still lower than PEG 4000 permeability, which is known as a non-absorbable drug. There was no significantly different permeation across rat, porcine and human mucosa. Through a mixture of chymotrypsin- and trypsin-inhibitors IGF-I permeability increased clearly, but in contrast to casein this mixture damaged the integrity of the mucosa indicated by the increased flux of PEG 4000. Saturable permeation kinetics was observed for IGF-I across porcine mucosa. IGF-I plasma concentrations were not significantly increased after single-pass perfusion compared to endogenous plasma concentrations, moreover, casein did not seem to have an
enhancing effect when this system was applied, which was in accordance with the permeation studies.

This study thus elucidated the permeation kinetics of IGF-I in relation to permeability markers. Moreover, the influence of various enhancers on the permeability of IGF-I was demonstrated. Also, the influence of target site in the gut on the IGF-I absorption was shown.

Generally, peroral delivery of IGF-I is clearly limited by its enzymatic stability and its low absorption. However, we could show several approaches to increase the stability and permeability of IGF-I.
Zusammenfassung


In Transportstudien wurde der Einfluss der Absorptionsbarriere des Magen-Darm-Trakts auf die Absorption nach peroraler Applikation von IGF-I untersucht. Neben in-vitro Permeationsstudien mit humaner Dickdarm-Mukosa und Mukosa von Schwein und Ratte wurden in-situ Perfusionsstudien an der Ratte durchgeführt (Kapitel IV). Dabei wurden


Assessment of Barriers for Proteinaceous Drug Uptake in the Intestine: Enzymatic Cleavage, Mucus and Epithelial Transport as Related to the Feasibility of IGF-I Absorption.
Because endogenous proteinaceous compounds play a predominant role in the regulatory processes of almost all body functions as enzymes, hormones, neuropeptides or neurotransmitters and cytokines, there is a rapidly growing interest in the therapeutic use of these compounds. Frequently such therapies require long-term treatments. For chronic therapies, however, the most convenient way for drug administration remains the oral route. However, in particular for peptide and protein drugs, this route poses major challenges for the development of efficient delivery systems. The enzymatic degradation in the lumen and brush border membrane, the low effective permeability of the involved epithelium, and to some extent the mucus layer are considered to represent the main barriers (cf. figure 1). By reducing the impact of these three barriers on the absorption of a selected compound, a higher absorption rate may be achieved.

A potentially interesting protein for oral drug delivery is insulin-like growth factor I (IGF-I) due to its demonstrated low liver metabolism (Kimura et al. 1994, Hill et al. 1997). IGF-I belongs to the family of the growth hormones (Stewart and Rotwein 1996) and carries chemical similarities to insulin (Rinderknecht and Humbel 1978; cf. figure 2).

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![Figure 1: An overview of the barriers encountered by peptide and protein drugs upon oral administration (TJ = tight junction)](image-url)
Because of its potentially chronic use, the feasibility of oral delivery of IGF-I is currently under investigation.

Based on multiple physiological effects (cf. figure 3) the potential of IGF-I for the treatment of a number of diseases is currently being discussed, e.g., for type II diabetes (Savage and Dünger 1996, Froesch 1994), Lou Gehrig’s disease (Piascik 1996, Festoff 1996) and for the treatment of growth hormone receptor deficiency in children (Guevara-Aguirre et al. 1995).

In this study we investigated the influence of the three typical intestinal barriers, the enzymatic cleavage barrier, the mucus barrier and the epithelial barrier, on the absorption of IGF-I in order to assess the feasibility of oral IGF-I delivery.

**Barriers to IGF-I absorption**

*The enzymatic barrier*

Various models have been established to test the stability of therapeutics in the gastrointestinal tract, reflecting the qualitative and quantitative composition of the digestive enzymes in the different intestinal segments.
Enzyme distribution in the gut

Lee (1988) describes the enzymatic barrier as by far the most important of the multitude of barriers limiting peptide and protein absorption from the gastrointestinal tract, as confirmed by other studies (Langguth et al. 1997).

By pancreatic excretion, chymotrypsin, trypsin, elastase and carboxypeptidase A and B are released into the duodenum. These enzymes are active throughout the small intestine, and partially also in the large intestine, and reach their highest activity at pH values around 8 (Lee 1988, Gibson et al. 1989, Langguth et al. 1997). The main metabolic activity in the large intestine is due to the activity of the colonic microflora which plays an important role in the degradation of peptides and proteins (Macfarlane et al. 1986, Tozaki et al. 1997). Proteolytic activity may either be found extracellularly, or associated to bacterial cells or due to disruption of bacterial cells (Gibson and Macfarlane 1988). The proteolytic activity of the colon is qualitatively different from that of the small intestine (Gibson and Macfarlane 1988).

In addition to luminal enzymes, the influence of cell bound proteases has to be considered. Langguth et al. (1997) gave an extensive
overview of important brush border membrane peptidases and their substrate requirement.

Based on the findings of other authors (Bai 1995, Kimura et al. 1997) it may be foreseen that IGF-I is degraded by chymotrypsin- and trypsin-like enzymes. The stability of IGF-I in the presence of cytosolic and luminal enzymes could be clearly increased by using chymotrypsin and trypsin inhibitors.

*Models to study enzymatic activity in the gut*

Various models have been described to determine the activity of luminal enzymes in the gut, such as incubations with pancreas extract, perfusate, extract of faecal slurries, brush border membrane vesicles, everted rings, excised mucosae and cell culture systems.

*Pancreas extract and purified pancreatic enzymes:* The physiologic effect of pancreatic enzymes on the stability of IGF-I is best assessed in incubation studies with crude pancreatic extract. However, this system is not useful to study specific enzyme kinetics because only apparent $K_m$ and $v_{max}$ values can be determined. Therefore, isolated and purified enzymes are employed to assess the enzyme specific kinetic parameters such as $K_m$ and $v_{max}$. Since the methods of extraction and purification may change the composition of pancreatic extract, standardisation of the extracts is essential due to variable enzyme activities of the components.

*Perfusates of the small intestine and faeces extract:* Since the composition of the enzymes changes qualitatively and quantitatively along the intestine (Lee 1988, Gibson et al. 1989), therefore, it is important to use appropriate test systems which take the distribution of intestinal enzymes in the lumen into account. The incubation with perfusates is a convenient test system and offers the opportunity to study the degradation of peptides and proteins in different intestinal segments. Routinely, the intestine is removed immediately after sacrifice of the animal, and rinsed with an appropriate volume of buffer. The perfusates are standardised with respect to the total protein concentration. In case of the large intestine an appropriate sample of colonic content is diluted with physiological buffer
at pH 4 and extracted for 30 min. After centrifugation the supernatant is used for incubation studies. The metabolism may be studied under anaerobic (Tozaki et al. 1997) or aerobic conditions (Kimura et al. 1997). An additional set-up was described by Breves et al. (1991) which permits to study specifically the anaerobic microbial metabolism. Under aerobic conditions the activities of pancreatic enzymes rather than the microbial metabolism (Macfarlane et al. 1986) can be investigated.

**Brush border membrane vesicles (BBMV):** Brush border membrane vesicles are used for both absorption (Bohner 1996) and enzymatic stability studies (Langguth et al. 1997). BBMV might be problematic due to the rather unphysiologically (Ganapatfry and Leibach 1983) high enrichment of brush border enzymes (Biber et al. 1981) and high variability in vesicle preparations (Osiecka et al. 1985). However, they are useful to investigate the influence of brush border membrane enzymes (Langguth et al. 1997, Heizmann et al. 1997) on the degradation of peptides and proteins and allow studies on the distribution of membrane bound enzymes along the various sites of the intestine. The BBMV technique, however, lacks the possibility to study the influence of mucus bound enzymes (Desai et al. 1991).

**Ussing chamber studies using excised mucosa and cell culture inserts:** Ussing chambers are an established tool to study metabolism. Cell culture inserts or excised mucosae might be used (Grass and Sweetana 1993). On-line electrical resistance measurements are applied to test the integrity of the cell membrane. Thus, with respect to metabolism this system allows the investigation of the influence of enzymes bound to the brush border membrane or to the epithelial mucus (Desai et al. 1991). Drug transport has to be taken into account because the decrease of drug concentration in the donor compartment is the net result of both enzymatic degradation and epithelial transport.

A simpler system is the technique of *everted rings*. Advantages of this models are the technical simplicity and, in contrast to the BBMV, the preservation of the epithelial barrier, i.e. its anatomical and functional polarisation, and a highly reproducible performance (Osiecka et al. 1985).
<table>
<thead>
<tr>
<th>Intestine</th>
<th>Duodenum</th>
<th>Jejunum</th>
<th>Ileum</th>
<th>Colon</th>
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<tr>
<td>Luminal Enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>chymotrypsin, trypsin, carboxypeptidase A/B, elastase</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>cytosolic bacterial enzymes</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Bound Enzymes</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>aminopeptidase M/A/P/W, carboxypeptidase A/P/M, endopeptidases</td>
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<tr>
<td></td>
<td>cysteine-, metalloproteases (bound to bact. cell membrane)</td>
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<tr>
<td>Methods</td>
<td></td>
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<tr>
<td></td>
<td>pancreatic porcine extract, purified pancreatic enzymes</td>
<td></td>
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<tr>
<td></td>
<td>BBMV</td>
<td></td>
<td></td>
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</tr>
<tr>
<td></td>
<td>perfusates, excised mucosa</td>
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</table>

Figure 4: Distribution of various proteases along the intestine and methods to study the enzymatic stability of IGF-I in the gut (Woodley 1994, Macfarlane et al. 1986, Gibson et al. 1989, Lee 1988, Langguth et al. 1996). Shading signalises the intensity of enzymatic activity.

However, the short life time of the tissue limits the use of this model (Fleisher 1995).

Therefore, in order to study the influence of luminal and brush border membrane bound enzymes on the enzymatic stability of IGF-I, we will combine incubation studies using pancreatic extract, purified enzymes, and perfusates collected from the intestine, with BBMV studies and incubation studies using excised intestinal mucosa (cf. figure 4).

**Mucus barrier**

Mucus forms a continuous viscoelastic gel layer covering the intestinal epithelia. It has been suggested that the mucus layer has important physiological functions, such as lubrication, protection of the
mucosa and maintenance of pH gradient (Saitoh et al. 1986, Forstner 1978). In particular, the mucus constitutes a barrier to diffusion of certain nutrients and, more importantly, may limit the access of therapeutic agents to the absorptive epithelial surface (Desai et al. 1992). The thickness of the mucus layer has been estimated to be 100 - 500 μm. Its major constituent is a $2 \times 10^6$ Da glycoprotein subunit (Carlstedt and Sheehan 1984), with a high amount of a carbohydrate component, mainly in the form of oligosaccharides. Additionally, DNA, inorganic material and plasma proteins are present in the mucus resulting from the metabolic breakdown of epithelial cells (cf. table 1).

The glycoprotein mentioned is thought to be responsible for both the physical and the structural properties of mucus (Slomiany, et al. 1983). Yet, Wikman Larhed et al. (1997) showed that lipids, rather than mucin glycoproteins, are the major components which contributes to reduced diffusion of lipophilic drugs in native intestinal mucus. Nevertheless, the authors also reported that protein binding effects may have to be taken into account when the influence of mucus on the diffusion of proteins is investigated. However, it seems undisputed that gel-forming and viscosity increasing agents which are important for the action of mucus as lubricant, mixing barrier and particle trap (Lee and Nicholls 1987 and Crowther et al. 1984) are more significant barriers to the diffusion of larger drugs such as peptides and proteins.

Various studies have demonstrated the influence of mucus on the diffusion of various drugs (Saitoh et al. 1996, Desai et al. 1992, Winne and Verheyen 1990). To what extent mucus represents a rate-limiting barrier in the sequence of drug absorption remains to be established. In order to overcome the mucus barrier in the framework of mechanistic studies on drug absorption, breakdown of the mucus gel might be the first choice. Livingstone et al. (1990) illustrated the influence of N-acetyl-L-cysteine on the cleavage of disulfide-bridge based cross-linkage of purified mucus glycoprotein.
Table 1: Components of the intestinal mucus layer according to Wikman et al. (1997). The contents were analysed by weighing the lyophilised fractions obtained after density gradient centrifugation.

<table>
<thead>
<tr>
<th>Component</th>
<th>% (w/w) of dry weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>mucin</td>
<td>5</td>
</tr>
<tr>
<td>lipids</td>
<td>37</td>
</tr>
<tr>
<td>proteins</td>
<td>39</td>
</tr>
<tr>
<td>DNA</td>
<td>6</td>
</tr>
<tr>
<td>other</td>
<td>13</td>
</tr>
</tbody>
</table>

Absorption barrier

The transport of peptide and protein drugs across the intestinal epithelium per se may occur via different pathways. Various model systems are available to assess the intrinsic permeability of IGF-I and its potential transport mechanisms.

Transport mechanisms

Various mechanisms may be involved in the transport of proteinacious drugs through the intestinal epithelium. A schematic overview on the preferential pathways for peptide and protein absorption are given in figure 5. Passive transport mechanisms via the paracellular or transcellular route, carrier-mediated and endocytotic/transcytotic mechanisms have been described. Bohner extensively reviewed (1996) a number of feasible transport routes for peptides, e.g., via the di- and tri-peptide transporter, passive transcellular and passive paracellular routes. A previous review by Burton et al. (1992) focused mainly on the passive aspects of transcellular peptide and protein absorption, whereas Nellans (1991) elaborated on the modulation of absorption via the paracellular pathway. Regarding transcytosis and endocytosis Shen et al. (1992) described in detail the aspects of enhancing peptide and protein absorption via these routes, and Rodmann and Mercer (1990) gave a comprehensive overview considering the cell biological aspects of these processes.
In case of IGF-I the endocytotic and transcytotic pathways are potential absorption routes. Many studies have shown the presence of IGF-I receptors in the gastrointestinal tract (Rouyer-Fessard et al. 1990, Young et al. 1990, Schober et al. 1990, Pillion et al. 1989) which led to the speculation of receptor-mediated transcytosis and/or receptor-mediated endocytosis. Moreover, receptor-mediated endocytosis and transcytosis of IGF-I have been described for fibroblasts and rat astrocytes (Zapf et al. 1994, Auletta et al. 1992). However, other active transport routes such as fluid-phase (Shen et al. 1992) and absorptive-mediated (Kimura et al. 1997) endocytosis also need to be taken into account.

Based on their typical saturation behaviour there is a distinction between receptor-mediated endocytosis on the one hand, and fluid-phase and absorptive-mediated endocytosis on the other hand. This difference is due to the fact that at therapeutic doses the two latter routes are less saturable than the former one (Shen 1990). While Quadros et al. (1994) reported a non-saturable transport of IGF-I across rat mucosa only at high concentrations, Kimura et al. (1997) demonstrated a saturable transport mechanism even at IGF-I concentrations that were lower than those applied by Quadros et al. (1994).

Though the range of the molecular weight cut-off in the intestine via the paracellular pathway is still hypothetical the possibility that IGF-I (7468 Da) might be passively transported via the paracellular pathway is very low. The cut-offs described in the literature range between 400 and 2000 Da (Artursson et al. 1993, Donovan et al. 1990, Pappenheimer 1993, Bowers et al. 1970, Lundin and Vilhardt 1986).

Many studies have been performed to investigate the factors responsible for passive transcellular transport. It has been shown that the permeability of water-soluble peptides depends mainly on their potential to form hydrogen bonds with their aqueous environment rather than on their lipophilicity (Conradi et al. 1992, Kim et al. 1993, Burton et al. 1992). In other words, poor absorption generally found with polypeptides is at least partly due to the large number of hydrogen bonding groups found in such molecules. Additionally, charge seems to have a significant impact on peptide permeability. Because of the passive and non-saturable character
of this pathway, mechanistic permeation kinetics studies may elucidate whether passive transport is involved in the transport of IGF-I across the epithelial mucosa.

More recent investigations have focused on the influence of a secretion transport mechanism in the gut, namely the P-glycoprotein (P-gp) pathway, which may affect the bioavailability of drugs (Hunter and Hist 1995). For many peptides the interaction with P-gp was demonstrated, such as the pentapeptide metkephamid (Bohner et al. 1996), the cyclic undecapeptide cyclosporin A (Ford et al. 1990, Fricker et al. 1996, Fricker and Drew 1996) AcPhe(N-MePhe)_{2}NH_{2} (Nerurarkar et al. 1996) or acetyl-leucyl-leucyl-norlucinal (ALLN; Sharma et al. 1992). Positive charge at neutral pH and a certain hydrophobicity are general molecular requirements for P-gp substrates (Higgins and Gottesman 1992). In addition, some water solubility is required for the recognition by P-gp (Kessel et al. 1989).

**Transport models**

To assess the oral bioavailability of a certain compound *in vivo* studies remain the ultimate method. However, due to the complexity of bioavailability, *in vitro* and/or *in situ* models provide important auxiliary information regarding the feasibility of oral absorption. Figure 6 shows relevant models which are listed in a pyramid with increasing complexity from bottom to top.

*Octanol/water partition coefficient*: The partitioning behaviour of a solute between n-octanol and water is a reasonable predictor of its permeability across a variety of cell membranes in case of passive and/or paracellular transport, and, thus, serves as a traditional reference (Smith et al. 1975, Austel et al. 1983). However, the n-octanol/water partition coefficient is a poor predictor for peptides and proteins. Therefore other models may be preferred in order to estimate the absorption of IGF-I.
Figure 5: Peptide and protein transport mechanisms in the gastrointestinal tract. Numbers indicate reference to literature reviews. Transport mechanisms which might be relevant for IGF-I absorption in the gut are given in grey.

Figure 6: Absorption models displayed with increasing complexity (from bottom to top).

Membrane vesicles, everted rings and everted sacs: The usage of membrane vesicles allows the investigation of the uptake of peptides and proteins uncoupled from their intracellular metabolism. Also discrimination between the different sites of the intestine and distinction between the apical and basolateral composition of the cell membranes are feasible in order to account for regional and polarisation-related differences of the epithelia involved (Bohner 1996, Del Castillo and Robinson 1982). However, the membrane vesicle model is inappropriate to study paracellular transport mechanism and, moreover, may pose problems for quantitative predictions when solutes undergo carrier-mediated transport (Matthews 1991). Additionally, the high enzymatic activity due to the artificial enrichment of membrane bound enzymes (Biber et al. 1981, Bohner et al. 1996) may limit the use of membrane vesicles. Thus, this model may be inappropriate to study transport mechanisms involved in IGF-I absorption. Everted rings and everted sacs completely preserve the structural integrity of the mucosal barrier. On the one hand, their technical simplicity and high reproducibility are advantages of these models (Osiecka et al. 1995); on the other hand, the time limited viability (≈10
min) of these preparations restricts their value as absorption model (Fleisher 1995). There are other limitations. In case of everted sacs, the mucosal compartment is large, whereas the serosal compartment small and poorly oxygenated; and in case of everted rings both the serosal and the mucosal membrane are exposed to the drug. Such unphysiological conditions may restrict the use of the two models to study the absorption of IGF-I.

**Cell culture systems:** Audus et al. (1990) reviewed in detail the advantages of cell culture systems over other techniques such as:

- rapid assessment of the potential permeability and metabolism of a drug
- opportunity to elucidate the molecular mechanisms of drug absorption under controlled conditions
- rapid evaluation of strategies for achieving drug targeting, enhancing drug transport, and minimising drug metabolism
- benefit of using human rather than animal tissue
- the opportunity to minimise time-consuming, expensive and sometimes controversial animal studies

Several cell lines have been described (Audus et al. 1990), among them Caco-2 and HT 29 as the most commonly used ones. Table 2 shows some characteristics of these two cell lines. Permeation studies can be easily performed in Ussing chambers (Grass and Sweetana 1993). On-line measurements of the transepithelial resistance (TEER), short circuit current ($I_{sc}$) and the use of radio-labelled markers, such as mannitol or PEG 4000 (Artursson et al. 1993, Grass and Sweetana 1993) allow to monitor the integrity of the monolayers throughout the permeation process.

The rational choice of a cell line has to be in accordance with the specific issue of each investigation. Because of the potential for over- or under-expression of a specific transporter in a cell line versus the natural tissue, cell culture systems need to be thoroughly validated when used for transport studies involving specific carrier-mediated pathways. A major problem of cell culture systems may also be the differences between
Table 2: Comparison of two intestinal cell lines, Caco-2 and HT 29

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Caco-2 cells</th>
<th>HT 29 cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I receptors</td>
<td>+1</td>
<td>unknown 15</td>
</tr>
<tr>
<td>Endocytotic transport of vitamin B12</td>
<td>+2, 3</td>
<td>unknown 15</td>
</tr>
<tr>
<td>Endocytotic transport of transferrin</td>
<td>+3, 4</td>
<td>+6</td>
</tr>
<tr>
<td>Receptor-mediated transport of epidermal growth factor</td>
<td>+3</td>
<td>unknown 15</td>
</tr>
<tr>
<td>Fluid-phase endocytosis of horseradish peroxidase</td>
<td>+5</td>
<td>unknown 15</td>
</tr>
<tr>
<td>P-glycoprotein expression</td>
<td>+7, 12</td>
<td>unknown 15</td>
</tr>
<tr>
<td>Origin</td>
<td>Human colon carcinoma cell line 8</td>
<td>Human colon carcinoma cell line 8</td>
</tr>
<tr>
<td>Brush border</td>
<td>+9</td>
<td>unknown 15</td>
</tr>
<tr>
<td>Morphology</td>
<td>Monolayer, small intestinal enterocytes 9</td>
<td>Multilayer in presence of glucose 8</td>
</tr>
<tr>
<td>Metabolism</td>
<td>Higher levels of aminopeptidases than HT 29 8, 9</td>
<td>Lower levels of aminopeptidases than Caco-2 cells 8, 9</td>
</tr>
<tr>
<td>Differentiation</td>
<td>Spontaneous differentiation 9, Type 1 10</td>
<td>Type 2 10</td>
</tr>
<tr>
<td>Mucus</td>
<td>No mucus 11</td>
<td>Mucus producing cells 6</td>
</tr>
<tr>
<td>Disadvantages</td>
<td>Interclonal differences 13, 14</td>
<td>Multilayer, Type 2</td>
</tr>
<tr>
<td>Advantages</td>
<td>Broad data base, brush border, P-gp expression, IGF- receptors, Type 1</td>
<td>Mucus layer</td>
</tr>
</tbody>
</table>


passage numbers, and the development of sub-clones (Caro et al. 1995, Delie and Rubas 1997). In addition, one has to take into account that Caco-2 cells and HT 29 cells are derived from malignant tissues and thus may carry unwanted features. Additionally, both cell lines are derived from
colonie tissue which may imply regional differences to small intestinal tissue, e.g., with respect to brush border associated hydrolases (Artursson 1991). Also culture conditions might have an influence. In contrast to normal tissue, cultured monolayers are not in contact with luminal enzymes or mucus (Conradi et al. 1992) with whatever consequences this may have on the relevance of the applied cell culture model. More recently, Chou and Rubas (1998) introduced an immortalised human small intestinal cell which might be an alternative.

In contrast to normal intestinal tissue the influence of M-cells and non-differentiated crypt cells cannot yet be studied in cell culture. In a novel approach, Kerneis et al. (1997) recently described a promising co-culture of Caco-2 and lymphocytes which allows to transform Caco-2 into M-cell like cell cultures. Moreover, the Caco-2 cell line is an already established model to study the influence of P-gp related excretion on the transport of drugs. On the one hand, cell culture systems allow long-term studies, e.g., the influence of drugs on cell differentiation, growth and morphology. On the other hand, due to its greater cellular complexity excised mucosa might be a more suitable model to estimate drug absorption than by an uncritical use of homogenous cell culture monolayers.

Excised mucosa model: This model represents a more complex model than the homogeneous cell monolayer systems with respect to the physiological and regional variability of the epithelial layer, the mucus layer, and the brush border associated and membrane associated luminal enzymes. In case of IGF-I absorption, for which mucus interaction and luminal enzymes may play a major role, excised mucosa might be a more suitable in vitro system to estimate the IGF-I absorption in the intestine than cell culture systems. Additionally, excised mucosa allows absorption studies in different segments of the intestine. Typically, permeation studies across the excised tissue are performed under airlift in Ussing chambers (Grass and Sweetana 1993). Since electrophysiological measurements are insufficient to check the integrity of the mucosa (Rubas et al. 1996), the additional use of paracellular transport markers is requested.
**Perfusion studies:** Single-pass perfusion studies allow to investigate solute uptake from the intestine into the blood circulation. The single-pass perfusion set-up supplies a constant-input drug concentration, \( C_0 \), that can maintain initial conditions over the time course of the experiment. Following a short (depending on flow- and absorption rate) non-steady-state period over which the intestinal segment is filled and drug absorption equilibrates, a constant output concentration, \( C_m \), will be achieved, and will permit the calculation of steady-state permeabilities (Fleisher 1995).

The quantitative evaluation of perfusion data is based on the following theory (Elliott et al. 1980, Langguth et al. 1994) assuming that no significant metabolism in the lumen occurs: Briefly, the effective resistance \( R_{\text{eff}} \) to solute transport through the intestinal wall can be written as a sum of the individual resistances of the wall and the aqueous resistance.

\[
R_{\text{eff}} = R_w + R_{\text{aq}} \tag{1}
\]

where \( R_{\text{eff}} \) is the effective resistance, \( R_w \) the wall resistance and \( R_{\text{aq}} \) the aqueous resistance. Because of the reciprocal relationships of resistance \( R \) and permeability \( P \):

\[
\frac{1}{P_{\text{eff}}} = \frac{1}{P_w} + \frac{1}{P_{\text{aq}}} = R_{\text{eff}} \tag{2}
\]

The dimensionless intrinsic wall permeability, \( P_w^* \), is estimated by a modified boundary layer approach. The dimensionless effective permeability, \( P_{\text{eff}}^* \) (effective permeability), can be expressed as:

\[
P_{\text{eff}}^* = \frac{1 - \frac{C_m}{C_0}}{4 G_z} \tag{3}
\]

where \( C_0 \) is the inlet solute concentration, \( C_m \) the outlet concentration corrected for water absorption and for secretion, and the \( G_z \) the Graetz number, which is a function of the perfusion flow rate, the aqueous diffusion coefficient of the solute and the length of the perfused segment.
The dimensionless $P_{aq}^*$ can be expressed as:

$$P_{aq}^* = \frac{1}{AG_z^{1/3}}$$

(4)

The dimensionless wall permeability is thus calculated from

$$P_w^* = \frac{P_{aq}^* \times P_{eff}^*}{P_{aq}^* - P_{eff}^*}$$

(5)

$P_{aq}^*$ is a function of perfusion flow rate. Hydrodynamically, the faster the flow rate, the higher $P_{aq}^*$ (permeability of the aqueous boundary layer) for drug transport. In fact, if drug wall permeability is low which is the case of IGF-I (Rubas et al. 1996, Quadros et al. 1994), $P_w^*$ will dominate $P_{aq}^*$ regardless of flow conditions. In this case, effective permeability approximates wall permeability. However, the effective permeability might be under-estimated if the drug is degraded in the intestine, e.g., by luminal enzymes, which will result in high difference between $C_o$ and $C_m$.

**Conclusions**

For peroral IGF-I absorption all three barriers described are important to consider. Therefore, in the subsequent chapters we will study the influence of luminal and membrane bound enzymes on the stability and the influence of the mucus on the diffusion of IGF-I across the mucus layer (Chapters III, IV). Additionally, we will focus on an investigation of the permeation mechanism and, moreover, quantify the transport of IGF-I relative to permeability markers (Chapter IV). Various *in vitro* and *in situ* models with different degrees of complexity will be applied to achieve a high degree of adaptation to the *in vivo* conditions. However, only *in vivo* studies can fully elucidate the clinical feasibility of peroral IGF-I delivery.
Literature


Chapter II

P-Glycoprotein (P-gp) Mediated Efflux in Caco-2 Cell Monolayers: The Influence of Culturing Conditions and Drug Exposure on P-gp Expression Levels.

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Introduction

Recent observations on intestinal absorption of drugs with peptide and non-peptide structure suggest that the bioavailability of some drugs after peroral administration may be limited by an intestinal secretion process that is mediated by P-glycoprotein (P-gp) (Saitoh and Aungst 1995). P-gp is a 170 kDa protein originally found to cause multidrug resistance in cancer chemotherapy upon treatments with anticancer drugs such as vinblastine, actinomycin D and daunomycin (Hunter et al. 1993a, Hunter et al. 1993b, Horio et al. 1989, Bartels et al. 1996). But also noncytostatic drugs are reported to be affected by the P-glycoprotein transporter, such as β-receptor antagonists (Karlsson et al. 1993, Wetterich et al. 1993) and peptides (Burton et al. 1993, Toppmeyer et al. 1994, Sarkadi et al. 1994). In the gastrointestinal tract the P-gp transporter may secrete drugs at low concentrations out of the epithelium back into the intestinal lumen. At high intestinal concentrations the secretion may be saturable, leading to an apparent increase in bioavailability. In addition to the gastrointestinal tract, P-gp is expressed in the liver, the pancreas, the kidneys and in reproductive organs as well as in the endothelia of the brain, testes and adrenal glands.

Previous studies showed that P-glycoprotein is also expressed in Caco-2 cells, a well established cell line for drug transport studies in the gastrointestinal tract (Hunter et al. 1993b). Since Caco-2 cells are frequently used to estimate the fraction of drug absorbed based on a compound's permeability in this culture system, P-gp expression has to be taken into account. It may be foreseen that in the case of a much higher P-gp expression in Caco-2 cells as compared to the human intestine, the risk of underestimation of human intestinal permeability by this in vitro test system may exist. In addition, since the P-gp expression level in Caco-2 cells is depending on the time in culture (Hosoya et al. 1996) as well as on the culturing conditions (Hoskins et al. 1993), variable expression levels must be taken into account. As interlaboratory differences, such as passage number, composition of the medium including the use of antibiotics, passaging procedure etc. may affect P-gp expression, there is a need for a rigorous standardisation of the Caco-2 model with respect to P-gp expression levels.
The aim of this investigation was to localise and quantify the expression of P-gp in Caco-2 cells and to identify factors that affect P-gp expression in Caco-2 cells, namely by confocal laser scanning microscopy (CLSM), flow cytometry (FCM) and drug permeation studies.

**Material and Methods**

Dulbecco's Modified Eagle's medium (DMEM) containing 5 µg mL⁻¹ (34.2 µM) L-glutamin and 4.5 mg mL⁻¹ D-glucose (25.0 mM), non essential amino acids (NEAA) solution, foetal calf serum (FCS), 5 µg mL⁻¹ trypsin/2.5 µg mL⁻¹ (7.4 µM) ethylenediaminetetraacetic acid (EDTA) solution (trypsin/EDTA solution, trypsin activity standardised by manufacturer by cell lift activity test), 2.5 µg mL⁻¹ (7.4 µM) EDTA solution, penicillin/streptomycin solution (penstrepl) (10000 U mL⁻¹ penicillin and 10000 µg mL⁻¹ streptomycin), Hanks' balanced salt solution (HBSS), phosphate buffered saline (PBS; 0.9 mM calcium chloride dihydrate, 2.6 mM potassium chloride, 1.5 mM potassium dihydrogen phosphate, 0.5 mM magnesium chloride hexahydrate, 13.7 mM sodium chloride, 6.5 mM di-sodium hydrogenphosphate) were obtained from Life Technologies (Basle, CH). Transwell Snapwell cell culture inserts, area of 1.13 cm², mean pore diameter of 0.4 µm and Transwell cell culture inserts, area of 4.7 cm² were supplied by Costar (Basle, CH). Triton X-100, glycine, n-propyl-galate and glycerol, vinblastine sulfate and 2-morpholinoethane-sulfonic acid (MES) were from Fluka Chemie AG (Buchs, CH), Hoechst 33342 from Hoechst (Frankfurt, D), Rhodamin-phalloidin from Molecular Probes (Leiden, NL) and goat anti-mouse IgG Cy5 antibody (affinity purified F(AB')₂ secondary antibody) from Chemicon (Leiden, NL). The anti P-gp MRK 16, a monoclonal IgG₂ mouse (150 µg mL⁻¹ in PBS, 1 µg mL⁻¹ NaN₃ (15.4 µM), 10 µg mL⁻¹ BSA, reacting specifically with a surface epitope of human mdr1 P-gp) was supplied by Kamiya Biomedical Company. As a negative control to MRK 16 (MRK 16 NC) a purified myeloma protein mouse IgG₂ (1 mg mL⁻¹ in 0.02M Tris-buffered saline, pH 8.1, Organon Teknica) was used. Goat anti-mouse Ig FITC (specific to purified mouse IgG₁, IgG₂α, IgG₂β, IgG₃) was obtained from Becton Dickinson. P-GlycoCheck C219, a FITC labelled murine monoclonal antibody IgG₂ (reacting with an internal epitope of P-
gp) and P-GlycoCheck C219 negative control, a FITC labelled murine monoclonal antibody IgG₂, were purchased from CIS bio international (GIF-sur-Yvette Cedex, France). Fluorescamine and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical and Co. (St. Louis, USA). \(^3\)H-mannitol was from Du Pont de Nemours International S.A. (Regensdorf, CH) and Ultima Gold from Packard (Croningen, NL). Trospium was a gift from Madaus AG (Köln, Germany). Talinolol, losartan and its metabolite EXP 3174 and celiprolol were a gift from the Martin-Luther University (Halle, Germany), metkephamid (MKA) was donated by Eli Lilly and Co. (Indianapolis, USA) and latex particles, Immuno-Brite Level II were obtained from Instrumentation Laboratory AG (Schlieren, Switzerland). Caco-2 cells, passage 22 were obtained from ATCC (Rockville, USA), passage 46 from ECACC (London, GB) and passage 68 from the Department of Physiology (University of Zurich, Switzerland).

**Cell culture**

Caco-2 cells passage 22 - 38, 46 - 59 and 68 - 83 were cultured in cell culture medium (DMEM, with 4.5 mg mL⁻¹ (25.0 mM) D-glucose which was supplemented with 0.165 mL mL⁻¹ FCS, nonessential amino acids (1 mL mL⁻¹), 5 µg mL⁻¹ (34.2 µM) L-glutamine). Confluent monolayers were subcultured every 7 days by treatment with trypsin/EDTA solution (trypsinisation) and seeding at a density of \(2 \times 10^5\) cells/25 cm² into plastic flasks. Cultures were incubated at 37 °C in a humidified atmosphere of 5 %/95 % CO₂/O₂. Special treatments of the cells will be covered in the subsequent paragraphs.

**Flow cytometry (FCM)**

Caco-2 cells were cultured in 80 cm² flasks at 37 °C in a 5 % CO₂ atmosphere using cell culture medium. Caco-2 cells were grown as monolayers by seeding \(4 \times 10^5\) cells/80 cm² bottle. For passaging and/or immunolabelling confluently or approx. 70 % conflually grown cell monolayers were trypsinised and suspended. Alternatively, conflently grown cell monolayers were trypsinised, suspended and seeded onto polycarbonate filters (Transwell cell culture inserts, area of 4.7 cm², mean pore diameter of 0.4 µm) at a density of 100'000 cells cm⁻². For labelling 4
× 10^6 cells/100 μL FCS/PBS (100 μL mL⁻¹) were incubated for 30 min with 5 μL anti P-gp MRK 16 per 1 mL PBS or with 2.5 μL MRK 16 negative control per 1 mL PBS, respectively. Thereafter, the cells were incubated with FITC labelled. For labelling with P-GlycoCheck C219 or P-GlycoCheck C219 negative control the cells were fixed with 36 μL mL⁻¹ formaldehyde solution (in PBS), permeabilised with acetone at 0 °C and incubated with the antibody for 60 min at 4 °C under light exclusion. After washing with PBS (3 times) the cells were suspended in 1 mL formaldehyde solution (3.6 μL mL⁻¹ in PBS) and measured. 10'000 cells were counted on the average by an EPICS Profile Analyser of Coulter (Miami, USA). Excitation was at 488 nm and emission at 520 nm. As an internal standard latex particles, Immuno-Brite Level II, were used.

**Confocal Laser Scanning Microscopy (CLSM)**

Caco-2 cells (passage 70) were cultured in 80 cm² flasks at 37 °C in a 5 % CO₂ atmosphere using cell culture medium. Caco-2 cells were grown as epithelial layers by seeding 4 × 10⁵ cells/80 cm² bottle. Confluently grown cell monolayers were trypsinised, suspended and seeded onto polycarbonate filters (Transwell Snapwell cell culture inserts, area of 1.13 cm², mean pore diameter of 0.4 μm) at a density of 100'000 cells cm⁻² for 28 days using cell culture medium and 0.011 μM vinblastine sulfate. The cell layers were washed with PBS, pH 7.4, which was also used for all subsequent washing steps. Fixation and labelling were performed at room temperature. The following standard protocol was applied: Cells were fixed in formaldehyde solution (36 μL mL⁻¹ in PBS) for 15 min and treated with 0.1 M glycine for 5 min. After washing three times they were permeabilised with Triton X-100 (1 μg mL⁻¹) for 15 min and washed again three times. Cell nuclei were labelled with Hoechst 33342 stain (1:100) and actin was labelled with rhodamine-labelled phalloidin (1:10). For labelling with antibodies the sandwich technique was used. Preparations were incubated for 1 h with the primary antibody (1:4), i.e. the specific anti P-gp MRK 16, or the non specific MRK 16 negative control. As a secondary antibody Cy5-labelled anti IgG (1:50) was applied for 1 h. After washing again (three times) the cells were
mounted in a mixture of 7 mL glycerol 100 %, 3 mL 0.1 N Tris-HCl, pH 9.5, and 0.5 g n-propyl-galate.

**Transport studies**

Caco-2 cells were grown in 80 cm² flasks at 37 °C in a 5 % CO₂ atmosphere using cell culture medium. Caco-2 cells were grown as epithelial layers by seeding 4 × 10⁵ cells/80 cm² bottle. Confluently grown cell monolayers were trypsinised and suspended, and seeded onto polycarbonate filters (Transwell Snapwell cell culture inserts, area of 1.13 cm², meanpore diameter of 0.4 μm at a density of 100,000 cells cm⁻²) for 15 to 16 days.

The integrity of the cells was checked before and after the experiment by measuring the TEERs. The permeation studies were performed by 5 % CO₂/O₂ airlift (15 mL/min) in Ussing type chambers (Precision instruments, Costar, NL). The diffusion cells were maintained at 37 °C by a water-heated jacket.

As a marker for the passive transport ³H-mannitol with an activity of 1 μCi mL⁻¹ was used. The activity of the stock solution was 15-30 Ci mmol⁻¹.

After washing twice with HBSS the inserts were placed into the diffusion cells; 5.0 mL transport buffer containing 1 mM MKA or talinolol, respectively, was filled either into the apical (apical to basolateral permeation direction; a → b) or the basolateral compartment (b → a); and 5.5 mL of transport buffer (10 mM MES in HBBS, pH 6.5) was filled either into the basolateral (a → b) or apical (b → a) compartment. For the studies with anti P-gp MRK 16 the inserts were first incubated for 15 min at 37 °C with 40 μL of 30 μL MRK 16/300 μL HBSS and treated as described above. At time zero, samples of 100 μL for the determination of the ³H-mannitol concentration and 400 μL for the determination of the drug concentration were taken from the donor and receiver compartment and 0.5 mL immediately replaced by transport buffer in the receiver compartment. Samples of 500 μL were taken after 15, 30, 45 and 60 min from the receiver compartment and immediately replaced, and one sample of 500 μL was taken after 60 min from the donor compartment. For ³H-mannitol analysis, samples (3.0 mL or 3.5 mL) were also taken after 60
min. All samples for the \(^3\)H-mannitol transport studies were continuously mixed with 7.5 mL or 4 mL of scintillation fluid (Ultima Gold). The samples from the drug transport studies were analysed by HPLC analysis according to Bohner et al. (1996, MKA) or to Wetterich et al. (1996, talinolol). The \(^3\)H-mannitol samples were counted in a multipurpose scintillation counter Beckman type LS 6500 (Beckman Instruments Inc., Fullerton, USA).

**Calculation of permeability**

Effective permeability coefficients, \(P_{\text{eff}} \, [\text{cm s}^{-1}]\) were calculated according to:

\[
P_{\text{eff}} = \left( \frac{\text{dC}}{\text{dt}} \right)_{ss} \frac{V}{A \cdot C_0}
\]

where \(\left( \frac{\text{dC}}{\text{dt}} \right)_{ss}\) is the steady-state change of concentration over time \([\mu\text{g mL}^{-1} \, \text{s}^{-1}]\), \(A\) is the diffusion area \([\text{cm}^2]\), \(V\) is the volume of the receiver compartment \([\text{mL}]\) and \(C_0\) the initial concentration in the apical (\(a \rightarrow b\)) or basolateral (\(b \rightarrow a\)) compartment \([\mu\text{g mL}^{-1}]\). Data are presented as means ± SD of \(n = 3-5\) Caco-2 cell monolayers.

**Statistics**

Standard F-tests and Student’s unpaired t test (two-tailed) were used to compare means of flow cytometry and permeability data. \(P < 0.05\) was considered statistically significant.

**Results**

**Flow cytometry (FCM)**

With both antibodies, MRK 16 as an external antibody and P-GlycoCheck C219 as an internal antibody, P-gp expression is recognised. Based on FCM of Caco-2 cells incubated with P-GlycoCheck C219 only a small shift of the fluorescence intensity was measured (data not shown).

When incubated with MRK 16 antibody clearly two populations are apparent: one population showing P-gp expression, whereas the other does
Figure 1: P-gp expression in Caco-2 cells. FCM of passage 73, incubated with the external antibody, anti P-gp MRK 16. Shown is the formation of two populations and the overall shift of the positive sample to higher fluorescence intensity as compared to the negative control. MRK 16 NC = negative control.

not, having the same mean fluorescence as the negative control (cf. figure 1). For all subsequent studies MRK 16 labelling was therefore preferred.

**Influence of the cell culture conditions**

In our preliminary studies we have found that no systematic effect of trypsinisation (trypsin/EDTA solution) versus EDTA alone (EDTA solution) and of incubation time (20 versus 40 min), respectively, on P-gp expression was detectable. Therefore, trypsinisation over 10 - 20 min was not expected to have any influence on the P-gp expression level. In order to evaluate the influence of the time in culture on P-gp expression, the starting passages 29, 49 and 72, after reaching confluence, were trypsinised, suspended and seeded for 9 or 10 times, respectively, resulting in passages 38, 59 and 82, respectively.

Starting with passage 72 passage 73 was also evaluated. In all cases a significant drop of the P-gp expression levels was observed (cf. figure 2).
Figure 2: P-gp expression in Caco-2 cells, 7 days after seeding in bottles of 80 cm$^2$ area; incubated with anti P-gp MRK 16; ■ starting passages, □ later passages. Throughout the cells were trypsinised after reaching confluence (means ± SD, n = 3).

However, trypsinising the cells before reaching confluence (ca. 70 % confluence) resulted in a significant increase of the P-gp expression levels (cf. figure 3).

Figure 3: P-gp expression in Caco-2 cells, 4 days after seeding in bottles of 80 cm$^2$ area; incubated with anti P-gp MRK 16; ■ starting passages, □ later passages. Throughout the cells were trypsinised before reaching confluence (approx. 70 % confluence) (means ± SD, n = 3).
Figure 4: Influence of time in culture on P-gp expression in passages 71 (■) and 73 (○). Cells were trypsinised at 10, 13, 16 and 21 days or 3, 7, 10 and 14 days, respectively, after seeding in bottles of 80 cm² area; incubated with anti P-gp MRK 16 (means ± SD, n = 3).

The time in culture was a major determinant for the P-gp expression level as demonstrated with passages 73 and 71 (following trypsinisation). The results are shown in figure 4.

Additionally, the P-gp expression as a function of time in culture starting with passage 71 was studied treating the cells with EDTA solution. The amount of measurable fluorescence declined significantly over time in culture, both upon trypsinisation of the cells and upon EDTA treatment alone (data not shown).

Figure 5: Influence of the time in culture on P-gp expression in passage 73. Cells were trypsinised 6, 13 and 21 days after seeding on polycarbonate filters; incubated with anti P-gp MRK 16 (means ± SD, n = 3).
On the other hand, cultivation of the cells on polycarbonate filters did not result in any statistically significant effect on P-gp expression levels over time (cf. figure 5).

Influence of previous drug exposure on P-gp expression levels

The influence of previous drug exposure on P-gp expression levels was tested. Cells were always trypsinised. The results are shown in table 1.

The P-gp expression levels were found to increase, decrease or remain constant depending on the drug added and its concentration in the medium. Vinblastine sulfate (0.011 μM) added to the culture medium increased the P-gp expression by a factor of seven, i.e. from a fluorescence of 0.84 V ± 0.40 (mean ± SD) without vinblastine sulfate and 6.25 V ± 0.41 when vinblastine sulfate was added. On the other hand, when trospium (1 mM) was added to the culture medium no increase in P-gp expression (0.65 V ± 0.08) was observed. Effects of previous drug exposure were also tested for several other drugs including talinolol, losartan and EXP 3174 (a metabolite of losartan), celiprolol, verapamil and MKA in concentrations of 10 μM and 100 μM. Only verapamil and MKA showed a significant effect on the P-gp expression level. Verapamil increased the P-gp expression level compared to control. The same was observed for celiprolol, though at the borderline of statistical significance. In contrast, MKA decreased P-gp expression in a concentration-dependant manner. Cells cultured in medium containing 100 μM verapamil did not proliferate.

Confocal laser scanning microscopy

Cell monolayers of passage 71 were grown for 28 days on a polycarbonate filter in pure medium containing vinblastine sulfate (0.011 μM). Cultures were fixed and processed for CLSM as described in the experimental section. A triple labelling was performed: for actin, for cell nuclei, and for P-gp, the latter with specific MRK 16 antibody. As a control the same protocol was applied except that the negative control antibody, MRK 16 negative control, was used instead of MRK 16. As illustrated in figure 6, Caco-2 cells form monolayers as judged from the arrangement of the cell nuclei in the x,z sections and the y,z sections.
Table 1: Influence of the culture medium on P-gp expression. Cells were trypsinised after reaching confluence having been seeded in bottles of 80 cm². Immunolabelling with MRK 16. Caco-2 cells were cultured with pure Dulbecco's modified medium, after adding trospium, vinblastine, talinolol, losartan, EXP 3174 or metkephamid (MKA), penstrep, celiprolol, and verapamil (means ± SD, n = 3).

<table>
<thead>
<tr>
<th>Medium</th>
<th>Concentration of drug in medium [μM]</th>
<th>Passage</th>
<th>Fluorescence [%]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td>71</td>
<td>100.0 ± 30.9</td>
</tr>
<tr>
<td>Trospium</td>
<td>10</td>
<td>71</td>
<td>78.3 ± 11.6</td>
</tr>
<tr>
<td>Vinblastine*</td>
<td>0.011</td>
<td>71</td>
<td>747.9 ± 6.5</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>72</td>
<td>100 ± 33.2</td>
</tr>
<tr>
<td>Talinolol</td>
<td>10</td>
<td>72</td>
<td>151.2 ± 11.4</td>
</tr>
<tr>
<td>Losartan</td>
<td>10</td>
<td>72</td>
<td>109.1 ± 25.3</td>
</tr>
<tr>
<td>EXP 3174</td>
<td>10</td>
<td>72</td>
<td>90.5 ± 27.3</td>
</tr>
<tr>
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<td></td>
<td>72</td>
<td>100 ± 32.3</td>
</tr>
<tr>
<td>Talinolol</td>
<td>100</td>
<td>72</td>
<td>90.8 ± 19.6</td>
</tr>
<tr>
<td>Losartan</td>
<td>100</td>
<td>72</td>
<td>115.8 ± 18.6</td>
</tr>
<tr>
<td>EXP 3174</td>
<td>100</td>
<td>72</td>
<td>153.8 ± 44.0</td>
</tr>
<tr>
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<td></td>
<td>72</td>
<td>100 ± 8.5</td>
</tr>
<tr>
<td>MKA*</td>
<td>10</td>
<td>72</td>
<td>76.4 ± 13.0</td>
</tr>
<tr>
<td>MKA*</td>
<td>100</td>
<td>72</td>
<td>61.6 ± 16.1</td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td>72</td>
<td>100 ± 9.8</td>
</tr>
<tr>
<td>Penstrep</td>
<td>100 U</td>
<td>72</td>
<td>112.9 ± 134.0</td>
</tr>
<tr>
<td>Celiprolol</td>
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<td>72</td>
<td>190.8 ± 21.0</td>
</tr>
<tr>
<td>Celiprolol*</td>
<td>100</td>
<td>72</td>
<td>322.1 ± 55.4</td>
</tr>
<tr>
<td>Verapamil*</td>
<td>10</td>
<td>72</td>
<td>299.7 ± 7.5</td>
</tr>
</tbody>
</table>

*Previous exposure of these drugs had a significant influence on the P-gp expression compared to the control.
Figure 6: Expression of P-gp at the apical membrane of Caco-2 cell monolayers. Caco-2 cell monolayers were grown for 28 days in culture medium containing vinblastine sulfate (0.011 μM) and processed for CLSM as described in the experimental section. The confocal data sets are represented as xy-, xz-, and yz-projections. A, C: labelling for actin (rhodamin-phalloidin) and cell nuclei (Hoechst 33342 stain); B: same data set as A showing the cell nuclei (Hoechst 33342 stain) and the specific labelling with anti P-gp MRK 16; D: same data set as in C showing the labelled nuclei and the labelling with the negative control antibody (MRK 16 negative control). Bar indicates 10 μm.
Table 2: Effect of transport direction on effective permeability coefficients (P_{eff}) of metkephamid (MKA) or talinolol across Caco-2 cell monolayers with or without MRK 16 (means ± SD, n = 3-5)

<table>
<thead>
<tr>
<th>Donor composition</th>
<th>Flux direction</th>
<th>P_{eff} [cm s^{-1} x 10^7] MKA</th>
<th>P_{eff} [cm s^{-1} x 10^6] Mannitol(^a)</th>
<th>P_{eff} [cm s^{-1} x 10^6] Talinolol</th>
<th>P_{eff} [cm s^{-1} x 10^6] Mannitol(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 mM drug</td>
<td>apical → basolateral</td>
<td>0.60 ± 0.23</td>
<td>3.15 ± 0.29</td>
<td>0.35 ± 0.24</td>
<td>2.61 ± 0.63</td>
</tr>
<tr>
<td></td>
<td>basolateral → apical</td>
<td>7.42 ± 1.46</td>
<td>2.76 ± 0.08</td>
<td>5.05 ± 1.58</td>
<td>2.56 ± 1.71</td>
</tr>
<tr>
<td>1 mM drug + MRK 16</td>
<td>apical → basolateral</td>
<td>1.67 ± 0.37</td>
<td>1.74 ± 0.41</td>
<td>5.02 ± 0.47</td>
<td>2.67 ± 0.37 2.72</td>
</tr>
<tr>
<td></td>
<td>basolateral → apical</td>
<td>6.18 ± 1.63</td>
<td>4.19 ± 3.36</td>
<td>3.77 ± 1.73</td>
<td>± 1.01</td>
</tr>
</tbody>
</table>

\(^a\)Mannitol flux recorded during MKA experiment.
\(^b\)Mannitol flux recorded during talinolol experiment.
The outlines of the cells are strongly marked with actin (cf. figure 6A and C). P-gp could clearly be localised in the apical membrane of the cells (cf. figure 6B). The specificity of this labelling was demonstrated with the negative control antibody which only resulted in a very weak and diffuse fluorescence (cf. figure 6D).

**Permeation studies**

The transport of MKA and talinolol across Caco-2 cell monolayers in the apical to basolateral direction and in the basolateral to apical direction with and without MRK 16 antibody was investigated. As a control \(^3\)H-mannitol was used.

The transport of MKA and talinolol from the apical to the basolateral side was significantly slower than in the reverse direction. Binding of MRK 16 antibody significantly increased the effective permeability of both drugs from apical to basolateral. In the case of MKA, the presence of MRK 16 antibody reduced slightly the difference of \(P_{\text{eff}}\) apical \(\rightarrow\) basolateral and \(P_{\text{eff}}\) basolateral \(\rightarrow\) apical, whereas in the case of talinolol, a stronger reduction of the difference of the two \(P_{\text{eff}}\) values was observed (cf. table 2).

**Discussion**

On the basis of the significant expression of P-glycoprotein in Caco-2 cells, this cell line appears to be suitable for the investigation of intestinal, P-gp dependant secretion processes which may be relevant for the intestinal absorption and bioavailability of drugs. For the elucidation of quantitative structure/secretion and structure/transport relationships, however, the variability of the P-gp expression level as a function of Caco-2 cell passage number, culturing conditions and previous drug exposure needs to be carefully monitored and possibly standardised. Only when standardised for reproducible P-gp expression can the Caco-2 model be applied for quantitative studies. In our study, the expression of P-gp in Caco-2 cells is demonstrated (i) visually by CLSM, (ii) functionally by transport studies with substrates of the efflux pump and (iii) quantitatively
by FCM using specific monoclonal antibodies. In addition, the variability of the expression level was investigated as a function of culturing conditions and exposure to drugs which are potential substrates of P-gp.

**Flow cytometry:** After immunolabelling of Caco-2 with P-GlycoCheck C219, the shift of fluorescence intensity as compared to the negative control was much smaller than after immunolabelling with MRK 16. Since MRK 16 binding to human mdr1 (multidrug resistance protein 1) (Lehel et al. 1993, Thiebaut et al. 1989) is very specific, but P-GlycoCheck C219 reacts additionally with human mdr3 and its murine analogues (Georges et al. 1990), and the shift of the fluorescence intensity using MRK 16 immunolabelling was more significant than with P-GlycoCheck C219, we applied MRK 16 for all subsequent studies.

**Influence of culturing:** No practically relevant differences were observed between treatments with trypsin/EDTA (trypsinisation) and with EDTA alone, from which can be concluded that trypsin does not seem to interact with the P-gp epitope reacting with the external (MRK 16) or internal (P-GlycoCheck C219) P-gp specific antibodies.

The time in culture, however, has a major influence on the P-gp expression in Caco-2 cells. P-gp expression decreased in higher passages compared to the starting passages when the cells were trypsinised after reaching confluence. On the contrary, the P-gp expression level increased significantly in higher passages compared to the starting passages when the cells were trypsinised before reaching confluence. It was also apparent that there may be a difference depending on whether the cells have been cultured in plastic flasks or on polycarbonate filters. When the cells were cultured in plastic flasks a significant decrease of the P-gp expression level was apparent whereas cultivation on polycarbonate filters lead to an increase of the P-gp expression level.

Using Western blots, Hosoya et al. (1996) demonstrated that the order of P-gp expression over a period of 4 weeks was 4w > 1w > 2w > 3w, while in permeation studies the function of P-gp as a transporter protein increased significantly from day 17 to day 27 when the cells were seeded on polycarbonate filters. In contrast to Hosoya et al. (1996), however, Wils et al. (1994a and b) using immunoblotting reported that no
increase of P-gp expression from day 4 to 22 occurred when Caco-2 cells were cultured in plastic flasks. Additionally, the authors observed that the capacity to express P-gp may be lost during long-term cultivation.

Obviously, P-gp expression in Caco-2 cells strongly depends on the individual culturing conditions. The time in culture and the material used for the culture primarily have an important influence on the P-gp expression levels. Certain conditions, e.g., trypsinisation after reaching confluence may even provoke a loss of P-gp expression after certain times of cultivation (cf. figure 2).

Influence of previous drug exposure on P-gp expression levels: It is well known that the transport of certain drugs across Caco-2 cells is affected by P-gp expression, e.g., with celiprolol (Karlsson et al. 1993), talinolol (Wetterich et al. 1996), vinblastine (Hunter et al. 1993a, Wils et al. 1994a) and MKA (Bohner et al. 1996). From all of the examined drugs in this study only some drugs, such as verapamil, celiprolol, vinblastine and MKA, influenced the P-gp expression levels. Culturing Caco-2 cells in medium containing verapamil, celiprolol and vinblastine induced the P-gp expression, whereas it was reduced by exposure to MKA as compared to the control. The penstrep combination studied did not have an effect on the P-gp expression levels.

In accordance with our studies, Hoskins et al. (1993) showed an induction of P-gp expression in Caco-2 cells which were previously exposed to desacetylvinblastine. Contrary to our findings indicating a stimulation of P-gp expression in Caco-2, when previously exposed to verapamil-containing medium, Muller et al. (1994) reported a reversible decrease of P-gp expression in human leukemic cell lines. No explanation is available to interpret this conflicting evidence.

Permeation studies: The permeation studies elucidated the sensitivity of talinolol and MKA transport to P-gp in Caco-2 cells. For both drugs a higher transport from the basolateral to the apical side as compared to the reverse direction could be measured. Incubation of the cell monolayer with the P-gp specific monoclonal antibody MRK 16 reduced the secretion process to the apical side, but did not influence 3H-mannitol flux. This result is in line with previously reported data (Rubas et al. 1993).
Both MKA and talinolol permeation across Caco-2 were previously reported to be affected by P-gp (Wetterich et al. 1996, Bohner et al. 1996). Corresponding to Hunter et al. (1993a) and in agreement with our studies, binding of MRK 16 seems to inhibit P-gp. Since there was no induction of the P-gp expression observed when Caco-2 cells were cultured in medium containing talinolol or MKA - for MKA even a decrease of P-gp expression could be detected - high concentrations might help to saturate the P-gp mediated secretion and increase bioavailability.

Further studies using immunoblotting should help to elucidate the relationship of various factors studied in this work on the level of P-gp expression monitored by FCM analysis. This question may also be studied on the basis of mRNA translation from the multidrug resistance gene. Other potential influences on secretion processes such as, e.g., expression of multidrug resistance associated protein (Endo et al. 1996) must not be neglected.

The observed variability of P-gp expression as a function of cell culture conditions and previous drug exposure raises several questions and issues. First, one may consider to develop a standard P-gp substrate that can serve as a reference in a transport study, but it has to be taken into account that by measuring transport activity with a standard P-gp substrate one determines only the overall functional expression. Second, P-gp expression may be normalised with respect to an internal standard, e.g., by monitoring mdr1 mRNA taking into account that variability can result from altered transcription, processing, stability and translation of mRNA, and functional insertion of the protein into the plasma membrane. Third, a stable transfected cell line may show less variation, but eventually lead to a change of expression levels due to selection pressures. And finally, changes of cell regulation may also be profitable. At this point, however, this aspect remains speculative.

**Literature**

the Transepithelial Fluxes. Presented at the 13th European International Transport Group (EITG) Congress, Sept. 22-26, Mikolayki, Poland.


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Chapter III

In Vitro Assessment of Intestinal IGF-I Stability

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Introduction

Insulin like growth factor I (IGF-I) is a 7648 Da growth hormone of 70 amino acid residues in a single chain containing three disulphide bridges. It shows homology with proinsulin but exerts its biological actions through specific IGF-I receptors (Rinderknecht et al. 1978, Kadowaki et al. 1986). It is predominantly produced in the liver and released into the circulation, but it is also produced locally in many tissues where it exerts a paracrine effect. IGF-I plays important roles in somatic growth, cellular proliferation, in development, differentiation and cell death as well as in different kinds of cancer (Stewart et al. 1996).

In clinical studies, IGF-I was found to lower blood glucose in insulin-resistant diabetic patients and, thus, might be of therapeutic value to patients with poorly controlled blood glucose levels (Schalch et al. 1991). It may also offer therapeutic advantages over insulin in terms of lipid metabolism. Many long-term complications seen in diabetes may be related to hyperinsulinemia, and the use of IGF-I may obviate these effects, particularly since the IGF-I binding proteins (BP) serve to modulate plasma levels of IGF-I (Stewart et al. 1996, Baxter 1993).

IGF-I is considered for treatment of various diseases, such as type II diabetes (Savage et al. 1996, Froesch et al. 1994), Lou Gehrig’s disease (Piascik 1996, Festoff 1996) and to treat growth hormone receptor deficiency in children (GueVara-Aguirre et al. 1995). Because of the chronic nature of these diseases, an alternative to subcutaneous injection, preferably the peroral delivery route is desirable. However, the peroral application of peptides and proteins encounters various problems such as low intrinsic permeability of the membrane and high metabolic cleavage (Langguth et al. 1997, Lee 1988, Woodley 1994) in the gastrointestinal tract. Different strategies to overcome the proteolytic barrier have been proposed such as (i) targeting to a particular part of the gut with low enzymatic activity, mainly the colon (Lee 1988, Yamamoto et al. 1994, Langguth et al. 1994a and b, Saffran et al. 1986), (ii) using specialised drug carrier systems which shuttle the peptide to its absorption site (Takeuchi et al. 1996, Tozaki et al. 1997, Damgé et al. 1988), (iii) lowering the proteolytic activity (Langguth et al. 1997, Yamamoto et al. 1994), or
(iii) enhancing the enzymatic resistance to breakdown by structural modification (Pauletti et al. 1997).

Because of the low enzymatic activity of the colon, colonic drug delivery may be an interesting approach for peptides and proteins, although the enzymatic activity of colonic contents can play an equally important role in protein metabolism (Tozaki et al. 1997, 1995). Whereas in the small intestine mainly serine proteases, e.g., trypsin and chymotrypsin, are active, cysteine- and metallo-proteases are predominantly present in the colon and the faeces. Moreover, as residues of pancreatic secretion, and as remnants of colonic degradation of bacteria (Gibson et al. 1989, Bai et al. 1995), chymotrypsin- and trypsin-like enzymes are present in the colon. Based on the chemical structure of IGF-I one would expect this polypeptide mainly to be degraded by serine proteases (Chapter I, Schilling and Mitra 1991).

The enzymatic cleavage of IGF-I in the gut has been examined in vivo as well as in vitro in rat gut flushings (Xian et al. 1995). Their studies reported that IGF-I was rapidly degraded in vitro in jejunal and ileal flushings. Additionally, the metabolic cleavage of IGF-I is much faster in rat jejunal and ileal contents than in colonic content (Kimura et al. 1997). Similarly, IGF-I was degraded in duodenal and stomach flushings, albeit at lower degradation rates. In all segments the stability of IGF-I could be significantly increased when casein or serine protease inhibitors, namely aprotinin and soybean trypsin inhibitor were added (Kimura et al. 1997, Xian et al. 1995). In contrast, IGF-I seems to be stable in flushings from newborn rats (Koldovsky et al. 1992), which probably reflects the poorly developed luminal digestion in the neonatal period (Britton and Koldovsky 1989) and the presence of natural protease substrates in the milk, such as casein (Rao et al. 1993).

The aim of the present investigation was to assess the feasibility of oral IGF-I delivery. For this purpose, we examined the stability of IGF-I throughout the intestine. Inter- and intra-species differences will be particularly emphasised. Furthermore, the half-lives of IGF-I in the various segments of the gastrointestinal tract were compared and, moreover, related to the half-lives of substrates of pancreatic and membrane bound enzymes. For comparison, N-acetyl-L-tyrosine ethyl ester (ATEE) and N-
benzoyl-L-tyrosine ethyl ester (BTEE) were also used as chymotrypsin substrates. Both represent established standards for measuring the chymotrypsin activity in vivo and in vitro (Sinko 1992, Kai et al. 1982, Kay et al. 1983, Senger et al. 1981, Interdelta 1997, personal communications). Finally, various serine protease inhibitors and casein were tested regarding their ability to protect IGF-I from cleavage by pancreatic enzymes.

**Materials and Methods**

N-acetyl-L-tyrosine ethyl ester (ATEE), N-acetyl-L-tyrosine (AT), N-benzoyl-L-tyrosine ethyl ester (BTEE), N-benzoyl-L-tyrosine (BT), p-nitroaniline, soybean trypsin inhibitor, puromycin dihydrochloride, aprotinin, Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK), leucine-aminopeptidase (microsomal, 5.6 mg protein mL⁻¹, 34 units mg⁻¹ protein) were purchased from Sigma (Buchs, CH). A combination of aprotinin, soybean trypsin inhibitor and TLCK was used as an enzyme inhibitor cocktail (GenMix, Genentech Inc., South San Francisco CA, USA). L-leucine-4-nitroanilide (LNA), casein sodium salt, ethylenediaminetetraacetic acid disodium salt (EDTA), trypsin from bovine pancreas, α-chymotrypsin from bovine pancreas, carboxypeptidase A, material for the buffers and material for Na⁺,K⁺ATPase determination were obtained from Fluka Chemie AG (Buchs, CH). Thymopentin (TP5) was a gift from Schwabe (Karlsruhe, D). Crude porcine pancreas extract (CPPE, Eurobiol®) was supplied by Interdelta AG (Fribourg, CH). DC Bio-Rad protein assay Kit and the Bio-Rad protein assay with IgG as a standard was purchased from Bio-Rad (Munich, D). Acetate buffer consisted of 99.9 mM sodium chloride, 42.8 mM sodium acetate trihydrate, 7.1 mM acetic acid, 2 mg mL⁻¹ Tween 20 (peroxide free, < 0.1 %, by Fluka, Buchs, CH), adjusted with 1 N sodium hydroxide to pH 6.5. TRIS buffer contained 49.9 mM tris(hydroxymethyl)aminomethane (TRIS) and 1.6 M calcium chloride dihydrate, adjusted to pH 7.9 with acidic acid. Krebs' phosphate buffer consisted of 95.1 mM sodium chloride, 4.8 mM potassium chloride, 2.5 mM calcium chloride dihydrate, 1.2 mM magnesium sulfate septahydrate, 24.8 mM sodium hydrgencarbonate, 1.2
mM potassium dihydrogenphosphate and 40 mM glucose, adjusted with 1 N acidic acid to pH 7.0. Recombinant human IGF-I (IGF-I) was a gift from Genentech Inc. (South San Francisco CA, USA). All materials used for HPLC were of analytical grade.

**Standardisation of crude porcine pancreatic extract (CPPE)**

Chymotrypsin activity of CPPE was determined in TRIS buffer (25 °C) according to the instructions given by Interdelta (1997). As IGF-I is more stable in pH 6.5 acetate buffer than in pH 7.9 TRIS buffer (Fransson 1996, Fransson and Hagman 1996), acetate buffer was the preferred buffer in our studies. For validation we compared the chymotrypsin activity of CPPE in both buffer systems, always using ATEE as substrate. The investigated factors were: buffer system, incubation temperature, and CPPE and ATEE concentrations (cf. table 1).

Furthermore, the influence of CPPE filtration on the proteolytic activity of the extract was examined. CPPE in TRIS buffer or acetate buffer, respectively, was incubated for 30 min at 25 °C or 37 °C. The extract was either used directly (unfiltrated extract) or after filtration through a 0.45 μm filter (filtrated extract). The extract was then incubated for another 15 min at 25 °C or 37 °C, respectively. ATEE was added to yield a final concentration as given in table 1. Samples of 200 μL were taken at various times until 60 min. Enzymatic activities were stopped by addition of 200 μL perchloric acid (1 M) followed by centrifugation (at 800 × g for 3 minutes) and storage at -23 °C until HPLC analysis.

Despite the differences in pH, electrolyte concentrations and temperature there was no influence of the buffer system on the chymotrypsin activity. Filtration did not seem to influence the chymotrypsin activity, which shows that dissolution of the proteolytic enzymes was complete after 30 min. Also adsorption to the filter could be excluded. Only in the TRIS buffer system at 25 °C a significant but modest influence of filtration on chymotrypsin activity was observed, which, however, was not considered to be relevant for this study.
Table 1: Experimental parameters for crude porcine pancreas extract (CPPE) standardisation by enzymatic cleavage of N-acetyl-L-tyrosine ethyl ester (ATEE) under various conditions.

<table>
<thead>
<tr>
<th></th>
<th>25 °C</th>
<th></th>
<th>37 °C</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TRIS buffer</td>
<td>Acetate buffer</td>
<td>TRIS buffer</td>
<td>Acetate buffer</td>
</tr>
<tr>
<td>ATEE [μg mL⁻¹]/[μM]</td>
<td>5000/20325</td>
<td>5000/20325</td>
<td>1.6/6.5</td>
<td>1.6/6.5</td>
</tr>
<tr>
<td>CPPE [μg mL⁻¹]</td>
<td>200</td>
<td>200</td>
<td>10</td>
<td>10</td>
</tr>
</tbody>
</table>

According to incubation conditions of Interdelta (1997)

Throughout and at all conditions, the chymotrypsin activities found were significantly lower than the activity of 150 μkatal post activ. in 2 g CPPE (cf. table 2) as specified by the manufacturer.

**Cleavage of IGF-I in the presence of CPPE**

The cleavage kinetics of IGF-I in the presence of CPPE was investigated. ATEE and BTEE were studied as controls. 10.0 mg of CPPE was suspended in 10 mL acetate buffer (pH 6.5) at 37 °C. To maintain the chemical stability of IGF-I all studies were performed in acetate buffer containing peroxide free Tween 20 (H₂O₂ ≤ 0.1 %). After a 30 min enzyme activation period the extract was diluted with buffer to a final concentration of 10 μg mL⁻¹ CPPE after filtration through a 0.45 μm filter. The mixture was pre-incubated for another 15 min at 37 °C and then IGF-I or the controls, respectively, were added to yield final concentrations of 1.5 μg mL⁻¹ to 152.9 μg mL⁻¹ (0.2 μM to 20 μM) IGF-I, 0.1 μg mL⁻¹ to 391.6 μg mL⁻¹ (0.6 μM to 1560 μM) ATEE and 31.3 μg mL⁻¹ to 306.7 μg mL⁻¹ (10 μM to 980 μM) BTEE. Final volume was 3 mL. The objective was to assess a stability profile under conditions relevant for the intestinal transit. Samples (200 μL) were taken at various time points until 60 minutes. In the case of IGF-I the enzymatic activity was concluded by cooling in ice-water for 10 min, as verified in preliminary studies. After centrifugation (800 × g for 3 min) the samples were stored at -23 °C. The enzyme reaction was terminated by adding 3 mL of 1 M perchloric acid.
Table 2: Standardisation of chymotrypsin activity of crude porcine pancreas extract (CPPE) by enzymatic cleavage of N-acetyl-L-tyrosine ethyl ester (ATEE) in TRIS buffer and acetate buffer at 25 °C versus 37 °C. After an activation period of 30 min both filtrated and unfiltrated extract was used. Data related to 2 g CPPE (n = 3, mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>TRIS buffer</th>
<th>Acetate buffer</th>
<th>TRIS buffer</th>
<th>Acetate buffer</th>
</tr>
</thead>
<tbody>
<tr>
<td>Filtrated extract</td>
<td>30.3 ± 1.4</td>
<td>28.2 ± 8.1</td>
<td>23.4 ± 0.8</td>
<td>25.7 ± 0.5</td>
</tr>
<tr>
<td>Unfiltrated extract</td>
<td>37.6 ± 2.7</td>
<td>29.6 ± 2.9</td>
<td>21.0 ± 5.1</td>
<td>25.0 ± 1.8</td>
</tr>
</tbody>
</table>

Precipitates were spun down (4 °C, 3 min, 800 × g) and stored at -23 °C until HPLC analysis.

All samples were analysed by HPLC. Peak areas were related to freshly prepared calibration curves of IGF-I, ATEE or BTEE, respectively, in acetate buffer covering ranges of 0.2 μg mL⁻¹ to 198.8 μg mL⁻¹ (0.026 μM to 26 μM), 0.1 μg mL⁻¹ to 459.3 μg mL⁻¹ (0.22 μM to 1830 μM) or 0.5 μg mL⁻¹ to 481.1 μg mL⁻¹ (1.6 μM to 1537 μM), respectively.

To characterise enzyme kinetics, the apparent Michaelis-Menten parameters were calculated by non-linear regression using Table Curve 2D (Jandel Scientific, AISN Software, 1989-1994, Corte Madera CA, USA). The total protein concentration of each CPPE batch was determined using the DC Bio-Rad protein assay.

**Enzyme inhibition studies**

The influence of enzyme inhibitors and casein on the stability of IGF-I in the presence of CPPE was investigated. ATEE, LNA and TP5 were used as positive controls for α-chymotrypsin, aminopeptidase M and carboxypeptidase A activities, respectively. 10.0 mg of CPPE was suspended in 10 mL acetate buffer (pH 6.5) at 37 °C. After a 30 minute enzyme activation period the extract was diluted with buffer to yield a final concentration of 10 μg mL⁻¹ CPPE in buffer after filtration through a 0.45 μm. For inhibition, puromycin (108.9 μg mL⁻¹ = 0.2 mM), EDTA (675.4 μg mL⁻¹ = 2 mM), casein (5 mg mL⁻¹) or GenMix (8.3 μg mL⁻¹ aprotinin,
7.6 μg mL⁻¹ soybean trypsin inhibitor and 8.0 μg mL⁻¹ = 8.0 μM TLCK), respectively, were added and the mixture incubated for 20 min. IGF-I or the controls, respectively, were added to yield final concentrations of 6.5 μM. The final volume was 3 mL. Samples (200 μL) were taken at various times until 90 minutes after the addition of IGF-I or controls, respectively. The enzymatic activity of the samples containing IGF-I was stopped by cooling for 10 min in ice-water. For the studies with ATEE, LNA and TP5 the reaction was stopped by adding an equal volume of chilled 1M perchloric acid. Sample preparation for HPLC was performed as described in the last section.

**Cleavage of IGF-I in the presence of purified pancreatic enzymes**

IGF-I, and ATEE, LNA and TP5 as positive controls, were studied in the presence of either aminopeptidase M, carboxypeptidase A, α-chymotrypsin or trypsin, respectively. The purified pancreatic enzymes were added (447 ng mL⁻¹ aminopeptidase M, 19.8 ng mL⁻¹ carboxypeptidase A, 1020 ng mL⁻¹ α-chymotrypsin, 980 ng mL⁻¹ trypsin) to the buffer, and after a 30 minute incubation period the substrates were spiked to yield final concentrations of 6.5 μM. Samples of 200 μL were taken at various times until 90 minutes after addition of the protein or the positive controls, respectively. Termination of the enzymatic activity and sample preparation for HPLC were performed as described above.

**Cleavage of IGF-I in the presence of jejunal and ileal flushings, faeces extract, brush border membrane vesicles (BBMV) and excised mucosa**

**Preparation of flushings, BBMV and faeces extract**

**Preparation of flushings:** Intestinal flushings were prepared according to Kessler et al. (1978). Briefly, specimens from porcine midjejunum, canine midjejunum and ileum (a = beagle and b = labrador), and rat midjejunum and ileum, respectively, were taken immediately after the animals were euthanised. 50 cm segments of porcine and canine tissue, or 10 cm segments of rat tissue were rinsed with 40, 50, or 10 mL, respectively, of ice-cold Krebs’ phosphate buffer. During transport to the laboratory the flushings were kept on ice, and then centrifuged (4 °C, 10 min and 2700 × g). Supernatants were used either immediately or stored at
-80 °C until further use. Rinsed intestinal specimens were stored in Krebs’ phosphate buffer (on ice) for subsequent BBMV preparation.

Preparation of porcine BBMV: Jejunal specimens of three animals were rinsed separately with chilled isotonic salt solution to remove intestinal contents and opened along their mesenteric border. BBMV preparation was according to Kessler et al. (1978) using mucosal scrapings, Ca\(^{2+}\)-precipitation and preloading with a solution containing 25 mM N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid (HEPES), 118 mM sodium chloride, 1.2 mM magnesium sulfate, 1.2 mM monobasic potassium hydrogenphsphate, 2.5 mM calcium chloride, adjusted to pH 7 with TRIS; osmolarity was adjusted to 300 mOsm/L by addition of adequate amounts of D-mannitol. Enzyme enrichment of BBMV was examined by determination of leucine-aminopeptidase activity in BBMV suspension and compared with the enzymatic activity in the homogenate. The test is based on the formation of p-nitroaniline from L-leucine-4-nitroanilide measured at 410 nm. The enrichments of the specific activity of leucine-aminopeptidase in the three parallel BBMV preparations were 27-, 18-, and 24-fold, respectively, with respect to the original homogenates. The specific activity of the basolateral membrane marker enzyme, Na\(^+\)/K\(^+\)-adenosintriphosphatase (Na\(^+\),K\(^+\)ATPase), was determined by the method reported by Berner and Kinne (1976) in which the resynthesis of the ATP split by ATPase is coupled via the pyruvate kinase and lactic dehydrogenase reaction to NADH oxidation. Cross-contamination with basolateral membranes was negligible as shown by the reduction (67 %, 25 % and 65 % reduction for the three vesicle preparations) of Na\(^+\)/K\(^+\)ATPase (Biber et al. 1981).

Preparation of faeces extract: Faecal material from the colon of pig, rat and dog was taken immediately after the animals were euthanised. 5 g was suspended in 30 mL chilled Krebs’ phosphate buffer and kept on ice for 30 min while being shaken every 5 min (10 strokes). The suspension was centrifuged (10 min, 2700 \( \times \) g and 4 °C) and the supernatant was used immediately.
Incubation studies in the presence of flushings, BBMV and faeces extract under aerobic conditions

Total protein concentrations of flushings, BBMV and faeces extract were determined using the Bio-Rad protein assay. The preparations were diluted in Krebs' phosphate buffer to yield a final protein concentration of 1 μg mL⁻¹. After 30 min pre-incubation at 37 °C, IGF-I, ATEE or BTEE (as positive controls), respectively, were added to yield final concentrations of 6.5 μM (final volume 3 mL). The subsequent incubation studies with (1 mg mL⁻¹ in Krebs' phosphate buffer) and without casein, termination of enzymatic activity and sample preparation for HPLC were performed as described above.

Loss of IGF-I after incubation in Ussing chambers

It is a common characteristics of proteins to adsorb irreversibly to surface materials (Oeswein and Shire 1991). Therefore, prior to using the Grass-Sweetana Ussing chamber studies we addressed protein recovery under experimental conditions but in the absence of tissue. The initial IGF-I concentration tested was 50 μg mL⁻¹ (6.5 μM) in Krebs' phosphate buffer. Ussing chambers were maintained at 37 °C by a water-heated jacket. 1 mL of IGF-I in pre-warmed (37 °C) Krebs’ phosphate buffer was added to each compartment. Stirring and oxygen supply was achieved by 5% CO₂/O₂ airlift at 15 mL/min. Samples of 100 μL were taken at time zero and after 10, 20, 30, 45 and 60 min. The samples were analysed by HPLC.

Cleavage of IGF-I in the presence of excised mucosa

Proteins which might be released from the tissues could potentially display peptidase and protease activities and, therefore, obscure the true stability of IGF-I in the presence of the tissue. Thus, to validate the stability studies in the presence of excised mucosae, the amount of protein released over time into the apical and basolateral chamber was determined using the Bio-Rad assay. The cleavage of IGF-I in the presence of excised mucosa from fresh porcine colon and midjejunum was tested. Fresh porcine colon and midjejunum (suis scrofa domestica) were obtained from the local slaughterhouse in Zurich. The intestine was rinsed with chilled
water (aqua potabilis) and kept for transport at 4 °C in Krebs’ phosphate buffer. After a ~40 min transport, intestinal specimens of ~4 cm² were stripped off their serosae and adjacent muscularis layers with a bent scalpel and used immediately for incubation studies. The mucosae were mounted in Ussing chambers (Physiologic Instruments, Inc., San Diego CA, USA) and 1 mL of prewarmed (37 °C) Krebs’ phosphate buffer was added to each side. Stirring and oxygen supply was achieved by a 5 % CO₂/O₂ airlift at 15 mL/min. The diffusion cells were maintained at 37 °C by a water-heated jacket. After 30 min equilibration the buffer on each side was replaced by 1.1 mL of IGF-I/Krebs’ phosphate buffer (50 µg mL⁻¹ = 6.5 µM IGF-I in buffer at 37 °C), samples of 100 µL were taken immediately and after 10, 20, 30, 45 and 60 min. Protein release was determined after 10 and 60 min from buffer incubations only. Removed volumes were replaced with fresh Krebs’ phosphate buffer. All samples were maintained on ice during the stability studies and stored at -23 °C until HPLC analysis.

*Calculation of kinetic parameters*

Half-lives (t₁/₂) were calculated according to first order kinetics, with k [min⁻¹] as degradation rate constant.

*Interaction of IGF-I and casein*

Several methods, such as equilibrium dialysis, ultrafiltration, gelfiltration and ultracentrifugation are commonly applied to determine protein binding. The potential interaction of IGF-I and casein was examined using ultrafiltration. Solutions of IGF-I in Krebs’ phosphate buffer with final concentrations of 50 µg mL⁻¹ (6.5 µM) and casein at a concentration range between 10 µg mL⁻¹ and 1000 µg mL⁻¹ in Krebs’ phosphate buffer were prepared and incubated overnight at 4 °C and afterwards at room temperature for 2 hours. This protocol was designed to provide enough time for the establishment of equilibrium conditions and to limit possible instabilities of IGF-I. After incubation, 400 µL samples were ultracentrifuged for 30 min (4 °C, 8000 × g; fixed angle rotor system) through a filter with a molecular cut-off of 10'000 Da (Centrisart-C4, Dr. Vaudaux, Basle, CH).
Furthermore, the potential loss of IGF-I in the filtrate due to non-specific adsorption to the device was determined and compared to the stock solution (cf. equation 1). For this purpose, solutions in a concentration range of 6.25 μg mL⁻¹ to 50 μg mL⁻¹ (0.8 μM to 6.5 μM) of IGF-I in Krebs’ phosphate buffer were freshly prepared. Their IGF-I concentrations (C_pre) were compared to the concentrations of the same solutions after ultracentrifugation (C_post). IGF-I concentrations in supernatants and filtrates were analysed by HPLC. Peak areas were related to a freshly prepared calibration curve of IGF-I in Krebs’ phosphate buffer at a range of 0.54 μg mL⁻¹ to 100 μg/L (0.1 μM to 13.1 μM). Non-specific adsorption [%] was calculated according to:

\[
\text{NSA} \% = \frac{C_{\text{pre}} - C_{\text{post}}}{C_{\text{post}}} \times 100\% 
\]  

where \( C_{\text{pre}} \) is the IGF-I concentration before ultracentrifugation, and \( C_{\text{post}} \) the IGF-I concentration after ultracentrifugation in the filtrate.

The fraction of IGF-I bound [%] at various casein concentrations was calculated according to:

\[
\text{IGF-I bound} \% = \frac{C_0 - C_{\text{fil}}}{C_0 \left( \frac{1}{1 - F} \right)} \times 100\% 
\]  

where \( C_0 \) is the IGF-I concentration before filtration, \( C_{\text{fil}} \) is the IGF-I concentration in the filtrate, and \( F \) is NSA/100%.

**Analytical Methods**

**DC Bio-Rad and Bio-Rad protein assays**

The concentration of total protein was determined for CPPE after equilibration period (30 min) and filtration or before dilution with buffer, respectively, using the DC Bio-Rad protein assay. 75 μl samples of CPPE in acetate buffer were prepared according to standard protocol of the DC Bio-Rad protein assay. All samples were monitored between 15 min and one hour. Maximum absorbance was measured at 720 nm using a UV-spectrophotometer. The absorbances of the samples were compared to a
freshly prepared calibration curve of IgG in acetate buffer pH 6.5 at a range of 0.2 mg mL\(^{-1}\) to 1.35 mg mL\(^{-1}\) IgG. 1 mg mL\(^{-1}\) CPPE corresponded to 0.12 ± 0.04 mg mL\(^{-1}\) of total protein concentration.

Total protein contents of flushings, faeces extract and brush border membrane vesicles were determined using the Bio-Rad protein assay. 100 μL samples were mixed with 5 mL of Bio-Rad reagent (Bio-Rad protein assay stock solution diluted with distilled water 1:4), gently vortexed, incubated for at least 5 min, but not more than 60 min at 25 °C. Maximum absorbance was measured at 595 nm using a VIS-spectrophotometer. The absorbances of the samples were compared to a freshly prepared calibration curve of IgG in distillated water at a range of 0.2 mg mL\(^{-1}\) to 1.35 mg mL\(^{-1}\) IgG.

**HPLC conditions**

The HPLC system consisted of a L-6200A pump, an AS-2000 autosampler and a L-4250 UV-VIS absorbance detector (Merck-Hitachi, Darmstadt, D). IGF-I was separated by reversed phase chromatography on a RP 18 VYDAC column, (5 μm, 300 Å) 25 cm × 4.6 mm (Buecher, Basle, CH) at room temperature and UV-detected at 210 nm. The mobile phase consisted of phosphate buffer (0.02 M sodium dihydrogen phosphate, 0.01 M propanesulfonic acid sodium salt, pH 2):acetonitrile, 90:10 (v:v) for A and phosphate buffer (0.02 M sodium dihydrogen phosphate, 0.01 M propanesulfonic acid sodium salt, pH 2):acetonitrile, 50:50 (v:v) for B. The flow rate was 1.0 mL min\(^{-1}\). Elution was by a gradient involving A and B, running from 64 % to 38 % A in 20 minutes.

ATEE, BTEE, LNA, and TP5 were separated from their metabolites by reversed phase chromatography on a LiChrospher 100 RP-18 column, (5 μm, 100 Å, encapped, 12.5 cm × 4 mm, Merck, Basle, CH). The mobile phases were: (A) 0.1 M monobasic sodium phosphate:orthophosphoric acid (85 %), 916:5 (v:v) and (B) 100 % acetonitrile. A flow rate of 1 mL min\(^{-1}\) and an isocratic system composed of 72% A and 28 % B, 82 % A and 18 % B, 78 % A and 22 % B, and 93 % A and 7 % B for ATEE, BTEE, LNA, and TP5, respectively. UV detection was at 214 nm.
Size exclusion chromatography (SEC) of IGF-I

A potential complex between IGF-I and casein should have a distinctly higher molecular weight as casein alone. Therefore, size exclusion chromatography was performed. The SEC system used consisted of a L-6200A pump, an AS-4000 autosampler and a L-4250 UV-VIS absorbance detector (Merck-Hitachi, Darmstadt, D). IGF-I was separated on a TSK 2000 column (10 µm) 30 cm x 7.5 mm (Buecher, Basle, CH) at room temperature. Detection was at 210 nm. The mobile phase consisted of 0.2 M potassium phosphate monobasic/0.15 M potassium chloride, pH 6.5. Eluent flow was 1.0 mL min⁻¹.

Statistics

Significance between mean values was determined by Student’s unpaired t test (two-tailed), P < 0.05.

Results

Cleavage of IGF-I in the presence of CPPE

Vₘₐₓ and Kₘ of IGF-I degradation due to pancreas enzymes was compared to established chymotrypsin substrates such as ATEE and BTEE. All substrates were degraded in the presence of CPPE (cf. table 3). The capacity factor, defined as the ratio vₘₐₓ/Kₘ, indicated a higher pancreatic clearance of BTEE compared to ATEE and IGF-I.

It appears that IGF-I has the highest affinity to the pancreatic enzymes followed by BTEE and ATEE. Based on the comparison of Kₘ and vₘₐₓ the pancreas enzymes display high affinity/low capacity towards IGF-I. This contrasts with the observation for ATEE and BTEE. In their case rather low affinity/high capacity conditions are to be considered. Comparing the capacity factors of BTEE, ATEE and IGF-I shows that the capacities of the CPPE enzyme cocktail for ATEE and IGF-I were similar, whereas the capacity for BTEE was five-fold higher.

The initial concentration of 6.5 µM applied in all following stability studies was lower than the determined Kₘ values of ATEE, BTEE and IGF-I. Thus, stability studies in acetate buffer were under strictly non-saturated conditions.
Table 3: Apparent Michaelis-Menten parameters of N-benzoyl-L-tyrosine ethyl ester (BTEE), N-acetyl-L-tyrosine ethyl ester (ATEE) and IGF-I in the presence of 10 µg mL⁻¹ crude porcine pancreas extract (CPPE) in acetate buffer (pH 6.5; 37 °C)

<table>
<thead>
<tr>
<th>Drug</th>
<th>( v_{\text{max}} ) [10³ × nmol min⁻¹ mg⁻¹ protein]</th>
<th>( K_m ) [µM]</th>
<th>Capacity factor ( ^a ) [10³ × mL min⁻¹ mg⁻¹ protein]</th>
<th>r</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATEE</td>
<td>6.6 ± 0.6</td>
<td>436.1 ± 110.1</td>
<td>0.015</td>
<td>0.966</td>
</tr>
<tr>
<td>BTEE</td>
<td>5.1 ± 0.3</td>
<td>91.8 ± 17.8</td>
<td>0.056</td>
<td>0.958</td>
</tr>
<tr>
<td>IGF-I</td>
<td>0.089 ± 0.011</td>
<td>8.4 ± 2.2</td>
<td>0.011</td>
<td>0.971</td>
</tr>
</tbody>
</table>

\(^a\) \( v_{\text{max}}/K_m \); Segel (1976)

Inhibition of IGF-I cleavage

The influence of protease inhibitors on the enzymatic cleavage of IGF-I by CPPE was tested. ATEE, TP5 and LNA were included as positive controls to assess chymotrypsin, aminopeptidase and carboxypeptidase/aminopeptidase activities, respectively. EDTA, as a metalloprotease inhibitor, puromycin as an aminoprotease inhibitor, GenMix as a mixture of serine protease inhibitors, and casein as a toxicologically safe nutrient additive were evaluated. The results are included in table 4.

The enzymatic stability of IGF-I was significantly improved when casein or GenMix were added. The presence of EDTA and puromycin stabilised both TP5 and LNA, but neither IGF-I nor ATEE were affected (cf. table 4). In conclusion, IGF-I is a substrate for serine proteases such as trypsin and chymotrypsin. In contrast, TP5 and LNA are susceptible to hydrolysis by both carboxypeptidase A and aminopeptidase M.

Interaction of IGF with casein

We hypothesise that protein binding of IGF-I to casein may explain the increased stability of IGF-I in the presence of tryptic and chymotryptic intestinal enzymes (cf. figure 1). The IGF-I fraction bound seems to depend on the casein concentration, because binding appears to increase with increasing casein concentrations reaching a plateau value of ~72 % binding above 1000 µg mL⁻¹ casein.
Table 4: Cleavage half-lives of IGF-I, N-acetyl-L-tyrosine ethyl ester (ATEE), N-benzoyl-L-tyrosine ethyl ester (BTEE), thymopentin (TP5) and L-leucine-4-nitroanilide (LNA) in the presence of 10 µg mL⁻¹ crude porcine pancreas extract (CPPE) in acetate buffer (pH 6.5; 37 °C). Effects of EDTA (675.4 µg mL⁻¹ = 2mM), puromycin (108.9 µg mL⁻¹ = 0.2 mM), casein (0.5 %), and GenMix (aprotinin: 8.3 µg mL⁻¹, soybean trypsin inhibitor: 7.6 µg mL⁻¹ and TLCK: 8.0 µg mL⁻¹ = 0.02 mM), and negative control. Substrates were studied at a concentration of 6.5 µM (n = 3, mean ± SD).

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Control [min]</th>
<th>EDTA [min]</th>
<th>Puromycin [min]</th>
<th>GenMix [min]</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>64.6 ± 9.0</td>
<td>71.7 ± 3.7</td>
<td>73.4 ± 7.2</td>
<td>388.7 ± 23.8</td>
<td>stable</td>
</tr>
<tr>
<td>ATEE</td>
<td>32.5 ± 7.5</td>
<td>34.2 ± 9.4</td>
<td>51.9 ± 4.9</td>
<td>137.3 ± 20.8</td>
<td>213.2 ± 79.8</td>
</tr>
<tr>
<td>BTEE</td>
<td>28.4 ± 5.0</td>
<td>N.D. b</td>
<td>N.D. b</td>
<td>N.D. b</td>
<td>N.D. b</td>
</tr>
<tr>
<td>TP5</td>
<td>25.7 ± 5.7</td>
<td>242.1 ± 61.1</td>
<td>32.6 ± 8.7</td>
<td>52.5 ± 4.8</td>
<td>129.0 ± 30.4</td>
</tr>
<tr>
<td>LNA</td>
<td>293.2 ± 52.1</td>
<td>stable a</td>
<td>472.6 ± 28.1</td>
<td>355.0 ± 145.4</td>
<td>stable a</td>
</tr>
</tbody>
</table>

*No significant degradation observed within 90 min.

Size-exclusion chromatography (SEC) of the IGF-I/casein mixture did not provide evidence for binding because no further peak with a higher molecular weight than casein was detectable under the experimental conditions (data not shown).

*Cleavage of IGF-I in the presence of purified pancreatic enzymes*

Because the incubation studies with pancreas extract cannot reveal the precise involvement of the respective enzyme(s), studies with purified pancreatic enzymes were performed to determine unequivocally the proteases involved in the enzymatic degradation of IGF-I. For comparison we included substrates such as ATEE, LNA, and TP5 (cf. table 5).

IGF-I was metabolised in the presence of purified α-chymotrypsin and trypsin. The result is in good agreement with the data in the presence of standardised CPPE (cf. table 4). In contrast, no significant degradation
Figure 1: Interaction between IGF-I and casein as a function of casein concentration (pH 7, room temperature). Throughout, total IGF-I concentration was 50 μg mL⁻¹ = 6.5 μM. Maximum binding of ~72 % was above 200 mg mL⁻¹ casein (non-mechanistic fit: y = 72 (1 - e⁻bx)).

of IGF-I took place in the presence of aminopeptidase M and carboxypeptidase A. As expected the cleavage of ATEE followed the same pattern, whereas TP5 and LNA were only substrates of aminopeptidase M and carboxypeptidase A, and were stable in the presence of trypsin and α-chymotrypsin. This study corroborates the data using EDTA, puromycin, and GenMix as enzyme inhibitors (cf. table 4).

Cleavage of IGF-I in the presence of porcine flushings, faeces extract, brush border membrane vesicles (BBMV) and excised mucosa

The cleavage of IGF-I was examined in the presence of porcine midjejunal flushings, colonic faeces extract, brush border membrane vesicles (BBMV) and excised mucosa. In control studies the protein release from tissue into the medium was assessed. Throughout, ATEE and
Table 5: Cleavage half-lives of IGF-I, N-acetyl-tyrosine ethyl ester (ATEE), thymopentin (TP5) and L-leucine-4-nitroanilide (LNA) in the presence of aminopeptidase M (448 ng mL\(^{-1}\), protein), carboxypeptidase A (19.8 ng mL\(^{-1}\) protein), \(\alpha\)-chymotrypsin (1020 ng mL\(^{-1}\)) and trypsin (980 ng mL\(^{-1}\)) in acetate buffer (pH 6.5, 37 °C). Throughout the study, substrate concentration was 6.5 \(\mu\)M. Results are means ± SD; (n = 3).

<table>
<thead>
<tr>
<th></th>
<th>Aminopeptidase M</th>
<th>Carboxypeptidase A</th>
<th>(\alpha)-Chymotrypsin</th>
<th>Trypsin</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I</td>
<td>stable(^a)</td>
<td>stable(^a)</td>
<td>2.7 ± 0.4</td>
<td>34.6 ± 4.2</td>
</tr>
<tr>
<td>ATEE</td>
<td>stable(^a)</td>
<td>stable(^a)</td>
<td>1.7 ± 0.1</td>
<td>51.0 ± 6.7</td>
</tr>
<tr>
<td>TP5</td>
<td>58.5 ± 5.9</td>
<td>83.9 ± 3.4</td>
<td>stable(^a)</td>
<td>stable(^a)</td>
</tr>
<tr>
<td>LNA</td>
<td>323.6 ± 98.5</td>
<td>538.2 ± 193.5</td>
<td>stable(^a)</td>
<td>832.9 ± 152.3</td>
</tr>
</tbody>
</table>

\(^a\) No significant degradation observed within 90 min.

BTEE were used as positive controls for chymotryptic activity. Flushings, faeces extract and BBMV were diluted and standardised to final protein concentrations of 1 \(\mu\)g mL\(^{-1}\). IGF-I was degraded by midjejunal flushings and faeces extract. In contrast, BBMV did not cause any degradation of IGF-I. Likewise, BTEE and ATEE were degraded in the presence of flushings and faeces extract, but not in the presence of BBMV. Casein enhanced the stability of IGF-I, ATEE and BTEE (cf. table 7). As expected, BBMV did not contain chymotryptic and tryptic activities (Langguth et al. 1997) which were only present in flushings and faeces extract (Lee 1988, Tozaki et al. 1997).

Table 6: Protein release from colonic and jejunal excised porcine mucosa in Ussing chambers (n = 2-3, mean ± SD)

<table>
<thead>
<tr>
<th>Protein concentration [(\mu)g mL(^{-1})]</th>
<th>Colon</th>
<th>Jejunum</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time [min]</td>
<td>Mucosal side</td>
<td>Serosal side</td>
</tr>
<tr>
<td>10</td>
<td>118 ± 30</td>
<td>159 ± 17</td>
</tr>
<tr>
<td>60</td>
<td>170 ± 52</td>
<td>192 ± 78</td>
</tr>
</tbody>
</table>

Flushings, faeces extract and BBMV were diluted and standardised to final protein concentrations of 1 \(\mu\)g mL\(^{-1}\). IGF-I was degraded by midjejunal flushings and faeces extract. In contrast, BBMV did not cause any degradation of IGF-I. Likewise, BTEE and ATEE were degraded in the presence of flushings and faeces extract, but not in the presence of BBMV. Casein enhanced the stability of IGF-I, ATEE and BTEE (cf. table 7). As expected, BBMV did not contain chymotryptic and tryptic activities (Langguth et al. 1997) which were only present in flushings and faeces extract (Lee 1988, Tozaki et al. 1997).
Figure 2: Comparison of IGF-I loss in Krebs’ phosphate buffer in the absence of tissue (■) versus cleavage in the presence of excised colonic porcine mucosa (●). $C_{IGF-I}$ = IGF-I concentration in the Ussing chamber (means ± SD, n= 3 - 4). Broken line shows the calculated IGF-I concentration decrease rate caused by the tissue only.

Protein release from excised porcine mucosa after 10 min and 60 min, respectively, was studied in Ussing chambers and is reported in Table 6. Generally, jejunal release was higher than colonic release. However, the observed protein release over time does not seem to have a major effect on the degradation rate of IGF-I (cf. Figure 2).

**IGF-I loss in Ussing chambers**

In the presence of excised porcine mucosa the half-life of IGF-I was between 20 and 30 min, regardless whether it was added to the mucosal or serosal sides of the tissue (Table 7). The half-life of IGF-I in Ussing chambers in the absence of tissue was 164.6 ± 57.4 min (mean ± SD, n = 3). The contrast between the loss in Krebs’ phosphate buffer versus the enzymatic cleavage in the presence of excised porcine colonic mucosa (mucosal side) is demonstrated in figure 2.
Table 7: Cleavage half-lives of IGF-I, N-acetyl-L-tyrosine ethyl ester (ATEE) and N-benzoyl-L-tyrosine ethyl ester (BTEE) in the presence of jejunal and ileal flushings, faeces extract and BBMVs (all standardised to 1 μg mL⁻¹ protein in Krebs’ phosphate buffer, pH 7.0) of three species (pig, rat, dog: dog a = Beagle, dog b = Labrador) at 37 °C, and in the presence of excised porcine mucosa in Ussing chambers on the mucosal (m) and serosal (s) side (Krebs’ phosphate buffer, pH 7.0, gas lift, 95% O₂/ 5% CO₂) at 37 °C (n = 2-3, mean ± SD).

<table>
<thead>
<tr>
<th></th>
<th>IGF-I</th>
<th>IGF-I/casein</th>
<th>ATEE</th>
<th>ATEE/casein</th>
<th>BTEE</th>
<th>BTEE/casein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Half-life [min]</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pig</td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Flushing, jejunum</td>
<td>225.9 ± 42.5</td>
<td>412.8 ± 35.9</td>
<td>321.0 ± 232.7</td>
<td>532.1 ± 133.9</td>
<td>85.2 ± 51.2</td>
<td>144.0 ± 52.26</td>
</tr>
<tr>
<td>Faeces extract</td>
<td>42.4 ± 8.4</td>
<td>2274.8 ± 397.2</td>
<td>594.3 ± 277.5</td>
<td>767.2 ± 178.2</td>
<td>249.7 ± 21.65</td>
<td>721.8 ± 205.38</td>
</tr>
<tr>
<td>BBMV stable</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ussing chamber, colon, m</td>
<td>21.7 ± 4.5</td>
<td></td>
<td>18.6 ± 4.1</td>
<td></td>
<td>20.1 ± 4.6</td>
<td></td>
</tr>
<tr>
<td>Ussing chamber, colon, s</td>
<td>24.7 ± 4.4</td>
<td></td>
<td>22.2 ± 4.2</td>
<td></td>
<td>24.2 ± 4.2</td>
<td></td>
</tr>
<tr>
<td>Ussing chamber, jejunum, m</td>
<td>29.7 ± 9.1</td>
<td></td>
<td>27.0 ± 9.0</td>
<td></td>
<td>28.5 ± 9.0</td>
<td></td>
</tr>
<tr>
<td>Ussing chamber, jejunum, s</td>
<td>27.4 ± 0.6</td>
<td></td>
<td>25.0 ± 0.6</td>
<td></td>
<td>26.5 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Dog a Flushing, jejunum</td>
<td>181.0 ± 107.8</td>
<td>stablea</td>
<td>183.1 ± 110.9</td>
<td>stablea</td>
<td>185.1 ± 110.9</td>
<td>stablea</td>
</tr>
<tr>
<td>Dog b Flushing, jejunum</td>
<td>13.6 ± 3.4</td>
<td>198.2 ± 21.0</td>
<td>5.1 ± 0.1</td>
<td>26.9 ± 23.8</td>
<td>&lt;2.6</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>Dog a Flushing, ileum</td>
<td>9.1 ± 0.4</td>
<td>50.4 ± 18.4</td>
<td>&lt;4.9</td>
<td>33.5 ± 3.2</td>
<td>&lt;2.6</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>Dog b Flushing, ileum</td>
<td>76.7 ± 16.4</td>
<td>177.2 ± 100.7</td>
<td>&lt;4.9</td>
<td>30.2 ± 10.8</td>
<td>&lt;2.6</td>
<td>&lt;2.6</td>
</tr>
<tr>
<td>Dog a Faeces extract</td>
<td>99.8 ± 15.8</td>
<td>109.9 ± 22.1</td>
<td>297.0 ± 25.0</td>
<td>411.7 ± 319.5</td>
<td>119.3 ± 30.0</td>
<td>107.9 ± 2.0</td>
</tr>
<tr>
<td>Dog b Faeces extract</td>
<td>4.2 ± 0.6</td>
<td>57.2 ± 6.0</td>
<td>102.1 ± 1.7</td>
<td>82.2 ± 0.2</td>
<td>27.0 ± 0.7</td>
<td>13.8 ± 5.3</td>
</tr>
<tr>
<td>Rat</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flushing, jejunum</td>
<td>43.1 ± 19.3</td>
<td>118.5 ± 37.3</td>
<td>4.0 ± 0.23</td>
<td>10.9 ± 1.3</td>
<td>4.8 ± 0.6</td>
<td></td>
</tr>
<tr>
<td>Flushing, ileum</td>
<td>30.4 ± 3.3</td>
<td>116.0 ± 55.6</td>
<td>4.5 ± 0.6</td>
<td>5.3 ± 3.9</td>
<td>4.6 ± 0.4</td>
<td></td>
</tr>
<tr>
<td>Faeces extract</td>
<td>33.9 ± 19.1</td>
<td>86.8 ± 42.1</td>
<td>4.0 ± 0.3</td>
<td>10.4 ± 3.4</td>
<td>&lt;1.0</td>
<td>&lt;1.0</td>
</tr>
</tbody>
</table>

aNo significant degradation observed within 90 min. bHalf-life not determined. cMean was determined using the material of three different animals.
Cleavage of IGF-I in the presence of flushings and faeces extract of different species

The cleavage of IGF-I, ATEE and BTEE after incubation with flushings from jejunum and ileum, faeces extracts from rat, dog and pig was determined (table 7). IGF-I, ATEE and BTEE degraded in all intestinal sections studied. Significant intra- and inter-species differences were observed. The general trends were as follows: The addition of casein increased the half-lives of all substrates in the presence of porcine and canine faeces extract, and canine jejunal and ileal flushings. Casein seems to enhance the stability of IGF-I much better than the stabilities of ATEE and BTEE.

Discussion

Two barriers play a major role for the often poor peroral absorption of therapeutic peptides and proteins: their degradation by intestinal enzymes in the gut, and their low intrinsic permeability in the intestinal mucosa (Lee 1988). In this study, we assessed the enzymatic stability of IGF-I in the various segments of the intestine with the objective to elucidate the potential for oral IGF-I delivery and absorption. Various in vitro models were applied to investigate the influence of luminal and membrane bound enzymes. The degradation of IGF-I by gastric enzymes was not studied since various established technologies are available (e.g., enteric coating) to protect proteins from their degradation in the stomach.

Cleavage of IGF-I

The cleavage of IGF-I in the presence of crude porcine pancreas extract (CPPE) was compared to established chymotrypsin substrates (Kantorski and Tchorzewski 1992), namely N-acetyl-L-tyrosine ethyl ester (ATEE) and N-benzoyl-L-tyrosine ethyl ester (BTEE). The data (table 3) demonstrate that the capacity factors of IGF-I and ATEE were similar. However, IGF-I has a 50-fold higher affinity to the pancreas enzymes. ATEE was considered a useful positive control for the chymotryptic degradation of IGF-I. For comparison, we also tested the cleavage of BTEE. The degradation of BTEE in various fluids was reported in the literature, such as in human and canine fluids (Sinko et al. 1992,
Rinderknecht et al. 1978). Thus, BTEE is considered an important reference to estimate the potential chymotryptic degradation of IGF-I in human intestine. In contrast to IGF-I, BTEE seems to be a low affinity/high capacity substrate for porcine pancreatic enzymes. The estimated capacity factors of IGF-I and BTEE may suggest that IGF-I degradation is 5 times slower than BTEE in the presence of human pancreatic enzymes.

Fasting chymotrypsin activity in humans (in duodenal juice) is approximately 2 BTEE units mL\(^{-1}\) (Rinderknecht et al. 1978). In our study, we found a chymotrypsin activity of 0.005 BTEE units mL\(^{-1}\) in 10 µg mL\(^{-1}\) CPPE in acetate buffer (cf. table 3), i.e. about 400 times lower. Accordingly we would expect the enzymatic cleavage of IGF-I in human duodenal juice to be at least 400 times faster (35.6 nmol min\(^{-1}\)) than in the standardised CPPE preparation used in this study (0.089 ± 0.011 nmol min\(^{-1}\); cf. table 3). Because of the differences seen for BTEE it is conceivable that in vivo IGF-I degradation might be faster when compared to in vitro conditions.

*Inhibition studies*

To determine the responsible proteolytic enzymes, IGF-I was incubated with CPPE in the presence of inhibitors such as puromycin (aminopeptidase inhibitor), EDTA (metalloprotease inhibitor) and aprotinin, soybean trypsin inhibitor and TLCK (serine protease inhibitor) (Fritz and Wunderer 1983, Kato et al. 1988, Shaw and Glover 1970, Heizmann et al. 1996).

Inhibitors of serine protease enhanced the stability of IGF-I whereas inhibitors of aminopeptidases and carboxypeptidases had no effect. To verify this observation IGF-I was also incubated with purified carboxypeptidase A, aminopeptidase M, chymotrypsin and trypsin. Only in the presence of chymotrypsin and trypsin IGF-I was degraded, which is consistent with the data derived from the inhibition studies corroborating our expectations. Thus, we conclude that chymotryptic and tryptic activities play a prominent role in the degradation of IGF-I confirming earlier work by Bai (1995).

As a central aspect of this study, the enzymatic cleavage of IGF-I was efficiently inhibited by adding casein, a physiologically safe nutrient
additive. In the presence of casein CPPE caused negligible degradation within 90 min incubation. The protection by casein could result from (i) a direct interaction with IGF-I and/or (ii) an interaction with the responsible enzyme(s) that cleave(s) IGF-I. To this point the possibility of a casein/IGF-I interaction has been explored. The results so far are inconclusive. Protein binding studies at various casein concentrations seem to support the idea of IGF-I binding to casein. Interestingly, IGF-I binding increased with increasing casein concentrations until a plateau was reached. Similar to our study, Rudy and Poynor (1990) examined protein binding of pyrimethamine. In close agreement with our findings the authors observed, with increasing protein concentration, a pronounced increase of binding which reached a plateau at high protein concentrations. However, the attempt to fit our IGF-I binding data in the way suggested by Rudy and Poynor (1990) was unsuccessful. Furthermore, size exclusion chromatography did not reveal an IGF-I/casein complex. Hypothetically, the IGF-I/casein complex may be unstable under the SEC conditions used, e.g., because of differences in pH, buffer systems and salt concentrations (Rudy and Poynor 1990). At this point, further studies would be needed to elucidate the hypothetical complex between these components.

Likewise, also the second hypothesis should be considered, i.e. the interaction of casein with the responsible enzyme(s) to cleave IGF-I. As compared to the two control substrates ATEE and BTEE, stabilisation of IGF-I was more enhanced, especially in case of faeces extract. Hence, we conclude that casein acts as a non specific inhibitor of serine proteases in case of both ATEE and BTEE, but additional protection is likely to be induced by direct interaction with IGF-I.

Previously, Xian et al. (1995) demonstrated that high salt concentrations could not neutralise the stability enhancing effect of casein on IGF-I. The authors concluded that other mechanism(s) than charge-charge interactions govern the interaction between IGF-I and casein. Kimura et al. (1997) observed the stabilising effects of casein only in the jejunum and ileum of rats. However, contrary to our findings no effect was observed in the colon. It remains to be seen whether this result is attributable to the non-specific analysis chosen by the authors, i.e.
radioanalysis of $^{125}$I-IGF-I after precipitation with trichloroacetic acid (TCA), whereas we analysed IGF-I by specific HPLC.

At the moment, the full mechanism(s) involved in the stabilisation of IGF-I in the presence of casein is (are) not understood. However, in accordance with our studies, the stabilising effect of casein on peptides and proteins in the gastrointestinal tract was previously recognised by other groups (Rao et al. 1990, Xian et al. 1995). They emphasised the physiological relevance of milk relative to its protective effect on intestinal peptide and protein uptake in suckling rats.

Cleavage of IGF-I with respect to the different intestinal segments

To evaluate its degradation by luminal versus membrane bound enzymes, IGF-I was also challenged in the presence of intestinal flushings and faeces extract of various species, and porcine BBMV. Typically, the stability of IGF-I was only affected by luminal enzymes of the small and large intestine, whereas membrane bound enzymes were not involved. The half-lives of IGF-I in the presence of large intestinal porcine and canine content were significantly lower than in small intestinal flushings. In rat tissue we saw no significant difference.

Kimura et al. (1997) observed an increase in stability along the intestine in the following order: ileum $<$ jejunum $<$ colon. Correspondingly, Xian et al. (1995) reported an increase in the half-lives from 2 min in the duodenum and ileum up to $>$ 60 min in the colon. In the present study, there was no statistical difference between flushings from jejunum and ileum. The degradation rates of IGF-I observed by Kimura et al. (1997) and Xian et al. (1995) were much lower as compared to our findings, taking the actual protein concentrations of their flushings into account. The contrast may be explained by major differences in the analytical protocols. Whereas Kimura et al. (1997) applied radioanalysis of $^{125}$I-IGF-I after precipitation by trichloroacetic acid (TCA), we analysed IGF-I directly by HPLC. We assume their analytical technique to be less specific than HPLC because upon TCA metabolites might co-precipitate with intact IGF-I.

Similarly to other authors (Kimura et al. 1997, Xian et al. 1995, Bai et al. 1995), we studied the influence of colonic content on the stability of IGF-I under aerobic conditions. According to Gibson et al. (1989) aerobic...
conditions favour chymotrypsin- and trypsin-like activities of colon content, whereas cysteine and metalloprotease activities dominate under anaerobic conditions. In contrast, Tozaki et al. (1997) measured under anaerobic conditions a significant serine protease activity. The authors reported that the stability of insulin which is significantly degraded by chymotrypsin and trypsin (Schilling and Mitra 1991) could be significantly increased in the presence of caecum content by adding serine protease inhibitors. The presence of chymotrypsin- and trypsin-like enzymes in the colon does not only originate from pancreatic secretion, but is also likely the result from bacterial remnants formed upon disruption of colonic bacteria (Gibson et al. 1989, Bai et al. 1995). Therefore, we conclude that the high cleavage of IGF-I in the large intestinal content might be explained by an increased release of bacterial chymotrypsin- and trypsin-like enzymes under the aerobic conditions applied. This may be different under the anaerobic conditions in vivo.

On the other hand, in the presence of porcine faeces extract, ATEE and BTEE were more stable than in the flushings, which leads to the conclusion that serine protease activity in the colon is reduced as previously described in the literature (Lee 1988, Langguth et al. 1997). Therefore, we assume that in addition to luminal serine proteases, other enzymes might be also involved in the cleavage of IGF-I in the presence of colonic content.

Similar to our studies, Kimura et al. (1997) showed that IGF-I was stable in the presence of ileal and jejunal brush border membrane fractions of the rat. This supports the concept that aminopeptidases, which are predominantly present in the brush border membrane, have little effect on IGF-I stability.

Cleavage of IGF-I in the presence of excised porcine mucosa: Incubation studies performed in Ussing chambers showed that IGF-I was likewise degraded in the presence of excised jejunal and colonic mucosa. In addition, there was no significant difference in IGF-I stability between the mucosal and serosal compartments.\(^3\)

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\(^3\) Protein release of the excised mucosae was studied for validation reasons. According to W. Rubas (personal communication) the Bio-Rad protein assay may overestimate protein concentrations in the presence of glucose by ~30% if the calibration curve is
The noticeable degradation of IGF-I indicated that enzyme activity was still present after washing the tissue. Moreover, controls in Ussing chambers demonstrated a decrease in IGF-I concentration even when incubated in Krebs’ phosphate buffer alone. Consequently, in addition to enzymatic cleavage, both adsorption to the Ussing chambers and/or instability in Krebs’ phosphate buffer need to be considered when half-lives of IGF-I in the presence of excised mucosa are determined.

In summary, this study demonstrates that IGF-I is severely degraded throughout the intestine, mainly by serine proteases such as chymotrypsin- and trypsin-like enzymes. Remarkable inter-species differences point out the importance of testing various species for the assessment of IGF-I stability in the gastrointestinal tract.

Rat and canine intestinal tissue are well-accepted models regarding their intestinal chymotryptic activity (Sinko 1992, Bai et al. 1995, Tozaki et al. 1997), and, thus, meaningful for comparison. On the other hand, there is increasing interest in the use of the porcine model because of its close similarity to the human physiology (Flores et al. 1998, Gestin et al. 1997). Moreover, intestinal permeability studies revealed good agreement between porcine and human colonic mucosae with respect to permeabilities of IGF-I and PEG 4000 (cf. Chapter IV).

There is no final conclusion as to a preferred intestinal segment concerning IGF-I stability. However, the variabilities of the half-lives in rat, porcine and canine small intestine were generally higher than those in the colon. This may be explained by the fluctuations in pancreatic enzyme secretion in the small intestine. There are two factors in favour of colon delivery: (i) the more regular enzymatic activity in the colon in contrast to the small intestine, and (ii) the fact that the binding activity of IGF-I to its receptors is higher in the colon (Laburthe et al. 1988). On the other hand, delivery to the small intestine should not be ruled out, because of the advantage of a large surface area and the observed stability of IGF-I in the presence of brush border membrane enzymes (Chadwick et al. 1977).

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obtained in the absence of glucose. Thus, data presented in table 6 needs to be looked at critically taking potential overestimation into account. Data are in accordance with protein release found for rabbit mucosa which was ~1 mg mL⁻¹ after 120 min (W. Rubas, personal communications)
Literature


Chapter IV

Transport of IGF-I across Intestinal Epithelium: Permeation Rates and Mechanisms Relative to Permeability Markers.

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Introduction

IGF-I is a 7648 Da polypeptide of 70 amino acids and considered for treatment of various diseases, such as type II diabetes (Savage et al. 1996, Froesch and Hussain 1994), Lou Gehrig's disease (Piascik 1996, Festoff 1996) and for the treatment of growth hormone receptor deficiency in children (Guevara-Aguirre et al. 1995). Because of the need to administer IGF-I chronically in these disease states, the oral route would be a highly desirable mode of administration as an alternative to the parenteral injection.

Various uptake mechanisms of IGF-I across epithelia and endothelia have been suggested, such as receptor-mediated endocytosis, as shown in rat astrocytes and fibroblasts (Auletta et al. 1992, Zapf et al. 1994) and paracellular transport of IGF-I across umbilical vein endothelial cell monolayers (Bastian et al. 1997). Moreover, based on size and charge absorptive-mediated endocytosis of IGF-I in the intestine was hypothesised (Kimura et al. 1997, Terasaki et al. 1989). In the intestine the absorption of IGF-I could be inhibited in the presence of colchicine, an inhibitor of microtubular assembly, and by polycations such as poly-L-lysine and protamine, but not by a polyanion such as poly-L-glutamic acid (Kimura et al. 1997), which indicates absorptive-mediated endocytosis.

The presence of IGF-I receptors in the jejunum as well as in the colon has been demonstrated for various species such as human, rat, pig and rabbit (Rouyer-Fessard et al. 1990, Young et al. 1990, Schober et al. 1990, Pillion et al. 1989). Additionally, in various intestinal cell lines the presence of IGF-I receptors was demonstrated such as in IEC-6, a cell line derived from rat jejunal crypts (Park et al. 1990). Domain selective biotinylation of the apical and basolateral surfaces of Caco-2 cells, a colon carcinoma cell line, grown on filter supports revealed a 3.6-fold enrichment of IGF-I receptors on the basolateral membrane (Oguchi et al. 1995, Hoeflich et al. 1994, Dahms et al. 1996).

In neonatal rats and pigs IGF-I may play an important role in the development of the intestinal mucosa. Burrin et al. (1996) showed that orally administered IGF-I increased the intestinal mucosal growth in formula fed neonatal pigs and the height of jejunal and ileal villi. At the
same time neither the concentration of circulating IGF-I nor IGF-binding proteins changed. Other studies demonstrated that the effect of IGF-I in gastrointestinal renewal in the weaned rat did not depend on IGF-I supply through the gastrointestinal fluids but rather through the supply via the circulation or by the local intestinal production (Fholenhag et al. 1997). This shows that peroral IGF-I may only elicit local effects, but may not be taken up into the systemic circulation.

In contrast to the findings by Fholenhag et al. (1997) Kimura et al. (1997) showed that after peroral intubation IGF-I reached the systemic circulation in an intact form, thus demonstrating the potential feasibility of an oral dosage of IGF-I. Only few studies on the permeation of IGF-I across intestinal tissue are available, e.g., in the colon of guinea pigs and rats (Quadros et al. 1994) as well as in humans (Rubas et al. 1996) and rabbits (Rubas et al. 1995).

In this study the absorption of IGF-I across the intestinal mucosa is investigated with an emphasis on the permeability of IGF-I in various animal species and in human, its mechanism of permeation and its permeability relative to commonly used intestinal permeability markers. Artursson (1991) gave an extensive overview of methods to discriminate between passive and active transport, such as direction dependency, inhibition of ATP-dependant transport, and transport rate dependency on donor concentration. To inhibit active transport, such as endocytosis, we use K+-depletion of the cells and cytochalasin D treatments. Both are well known methods to inhibit endocytosis (Altankov and Grinnell 1993, Cremaschi et al. 1996). Additionally, the effect of proteolytic enzyme inhibitors and casein on the permeability of IGF-I is studied. Finally, IGF-I permeability in the small intestine is compared to the large intestine.

**Materials and Methods**

Cytochalasin D, 2-[Morpholino]ethane-sulfonic acid (MES), soybean trypsin inhibitor, aprotinin, Nα-p-tosyl-L-lysine chloromethyl ketone hydrochloride (TLCK) and N-acetyl-L-cysteine were purchased from Sigma (Buchs, CH). A combination of soybean trypsin inhibitor, aprotinin and TLCK was used as an enzyme inhibitor cocktail (GenMix).
Casein sodium salt, ethylenediaminetetraacetic acid disodium salt (EDTA), and buffer materials were obtained from Fluka Chemie AG (Buchs, CH). Metkephamid (MKA) was donated by Eli Lilly and Co. (Indianapolis, USA). $^3$H-Mannitol (1 mCi mL$^{-1}$; mannitol), $^{14}$C-PEG 4000 (11 mCi mL$^{-1}$; PEG 4000) and $^3$H-D-glucose (1 mCi mL$^{-1}$; D-glucose) were from Du Pont de Nemours International S.A. (Regensdorf, CH) and Ultima Gold scintillation fluid from Packard (Croningen, NL). Metoprolol and atenolol were a gift from Astra Hässle (Mölndal, Sweden). Calcein AM was purchased from Molecular Probes Europe BV (Leiden, NL), DSL Active IGF-I coated-tube IRMA kit was obtained from Labodia SA (Yens, CH). Fluorescamine and sodium dodecyl sulfate (SDS) were obtained from Sigma Chemical and Co. (St. Louis, USA). Krebs’ phosphate buffer consisted of 95.1 mM sodium chloride, 4.8 mM potassium chloride, 2.5 mM calcium chloride dihydrate, 1.2 mM magnesium sulfate septahydrate, 24.8 mM sodium hydrogen carbonate, 1.2 mM potassium dihydrogen phosphate and 40 mM glucose, adjusted with 1 N acidic acid to pH 7.0. Hanks’ balanced saline solution (HBSS), foetal calf serum (FCS) and rat serum, Dulbecco’s modified phosphate buffered saline (PBS; 0.9 mM calcium chloride dihydrate, 2.7 mM potassium chloride, 1.5 mM potassium dihydrogen phosphate, 0.5 mM magnesium chloride hexahydrate, 136.9 mM sodium chloride, 8.1 mM di-sodium hydrogenphophosphate) were obtained from Life Technologies (Basle, CH). Recombinant human IGF-I (IGF-I) was a gift from Genentech Inc. (South San Francisco CA, USA). All materials used for HPLC analysis were of analytical grade.

**Permeation studies**

*Preparation of porcine, rat and human intestinal mucosae*

*Porcine mucosa:* Fresh porcine colon and midjejunum (suis scrofa domestica) were obtained from the local slaughterhouse in Zurich. The intestine was rinsed with chilled water (aqua potabilis) and kept for transport at 4 °C in Krebs’ phosphate buffer. After the transfer to the laboratory (~40 min) the tissue samples of ~4 cm$^2$ were stripped off their
serosae and adjacent muscularis layers with a bent scalpel and used immediately for permeation studies.

*Rat mucosa:* Male Sprague-Dawley rats (Institut für Zuchthygiene, Universität Zürich), weighing 280 - 320 g were sacrificed, the intestines immediately removed and washed with chilled Krebs' phosphate buffer. 2.5 to 3 cm strips of intestinal tissue were excised, then stretched onto a plastic rod, the serosae and adjacent layers were dissected away, and the intestinal tissues were used immediately.

*Human mucosa:* Normal, i.e. tumour-free human tissue adjacent to localised tumours was received from bowel resections and immediately placed in ice-cold Krebs' phosphate buffer for transport. Informed written consent was obtained from the patient before the operation. After ~30 min transport the intestinal tissue was prepared as described above for porcine tissue.

*Transport buffer*

For all permeation studies transport buffer consisting of freshly prepared Krebs' phosphate buffer and 5 µL mL⁻¹ FCS was used. Except when specifically mentioned, the concentration of the permeating compounds was 500 µg mL⁻¹ in transport buffer (drug solution).

To assess the integrity of the mucosa the marker combinations mannitol/PEG 4000 or D-glucose/PEG 4000 were added to the drug solution. Concentration of both labelled mannitol and labelled D-glucose was 0.06 mM. PEG 4000 was used as provided by the manufacturer.

*IGF-I permeation involving GenMix and casein:* GenMix or casein, respectively, were added to the transport buffer as well as to the drug solution. In case of casein, 100 µg of casein was dissolved overnight in 100 mL transport buffer. For GenMix studies 7.5 µg mL⁻¹ aprotinin, 8.1 µg mL⁻¹ soybean trypsin inhibitor and 6.0 µg mL⁻¹ (15 µM) TLCK were added.

*Permeation kinetics:* The flux of IGF-I across porcine colonic mucosa was studied as a function of IGF-I concentration. The donor concentration range was 6.1 µg mL⁻¹ - 442.1 µg mL⁻¹ (0.8 µM - 57.8 µM).

*IGF-I permeation in the presence of endocytosis inhibitors:* Permeation in the presence of cytochalasin D or in K⁺-free transport buffer
system was studied to elucidate the potential of endocytosis. 40 ng mL\(^{-1}\) (0.08 \(\mu\)M) cytochalasin D and 1 mg mL\(^{-1}\) of casein (for IGF-I stabilisation) were added to the drug and receiver solution. In case of K\(^{+}\)-free transport buffer, potassium chloride and potassium dihydrogenphosphate in transport buffer were replaced by sodium chloride and sodium dihydrogenphosphate (Altankov and Grinnell 1993). Pre-equilibration of the tissue (30 min) was in transport buffer containing inhibitors or in K\(^{+}\)-free transport buffer, respectively.

**IGF-I permeation in the presence of N-acetyl-L-cysteine (AC):**
Permeability studies in the presence of AC, a mucolytic agent, were performed to test the influence of mucus on IGF-I permeability. Transport buffer and drug solution contained 1 mg mL\(^{-1}\) casein. A stock solution of 500 mg AC and 20 mg casein in 20 mL distilled water was prepared. For pre-equilibration of the mucosae 20 mL of the stock solution was diluted with 20 mL casein/Krebs' phosphate buffer to yield a final AC concentration of 12.5 mg mL\(^{-1}\) (76.5 mM) and a final casein concentration of 1 mg mL\(^{-1}\) (AC solution).

**IGF-I stability under control conditions**
To exclude artefacts due to chemical degradation and physical adsorption the stability of IGF-I was tested in Krebs' phosphate buffer and in transport buffer using Ussing chambers, in the presence (1 mg mL\(^{-1}\)) or absence of casein. Effects were studied at an initial concentration of 1 \(\mu\)g mL\(^{-1}\) (0.13 \(\mu\)M) IGF-I. Ussing chambers were maintained at 37 °C by a water-heated jacket. 1 mL of prewarmed (37 °C) IGF-I/buffer solution was added to each compartment. Stirring and oxygen supply was achieved by 5 % CO\(_2\)/O\(_2\) airlift at 15 mL/min. Samples of 100 \(\mu\)L were taken at time zero and after 10, 20, 30, 45 and 60 min. The samples were analysed by HPLC.

**In vitro permeation**
Mucosae were mounted in Ussing chambers (Physiologic Instruments Inc., San Diego CA, USA) and 1 mL of pre-warmed (37 °C) transport buffer was added to each side. Stirring and oxygen supply was achieved by a 5 % CO\(_2\)/O\(_2\) airlift at 15 mL/min. The diffusion cells were
maintained at 37°C by a water-heated jacket. After a 30 min equilibration period 1.2 mL drug solution containing 500 μg mL⁻¹ of IGF-I, metoprolol, atenolol or MKA, respectively, was filled into the respective donor compartment, and 1 mL transport buffer into the receiver compartment. Both mucosal-to-serosal (m → s) and serosal-to-mucosal (s → m) permeation was studied.

For the studies with AC, 1 mL of AC solution was added to the mucosal compartment and 1 mL of transport buffer to the serosal compartment. After a 30 minute equilibration period, AC solution and transport buffer were replaced by 1.2 mL of drug solution (500 μg mL⁻¹ IGF-I in transport buffer) for the mucosal compartment (donor) or 1 mL of transport buffer for the serosal compartment (receiver), respectively.

At time zero, 200 μL was sampled from the donor and split for the determination of mannitol, D-glucose or PEG 4000, respectively, and for drug analysis. Samples of 100 μL were taken after 10, 20, 30, 45 and 60 min from the receiver and immediately replaced by fresh transport buffer. One sample of 100 μL was taken after 60 min from the donor, i.e. at the end of the experiment.

For radio isotop analysis, samples of 100 μL were taken after 10 and 30 min from the receiver compartment and immediately replaced by transport buffer. At 60 min, 300 μL from the receiver and 100 μL from the donor compartment were collected. Samples for the mannitol, D-glucose and PEG 4000 transport studies were mixed with 4.5 mL of scintillation fluid. Drug analysis was by HPLC. Mannitol, D-glucose and PEG 4000 samples were counted in a multipurpose scintillation counter (Beckman LS 6500, Beckman Instruments Inc., Fullerton CA, USA

Calculation of kinetic parameters

Effective permeability coefficients, \( P_{\text{eff}} \) [cm s⁻¹] were calculated according to:
where \((dC/dt)_{ss}\) is the steady-state change of concentration over time \([\mu g \text{ mL}^{-1} \text{ s}^{-1}]\), \(A\) is the diffusion area \([0.636 \text{ cm}^{2}]\), \(V\) is the volume of the receiver compartment \([\text{mL}]\) and \(C_0\) is the initial concentration in the donor compartment.

The flux \((P_{\text{eff}} \times C)\) versus IGF-I concentration profile was fitted according to Michaelis-Menten kinetics. The kinetic parameters \((V_{\text{max}}\) and \(K_m)\) were calculated using Table Curve 2D (Jandel Scientific, AISN Software, 1989-1994, Corte Madera CA, USA).

Determination of transepithelial electrical resistance (TEER) and short-circuit current (Isc)

Experimental measurements of the electrical parameters were made by silver/silver chloride reference electrodes (Physiologic Instruments, Inc., San Diego, CA, USA) consisting of silver wires, which were contained in a glass barrel, terminating in a ceramic tip. Glass barrels were filled with 3 M potassium chloride solution. Two sets of electrodes which bridged the serosal and mucosal compartment were placed at the mucosal and serosal membrane under voltage clamp conditions. Voltage across the tissue was zeroed (Model VCC MC6 multichannel clamp. Physiologic Instruments Inc., San Diego CA, USA) by completing the feedback loop. Pulses of 5 mV were applied for 994 ms with an interval of 142 ms (time separating the two monopolar pulses forming the bipolar waveform) at a frequency of 20 s. Prior to measurements, compensations were made for fluid resistance.

The transepithelial electrical resistance (TEER) was calculated according to:

\[
\text{TEER} = \frac{\Delta P.D.}{I_{sc}}
\]

where \(\Delta P.D.\) is the potential difference and \(I_{sc}\) is the short circuit current. TEER and \(I_{sc}\) were monitored throughout the permeation experiments.
Viability of excised mucosa

Jejunal and colonic porcine serosa and the adjacent muscularis layer were stripped off as described above. Mucosa samples of ~1 cm² were washed three times with 2 mL of filtrated Dulbecco’s modified PBS. 100 µL of 16 µM calcein AM was added to the mucosal surface. The tissues were incubated for 30 min at 37°C in a covered petri dish to prevent drying of samples. After washing three times with 2 mL of filtrated Dulbecco’s modified PBS tissue samples were examined by fluorescence microscopy. Fluorescence detection of polyanionic calcein was performed at an excitation wavelength of 490 nm and an emission wavelength of 520 nm. Stripped mucosa samples showed strong fluorescence indicating preservation of enzymatic activity of the cells fluorescence was detected indicating full enzymatic activity of the cells. However, based on the fluorescence of calcein alone, clear distinction between extracellular and cytosolic fluorescence was unfeasible.

Perfusion studies

Single-pass intestinal perfusions were performed in male Sprague-Dawley rats (Institut für Zuchthygiene, Universität Zürich). Perfusion buffer consisted of Hanks’ balanced salt solution, 10 mM MES, PEG 4000 (0.1 µL mL⁻¹) and rat serum (5 µL mL⁻¹) or FCS (5 µL mL⁻¹), respectively. Additionally, either GenMix (7 µg mL⁻¹ aprotinin, 5 µg mL⁻¹ soybean trypsin inhibitor and 5 µg mL⁻¹ (12.5 µM) TLCK) or casein (1 mg mL⁻¹), respectively, was added to the perfusion buffer. The pH was adjusted to 6.5 with 1 N sodium hydroxide solution. All studies were performed at 100 µg mL⁻¹ (13.1 µM) IGF-I in the perfusion solutions.

Perfusion studies were performed using the single-pass in situ perfusion technique according to Langguth et al. 1994. Male rats (Institut für Zuchthygiene, Universität Zürich), weighing 280 - 320 g, were anaesthetised by i.m. urethane injection of 1.5 g/kg body weight. The rats were put on a heating pad to maintain body temperature. The peritoneum was opened by midline incision. Segments of the jejunum, about 10 cm in length, and of the ascending colon, about 4 - 6 cm in length, starting approximately 1 cm caudal of the ileocaecal valve, respectively, were cannulated with a silicon tube (OD 4 mm, ID 2 mm) following a midline
longitudinal incision. Blood supply to the perfused segments was maintained during the study. The segments were thoroughly cleaned of faecal matter by passing appropriate volume of plain perfusion buffer through the segment. Perfusion was from cranial to caudal. The inlet cannula was attached to a 50 mL syringe (Becton-Dickinson, Basle, CH) which was placed on a perfusion pump (Perfusor® VI, Braun Melsungen, Neuhausen, CH). Perfusion solution, which was always freshly prepared on the day of the trial, was delivered continuously at a rate of 0.2 mL/min for 90 min through the segment. The inlet tubing was thermostated at 39°C by a water bath so that the perfusate entered the intestinal segment at body temperature. After surgery the intestinal segment was placed back to the abdominal cavity which was then covered with moist tissue to prevent dehydration of the animals. Care was taken to avoid any kinks. Initially the eluate from the segments was collected in 15 min intervals to determine the time necessary to reach a steady state flow in terms of peptide, water and electrolyte absorption. In later studies, the eluates from t = 0 min to t = 30 min were discarded and from t = 30 min to t = 90 min collected in polypropylene tubes in 15 min intervals, centrifuged at 1310 g and the supernatant stored in polypropylene tubes at -80°C until analysis. After each perfusion one sample of 5 mL was collected directly from the inlet into the intestinal segment to determine the initial IGF-I concentration C_0. For PEG 4000 determination 100 μL of each eluate was continuously mixed with 4.5 mL of scintillation fluid. After 90 min perfusion 1 mL blood was sampled from the portal vein, the venae hepaticae and the aorta abdominalis. Samples were immediately stored in EDTA coated tubes on ice, centrifuged and the plasma kept at -80°C. The concentration of IGF-I in the perfusate of the colon and jejunum was analysed by HPLC. Peak areas were related to a freshly prepared calibration curve of IGF-I in Krebs’ phosphate buffer at a range of 35 μg mL⁻¹ - 500 μg mL⁻¹ (4.6 μM - 65.3 μM). The concentration of IGF-I in blood was measured by RIA (Assay Services, Genentech Inc., South San Francisco CA, USA) or two-site IRMA, respectively. PEG 4000 samples were counted in a multipurpose scintillation counter (Beckman LS 6500, Beckman Instruments Inc., Fullerton CA, USA).
In preliminary experiments we verified that adsorption of IGF-I to the tubing of perfusion equipment was insignificant, i.e. IGF-I concentrations taken from the freshly prepared perfusion solution and after perfusion of the tubing at the inlet into the intestinal segment were comparable.

**Determination of water absorption**

Water absorption was determined by measuring the radioactivities of PEG 4000 in the perfusion solution initially and in the eluate. Water absorption was described by factor F:

\[
F = \frac{R_{A0}}{R_{A_{30-90}}} \tag{3}
\]

where \(R_{A0}\) is the initial radioactivity of PEG 4000 in the perfusion solution, and \(R_{A_{30-90}}\) is the mean steady state radioactivity measured in the eluate from 30 to 90 min. Factor F was used to correct the IGF-I concentrations of the collected eluates for water absorption relative to the initial IGF-I concentrations:

\[
\frac{C_0 - C_{30-90} \times F}{C_0} \times 100 \tag{4}
\]

where \(\Delta\text{IGF-I} \, [%]\) is the fraction of IGF-I eliminated (e.g., by metabolism) from the perfusion solution as compared to the initial concentration \(C_0\), \(C_{30-90}\) is the mean steady state IGF-I concentration in the eluate from 30 to 90 min, and F is the factor as described in equation 3.

**HPLC of IGF-I, atenolol, MKA, and metoprolol**

The HPLC system for IGF-I samples consisted of a L-6200A pump, an AS-2000 autosampler and a L-4250 UV-VIS absorbance detector (Merck-Hitachi, Darmstadt, D). IGF-I was separated by reversed phase chromatography on a RP 18 VYDAC column (5 μm, 300 Å) 25 cm × 4.6 mm (Buecher, Basle, CH) at room temperature and detected in the UV at 210 nm. The mobile phase consisted of phosphate buffer (0.02 M sodium dihydrogen phosphate, 0.01 M propanesulfonic acid sodium salt, pH
2):acetonitrile, 90:10 (v:v) for A and phosphate buffer (0.02 M sodium dihydrogen phosphate, 0.01 M propanesulfonic acid sodium salt, pH 2):acetonitrile, 50:50 (v:v) for B. The flow rate was 1.0 mL min$^{-1}$. Elution was accomplished by changing the mobile phases, A and B, according to a gradient running from 64 % to 38 % of solution A in 20 minutes.

Atenolol and metoprolol were analysed by reversed phase chromatography on a LiChrospher 100 RP 18 column, (5 μm, 100 Å) 25 × 4 mm (Merck, Basle, CH) according to published procedures (Rubas et al. 1996). The same HPLC system as described above was applied. The mobile phase A consisted of 0.1 % TFA in water and the mobile phase B of 0.1 % TFA in acetonitrile. Elution of atenolol was accomplished by changing the mobile phases, A and B, according to a gradient running from 5 % to 35 % of solution B in 9 minutes applying a flow rate of 1 mL min$^{-1}$, whereas elution of metoprolol according to a gradient running from 10 % to 50 % of solution B. Detection occurred at 214 nm.

Metkephamid of the donor compartment was separated by reversed phase chromatography on a LiChrospher RP 18, (5 μm, 100 Å) 25 cm × 4 mm (Merck, Basle, CH) and detected in the UV at 210 nm. The HPLC system was the same as described above; however, a F-1050 fluorescence detector (Merck, Basle, CH) was used. The mobile phase consisted of 0.01 M sodium heptanesulfonic acid in 50 mM monobasic potassium phosphate (pH 4):acetonitrile, 94:6 (v:v) for A and 65:35 (v:v) for B, the flow rate was 1.5 mL min$^{-1}$. The gradient program was as follows: 0-2 min: 100 % A; 2-20 min: linear gradient from 100 % to 30 % A; 20 - 25 min: linear gradient from 30 % to 0 % A and 25 - 30 min: 0 % A.

The analysis of metkephamid in the receiver compartment was performed by an automated precolumn derivatization with fluorescamine in the autosampler AS-4000 according to Bohner et al. (1996). Briefly, a LiChrospher RP 8 (5μm, 100 Å) column 25 cm × 4 mm (Merck, Basle, CH) was applied. The mobile phases consisted of 50 mM monobasic potassium phosphate (pH 4):acetonitrile, 75:25 (v:v) for A and 55:45 (v:v) for B. The flow rate was 1.5 mL min$^{-1}$. A linear gradient from 100 % to 30 % A within 12 min was applied followed by 8 min isocratic flow of 30 % A.
Fluorescence detection was performed at excitation 390 nm and emission 470 nm.

**IRMA (two-site immunoradiometric assay) of IGF-I**

The kit used to determine IGF-I in blood samples consisted of IGF-I standards in a concentration range of 5 ng mL$^{-1}$ - 600 ng mL$^{-1}$ (0.65 nM - 78.0 nM), anti-IGF-I reagent with a radioactivity of < 10 μCi ($^{125}$I) per 22 mL, anti-IGF-I-coated tubes, extraction solution (ethanolic HCl solution), neutralising solution and IGF-I controls (lyophilised). Two replicates of each plasma sample were analysed. For extraction 50 μL of the sample were added to 200 μL of extraction solution, vortexed and incubated for 30 - 60 min. After centrifugation at 10'000 rpm for 3 min at RT, 100 μL of the clear supernatant were mixed with 500 μL of the neutralising solution. This neutralised sample extract was used for the assay, for which 50 μL of neutralised sample extracts, controls or standards, respectively, were pipetted to the bottom of the coated tubes and 200 μL of anti-IGF-I reagent was added immediately. The solutions were gently mixed for 3 hours by shaking the test tubes on a shaker set at 180 rpm. All tubes were decanted for 1 to 2 min and washed 3 times by adding 5 mL of deionized water. Samples were analysed by a gamma counter (Beckmann, Instruments Inc., Fullerton CA, USA). The radioactivity measured of extracted unknowns was compared to the calibration curve of known standards.

The percentage of IGF-I recovery in rat serum was examined in a concentration range of 50 ng mL$^{-1}$ -10 μg mL$^{-1}$ (6.5 nM - 1.3 μM). Additionally, rat serum and bovine serum were analysed to check for potential cross-reaction of rat IGF-I and bovine IGF-I with human specific anti-IGF-I immunoglobulin. Furthermore, to test the reproducibility of the kit, blood samples of 4 volunteers (29 - 67 years) were analysed.

**Statistics**

The data are presented as means ± SD. Standard F-tests and Student’s unpaired t test (two-tailed) were used to compare means of permeability coefficients. P < 0.05 was considered statistically significant.
Statistical analysis for blood concentrations in perfusion studies was by analysis of variance (ANOVA) at a 95 % confidence level.

Results

Permeation studies

Integrity and viability of excised mucosae

Integrity: PEG 4000 was used as a marker to test the integrity of the excised mucosae. PEG 4000 permeabilities in porcine, rat and human tissue are given in table 1. As an additional control of mucosal integrity, electrophysiological parameters were monitored on-line throughout the permeation studies. The range of $I_{sc}$ values [$\mu$A cm$^{-2}$] was 30 - 125 in porcine colon, 30 - 120 in porcine jejunum, 8 - 40 in rat colon, 47 - 110 in rat jejunum and 12 - 36 in human colon. There were no significant differences between initial and final values. TEERs [$\Omega$ cm$^2$] of porcine and human tissues were 12 - 25 (porcine jejunum: 12 - 20; porcine colon: 12 - 16; human colon: 16 - 25), whereas in rat TEERs were significantly higher (jejunum: 50 - 100; colon: 40 - 90). Significant differences of PEG 4000 permeabilities $m \rightarrow s$ versus $s \rightarrow m$ occurred in porcine and rat colon, but were not considered relevant. In conclusion, PEG 4000 permeabilities and electrophysiological parameters indicated intact barrier function of the mucosae.

IGF-I stability in Ussing chambers

The stability of IGF-I in Krebs’ phosphate buffer in Ussing chambers was investigated under the conditions used for permeation studies. Moreover, the influence of FCS and casein on the IGF-I stability was examined. The degradation rate in Krebs’ phosphate and in the presence of FCS was 0.9 ± 0.2 and 1.3 ± 0.6 [10$^3$ × nmol mL$^{-1}$ min$^{-1}$], respectively and was statistically equivalent. IGF-I was stable in presence of 0.1 % casein within a 60 min period.
Figure 1: Cumulative mass vs. time profile. Permeabilities were calculated from the pseudo steady-state phase (30-60 min). IGF-I across colonic porcine mucosa. • m → s; ▣ s→m (means ± SD, n = 5 - 6). Correlation coefficients were \( r = 0.977 \) for m → s and \( r = 0.968 \) for s → m.

**In vitro permeation**

Permeability coefficients \( P_{\text{eff}} \) were calculated by regression from the linear sections of mass permeated versus time profiles at pseudo steady-state. An example for porcine mucosa is illustrated in figure 1.

**Permeability in porcine mucosa:** Permeability coefficients of IGF-I and PEG 4000 in jejunal versus colonic mucosae were calculated. As compared to PEG 4000 the permeability of IGF-I in the jejunum as well as in the colon was low. There were no significant differences between m → s and s → m transport of IGF-I. Furthermore, the permeability of D-glucose in the porcine jejunum was studied. The transport m → s and s → m were not significantly different (cf. table 1).

The ratio of D-glucose/mannitol permeability coefficients (cf. table 1) in the porcine jejunum was calculated according to:

\[
\frac{P_{\text{eff D-glucose}}}{P_{\text{eff mannitol}}} = F
\]  

where \( P_{\text{eff mannitol}} \) is the permeability coefficient of mannitol and \( P_{\text{eff D-glucose}} \) the permeability coefficient of D-glucose in jejunal porcine mucosa. The
factors $F_{ratio}$ calculated according to equation 5 were 1.92 for $m \rightarrow s$ transport and 1.85 for $s \rightarrow m$ transport, respectively, resulted accordingly to equation 5. The non-significant difference between these two factors demonstrates that passive paracellular transport of D-glucose is predominant over active transport. In light of the high statistical variability of the data ($m \rightarrow s$ and $s \rightarrow m$) the contribution of the absorptive carrier-mediated transport of D-glucose $m \rightarrow s$ cannot be factored out.

Species differences: Porcine, human and rat colonic mucosae were compared regarding their permeabilities to IGF-I. Porcine, human and rat colonic mucosae were compared regarding their permeabilities of IGF-I. The permeabilities of IGF-I and PEG 4000 in the rat were slightly lower as compared to human and pig tissue (cf. table 1).

IGF-I permeability relative to permeability standards: Figure 2 relates the permeability coefficients of IGF-I in porcine colonic tissue with those of various permeability standards. Metoprolol, a high permeability drug, atenolol, mannitol and the pentapeptide metkephamid (MKA), all having low permeabilities, and PEG 4000, a non-absorbable marker, were included.

The magnitude of IGF-I permeability was similar to PEG 4000 a non-absorbable marker and one order of magnitudes lower than that of the low permeability standard mannitol or atenolol. The permeability coefficients of the paracellularly transported standards, namely mannitol, atenolol, and PEG 4000, decreased with increasing molecular weight. As expected, metoprolol had the highest permeability coefficient. physiological fluctuations, the statistical differences were not considered relevant.

There were no significant differences regarding the permeabilities $m \rightarrow s$ versus $s \rightarrow m$ except for mannitol and PEG 4000. In the light of normal physiological fluctuations, the statistical differences were not considered relevant.

Saturable permeation kinetics: Permeation studies with various initial concentrations of IGF-I in the donor-compartment showed that the flux of IGF-I across porcine colonic mucosa was saturable. The effective Michaelis-Menten parameters, as derived by non-linear regression analysis,
Table 1: Comparison of permeability coefficients in the absorptive (m → s) and secretory direction (s → m) of IGF-I, PEG 4000 and D-glucose in jejunal and colonic porcine and in rat colonic and human colonic mucosa (means ± SD, n = 3 - 6).

<table>
<thead>
<tr>
<th>Compound</th>
<th>Permeation direction</th>
<th>Pig</th>
<th>Rat</th>
<th>Human</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Colon</td>
<td>Jejunum</td>
<td>Colonic</td>
</tr>
<tr>
<td>IGF-I</td>
<td>m → s</td>
<td>1.46 ± 0.52</td>
<td>1.04 ± 1.05</td>
<td>0.82 ± 0.44</td>
</tr>
<tr>
<td></td>
<td>s → m</td>
<td>2.08 ± 0.57</td>
<td>2.17 ± 2.44</td>
<td>3.13 ± 3.06</td>
</tr>
<tr>
<td>PEG 4000</td>
<td>m → s</td>
<td>5.20 ± 1.09&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>10.43 ± 5.82</td>
<td>2.44 ± 0.12&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td></td>
<td>s → m</td>
<td>11.82 ± 4.27&lt;sup&gt;a,b&lt;/sup&gt;</td>
<td>7.72 ± 5.73</td>
<td>6.50 ± 0.97&lt;sup&gt;a,b&lt;/sup&gt;</td>
</tr>
<tr>
<td>D-Glucose</td>
<td>m → s</td>
<td>-</td>
<td>47.44 ± 20.64</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>s → m</td>
<td>-</td>
<td>39.45 ± 23.34</td>
<td>-</td>
</tr>
<tr>
<td>Mannitol</td>
<td>m → s</td>
<td>c</td>
<td>24.69 ± 8.35</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>s → m</td>
<td>c</td>
<td>21.25 ± 3.00</td>
<td>-</td>
</tr>
</tbody>
</table>

<sup>a</sup>Significant difference between pig and rat.
<sup>b</sup>Significant difference of permeation direction
<sup>c</sup>cf. Figure 2

Figure 2: Double logarithmic plot of permeability coefficients in porcine colon of mannitol, atenolol, metoprolol, metkephamid (MKA), PEG 4000 and IGF-I. ○ m → s and ■ s → m in the order of increasing molecular weights (means ± SD, n = 3 - 6).
were $K_m$ of $1.04 \pm 0.58 \, [\mu M]$ and a $V_{\text{max}}$ of $7.10 \pm 0.71 \, [10^3 \times \text{nmol cm}^{-2} \text{min}^{-1}]$ (cf. figure 3). However, only two IGF-I concentrations below $K_m$ were studied, therefore, the calculated $K_m$ had to be looked at critically.

Influence of casein and GenMix: Either casein or a mixture of chymotrypsin- and trypsin-inhibitors (GenMix), respectively, were added to the donor and receiver compartment to protect IGF-I from enzymatic degradation, and to study the influence on the effective IGF-I permeability. Adding either casein or GenMix significantly increased the permeability of IGF-I in porcine colonic mucosa in the direction m $\rightarrow$ s (cf. table 2). In case of casein the permeability of IGF-I could be increased up to $\sim 10 \%$ of the permeability measured for atenolol (cf. figure 2). Casein had no influence on tissue integrity with respect to the permeability of PEG 4000 and TEER, while GenMix significantly increased the permeability of mannitol and PEG 4000 in the mucosa and it also decreased the TEER (cf. table 2). TEERs of control and casein groups stayed constant, whereas the TEERs of the GenMix group decreased progressively during the experiment.

Figure 3: Relationship between flux and donor concentration of IGF-I. Flux experiments were performed across porcine colonic mucosa in the presence of 0.1 % casein. Curve fitting according Michaelis-Menten revealed a $K_m$ of $1.04 \pm 0.58 \, [\mu M]$ and a $V_{\text{max}}$ of $7.10 \pm 0.71 \, [10^3 \times \text{nmol cm}^{-2} \text{min}^{-1}]$, $r = 0.768$. 
Permeation in the presence of endocytosis inhibitors: The influence of cytochalasin D or a potassium free buffer system, respectively on the IGF-I permeability in colonic porcine mucosa was investigated. Cytochalasin D and the K⁺-free buffer system led to a significant increase of the permeability of IGF-I and PEG 4000 in porcine colonic mucosa. In the presence of cytochalasin D permeability coefficients (P_{eff}) for IGF-I, mannitol and PEG 4000 were 43.36 ± 22.94, 153.52 ± 70.35 and 75.92 ± 41.47 [10^{6} \times \text{cm s}^{-1}], respectively. The P_{eff} [10^{6} \times \text{cm s}^{-1}] measured for IGF-I, D-glucose and PEG 4000 in K⁺-free buffer system were 53.86 ± 45.02, 108.21 ± 16.28 and 78.89 ± 16.62. Additionally, the TEERs [\Omega \text{cm}^2] were decreased to 3 - 12 in the presence of cytochalasin D, and to 3 - 12 in K⁺-free buffer system. The present data suggests that the integrity of the mucosa was negatively affected by the inhibitor cocktail used.

Permeation studies in presence of N-acetyl-L-cysteine (AC): Permeation studies in the presence of AC were performed to elucidate the influence of mucus on the absorption of IGF-I in the small and large intestine. AC had no positive effect on the permeabilities of IGF-I and PEG 4000, neither in the jejunum nor in the colon. Permeability coefficients of mannitol were slightly increased after adding AC. However, there was no significant difference from the controls (cf. table 3).

Perfusion studies
The in situ single-pass perfusion technique for rat colon and rat jejunum was validated on the basis of PEG 4000, phenol red and potassium, sodium and chloride ion fluxes (Langguth et al. 1994). Water absorption, potassium and sodium transports were in accordance with the data presented by Stöckli (1993) investigating various buffer systems. The steady state was reached after 30 min perfusion time. IGF-I studies were performed to quantify the amount of intact IGF-I reaching the blood circulation after single-pass perfusion. Additionally, the effects of casein and GenMix on IGF-I plasma concentration levels were investigated. In the first series, total plasma IGF-I (endogenous rat IGF-I plus human IGF-I) was analysed by a non-specific RIA.
Table 2: Comparison of the IGF-I permeabilities in the presence of casein or GenMix across porcine colonic mucosae. (means ± SD, n= 3 - 6).

<table>
<thead>
<tr>
<th>Permeation Direction</th>
<th>Control</th>
<th>GenMix</th>
<th>Casein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Peff ( \times 10^6 \text{ cm s}^{-1} )</td>
<td>PEff ( \times 10^6 \text{ cm s}^{-1} )</td>
<td>TEER ( \Omega \text{ cm}^2 )</td>
</tr>
<tr>
<td>IGF-1</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-s</td>
<td>1.38 ±0.51b,d</td>
<td>33 ± 4</td>
<td>133.31 ±114.60b,e</td>
</tr>
<tr>
<td>s-m</td>
<td>2.00 ± 0.61b,d</td>
<td>31 ± 3</td>
<td>61.62 ± 70.90b,e</td>
</tr>
<tr>
<td>PEG 4000</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-s</td>
<td>5.20 ± 1.05a,b</td>
<td>33 ± 4</td>
<td>220.21 ±175.70b,e</td>
</tr>
<tr>
<td>s-m</td>
<td>11.82 ± 4.27a,b</td>
<td>33 ± 3</td>
<td>252.88 ±142.39b,c</td>
</tr>
<tr>
<td>Mannitol</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>m-s</td>
<td>11.33 ± 5.27b</td>
<td>33 ± 4</td>
<td>179.94 ±154.46c</td>
</tr>
</tbody>
</table>

*Significant difference of permeation direction
bSignificant difference between control and GenMix
cSignificant difference between GenMix and casein
dSignificant difference between control and casein
eDecrease of TEER with increasing time, TEER values given at t = 30 min
Table 3: Effect of N-acetyl-L-cysteine on the permeability of IGF-I in porcine colonic and jejunal mucosa. Permeability studies were performed in the presence of 0.1 % casein (means ± SD, n = 5 - 6).

<table>
<thead>
<tr>
<th>Compound Permeation Direction</th>
<th>Control Colon</th>
<th>Jejunum</th>
<th>N-acetyl-L-cysteine (AC) Colon</th>
<th>Jejunum</th>
</tr>
</thead>
<tbody>
<tr>
<td>IGF-I m→s</td>
<td>1.38 ± 0.51</td>
<td>1.79 ± 4.72</td>
<td>1.50 ± 0.94</td>
<td>3.02 ± 2.12</td>
</tr>
<tr>
<td>PEG 4000 m→s</td>
<td>5.20 ± 1.09</td>
<td>10.43 ± 5.82</td>
<td>5.38 ± 2.74</td>
<td>6.90 ± 2.64</td>
</tr>
<tr>
<td>Mannitol m→s</td>
<td>18.63 ± 5.29</td>
<td>18.42 ± 1.99</td>
<td>29.05 ± 12.89</td>
<td>24.69 ± 8.35</td>
</tr>
</tbody>
</table>

In the second series, a human IGF-I specific two-site IRMA was applied to identify human IGF-I separately from endogenous rat IGF-I. Perfusion studies were performed in MES/HBSS containing GenMix. The IGF-I plasma concentration levels after 90 min perfusion period were analysed with RIA (cf. figure 4).

There was no significant difference between IGF-I plasma concentrations following perfusion with IGF-I containing buffer and controls.

Figure 4: Plasma concentrations of IGF-I in the portal vein (☐), venae hepaticae (Ⅲ) and aorta (■) after 90 min of in situ perfusion of rat jejunum and colon. Analysis by non-specific RIA. Throughout, all perfusion studies were performed in the presence of GenMix (means ± SD, n= 2 - 4).
Figure 5: Plasma concentrations of IGF-I in the portal vein (□), venae hepaticae (■) and aorta (■) after 90 min of *in situ* perfusion of the rat colon. Samples were analysed by IRMA. With casein: perfusion was performed in the presence of 0.1 % casein in perfusion buffer MES/HBSS; without casein: perfusion buffer MES/HBSS only was used (means ± SD, n= 2 - 4).

Figure 6: Plasma concentrations of IGF-I in the portal vein (□), venae hepaticae (■) and aorta (■) after 90 min of *in situ* perfusion of rat jejunum. Samples were analysed by IRMA. With casein: perfusion was performed in the presence of 0.1 % casein in perfusion buffer MES/HBSS; without casein: perfusion buffer MES/HBSS buffer only was used (means ± SD, n= 2 - 4).
Figure 7: Loss of IGF-I upon jejunal and colonic perfusion. ΔIGF-I [%] is the fraction of IGF-I eliminated from the perfusion solution as compared to the initial concentration $C_0$ after 90 min perfusion of jejunum and colon, respectively. White bars show decrease of IGF-I concentration in the presence of casein, filled bars the decrease of IGF-I in MES/HBSS without casein (means ± SD, n= 3 - 4).

The second series was performed to study the influence of casein on intestinal absorption. A specific IRMA was used to analyse plasma samples. Plasma concentration levels were slightly increased after perfusion with IGF-I in the presence of casein in both the colon (figure 5) and the jejunum (figure 6), as compared to the controls. However, statistical analysis by ANOVA showed that these differences were not statistically significant.

In conclusion, a significant decrease of IGF-I concentration in the eluate compared to the inlet concentration $C_0$ occurred, which was reduced in the presence of casein (cf. figure 7). As suggested by the occurrence of additional HPLC peaks after perfusion, the loss of IGF-I appears to be caused by metabolism (data not shown). Effective permeabilities upon perfusion were not calculated because of the unknown fraction of initial IGF-I metabolised per centimetre of perfused intestine (cf. Chapter I).
Discussion

Enzymatic cleavage and low permeability in the intestine are the main factors that restrict the absorption of peptides and proteins in the gut. Additionally, the mucus layer of the intestine may have a limiting effect (Wikmann et al. 1998). Previously, we reported on the metabolism of IGF-I in rat, pig and dog intestinal tissue (Chapter III). The purpose of this study was to assess the intestinal absorption of IGF-I by means of permeation studies in Ussing chambers (rat, porcine and human tissue) and by in situ single-pass perfusion in rats.

Permeation studies

Integrity of excised mucosae: On-line measurements of TEER and I_sc of all excised mucosae during permeation showed that their preparation, e.g., transport or stripping, had no negative effect on mucosal integrity (Sutton et al. 1992). Moreover, tests in porcine mucosa suggested mucosal viability with respect to the preservation of normal intracellular esterase activity. However, extracellular esterase activity may confound the present data. The I_sc values measured for human, rat and porcine mucosa were within the variability range of the values reported by Quadros et al (1994) for rat colon and by Grotmol et al. (1996) for human and porcine mucosa. The range of the TEERs reported in the literature for human colonic tissue (Rubas et al. 1994), porcine colonic tissue (Engelhardt et al. 1995), rat colon tissue (Quadros et al. 1994) and rat ileal tissue (Artursson 1991) was also similar to our findings.

In addition, the permeability coefficients (P_eff) of labelled paracellular markers such as mannitol and PEG 4000 were determined as controls for the integrity of the mucosa. D-glucose was used in order to assess the active transport system. The permeabilities of the paracellular markers in porcine mucosa were generally higher than those in other mucosae such as rabbit, monkey or dog mucosa (Jezyk et al. 1992 and Grass et al. 1993). However, taking interspecies variations into account the measured permeabilities of the paracellular markers were in an acceptable range (He et al. 1998).
Surprisingly, no direction-specific, carrier-mediated D-glucose transport was observed in porcine jejunum. On the one hand, this might be ascribed to the high statistical variability of the data $m \rightarrow s$ versus $s \rightarrow m$ (cf. table 1). On the other hand, we suggest to view the data in light of high PEG 4000 and mannitol fluxes observed in this tissue. Therefore, the tissue appears to be leakier than the other tissues mentioned (He et al. 1998). As a consequence, paracellular transport of D-glucose is predominant and the additional contribution of carrier-mediated transport difficult to factor out.

**Stability of IGF-I in Ussing chambers**

A marked drop in IGF-I concentrations occurred when IGF-I was incubated in Ussing chambers in the presence of Krebs’ phosphate buffer. Addition of FCS (0.5 %) did not affect the loss in IGF-I. In contrast, casein (0.1 %) was able to protect IGF-I in the presence of FCS. Decrease of IGF-I may have occurred due to physical adsorption to the Ussing chambers and/or physico-chemical degradation in Krebs’ phosphate buffer. Similar to our studies with casein, Quadros et al. (1994) showed that addition of bovine serum albumin stabilised completely IGF-I both in the donor and the receiver compartment over a period of five hours. According to Rubas (W. Rubas, personal communication, 1998) IGF-I stability is compromised by Krebs’ phosphate buffer, but can be maintained by BSA.

In conclusion, both physical adsorption and/or physico-chemical degradation of IGF-I in the Ussing chambers need to be considered when evaluating the results of the permeation experiments in the presence of Krebs’ phosphate buffer or in Krebs’ phosphate buffer containing FCS. On the other hand, in the presence of casein as a stabiliser, adsorption and/or degradation can be excluded as a factor influencing the assessment of permeability coefficients.

**Permeation rates relative to intestinal markers:** The permeability of IGF-I in rat, porcine and human mucosae was much lower when compared to other reference compounds such as metoprolol (Amidon et al. 1995), atenolol (Rubas et al. 1996, Amidon et al. 1995), MKA (Langguth et al. 1994) and mannitol. In all species IGF-I permeability was always lower than that of PEG 4000, a non absorbable marker (Lennernäs et al. 1997).
The permeabilities of IGF-I found in human and porcine tissue were similar, yet higher, to that reported by Rubas et al. (1996) in excised human mucosa which was $0.7 \times 10^6$ cm s$^{-1}$. In contrast, Quadros et al. reported much lower permeabilities in the colon of guinea pig and rat, i.e. 0.04 and 0.08 $\times 10^6$ cm sec$^{-1}$, respectively. At this time, no mechanistic explanations for these differences can be given, except that Quadros et al. (1994; cf. below) used much higher IGF-I concentrations which may have changed cell physiology.

Influence of casein and GenMix: Previously, we demonstrated that the stability of IGF-I in the presence of pancreatic enzymes was significantly increased by adding GenMix or casein (Chapter III). GenMix was concluded to act by enzyme inhibition, whereas protection by casein resulted from a yet unknown interaction at pH 7. Consequently, both effects were further investigated with respect to IGF-I absorption. GenMix strongly enhanced the permeability of IGF-I, but compromised the integrity of the mucosa as indicated by decreased TEER and increased PEG 4000 permeability. Variable toxicity was indicated by particularly large variability of IGF-1 permeability. Thus, toxicity related alterations of the paracellular pathway are likely to be explained as part of the observed increase in IGF-I permeability in the presence of GenMix.

In case of casein, permeability was also increased but without measurable negative effects on mucosal integrity. Hypotheses as to the permeability increase are as follows. Firstly, an interaction between the two proteins, e.g., by charge interaction, may stabilise IGF-I in both the receiver and the donor compartments, yielding higher effective permeabilities. Secondly, a preferential uptake of a potential IGF-I/casein complex versus IGF-I alone may be hypothesised. However, at the moment the mechanisms involved are still unknown and require further investigations.

Transport mechanisms: On the one hand, the rat is an established model to predict drug absorption in humans (Fagerholm et al. 1996). On the other hand, the pig model is considered the most suitable animal model for oral drug delivery since it closely resembles the human situation with respect to anatomy and physiology (Kararli 1995, Gardner et al. 1996,
Augustijins et al. 1998). Thus, we compared permeabilities of IGF-I and PEG 4000 in rat, human and porcine mucosa.

We observed a close similarity of porcine mucosa and human mucosa mainly with respect to TEER, PEG 4000 permeability and IGF-I permeability. In contrast, the permeability features of rat tissue were largely different from both human and porcine tissue. Therefore, porcine tissue was selected as the main model to study mechanistic aspects of IGF-I permeation. On the one hand, the permeabilities of the paracellularly transported markers in porcine mucosae were quite high. Thus, in some cases the porcine model may be exceptional. In contrast to our findings, He et al. (1998) and Chiou and Barve (1998) showed a close relationship between the intestinal absorption in human and in rat. However, both studies focused on bioavailability rather than permeability as in our study, in particular on oral fraction absorbed in human versus rat. By comparison of porcine, rabbit and rat mucosa we found that permeabilities in rat and likewise in rabbit are generally lower than in pig (cf. figure 8). In conclusion, the high permeabilities found in colonic porcine mucosae need to be looked at with caution: For instance, the linearity extends up to metoprolol, a highly lipophilic compound, which is rather unusual (Lennernäs et al. 1997). At the moment, we cannot fully explain these exceptional findings.

As an hypothesis active transcellular transport by endocytosis, as suggested by the saturable flux of IGF-I (cf. figure 3), may be assumed. However, control studies with cytochalasin D and K+-free buffer, well known inhibitors of endocytosis (Cremaschi et al. 1996, Larkin et al. 1983), were inconclusive, since in both cases the integrity of the mucosa was compromised as measured by a decrease in TEER and an increase of mannitol and PEG 4000 flux. In accordance with these findings, Madara et al. (1986) showed a negative effect of cytochalasin D on the integrity of guinea pig ileal mucosa.
Zapf et al. (1994) demonstrated that after cellular internalisation of IGF-I, 80% of intracellular IGF-I remained intact for 120 min. This shows that, once internalised by endocytosis, IGF-I seems to be quite stable. Consequently, interaction with casein is concluded to be more crucial to protect its stability in the intestinal lumen rather than in the proteolytic, low pH environment of the endosomal compartment (Rodman et al. 1990).

In the literature other transport mechanisms were also described which may play a role in the absorption of IGF-I. Young et al. (1990) suggested that IGF-I dispersed in milk may be taken up by non specific fluid-phase endocytosis which is typical for the absorption of intact milk. Rubas and co-workers observed in excised rabbit small intestinal mucosa a maximal m → s flux for IGF-I at donor concentrations of 200 - 250 μg mL⁻¹. At lower and higher IGF-I concentrations the flux was reduced (W. Rubas, personal communication, 1998). In parallel, a marked increase in cell size was observed, suggesting that IGF-I flux might be influenced by surface enlargement of the cells as a biological effect of IGF-I (Rubas et al. 1998). In rat fibroblasts, Zapf et al. (1994) described receptor-mediated endocytosis as the uptake mechanism of IGF-I, which was also found for astrocytes (Auletta et al. 1992). However, the large difference between the
high effective $K_m$ value resulting from saturable IGF-I permeation (~1.1 µM, cf. figure 3) versus the much lower dissociation constants of the IGF-I/IGF-I receptor interaction reported for various tissues (3.1 nM to 6.9 nM; Park et al. 1990, Oguchi et al. 1995, Laburthe et al. 1988) does not support the hypothesis of receptor-mediated endocytosis as the main transport mechanism in our study. However, a direct mechanistic comparison of an effective $K_m$ derived from (non-equilibrium) permeation kinetics versus equilibrium derived dissociation constants is highly controversial. Both distributive and diffusional contributions to the observed effective $K_m$ cannot be ruled out and make mechanistic conclusions speculative.

A physiological aspect also needs to be considered in this discussion. Laburthe et al. (1988) observed a particularly high IGF-I receptor density in the colon. Despite this we could not find a remarkably increased IGF-I permeability in porcine colon versus jejunum, which would again argue against receptor-mediated endocytosis as a major mechanism. Based on our study, however, a fractional contribution of receptor-mediated endocytosis as a potential transport pathway cannot be completely excluded.

The involvement of the paracellular pathway as a potential transport mechanism for IGF-I also needs to be considered. Several observations are in favour of this pathway. Firstly, there was no significant difference between the m → s and s → m permeabilities, indicating a direction-independent passive pathway. Secondly, IGF-I permeability was significantly increased in the presence of cytochalasin D. According to Madara et al. (1986) cytochalasin D affects the peri-junctional contractile ring in intestinal absorptive cells and thus enhances paracellular permeability. Thirdly, Bastian et al. (1997) demonstrated the paracellular transport of IGF-I in endothelial cell monolayers. By combination, these observations support a paracellular contribution to the overall transport of IGF-I.

Influence of mucus on IGF-I permeability: In order to estimate the influence of the epithelial mucus layer as a potential absorption barrier, permeation studies were also performed in the presence of N-acetyl-L-cysteine (AC; 0.08 M, 30 min). AC is known for its reduction of mucus glycoprotein to smaller subunits and breakdown of the gel structure.
(Livingstone et al. 1990). Under the study conditions no significant effect on the permeability of IGF-I was observed. Likewise, mannitol and PEG 4000 permeabilities were not affected.

Previously, several studies demonstrated a significant influence of the intestinal mucus (Allen et al. 1983, Sakata and von Engelhardt 1981) on the diffusion of various compounds, especially for larger hydrophilic drugs, such as proteins and peptides (Saitoh et al. 1986, Desai et al. 1992/1991, Wikman et al. 1998), while diffusion of smaller substrates such as mannitol was unaffected (Wikman et al. 1998). These reports support our findings regarding the transport of mannitol, but contrast to unaffected IGF-I and PEG 4000 permeabilities in the presence of AC. After nasal application in rats, O'Hagan et al. (1990) found only a modest increase in growth hormone bioavailability when applying a 20 % solution of AC. Livingstone et al. (1990), using 0.06 M AC for 2 hours, found a marked effect of AC on the macroviscosity of collected mucus and the concentration of hexose present in the mucus, a general marker for glycoprotein. The contrast of this data to our findings could result from the much shorter incubation time used in our study (30 min versus 2 hours). On the other hand, in light of the frailty of excised intestinal tissue an additional two hour pre-treatment with AC is likely to compromise its viability. So far, to our knowledge, there is no permeation study available that gives unequivocal support of the relevance of mucus microviscosity for the intestinal absorption of proteins.

**Single-pass perfusion studies**

In combination with the *in vitro* permeation studies, *in situ* single-pass perfusion studies in rats were also performed to assess IGF-I absorption in the intestinal tract. A noticeable decrease in IGF-I concentration in the eluates compared to the initial concentration was observed. Analyses of the collected eluates by HPLC showed that high amounts of metabolites were present. Addition of casein significantly reduced the degradation. In the absence of casein no statistically significant increase in IGF-I plasma concentrations was found after 90 min of perfusion, which was in accordance with the permeation studies in the excised rat mucosae where we could show that IGF-I permeability was
lower than the permeability of PEG 4000. Moreover, in the presence of casein no significant increase in IGF-I plasma concentrations was observed, which is in accordance with the permeability studies. In the excised porcine mucosa model addition of casein increased IGF-I permeability significantly, yet permeability was still lower than PEG 4000 permeability.

Contrasting to our findings, Kimura et al. (1997) found evidence for an increase in $^{125}$I-IGF-I plasma concentrations after gastric intubation of rats, and after bolus administration into both jejunal and colonic loops. Addition of casein and aprotinin improved significantly the bioavailability of $^{125}$I-IGF-I. Likewise, Nakagawa et al. (1997) observed a substantial uptake of $^{125}$I-IGF-I from the gastrointestinal tract in the mouse after gastric gavage. In contrast to our short term studies, Kimura et al. (1997) followed $^{125}$I-IGF-I plasma concentrations over a period of $>6$ hours. Also, both other groups tested the effect of a bolus administration either into the stomach or various intestinal loops, versus a constant delivery of IGF-I by single-pass perfusion in our study. Contrasting to the specific IRMA used in our study, the analysis of $^{125}$I-IGF-I blood levels was by TCA-precipitable radioactivity only, i.e. by a relatively non-specific technique, and without considering the potential of metabolic cleavage in the intestine or in the liver. Clearly, the results of Kimura et al. (1997) and Nagakawa et al. (1997) should be interpreted under these aspects.

After peroral administration over a period of 7 to 14 days only a minor increase in concentrations was observed, i.e. from 500 to 587 ng mL$^{-1}$ after a high caloric diet, or 256 to 320 ng mL$^{-1}$ after a low caloric diet, respectively, when 2 mg/kg body weight were dosed once a day (Fholenhag et al. 1997). Similarly, Donovan et al. (1997) reported that IGF-I orally administered is poorly absorbed by newborn piglets.

Metabolism in the blood: Metabolism of absorbed IGF-I in the blood might play an important role. Lewitt et al. (1993) showed that an intravenous bolus of IGF-I disappeared from the circulation with an initial $t_{1/2}$ of 1.2 min which increased to 5.3 min when hIGF binding protein-I (IGFBP-I) was co-administered. The second phase of disappearance of IGF-I indicated an apparent $t_{1/2}$ of 35 min which was not significantly altered by the co-infusion of IGFBP-I. The authors proposed that the
insulin-like activity of unbound IGF in the circulation may be regulated by fluctuating endogenous IGFBP-I levels. Likewise, other IGF binding proteins may also have a beneficial effect on the IGF-I stability in the blood (Ballard et al. 1993, Lewitt et al. 1993). Accordingly, Stewart Rotwein (1996) described extensively the importance of the IGF binding proteins for maintaining a IGF-I reservoir in the circulation.

At the moment, we cannot finally explain the significant difference between the findings reported by Kimura et al. (1997) and ours. So the following explanation is hypothetical: On the one hand, IGF-I has a high plasma clearance (Lewitt et al. 1993, McMurtry et al. 1996), on the other hand, endogenous IGF-I seems to be bound to various tissues, e.g., cerebral cortex, kidney, testis, mammary gland and adipose tissue (Maheshwari et al. 1997, Price et al. 1997, Zhou and Bondy 1993, Lavandero et al. 1991, Lewitt et al. 1993). We suppose that once taken up from the intestine into the blood circulation IGF-I is rapidly cleared due to metabolism in the blood and/or transport to peripheral tissues. Thus, the extremely increased IGF-I plasma concentrations upon peroral delivery as shown by Kimura et al. (1997) may be rather exceptional and more likely to be explained by the choice of their analytical protocol. Also, our findings, namely for excised rat mucosa, where the permeability of IGF-I was even lower than of the non-absorbable marker PEG 4000, also emphasise that high plasma concentrations after intestinal perfusion are unlikely.

In conclusion, based on in vitro studies with excised mucosae in combination with literature data, we are unable to assess the exact contribution of an active transport mechanism to the flux of IGF-I. A paracellular pathway may also be involved, as stated by other authors. Furthermore, we could demonstrate that co-administration of casein led to a significant enhancement of IGF-I permeation, potentially by interaction, without affecting tissue viability. However, IGF-I permeability in the presence of casein was still lower than the permeability of PEG 4000, a non absorbable marker. A mixture of chymotrypsin- and trypsin-inhibitors also enhanced permeation through excised mucosae, but significantly compromised viability. In contrast, in situ single-pass perfusion studies in rats did not show significant IGF-I plasma levels above the endogenous
concentration, possibly because of tissue binding. In conclusion, although intestinal IGF-I absorption is undetectable in the systemic circulation its uptake by the intestinal mucosa is significant and may be therapeutically relevant. Protection of IGF-I while exposed to the proteolytic environment of the intestinal lumen and the use of biocompatible absorption enhancers have potential in order to achieve relevant local IGF-I uptake upon peroral delivery.

**Literature**


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Permeability measurements in human excised mucosa:
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Development of an ocular delivery system applicable for pharmacokinetics studies:
Prof. Dr. Robert and Dr. Caroline Albach, Institute of Ophthalmology, University of Zurich, Zurich

Development of a muco-adhesive gel:
Prof. Dr. Orhan Vaizoglu, University of Ankara, Turkey

Publications


P. Anderle, E. Niederer, H. Spahn-Langguth, H. Wunderli-Allenspach, H.P. Merkle, P. Langguth: The Influence of Culturing Conditions and


Experience is not what happens to a man. It is what a man does with what happens to him.

Aldous Huxley