Modulation of Intestinal Tight Junctions by Zonula Occludens Toxin Permits Enteral Administration of Insulin and Other Macromolecules in an Animal Model

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Abstract

The intestinal epithelium represents the major barrier to absorption of orally administered drugs and peptides into the systemic circulation. Entry of molecules through the paracellular pathway is restricted by tight junctions. We have previously reported that these structures can be modulated by Zonula occludens toxin (Zot). In the present report, we show that Zot reversibly increases rabbit intestinal permeability to insulin by 72% (P = 0.034) and immunoglobulins by 52% (P = 0.04) in vitro. When tested in vivo, Zot induced a 10-fold increase of insulin absorption in both the rabbit jejunum and ileum, whereas no substantial changes were detected in the colon. Similar results were obtained with immunoglobulins, whereby Zot induced twofold and sixfold increases of IgG absorption in the jejunum and ileum, respectively. In diabetic rats, bioavailability of oral insulin coadministered with Zot was sufficient to lower serum glucose concentrations to levels comparable to those obtained after parenteral injection of the hormone. The survival time of diabetic animals chronically treated with oral insulin + Zot was comparable to that observed in parenterally treated rats. These studies offer an innovative strategy for the oral delivery of drugs and proteins normally not absorbed through the intestine. (J. Clin. Invest. 1997, 99:1158–1164.) Key words: permeability • oral delivery • drugs • diabetes • immunodeficiency

Introduction

Intestinal absorption of numerous compounds routinely used for the treatment of common diseases is profoundly limited by their physicochemical characteristics. With the exception of those molecules which are transported by active or facilitated transcellular mechanisms, absorption of large and more hydrophilic macromolecules is almost exclusively limited to the paracellular pathway (1). Under normal conditions, however, this pathway is restricted to molecules with molecular radii < 11 Å (2). The utility of the paracellular route for oral drug delivery has remained unexplored due to a limited understanding of tight junction (tj) physiology and the lack of substances capable of increasing the tj permeability without irreversibly compromising intestinal integrity and function (3–6).

We have recently demonstrated that Zonula occludens toxin (Zot), a protein elaborated by Vibrio cholerae (7, 8), induces modifications of cytoskeletal organization that lead to the opening of tj related to the PKCo-dependent polymerization of actin monomers into actin filaments strategically localized to regulate the paracellular pathway (9). We have also shown that this modulation is reversible, time- and dose-dependent, and is confined to the small intestine, since Zot does not affect the colon permeability (7, 10). The selective effect of the toxin on the small intestine seems related to the regional distribution of the Zot receptors that are present in the jejunum and ileum, but not in the colon (10). The aforementioned properties make this moiety a potential tool for modulating the tj permeability. In the present report, we have demonstrated that Zot may be used to enhance the intestinal absorption of orally administered macromolecules through the paracellular pathway.

Methods

Zot purification. Purified Zot was obtained as previously described (10). Briefly, zot gene was fused in frame with the maltose binding protein (MBP) malE gene, using vector pMal-c2 (10) to create a MBP-Zot fusion protein. The fusion product was expressed in Escherichia coli, and obtained by disrupting the cells using a french press. The MBP-Zot fusion protein was then purified by affinity chromatography using an amylose column (MBP-fusion purification system, New England Biolabs, Beverly, MA). Purified Zot was finally obtained by cleaving the fusion protein with factor Xa (10). The amount of toxin obtained was assessed by the Bradford method (11).

In vitro Ussing chambers experiments. Experiments were carried out as previously described (12) following an experimental protocol approved by our institutional animal welfare committee. Briefly, adult male New Zealand white rabbits (2–3 kg) were killed by cervical dislocation. Segments of rabbit intestine (including jejunum, ileum, and colon) were removed, rinsed free of the intestinal content, opened along the mesenteric border, and stripped of muscular and serosal layers. Eight sheets of mucosa so prepared were then mounted in Lucite Ussing chambers (1.12 cm² opening), connected to a voltage clamp apparatus (EVC 4000 WPI, Sarasota, FL), and bathed with freshly prepared buffer containing (mM): NaCl, 53; KCl, 5; NaH2PO4, 30.5; mannitol, 30.5; Na2HPO4, 1.69; NaH2PO4, 0.3; CaCl2, 1.25; MgCl2, 0.04) in vitro. When tested in vivo, Zot induced a 10-fold increase of insulin absorption in both the rabbit jejunum and ileum, whereas no substantial changes were detected in the colon. Similar results were obtained with immunoglobulins, whereby Zot induced twofold and sixfold increases of IgG absorption in the jejunum and ileum, respectively. In diabetic rats, bioavailability of oral insulin coadministered with Zot was sufficient to lower serum glucose concentrations to levels comparable to those obtained after parenteral injection of the hormone. The survival time of diabetic animals chronically treated with oral insulin + Zot was comparable to that observed in parenterally treated rats. These studies offer an innovative strategy for the oral delivery of drugs and proteins normally not absorbed through the intestine. (J. Clin. Invest. 1997, 99:1158–1164.) Key words: permeability • oral delivery • drugs • diabetes • immunodeficiency

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1. Abbreviations used in this paper: MBP, maltose binding protein; Rt, tissue resistance; tj, tight junctions; Zot, Zonula occludens toxin.
1.1; NaHCO₃, 25. The bathing solution was maintained at 37°C with water-jacketed reservoirs connected to a constant-temperature circulating pump and gassed with 95% O₂/5% CO₂. Potential difference (PD) was measured, and short-circuit current (Isc) and tissue resistance (Rt) were calculated as previously described (7). Once the tissue samples were prepared, the short-circuit current (Isc) and tissue resistance (Rt) were simultaneously measured at a rate of 0.4 ml/min with the perfusion solution (1.5 g/100 cm² Ringer’s solution containing either 143 pmol/ml of oral Humulin® U 10 IU + Zot 1.1 × 10⁻¹⁰ mol, and (c) oral Humulin® U 10 IU. The body weight, temperature, and blood glucose level (before treatment and 60 min thereafter) were daily monitored. Once the rats died, segments of the small and large intestine were isolated, fixed in paraformaldehyde 4%, and processed for light microscopy examination.

**Results**

**In vitro animal model**

Insulin and immunoglobulin G (IgG) were selected as prototype molecules to be tested for oral delivery, based on the relative size and structure, biological activities, and therapeutic relevance of these proteins. Rabbit ileum was mounted in Ussing chambers, and in vitro transepithelial transport of 125I-insulin and 125I-IgG from the mucosal (i.e., intestinal lumen) to the serosal (i.e., bloodstream) boundaries in the presence and absence of purified Zot was measured. Zot (1.1 × 10⁻¹⁰ M) reversibly increased the intestinal absorption of both insulin (3.84±0.41 fmol/cm² per min vs. 6.60±1.4 fmol/cm² per min, untreated vs. Zot-treated tissues, respectively, \( P = 0.034, n = 4 \)) and IgG (7.97±1.17 pg/cm² per min vs. 12.1±1.8 pg/cm² per min, \( P = 0.04, n = 4 \)) in a time-dependent manner. Zot permeabilizing effect peaked at 80 min, and was completely reversible within 20 min from the withdrawal of the toxin from the Ussing chambers (see Fig. 1). This Zot-induced increase in absorption coincided with a reduction in tissue resistance (Fig. 1, A and B). No significant changes of Rt were observed in control tissues after 80 min incubation, however, the tissues exposed to insulin alone showed an increase of Rt above the baseline when the solutions were changed (see Fig. 1 A).

**In vivo model**

To study the effect of Zot in the intact host, we used the in vivo perfusion assay (13). We have recently demonstrated that the effect of Zot on tissue permeability is limited to the small bowel, and this distinct permeabilizing effect of the toxin coin-
cides with the regional distribution of Zot receptor(s) within the intestine (10). Therefore, three distinct segments of rabbit intestine—the jejunum, the distal ileum, and the colon—were simultaneously perfused in the same animal. When added to the perfusion solution, Zot (1.1 × 10⁻¹⁰ M) increased the passage of ¹²⁵I-insulin across both the jejunum and distal ileum 10-fold, whereas no substantial changes were observed in the colon (Fig. 2). The increased absorption of insulin was reciprocal with a shift of water absorption toward secretion (Fig. 2), a change that has been related to the permeabilizing effect of Zot on the paracellular pathway in vivo (10). This effect was detectable as soon as 20 min after Zot perfusion in the small intestine, and was completely reversible within 60 min of its withdrawal (Fig. 2). Zot also reversibly increased the concentration of both ¹²⁵I-IgG and ¹⁴C-PEG-4000 in the efferent mesenteric vein of the perfused small intestinal segments (data not shown).

Oral delivery of insulin in BB/Wor diabetic rats

Acute treatment. We subsequently evaluated the bioactivity of insulin after enteral co-administration with Zot to acute type 1 diabetic male BB/Wor rats (15, 16). Insulin was orally administered to animals with or without Zot, and the blood glucose levels of the rats were serially measured. After oral administration of insulin alone (given at doses between 5 and 30 IU), blood glucose levels of treated animals were not appreciably lowered (Table I). In contrast, when insulin at doses as low as 10 IU was orally coadministered with Zot 1.1 × 10⁻¹⁰ mol (5 μg), a significant reduction in blood glucose concentration was observed (Table I and Fig. 5). This decrement was comparable to that seen with a conventional dose of SQ insulin (Ta-

Figure 1. Reversible effect of purified Zot on tissue resistance (A and B) and transepithelial transport of insulin (C) and IgG (D) in rabbit ileum in vitro. Paired tissues, matched on the basis of their resistance, were exposed luminally to either 10⁻¹¹ M ¹²⁵I-insulin (Amersham) (left) or 156.25 ng ¹²⁵I-IgG (right), alone (○) or in the presence of 1.1 × 10⁻¹⁰ M Zot (□). After 80 min of incubation, the Ringer's solutions were replaced with solutions of identical composition but without Zot. Zot reversibly increased the transepithelial absorption of both insulin and IgG. These effects paralleled the Rt decrement induced by the toxin. n = 4.
ble I), and returned to baseline by 6 h post-administration (Fig. 5). There was a significant difference in mean glucose level between the oral insulin treatment and the oral insulin + Zot treatment ($P = 0.002$), as well as a significant time x group interaction ($P = 0.03$), reflecting the drop in glucose level in the presence of Zot. By t tests, the difference in glucose level reached statistical significance between 60 and 120 min post administration (Fig. 5). Administration of Zot alone did not al-
ter the blood glucose levels (Table I and Fig. 5). Mean ($\pm$SE) time to reach the blood glucose nadir after oral treatment with insulin + Zot (97$\pm$12 min) was not different from that observed in the rats receiving parenteral insulin (90$\pm$19 min). Furthermore, increased amounts of either Zot (up to $4.4 \times 10^{-10}$ mol) or insulin (up to 30 IU) each induced a dose-depen-
dent decrement of blood glucose (Table I). No toxicity was ob-
served in the treated animals.

**Prolonged treatment.** To establish whether repeated Zot administration had any adverse effect on the survival of instru-
mented animals, survival times were compared in diabetic rats daily treated with oral insulin 10 IU, with or without $1.1 \times 10^{-10}$ mol Zot. After the administration of insulin + Zot, post-
operative survival time (average 84 h) and decrease in blood glucose levels 60 min after treatment (average 31%) were com-
parable to those observed in parenterally treated animals (96 h survival and 44% blood glucose decrement, respectively). In con-
trast, rats treated with oral insulin alone (10 IU) survived for only 60 h after jugular vein cannulation, and their blood glucose levels increased by 19% 60 min after treatment. None of the animals treated with insulin + Zot experienced diar-
rhea, fever, or other systemic symptoms, and no structural changes could be demonstrated in the small intestine on histo-
logical examination (data not shown).

**Discussion**

The intestinal epithelium represents the largest interface (more then 2,000,000 cm$^2$) between the external environment and the internal host milieu, and constitutes the major barrier through which molecules either can be absorbed or secreted. The para-
cellular route is the dominant pathway for passive transepithel-
ial solute flow in the small intestine, and its permeability de-

pends on the regulation of intercellular tj (17). A century ago, these structures were thought to be a secreted extracellular cement forming an absolute and unregulated barrier within the paracellular space (18). Physiological studies of the past several decades have shown that the tj is a dynamic structure whose physiological regulation remains largely undefined (17).

In the past few years, we have witnessed an explosion in research aimed at creating new drug delivery systems (19). Unfortunately, attempts so far to find ways to increase paracellular transport by loosening intestinal tj have been hampered by unacceptable side effects induced by the potential absorption enhancing agents. (3–6). For the most part, these agents fall within two classes: (a) calcium chelators, and (b) surfactants (5). Both types have properties which limit their general utility as a means to promote absorption of various molecules. In the case of calcium chelators, Ca$^{2+}$ depletion induces global changes in the cells, including disruption of actin filaments, disruption of adherent junctions, and diminished cell adhesion (6). In the case of surfactants, the potential lytic nature of these agents may cause exfoliation of the intestinal epithelium, irreversibly compromising its barrier functions (5).

Considering these limitations, it was worth examining whether our previous findings on Zot modulation of tj permeability could be applied to develop alternative approaches to enhancing drug absorption through the paracellular route. We have recently demonstrated that Zot activates a complex intracellular cascade of events that regulate intestinal permeability (9). Zot induces a dose- and time-dependent PKC-$\alpha$-related polymerization of actin filaments strategically localized to regulate the paracellular pathway (9). These changes are a prerequisite to opening of tj, and are evident at a toxin concentration as low as 1.1 $\times 10^{-13}$ M (10). The toxin exerts its effect by interacting with a specific surface receptor that is present in the small intestine, but not in the colon (10). Our previous data suggest that the regional distribution of Zot receptors coincides with the different permeabilizing effect of the toxin on the various tracts of intestine tested (10). We have also demonstrated, both in vivo (10 and present paper) and in vitro (7, 10,
Oral insulin, when orally coadministered with Zot, decreases serum glucose concentration of diabetic rats to levels comparable to those obtained with the parenteral administration of the hormone.\(^*\) Oral insulin, when orally coadministered with Zot, decreases serum glucose concentration of diabetic rats to levels comparable to those obtained with the parenteral administration of the hormone.\(^*\)

**Table I. Effect of Oral Insulin, Alone or Coadministered with Purified Zot on the Blood Glucose Levels of BB/Wor Diabetic Rats**

<table>
<thead>
<tr>
<th>Treatment</th>
<th>n</th>
<th>Animal weight</th>
<th>Baseline blood glucose</th>
<th>Blood glucose decreament</th>
<th>Time to reach nadir blood glucose</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>g</td>
<td>mg/dl</td>
<td>mg/dl</td>
<td>min</td>
</tr>
<tr>
<td>No treatment</td>
<td>3</td>
<td>217.0±34</td>
<td>235.1±41.7</td>
<td>29.1±20.1</td>
<td>180±60</td>
</tr>
<tr>
<td>Parenteral insulin (SO) (1.2–2.4 IU)</td>
<td>5</td>
<td>237.0±17</td>
<td>275.6±26.7</td>
<td>182.4±29.3(^*)</td>
<td>90±19</td>
</tr>
<tr>
<td>Oral Zot (1.1 ( \times 10^{-10} ) mol)</td>
<td>4</td>
<td>248.8±31</td>
<td>248.0±43.9</td>
<td>26.0±12.7</td>
<td>75±9</td>
</tr>
<tr>
<td>Oral insulin (5 IU)</td>
<td>2</td>
<td>247.5±13</td>
<td>229.0±48.0</td>
<td>15.0±7.5</td>
<td>78±17</td>
</tr>
<tr>
<td>Oral insulin (5 IU) + Zot 1.1 ( \times 10^{-10} ) mol</td>
<td>2</td>
<td>239.5±15</td>
<td>273.8±12.5</td>
<td>85.5±33.5</td>
<td>75±15</td>
</tr>
<tr>
<td>Oral insulin (10 IU)</td>
<td>3</td>
<td>238.0±20</td>
<td>249.0±9.5</td>
<td>26.0±4.0</td>
<td>110±50</td>
</tr>
<tr>
<td>Oral insulin (10 IU) + Zot 1.1 ( \times 10^{-10} ) mol</td>
<td>3</td>
<td>223.0±22</td>
<td>262.0±67.1</td>
<td>161.0±24.6(^i)</td>
<td>93±28</td>
</tr>
<tr>
<td>Oral insulin (20 IU)</td>
<td>3</td>
<td>267.5±35</td>
<td>318.5±15.5</td>
<td>66.5±38.5</td>
<td>140±70</td>
</tr>
<tr>
<td>Oral insulin (20 IU) + Zot 1.1 ( \times 10^{-10} ) mol</td>
<td>3</td>
<td>267.5±36</td>
<td>364.5±64.5</td>
<td>168.0±41.0</td>
<td>75±45</td>
</tr>
<tr>
<td>Oral insulin (30 IU)</td>
<td>6</td>
<td>211.2±9</td>
<td>244.7±66.7</td>
<td>70.2±33.5</td>
<td>116±33</td>
</tr>
<tr>
<td>Oral insulin (30 IU) + Zot 2.2 ( \times 10^{-10} ) mol</td>
<td>4</td>
<td>247.0±12</td>
<td>308.0±98.0</td>
<td>193.5±40.5(^i)</td>
<td>106±75</td>
</tr>
<tr>
<td>Oral insulin (30 IU) + Zot 4.4 ( \times 10^{-10} ) mol</td>
<td>5</td>
<td>208.0±4</td>
<td>362.4±53.4</td>
<td>214.3±7.5(^f)</td>
<td>139±30</td>
</tr>
</tbody>
</table>

Oral insulin, when orally coadministered with Zot, decreases serum glucose concentration of diabetic rats to levels comparable to those obtained with the parenteral administration of the hormone.\(^*\) Oral insulin, when orally coadministered with Zot, decreases serum glucose concentration of diabetic rats to levels comparable to those obtained with the parenteral administration of the hormone.\(^*\)

and present paper) that the effect of Zot on tissue permeability occurs within 20 min of the addition of the protein to the intestinal mucosa, and is readily reversible once the toxin is removed. The in vivo experiments in BB/wor diabetic rats presented herein also showed a transient permeabilization effect of the toxin; the blood glucose levels of the animals orally treated with insulin + Zot decreased within 30 min of co-administration of the compounds, reached its nadir after 90 min, and returned to baseline values after 6 h (see Fig. 5).

Zot displays multiple properties that make it the most promising tool currently available to enhance drug and peptide transport through the intestinal mucosa. Zot (a) is not cytotoxic and does not affect the viability of the intestinal epithelium ex vivo (7, 9); (b) fails to completely abolish the intestinal transepithelial resistance (7, 9, and present paper); (c) interacts with a specific intestinal receptor whose regional distribution within the intestine varies (10); (d) is not effective in the large intestine where the presence of the colonic microflora could be potentially harmful if the mucosal barrier was compromised (10 and present paper); (e) could be potentially harmful if the mucosal barrier was compromised (10 and present paper); (f) does not induce acute systemic side-effects (for at least 80–90 h) when orally administered (present paper); and (g) induces a reversible increase of tissue permeability (7, 9, and present paper). Our results demonstrate that coadministration of Zot with biologically active ingredients enhances intestinal absorption of the active molecule, and that this enhancement is effective for both relatively small (insulin, 5733 D) and large molecules (IgG, 140–160 kD). Furthermore, the experiments in BB/Wor diabetic rats demonstrate that orally delivered insulin can retain its biological activity without provoking severe hypoglycemia within the range of the insulin administered (i.e., up to 15 times more than the effective parenteral insulin dose). These findings have important practical implications, since the insulin therapeutic index (i.e., the ratio between the median toxic dose and the median therapeutic dose) is relatively low.

Our data suggest that the modulation of intestinal TJ may be used for the oral administration of molecules normally not absorbed through the intestine. Further studies are needed, however, to establish the possible clinical application of this system for the treatment of diseases that currently require frequent and long-life parenteral drug administration.

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