Effects of peptides derived from dietary proteins on mucous secretion in rat jejunum

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A MAJOR FACTOR OF THE FUNCTIONAL barrier of intestine is luminal mucus gel. The mucus coat separates mucosal cells from the exterior milieu and provides protection from noxious substances, allows lubrication of the cell surface, and regulates ion fluxes (10). Mucus, therefore, plays an important role for the intestinal surface integrity in health, and dysfunction of mucus secretion and/or mucin expression could be involved in several pathologies such as inflammatory intestinal diseases and cancer (16, 41). For these reasons, there is a need for better knowledge of the regulation of mucous secretion. In particular, very little is known about the modulation of intestinal mucous secretion by dietary factors, although the interactions of dietary components with the secretory activity of goblet cells could represent new interesting possibilities for the manipulation of this important protective function.

Mucins, the predominant components of the mucous gel, are high-molecular weight glycoproteins with oligosaccharides attached by O-glycosidic bonds to serine or threonine residues on a peptide backbone. In rat small and large intestine, secreted mucins are primarily accounted for by rMuc2 (40) and are mainly synthesized by epithelial goblet cells (or mucus cells) (36). Previous experiments performed either in vivo or ex vivo have demonstrated that dietary fibers and short-chain fatty acids may alter the dynamics of mucous through increased secretion, tissue content, or even altered mucus cell number (2, 18, 27, 28, 35). In contrast, no information on the impact of proteins is available. Dietary proteins and their degradation products are yet involved in the physiological regulation of digestion. In particular, proteins or digested proteins stimulate cholecystokinin release and interact with intestinal endocrine L cells to modulate glucagonlike peptide-1 secretion (4–6, 17). Partial hydrolysates of proteins are also potent stimulants of gastric acid and pancreatic secretion and participate in the regulation of gastrointestinal motility through stimulation of the adrenergic pathway (11, 15, 20). Furthermore, Roberts et al. (26) demonstrated that some dietary peptides may improve wound healing in rats.

The present study was undertaken to determine the effects of dietary proteins or their enzymatic hydrolysates on intestinal goblet cell secretion. For this purpose, we used the preparation of isolated vascularly perfused rat jejunum. This model provides a unique opportunity to study the secretion of mucus in response to well-defined luminal stimuli in a manner that eliminates extraneous influences potentially encountered in vivo. The polarized mucus cells may thus be submitted to specific stimulation with dietary proteins or their products of hydrolysis at a site where nutrient

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derivatives interact physiologically with the intestinal mucosa. The subsequent jejunal mucin discharge was evaluated by ELISA.

**MATERIALS AND METHODS**

**Materials**

BSA was purchased from Biomedia (Boussens, France). Nutrilamine 25, a mixture of amino acids, was obtained from Braun Medical (Boulogne, France). Pancreatic digest of lactalbumin (LH, peptone n°60, ref 20025–037) was from GIBCO-BRL (life Technologies, Cergy Pontoise, France). β-Casomorphin (β-CM)-7 was obtained from Bachem (Bubendorf, Switzerland). TTX was supplied by Calbiochem (Darmstadt, Germany). Naloxone was purchased from ICN pharmaceuticals (Costa Mesa, CA). Microtitr plates (NUNC-Immunoplate) were obtained from Polylabo (Paul Block & Cie, Strasbourg, France). Cesium Chloride and DIG glycan detection kit were supplied by Boehringer (Mannheim, Germany). Electrophoresis products were obtained from Bio-Rad (Hercules, CA). Other reagents, including casein from bovine milk (sodium salt, ref C-8654), casein enzymatic hydrolysate from bovine milk (type II, ref C-4523), chicken egg albumin (crude, grade II, ref A-5253), chicken egg albumin hydrolysate (ref A-3154), and type I enzymatic hydrolysate from meat (peptone, ref P-7750) were purchased from Sigma (Saint Louis, MO).

**Animal Model and Experimental Protocol**

**Surgical preparation.** The operative procedure to prepare an isolated vascularly perfused rat jejunum was previously described (7). Briefly, male Wistar rats (250–350 g) purchased from Le Centre d’Elevage Dépré (Saint Doulchard, France) were anesthetized with pentobarbital sodium (50 mg/kg ip). The right and middle colic veins and arteries were tied and cut off near the serosa of the colon. Both ends of the jejunal loop (12 cm in length, 2 cm beyond the ligament of Treitz) were then equipped with Silastic tubing. The jejunal lumen was flushed out with prewarmed isotonic saline and then with air. After the air was gently emptied, both ends of the intestinal loop were ligated. A metal cannula and a Silastic one were then quickly inserted in the superior mesenteric artery and portal vein, respectively. The arterial and venous Silastic one were then quickly inserted in the superior mesenteric artery and portal vein, respectively. The arterial and venous Silastic tubing was then connected to the arterial and venous Silastic tubing. The jejunal loop was then homogenized (30 min, 18,500 rpm, 4°C) and centrifuged. The jejunal loop homogenates were then analyzed for DNA content. This analysis served as an indirect measure of tissue viability, and loops were discarded if luminal DNA content represented >2% of the total DNA (tissue DNA + luminal DNA).

**Preparation of rat intestinal mucin.** Rat intestinal mucins (RIM) were purified using essentially the same procedure as described for rat colonic mucins (24, 40). Briefly, male Wistar rats were killed by a lethal dose of pentobarbital sodium. Their small intestines were rapidly excised lengthwise and rinsed in cold PBS (4°C). The mucosa was then gently scraped off, and scrapings were homogenized by moderate stirring in the dark for 24 h at 4°C in 50 mM Tris buffer (pH 7.5) containing 6 M guanidinium hydrochloride, protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 mM EDTA, 10 mM benzamidine hydrochloride, 0.1 mg/ml soybean trypsin inhibitor, and 10 μg/ml pepstatin) and 100 mM DTT. Sulfhydride groups were stabilized by carboxymethylation with 250 mM iodoacetamide and stirring in the dark for 24 h. Insoluble material was removed by centrifugation (30 min, 15,500 rpm, 4°C). Mucins were then purified by equilibrium centrifugation on three consecutive CsCl density gradients (Centrifkon T-2055 ultracentrifuge, T70.38 rotor, 40,000 rpm, 70 h, 12°C) as described for rat colonic mucins (24). The high-molecular weight nature of purified mucins was verified by SDS-PAGE and Western blot using an immunological detection system for glycoproteins (DIG Glycan detection kit) (13).
Blots were incubated with anti-RIM (1:1,000 or 1:5,000) and then with goat anti-rabbit IgG conjugated to horseradish peroxidase (1:1,000). Blots were developed using the enhanced chemiluminescence method (chemiluminescence, Pierce, Rockford, IL). The serum with the highest antibody titer (59C) was selected. On dot blot, no reaction was observed with bovine albumin, bovine thyroglobulin, bovine apolipoprotein A-II, bovine apolipoprotein B, human immunoglobulin, bovine glycophorin α, bovine submaxillary mucin, pectin, and gum arabic. The antiserum 59C was also studied for epitope specificity. Purified mucins were digested with 0.1 mg/ml proteinase K in 50 mM Tris buffer, pH 7.4 (1 h, 37°C), to assess the reactivity toward protease-sensitive epitopes. The digested mucins were blotted on nitrocellulose and processed as described previously. Proteinase K digestion of the purified mucins with proteinase K resulted in a very strong decrease in reactivity on dot blot (data not shown), thus suggesting that protease-sensitive domains in purified mucins were required for antibody binding.

Recognition of intestinal mucins by anti-RIM was controlled histologically. Sections (5 μm) of paraffin-embedded jejunal tissue were incubated with diluted normal blocking serum and then with anti-RIM antiserum (1:500 dilution). After sections were washed in PBS, they were sequentially exposed to biotinylated goat anti-rabbit antibody, to avidin/biotinylated peroxidase complex (Vectastain Elite ABC Reagent), and 3,3′-diaminobenzidine solution (all from Vector Laboratories, Burlingame, CA). The sections were then counterstained, cleared, and mounted. Antiserum 59C reacted with intestinal goblet cells as well as with the mucus layer, as shown in Fig. 1. In control experiments, substituting antiserum for a preincubated antigen-antiserum mix gave no reaction toward mucus cells (Fig. 1).

An immunoglobulin-rich fraction of serum 59C was purified on Protein A Sepharose CL-4B column (Pharmacia, Uppsala, Sweden) and used to prepare labeled antibody (biotinylated-59C) for the ELISA using a succinimide ester of biotin (12).

**Immuunoassay.** An ELISA for rat intestinal mucin glycoproteins was then established. Wells of a microtiter plate were coated with 100 μl of purified mucins or with sample diluted in carbonate buffer (pH 9.6) and then incubated overnight at 4°C. On the following day, the microtiter plate was washed four times with PBS containing 0.1% Tween (PBS-Tween, pH 7.2). The remaining binding sites in the wells were blocked by addition of 250 μl of PBS-Tween-bovine albumin (0.2 g albumin in 100 ml PBS-Tween) (PBS-Tween-BA) for 1 h at 37°C, and the plate was washed again. At this stage, the wells were incubated with 100 μl of biotinylated-59C (7.5 μg/ml) diluted in PBS-Tween-BA for 1 h at 37°C. After the wells were washed, 100 μl of avidine-peroxidase conjugate were added and allowed to bind for 1 h at room temperature. The plate was washed five times. One hundred microliters of 0-phenylenediamine dihydrochloride solution were then added, the color was allowed to develop in the dark for 5–10 min, and the reaction was stopped with 25 μl of 3 M sulfuric acid. The absorbance was read at 492 nm on a microELISA plate reader. A typical standard curve was obtained by using increasing concentrations (0–1,000 ng dry wt/ml) of purified mucins. The curve was linear in a 62.5- to 1,000-ng/ml range. Luminal contents were tested at three dilutions (1:1,000, 1:2,000, and 1:4,000), and all the assays were performed in duplicate. The coefficient of variation of the ELISA was 4.5%. Serial dilutions of luminal samples taken from control or from stimulated loops gave results in ELISA that could be superimposed on the standard curve (data not shown). This immunoreactive material was distributed like purified rat intestinal mucins on a CsCl density gradient, with a peak at a density of 1.4 g/ml, which is characteristic of mucins (40, 42).

**Statistical Analysis**

Data are presented as means ± SE and were compared using repeated-measures ANOVA followed by paired t-test when appropriate. *P < 0.05* was considered significant.

**RESULTS**

**Effects of Native and Enzymatically Hydrolyzed Dietary Proteins on Mucin Secretion in Rat Jejunum**

The casein hydrolysate (CH) we used is a highly refined trypsin hydrolysate from Sigma (ref C-4523). In the isolated vascularily perfused rat jejunum, luminal
administration of CH [5% (wt/vol)] induced mucin release (response at 273 ± 54% of control loops, n = 6, P < 0.05). As shown in Fig. 2, the first significant response was observed with 0.1% (wt/vol) CH (277 ± 62% of controls, n = 6), and the maximal response was obtained with 0.5% (wt/vol) CH (417 ± 54% of controls, n = 6).

In contrast with CH, luminal administration of native casein [5% (wt/vol)] did not significantly stimulate mucin secretion in the isolated perfused rat jejunum. Similarly, neither the amino acid mixture [0.5 and 4% (wt/vol)] nor glutamine (10–100 mM) or glutamic acid (100 mM) induced any discharge of mucin.

Enzymatic lactalbumin hydrolysate [LH; 5% (wt/vol)] led to a significant increase in luminal mucin content (335 ± 56% of control loops, n = 5). On stimulation with 0.5% (wt/vol) LH, the rise in luminal mucin was less pronounced and did not reach significance (Fig. 3).

The chicken egg albumin hydrolysate [CEAH; 5% (wt/vol)] only showed a tendency to increase mucin release, and this increase did not achieve statistical significance (140 ± 18% of control loops, n = 6). Administration of 0.5% (wt/vol) CEAH or of native chicken egg albumin [5% (wt/vol)] was also without effect on mucin secretion in rat jejunum.

Luminal administration of an enzymatic meat hydrolysate [5% (wt/vol)] failed to induce any significant increase in the discharge of mucin in rat jejunum (129 ± 17% of control loops, n = 5).

**Mechanisms Involved in CH-Induced Mucin Secretion**

The effect of the neuronal blocker TTX (10^{-6} M) was tested on the mucin secretion induced by intraluminal
administration of CH (0.5% wt/vol). As shown in Fig. 4, intra-arterial pretreatment with TTX abolished CH-induced jejunal mucin secretion (P < 0.05 vs. CH alone). In experiments without CH, TTX (10⁻⁴ M) had no effect on mucin discharge (response at 108 ± 10% of controls, n = 6, P > 0.05).

Because some casein fragments were shown to behave as opioid receptor ligands, the effect of intra-arterial infusion of naloxone (antagonist at μ-, κ-, and δ-receptors) on CH-induced mucin secretion was investigated. Interestingly enough, mucin secretion induced by 0.5% CH was inhibited by pretreatment with 10⁻⁵ M naloxone. Naloxone alone, in contrast, was without effect on the mucin secretion in isolated rat jejunum (response at 116 ± 15% of controls, n = 6; P > 0.05).

Effect of β-CM-7 on Mucin Secretion in the Isolated Perfused Rat Jejunum

Bioactive peptides, including opioid peptides, antihypertensive peptides, antithrombotic peptides, immunostimulants, or mineral carriers, are encrypted in the polypeptide chains of caseins and are produced by in vitro or in vivo enzymatic proteolysis. The opioid peptides derived from β-casein are designated as β-CMs and include β-CM-7 (β-CM-7), the 60–66 fragment of β-casein.

Luminal administration of 1.2 × 10⁻⁴ M β-CM-7 produced a sharp rise in the discharge of immunoreactive material (563 ± 65% of control preparations, n = 6; P < 0.05). This concentration of β-CM-7 is equivalent to what could be obtained from a 0.5% (wt/vol) CH. The effect of β-CM-7 was concentration dependent over the range 1.2 × 10⁻⁵–1.2 × 10⁻⁴ M (Fig. 5A), with the first significant response obtained at 6 × 10⁻⁵ M (response at 300 ± 49% of control loops, n = 6; P < 0.05).

As can be seen from Fig. 5B, pretreatment with 10⁻⁵ M naloxone inhibited the β-CM-7-induced mucin release (response at 146 ± 36% of controls, n = 7 vs. 493 ± 87% of controls for β-CM-7 alone, n = 7; P < 0.05).

Fig. 5. Effect of β-casomorphin-7 (β-CM-7) on jejunal mucin secretion. A: jejunal mucin secretion on luminal administration of increasing amounts of β-CM-7 in the isolated perfused rat jejunum. Mucin release (% controls) is given as means ± SE. *P < 0.05 vs. controls. A control experiment was produced for every stimulated loop. In related control preparations, the mucin secretion measured at the end of the experiments was 220 ± 16 μg/mg DNA.

B: effect of luminal β-CM-7 (1.2 × 10⁻⁴ M) alone or in combination with intra-arterial naloxone (10⁻⁵ M) on mucin glycoprotein secretion in rat jejunum. *P < 0.05 vs. controls. &P < 0.05 vs. β-CM-7 (1.2 × 10⁻⁴ M). In related control preparations, the mucin discharge measured at the end of the experiments was 220 ± 16 μg/mg DNA. C: effect of intra-arterial perfusion of increasing amounts of β-CM-7 in the isolated perfused rat jejunum. Mucin release (% controls) is given as means ± SE. *P < 0.05 vs. controls. In related control preparations, the mucinlike immunoreactivity observed at the end of the experiments was 197 ± 20 μg/mg DNA for controls.
On intra-arterial infusion, $1.2 \times 10^{-6}$ M $\beta$-CM-7 induced jejunal mucin glycoprotein discharge (response at $410 \pm 74\%$ of control preparations, $n = 7; P < 0.05$). On infusion of $\beta$-CM-7 at the $6 \times 10^{-7}$ M concentration, the rise in mucin discharge was also significant (Fig. 5C).

**DISCUSSION**

Studies providing direct evidence for intestinal mucin discharge by nutrients are scarce. Previous investigations showed that dietary fibers and short-chain fatty acids evoke intestinal mucus secretion (2, 35). In the present study, we demonstrate, for the first time, that another class of nutrients might regulate the secretory function of rat intestinal goblet cells. Indeed, an enzymatic CH elicited a strong mucin discharge in the rat jejunal lumen. Beside casein (80%), milk proteins consist of whey proteins (20%) (30), chiefly lactalbumin, $\beta$-lactoglobulin, and immunoglobulins. Our data establish that LH also increased mucin discharge in the lumen of isolated perfused rat jejunum. Interestingly enough, the two other dietary protein hydrolysates we tested (meat and chicken egg albumin hydrolysates) failed to elicit jejunal mucin discharge. Thus all protein hydrolysates are not equally potent in triggering mucin glycoprotein secretion, and mucin discharge in rat jejunum appears to be critically dependent on the source of dietary protein.

CH produced a dose-dependent release of mucin in the 0.05–0.5% range, with the first significant response at 0.1%. Because the casein content of bovine milk is 2.7%, the CH-induced intestinal mucin secretion may be physiologically relevant not only in the neonate but also in the adult. In this context, it is worth noting that milk proteins constitute the only source of protein for the neonate and up to 20% of a human’s average food protein intake in the European community and in the United States (30).

Oligopeptides derived from casein seem specific for mediating the effect of this hydrolysate, because native casein and mixed free amino acids were ineffective to induce mucin release in the isolated rat jejunum. Glutamine is one of the most abundant amino acids in casein and is also the prominent metabolic fuel for small intestinal epithelium. In this study, we show that glutamine did not stimulate mucin discharge. Glutamic acid, the other amino acid tested in this study, was also without effect. These results are meaningful because the enzymatic CH is more likely to mimic dietary protein-derived components found in jejunal chyme than native casein or amino acids.

CH may stimulate intestinal mucin secretion either directly, namely oligopeptides making contact with the jejunal goblet cells, or indirectly, e.g., via the enteric nervous system. Because several authors previously showed that intestinal mucus cells are under the control of the enteric nervous system (22, 24), we addressed the possibility that this pathway could trigger CH-induced mucin secretion. In the present study, the CH-evoked mucin discharge was fully blocked by intra-arterial infusion of TTX, thus suggesting the involvement of intramural neurones. The effect of a synthetic analog of casomorphins, $\beta$-[DAla2,4, Tyr5] CM-5-NH2 on ion transport in rabbit ileum in vitro was also found to be mediated neurally (39). A striking finding was that naloxone, an antagonist at opioid receptors, completely inhibited the mucin release induced by 0.5% CH. The opioid pathway is thus involved in mediating the secretion of intestinal mucin produced by CH administration.

The CH used in our experiments is a highly refined trypsin hydrolysate, and the molecular weights of its oligopeptides are in the 100–1,000 range (informations from the provider). Interestingly, caseins are the source of numerous biologically active peptides such as opioid agonists, antihypertensive peptides, antithrombotic peptides, immunostimulants, and mineral carriers. $\beta$-CMs thus represent the family of exogenous opioid peptides originally isolated from enzymatic digests of bovine milk $\beta$-casein (37, 38). They are fragments of the $\beta$-casein sequence 60–70 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile-Pro-Asn-Ser-Leu) (21), and all contain the NH$_2$-terminal amino acid sequence Tyr-Pro-Phe-Pro-$\beta$-CM-5 (fragment 60–64) and -7 (fragment 60–66) were among the first food-derived opioid peptides described and were found in intestinal chyme or in trypsin hydrolysates (21). In a final analysis, it can be noticed that molecular weights of these $\beta$-CMs (580 and 790 for $\beta$-CM-5 and -7, respectively) are in adequacy with those observed in our CH.

$\beta$-CMs, first described in the bovine $\beta$-casein sequence, are also found in analogous position in sheep, water buffalo, and human $\beta$-casein (21, 25). The well-preserved primary structure of these peptides suggests that $\beta$-CMs are important biologically active molecules and raises the question of the significance of these opioid peptides in diet. In fact, these $\beta$-CMs are resistant to the action of gastrointestinal proteolytic enzymes (25) and could elicit physiological effects in the intestine. Previous studies showed indeed that $\beta$-CMs modulate postprandial release of insulin, glucagon, somatostatin, and pancreatic polypeptide in dogs, stimulate intestinal absorption of electrolytes in rabbit ileum, and significantly prolong intestinal transit time in human and rat (3, 8, 31–34). $\beta$-CMs have also been proposed for the treatment of diarrhea in calves (31). In the present study, we demonstrate for the first time that $\beta$-CM-7 evokes intestinal mucin secretion in a dose-dependent manner when added to the luminal side of rat jejunum. Together, $\beta$-CMs appear to be important bioactive peptides in the regulation of intestinal function. Luminal concentrations of $\beta$-CMs after ingestion of milk have not been determined, but the highest dose of $\beta$-CM-7 we used in this study ($1.2 \times 10^{-4}$ M) is equivalent to what could be theoretically obtained from a 0.5% CH. In the neonate, such an effect of milk protein may be a part of the control of mother on defence mechanisms in the infant’s organism, and similar protective effects may be expected in the adult (29).
The mucin secretion induced by β-CM-7 was completely reversed by naloxone, thus confirming the opioid nature of this effect. Opioid receptors can be classified into three types (referred to as μ, κ, and δ), and β-CMs may represent agonists for μ-receptors detected in the gut (30, 38). Because numerous μ-receptor-immunoreactive nerve fibers have been detected around crypts and blood vessels in the mucosal and submucosal layers of rat intestine (1), a physiological action of β-CMs requires the passage of active sequences from the lumen to the basolateral side of the epithelium. In keeping with this view, intra-arterial β-CM-7 administered at a concentration even 100-fold lower than those used for luminal administration evoked a sharp mucin discharge in rat jejunum. The mechanism of β-CMs absorption is unknown, but studies with β-lactoglobulin or with insulin (19, 43) support the hypothesis of a transcytotic transport of peptides through intestinal enterocytes. The peptides could also be transported intact across Peyer’s patches (9). It was recently shown that the major whey proteins lactalbumin and β-lactoglobulin also contain bioactive sequences with opioid (lactorphins) or angiotensin I-converting enzyme inhibitory activity (lactokinins) (29). An attractive hypothesis that could explain mucin secretion induced by LH is the implication of one of these bioactive peptides. Additional experiments with isolated perfused rat jejunum are thus required to determine which of these peptides is implicated in mucus discharge induced by the pancreatic digest of lactalbumin used in our study.

In conclusion, we provide evidence that enzymatic hydrolysates of casein or lactalbumin may modulate mucin secretion in rat jejunum. In contrast, native casein, amino acids, chicken egg albumin and its hydrolysate, or meat hydrolysate does not significantly stimulate mucin discharge. The secretion of mucin induced by CH or by one of its well-defined components, β-CM-7, is triggered by opioid receptors and involves a nervous pathway. Our findings extend the list of nutrients or luminal factors liable to stimulate mucus secretion, which may be of interest in preventive nutrition and in gastrointestinal pharmacology.

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