Milk Bioactive Peptides and β-Casomorphins Induce Mucus Release in Rat Jejunum

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ABSTRACT Intestinal mucus is critically involved in the protection of the mucosa. An enzymatic casein hydrolysate and β-casomorphin-7, a μ-opioid peptide generated in the intestine during bovine casein digestion, markedly induce mucus discharge. Because shorter μ-opioid peptides have been described, the effects of the opioid peptides in casein, β-casomorphin-7, -6, -4, -4NH_2 and -3, and of opioid neuropeptides met-enkephalin, dynorphin A and (D-Ala2,*N*-Me-Phe4,glycinol5)enkephalin (DAMGO) on intestinal mucus secretion were investigated. The experiments were conducted with isolated perfused rat jejunum. Mucus secretion under the influence of β-casomorphins and opioid neuropeptides administered intraluminally or intra-arterially was evaluated using an ELISA for rat intestinal mucus. Luminal administration of β-casomorphin-7 (1.2×10^{-4} mol/L) provoked a mucus discharge (500% of controls) that was inhibited by naloxone, a specific opiate receptor antagonist. Luminal β-casomorphin-6, -4 and -4NH_2 did not modify basal mucus secretion, whereas intra-arterial administration of β-casomorphin-4 (1.2×10^{-6} mol/L) induced a mucus discharge. In contrast, intra-arterial administration of the nonopioid peptide β-casomorphin-3 did not release mucus. Among the opioid neuropeptides, intra-arterial infusion of Met-enkephalin or dynorphin-A did not provoke mucus secretion. In contrast, β-endorphin (1.2×10^{-8} to 1.2×10^{-6} mol/L) induced a dose-dependent release of mucus (maximal response at 500% of controls). DAMGO (1.2×10^{-6} mol/L), a μ-receptor agonist, also evoked a potent mucus discharge. Our findings suggest that μ-opioid neuropeptides, as well as β-casomorphins after absorption, modulate intestinal mucus discharge. Milk opioid–derived peptides may thus be involved in defense against noxious agents and could have dietary and health applications. J. Nutr. 133: 3499–3503, 2003.

KEY WORDS: • *mucus* • *intestine* • *milk* • *casomorphin* • *opioid*

The intestinal mucus gel covering the mucosal surface is a major component of physiologic defense mechanisms. Mucus separates mucosal cells from the exterior milieu, provides protection from noxious substances (e.g., acidity, proteolytic enzyme activities or toxins) and constitutes a local physical barrier against bacteria and pathogens (1). It also regulates epithelial hydration, allows lubrication of the cell surface and participates indirectly in the immune response due to interactions with secretory immunoglobulins (2). The intestinal mucus gel owes its properties to secreted mucins, which are high-molecular-weight glycoproteins produced by goblet cells of the epithelium (3). Velcich et al. (4) demonstrated that inactivation of the major intestinal mucin gene Muc2 in mice causes intestinal tumor formation with spontaneous progression to invasive carcinoma.

The protective role of intestinal mucus requires the regulation of mucin release, and any quantitative change in mucus secretion may modify this protective barrier. In particular, the interactions of nutritional factors with the secretory activity of mucus cells may represent new, interesting possibilities for the manipulation of this important protective function. In view of this fact, we demonstrated in a previous study that luminal administration of a casein hydrolysate or of β-casomorphin-7 induced a strong jejunal mucus secretion (5). Caseins constitute ~80% of the total milk protein fraction and consist of four principal proteins (αS1-, αS2-, β- and κ-caseins) in the ratio 38:11:38:13 (6–8). Although none of the casein proteins has a bioactive role, peptides with biological activities are generated during casein digestion (8). β-Casomorphins (β-CM)[2] are a family of opioid peptides derived from bovine β-casein. They possess preferentially μ-receptor agonist activity and consist of β-CM-4, -5, -6 and -7, which are obtained by successive C-terminal cleavage of the 60–66 (Tyr-Pro-Phe-Pro-Gly-Pro-Ile) fragment in bovine β-casein (9). Identical sequences exist in ovine β-casein, and peptides with similar sequence activity are derived from human β-casein (9). Interestingly, β-casomorphins, which have been detected in the small intestine of adult humans and in the plasma of newborn

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[2] Abbreviations used: BSA, bovine serum albumin; CM, casomorphin; DAMGO, (D-Ala2,*N*-Me-Phe4,glycinol5)enkephalin.

0022-3166/03 $3.00
Manuscript received 21 March 2003. Initial review completed 24 May 2003. Revision accepted 7 July 2003.

calves after ingestion of bovine milk (10), are resistant to the actions of gastrointestinal enzymes due to a high content of proline residues. They could thus elicit physiologic effects and may represent natural agonists for the opioid receptors detected in the gut. Because these receptors have been detected mainly in the nervous plexus of rat intestine (11), it seems necessary that β-casomorphins be absorbed as active sequences across the intestinal mucosa. The present study was thus undertaken to characterize the effects of β-casomorphins on rat mucus discharge after luminal or arterial administration of bovine β-casomorphin-7, -6, -4, -4 amidated and -3 and to compare these effects with those of opioid neuropeptides. For this purpose, we used a preparation of isolated vascularly perfused rat jejunum. In this model, mucus cells keep their physiologic orientation and environment and are thus subjected to well-defined luminal, neural and blood-borne stimuli. The secretion of mucus was evaluated by an ELISA.

MATERIALS AND METHODS

Materials. Bovine serum albumin (BSA) was purchased from Biomedia (Boussens, France). Nutrilamine 25, a mixture of amino acids, was obtained from Braun Medical (Boulogne, France). β-Casomorphin-7, β-casomorphin-6, β-casomorphin-4, β-casomorphin-4NH_2, β-casomorphin-3, Met-enkephalin, dynorphin A, β-endorphin and (D-Ala2,*N*-Me-Phe4,glycinol5)enkephalin (DAMGO) were obtained from Bachem (Bubendorf, Switzerland). Naloxone was purchased from ICN pharmaceuticals (Costa Mesa, CA). Microtiter plates (Nunc-Immunoplate) were obtained from Polylabo (Paul Block, Strasbourg, France). Other reagents were purchased from Sigma Chemical (St. Louis, MO).

Animal model and experimental protocol

Surgical preparation. The surgical procedure to prepare an isolated vascularly perfused rat jejunum was described previously (5,12). Briefly, male Wistar rats (250–350 g), purchased from Le Centre d'Elevage Dépré (Saint Doulchard, France), were anesthetized with an intraperitoneal injection of sodium pentobarbital (50 mg/kg). 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 gently emptying the air, both ends of the intestinal loop were ligated. Metal and Silastic cannulae were then quickly inserted into the superior mesenteric artery and portal vein, respectively. The arterial perfusion started immediately at a rate of 2.5 mL/min with a Krebs-Henseleit buffer (2 mmol/L $CaCl_2$, 6 mmol/L KCl, 3.18 mmol/L NaH_2PO_4, 104 mmol/L NaCl, 1 mmol/L $MgSO_4$, 41.6 mmol/L $NaHCO_3$, pH 7.4) containing 25% washed bovine erythrocytes, 3% BSA, 5 mmol/L glucose and 1% Nutrilamine 25 (v/v), and continuously gassed with O_2/CO_2 (95:5). The preparation was then removed and transferred to a bath containing isotonic saline at 37°C. The pressure of perfusion, continuously recorded with a mercury manometer, ranged from 40 to 55 mm Hg. In preliminary experiments, the viability of the preparation was found to be 1–2 h.

Experimental protocol. The experiments consisted of a 5-min equilibration period, followed by a 30-min stimulation period. Immediately after the equilibration period, the loops were filled by injection as a bolus of 0.8 mL of prewarmed isotonic saline (control preparations or arterial stimulations) or of 0.8 mL of luminal factors at 37°C. When required, the pH of luminal compounds to be tested was adjusted to 7–7.5 with diluted hydrochloric acid, and the osmolarity was adjusted to 300 mosmol/kg with sodium chloride. A control experiment was performed for each stimulated loop.

In some experiments, 10^{-5} mol/L naloxone was administered intra-arterially after 2 min of equilibration and maintained over the 30-min period of stimulation. All vascularly perfused drugs were dissolved in Krebs-Henseleit buffer supplemented with 30 g/L BSA and were delivered at a rate of 0.25 mL/min through a catheter close to the vascular inflow.

At the end of the experimental period, loops were cut at both ends and fluid content was collected. To remove the mucus adherent to the mucosal surface, loops were carefully emptied by manual massage, flushed with 4 mL isotonic saline (37°C) and subsequently with air, and then drained. Luminal content (fluid content + adherent mucus gel) was weighed, homogenized by sonication, and frozen at −20°C for subsequent determination of mucin-like immunoreactivity and luminal DNA content. The adherent mucus gel was taken in account because it represents a substantial part of the secreted immunoreactive material. The empty jejunal loops were weighed and the length measured (cm). They were then homogenized (Ultra-Turrax, Janke and Kundel, Staufen, Germany) for 1 min in PBS and stored at −20°C. Tissue homogenates were then analyzed for DNA content.

Analysis of samples. Samples of luminal contents were incubated for 24 h with 100 mmol/L 1,4-dithiothreitol at 4°C for reduction and then assayed for mucin glycoproteins by an ELISA as previously described (5). Briefly, the polyclonal antiserum 59C was raised in a rabbit against rat intestinal mucus glycoproteins obtained from CsCl gradient purified material. An immunoglobulin-rich fraction was prepared using DEAE-Sephadex A50 (pH 7.2), and then used to produce biotinylated labeled antibody using a succinimide ester of biotin (13). The mucin content of samples was determined from standard curves prepared from purified rat intestinal mucin. The luminal contents were tested at three dilutions (1:1000, 1:2000 and 1:4000). All assays were performed in duplicate. The CV was 4.5%. Samples of luminal stimulants were also assayed for checking the absence of interference in the ELISA.

Jejunal loop homogenates were thawed and briefly homogenized. Aliquots were sonicated for 20 s, and then analyzed for DNA content using the fluorometric method of Hinegardner (14). The amount of mucin glycoproteins secreted from each loop was expressed as μg mucin/mg tissue DNA. Samples of luminal content were also 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).

Statistical analysis. Data were compared using repeated-measures ANOVA, followed by paired *t* test or the Scheffé test when appropriate. Differences with $P < 0.05$ were considered significant. Data were analyzed using Statview 4.57 for Windows (Abacus Concepts, Berkeley, CA). Data are expressed as a percentage of control experiments (means ± SEM).

RESULTS

Luminal administration of β-casomorphins. Luminal administration of β-casomorphin-7 (1.2×10^{-4} mol/L) in the isolated vascularly perfused rat jejunum induced a strong mucin secretion (555 ± 54% of control loops). Pretreatment with arterial naloxone (opioid antagonist at μ, δ and κ receptors, 10^{-5} mol/L) abolished the β-casomorphin-7–induced mucin release (**Fig. 1**). In experiments without β-casomorphin-7, naloxone alone (10^{-5} mol/L) did not affect baseline mucin secretion in the isolated rat jejunum, as assessed by ELISA (data not shown).

The amidated tetrapeptide morphiceptin (β-casomorphin-4NH_2), is the most active opioid agonist in the bovine β-casomorphin family (15). Luminal administration of β-casomorphin-4NH_2 or of β-casomorphin-4 (1.2×10^{-4} mol/L) did not induce the release of jejunal mucus (Fig. 1). β-Casomorphin-6 also did not produce mucin discharge (153 ± 18% of control loops).

Arterial β-casomorphins. β-Casomorphin-7 had a large stimulatory effect on mucus secretion in the 1.2×10^{-7} to 1.2×10^{-6} mol/L range ($P < 0.05$, **Table 1**). Intra-arterial administration of β-casomorphin-4 ($1.2\ 10^{-6}$ mol/L) also produced a rise in the secretion of mucin (**Fig. 2**). β-Casomorphin-3 (1.2×10^{-6} mol/L), which is not considered an opioid agonist, had no effect (120 ± 24% of control loops). Amida-

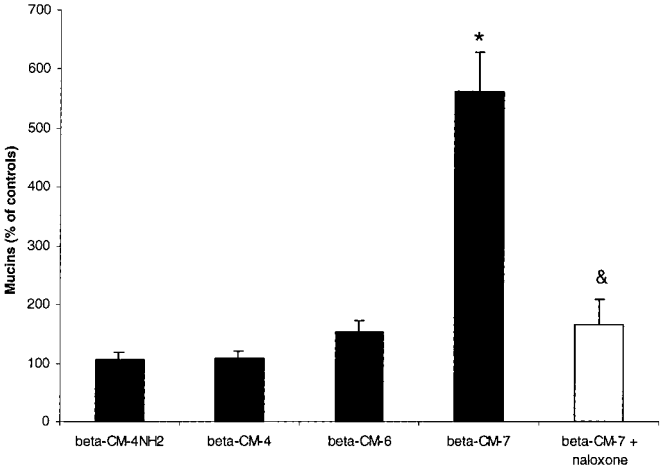

FIGURE 1 Effect of luminal administration of different β-casomorphins (β-CM, 1.2×10^{-4} mol/L) on mucus secretion by isolated rat jejunum and the effect of naloxone (10^{-5} mol/L) on the secretory response induced by β-CM-7. A control experiment was produced for every stimulated loop. In blockade experiments, naloxone was administered intra-arterially 3 min before and during β-CM-7 stimulation. Results are expressed as a percentage of controls given as means ± SEM of 6–8 experiments. *Different from controls, $P < 0.05$; &different from β-CM-7, $P < 0.05$. In related control preparations, the mucin discharge measured at the end of the experiment was 170 ± 49 μg/mg DNA.

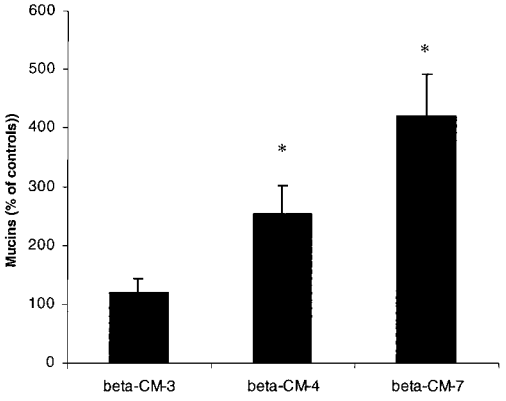

FIGURE 2 Effect of intra-arterial infusion of different β-casomorphins (β-CM, 1.2×10^{-6} mol/L) on mucin release by rat jejunum. A control experiment was produced for every stimulated loop. Mucin release (% of controls) is given as means ± SEM of 6–8 experiments. *Different from controls, $P < 0.05$. In related control preparations, the mucin discharge measured at the end of the experiment was 187 ± 42 μg/mg DNA.

tion of β-casomorphin-4 to morphiceptin (β-casomorphin-4NH_2) resulted in a dramatic mucus secretion (445 ± 75% of control loops, **Fig. 3**), of the same order of magnitude as obtained with intra-arterial β-casomorphin-7. On stimulation with 1.2×10^{-7} mol/L morphiceptin, the rise in mucin release was also significant but less pronounced (Fig. 3).

Arterial perfusion of endogenous opiates. Neuropeptides representing each of the three endogenous opioid peptide families have been detected in the gastrointestinal tract. Intra-arterial perfusion of 1.2×10^{-6} mol/L β-endorphin (proopiomelanocortin family) induced a sharp rise in the secretion of mucin-like immunoreactivity (response at 509 ± 111% of control loops, **Fig. 4**). The effect of β-endorphin was concentration dependent over the range 1.2×10^{-8} to 1.2×10^{-6} mol/L ($f = 7.94$, $P < 0.05$), with the first significant response obtained with the $1.2\ 10^{-7}$ mol/L concentration. Treatment with 1.2×10^{-6} mol/L DAMGO, a specific μ-opioid-receptor agonist, resulted in mucus release from jejunal preparation (response at 305 ± 62% of control loops).

Met-enkephalin (proenkephalin family) and dynorphin-A (prodynorphin family), infused in the 1.2×10^{-6} to 1.2×10^{-8} mol/L range, did not elicit any release of mucus (data not shown).

DISCUSSION

We recently demonstrated that luminal administration of an enzymatic hydrolysate of casein (0.5–5%) from bovine milk strongly stimulated rat intestinal mucus secretion, which was mediated by opioid receptor activation (5). We also showed that β-casomorphin-7 (1.2×10^{-4} mol/L), a fragment of β-casein with opiate activity, reproduced this mucin release in rat jejunum.

In the course of characterizing effects of several luminally administered β-casomorphins on jejunal mucus secretion, β-casomorphin-7 (1.2×10^{-4} mol/L) was the only one that

TABLE 1

Intra-arterial infusion of increasing amounts of β-casomorphin-7 and mucus secretion in the isolated vascularly perfused rat jejunum[1,2]

	Mucin (% of controls)	*n*
β-Casomorphin-7 (1.2×10^{-8} mol/L)	134 ± 17	6
β-Casomorphin-7 (1.2×10^{-7} mol/L)	291 ± 60*	6
β-Casomorphin-7 (1.2×10^{-6} mol/L)	420 ± 72*	8

[1] Values are means ± SEM. * Different from paired control loops, $P < 0.05$.

[2] In related control preparations, the mucin discharge measured at the end of the experiment was 198 ± 57 μg/mg DNA.

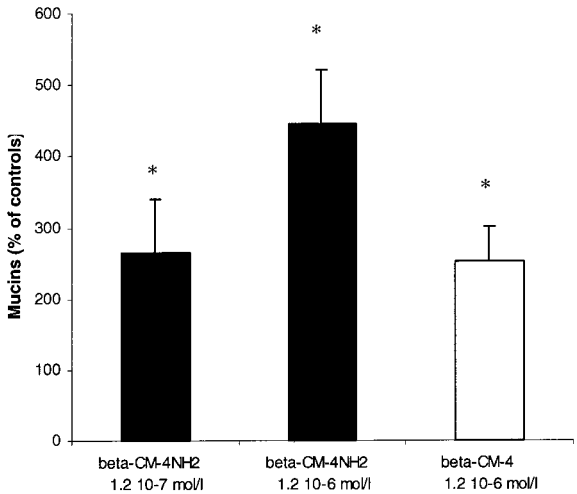

FIGURE 3 Effect of intra-arterial infusion of nonamidated and amidated forms of β-casomorphin (β-CM)-4 on mucin secretion in rat jejunal preparations. Mucin release (% of controls) is given as means ± SEM of 6–8 experiments. *Different from controls, $P < 0.05$. In related control experiments, the mucin-like immunoreactivity observed at the end of the experiments was 194 ± 31 μg/mg DNA.

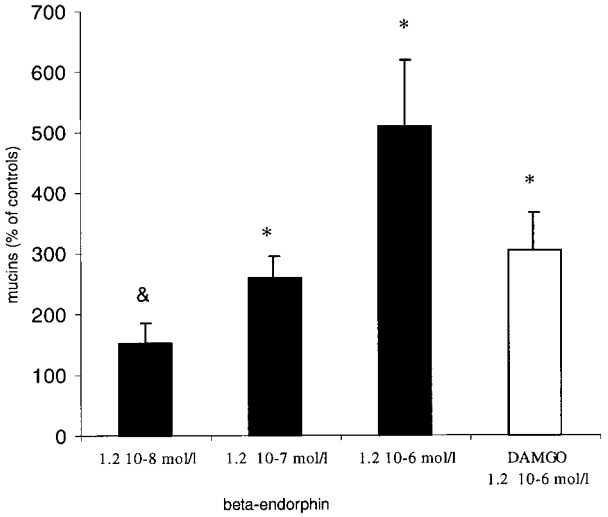

FIGURE 4 Effect of intra-arterial administration of β-endorphin and of D-Ala2,*N*-Me-Phe4,glycinol5)enkephalin (DAMGO) on mucin release in rat jejunum. A control experiment was produced for every stimulated loop. Mucin release (% of controls) is given as means ± SEM of 6–8 experiments. *Different from controls, $P < 0.05$; &different from 1.2×10^{-6} mol/L β-endorphin. In related control preparations, the mucin discharge measured at the end of the experiment was 201 ± 47 μg/mg DNA.

elicited a response. In fact, β-casomorphin-4, β-casomorphin-4NH_2 (morphiceptin) and β-casomorphin-6 were not luminal stimulants of mucus discharge. Thus, despite their opioid-agonist properties (8), luminal administration of β-casomorphins that were smaller in size than β-casomorphin-7, was not effective at stimulating intestinal mucus release. It is noteworthy that β-casomorphin-7 is unique in that it contains the minimal sequence required for luminal stimulation of mucus release and it contains a cathepsin B inhibitory peptide that corresponds to the 61–66 fragment in bovine β-casein (16). Cathepsin B is a major lysosomal protease widely distributed in gastrointestinal epithelial cells (17). It exhibits both endopeptidase and exopeptidase activities with a strong proteolytic activity (18) and has been implicated in intracellular proteolysis (19). Interestingly, Davies and Messer (20) suggested that cathepsin B plays a role in the digestion of milk proteins, and it is believed that milk peptides, which are resistant to enzymatic hydrolysis, are transcytosed by the enterocytes and subjected to lysosomal degradation (21–23). Accordingly, an attractive hypothesis that could explain the specific effect of β-casomorphin-7 on mucus secretion is that this bioactive peptide contains an inhibitory sequence to its intracellular hydrolysis. It must be also emphasized that considerable amounts of β-casomorphin-7, but not β-casomorphin-5 and only very small amounts of β-casomorphin-4 or -6 were found in intestinal contents of adult humans that ingested bovine milk (24). Together, these data suggest the importance of β-casomorphin-7 as a "food hormone" for intestinal functions. We can also noted that β-casomorphin precursors with substantial opioid activity such as β-casomorphin-9, -13 and -21 or β-casomorphin-11 (25,26) could be produced during milk digestion. Thus, in addition to β-casomorphin-7, other longer opioid peptides may be responsible for the effect of casein hydrolysate on mucus discharge.

In our study, we also demonstrated that jejunal mucus secretion induced by luminal β-casomorphin-7 was abolished by an opiate antagonist (naloxone). These results indicate that luminal β-casomorphin-7 activity on mucus secretion is mediated by opiate receptors. Thus, β-casomorphins may represent agonists for the μ-receptor detected in the gut (9,15). Because the μ-receptors are present mainly in the myenteric and submucosal plexus of rat intestine (11), a physiologic action of β-casomorphins will require the passage of active sequences from the intestinal lumen to the basolateral side of the epithelium. In support of this idea, intra-arterial β-casomorphin-7 administered at a concentration as low as 1% of those used for luminal administration evoked a sharp mucin discharge in rat jejunum. Also, it was a novel finding that β-casomorphin-4 specifically induced mucus secretion after intra-arterial but not after luminal administration in the isolated vascularly perfused rat intestine model. This secretion induced by intra-arterial infusion was further increased when the β-casomorphin-4 was amidated to morphiceptin in agreement with the increased potency observed for inhibiting guinea pig smooth muscle contraction (9). Our results suggest that the limitation of the effect of luminally administered casomorphins on mucus secretion is their entry in the submucosal layer, an activity performed only by β-casomorphin-7.

Because nothing is known about intestinal mucus secretion in response to endogenous opioid neuropeptides, our interest focused then on such ligands. Endogenous opiates, which are widely distributed in the gastrointestinal tract, are grouped into three major families according to their precursor proteins: proopiomelanocortin (endorphin), proenkephalin (enkephalin) and prodynorphin (dynorphin) (27). These endogenous opioid peptides have the same N-terminal amino acid sequence Tyr-Gly-Gly-Phe and are implicated in the control of gastrointestinal functions including regulation of transit time as well as mucosal transport of fluids and electrolytes (28,29). In this study, we demonstrated for the first time that arterial administration of β-endorphin, a preferential μ-receptor agonist, produced a strong mucus release in the model of isolated vascularly perfused rat jejunum. In keeping with this finding, the specific μ-receptor agonist DAMGO was also a potent mucus secretagogue. In contrast, Met-enkephalin (a preferential δ-receptor agonist) and dynorphin-A (a preferential κ-receptor agonist) did not affect mucus secretion at up to 10^{-6} mol/L. These data suggest that endogenous opioid peptides may be involved in defense mechanisms of the gastrointestinal tract through activation of the μ-receptors. In agreement with this result, Speroni et al. (30) demonstrated that the gastroprotective effect of opioid peptides against necrotizing agents (HCl, ethanol) involved the mucus layer. These results are also consistent with those of Zhang et al. (31) who showed that pretreatment with morphine (a preferential μ-opioid receptor agonist) before ischemia-reperfusion could induce protection in rat intestine.

In conclusion, we provided evidence that nutrient-derived opiate materials and opioid neuropeptides may participate in the regulation of intestinal mucus discharge. Because the mucus layer is the first barrier encountered by enteric pathogens, bioactive peptides from dairy products may provide new dietary prospects for improving gastrointestinal protection.

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