Glucagon-like peptide-2 relaxes mouse stomach through vasoactive intestinal peptide release

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Amato A, Baldassano S, Serio R, Mule`F. Glucagon-like peptide-2 relaxes mouse stomach through vasoactive intestinal peptide release. J Physiol Gastrointest Liver Physiol 296: G678–G684, 2009. First published December 24, 2008; doi:10.1152/jpgi.90587.2008.—Glucagon-like peptide-2 (GLP-2) influences different aspects of the gastrointestinal function, including epithelial growth, digestion, absorption, motility, and blood flow. Intraluminal pressure from isolated mouse stomach was recorded to investigate whether GLP-2 affects gastric tone and to analyze its mechanism of action. Regional differences between diverse parts of the stomach were also examined using circular muscular strips from fundus and antrum. In the whole stomach, GLP-2 (0.3–100 nM) produced concentration-dependent relaxation with a maximum that was about 10% of relaxation to 1 μM carbachol (IC50 = 2.5 nM). This effect was virtually abolished by desensitization of GLP-2 receptors or by α-chymotrypsin. The relaxant response to GLP-2 was not affected by tetrodotoxin, a blocker of neuronal voltage-dependent Na+ channels, but it was significantly reduced by ω-conotoxin GVIA, a blocker of neuronal N-type voltage-operated Ca2+ channels. Nω-nitro-L-arginine methyl ester, a blocker of nitric oxide synthase, or apamin, a blocker of Ca2+-dependent potassium channels, failed to affect the gastric response to the peptide. However, the relaxation was significantly antagonized by [Lys1,Pro2,5,Arg3,4,Tyr6]VIP7–28, a vasoactive intestinal peptide (VIP) receptor antagonist (GLP-2 maximum effect = 45% of relaxation to 1 μM isoproterenol), and virtually abolished by desensitization of the VIP receptors. GLP-2 induced concentration-dependent relaxation in carbachol-precontracted fundic strips but not in antral strips. These results provide the first experimental evidence that GLP-2 is able to induce gastric relaxation acting peripherally on the mouse stomach. The effect appears to be mediated by presynaptic neural release of VIP and confined to fundic region.

enteric nervous system; gastrointestinal hormones; gastric motility; gastric fundus

GLUCAGON-LIKE-PEPTIDE-2 (GLP-2) is a proglucagon-derived peptide produced in the “L” cells, a subset of endocrine cells that are abundant in the distal jejunum, ileum, and colon, and it is secreted in a nutrient-dependent manner (6, 25). GLP-2 is a pleiotropic hormone affecting multiple aspects of gastrointestinal physiology. Foremost among these is the ability to increase small and large intestinal weight through stimulation of epithelial cell proliferation and inhibition of apoptosis, leading to enlarged crypts and villi and, consequently, enhanced intestinal digestive and absorptive capacity (6, 25). For this reason, GLP-2 is in the late-stage clinical trials for the treatment of short bowel syndrome (10). In addition, GLP-2 stimulates the intestinal glucose transport and the blood flow, inhibits the gastric acid secretion (9, 32), and is involved in the regulation of food intake (23). Although morphological changes attributable to the peptide are not reported in intestinal muscle, GLP-2 has been shown to have inhibitory effects on gastrointestinal motility. In particular, it reduces the vagally induced antral motility in pigs (27). However, results on the stomach motor activity from human studies are conflicting because GLP-2 has been demonstrated to have either no influence (14, 20) or to slow gastric emptying (11, 18). Moreover, GLP-2 seems to act in concert with GLP-1 to inhibit rat small bowel myoelectric activity in the fasted state (2). Either way, because preclinical studies in patients with short-bowel syndrome have shown that administration of GLP-2 increases their capacity to absorb enteral nutrients (11), a reduced gastrointestinal motor function could contribute and promote the intestinal adaptive growth.

The biological actions of GLP-2 are mediated by a specific G protein-coupled receptor belonging to the class B glucagon-secretin receptor family (17). GLP-2 receptor expression is restricted to the gastrointestinal tract and to the central nervous system although, in a lesser extent, it has been found in lung, cervix, and in vagal afferents (6). The precise cellular location of GLP-2 receptor within the gastrointestinal tract has not yet been well defined, and there are controversial results related probably to methodological problems or species-specific expression (6). In particular, within the intestine, the GLP-2 receptor has been localized in diverse mucosal enteroendocrine cells (8, 29) and subepithelial myofibroblasts (19) as well as in enteric neurons situated in the muscularis layer (1, 8), suggesting that many of the GLP-2 actions may be indirect through release of not yet identified secondary mediators. So far, it has not yet been clarified whether GLP-2 has a peripheral direct influence on the gastric emptying or intestinal motor activity because the few experimental studies about its involvement in the regulation of gastrointestinal motility have been conducted in vivo conditions (2, 18, 27). However, because the GLP-2-related increase of intestinal mass is accompanied by enhanced intestinal digestive and absorptive functional capacity, it is likely to hypothesize that this peptide may also alter the gut motility acting peripherally. Therefore, the purpose of the present investigation was to examine whether GLP-2 may induce peripheral effects with the use of an in vitro preparation from mouse gastrointestinal tract. In particular, we analyzed the influence of exogenous GLP-2 on the gastric spontaneous mechanical activity and examined the mechanism of action responsible for the observed effects.
Whole stomach was used to examine the muscle function under conditions where the influence of external factors is removed, but the muscle performs in a manner analogous to its in vivo capacity and is able to relax in absence of contractile agents (15, 16). In addition, experiments using circular muscular strips obtained from gastric fundus and antrum were performed to clarify the regional activity of GLP-2.

**MATERIALS AND METHODS**

Adult male mice (C57BL/10SnJ; weighing 25.5 ± 0.5 g), obtained from Charles River Laboratories (Calco-Lecco, Italy) were used for the study. Animals were housed in standard conditions under a constant light-dark cycle, constant temperature (22 ± 1°C) and humidity (55 ± 5%), with free access to food and water. Experimental protocols were approved by Ministero della Sanità (Rome, Italy), following the guidelines of the European Communities Council Directive of 24 November 1986. Animals were euthanized by cervical dislocation. The abdomen was immediately opened, the esophagus was tied just below the lower esophageal sphincter, and the entire stomach was excised.

**Preparation of the isolated stomach.** The stomach was mounted in a custom-designed organ bath (volume = 5 ml), which was continuously perfused with oxygenated (95% O₂ and 5% CO₂) and heated (37°C) Krebs solution with the following composition (in mM): 119 NaCl, 4.5 KCl, 2.5 MgSO₄, 25 NaHCO₃, 1.2 KH₂PO₄, 2.5 CaCl₂, and 11.1 glucose. The pyloric end was cannulated and connected to a standard pressure transducer (Statham Mod. P23XL; Grass Medical Instruments, Quincy, MA). The mechanical activity, monitored as changes of endoluminal pressure, was recorded on ink-writer polygraph (Grass model 7D, Grass Medical Instruments). Preparations were allowed to equilibrate for about 60 min before starting the experiment. At the beginning of each experiment, the preparation was challenged with isoproterenol (1 μM) until reproducible responses were obtained to ensure that a stable and acceptable level of sensitivity had been reached before the experimental procedure was begun. Concentration-response curves to the peptide were obtained to ensure that a stable and acceptable level of sensitivity had been reached before the experimental procedure was begun. At the beginning of each experiment, the preparation was

**RESULTS**

**Effects of GLP-2 on the whole stomach.** GLP-2 (0.3–100 nM) induced a relaxation that developed slowly, persisted throughout the contact time, and was reversible after washing out (Fig. 1). The effect enhanced by increasing the concentration and the maximal response was obtained at 30 nM of GLP-2 (about 75% of the relaxation to 1 μM isoproterenol; IC₅₀ = 2.5 nM; CIs 0.8–7.3 nM), and then the response started to decline (Fig. 2).

To assess the specificity of the effect, the preparation was pretreated for 30 min with GLP-2 (100 nM) to desensitize the receptors. In these conditions, the relaxation induced by the peptide was abolished over the full concentration range (Fig. 2), whereas the isoproterenol-induced relaxation was not modified being 4.1 ± 0.3 cm H₂O in the control and 4.0 ± 0.5 cm H₂O after GLP-2 desensitization (n = 4; P > 0.05). Moreover, changes in the gastric spontaneous mechanical activity were not observed after GLP-2 desensitization. The peptidase α-chymotrypsin (10 U/ml) was mixed with stock solution of GLP-2 (1 μM) and then tested on the preparation. In these conditions, the inhibitory effect induced by GLP-2 was abol-

**Drugs.** The following drugs were used: rat GLP-2 (Tocris-Bioscience, Bristol, UK), isoproterenol hydrochloride, CCh, apamin, α-chymotrypsin, l-NAME, VIP (Sigma-RBI, Milano, Italy), TTX, α-conotoxin GVIA (Alomone Labs, Jerusalem, Israel), and [Lys¹,Pro²,Arg³,4,Tyr⁶]VIP²⁻⁸ (California Peptide Research, Napa, CA). Each compound was prepared as a stock solution in distilled water. The working solutions were prepared fresh the day of the experiments by diluting the stock solutions in Krebs.
Fig. 1. Typical tracings showing the inhibitory effects induced by increasing concentrations of glucagon-like peptide-2 (GLP-2) in isolated mouse gastric preparations.

Fig. 2. Concentration-response curves for the relaxation induced by GLP-2 before and after treatment of the stock solution of GLP-2 (1 μM) with α-chymotrypsin (10 U/ml). The values are means ± SE (n = 4) and are reported as a percentage of the relaxation induced by isoproterenol (1 μM), taken as 100%. *P < 0.05 compared with the respective control conditions.

Fig. 3. Relaxation induced by GLP-2 before and after treatment of the stock solution of GLP-2 (1 μM) with α-chymotrypsin (10 U/ml). The values are means ± SE (n = 4) and are reported as a percentage of the relaxation induced by isoproterenol (1 μM), taken as 100%. *P < 0.05 compared with the respective control conditions.

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ished (Fig. 3). α-Chymotrypsin at the final concentration reached in the bath (0.01 U/ml) per se did not modify the gastric tone, and it failed to affect the relaxation induced by isoproterenol (1 μM) or GLP-2 (10 nM).

To investigate eventual neural mechanisms, we tested the effect of GLP-2 in presence of TTX, blocker of neuronal voltage-dependent Na⁺ channels or α-conotoxin GVIA, a blocker of neuronal N-type voltage-operated Ca²⁺ channels. These drugs did not cause significant changes in the gastric tone. TTX (1 μM) abolished the EFS-evoked response, but it failed to affect the relaxation to a submaximal concentration of GLP-2 (10 nM) (Fig. 4). α-Conotoxin GVIA (0.3 μM) greatly reduced the EFS-induced response, with the early rapid relaxation reduced by 81.5 ± 5.3% (n = 4) and the slow relaxation virtually abolished (Fig. 4). In the presence of α-conotoxin GVIA (0.3 μM), the gastric response to GLP-2 was significantly reduced (Figs. 4 and 5). Indeed, the relaxation induced by isoproterenol (1 μM) was not modified (4.2 ± 0.3 cm H₂O in control and 4.1 ± 0.4 cm H₂O after α-conotoxin GVIA, n = 4; P > 0.05).

Because nitric oxide (NO), ATP, and VIP have been already shown as neurotransmitters mediating the relaxation induced by activation of intrinsic neurons in the mouse gastric preparations (15), their potential role as responsible for GLP-2 effect was investigated with the use of l-NAME, a blocker of the NO synthesis, apamin, a blocker of small conductance Ca²⁺-dependent K⁺ channels, which antagonizes the relaxation to ATP in this preparation (15), or [Lys1,Pro2,5,Arg3,4,Tyr6]VIP⁷⁻²⁸, a specific VIP antagonist. l-NAME (300 μM) induced an increase in the basal pressure (about 1 cm H₂O), whereas apamin (0.1 μM) or [Lys1,Pro2,5,Arg3,4,Tyr6]VIP⁷⁻²⁸ (300 nM) were without any effect. l-NAME (300 μM) or apamin (0.1 μM) failed to affect the response to GLP-2 (10 nM) (Fig. 6), whereas [Lys1,Pro2,5,Arg3,4,Tyr6]VIP⁷⁻²⁸ (300 nM) significantly reduced the inhibitory effect of GLP-2 (Fig. 7). VIP involvement in GLP-2 inhibitory response was also investigated, testing the peptide after VIP receptor desensitization. Administration of VIP (100 nM) to desensitize the receptors produced a slowly developing relaxation, which was followed by a slow decline in the response up to recovery of the initial tone. The peak relaxation was reached after about 5 min of VIP exposure, whereas the recovery of the basal tone was obtained after about 1 h. The stomach treated with a priming VIP concentration (100 nM) was not able to respond at the second 100 nM VIP administration (carried out as soon as the effect of the priming dose had vanished), indicating the occurrence of VIP desensitization. In desensitized tissue, the GLP-2 (10 nM) relaxation was virtually abolished (Figs. 6 and 8), whereas the isoproterenol (1 μM) relaxation was not modified (Fig. 8).

Effects of GLP-2 on antral and fundic muscular strips. Antral but not fundic circular muscular strips showed spontaneous contractile activity (mean amplitude 0.48 ± 0.04 g at a frequency of 6.5 ± 1 contractions/min, n = 3). Since GLP-2 (0.3–100 nM) did not induce any effect on muscular strips but caused scant relaxation just in the fundus, gastric muscular...
strips were precontracted with CCh (10 µM). Addition of the muscarinic receptor agonist CCh (10 µM) to the bath medium of both antral and fundic strips caused a sustained contraction that reached a plateau that persisted until washout. In CCh-precontracted fundic strips, GLP-2 (0.3–100 nM) was able to induce concentration-dependent inhibition of the response to CCh (IC₅₀ 2.5 nM; Cls 1.1–5.4 nM, n = 5), whereas it was without any effect in CCh-precontracted antral strips (Fig. 9). The GLP-2-induced effects on fundic strips were prevented by ω-conotoxin GVIA (0.3 µM) but not by TTX (0.1 µM) (Fig. 9).

**DISCUSSION**

The results of the present study demonstrate that the hormone peptide GLP-2 is able to induce relaxation of mouse gastric preparation through VIP neural release. However, the peptide appears to exert region-specific effects, being active in the gastric fundus but not in the antrum.

It is well accepted that GLP-2 is an important factor in the regulation of mucosal morphology, function, and integrity, and it is emerging as a novel treatment modality in a variety of conditions with intestinal injury (25). Relatively little is known about its role in the regulation of gastrointestinal motility even if it is likely that this hormone, besides its trophic effects, may improve intestinal absorption and nutritional status also through gut motor changes.
To date, peripheral effects on bowel smooth muscle have not been described. To our knowledge, only one previous study examined the action of GLP-2 on isolated rat colon, showing that GLP-2 induced direct inhibitory effects on contractility of the smooth muscle (5). Therefore, our information on the motor action of GLP-2 fundamentally derives from in vivo studies. In anesthetized splanchnicotomized pigs in which insulin-induced hypoglycemia strongly stimulates antral motility by a purely vagal mechanism, GLP-2 abolishes this motor response, acting as an inhibitor of gastric motility (27). However, results about the ability of GLP-2 to suppress gastric motility in humans are conflicting, and it could depend on the methodology used to assess antral emptying or on the type of administered test meal (low-calorie liquid meal or high-calorie solid meal) (11, 14, 18, 20).

The present study provides the first experimental evidence in vitro about the inhibitor role of the proglucagon-derivative peptide GLP-2 on mouse gastric motility. In fact, in the whole stomach, exogenous GLP-2 relaxed the smooth muscle of mouse stomach in a concentration-dependent manner, suggesting that the peptide is also able to act peripherally to control gastric motility. The potency of GLP-2 in inducing gastric relaxation was $IC_{50} = \approx 2 \text{nM}$, which is in agreement with that reported for the human and/or rat GLP-2 ability to stimulate GLP-2 receptor in various cell types ($EC_{50}$ ranging from 0.04 to 14 nM) (7, 17, 24, 26). Nevertheless, we cannot state whether the range of effective concentrations of GLP-2 is physiological or pharmacological because so far the murine plasma GLP-2 levels have not been determined because of high variations in the analyzed samples (21). Indeed, the circulating concentrations of GLP-2 found in plasma from humans ($\approx 20 \text{ pM}$) (9, 28) or rat ($\approx 0.2 \text{ nM}$) (3) are less than the concentration range effective in our experiments although they increased several fold after ingestion of meals (3, 9, 28). The comparison between human and rat concentrations could indicate an inverse correlation between the size of the animal and the circulating GLP-2 levels. Consequently, we do not exclude a physiological role for the hormone in the control of mouse gastric motility although further experiments need to clarify this point.

The induced gastric relaxation is mediated by GLP-2 receptor activation because the agonist-induced desensitization abolished the GLP-2 responses without affecting the isoproterenol relaxation. In fact, in the absence of well-characterized GLP-2 receptor antagonists, desensitization can represent a useful pharmacological tool in the study of receptors, and GLP-2R has been shown to undergo rapid and sustained homologous desensitization induced by the agonist (7, 26). Indeed, GLP-23–33 has been indicated as a murine GLP-2 receptor antagonist (21), but it works as well as a weak partial agonist (24). The desensitization of the GLP-2 receptors did not modify the spontaneous mechanical activity, suggesting that these receptors are not involved in an ongoing control on the mouse gastric tone. In addition, the gastric relaxation to GLP-2 was...
abolished when the stock solution of GLP-2 was pretreated with α-chymotrypsin, a peptidase that cleaves peptides at the carboxyl side of amino acids containing phenyl rings (present in the sequence of the hormone), confirming the fact that the effects observed were due to GLP-2. On the other hand, it is unlikely that the enzyme can act by degrading membrane receptors or other extracellular effector proteins involved in the relaxation induced by GLP-2 because the responses of the preparation to isoproterenol or to GLP-2 were not affected by the final concentration of α-chymotrypsin reached in the bath.

The second step was to investigate whether GLP-2 induces gastric relaxation via a direct action on the smooth muscle cells and/or via an indirect action involving neural pathways. In fact, the exact cellular localization of the GLP-2 receptor in the gastrointestinal tract has been a source of controversy, with variable reports demonstrating expression in endocrine cells, enteric neurons of both submucosal and myenteric plexus, and/or subepithelial myofibroblasts (1, 8, 19, 29), which lead to the hypothesis that GLP-2 can act through an indirect action involving neural and/or endocrine mechanisms (6). In particular, Bjerknes and Cheng (1) have shown that, in mouse intestine, GLP-2 receptor is not expressed in the epithelial enteroendocrine cells but in enteric neurons, where it seems to be involved in the control of crypt epithelial function through a TTX-sensitive mechanism. Our results showed that in mouse stomach the GLP-2-induced concentration-dependent reduction of the gastric tone was not affected by TTX, but rather it was significantly reduced by ω-conotoxin GVIA. Because TTX blocks the voltage-dependent sodium channel responsible for genesis and propagation of neural action potential and ω-conotoxin GVIA blocks the voltage-dependent N-type calcium channels involved exclusively in the neurotransmitter release, the peptide may act prejunctionally in a manner that is independent of action potential. Although ω-conotoxin GVIA-sensitive channels are not uniformly represented on mammalian autonomic nerve terminals (4, 12), this toxin has been reported to suppress the inhibitory junction potential in circular smooth muscle of mouse gastric fundus (13). Consistent with the presynaptic role of N-type Ca2+ channels in mediating neurotransmitter release, in our experiments the response evoked by EFS was sensitive to ω-conotoxin GVIA. The observation that the toxin significantly reduced the response to GLP-2 suggests that Ca2+ influx through N-type channels is a step in the mechanism of GLP-2 action, and the relaxation is likely mediated by the release of some inhibitory neurotransmitter from nerve terminals.

Therefore, we focused our attention on NO, ATP, and VIP as possible neurotransmitters involved in the GLP-2 effects in the mouse gastric preparation because they have been reported to be involved in the relaxation induced by activation of intrinsic neurons (15). On the other hand, in human and porcine intestine, GLP-2 receptor is expressed in nitrergic enteric neurons, participating in the regulation of intestinal blood flow and mucosal epithelial growth caused by GLP-2 (8). However, in our experiments, GLP-2-induced relaxation was unaffected by the NO synthase inhibitor, L-NAME, ruling out a role of NO in the action of GLP-2. In mouse stomach, ATP has also been involved in the mechanical relaxation in response to nerve nonadrenergic noncholinergic activation by acting through the opening of apamin-sensitive K+ channels (15). In our preparation, apamin was without any effect on the GLP-2 relaxation, leading us to rule out any involvement of ATP and any opening of small-conductance calcium-dependent potassium channels in the response induced by GLP-2.

It has to be underlined that the GLP-2 anti-inflammatory effects are mediated by VIP release from enteric neurons in a rat model of intestinal bowel disease (22) and that GLP-2 receptor has been localized to VIP-positive neurons in submucosal and myenteric plexus in human and porcine intestine (8). Consistent with these findings, in our experimental model, VIP receptor antagonist significantly reduced the relaxation induced by GLP-2, suggesting that GLP-2 relaxant effect is mediated by VIP. [Lys1,Pro2,5,Arg3,4,Tyr6]VIP7–28 was chosen because previously it was demonstrated to be effective against the anti-inflammatory action of GLP-2 in rat intestine (22). However, a VIP desensitization procedure was used as well. When VIP desensitization was induced, the response to GLP-2 was virtually abolished, confirming VIP participation in the gastric relaxation to GLP-2. Our results indicate that VIP desensitization involves VIP receptors specifically (and not other receptor types or postreceptor mechanisms) because isoproterenol-induced relaxation was not affected by the desensitization procedure.

Lastly, we attempted to differentiate the action of GLP-2 on fundus or antrum of the stomach by testing the peptide on muscular strips from both gastric regions. Because the strips did not develop muscular tone sufficient to detect relaxation, they were precontracted by CCh. Our results pointed out the GLP-2 ability to relax precontracted fundic strips but not precontracted antral strips, suggesting that the peptide exerts region-specific effects in the stomach according to the receptive and propulsive functions of the proximal and the distal part, respectively. The GLP-2-induced effect, as in the whole stomach, was ω-conotox GVIA sensitive and TTX insensitive. Therefore GLP-2, likely through VIP release, could facilitate accommodation of the proximal stomach concurring to its reservoir function.

In summary, the results of the present study show for the first time that the GLP-2 is able to induce gastric relaxation acting peripherally on the mouse stomach. This effect appears to be mediated by presynaptic neural release of VIP and to be confined in the fundus. This new role of GLP-2 could represent a satiety signaling, which fits well with the finding that GLP-2 is a chemical mediator inhibiting rodent feeding behavior (23). In addition, decreasing tone in the proximal stomach would delay the flow through the pylorus, prolonging the gastric emptying time, and this effect, in turn, might be advantageous to improve the absorptive or proliferative intestinal activity.

GRANTS

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