Exogenous glucagon-like peptide 1 reduces contractions in human colon circular muscle

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Abstract
Glucagon-like peptide 1 (GLP1) is a naturally occurring peptide secreted by intestinal L-cells. Though its primary function is to serve as an incretin, GLP1 reduces gastrointestinal motility. However, only a handful of animal studies have specifically evaluated the influence of GLP1 on colonic motility. Consequently, the aims of this study were to investigate the effects induced by exogenous GLP1, to analyze the mechanism of action, and to verify the presence of GLP1 receptors (GLP1Rs) in human colon circular muscular strips. Organ bath technique, RT-PCR, western blotting, and immunofluorescence were used. In human colon, exogenous GLP1 reduced, in a concentration-dependent manner, the amplitude of the spontaneous contractions without affecting the frequency and the resting basal tone. This inhibitory effect was significantly reduced by exendin (9–39), a GLP1R antagonist, which per se significantly increased the spontaneous mechanical activity. Moreover, it was abolished by tetrodotoxin, a neural blocker, or Nω-nitro-L-arginine – a blocker of neuronal nitric oxide synthase (nNOS). The biomolecular analysis revealed a genic and protein expression of the GLP1R in the human colon. The double-labeling experiments with anti-neurofilament or anti-nNOS showed, for the first time, that immunoreactivity for the GLP1R was expressed in nitrergic neurons of the myenteric plexus. In conclusion, the results of this study suggest that GLP1R is expressed in the human colon and, once activated by exogenous GLP1, mediates an inhibitory effect on large intestine motility through NO neural release.

Key Words
- GLP-1 receptor expression
- intestinal peptides
- colonic motility
- nitric oxide

Introduction
Subjects and methods

Human tissue excision and preparation

Specimens of human colon (n=18) were obtained from patients (aged 40–88 years, 35% females) with no symptoms of major clinical motility disorders, and who underwent surgery for neoplastic conditions at the Mediterranean Institute for Transplantation and Advanced Specialized Therapies (ISMETT), Palermo, Italy. The experimental protocol was approved by the Institutional Ethics Committee and written informed consent was obtained from all individuals before surgery. Samples were frozen and stored at −80 °C for subsequent GLP1R expression analysis (RT-PCR and western blot) or fixed in cold 4% paraformaldehyde, diluted in PBS for immunohistochemistry (n=3).

Functional experiments

Following the overnight storage, the circular muscle strips (4 mm wide by 10 mm long) were suspended in a four-channel organ bath containing 8 ml of heated (37 °C) and oxygenated (95% O2 and 5% CO2) Krebs solution with the following composition (mM): NaCl 119, KCl 4.5, MgSO4 2.5, NaHCO3 25, KH2PO4 1.2, CaCl2 2.5, and glucose 11.1. One end of each strip was tied to organ holders, while the other end was secured with a silk thread to an isometric force transducer (FORT 25, Ugo Basile, Biological Research Apparatus, Comerio, VA, Italy). Mechanical activity was digitized on an analog-to-digital converter, visualized, recorded, and analyzed on a personal computer using the PowerLab/400 System (Ugo Basile). A tension of 1 g was applied, and the tissue was allowed to equilibrate for 1 h. During this period, the strips developed spontaneous phasic contractions.

After the equilibration period, the effects induced by non-cumulative concentrations of GLP1 (1 nM–1 μM) on the spontaneous mechanical activity were examined. The peptide was added to the bath at increasing concentrations in volumes of 80 μl, and left in contact with the tissue for 7 min. The response to GLP1 was also tested in the presence of exendin (9–39) (100 nM), a GLP1R antagonist; tetrodotoxin (TTX; 1 μM), a voltage-dependent Na+ channel blocker; and Nω-nitro-L-arginine (L-NNA; 300 μM), an inhibitor of neuronal NO synthase (nNOS).

GLP1R expression analysis and immunofluorescence

Total RNA from human colon specimens was extracted with the PureLink RNA Mini Kit (Invitrogen) according to manufacturer’s instructions. After quantification by spectrophotometry, 1 μg of total RNA was reverse transcribed with the High-Capacity c-DNA RT Kit (Applied Biosystems). The GLP1R and β-actin were amplified using 5 μl of cDNA (30 ng total RNA equivalents) per reaction. The oligonucleotide primers for human GLP1R and β-actin were as follows: F, 5′-ctctgtcttgtatatgcctct-3′ and R, 5′-agataagacgaaagcc-3′ to generate a 317 bp product from human GLP1R; and F, 5′-gggacacccctgactg-3′ and R, 5′-ccacagctcttctagtgc-3′ to generate a 396 bp product from human β-actin, as described previously (Suen et al. 2008). The primers were

(Amato et al. 2010, Rotondo et al. 2011). In fact, previous studies have shown that the GLP1 receptor (GLP1R) is expressed in the CNS (Göke et al. 1995) and in the nodose ganglion (Nakagawa et al. 2004), and that exogenous GLP1 can activate vagal afferents (Bucinskaite et al. 2009). More recent evidence has suggested that the activation of enteric neurons mediates GLP1 inhibitory effects by nitric oxide (NO) release (Amato et al. 2010, Rotondo et al. 2011). Actually, GLP1R expression has been found in the enteric neurons of the small and large intestine in different species, including humans (Amato et al. 2010, Baldassano et al. 2012, Mamdouh et al. 2013, Richards et al. 2013).

The GLP1 effects on the stomach and small intestinal motility have been investigated, although the action on lower gut motility is less clear (Marathe et al. 2011). Few studies have analyzed the role of GLP1 in the regulation of colonic contractility, and the results remain controversial because both GLP1 stimulatory (Gülpinar et al. 2000, Ayachi et al. 2005) and inhibitory (Byrne et al. 2001, Amato et al. 2010) effects have been described. Interestingly, constipation has been reported among the side effects in treatment with liraglutide, a human GLP1R agonist, successfully used for the treatment of type 2 diabetes mellitus (Seino et al. 2010, Jeong & Yoo 2011).

This study was undertaken to examine the possible presence of the GLP1R in the human colon, to analyze the effects of GLP1 on spontaneous mechanical activity, and to determine the GLP1’s mechanism of action.

The oligonucleotide primers for human GLP1R and β-actin were as follows: F, 5′-tgagaatgcgaagctgagc-3′ and R, 5′-agataagacgaaagcc-3′ to generate a 317 bp product from human GLP1R; and F, 5′-tgagggactcctcctactatc-3′ and R, 5′-ccacagctcttctagtgc-3′ to generate a 396 bp product from human β-actin, as described previously (Suen et al. 2008). The primers were
verified on gene sequence by public databases (Homo sapiens GLP1R mRNA GenBank accession no.: NM_002062.3 and Homo sapiens β-actin mRNA GenBank accession no.: NM_001101.3).

The thermal cycle profile employed a 5 min denaturing step at 94 °C followed by 40 cycles at 95 °C for 15 s, 60 °C for 60 s, and 72 °C for 30 s, and a final extension step of 10 min at 72 °C. The amplimers were separated on a 1% agarose gel containing 0.5 μg/ml of ethidium bromide for visualization, and the gel was scanned under u.v. light. Hep-G2 cell line (purchased from ATCC, Manassas, VA, USA) was used as positive control (Gupta et al. 2010, Svegliati-Baroni et al. 2011).

For western blotting, 30 mg of tissue from the colon was incubated on ice in RIPA buffer (50 mM Tris–HCl, pH 7.4; 150 mM NaCl, 1% Nonidet P-40) containing protease inhibitors (2 mM phenylmethlysulphonyl fluoride, 2 mM ethylenediaminetetraacetic acid, 10 mM sodium fluoride, 1 mM phenylmethylsulphonyl fluoride, 10 μM leupeptin, 10 μM aprotinin, 10 μM pepstatin) and 0.5× protease inhibitors. The tissue permeability. The tissues were incubated overnight at 4 °C with primary antibodies for GLP1R or a mixture of primary antibodies for double labeling. After being washed with PBS, the tissues were incubated with a single secondary antibody or a mixture of appropriate secondary antibodies conjugated with fluorescein isothiocyanate or indocarbocyanine (Cy3), at room temperature for 1 h, followed by washing in PBS and mounting with VECTASHIELD (Vector, Burlingame, CA, USA). Negative controls were created by omitting the primary antibodies or substituting it with a no-immune rabbit serum and no labeling was observed (data not shown).

Fluorescence labeling was examined with an Olympus BX 50 fluorescence microscope (Olympus Italy, Milano, Italy). Photomicrographs were acquired with a Nikon DS-U1 Monochrome digital camera and NIS-Elements Software (Nikon Instruments, Melville, NY, USA), stored on disk and analyzed with NIS-Elements. Images were minimally adjusted for brightness and contrast using NIS-Elements. nNOS-immunoreactive (IR)–neurons that expressed GLP1R-IR were counted on three adjacent slices (Puddu et al. 2013).

For the immunohistochemical study, primary and secondary antibodies were used, as listed in Table 1. The specimens (n = 3) were included in Killik cryostat embedding medium (Bio-Optica, Milan, Italy) and frozen at −80 °C. Transverse sections, 10 μm thick, were cut, collected on polylysine-coated slides, and fixed in Zamboni’s fixative (4% formaldehyde plus 0.2% picric acid in 0.1 M sodium phosphate buffer, pH 7.0) for 15 min at room temperature. After fixation, the tissues were washed (3×10 min) in PBS (0.9% NaCl in 0.1 M sodium phosphate buffer, pH 7.0) and placed in PBS containing 20% fetal bovine serum, 5% BSA, and 0.1% Triton X-100 (Sigma–Aldrich) for 30 min at room temperature to minimize nonspecific binding and increase tissue permeability. The tissues were incubated overnight at 4 °C with primary antibodies for GLP1R or a mixture of primary antibodies for double labeling. After being washed with PBS, the tissues were incubated with a single secondary antibody or a mixture of appropriate secondary antibodies conjugated with fluorescein isothiocyanate or indocarbocyanine (Cy3), at room temperature for 1 h, followed by washing in PBS and mounting with VECTASHIELD (Vector, Burlingame, CA, USA). Negative controls were created by omitting the primary antibodies or substituting it with a no-immune rabbit serum and no labeling was observed (data not shown).

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**Table 1** Codes and sources of the primary and secondary antibodies used in the study

<table>
<thead>
<tr>
<th>Antigen</th>
<th>Host</th>
<th>Code</th>
<th>Dilution</th>
<th>Source</th>
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<tbody>
<tr>
<td>Anti-NF GLP1R</td>
<td>Mouse</td>
<td>Rabbit</td>
<td>sc-66911</td>
<td>1:100</td>
</tr>
<tr>
<td>nNOS</td>
<td>Mouse</td>
<td>Mouse NOS-3F7-B11-B5</td>
<td>111-095-003</td>
<td>1:100</td>
</tr>
<tr>
<td>Rabbit IgG</td>
<td>Goat</td>
<td>FTC</td>
<td>115-165-003</td>
<td>1:500</td>
</tr>
<tr>
<td>Mouse IgG</td>
<td>Goat</td>
<td>Cy3</td>
<td></td>
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</tr>
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Anti-NF, anti-neurofilament; GLP1R, glucagon-like peptide 1 receptor; nNOS, neuronal nitric oxide synthase.

**Drugs**

The following drugs were used: L-NNA (Sigma–Aldrich), TTX (Alomone Labs, Jerusalem, Israel), GLP1 (7–36) amide, and exendin (9–39) (Tocris Biosciences, Bristol, London, UK).
Each compound was prepared as a stock solution in distilled water. The working solutions were prepared fresh on the day of the experiments by diluting the stock solutions in Krebs.

**Data and statistical analysis**

The inhibitory response of the circular muscle to GLP1 was taken as the change in percentage from the resting spontaneous activity (e.g. 100% corresponds to the abolition of spontaneous activity). The mean amplitude of spontaneous contractions was measured before and following the administration of a drug when a new steady state was reached. All data are expressed as mean values ± S.E.M. The letter n indicates the number of human colonic specimens. The concentration–response curves were fitted to a sigmoidal curve using nonlinear regression (Prism 4.0, GraphPad Software, San Diego, CA, USA). Statistical analysis was done with the Student’s t-test or ANOVA, followed by the Bonferroni’s post hoc test when appropriate. A probability value of <0.05 was regarded as significant.

**Results**

**Functional studies**

Circular muscle strips of human colon exhibited spontaneous mechanical activity consisting of phasic contractions at a frequency of 2.7 ± 0.3 contractions/min (n=15) and an amplitude of 4 ± 0.5 g (n=15). GLP1 (1 nM–1 μM) produced a decrease in the mean amplitude of spontaneous contractions without affecting the frequency and the resting basal tone (Fig. 1A). The inhibitory effect was enhanced by increasing the concentration of the peptide and was completely reversible after washout with normal Krebs solution. As shown by the concentration–response curves (Fig. 1B), the response obtained at the maximal tested concentration (1 μM GLP1) corresponded to 60% reduction in the amplitude of spontaneous contractions. To assess the specificity of the effect, the preparations were pretreated for 30 min with exendin (9–39) (100 nM), a GLP1R antagonist, which per se significantly increased the spontaneous mechanical activity (+ 41.5 ± 2.0%, n=5; P <0.01) (Fig. 1C). Exendin (9–39) markedly reduced the inhibitory effect induced by GLP1 (Fig. 1B). Moreover, the response to GLP1 was abolished by TTX (1 μM), a blocker of neural voltage-dependent Na+ channels (Fig. 2A), suggesting its neural origin, and by 1-NNa (300 μM), a blocker of nNOS (Fig. 2B). TTX failed to affect spontaneous contractions, indicating its balanced effect on excitatory and inhibitory nerves, while 1-NNa increased the contraction amplitude suggesting that NO exerts a tonic inhibitory action on human circular muscle.

**Molecular analysis of GLP1R expression**

In the colonic specimens, RT-PCR revealed the presence of a 317 bp mRNA encoding the GLP1R, with the expression of a β-actin PCR product (396 bp) as standard. The gene was also expressed in Hep-G2 cell line, used as positive control (Fig. 3A). Western blot analysis confirmed that the colonic human preparations express GLP1R at the protein level. A single 56-kDa band was detected in human colonic tissue and in Hep-G2 cells used as a positive control (Fig. 3B).

**Immunofluorescence**

We used immunohistochemical staining to identify the location of GLP1R in the myenteric plexus area of our human colon tissue samples. Double labeling with anti-neurofilament (anti-NF) showed that IR for GLP1R is present in the neurons of the myenteric plexus.
Inhibition (%)

Moreover, we found that neurons with IR for nNOS coexpressed GLP1R-IR (Fig. 4). Among the nNOS-IR neurons, 34.6 ± 2.5% co-expressed GLP1R. Muscle cells did not express GLP1R-IR.

Discussion

This study shows that GLP1R is expressed in the human colon and, once activated by exogenous GLP1, mediates an inhibitory effect on large intestinal motility through NO neural release. In addition to its well-known glucoregulatory effects, GLP1 can affect the motor functions of the gastrointestinal tract in different species, including humans (Marathe et al. 2011). In fact, it inhibits gastric emptying, relaxes the antral region of the stomach, and reduces the motility of the upper intestine (Wettergren et al. 1993, Giralt & Vergara 1999, Miki et al. 2005, Nagell et al. 2006, Schirra et al. 2006, Amato et al. 2010, Rotondo et al. 2011). Despite the clear inhibitory effects exerted by the peptide on the motility of the upper gastrointestinal tract, a potential regulatory effect on large intestine mechanical activity has yet to be clarified. On the other hand, it has been shown that the enteroendocrine cells of the human colon fully process biologically active GLP1 in concentrations similar to that reported for the small intestine, and that are high enough to have a physiological role (Deacon et al. 1995).

Indeed, current knowledge of the GLP1R expression in human tissue is incomplete and based largely on receptor mRNA investigations in whole-organ preparations (Wei & Moisov 1995, Satoh et al. 2000). The protein has been found in a variety of tissues, including the brain and pancreas (Baggio & Drucker 2007, Tornehave et al. 2008), hepatic cells (Gupta et al. 2010), adipocytes (Vendrell et al. 2011), and gastric mucosa (Broide et al. 2013). Concerning the bowel, low levels of GLP1R protein expression have been found in duodenal Brunner’s glands and the myenteric plexus of the human gut, including the colon, by autoradiography (Körnet et al. 2007), but the possible functions mediated by GLP1R in these regions remain purely hypothetic.

This study provides further evidence of the presence of GLP1R in the human distal colon, supported by conventional analysis (RT-PCR, immunoblot). Furthermore, the activation of the receptor by exogenous GLP1 induces a reduction of the spontaneous mechanical activity of human circular smooth muscle, suggesting, for the first time, an inhibitory role of GLP1 on human colonic motility. This mechanism is consonant with the GLP1 capacity of delaying gastrointestinal transit not only in humans but also in rodents (Tolessa et al. 1998a,b, Näslund et al. 2001, Miki et al. 2005, Schirra et al. 2009, Hellström 2011). Moreover, our results could provide a mechanistic explanation for the observations that GLP1-secreting tumors are associated with severe constipation and delayed colonic transit (Byrne et al. 2001,
Our finding seems in contrast with previous findings that showed weak contractions in response to GLP1 in isolated circular smooth muscle cells from the human colon, which are likely due to an increase in the availability of glucose energy (Ayachi et al. 2005). However, our preparations consisted of full thickness muscular strips, in which different cellular types, including enteric neurons, are present. Then, as discussed below, a GLP1 inhibitory action mediated by the enteric nervous system may lead to a decrease in contractions.

The inhibitory responses induced by GLP1 were significantly antagonized by exendin (9–39), a specific GLP1R antagonist (Schirra et al. 1998), indicating the specificity of the observed effect. Moreover, it is worth noting that exendin (9–39) per se increased the spontaneous contractions of circular colonic smooth muscle. This observation could be interpreted as suggested that exendin (9–39) is an inverse agonist of the GLP1R, as reported in other experimental studies (Serre et al. 1998, De León et al. 2008, Calabria et al. 2012). We retain that it is an unlikely hypothesis that exendin (9–39) can disclose a tonic action of endogenous GLP1, as suggested in previous in vivo studies (Schirra et al. 2006, 2009), because we used mucosa-free muscular strips in vitro, so our preparations were lacking the primary source of GLP1.

One still open question concerns the relative importance of the local enteric nervous system in mediating the actions of GLP1 in the gastrointestinal tract. Although some GLP1 gastrointestinal motor effects appear to be centrally mediated (Gülpinar et al. 2000), with the involvement of vagal nerves (Imeryüz et al. 1997, Delgado-Aros et al. 2002, 2003, Bucinskaite et al. 2009, Schirra et al. 2009), the hypothesis that GLP1 may act directly on the enteric nervous system has also been advanced (Tolessa et al. 1998a,b, Daniel et al. 2002, Chan et al. 2007, Amato et al. 2010, Rotondo et al. 2011, Mamdouh et al. 2013).

The observation that TTX, a blocker of neural voltage-dependent Na\(^+\) channels, abolishes the inhibitory effects induced by GLP1 suggests that neurons within the intramural plexus are responsible for the action of the peptide in human colon circular muscle strips. Furthermore, the inhibitory effect of GLP1 was also abolished in the presence of L-NNA, a blocker of NO synthesis, suggesting that it is mediated by NO production. Indeed, the involvement of nitrergic neural pathways has been reported in the effects induced by exogenous GLP1 on gastrointestinal motor function in different animal species, including humans (Tolessa et al. 1998b, Näslund et al. 2002, Andrews et al. 2007, Amato et al. 2010, Rotondo et al. 2011).

Our immunofluorescence results showing co-expression of GLP1R and nNOS in myenteric neurons are

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**Figure 4**

Expression of immunoreactivity (IR) for the GLP1 receptor (GLP1R) in the myenteric plexus area of the human colon tissue sample. (A1, A2, and A3). Coexpression of GLP1R-IR with anti-NF-IR. (B1, B2, and B3) Coexpression of GLP1R-IR with nNOS-IR. Scale bars, 20 μm.
the first report on the presence of GLP1R on the surface of human colon nitregeric neurons, and are consistent with the conclusion that GLP1R activation reduces human colon contractility through neural release of NO.

The concentrations found in our experiments to be effective are higher than the plasmatic level reported in humans (Ørskov et al. 1994), thus our data do not support the idea that GLP1 effects may be mediated via circulating GLP1 in an endocrine manner. Nevertheless, it remains possible that GLP1 acts with a paracrine mechanism near its site of production (colonic enteroendocrine L-cells), reaching concentrations sufficient to activate GLP1R.

In conclusion, in the human colon, GLP1R activation is able to inhibit spontaneous mechanical activity through NO neural production. This action might contribute to delaying intestinal propulsion, thus increasing absorption of water and electrolytes, and triggering constipation.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement
A A performed the functional and molecular biology experiments, statistical analysis, and data interpretation; S B carried out the immuno-histochemistry experiments; R L participated in the conception of the study, provided samples, and reviewed the manuscript; R S participated in the conception of the study and data interpretation; and F M designed the study and wrote the paper.

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References
Calabria AC, Li C, Gallagher PR, Stanley CA & De Leon DD 2012 GLP-1 receptor antagonist exendin-(9–39) elevates fasting blood glucose levels in congenital hyperinsulinism owing to inactivating mutations in the ATP-sensitive K+ channel. Diabetes 61 2585–2591. (doi:10.2337/db12-0166)


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