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The glucagon-like peptide 2 receptor is expressed in enteric neurons and not in the epithelium of the intestine

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ABSTRACT

Glucagon-like peptide 2 (GLP-2) is a potent intestinotrophic growth factor with therapeutic potential in the treatment of intestinal deficiencies. It has recently been approved for the treatment of short bowel syndrome. The effects of GLP-2 are mediated by specific binding of the hormone to the GLP-2 receptor (GLP-2R) which was cloned in 1999. However, consensus about the exact receptor localization in the intestine has never been established.

By physical, chemical and enzymatic tissue fragmentation, we were able to divide rat jejunum into different compartments consisting of: (1) epithelium alone, (2) mucosa with lamina propria and epithelium, (3) the external muscle coat including myenteric plexus, (4) a compartment enriched for the myenteric plexus and (5) intestine without epithelium. Expression of *Glp2r*; *chromogranin A*; *tubulin*, *beta 3*; *actin*, *gamma 2*, *smooth muscle*, *enteric* and *glial fibrillary acidic protein* in these isolated tissue fractions was quantified with qRT-PCR. Expression of the *Glp2r* was confined to compartments containing enteric neurons and receptor expression was absent in the epithelium.

Our findings provide evidence for the expression of the GLP-2R in intestinal compartments rich in enteric neurons and, importantly they exclude significant expression in the epithelium of rat jejunal mucosa.

Introduction

The dietary process is highly regulated, as reflected in the numerous hormones secreted by the gut, which are all involved in the regulation of digestion and absorption of nutrients. The proglucagon-derived sister hormones, glucagon-like peptide 1 (GLP-1)[2] and glucagon-like peptide 2 (GLP-2), are both highly involved in the dietary processes and are co-secreted from the intestinal L-cells [1]. GLP-1 is mainly involved in glucose metabolism and regulation of appetite, gastrointestinal motility and digestive secretions, actions which are now exploited in treatment of type 2 diabetes [2]. GLP-2 is involved in the regulation of many intestinal adaptive processes including epithelial proliferation, epithelial apoptosis, intestinal nutrient uptake, intestinal permeability, motility, blood flow and inflammation. Additionally, GLP-2 may be involved in appetite regulation and bone metabolism [3–6]. The intestinal effects of GLP-2 have been shown to benefit patients suffering from short bowel syndrome [7–10] and GLP-2 is

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[2] GLP-1: Glucagon-like peptide 1, GLP-2: Glucagon-like peptide 2, GLP-2R: GLP-2 receptor, 5-HT: Serotonin, qRT-PCR: Real-time quantitative reverse transcriptase-PCR, HBSS-CMF: Calcium and magnesium-free Hank's balanced salt solution, Sdha: Succinate dehydrogenase complex, subunit A, Ywhaz: Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide, Gapdh: glyceraldehyde-3-phosphate dehydrogenase, Chga: Chromogranin A, Actg2: Actin, gamma 2, smooth muscle, enteric, Gfap: Glial fibrillary acidic protein, Tubb3: Tubulin, beta 3, Hprt: Hypoxanthine guanine phosphoribosyltransferase, Rpl13a: Ribosomal protein L13A, Igf1: Insulin-like growth factor 1, Fgf7: Keratinocyte growth factor/fibroblast growth factor 7, Nos3: Nitric oxide synthase 3, endothelial cell, Tgfb1: Transforming growth factor, beta 1, Vegfa: Vascular endothelial growth factor A, Vip: Vasoactive intestinal polypeptide, H & E: Hematoxylin and eosin, SEM: Subepithelial myofibroblast.

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being used for treatment of patients suffering from intestinal failure [8,11]. Recently, the degradation-resistant GLP-2 analog Gly^2-GLP-2 (teduglutide) was approved as a drug for patients with short bowel syndrome.

The effects of GLP-2 are mediated by its binding to the GLP-2 receptor (GLP-2R) which, based on sequence homology, belongs to the glucagon/secretin family B of G protein-coupled receptors, known to signal through G_s proteins [12]. The GLP-2 mediated effects have been shown to involve *insulin-like growth factor 1* (*Igf1*), *keratinocyte growth factor/fibroblast growth factor 7* (*Fgf7*), *nitric oxide synthase 3*, *endothelial cell* (*Nos3*), *transforming growth factor*, *beta 1* (*Tgfb1*), *vascular endothelial growth factor A* (*Vegfa)* and *vasoactive intestinal polypeptide* (*Vip*) [13–19] and recently it was also shown that ErbB activation also is involved [20,21]. Though the receptor was cloned in 1999 and was shown to be predominantly expressed in the small intestine [12], there is ongoing debate regarding the exact localization of the receptor in the intestine. Yusta et al. reported in a study based on immunohistochemistry and RNA expression that the receptor was localized in serotonin (5-HT)-positive enteroendocrine cells in the epithelium [22], whereas Bjerknes and Cheng could not find expression of the receptor in the epithelium, but found expression of the receptor in intestinal ganglion cells [23]. Also, Ørskov et al. were unable to find receptor expression in the enteroendocrine cells, but identified it in subepithelial myofibroblasts [13]. Guan et al. studied the receptor localization in pigs and humans and localized the receptor to 5-HT-positive enteroendocrine cells, and to VIP and eNOS-positive enteric ganglion cells [24], findings that were later reproduced in normal [25] and jejunum-resected rats [26]. GLP-2R has been immunohistochemically detected in neurons in the submucosal plexus of ileum from guinea pigs [27] and similarly, it was demonstrated by in vitro receptor autoradiography of human intestinal tissue specimens that the GLP-2R could be localized to myenteric plexus in healthy individuals and more so in individuals suffering Crohn's disease, whereas other intestinal tissues did not show GLP-2R reactivity, including the epithelium [28].

The goal of this study was to establish evidence of the cellular localization of the GLP-2R with the use of real-time quantitative reverse transcriptase-PCR (qRT-PCR) applied to tissue fractions isolated from rat proximal jejunum. In addition, we analyzed the relative expression of various growth factors not known to be a part of the ErbB pathway in the jejunum of mice treated with a GLP-2R agonist for five days.

Materials and methods

Animals

All animal studies conformed to international guidelines (National Institutes of Health Publication No. 85-23, revised 1985, and Danish legislation governing animal experimentation, 1987), and were approved by the Animal Experiments Inspectorate, Ministry of Justice, Denmark.

Animals were maintained on water and chow (Altromin no. 1314), housed in plastic-bottom wire-lidded cages, and allowed to acclimatize for at least one week before they were used for tissue isolation.

Male Wistar rats weighing between 200 and 250 g were used for isolation of intestinal tissue fractions.

8 weeks old C57BL/6 mice were used for the stimulation study.

In vivo stimulation

Mice were divided into two groups ($n = 8$) matched by bodyweight and received a subcutaneous injection of either 100 μL PBS containing 3.5 mg/mL polygelinum (Haemaccel®, Behringwerke, Marburg, Germany) or 100 μL PBS with 3.5 mg/mL Haemaccel® and 25 μg of recombinant h[Gly_2]GLP-2, a long-acting GLP-2 analog, a kind gift from Lars Thim (Novo Nordisk) as previously described [29,30]. Injections were given twice daily at 8 a.m. and 8 p.m. for 5 days, after which the mice were euthanized and the small and large intestine were removed and flushed with PBS. Afterwards wet weight was measured. Tissue samples from the proximal intestine (just distal to the major duodenal papilla) were removed and immersed in either RNA-later or in 4% paraformaldehyde overnight followed by 70% EtOH.

Tissue isolation

For tissue isolation, 10 cm pieces of the intestine were removed 8 cm from the pyloric sphincter. One centimeter pieces of the intestine, distally to the removed part, were saved as reference tissue for normalization and immersed in RNA-later or immediately homogenized in TRI REAGENT™ (Molecular Research Center Inc., Cincinnati, OH, USA) for subsequent RNA isolation.

Isolation of the epithelium/epithelial cells ($n = 7$) was carried out as previously described by Bjerknes and Cheng [31]. In brief, the pieces of intestine were placed in calcium and magnesium-free Hank's balanced salt solution (HBSS-CMF – Invitrogen Cat No. 14180-046), then flushed with 10 mL HBSS-CMF and everted over a 4 mm glass rod followed by a 5 s vibration in ice cold HBSS-CMF to remove debris. The everted intestine was then incubated at 37 °C for 25 min in HBSS-CMF +30 mM EDTA. The epithelial cells were collected by vigorous rotation and vibration of the everted intestine in HBSS-CMF, and thereafter washed 3 times in Hank balanced salt solution (HBSS – Invitrogen cat. no. 24020-091). A small sample was used for preparation of a smear and fixed in 96% ethanol for 5 min. The rest was used for RNA isolation.

The everted intestine, now without epithelium, was cut into small pieces (5 mm × 5 mm) and washed 3 times in HBSS. Subsequently, the tissue was immersed into 4% paraformaldehyde in 0.1 M phosphate buffer pH 7.4 or RNA-later.

Isolation of smooth muscle layers and mucosa ($n = 7$): excized intestine was washed with HBSS and while immersed in HBSS, the smooth muscle layers and the mucosa were mechanically separated using tweezers. The mucosa and muscle fraction were immersed in TRI REAGENT™, immediately homogenized and used for RNA purification.

Isolation of neurons was carried out as previously described by Schäfer et al. [32]. In short, isolated smooth muscle layers (*not* including subepithelial myofibroblasts) were digested in 2 mL freshly prepared collagenase buffer (Worthington Collagenase type II, 300 Kunitz/mL; DNase Sigma DN-25, 34 Kunitz/mL in 0.025 M Hepes-buffered MEM (Invitrogen)) for 30–50 min at 37 °C. After 15 s of vortex mixing, the solution was centrifuged at 100 × *g*, 4 °C for 3 min and the supernatant was gently removed. The pellet was either vapor-fixed with 4% paraformaldehyde (1 h at 37 °C), or added 1 mL TRI REAGENT™ and homogenized for RNA isolation.

RNA isolations

The tissues (either stored in RNAlater or TRI REAGENT™) were homogenized in 1 mL TRI REAGENT™. RNA isolations were carried out according to the manufacturer's protocol. The concentrations were determined spectrophotometrically and the RNA quality was examined by agarose gel electrophoresis. The samples were stored at −80 °C.