

Chapter 21

Murine GLUTag Cells

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Abstract The incretin hormone, glucagon-like peptide-1 (GLP-1) serves as a link between ingested nutrients and insulin secretion. Because of the anti-diabetic properties exerted by GLP-1, several derivative drugs are currently in the market for the treatment of patients with type 2 diabetes. Over the past few years, several cell lines have been established as useful models for the study of the GLP-1-secreting enteroendocrine L-cells. This review focuses on the murine GLUTag cell line, derived from colonic tumours of transgenic mice expressing large T antigen under the control of the *proglucagon* promoter. These cells are widely used to examine the effects of food components on GLP-1 secretion, as well as the signaling pathways underlying nutrient-induced GLP-1 release. The effects of different food components, as well as of nutrient-related signals, on GLP-1 secretion, and the protocols for the maintenance and use of the murine GLUTag L-cell line will be discussed.

Keywords Cell line • Cell culture • Endocrine hormone • Enteroendocrine • Food components • GLP-1 • L-cell • Murine • Nutrient • Neurotransmitter • Receptor • Secretion

21.1 Introduction

The potent insulinotrophic activity of the intestinal hormone, glucagon-like peptide-1 (GLP-1), in the perfused rat pancreas was first described almost 30 years ago. This finding was rapidly followed by the demonstration that physiological levels of the peptide also stimulate insulin secretion in a glucose-dependent manner in humans. Since then, our knowledge of the physiological effects of GLP-1 has vastly increased and several anti-diabetic properties such as suppression of glucagon

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secretion, induction of satiety, reduction of gastric activity and potentiation of pancreatic β -cell mass have also been attributed to the peptide. Overall, these effects made GLP-1 a promising novel therapeutic tool for the treatment of type 2 diabetes mellitus, and GLP-1 derivative drugs have been successfully used to reduce glycemia in patients with type 2 diabetes since 2005. These anti-diabetic agents include agonists of the GLP-1 receptor long-lasting analogues of the native peptide and inhibitors of the enzyme responsible for the rapid degradation of the active forms of GLP-1 (Dong and Brubaker 2012). Interestingly, potentiation of the endogenous secretion of the peptide is another possible therapeutic approach which has gained much attention lately; however, a better understanding of the processes regulating GLP-1 secretion by the intestinal L-cell (an enteroendocrine type of cell which is the major source of circulating GLP-1 in the body) is still required.

Over the past few years, the establishment of different enteroendocrine cell lines has markedly improved our understanding of the different signals that modulate GLP-1 expression and secretion, as well as the intracellular pathways activated by these signals. The use of cell lines has also helped to troubleshoot the inherent difficulties derived from the use of intestinal primary cell cultures, such as low L-cell density and a high risk of bacterial contamination. Among all cell line models for the study of GLP-1 secretion, the murine GLUTag cells are the most widely used as well as the most specific. Thus, GLUTag cells not only have been demonstrated to appropriately respond to the same stimuli that trigger GLP-1 secretion *in vivo*, but they also have been shown to closely, although not fully, mirror the profile of genes and proteins expressed in non-immortalized L-cells. The present chapter will cover the modulation of GLP-1 secretion by the GLUTag cells, primarily focusing on the effect of food components, as well as the methodology for the use of this cell line.

21.2 Origin

GLUTag cells derive from a colonic tumour of a transgenic mouse expressing SV40 large T antigen under the control of approximately 2,300-base pairs of the promoter for *proglucagon*, the gene that encodes GLP-1 amongst other bioactive peptides. The mice developed endocrine carcinoma of the large bowel, the cells of which were positive for immunoreactive GLP-1 and cholecystokinin but were negative for other hormones, including adrenocorticotrophin, β -endorphin, calcitonin, corticotrophin-releasing hormone, gastrin, growth hormone-releasing hormone, insulin, pancreatic polypeptide, serotonin, somatostatin and vasoactive intestinal peptide, suggesting an L-cell lineage for these tumour cells. Fragments of the colonic tumours were subcutaneously propagated in nude mice, and individual cells from one of these tumours were isolated and single-cell cloned (Drucker et al. 1994). The cultured cells displayed pleomorphic nuclei with prominent nucleoli, and well-developed cytoplasmic organelles along with abundant secretory granules. GLUTag cells were found to express both *proglucagon* and cholecystokinin transcripts. The cells secrete GLP-1 in response to incubation with protein kinase A and protein kinase C activators, whereas *proglucagon* expression was only induced by

activators of protein kinase A, confirming previous studies in primary cells (Drucker et al. 1994). As described for the normal intestinal L-cell, the predominant GLP-1 form produced by the GLUTag cells is GLP-1(7-36)-amide (Drucker et al. 1994). Importantly, similar profiles in the expression of genes that encode mediators of nutrient-induced GLP-1 secretion, including glucokinase, sodium-glucose linked transporter 1 (SGLT1) and G protein-coupled receptors such as GPR40, GPR41, GPR119, GPR120 and GPR131 (TGR5), have been found in GLUTag and non-immortalized L-cells (Reimann et al. 2008; Tolhurst et al. 2012). The GLUTag cells are thus widely considered as the best in vitro model of the murine L-cell.

21.3 Features and Mechanisms

21.3.1 *Regulation of GLUTag Cell Secretory Activity by Nutrients*

Nutrient ingestion is the most powerful mechanism for triggering GLP-1 secretion by the intestinal L-cell (Dong and Brubaker 2012). Since the L-cells are open-type enteroendocrine cells residing in the intestinal epithelium, with their apical surfaces and associated microvilli open into the gut lumen, they can directly sense and respond to luminal nutrients. The effects of the major groups of food components on GLUTag cell physiology are discussed below.

Carbohydrates GLUTag cells are highly sensitive to glucose, such that cells that are propagated at 5 mmol/L concentration of glucose become quiescent and hyperpolarized when this monosaccharide is removed from the medium. Accordingly, when glucose is added back to the bath solution, the membrane conductance decreases, and the frequency of action potentials in the cells, as well as the secretion of GLP-1 are strongly enhanced, even at very low glucose concentrations (Reimann and Gribble 2002). Initial studies demonstrated that glucose metabolism was associated with the closure of K^+ channels sensitive to ATP (K_{ATP}), resulting in increases in membrane conductance and enhanced GLP-1 release (Reimann and Gribble 2002). However, recent studies have indicated a more important role for a metabolism-independent glucose-sensing pathway in GLUTag cells (Parker et al. 2012a). Hence, GLUTag cells express two sodium-glucose co-transporters, SGLT1 and SGLT3a, which have been found to increase glucose uptake inducing an inward current of Na^+ ions that results in cell depolarization and stimulation of GLP-1 secretion. These effects are appropriately diminished by SGLT inhibitors (Parker et al. 2012a). SGLTs also appear to be involved in the effects of non-metabolisable sugars, such as methyl- α -glucopyranoside, on GLP-1 secretion by the GLUTag cell line, but not in the effects of dietary sugars such as fructose that are not substrates for these transporters. Expression of SGLT1 in the intestinal epithelium is enhanced by dietary carbohydrates, a process believed to be mediated by type 1 taste receptors and gustducin (Margolskee et al. 2007). Interestingly, these receptors are also expressed by the

GLUTag cells and they seem to mediate the effects of artificial sweeteners in this cell line, although they may not be relevant *in vivo* (Fujita et al. 2009). The facilitative glucose transporters, GLUT, are also involved in the stimulatory effects of glucose on GLP-1 secretion in the GLUTag cell line, while SGLT1 appears to be the most important glucose-sensing mechanism for the L-cells *in vivo*. The different glucose-sensing mechanisms exhibited by GLUTag and non-immortalized L-cells may explain why GLUTag cells respond to small changes in glucose levels when they are incubated at very low concentrations of glucose (<1 mmol/L) but they are not sensitive to additional increments in glucose concentration (5–25 mmol/L; Reimann and Gribble 2002), as opposed to the dose-dependent effect of glucose in primary L-cells (Reimann et al. 2008).

Lipids Free fatty acids are potent stimulators of GLUTag cell activity, the magnitude of the effects being proportional to their chain length (Iakoubov et al. 2007). Furthermore, GLP-1 secretion is stimulated by long-chain monounsaturated fatty acids, such as oleic acid, but not by similar length saturated fatty acids such as palmitic acid, in agreement with previous observations in primary L-cell cultures. The effects of oleic acid on GLP-1 secretion are known to be mediated by the atypical isozyme protein kinase C ζ in GLUTag cells, as also found *in vivo* (Iakoubov et al. 2007; Dong and Brubaker 2012), while a role for fatty acid transport protein 4 in the uptake of oleic acid by GLUTag cells has also been demonstrated (Poreba et al. 2012).

The activation of the Gq-coupled receptors, GPR40 and GPR120, by long-chain unsaturated fatty acids has also been associated with enhanced GLP-1 release *in vivo* (Edfalk et al. 2008; Hirasawa et al. 2005). However, although GLUTag cells have also been reported to express these receptors (Lauffer et al. 2009), their role in the physiology of this cell line has not been extensively studied to date. In contrast, the Gs-coupled receptor, GPR119, has been shown to mediate the stimulatory effects on GLP-1 secretion of oleoylethanolamide, an endogenously-occurring oleic acid derivative (Lauffer et al. 2009). Furthermore, synthetic GPR119 agonists have been found to stimulate GLP-1 secretion both *in vivo* and in GLUTag cells (Chu et al. 2008). Another G-protein-coupled receptor, TGR5, has also been recently demonstrated to play a role in the stimulatory effects of bile acids on GLP-1 secretion in GLUTag cells, as previously also found *in vivo* (Parker et al. 2012b; Dong and Brubaker 2012).

Finally, short-chain fatty acids (SCFAs), including acetate, butyrate, and propionate, are generated by the gut microbiota through the digestion of complex carbohydrates, such as dietary fibers. It has been recently reported that SCFAs stimulate GLP-1 secretion via the G-protein-coupled receptor GPR43 (Tolhurst et al. 2012), establishing a novel and exciting link between gut microflora and gastrointestinal hormones. Unfortunately, the GLUTag cell line appears to be an inappropriate model to study the effects of SCFAs, since they express barely detectable levels of GPR43 and do not release GLP-1 in response to 1 or 10 mmol/L SCFAs (Tolhurst et al. 2012).

Proteins Protein hydrolysates stimulate GLP-1 secretion and production from a variety of cell lines, including GLUTag cells (Hira et al. 2009). When the effects of individual amino acids were examined, several were found to stimulate GLP-1

secretion, with a particularly robust effect promoted by glutamine. Two major mechanisms have been invoked in the stimulatory effects of glutamine on GLUTag cells: electrogenic sodium-coupled amino acid uptake which leads to membrane depolarization and voltage-gated calcium entry, and an elevation of intracellular cAMP levels (Tolhurst et al. 2011). The synergy of these two mechanisms appears to be a particularly strong stimulus for GLP-1 release *in vitro*. Other amino acids, such as alanine and glycine, have been found to trigger GLP-1 release from GLUTag cells via a pathway involving ionotropic glycine receptors (Gameiro et al. 2005), whereas the G protein-coupled receptor family C group 6 subtype A receptor has been implicated in the effects on GLP-1 secretion elicited by L-ornithine (Oya et al. 2013).

21.3.2 Regulation of GLUTag Cell Secretory Activity by Other Signals

Despite the predominant role of nutrients in the regulation of GLP-1 release, a number of other signals have been identified that exert stimulatory effects on the primary L-cell as well as on GLUTag cells. As many of these signaling mediators are induced by nutrient ingestion, this constitutes an indirect pathway that links food components to the release of GLP-1. Such indirect actions are particularly important to GLP-1 release, since L-cells are mainly concentrated in the distal ileum and colon (Dong and Brubaker 2012), and nutrients have not reached this area of the gastrointestinal tract when the initial phase of prandial GLP-1 secretion is triggered. Initially, a neurohormonal mechanism, involving the vagus nerve, was shown to mediate the very rapid rise in GLP-1 levels after nutrient ingestion (Dong and Brubaker 2012). Consistent with this hypothesis, a stimulatory effect of two muscarinic agonists, bethanechol and carbachol, has been found in GLUTag cells (Brubaker et al. 1998), although expression of muscarinic receptors by this cell line has not been reported to date. Notwithstanding, direct activation of downstream protein kinase C signaling does stimulate GLP-1 release from the GLUTag cells (Brubaker et al. 1998). Similarly, the incretin hormone, glucose-dependent insulinotropic peptide (GIP), secreted by the enteroendocrine K cells in response to carbohydrates and fats, has been reported to trigger GLP-1 secretion at a concentration range of 30–100 nM (Brubaker et al. 1998). The effects of GIP on GLUTag cells are mediated through activation of the protein kinase A signaling pathway (Simpson et al. 2007). Other agents capable of raising the intracellular levels of cAMP, such as forskolin plus the cAMP phosphodiesterase inhibitor, 3-isobutyl-1-methylxanthine, have also been found to stimulate both GLP-1 secretion and production in GLUTag cells (Drucker et al. 1994) and are commonly used as positive controls when the secretory activity of this cell line is examined. Supporting the prominent role of intracellular cAMP levels in GLP-1 secretion, it has been reported that phosphodiesterase inhibition enhances the GLP-1 response to physiological secretagogues such as GIP (Simpson et al. 2007). Finally, other nutrient-dependent bioactive peptides, including leptin and

insulin, have also been shown to stimulate GLP-1 secretion from GLUTag cells. The effects of leptin are believed to be produced via activation of the Janus-activated kinase/signal transducers and activators of transcription signaling pathway (Anini and Brubaker 2003), whereas the effects of insulin are mediated through the activation of the extracellular signal-regulated kinases and are dependent upon the presence of glucose (Lim et al. 2009).

21.4 Relevance to the Human Situation (L-Cell In Vivo)

Despite the rodent origin of GLUTag cells, they are the most extensively used in vitro model for the study of GLP-1 release. They have been demonstrated to respond to the same stimuli that trigger GLP-1 secretion in humans and they have been used to test potential therapeutic agents (i.e. GPR119 agonists). The difficulties in obtaining primary L-cell cultures from humans, as well as the homogeneity of the GLUTag cells (i.e. single-cell clone) as compared to other L-cell models, have made GLUTag cells an invaluable tool for the characterization of the intra-cellular pathways that govern GLP-1 secretion.

21.5 General Protocol

21.5.1 Maintenance of the GLUTag Cell Line

GLUTag cells are routinely grown in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10 % fetal bovine serum (FBS), whereas supplementation with antibiotics, normally penicillin and streptomycin, is optional but not required. Cells must be trypsinized and passaged every 3–4 days when they reach 60–80 % confluence. The presence of floating cells may be indicative of contamination or overgrowth. There is no standard glucose concentration to grow the cells but a final concentration within the range 5–25 mmol/L is commonly used. It has recently been reported that cells grown with 25 mmol/L of glucose show increased reactive oxygen species production, upregulated *proglucagon*, prohormone convertase 1/3 and glucokinase content, and elevated basal secretion of GLP-1 as compared to cells maintained under 5 mmol/L conditions, suggesting enhanced metabolic activity. However, no difference in cell viability was found between the two conditions, and the cells grown with 25 mmol/L glucose were more resistant to a further metabolic insult (Puddu et al. 2014). Moreover, cells grown at either a low (5 mmol/L) or high (25 mmol/L) concentration of glucose have been demonstrated to respond to stimuli in a similar way, and no differences in GLP-1 secretion or production were found in cells exposed to low or high glucose for 2 h (Reimann and Gribble 2002), which is the standard incubation period to examine the GLUTag cells secretory response to stimuli.

21.5.2 Performance of Secretion and Expression Assays

To study the effects of different treatments on GLP-1 release (secretion assays), cells can be cultured in 6- or 24-well plates coated with either poly-D-lysine or Matrigel® to avoid detachment of cells during the washes. A 2-day period between plating and the secretion assay is recommended in order to allow the cells to recover from trypsin and to reach the proper size and confluence. When the cells are too confluent (over 80 %), they tend to grow on top of each other, forming clumps. This should be avoided since it is associated with higher basal levels of secretion (personal observations). During the test period of 2 h, GLUTag cells should be maintained in either FBS-free DMEM or DMEM with a very low concentration of FBS (0.5 %), containing the treatments tested at different doses; buffers such as Krebs–Ringer are also acceptable. To study the effects of treatments on gene expression or protein content, larger surface areas, such as 10 cm culture plates, may be required and longer incubation periods are recommended.

A recent study has shown that GLUTag cells possess an endogenous metabolic clock and that the activity of the cells can be synchronized after overnight incubation with a low concentration of FBS (0.5 %) followed by a 1-h shock with 10 % FBS and 10 $\mu\text{mol/L}$ forskolin (Gil-Lozano et al. 2014). Synchronized GLUTag cells show a rhythm in clock genes expression as well as in their GLP-1 response to secretagogues, with higher responses at 4 h after synchronization and lack of effect at 16 h. This protocol may be adopted to obtain maximum secretory responses to a particular treatment.

21.6 Assess Viability

Some treatments can affect viability in GLUTag cells, inducing abnormally high levels of GLP-1 secretion that can lead to misinterpretation of the results. Both the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and the neutral red uptake assays have been routinely performed in our laboratory to test the effects of new treatments on cell viability. Both are colorimetric assays and a reduction in absorbance corresponds with loss of cell viability. These tests are not quantitative and the addition as a reference of a positive control, such as hydrogen peroxide, is required.

21.7 Experimental Readout of the System

In secretion assays, GLP-1 content in medium and cells should be determined independently. Both medium and cell lysate should be collected and acidified to ensure preservation of peptides and facilitate the subsequent purification step. Our laboratory

routinely extracts peptides from the medium and cell lysate by reversed-phase adsorption to C18 silica columns after acidification with trifluoroacetic acid. GLP-1 levels can then be determined by radioimmunoassay, ELISA or alternative quantification methods. GLP-1 secretion must be calculated as the total GLP-1 content of the medium divided by the total GLP-1 content of medium plus cells. A ratio of secretion is then obtained, which can be normalized to the percentage of secretion of the control group. A two- to fourfold increment in secretion can be found for the most potent secretagogues, such as the combination of forskolin and 3-isobutyl-1-methylxanthine, which are often included in the study as positive controls.

In expression assays, medium is discarded and cells are scraped in a solution containing lysis buffer and β -mercaptoethanol to inactivate RNAses. Our laboratory has found that extraction of RNA using RNeasy Plus Mini Kit with QIAshredder (Qiagen, MD) permits collection of high quality RNA with a 260/280 nm ratio over 2 and 30–100 μ g of RNA per 10 cm plate. In real-time PCR analysis involving cDNA derived from GLUTag cells, *H3f3a* amplicon (H3 histone gene) is strongly recommended as an internal control, since it shows much better consistency than other primers commonly used as housekeeping genes, such as *18S* amplicons (personal observations). For protein extraction, medium is discarded and cells are scraped in a lysis buffer such as RIPA (radioimmunoprecipitation assay) and then sonicated. As much as 100 μ g of protein per well can be collected from a 12-well plate.

21.8 Conclusions

Since the original generation of the GLUTag cell line in 1994 by Drucker, Brubaker and colleagues, these cells have been instrumental in the studies addressing the role of nutrients in L-cell physiology, and particularly in GLP-1 secretion. The easy maintenance of the cells, their close similarity in phenotype with non-immortalized L-cells and the ability to apply advanced techniques such as patch-clamp recording and gene silencing are clear advantages for the use of this cell line. Yet, as is common for many tumoural cell lines, some phenotypic differences are found between GLUTag cells and the normal L-cell, notably, the former presenting a higher diversity of glucose-sensing mechanisms and, apparently, insensitivity to SCFAs. Nevertheless, the use of GLUTag cells has been crucial to develop potential novel therapies for diabetes, such as GPR119 agonists. It is highly likely that GLUTag cells will continue to be intensively used as a model of the enteroendocrine L-cell until the isolation of primary L-cells becomes an easier and more standardized procedure.

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