Chapter 20
NCI-H716 Cells

Jeffrey Gagnon and Patricia L. Brubaker

Abstract The endocrine response to nutrient ingestion is vital to the maintenance of energy homeostasis in the body. Glucagon like peptide-1 (GLP-1) is one such hormone that is released from L-cells of the distal small intestine and colon in response to meal ingestion. GLP-1 acts on various systems in the body to enhance glucose-stimulated insulin secretion, delay gastric emptying and promote satiety. As such, elevating the levels of active GLP-1 in the circulation, as well as enhancing GLP-1 bioactivity, is the basis of several recent anti-diabetic medications. Gaining an understanding of how GLP-1 secretion is regulated at the cellular level requires in vitro L-cell models. NCI-H716 is a cell line derived from ascites fluid of a colorectal adenocarcinoma from a 33 year old Caucasian male. This cell line is currently the only human model available for the in vitro study of GLP-1 regulation and is the topic of the following chapter. This chapter will cover the origin, characteristics and methods for using this model. Comparisons are then made between other available in vitro GLP-1 models.

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

20.1 Introduction

Glucagon like peptide 1 (GLP-1) is a key hormone in the regulation of nutrient metabolism. Its release from intestinal enteroendocrine L-cells is stimulated by the ingestion of nutrients (both directly, through luminal nutrient sensing, and indirectly, via parasympathetic innervation). The L-cells are distributed primarily in the distal

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small intestine and colon. Once released into the circulation, GLP-1 acts to promote satiety as well as the storage of ingested nutrients. A well-established and pharmacologically-targeted aspect of GLP-1 action is its role in potentiating glucose-stimulated insulin secretion from the β cells of the pancreas. This action creates a larger insulin response to ingested glucose as compared to isoglycemic levels of glucose delivered intravenously, and is known as the incretin effect. The GLP-1 responses to various dietary nutrients and their respective intracellular mechanisms of action on the L-cell have been, and continue to be, intensively studied using in vitro GLP-1-secreting cell models. One such model is the human colonic cell line, NCI-H716. This review will describe the origin, utility and associated methods of use of this cell line in the study of GLP-1 biology.

20.2 Origin

NCI-H716 cells were one of several colorectal cancer cell lines originally described in 1987 (Park et al. 1987). This particular cell line was developed from cells harvested in ascites fluid of a 33-year old Caucasian male with colorectal cancer. Interestingly, of the 14 cell lines described in this study, NCI-H716 was the only line that contained dense-core granules, which are characteristic of endocrine cells. While it was unclear as to the contents in these granules at the time of discovery, later work in several laboratories elucidated the hormones produced by these cells. Early studies with these cells demonstrated the expression of both the secretory granule marker, chromogranin A, and mucin, as well as receptors for gastrin, somatostatin and serotonin (de Bruine et al. 1992). This not only confirmed that these cells had an endocrine phenotype but suggested that they were also sensitive to other gastrointestinal hormones. After several years of being used as model of tumour/endocrine differentiation, Reimer et al. demonstrated both expression and regulated secretion of glucagon-like peptide-1 (GLP-1) by these cells (Reimer et al. 2001). In that study, NCI-H716 cells were found to secrete high levels of active GLP-1 in amounts that could be easily measured by collecting cell culture medium and cell lysates. They also showed that nutrients, including fatty acids and protein hydrolysate, dose-dependently stimulated GLP-1 secretion by these cells (Reimer et al. 2001). Following this, work by our group elucidated roles for several hormones (Anini and Brubaker 2003b; Lim et al. 2009), neurotransmitters (Anini and Brubaker 2003a), fatty acids (Lauffer et al. 2009) and anti-diabetic medications (Mulherin et al. 2011) in the regulation of GLP-1 secretion. To date, the NCI-H716 cells remain the only characterized model of the human intestinal L-cell.

20.3 Features and Mechanisms

NCI-H716 cells are considered pseudo-diploid with an average chromosome count of 61 (Park et al. 1987). Electron micrographs shown in this initial study clearly demonstrate many dense-core granules. In culture, these cells appear spherical and

undifferentiated, and they grow in suspension, occasionally forming clumps. Plating the cells on a basement membrane-coated surface allows the cells to attach and grow horizontally. Although this plating strategy was initially reported to allow the cells to become differentiated towards an endocrine lineage (Reimer et al. 2001), studies by our laboratory and other groups have indicated that plating does not change the levels of GLP-1 in these cells (Anini and Brubaker 2003a; Cao et al. 2003).

Anini and Brubaker demonstrated expression of the leptin receptor in NCI-H716 cells by western blot and fluorescent immunocytochemistry (Anini and Brubaker 2003b). This study further showed that leptin stimulates GLP-1 secretion by these cells, and that the hyperleptinemia that occurs during obesity leads to leptin resistance and impaired GLP-1 secretion in this condition. In that same year, Anini and Brubaker also demonstrated expression of the M1, M2 and M3 muscarinic receptors in the NCI-H716 cells by western blot and fluorescent immunocytochemistry (Anini and Brubaker 2003a). Herein, they also established a role for M1 muscarinic regulation of GLP-1 secretion. Shortly after, Lim et al. showed that NCI-H716 cells express the insulin receptor, by semi-quantitative reverse transcriptase PCR and fluorescent immunocytochemistry (Lim et al. 2009). They further demonstrated that, while insulin was able to stimulate GLP-1 secretion acutely, extended pre-incubation with insulin led to a loss in the ability of these cells to respond to other GLP-1 secretagogues. Hence, the hyperinsulinemia that occurs in patients with type 2 diabetes mellitus (T2DM) may also result in impaired GLP-1 secretion.

The ability of the L-cell to respond to luminal nutrients is a vital component of the incretin system. GLP-1 regulation by ingested nutrients through specific receptors has been demonstrated using the NCI-H716 cell line. In the initial NCI-H716 and GLP-1 secretion study, the authors showed that meat hydrolysate stimulated GLP-1 release (Reimer et al. 2001). Several years later, they also demonstrated mRNA expression of several amino acid transporters by these cells, including Y^+LAT2, ASCT2, and ATA-2 (Reimer 2006).

Luminal glucose sensing by the L-cells has also been demonstrated through the sweet taste receptor system. NCI-H716 cells express several of the type 1 taste receptors and α-gustducin, which is a key component of the taste transduction pathway (Jang et al. 2007). In this study, knockdown of α-gustducin prevented the glucose-stimulated GLP-1 response by NCI-H716 cells. While this study and others (Zhang et al. 2012) have demonstrated that glucose can stimulate GLP-1 secretion from NCI-H716 cells, the presence of a glucose transporter has yet to be demonstrated.

Lipid sensing by the NCI-H716 cell line has been demonstrated by several groups (Chen et al. 2012; Lauffer et al. 2009). Expression of the monoacylglycerol-sensing G-protein coupled receptor, GPR119, by RT-PCR in NCI-H716 cells was shown by Lauffer et al. (2009). While examining the effects of milk products on GLP-1 secretion, Chen et al. also demonstrated expression of fatty acid transport protein 4 in the NCI-H716 cells by quantitative RT-PCR (Chen and Reimer 2009).

The above studies clearly indicate that NCI-H716 cells possess many of the receptors and intracellular machinery required to sense hormones, neurotransmitters and nutrients, and to release GLP-1 in response to these agents.

20.4 Stability/Consistency/Reproducibility

The increasing use of the NCI-H716 cells as a model of the human intestinal L-cell necessitates an examination of the consistency of findings between different research groups using these cells. In terms of GLP-1 secretion, many groups now present secretion data relative to an untreated control, making interpretation of actual percent secretion difficult. However, early studies with the NCI-H716 cells demonstrated percent secretion to be in the range of 1–15 % of the total well/plate content of GLP-1 over a 2 h incubation period (Reimer et al. 2001; Anini and Brubaker 2003a; Lim et al. 2009; Lauffer et al. 2009). Regarding the error in replicates of GLP-1 secretion, these studies report standard error to be ~10 % (of the mean).

Some groups have examined the effect of similar treatments on GLP-1 secretion. Anini et al. found that the protein kinase C activator, phorbol 12-myristate 13-acetate (PMA) caused a 2.5-fold stimulation in GLP-1 secretion (Anini and Brubaker 2003a), whereas Reimer et al. saw a 4.3-fold stimulation with the same dose and duration of incubation (Reimer et al. 2001). While both studies showed a robust stimulation of GLP-1 secretion, the difference in magnitude may be due to differences in media/buffers used during the secretion experiment or in sample preparation (both subjects of further discussion below).

20.5 Relevance to the Human L-Cell In Vivo

Since NCI-H716 cells were derived from a human sample, they are of particular significance to the study of human GLP-1 secretion. However, while these cells do secrete GLP-1 in response to nutrients, as found in vivo, it must be recognized that they are derived from a tumour cell which was likely to have been relatively undifferentiated initially. Furthermore, one study examined the ability of NCI-H716 cells to regulate expression of reporter plasmids encoding human and rat *proglucagon* (i.e. the GLP-1 prohormone gene). Surprisingly, no basal promoter activity was observed even in the presence of several positive regulators of rodent *proglucagon* gene expression (Cao et al. 2003). In addition, compounds that are known to enhance *proglucagon* gene expression in rodent models failed to stimulate the endogenous human *proglucagon* gene in NCI-H716 cells. These findings suggest that either the NCI-H716 cells do not regulate *proglucagon* gene expression normally or that there are marked species-specific differences in the regulation of *proglucagon* expression.

20.6 General Protocol

20.6.1 Cell Maintenance Protocol

NCI-H716 cells are normally grown in suspension culture. Our group uses Roswell Park Memorial Institute (RPMI) media containing L-glutamine and Phenol Red. This media is further supplemented to contain 10 % fetal bovine serum (FBS) and

100 units/ml each of penicillin and streptomycin ('complete RPMI'). Cells can be grown in suspension in cell culture flasks and should be kept at a density of ~500,000 cells/ml to ensure a rapid growth rate; lower densities may cause stalled growth. Under these conditions, cells double every ~3 days and should be passaged once a week. To passage, cells do not require any enzymatic digestion; any small clumps of cells are simply dissociated using repeated serological pipetting.

20.6.2 Experimental Protocol for Test Compounds

As NCI-H716 cells require a basement membrane matrix coating to attach to cell culture plates, preparation must occur before seeding cells for an experiment. Matrigel (BD Biosciences) is provided as a concentrate with lot-dependent concentration. It is stored frozen and gels at 15 °C. Matrigel is therefore thawed on ice (stock aliquots of the concentrate can be thawed overnight on ice in a 4 °C fridge, as per manufacturer's directions). Plates or wells are then coated with 0.5 g/ml Matrigel diluted in ice-cold sterile Hanks Buffered Saline Solution (HBSS), with the area of the surface being coated dictating how much diluted Matrigel is required (generally ≥200 µl/well in a 24-well plate, ≥ 800 µl/well in a 6-well plate, etc.). After allowing the Matrigel to gel for 1 h at room temperature, the plates/wells are then rinsed with HBSS and allowed to dry for 30 min; the surface is then ready for seeding of the cells. Cells should be reconstituted in complete RPMI to ~200,000 cells/ml (1 ml for each well in a 24-well plate, 2 ml for each in a 12-well plate, etc.). This density is optimized to allow ~2 days of growth before experimental treatments. On the day of treatment, cells are rinsed with low-serum RPMI media (i.e. 0.5 % FBS) then incubated with treatments in low-serum RPMI. For GLP-1 secretion studies, 2-h of incubation time is ideal as any possible effects on de novo GLP-1 synthesis are unlikely to be observed in this relatively short time-frame. Over a 2-h incubation period, a 24-well plate treated with vehicle (control) will have ~300 pg of GLP-1 (total) per well in the collected media, with ~7,000 pg of GLP-1 in the cell lysate. Based on these values, the percent secretion (i.e. pg in media/(pg in media + cells) × 100) is 4.1 %.

Similar plating densities can be used for RNA and protein extraction experiments. A larger surface area can be used for such studies (i.e. 6-well, 10-cm), depending on expression levels for the mRNA transcript or protein of interest. From a 6-well plate containing ~800,000 cell/well, ~20 µg of RNA and ~60 µg of protein can be collected.

20.7 Assess Viability

To ensure treatments do not affect cell health or viability, standard tests should be employed. These tests include the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium (MTT) assay and neutral red uptake assay. Both of these tests can be done within a few hours of an experimental treatment, and should include a positive control, such as hydrogen peroxide, to ensure that the assay is reliable.

20.8 Experimental Readout

Sample processing from NCI-H716 cells is dependent on the nature of the experiment being conducted. Samples collected for GLP-1 secretion assay, as described in the experimental protocol above, are processed as follows.

Immediately after the 2-h incubation with treatments is complete, media is collected and centrifuged for 5 min at 1,000×*g* and 4 °C to remove any floating cells. This media is then decanted into a new tube containing 1 % trifluoroacetic acid (TFA) to make a final TFA concentration of 0.1 %. This acidic environment prevents protease activity and prepares the sample for the subsequent peptide purification step. The culture plate with the cells is simultaneously placed on ice and then 1 ml of ice-cold acidic cell lysis solution (i.e. 1 % TFA, 1 N hydrochloric acid, 5 % formic acid, and 1 % NaCl) is added. The cells are scraped and wells are rinsed with an additional volume of lysis buffer. These cell lysates are then sonicated, pelleted by centrifugation, and the supernatants collected on ice. However, of note, media and cells may be collected in a variety of different buffers, as indicated by the desired assay protocol, as long as proteolysis is prevented.

Depending on the method of hormone assay, samples may require additional purification. Using a total GLP-1 radio-immuno assay (RIA; i.e. Millipore), we have found that additional purification by C18 solid phase peptide extraction removes salts and proteases that may interfere with the assay. Peptides are thus purified using Sep Pak C18 Classic columns (Waters Associates) with 0.1 % TFA water (Solution A) and 0.1 % TFA/80 % isopropyl alcohol (Solution B), as follows: (1) 4 ml Solution B (wetting/clearing); (2) 4 ml Solution A (equilibrating); (3) sample loading; (4) 4 ml Solution A (washing); and (5) 4 ml Solution B (sample elution). A portion of the total 4 ml peptide eluate is then dried under vacuum in preparation for the RIA. Once samples have been purified using this method, they can be sealed and stored at −20 °C for later analysis.

Media and cell GLP-1 content determined from the assay can be used to calculate percent GLP-1 secretion, as described above. These percent secretion values may differ slightly from 1 week to the next due to inter-assay variability. To control for differences in percent secretion, treatment groups are often normalized to a vehicle-treated control; as such, data often appears “relative to control” in papers examining GLP-1 secretion, although absolute values should always be included in the publication to facilitate comparison between studies. The use of an appropriate control in each experiment is critical. The control treatment group must thus contain the solvent in which the treatment group is dissolved at an identical final concentration. This may include dimethyl sulfoxide, ethanol or a buffer solution. In addition, experiments should also be designed to include a positive control for GLP-1 secretion. Strong activators of cAMP signaling, such as forskolin, have thus been used by several groups to demonstrate cell responsiveness to a known secretagogue in parallel with experimental treatment groups.

In GLP-1 secretion experiments with NCI-H716 cells, at least three biological replicates should be produced for each treatment and control group. Additionally, any given experiment should be repeated at least twice to ensure statistical power for subsequent analysis, thereby constituting a minimum of six replicates.

For RNA and protein extraction, standard methods apply. For RT-PCR and qRT-PCR studies, the target gene of interest should be determined relative to an internal control, such as 18S ribosomal RNA. However, quantification of appropriate reference genes for a particular treatment must also be done to ensure that the internal control does not change in response to the experimental treatment. Similarly, protein expression analysis by immunoblot should also include a suitable validated control, such as actin, for protein loading.

20.9 Conclusions

The ability of the L-cell to respond to nutrient, hormonal and neurotransmitter cues is vital in the regulation of GLP-1 secretion and, ultimately, glucose homeostasis. Strategies targeting the GLP-1 system in humans have garnered great interest and application in the treatment of T2DM (Drucker 2011). Cell systems that enable a direct mechanistic examination of GLP-1 regulation have been and continue to be essential tools in developing novel GLP-1 therapies. The NCI-H716 cell line is the only human cell line currently available to study GLP-1. This places it in an important position to relate the effects of various treatments to human biology, although experimental effects should always be cross-examined through the use of additional in vitro and in vivo models to strengthen the hypothesis. The NCI-H716 cells are thus a useful tool to study the control of GLP-1 release and the underlying cell signaling pathways as well as, potentially, *proglucagon* gene expression. Overall, they are an excellent model to investigate the regulation of human GLP-1.

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