***In-vitro* GLP-1 Release Assay Using STC-1 Cells**

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[Abstract] Enteroendocrine cells (EECs) are known chemosensors in the gastrointestinal (GI) epithelium. They release a diversity of gut hormones in response to various stimuli. Here, we report an in-vitro assay to measure GLP-1 release from cultured murine EEC's under fatty acid stimulation.

Keywords: Enteroendocrine cells, L-cells, STC-1, Docosahexaenoic acid (DHA), Glucagon-like peptide-1 (GLP-1)

[Background] The intestinal epithelium is the largest interface between the human body and the environment. This interface consists of a single epithelial layer containing several distinct cell-types with unique functional roles. Differentiated from LGR5-positive epithelial stem cells, enteroendocrine cells (EECs) are scattered throughout the epithelium along the entire GI tract. EECs play a critical role in responses to nutrient and other stimuli by producing and secreting a variety of hormones. Traditionally, these cells are classified by their hormonal profile (Nausheen *et al.*, 2013; Latorre *et al.*, 2016; Verhoeckx *et al.*, 2015a), although with the advent of single cell RNA-sequencing, we have come to appreciate that most of these cells secrete multiple hormones (Haber *et al.*, 2017; Billing *et al.*, 2019). A subtype of EECs, L-cells, are defined by the production of glucagon-like peptides 1 and 2 (GLP-1 and GLP-2), and may also secrete peptide YY (PYY) and insulin-like peptide 5 (INSL5). Most L-cells are found in the distal ileum and colon, while some are also found proximally in the duodenum and jejunum. The receptors on the surface of L-cells allow them to directly sense luminal components and produce appropriate hormone responses. The most notable receptors of nutrients and food components on L-cells are the classic taste receptors, free fatty acid receptors and receptors for protein and their products (Drucker and Nauck, 2006; Furness *et al.*, 2013; Spreckley and Murphy, 2015; Gribble and Reimann, 2016; Parada Venegas *et al.*, 2019). L-cells can also sense LPS through expressing Toll-like receptors (TLRs) (Lebrun *et al.*, 2017). By producing hormones, L-cells are able to stimulate carbohydrate uptake and insulin release, slow down intestinal transit and regulate appetite.

To study the role of L cells in-vitro, numerous human and rodent mouse cell lines have been derived. The NCI-H716 cell line, the only human EEC line, was derived from a spontaneous colorectal tumor. The NCI-H716 cell line produces GLP-1, PYY and mucin and is thought to represent distal L-cells. They

require a basement membrane to attach to culture plates and only 30% of NCI-H716 cells produce PYY, thus limiting their use in routine *in-vitro* experiments (Park *et al.*, 1987; Verhoeckx *et al.*, 2015b). To overcome the limitations posed by this cell line, non-human EECs have been derived. Notably, GLUTag and STC-1 are of great utility in the study of GLP-1 secretion. The GLUTag cell line is a single-cell subcloned population of L-cells, established from a large bowel tumor in proglucagon-SV40 Large T antigen transgenic mice. They are easy to transfect and secrete GLP-1 and CCK. Like the NCI-H716 cells, GLUTag cells also need attachment matrices (Chang *et al.*, 1994; Drucker *et al.*, 1994; Verhoeckx *et al.*, 2015b). In contrast to GLUTag cells, the STC-1 cell line was established from a secretin producing tumor, which arose in the duodenum of double transgenic mice expressing proinsulin-SV40 Large T antigen/proinsulin-polyoma x Small T antigen. Like native intestinal L-cells, STC-1 cells produce GLP-1, CCK, GIP, PYY, pancreatic polypeptide, neurotensin, GLP-2 and oxyntomodulin, in varying amounts (Rindi *et al.*, 1990; Verhoeckx *et al.*, 2015b). They are easy to culture without the need for an attachment matrix. These features make STC-1 cells a good model to study hormonal secretion mechanisms.

Like EECs of the large intestine, STC-1 cells harbor a variety of nutrient receptors. Prior work has demonstrated that various substances binding to specific cell-surface receptors on STC-1 cells results in hormone secretion (McCarthy *et al.*, 2015). Notably, fatty acids elicit strong CCK secretion from STC-1 cells, thus they have been extensively used to study CCK secretion dynamics. However, few studies have focused on fatty acid induced GLP-1 secretion or have failed to address variation in cellular density (McLaughlin *et al.*, 1998; Sidhu *et al.*, 2000; Benson *et al.*, 2002; Hirasawa *et al.*, 2005). Here we describe a method to measure GLP-1 secretion from STC-1 cells upon fatty acid stimulation.

Materials and Reagents

1. 10 ml Glass bottles
2. 50 ml Glass beaker
3. 5 ml Glass pipettes (Corning, catalog number: 7077-5N)
4. Capillary Micropipettes (Drummond Scientific Company, catalog number: 5-000-2005)
5. 6-cm dishes (Corning, catalog number: 430166)
6. 10 ml and 50 ml sterile plastic pipettes with filter (Corning, catalog numbers: 4488, 4490)
7. Sterile cell lifts (Corning, catalog number: 3008)
8. 1.5 ml microcentrifuge tubes (Fisherbrand, catalog number: 05-408-129)
9. 1.5 ml disposable cuvettes (BRAND, catalog number: 759076D)
10. STC-1 cell line (ATCC, catalog number: CRL-3254™)
11. Phosphate-buffered saline (Sigma, catalog number: D8537)
12. Trypsin (Corning, catalog number: 25-053-CI)
13. DMEM, High Glucose (Hyclone, catalog number: SH30022.02)
14. Fetal Bovine Serum (Gibco, catalog number: 35050-061)
15. Enrofloxacin (Sigma, catalog number: 17849-5G-F)
16. Docosahexaenoic acid, DHA (Nu-chek-policy, U-84-A DHA, 1GM), DHA comes as a solution

17. Pure ethanol (Pharmco Products, catalog number: 111000200C1GL)
18. Nitrogen gas
19. HEPES (Sigma, catalog number: H3375)
20. NaCl (Fisher Scientific, catalog number: BP358-10)
21. KCl (Sigma, catalog number: P9333)
22. CaCl₂ (Sigma, catalog number: C1016)
23. MgCl₂ (Sigma, catalog number: M8266)
24. EDTA (OmniPur, catalog number: 4050)
25. Triton X-100 (Fisher Scientific, catalog number: BP151-500)
26. Tris-base (Fisher Scientific, catalog number: BP152-5)
27. PMSF (MP biomedical, catalog number: 195381)
28. Halt™ Protease and Phosphatase Inhibitor Cocktail (100x) (Thermo Fisher Scientific, catalog number: 78444)
29. Sterile water
30. Protein assay dye reagent concentrate (Bio-Rad, catalog number: 5000006)
31. High sensitive glucagon-like peptide(GLP-1) active chemiluminescent ELISA kit (Millipore, catalog number: EZGLPHS-35K)
32. 10 mg/ml DHA stock solution (see Recipes)
33. HEPES buffer (see Recipes)
34. 100 μM DHA Stimulation buffer (see Recipes)
35. Cell lysis buffer (see Recipes)
36. 100 mM PMSF stock solution (see Recipes)

Equipment

1. Portable Pipet-Aid® XP Pipette Controller (Drummond Scientific Company, catalog number: 4-000-101)
2. Tissue culture incubator
3. Refrigerated microcentrifuge (Eppendorf, model: 5424R)
4. Sonicator (Branson 250 Sonifier)
5. ELISA plate reader (TECAN, Sunrise absorbance microplate reader)

Procedure

A. Prepare 10 mg/ml docosahexaenoic acid (DHA) stock solution (see Recipe)

1. Dilute DHA solution with pure ethanol at 10 mg/ml.

Note: DHA will stick to plastic material. Always remember to use glass pipettes when you work with fatty acids.

2. Aliquot DHA stock solution into small glass bottles, 10 ml each.

3. Ambient air above the solution should be displaced to avoid oxidation of DHA by priming pure nitrogen gas on the surface of the solution prior to closing the container.
4. Store at -20 °C.

B. DHA Stimulation assay

1. Seed 2×10^6 STC-1 cells in 6-cm dishes two days prior to experiment in duplicates. Culture the cells in tissue culture incubator at 37 °C, 5% CO₂, using DMEM with 10% FBS and 20 ng/ml enrofloxacin.
2. On experimental day cells should be 80% confluent.
3. Aspirate media and wash the cells twice with 3 ml of HEPES buffer.
4. Add 3 ml of HEPES buffer into each dish.
5. Put cells back to tissue incubator for 30 min. The lack of nutrients in the buffer is meant to serve as a 'starvation' period prior to nutrient stimulation.
6. Prepare 100 µM DHA stimulation buffer.
 - a. To prepare 20 ml of 100 µM DHA stimulation buffer, add 66 µl of 10 mg/ml DHA (30.4 mM) stock solution into a glass beaker using glass capillary micropipettes. Then add 20 ml of HEPES buffer.
 - b. Sonicate the solution immediately at output level 5 and constant duty cycle for 3 min prior to stimulation to prevent DHA precipitation. If using a different sonicator, set the amplitude as 40%. After sonication, the solution should be clear, with minimal bubbling.

Note: Always prepare this DHA solution freshly, just prior to use.

7. Aspirate HEPES buffer gently. Then add 1 ml of HEPES buffer into each control dish and 1 ml of DHA stimulation buffer into each stimulation dish. Put all dishes back to tissue culture incubator for 15 min.
8. Collect 600 µl of media from each dish and put into a fresh 1.5 ml microcentrifuge tube, then place them on ice.
9. Add 0.6 µl of 100 mM PMSF (see Recipe) into the 600 µl of media at final concentration of 100 µM.
10. Spin the microcentrifuge tubes at 850 x g for 5 min, at 4 °C.
11. Transfer 500 µl of media into a fresh tube.

Note: Samples are now ready for ELISA measurement. One can also store samples at -80 °C up to two weeks.

12. Measure GLP-1 concentration in samples by ELISA kit according to manufacturer's instructions. Make sure to set the standard as suggested by the manufacturer.

C. Lyse the cells in the culture and measure protein concentration from all dishes by Bradford assay

Note: This is meant to account for differences in the number of cells in each dish in these experiments.

1. Carefully remove any residual HEPES buffer in each dish.

2. Add 1 ml of cold PBS into each dish.
3. Scrape cells down very carefully using a cell lift.
4. Collect cells into a 1.5 ml microcentrifuge tube and put on ice.
5. Spin the tubes at 850 x g for 5 min, at 4 °C.
6. Aspirate supernatants, then add 50 µl of cell lysis buffer and pipette up and down gently to homogenize. Put tubes on ice.
7. Lyse the cells for 20 min. Vortex every 5 min.
8. Spin the tubes at 21,130 x g for 15 min, at 4 °C.
9. Transfer supernatants into a fresh tube.
10. Measure protein concentration using protein assay dye reagent concentrate according to manufacturer's instructions.

Data analysis

Calculate measured GLP-1 concentration of each sample appropriately from three independent experiments, duplicates in each experiment. Then normalize the value to its corresponding protein concentration value as measured by Bradford assay (Table 1). Use any software package with 4-parameter logistic regression for ELISA analysis.

Table 1. Adjusted GLP-1 concentration

	Adjusted GLP-1	Experiment	No FA (average GLP-1)	54.63834	
No FA Parental	56.75393031	1	DHA (average GLP-1)	492.2011	
No FA Parental	55.23913626	2			
No FA Parental	51.92193876	3		No FA	DHA
DHA 100 Parental	439.799523	1	SD	2.471388	137.8699
DHA 100 Parental	388.2149664	2	SEM	1.426856	79.5992
DHA 100 Parental	648.5889426	3	P value	0.005341	

Notes

Using healthy cells is critical for experiments to work. One should check cell growth status and mycoplasma routinely. Always remember to fill DHA stock solution with nitrogen and prepare stimulation buffer fresh prior to use. If one freezes samples at -80 °C, do not freeze and thaw repeatedly.

Recipes

1. 10 mg/ml DHA stock solution
 - a. 600 µl of DHA liquid coming from the manufacturer (1 g, 1.2 ml in total)

- b. 49.4 ml of 100% pure ethanol
 - c. Aliquot into small glass bottles and fill with nitrogen gas. Store at -20 °C
2. HEPES buffer
 - 20 mM HEPES, pH 7.9
 - 140 mM NaCl
 - 4.5 mM KCl
 - 1.2 mM CaCl₂
 - 1.2 mM MgCl₂
3. 100 µM DHA Stimulation buffer
 - 66 µl of 10 mg/ml DHA stock solution
 - 20 ml of HEPES buffer
 - Sonicate the solution at output level 5 constant duty cycle for 3 min prior to use
4. Cell lysis buffer
 - 25 mM HEPES, pH 7.9
 - 100 mM NaCl
 - 1 mM EDTA
 - 10% Glycerol
 - 1% Triton X-100
5. 100 mM PMSF stock solution
 - 174.2 mg PMSF
 - 10 ml of 100% ethanol

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Competing interests

The authors declare no competing interests.

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