Physiological gut oxygenation alters GLP-1 secretion from the enteroendocrine cell line STC-1

Alina Kondrashina[1], Dmitri Papkovsky[2], Linda Giblin[1]

[1] Food for Health Ireland, Teagasc Food Research Centre, Moorepark, Fermoy, Co.Cork, Ireland

[2] School of Biochemistry and Cell Biology, University College Cork, Cork, Ireland.

Correspondence: Dr. Linda Giblin

Teagasc Food Research Centre, Moorepark, Fermoy, Co.Cork, Ireland, Tel. +3532542614.

(linda.giblin@teagasc.ie)

Abbreviations:

AEBSF, 4-(2-aminoethyl)benzenesulfonyl fluoride hydrochloride; **cAMP**, cyclic adenosine monophosphate; **CCK**, cholecystokinin; **DPP-IV**, dipeptidyl peptidase IV; **FA**, fatty acids; **Fsk**, forskolin; **GI tract**, gastro-intestinal tract; **GLP-1**, glucagon-like-peptide-1; **GPCR**, G-protein coupled receptor; **HIF-1α**, hypoxia inducible factor 1 α; **IBMX**, 1-mathyl-3-(2-mathylpropyl)-7H-purine-2,6-dione; **MW-SPPS**, microwave-assisted solid phase peptide synthesis; **PYY**, peptide YY; **ROS**, reactive oxygen species; **RP-HPLC**, Reversed phase HPLC; **SGF**, simulated gastric fluid; **SIF**, simulated intestinal fluid; **SSF**, simulated salivary fluid; **VAS**, visual analogue scale.

Keywords: Glucagon-Like Peptide-1, intestinal nutrient sensing, physiological gut oxygenation, satiety hormones, simulated digestion.

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Abstract

Scope: Enteroendocrine cell lines are routinely assayed in simple buffers at ~20% oxygen to screen foods for bioactives that boost satiety hormone levels. However, i*n vivo*, enteroendocrine cells are exposed to different phases of food digestion and function at low oxygen concentration ranging from 7.5% in the stomach to 0.5% in the colon-rectal junction.

Methods and results: The objective of this study was to investigate the effect of physiologically relevant O_2 concentrations of the gut on the production and secretion of the satiety hormone, GLP-1, from the murine enteroendocrine cell line, STC-1, in response to dairy macronutrients as they transit the gut. GLP-1 exocytosis from STC-1 cells is influenced by both oxygen concentration and by individual macronutrients. At low oxygen STC-1 cell viability was significantly improved for all macronutrient stimulations and cAMP levels were dampened. GLP-1 secretion from STC-1 cells was influenced by both the phase of yogurt digestion and corresponding O_2 concentration. Atmospheric oxygen at 4.5% combined with upper gastric digesta, which simulates ileum conditions, yields the highest GLP-1 response.

Conclusion: This demonstrates the importance of considering physiological oxygen levels and food digestion along GI tract for reliable *in vitro* analysis of gut hormone secretion.

1 Introduction

Healthy foods that increase feeling of fullness may reduce food intake and therefore help to manage weight over time. As such there has been considerable scientific interest in screening for food bioactives that modulate satiety signals in the gut. To be physiologically relevant, such *in vitro* screening platforms must closely resemble *in vivo* conditions.

Specialised endocrine L cells in the gastro-intestinal (GI) tract respond to food ingestion by secreting a range of satiety hormones, including Glucagon-like peptide-1 (GLP-1), cholecystokinin (CCK) and peptide YY (PYY) [1, 2]. GLP-1 has effects on the ileal brake, gastric emptying, the hypothalamic circuits that regulate food intake and insulin secretion via its receptor GLP-1R. Secretion of GLP-1 in the GI tract occurs via elevation of cAMP and/or cytoplasmic Ca^{2+} concentrations, with both of these

signalling pathways highly influenced by macronutrients in contact with L-cells [3, 4]. Some studies show that carbohydrates are the main stimulants of GLP-1 secretion, whereas other studies favour proteins [5, 6]. Fatty acids (FA) also play an important role in GLP-1 release via the G-coupled protein receptors GPR40 and GPR120 [7, 8]. To date *in vitro* studies of GLP-1 production, secretion and the underlying mechanisms involved have been focused on individual ingredients and macronutrients rather than whole foods or digesta [9].

Enteroendocrine open type L cells are found throughout the length of the GI tract, with their numbers increasing towards the distal gut [10, 11]. As such, these cells are exposed to the lumen contents, from whole foods to digesta and to varying O_2 concentrations. O_2 level drops from 10-7.5% in the stomach to 1.5-0.5% in the colon-rectal junction [12, 13]. Together with nutrient content of the food, stage of food digestion and pathological conditions, O_2 tension most likely plays a significant role in GLP-1 production and secretion [14]. Physiological normoxia (2-5% O_2 in cellular environment) has attracted significant attention recently, as it affects many aspects of cell activities including secretory function as metabolism switches from oxidative phosphorylation to anaerobic glycolysis with subsequent increase in glucose consumption [15-17]. Cell adaptation to the low O_2 is mediated by hypoxia inducible factor 1 (HIF-1). Its α subunit is rapidly degraded at normoxia, however at lower O_2 it stabilises and regulates expression of genes, involved in cellular response [18]. Recently there is evidence that hypoxia decreases levels of *proglucagon* mRNA transcript and secreted GLP-1 in GLUTag cells exposed to glucose and the cAMP stimulator forskolin (Fsk) [19].

The murine and human L cell lines STC-1, GLUTag, NCI-H716, are popular models for studying gut hormone expression and secretion in response to food components [20-22]. However differences do exist with native L cells [23, 24]. These cell lines are routinely tested with intact individual macronutrients as well as monosaccharides, amino acids and fatty acids, at ambient O_2 (~20% in air), and not with food digesta at physiologically relevant O_2 concentrations.

The objective of this study was to investigate the effect of physiologically relevant O_2 concentrations (i.e. atmospheric hypoxia) on the production and secretion of GLP-1 from the murine enteroendocrine cell line, STC-1, in the presence of dairy components as they transit the GI tract. In epidemiological

and intervention studies consumption of dairy products has been associated with short and long term satiety [25] and has demonstrated positive effects on appetite suppression, energy intake and body weight [26, 27]. Fermented dairy beverages and yogurts have the highest satiety by visual analogue scales (VAS) and/or by ad libitum energy intake [28, 29]. There is evidence that the satiety effect of individual milk ingredients is mediated via secretion of GLP-1 [30]. For instance the major milk protein component, casein, bolsters GLP-1 secretion *in vitro* [31, 32] and *in vivo* [33]. Oleic and butyric acids, which represents 23.8% and 4.4% w/w of the total fatty acids in milk, increase GLP-1 exocytosis from the GLUTag cell line and from primary murine L cells [7, 34, 35]. Possible effects of milk disaccharide, lactose, on GLP-1 release have not been investigated but digestion of lactose releases galactose and glucose, which are effective secretory stimulants of GLP-1 [3, 36].

We hypothesised that O_2 availability and hypoxia signalling pathways will impact on GLP-1 exocytosis in response to food, using enteroendocrine cells and dairy macronutrients as *in vitro* model system.

2 Materials and Methods

2.1 Materials

Krebs-Ringer buffer was sourced from Sigma Aldrich (Ireland). 100X Halt Protease and Phosphatase Inhibitor was purchased from Thermo Fisher Scientific (MSC, Ireland). 10X Gel Prep/Running buffer was sourced from Ambion (Applied Biosystems, USA). Mouse Metabolic Magnetic Bead Panel for active GLP-1 (#MMHMAG-44K) was from Millipore (Ireland). RNeasy RNA extraction kit, on-column DNase digestion kit and cell lysis buffer were from Qiagen (UK). Tetro cDNA synthesis kit was from Bioline (MSC, Ireland). LightCycler 480 SYBR Green I Master mix for tPCR was from Roche (Roche Products Ireland Limited). CellTitter One solution reagent and CellTox Green viability assay kit were from Promega (MyBIO, Ireland). Fluo-4 AM calcium indicator was from Molecular probes (UK). Direct cAMP ELISA kit and forskolin were from Enzo Life Sciences (UK). STC-1 was purchased from the American Tissue Culture Collection (ATCC code SD5482, supplied by LGC Standards, Teddington, UK). Casein (sodium caseinate from bovine milk, 95.6% protein on dry

matter) was from Arrabawn (Ireland). Low fat Greek style natural yogurt (2.8g fat, 5.6 g carbohydrates, 5.2 g protein per 100g) was sourced from Tesco, Ireland. Oligonucleotide primers were synthesized by IDT (Belgium). LRF tripeptide was synthesised by microwave-assisted solid phase peptide synthesis (MW-SPPS) and purified using RP-HPLC. All other reagents were from Sigma Aldrich (Ireland). Filter-tubes with threshold 10 kDa were from Satorius (Ireland). Other plastic ware was from Sarstedt (Ireland).

2.2 Cell culture

Murine intestinal neuroendocrine tumour cell line STC-1 was cultured in Dulbecco's Modified Eagle Medium (DMEM) containing 4.5 g/L of glucose and L-glutamine, supplemented with 10% Foetal Bovine Serum (FBS), 100 U/mL penicillin and 100 μg/mL streptomycin. Cells (between passage numbers 25-33) were grown at 37ºC and 5% CO_2 up to a confluence 80-90%, media was changed every 2 days and cells were passaged every 2-3 days.

2.3 STC-1 cell exposure

STC-1 cells were seeded in 6 well plates at concentration 1.5×10^6 cells/well in standard growth media and incubated for 18 hours at 37ºC, 5% CO_2. For cell viability, cytotoxicity and intracellular Ca^{2+} assays, STC-1 cells were seeded at a concentration of 0.1×10^6 cells/mL in 96 well plates. For cAMP accumulation assay, 0.5×10^6 cell/well were seeded in 24 well plates. The following day, media was aspirated and cells were washed once with modified Krebs-Ringer buffer (without bicarbonate but with 10 mM HEPES) and then pre-incubated in the same buffer for 1 hour to acclimatise. Buffer was then aspirated and replaced with 1 mL (0.1 mL for 96 well plate) of macronutrients (100 μM oleic acid, 100 μM butyric acid, 0.5 mg/mL tripeptide LRF, 10 mg/mL casein, 40 mM glucose, 40 mM lactose) prepared in modified Krebs-Ringer buffer. Yogurt and yogurt digesta were diluted in the modified Krebs-Ringer buffer for a final concentration of 10 mg/mL. Phenol red free DMEM, supplemented with 10 mM HEPES was used as a control for cell viability assays. For cAMP accumulation assays, modified Krebs-Ringer buffer was supplemented with 1 mM

3-Isobutyl-1-methylxanthine (IBMX); and 10 μM Fsk was used as a positive control. All non-sterile solutions were filter-sterilised (0.45 μm) prior to cell exposures for 4 hours at 37ºC.

2.4 Hypoxic and normoxic conditions

For the experiments at ambient (~20%) O_2, standard 37ºC incubator was used without CO_2 supply. For experiments under hypoxic atmosphere, cells were placed in a flushing hypoxia chamber (Billups-Rothenberg, Inc., USA), equilibrated at 0.5-7.5% O_2 (verified by Optech® Platinum O_2 sensor, Mocon, USA) and inserted in a 37ºC incubator [37].

2.5 GLP-1 (active) secretion

Following the incubation period, 10 μL of 100X Halt Protease and Phosphatase Inhibitor were used to inactivate endogenous DPP-IV activity, cell supernatants were collected to 1.5 mL tubes and centrifuged at 900 g and 4ºC for 5 min to remove cellular debris. Supernatants were stored at -80ºC prior to quantification of GLP-1 (active) levels. This was performed with Milliplex Map Kit and MagPix fluorescent detection system according to the manufacturer's instructions. Milliplex assay allows detection of active GLP-1 in the range 41-30000 pg/mL, by using 5-parameter logistic curve fitting method and quantifying of samples according to the standard curve.

2.6 RNA extraction and real-time PCR

After 4 hours exposure to macronutrients, STC-1 cell monolayers were washed once with PBS buffer, pelleted, re-suspended in 0.35 mL of lysis buffer and stored at -80ºC prior to RNA extraction. RNA extraction was performed with RNeasy Mini Kit, including on-column DNase digestion, following the manufacturer's instructions. Total RNA was quantified spectrophotometrically, using the Nanodrop 1000 (Thermo Fisher Scientific, USA). RNA integrity was assessed by electrophoresis (BioRad, USA) in a 1.5% glyoxyl agarose gel and glyoxyl buffer. Tetro cDNA synthesis kit was used to prepare cDNA from 1 μg of RNA according to the manufacturer's instructions. Real time PCR was performed with a Light Cycler 96 instrument (Roche Diagnostics, Germany) and LightCycler 480

SYBR Green I Master kit to quantify *proglucagon*, *PYY*, *CCK* and *HIF1a* mRNA levels. All primers were designed across intron-exon boundaries. Primers for murine *proglucagon* (Z46845.1): forward CCTTCAAGACACAGAGGAGAAC; reverse GGAGTCGAGGTATTTGCTGTAG, annealing temperature 56ºC. Primers for murine *CCK* (NM_001284508): forward CTGTCTGCATTTGGCTTGAC; reverse GCCCACTACGATGGGTATTC, annealing temperature 55ºC. Primers for murine *PYY* (NM_145435.1): forward AACTGCTCTTCACAGACGAC; reverse GTGCCCTCTTCTTAAACCAAAC, annealing temperature 55ºC. Primers for murine *ActB* (NM_007393.5): forward TGTGACGTTGACATCCGTAAAG; reverse TCAGTAACAGTCCGCCTAGAA, annealing temperature 51ºC. Primers for murine *HIF1a* (NM_001313919.1): forward CCCATTCCTCATCCGTCAAATA, reverse CCTGTGGTGACTTGTCCTTTAG, annealing temperature 55ºC. Efficiency of amplification was measured by performing PCR for 4 dilutions of cDNA (1:1, 1:10, 1:100, 1:1000) and was in the range of 1.87-2.15. For each PCR reaction 8 μL of pre-mix were mixed with 0.5 μL of each forward or reverse primer and 1 μL of cDNA. Inter-assay and intra-assay variation was less than 5%. For each sample, the relative amount of target was calculated by the $2^{-\Delta\Delta C}{}_T$ method, where $\Delta\Delta C_T = (C_{T,\ Target} - C_{T,\ Actin})_x - (C_{T,\ Target} - C_{T,\ Actin})_0$, where 0 corresponds to the control conditions and x - to experimental conditions.

2.7 Cell viability and cytotoxicity assays

After 2 hours of cell exposure, 20 μL of Cell Titter One solution were added to wells, and STC-1 cells were incubated for another 2 hours. Absorbance at 490 nm in each well was recorded with Synergy plate reader (BioTek, USA) and viability was calculated as percentage of positive control, which was measured in supplemented with 10 mM HEPES DMEM at 20% O_2. For cytotoxicity assay, CellTox™ Green compound was added directly to the cell exposure solution (1:500 dilution in 50 μL per well) and fluorescence signal (ex. 485 nm, em. 520 nm) was measured after 4 hours of incubation. Cell lysate was used as the positive control and assigned a value of 100% cytotoxicity.

2.8 cAMP accumulation assay

Intracellular cAMP levels were measured using a Direct cAMP ELISA kit, according to manufacturers' instructions. After cell exposure to control or 10 mg/mL casein supernatant was removed and cells were lysed with 0.2 mL of 0.1 M HCl for 10 minutes at room temperature. Cellular debris was removed by centrifugation at 900 g for 5 minutes and samples were stored at -80ºC prior to analysis. Results were generated at optical density of 405 nm using a Synergy plate reader. This ELISA kit allows detection of cAMP with sensitivity 0.39 pmol/mL, using standard curve for the range 0.78-200 pmol/mL.

2.9 Intracellular Ca^{2+} assay

After 3 hours exposure to control or 10 mg/mL casein in physiological normoxic/hypoxic conditions, cells were loaded with 2.5 μM Fluo-4 AM intracellular Ca^{2+} probe and incubation was continued for another 1 hour. Following this period, cell monolayers were washed 2 times with Krebs-Ringer buffer supplemented with 0.1 mM sulfinpyrazone, to prevent changes in intracellular Ca^{2+} levels and leakage of de-esterified indicator. Fluorescent signals of intracellular Fluo-4 AM probe were collected with Synergy plate reader, using excitation 488 nm and emission 520 nm.

2.10 Simulated gastro-intestinal digestion (SGID)

SGID of yogurt sample was performed based on the detailed protocol by Minekus et.al. [44]. In short, 2 g of yogurt were mixed with simulated salivary fluid (SSF, 1.25x concentrate), H_2O, $CaCl_2$ and human salivary α-amylase for a final activity of 75 U/mL. The sample was mixed for >2 min at 37ºC to mimic oral phase. Half of the volume was removed and inactivated (amylase activity) by reducing pH to 3 and re-adjusting to 7. Remaining bolus was mixed with simulated gastric fluid (SGF, 1.25x concentrate) for the final ratio 50:50 v/v, $CaCl_2$ and porcine pepsin (2000 U/mL in the final mixture). The pH was reduced to 3 and water was added to dilute SGF. The mixture was incubated for 2 hours at 37ºC on rotor shaker to simulate gastric movement. Pepsin was inactivated in half of the volume by re-adjusting pH to 7. Remaining gastric chime was mixed with simulated intestinal fluid (SIF, 1.25x

concentrate) for the final ratio 50:50 v/v and neutralised to pH 7. Pancreatin was added according to its trypsin activity (100 U/mL in the final mixture). Bile salts were added to the final concentration 10mM, $CaCl_2$ was then added to reach 0.3mM in the final mixture and pH was re-adjusted with 1M NaOH. SIF was diluted with H_2O and digestion was performed for 2 hours at 37ºC on rotor mixer. Pancreatic activity was stopped by 2.4 mg of AEBSF (4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride) per 10 mL of digesta. Digesta was then diluted with distilled H_2O at a ratio of 1:5. Trypsin was removed by filtration with centrifuge tube concentrator (molecular weight cut-off 10000Da at 5000g, 10 min, 4ºC). Aliquots of digesta after each phase (oral, gastric and intestinal) were immediately snap-frozen in liquid nitrogen for 30 sec and stored at -80ºC prior to STC-1 cell exposures.

2.11 Data assessment and statistics

Cell viability, cytotoxicity and intracellular Ca^{2+} data were obtained from 2 experimental repeats on different days and 3 technical repeats within each experiment. Active GLP-1, cAMP and PCR data were obtained from at least 2 experimental repeats on different days and 2 technical repeats within each experiment. Data is presented as means with SD as error bars. Statistical analysis was performed by Paired-samples t-test or One-Way analysis of variance (ANOVA) in PASW statistics 18 software. Means without a common letter differ significantly from each other ($P<0.05$).

3 Results and Discussion

3.1 Low O_2 conditions decrease GLP-1 secretion from STC-1 cells

To determine how GLP-1 secretion in response to individual dairy macronutrients is influenced by hypoxic conditions (0.5% O_2 in the atmosphere) protein (casein), carbohydrate (lactose) and fat (oleic acid) components of milk were selected. *In vivo* transit of food through the length of the GI tract is accompanied not only by changes in O_2 tension, but also results in the digestion of food and appearance of macronutrient breakdown products. To mimic the typical food components that L cells, located in the distal gut, would be exposed to, a sodium caseinate tripeptide LRF, glucose (the

breakdown product of lactose) and butryic acid (a short chain fatty acid which is found in milk but also produced by gut bacteria [38]) were also selected. The final concentrations for the food components were as previously described: 10 mg/mL casein, 40 mM lactose, 100 μM oleic acid, 0.5 mg/mL tripeptide LRF, 40 mM glucose and 100 μM butyric acid [39-42]. Macronutrients were prepared in modified Krebs Ringer buffer with buffering system based on 10 mM HEPES and without bicarbonate to maintain the required pH of 7.2-7.4 in the sealed flushing hypoxia chamber without CO_2 supply [37]. Hypoxic conditions were reached, as determined by a significant fold increases in *HIF1a* mRNA transcript levels compared to ambient air conditions for all macronutrient exposures (data not shown). Low O_2 significantly decreased ($P<0.05$) levels of active GLP-1 secreted from STC-1 cells in response to glucose, oleic acid, tripeptide and casein compared to ambient O_2 (Fig.1A). None of the nutrients tested, except intact casein, were capable of stimulating GLP-1 above levels observed for 11mM glucose (modified Krebs-Ringer buffer control). This concentration of glucose is necessary for cell viability, however it may mask the stimulatory effects of other macronutrients. GLP-1 production, as measured by *proglucagon* mRNA transcript levels, was unaffected by hypoxia for all macronutrients tested except butyric acid, where mRNA transcript levels were significantly increased compared to ambient O_2 ($P<0.05$) (Fig. 1B). Treatment of STC-1 cells with glucose, tripeptide, butyric and oleic acids increased production of GLP-1 compared to the control in hypoxia, while no effect of treatments was observed in normoxia. This absence of correlation between production and secretion of active GLP-1 hormone suggests that regulation of its secretion in hypoxia occurs at post-transcriptional level. For instance other products of *proglucagon*, such as oxyntomodulin, GLP-2 and GLP-1 (1-37) may be released and the ratio of total to active GLP-1 (7-37) may differ [6]. Indeed, the significant stimulatory effect of casein could be a result of the release of active GLP-1 from secretory granules, without changes in the gene expression level. Kihiria et al. reported that hypoxia conditions reduced *proglucagon* mRNA transcript level in GLUTag cells but these observations were in response to cAMP stimulator Fsk in the presence of 2.5 mM glucose [19]. Similarly, levels of *PYY* and *CCK* mRNA transcripts were not significantly different ($P>0.05$) in

hypoxic conditions compared to ambient O_2 for each of the test food components except for butyric acid (1.86 and 1.82 vs. 1.42 and 1.39 fold change hypoxia versus ambient O_2).

Secretion of GLP-1 by pGIP/Neo STC-1 and their viability in the presence of 0-10 mg/mL of whole casein, casein proteins and their hydrolysates were studied before [42]. How the cells survive and proliferate in hypoxia for 4 hours in the presence of casein and other food components was measured by CellTox^{TM} cytotoxicity assay and Cell Titter cell proliferation assay. STC-1 cell line prefers the hypoxic environment with remarkably higher viability ($P<0.01$) at 0.5% O_2 than at 20% O_2 for all treatments against a backdrop of modified Krebs-Ringer buffer (Fig. 2A). At the same time, cytotoxicity was comparable between ambient O_2 and hypoxic conditions with significant differences ($P<0.05$) only observed for the treatments with glucose and casein (Fig. 2B). Only treatment with casein in normoxia was able to improve cell viability compared to the control. This combined result indicates that, not surprisingly, the STC-1 cell line derived from the small intestine prefers hypoxia conditions. We would therefore suggest the use of hypoxic conditions for future studies where complex media is unsuitable [5].

3.2 GLP-1 secretion over a range of oxygen concentrations

O_2 concentration in the gastro-intestinal epithelium decreases distally, with reports of 7.5% in the stomach, 4.5% in the duodenum, 1.5% in the colon and small intestine and 0.5% in the colon-rectal junction of mice [12]. To study if GLP-1 release linearly depends on the O_2 concentration, STC-1 cells were exposed to O_2 levels resembling physiological conditions of the GI tract. As casein significantly increased active GLP-1 release above vehicle control and resulted in a large difference between hypoxic and ambient conditions, it was chosen to evaluate GLP-1 release at various concentrations of oxygen (Fig. 3A). Secreted GLP-1 levels in response to intact casein depend on O_2 concentration, with an inverted bell curve response. The highest GLP-1 levels were observed at 20% O_2 (9.15 +/- 0.03 ng/mL) and the lowest levels at 7.5% O_2 (2.58 +/- 0.04 ng/mL) with hypoxic conditions resulting in 6.27 +/- 0.15 ng/mL. Interestingly the GLP-1 levels in response to 11mM

glucose (control) varied from 0.58 to 1.49 ng/mL and were significantly higher at 7.5% and 20% O_2 than at 0.5% and 1.5% O_2.

3.3 Mechanism of hypoxia influence on GLP-1 production.

In vivo, enteroendocrine cells sense (a) amino acids and proteins via taste receptors T1R1/T1R3 and pept1/pept2 channels [8], (b) fatty acids via GPCRs [7] and (c) carbohydrates via sweet taste receptors T1R2/T1R3 and transporters GLUT1 or GLUT5 [43]. Such interactions lead to electrogenic (Ca^{2+} flux) and electroneutral (cAMP) governed release of GLP-1. Figure 3B details cAMP and intracellular Ca^{2+} levels in STC-1 cells, exposed for 4 hours to 10 mg/mL casein and modified Krebs-Ringer buffer as a control. Intracellular cAMP levels are significantly lower ($P<0.05$) in response to intact casein under hypoxic conditions compared to normoxic conditions (8.65 and 10.92 pmol/mL respectively). Interestingly, the ability of STC-1 cells to increase cAMP levels in response to the cAMP stimulator Fsk is significantly attenuated under hypoxia (60.7 pmol/mL in hypoxia vs. 146.3 pmol/mL cAMP in normoxia). This is in good agreement with Kihara et al. who observed a 2 fold decrease in ATP levels when GLUTag cells were exposed to Fsk under hypoxia [19]. In STC-1 cells Ca^{2+} levels in response to casein are not influenced by hypoxia (Fig. 3B). In contrast, intracellular Ca^{2+} levels rise in pulmonary arterial myocytes and in rat neonatal carotid body type I cells in response to hypoxia via generation of mitochondrial reactive oxygen species (ROS) [44, 45]. The reduction in GLP-1 exocytosis in response to intact casein in hypoxic conditions may therefore be a symptom of the dampening of the cAMP signalling pathway rather than Ca^{2+} involvement.

3.4 Food digestion in study of gut hormone regulation

To study the effect of physiological O_2 concentrations together with different phases of food digestion on enteroendocrine secretion of active GLP-1, commercially available yogurt (2.8 g fat, 5.6 g carbohydrates, 5.2 g protein per 100g) was subjected to oral, gastric and intestinal SGID [46]. STC-1 cells were then incubated at 7.5% O_2 to represent typical conditions for orally digested food, 4.5% O_2 for gastric and 1.5% O_2 for intestinal digestas. Secreted GLP-1 levels in response to yogurt digestas

compared to whole yogurt are detailed in Figure 4A. GLP-1 levels secreted in response to oral, gastric and intestinal yogurt digestas at their matching O_2 concentrations ranged from 4.41 to 21.62 ng/mL, with levels for gastric yogurt digesta at 4.5% O_2 significantly higher than for either oral or intestinal digesta samples ($P<0.05$). In contrast, GLP-1 levels secreted in response to whole yogurt at different O_2 concentrations had a narrower range from 6.47 to 8.08 ng/mL. Interestingly, STC-1 cells exposed to intestinal yogurt digesta at 1.5% O_2 secreted significantly lower levels ($P<0.05$) of GLP-1 than any of the other test samples including whole yogurt. GLP-1 levels in response to yogurt digesta at each SGID stage with corresponding O_2 concentration were significantly different ($P<0.05$) compared to levels with yogurt at 20% O_2 (shown with dashed line).

The density of L cells increases from duodenum to colon, as O_2 concentration decreases [11]. Theoretically predicted efficiency of GLP-1 production was calculated from the GLP-1 levels obtained at each physiological condition (Figs. 3A, 4A) as a function of the predicted number of cells at each location within the GI tract [10, 11]. Limitations of this theoretical model include the use of STC-1 cell monolayer versus heterogeneous 3D structure of the GI tract and the difficulty to predict sensitivity of L cells to treatment and absorption of nutrients at different locations of GI tract. However, the model benefits from including O_2 concentration and the stage of food digestion as factors, influencing GLP-1 secretion. This model predicts that yogurt digesta would be a more potent stimulant of GLP-1 secretion than glucose in the gut and peak levels of secreted GLP-1 would occur in the ileal and colon (Fig 4B).

4 Concluding Remarks

In conclusion, intact casein can increase levels of GLP-1 secretion from glucose- stimulated STC-1 cells, probably via intracellular Ca^{2+} signalling. At low oxygen concentrations this secretion is attenuated possibly by a decrease in cAMP levels. Exposing enteroendocrine STC-1 cells to oxygen concentrations and food digesta relevant to specific GI locations significantly alters the level of active GLP-1 secreted. As yogurt transits the gut, 4.5% oxygen and digesta of the gastric phase generate peak GLP-1 levels. Transcription of satiety hormones is not affected by hypoxia for variety of

stimulations with macronutrients except for butyric acid, which is produced by the bacteria of the hypoxic colon. Where cells are assayed in buffers, cell viability is an issue and can be improved by the use of hypoxic conditions. Altogether, physiological O_2 levels and food digestion have significant influence on gut hormone secretion and their impacts on satiety hormones warrant further investigation.

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Figure 1. GLP-1 production and secretion in STC-1 cells exposed to different macronutrients at atmospheric normoxia (20% O_2) and hypoxia (0.5% O_2). STC-1 cells were exposed to negative control (modified Krebs-Ringer), 40 mM glucose, 40 mM lactose, 100 µM butyric acid, 100 µM oleic acid, 0.5 mg/mL tripeptide or 10 mg/mL casein for 4 hours. **A**. Levels of secreted GLP-1 (active) in STC-1 cell supernatants. **B**. Levels of *proglucagon* mRNA transcripts in STC-1 cell lysates. *ActB* was used as a reference gene. Asterisks indicate significant differences for each food component between normoxic and hypoxic conditions ($P<0.05$). Number signs indicate significant differences for each O_2 concentration between control and treatment ($P<0.05$).

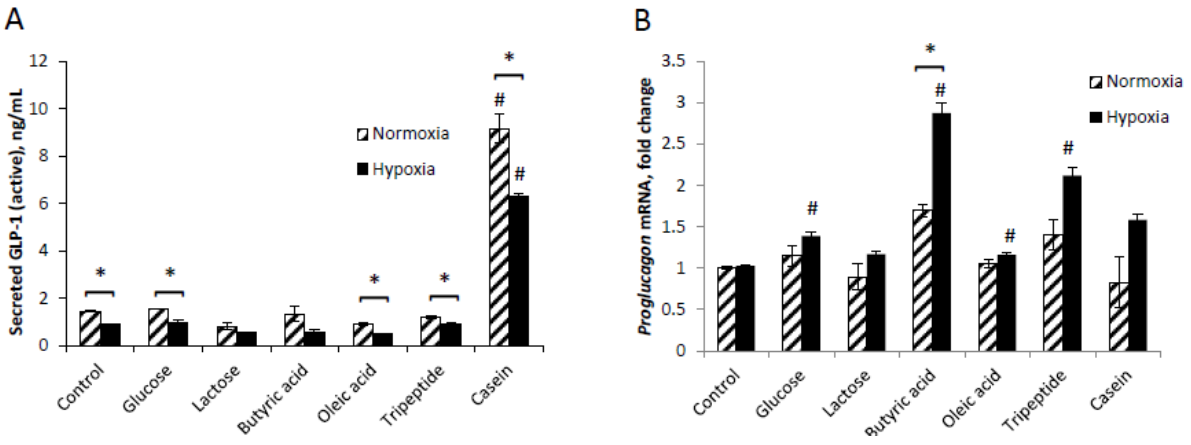


Figure 2. Effect of O_2 concentration on STC-1 cell viability and toxicity after 4 hours of exposure to different macronutrients at atmospheric normoxia (20% O_2) and hypoxia (0.5% O_2). STC-1 cells were exposed to negative control (modified Krebs-Ringer), 40 mM glucose, 40 mM lactose, 100 μM butyric acid, 100 μM oleic acid, 0.5 mg/mL tripeptide or 10 mg/mL casein for 4 hours. **A.** STC-1 cell viability is presented as percentage, where exposure to DMEM at 20% O_2 represents 100% cell viability. **B.** STC-1 cell toxicity is presented as percentage, where cell lysate at normoxia represents 100% cytotoxicity. Asterisks indicate significant differences for each food component between normoxic and hypoxic conditions (* $P<0.05$, ** $P<0.01$). Number signs indicate significant differences for each O_2 concentration between control and treatment ($P<0.05$).

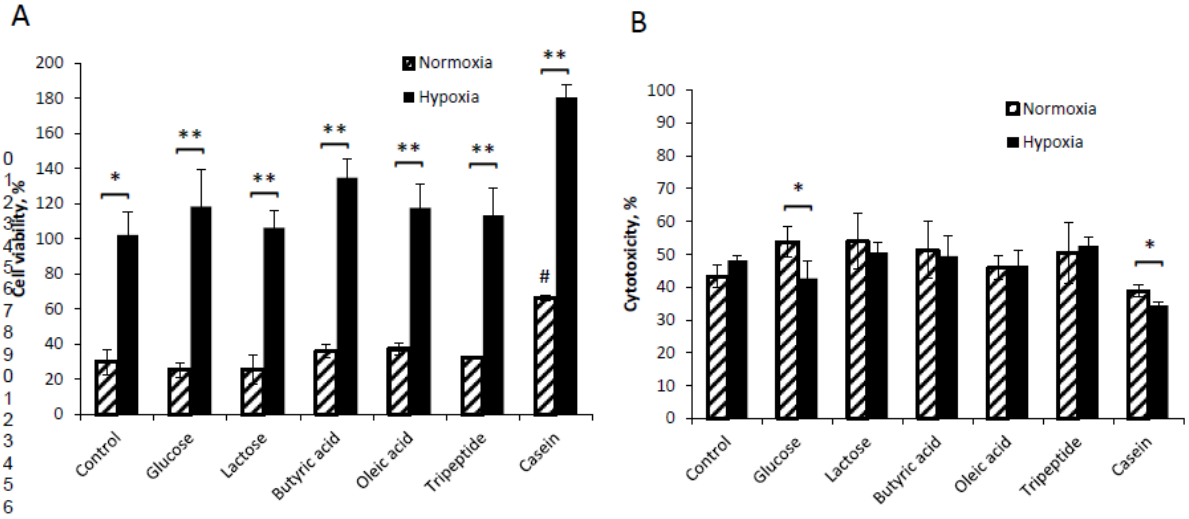


Figure **3 A. Secretion of active GLP-1 from STC-1 cells at various O_2 concentrations, corresponding to the different parts of GI tract.** STC-1 cells were exposed to negative control (modified Krebs-Ringer) or 10 mg/mL casein at 0.5, 1.5, 4.5, 7.5 and 20% O_2 for 4 hours. Different superscript letters (generated with One way ANOVA test) indicate significant difference in active GLP-1 ($P<0.05$) at different oxygenation for a treatment. **B. Effects of O_2 on GLP-1 secretion pathways.** STC-1 cells were exposed to control (supplemented with 1 mM IBMX modified Krebs-Ringer buffer) or 10 mg/mL casein. Intracellular cAMP and Ca^{2+} levels in STC-1 cells were measured after 4 hours exposure. Results of cAMP assay were generated at optical density of 405 nm. For Ca^{2+} assay fluorescence signals were collected with excitation 488 nm and emission 520 nm and presented as relative to control (modified Krebs-Ringer at 20% O_2). Asterisks indicate significant differences between normoxic and hypoxic conditions ($P<0.05$).

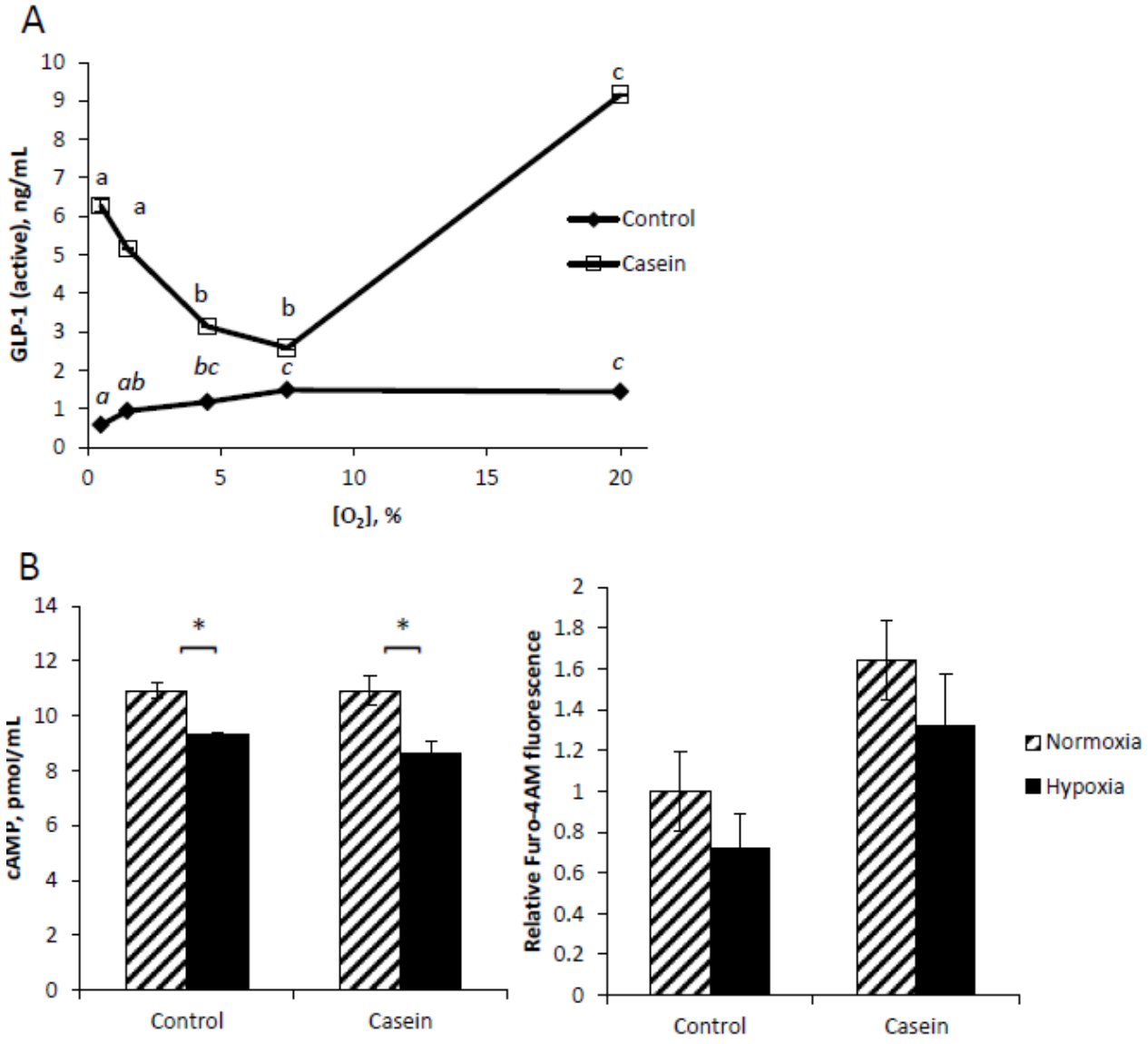
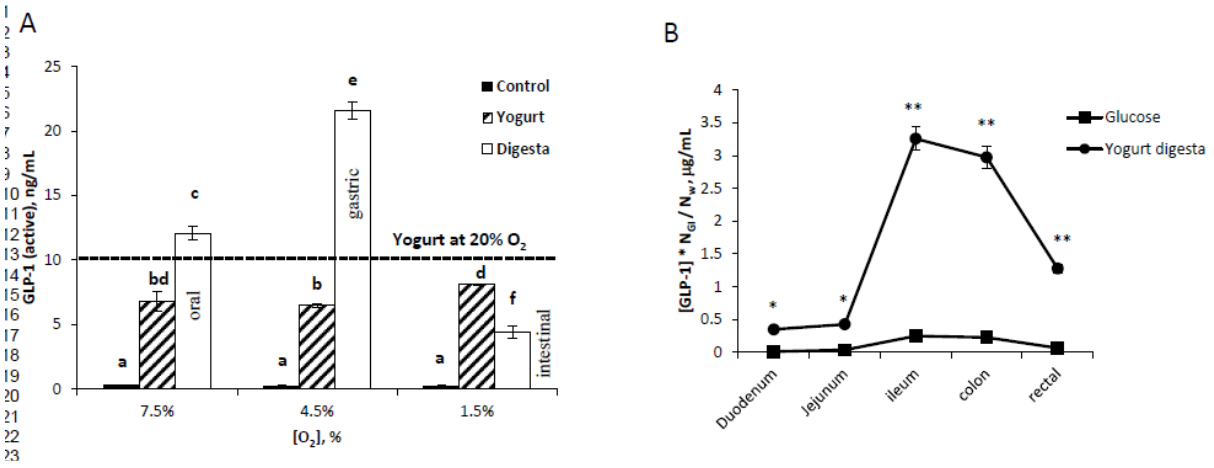


Figure **4. A. Effect of food digestion and O_2 concentration on active GLP-1 secretion from STC-1 cells.** STC-1 cells were exposed to commercial yogurt, at different phases of SGID (oral, gastric and intestinal) with physiologically relevant O_2 concentrations. Specifically, cells were treated with yogurt orally digested at 7.5%, gastrically digested - at 4.5% and intestinally digested - at 1.5% O_2. Cells were exposed to modified Krebs-Ringer buffer as negative control and whole yogurt for comparison purposes. Level of GLP-1 secretion after exposure to yogurt at 20% O_2 is shown with dashed line. **B. Predicted efficiency of GLP-1 secretion at different parts of GI tract**. Predicted GLP-1 secretion = [GLP-1]*(N_{GI} / N_w) where [GLP-1] is concentration of active GLP-1 secreted by STC-1 in response to stimulation, N_w - number of STC-1 cells in the well and N_{GI} – predicted number of L cells in the corresponding part of GI tract [11]. Different superscript letters (generated with One way ANOVA test) indicate significant difference ($P<0.05$) in active GLP-1 secretion within an O_2 concentration. Asterisks indicate significant differences for each treatment between normoxic and hypoxic conditions (* $P<0.05$, ** $P<0.01$).



Graphic Abstract

Secretion of hormones in the gut is accompanied by food digestion, decreasing oxygen concentration and increasing number of L cells towards the distal gut. These parameters are missing in exisiting *in vitro* models and their effects on study outcomes are not estimated. In this paper we report on the significant influence of low oxygen and food digestion on secretion of satiety hormone GLP-1 from enteroendocrine STC-1 cells.