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***INTESTINAL CELLULAR BIOENERGETICS,
GLUCAGON-LIKE PEPTIDE-1 AND CONTROL OF EATING***

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Summary

Western diet and sedentary lifestyle are strongly associated with the epidemic increase of obesity and its associated comorbidities. The small intestine is the first organ exposed to nutrients coming from the food and has been shown to metabolically respond to the composition of the diet. Indeed, its position is ideal to sense the energy availability in the intestinal lumen and to convey this information to the brain. With respect to this, it has been reported that the intestine is metabolically highly flexible, in particular as far as its reaction to high-fat-diet (HFD) is concerned. Also fat, and in particular monounsaturated fatty acids, potently stimulate the release of the eating-inhibitory peptide glucagon-like peptide-1 (GLP-1) from enteroendocrine cells. Studies with chronically HFD-fed rats showed that a pharmacological modulation of fatty acid metabolism in the intestinal epithelial layer enhanced the metabolic response of the jejunum, but not of the duodenum or the liver, to HFD as well as increased the concentration of GLP-1 in the hepatic portal vein (HPV) of the same rats after a HFD meal.

Based on these findings, in the first set of experiments we assessed the metabolic response of enterocytes derived from the duodenum, the jejunum or the liver of mice exposed for 3 or 14 days to HFD. As control we fed mice with control diet for the same periods of time. We could show that already a 3-day exposure to HFD potently increased the protein levels of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoAS2), the key enzyme of ketogenesis, in the jejunum. In the duodenum or the liver, the protein of HMG-CoAS2 was barely upregulated even after a 14-day HFD exposure. In contrast, although HFD exposure did not influence the protein level of HMG-CoAS2 in the duodenum, it did upregulate the expression of the *HMGCS2* gene in the duodenum. This was not the case for the jejunum or the liver. With respect to glucose metabolism, already a 3-day HFD exposure downregulated genes related to glucose metabolism in the small intestine. In the liver, a 3-day exposure to HFD influenced neither the protein levels of HMG-CoAS2 nor the expression of genes related to fatty acid or glucose metabolism, and a 14-day HFD exposure upregulated only genes involved in glucose metabolism.

The ability of the intestine, especially the jejunum, to respond to HFD via modulating its bioenergetics raised the question about the metabolic properties of enterocytes isolated from the different sections of the intestine and the hepatocytes from the liver. Experiments with the Extracellular Flux Analyzer (XF24) indicated that cell lines from the jejunum had a higher capacity to activate oxidative metabolism than cells from the duodenum. In contrast, duodenal cells more efficiently metabolized glucose to lactate than jejunal or hepatic cells.

With the last set of experiments we analyzed the possible role of mitochondrial fatty acid oxidation in the release of GLP-1 from an enteroendocrine cell line model. We could clearly show that oleic acid (OA) did not stimulate the release of GLP-1 because of its oxidation. Moreover, measurements of cellular bioenergetics with the XF24 showed that OA potentially uncoupled mitochondrial respiration and induced a switch of cellular metabolism towards aerobic glycolysis. An inhibition of glycolysis potentially inhibited the OA-induced release of GLP-1, suggesting that in the cell culture model the final trigger for the OA-induced GLP-1 release may involve the ATP produced during glycolysis and not the oxidation of OA in the mitochondria. In rats, an intragastric (IG) infusion of OA, induced a peak of HPV plasma GLP-1 concentration 5-10 min after infusion. We could, however, not completely inhibit this transient peak via an IG infusion of 2-deoxy-D-glucose, a potent inhibitor of glycolysis.

Together, these results indicate that a substantial dietary fat load induces an early adaptation of the jejunum rather than the duodenum or the liver to the diet. The findings underline the importance of the intestine, and in particular the jejunum, in sensing the composition of the diet and in adapting its own metabolism accordingly. Thus, the study of early diet-induced metabolic changes in the small intestine may reveal ways to help counteract obesity by nutritional interventions. Also, studying the pathways that mediate the OA-induced release of GLP-1 may raise the possibility to promote nutritional interventions as a valuable alternative to the use of pharmacological GLP-1 receptor agonists in the treatment of type-2-diabetes and obesity.

Zusammenfassung

Unser westlicher Lebensstil mit wenig körperlicher Bewegung und einem Überfluss an Nahrung ist mit der epidemisch zunehmenden, morbiden Adipositas assoziiert. Der Dünndarm ist direkt den Nährstoffen der aufgenommenen Diät ausgesetzt und resorbiert diese in den Blutkreislauf. Damit ist der Dünndarm in einer idealen Position, um das Vorhandensein von Energie im Darmlumen quantitativ und qualitativ zu überwachen und diese Information an das Gehirn weiterzuleiten.

In früheren Studien wurde gezeigt, dass das Jejunum metabolisch sehr flexibel auf die Zusammensetzung der Nahrung reagieren kann, insbesondere wenn die Nahrung fettreich ist. Weitere Studien zeigten, dass bei chronischer Aufnahme einer Hochfettdiät (HFD) eine pharmakologische Modulation des Fettsäuren-Metabolismus in den Epithel-Zellen des Dünndarms die metabolische Antwort des Jejunums auf die Diät verstärkt und die Konzentration des Sättigungshormons Glucagon-like-peptide 1 (GLP-1) in der Pfortader von Ratten erhöht.

Basierend auf diesen Ergebnissen, untersuchten wir in der ersten Serie von Experimenten die metabolische Reaktion des Duodenums, Jejunums und der Leber von Mäusen auf den 3- oder 14-tägigen Verzehr einer HFD. Als Kontrolle dienten Mäuse, denen jeweils für die gleiche Zeit eine Kontrolldiät (KD) gefüttert wurde. Wir konnten zeigen, dass im Vergleich zum Duodenum oder zur Leber, das Jejunum sich metabolisch stark an die Diät anpassen konnte. So war im Vergleich zu Mäusen die mit KD gefüttert wurden bereits nach 3-tägiger HFD-Fütterung der Proteingehalt von 3-Hydroxy-3-methylglutaryl-coenzym-A (HMG-CoAS2), dem Schlüsselenzym der Ketonkörpersynthese, im Jejunum deutlich hochreguliert. Der Gehalt desselben Proteins war im Duodenum oder in der Leber auch nach einer 14-tägigen Fütterung mit HFD kaum verändert. Im Gegensatz zum Proteingehalt, regulierte die 3-tägige Fütterung der HFD im Duodenum, aber nicht im Jejunum oder in der Leber die Expression des *HMGCS2* Gens stark hoch. Die Gene des Glukosemetabolismus wurden nach 3-tägiger Fütterung der HFD im ganzen

Dünndarm herunter- und nach 14-tägiger Fütterung der HFD in der Leber hochreguliert.

Da das Jejunum anders als das Duodenum oder die Leber sich bereits nach einer 3-tägigen Fütterung von HFD metabolisch an die Diät anpasste, untersuchten wir als nächstes die metabolischen Eigenschaften von Zelllinien, welche spezifisch vom Duodenum, Jejunum und der Leber isoliert wurden, mit dem Seahorse Extracellular Flux Analyser (XF24). Diese Experimente zeigten, dass die jejunale Zelllinie eine viel höhere Kapazität hatte, die oxidative Phosphorylierung zu benutzen, als die duodenale Zelllinie. Im Gegensatz dazu wandelte die duodenale Zelllinie deutlich mehr Glukose in Laktat um als die Zelllinien von Jejunum oder Leber.

In der letzten Serie von Experimenten überprüften wir den Einfluss der Fettsäureoxidation in den enteroendokrinen Zellen auf die Sekretion von GLP-1. Dabei konnten wir zeigen, dass Ölsäure die Sekretion von GLP-1 stimulierte, dass dieser Effekt aber unabhängig von der Oxidation der Ölsäure war. Vielmehr führte die Ölsäure zu einer Entkopplung der mitochondrialen Atmungskette und bewirkte dadurch eine Verschiebung des Zellstoffwechsels in Richtung aerober Glykolyse. Eine Hemmung der Glykolyse mit 2-Desox-Glukose reduzierte die durch die Ölsäure stimulierte Sekretion von GLP-1. Bei in-vivo Versuchen an Ratten konnten wir diese Ergebnisse jedoch nur zum Teil reproduzieren.

Insgesamt unterstreichen diese Ergebnisse die Rolle des Dünndarms bei der Erfassung der Diätzusammensetzung und bei der Aufrechterhaltung der Energiehomöostase. Das Verständnis der Mechanismen, welche den diätinduzierten metabolischen Veränderungen im Dünndarm zugrunde liegen, könnte eine Möglichkeit eröffnen, die Entstehung der Adipositas durch personalisierte Ernährung zu beeinflussen.

Chapter 1:

General introduction

1 General introduction

The profound changes in life style that occurred during the last decades led to a marked worldwide increase in the prevalence of obesity and the related comorbidities [1]. Although the quality of life has substantially improved, the population is now driven towards a life style that is mainly sedentary and reinforces the intake of palatable high caloric and low nutritional food [2]. As a consequence, obesity and the ensuing metabolic disorders are strongly promoted [3, 4]. Fat is a major component of the western diet [5] and has been shown to have a potent impact on the development of obesity in mice [6 - 8]. The intestine is the first organ exposed to the food-derived nutrients, and it reacts in several ways to the composition of the food [9, 10]. Further, pharmacological analogs of GLP-1, an eating-inhibitory and incretin peptide released from intestinal endocrine cells in response to a meal [11], are widely used to treat obesity and typ-2-diabetes (T2D) [12]. Thus, an understanding of the intestine's role in the control of eating and in the regulation of whole body energy homeostasis has been an attractive research target for a long time.

1.1 Control of food intake

Why do we eat and why do we stop eating? For humans as for all living animals, food is the main source of energy [13]. Despite day to day fluctuation in energy intake and energy expenditure, the overall body weight (BW) remains relatively stable over years [14]. This implies that food intake is accurately controlled by a complex process that is based on the continuous communication between brain and peripheral organs [15, 16]. A dysregulation of this system, i.e., prolonged increases in energy intake or decreases in energy expenditure lead to obesity, T2D and numerous other associated diseases [17]. The almost ridiculous question, "Why do we eat and why do we stop eating?" therefore requires a very complex answer and the underlying mechanisms are up to now not completely understood.

The control of food intake involves both, the processing of information coming from internal stimuli such as ingested nutrients, circulating metabolites, or stored energy as well as from the external environment [13]. The brain, in particular the brain stem and the hypothalamus, play a crucial role in this context. Indeed, it is generally accepted that the nucleus tractus solitarii and the area postrema of the brain stem as well as the arcuate and paraventricular nuclei of the hypothalamus, the lateral hypothalamus and the dorsomedial hypothalamic nucleus are major brain areas involved in the homeostatic control of food intake. Moreover, these areas are intricately connected to each other and to other areas of the brain to constantly integrate the peripheral signals regarding energy status and “translating” them into changes of eating behavior [15, 18].

In more detail, the signals from the periphery are conveyed to the brain via (a) neural transmission from peripheral tissues, (b) circulating nutrients as well as (c) endocrine signals secreted by peripheral organs. (a) Neuronal transmission of signals regarding the availability of energy in the periphery mainly occurs via vagal or spinal afferent fibers ending in the brain stem [19]. The gastrointestinal tract with its intrinsic enteric nervous system as well as the hepatic portal vein are densely innervated by the vagal and spinal afferents and efferents [20 - 22]. During food digestion, the qualitative and quantitative composition of the diet is sensed by the afferent fibers via gastric distention [23], peptide receptors present on vagal afferent fibers in close proximity of the enteroendocrine cells (ECs) [24] as well as via nutrient sensors present in the hepatic portal vein [22 , 25].

In addition to the neuronal transmission, (b) also circulating nutrients themselves can directly act in the area postrema of the brain stem and in the arcuate nucleus of the hypothalamus. Nowadays it is well accepted that the nutrient sensing neurons in the brain alter their activity in response to a range of circulating metabolites [26]. Also, the rapid uptake and metabolization of circulating glucose and fatty acids (FA) into the brain has been implicated in the regulation of whole body energy homeostasis [27 - 32]. (c) The last known route of conveying signals regarding whole body energy status to the brain is through the secretion of peptides by abdominal organs, such as the stomach, the intestine, the pancreas and/or the adipose tissue. These peptides can transfer

information regarding the availability of energy to the brain via activation of their receptors present in the brain as well as on vagal afferents that terminate in close proximity to the endocrine cell [24, 90]. The peripheral signals are classified into eating-stimulatory and eating-inhibitory peptides [13]. Up to now, only two peptides are known to stimulate food intake: ghrelin and insulin-like peptide 5. The first one is released from the gastric mucosa [33], whereas the second one is derived from ECs of the colon [34]. The circulating levels of these peptides are elevated during fasting, and they potently stimulate eating when administered exogenously [34, 35]. In contrast, many eating-inhibitory peptides are known. These are summarized into adiposity and satiation signals [13]. Adiposity signals, i.e., mainly the adipose tissue hormone leptin and the pancreatic hormone Insulin [36] are released into the circulation in proportion to the total body fat content [37, 38] and deliver information regarding the long-term availability and needs of energy to the brain [13]. Satiation signals, i.e., gastrointestinal peptides such as GLP-1, cholecystokinin (CCK), peptide tyrosine tyrosine (PYY) and many others, are released into the circulation in response to a meal. These peptides deliver information about the current energy intake to the brain and lead to meal termination [39, 40]. Among all satiation signals, GLP-1 is indisputably currently the most studied one [11], and it will be discussed in more detail in a later section of this thesis.

1.2 Role of the intestine in the control of food intake

With respect to peripheral organs related to the control of food intake, the small intestine regained importance over the last years [41 - 44]. The intestine plays an indispensable role in the digestion and absorption of food contents coming from the stomach as well as a barrier against potential pathogens present in the intestinal lumen [45]. Up to now, however, the major scientific challenge resides in the understanding of how this multitasking organ senses the fluctuations of energy availability in the lumen and translates this information into corresponding signals for the brain.

Anatomically, the extensive surface of the small intestine is composed by villi, the finger like projections into the gut lumen, and crypts of Lieberkühn [46].

The almost infinitely self-renewing stem cells migrate from the bottom of the crypt towards the top of the villi. During this trip, stem cells differentiate into fully functional enterocytes (or absorptive cells), goblet cells, and enteroendocrine or Paneth cells. At this differentiated stage, all these cells are finally exposed to the food components present in the intestinal lumen [45 - 47]. The turnover of the fully functioning and differentiated epithelial cells is between 2 and 7 days [48], underlining therefore the potential and the necessity of the intestine to rapidly adapt to changes in environmental stimuli, such as the diet [9, 10].

1.2.1 Metabolic characteristics of the small intestine

It has been shown that the diet can modulate the absorptive capacities of the intestine [9, 10, 49], thus making it an attractive organ for the prevention and treatment of obesity. In this context, enterocytes, the absorptive cells of the epithelial layer, are of particular interest [50]. As all mammalian cells, the enterocytes are capable of oxidizing amino acids, glucose and FA to produce ATP. For enterocytes these substrates may come from the lumen as well as from the plasma. Almost 20% of the whole body oxygen consumption is concentrated in the gastrointestinal tract, emphasizing the high metabolic activity of this organ.

Quantitatively, the major nutrients for the intestine are glutamine or ketone bodies [51, 52]. The use of glucose as metabolic fuel is not extensive, although the intestine is able to metabolize glucose at the same extent as glutamine and has a greater potential to oxidize it than the brain or the muscle [50, 53 - 55]. The discrepancy between the high capacity of the intestine to oxidize glucose and glutamine and the not extensive use of glucose as energy yielding substrate, suggests that a regulatory mechanism might stimulate the oxidation of glutamine rather than the one of glucose [50]. Recently, light has been shed on the connection between glucose and glutamine metabolism in the intestine [25]. Mithieux and colleagues showed that the gene conferring the gluconeogenesis function, the glucose-6-phosphatase (G-6-Pase), to liver and kidney is also highly expressed in the intestine [56], in particular during prolonged fasting [57]. In contrast, also a high protein diet induces the expression of G-6-Pase as well as the cytosolic form of the pyruvate

carboxylase and the glutaminase, all three key enzymes of gluconeogenesis [25]. Glutamine could therefore be used as a substrate for NADH and FADH₂ synthesis in the tricarboxylic acid (TCA) cycle and as the main substrate for intestinal gluconeogenesis [58]. Glutamine spares therefore the use of glucose as metabolic substrate for the enterocytes, thus making it available for other tissues in circulation [59].

Fatty acids are an important fuel for the intestinal epithelial cells during suckling [60], when the fat content of the diet is comparatively high. The use of fat as energy yielding substrate was supposed to be lost after weaning [61]. Recent studies showed, however, that the capacity to oxidize fat and even to produce ketone bodies can be reestablished by feeding a high-fat-diet (HFD) also during adulthood [41, 62, 63]. Also, intestinal and especially jejunal fatty acid oxidation (FAO) and ketogenesis have been implicated in the control of food intake [25, 42, 64] challenging the originally presumed role of the liver as a regulator of whole body energy homeostasis [65].

1.2.2 Intestinal fatty acid metabolism in the control of food intake

Western diets contain approximately 35% fat [1] that is mostly present in form of triglycerides (TG). The major role of the intestine in whole body lipid metabolism is to transfer the TG-derived FA from the intestinal lumen through the lymph duct into the blood circulation [66]. TG are hydrolyzed in the duodenum by the pancreatic lipase into two FA and one sn-2-monoacylglycerol (2MG). Once absorbed via the passive flip flop and/or the active protein dependent (mainly fatty acid transport proteins 1-4 (FATP 1-4)) mechanisms [67, 68], FA and 2MG are re-esterified via acyl-CoA:monoacylglycerol acyltransferase (MGAT) and acyl-CoA:diacylglycerol acyltransferase (DGAT) 1 and 2 into TG [69], temporally stored in the enterocyte [70], oxidized via beta oxidation [42] or assembled to lipoproteins and released into the lymph duct and the blood circulation, which is quantitatively the most important pathway [71]. Although these are well established facts, the exact mechanisms that govern this multistep process are still not well understood [71]. Although the mechanisms are not known, HFD potently can modulate the transit of fat through the enterocyte [10, 41, 62, 70, 72]. In more detail, in the 70ties, Singh

was the first to observe an influence of HFD on the morphological properties of the intestine [10]. Nowadays it is well accepted that exposure to a HFD upregulates the expression of genes and proteins related to lipid metabolism throughout the intestine already after 2 weeks of diet exposure [41, 49, 63].

A breakthrough with respect to the presumed influence of intestinal FAO on food intake was the recent realization that a modulation of intestinal fat metabolism was associated with an alteration of food intake. [73 - 75]. In more detail, an inhibition of DGAT1 was associated with an increase in intestinal FAO and ketogenesis as well as an inhibition of food intake [74, 76]. Further, an increase in intestinal FAO via specific activation of the nuclear receptor Peroxisome proliferator-activated receptor alpha (PPAR α), was also associated with an enhanced intestinal ketogenesis and with a decrease in food intake [75, 77]. The exact molecular mechanisms as well as the metabolic candidates directly responsible for these observations remained unknown.

1.2.3 Gastrointestinal peptides

Dietary FA are potent stimuli for the release of gastrointestinal peptides from ECs [78, 79]. Although only 1% of all intestinal epithelial cells are ECs, the small intestine is considered to be one of the largest endocrine organs of the body [80]. Traditionally, ECs have been categorized based on their location and hormone release in L, K, M and I cells [81]. Recent studies with transgenic enteroendocrine reporter mice on transcriptional elements of the GI anorexic peptides precursors provided new insights into the location and function of these cells [82]. Namely, ECs are capable of coexpressing different peptides and are scattered, with different density, over the entire small intestine [83 - 86]. Independent of the location of these cells, however, ECs sense the nutrient content of the lumen via different sensing mechanisms [87 - 89] and translate the availability of energy and nutrients in the gut into a peptide that is relayed to the brain via the blood stream or triggers an afferent nerve signal for the brain. Thus, the peptides released activate their cognate receptors on primary vagal afferents that terminate in close proximity to the ECs [24, 90] or they travel via the lymph and blood stream to the brain and act directly on the area postrema in the hindbrain or the arcuate nucleus of the hypothalamus [19, 80].

1.2.3.1 Glucagon like peptide one

Among the variety of known gastrointestinal peptides, GLP-1 is by far the most studied and reviewed anorexigenic gastrointestinal peptide [11, 91 - 93]. Together with glicentin, oxyntomodulin, intervening peptide 2 and GLP-2, GLP-1 is the result of the tissue-specific posttranslational processing of the proglucagon peptide in the ECs of the gut and a small group of neurons in the hindbrain [11, 94, 95]. In humans, the GLP-1 plasma concentrations range from 5 - 10 pmol/L in the fasted to 10 - 30 pmol/L in the fed state. This concentration is, however, largely dependent on the size and composition of the meal [96].

Once in the circulation, GLP-1 acts through the N-terminal extracellular region of the GLP-1 receptor (GLP-1R) [97], a G-protein-coupled receptor widely distributed on pancreatic cells, brain, kidney, heart, and the gastrointestinal tract [98]. Studies with GLP-1R agonists (e.g., exendin 4), GLP-1R antagonists (e.g. exendin-9) [99, 100] and GLP-1R knock-out mice (GLP-1^{-/-}) [101] characterized the physiological roles of this peptide. GLP-1 potently inhibits food intake [102], reduces gastric emptying [103], inhibits glucagon release from the endocrine α -cells of the pancreas [104] and acts as a potent incretin hormone, i.e., it stimulates the glucose-induced insulin release from the β -cells of the pancreas [105, 106]. The half-life of the active GLP-1 isoforms (GLP-1₍₇₋₃₇₎ and GLP-1₍₇₋₃₆₎) in the plasma is, however, less than 2 min. The proteolytic activity of the ubiquitously present enzyme dipeptidyl peptidase-IV (DPP-IV) cleaves the amino terminus of the protein at position 2, which leads to a deactivation of the peptide to its inactive form GLP-1₍₉₋₃₇₎ or GLP-1₍₉₋₃₆₎NH₂ [107, 108]. Approximately 5 min after its inactivation, the inactive GLP-1 is excreted through the kidney [109]. Because of the numerous physiological actions of GLP-1, GLP-1R agonists, resistant to DPP-IV cleavage, are widely used as treatment for obesity and T2D [12, 110].

1.2.3.1.1 Pathways regulating the secretion of GLP-1

Understanding the mechanisms of GLP-1 release may provide a valuable alternative to the use of pharmacological GLP-1 receptor agonists.

First attempts to understand the mechanism that induces the nutrient-stimulated GLP-1 release date back to 1963, when Unger *et al.* observed the incretin effect of GLP-1, i.e., that plasma insulin rose in response to enteric but not intravenous glucose loads [111]. Based on these observations and the potent influence of GLP-1 on glucose homeostasis, the pathways that control the glucose-induced GLP-1 release are the best studied. The main players in this context are the luminal glucose/Na⁺ co-transporter, the K_{ATP} channels and the basolateral glucose transporter glucose transporter 2 (GLUT2). Inhibition of sodium-glucose linked transporter 1 (SGLT1) with phloridzin or of the GLUT2 transporter with phloretin potently reduced or even abolished the glucose-induced GLP-1 release [112]. The same effect was observed in SGLT1 (SGLT1^(-/-)) or GLUT2-deficient mice (GLUT2^(-/-)) [113 - 115]. In perfused rat intestine, closure of K_{ATP} channels with sulfonylurea (gliclazide and tolbutamide) potently stimulated the release of GLP-1 [115, 116]. Recently, with transgenic mice models it was possible to demonstrate the presence of SGLT1, GLUT2 and K_{ATP} channels specifically in the EC [117]. Up to now it is still not clear, however, whether it is the oxidation of glucose or the Na⁺-induced modulation of the electrochemical properties of the membrane that finally triggers glucose-induced GLP-1 release [118].

In addition to glucose, FA are known to be potent stimuli for the release of GLP-1 [79, 119]. The fatty acid receptor-1 (GPR40), FFAR2 (GPR43), FFAR3 (GPR41) [120 - 123] as well as GPR120 have been implicated in the sensing of short-, medium- and long-chain FA by the EC [124]. Binding of the FA to the extracellular domain of the GPR induces a conformational change of the receptor which leads to an intracellular cascade of events that ultimately results in GLP-1 release. Dependent on the cascade of events G protein coupled receptors (GPRs) are classified into activators (G_{αs}) or inhibitors (G_{αi}) of the adenylate cyclase, stimulators of the phospholipase C (G_{αq}) or activators of the protein Rho (G_{α12/13}) [125]. In general, upon activation of the adenylate cyclase, cyclic adenosine monophosphate (cAMP) is synthesized from ATP and interacts with the protein kinase A (PKA) or with the exchange protein directly activated by cAMP (EPAC), inducing an influx of Ca²⁺ and a subsequent release of the GLP-1. On the other hand, activation of the phosphoinositide-hydrolyzing phospholipase C (PLC) induces the synthesis of 1,4,5-

trisphosphate (IP₃) and diacylglycerol (DAG), thus stimulating the release of GLP-1 vesicles via increasing the intracellular Ca²⁺ as well as via activating the protein kinase C (PKC) [119, 126 - 128]. In addition to GPR, also a modulation of energy metabolism can affect GLP-1 release [129]. More precisely, an inhibition of the mitochondrial respiratory chain with Indole [130] inhibited the release of GLP-1. An inhibition of intestinal re-esterification of FAs through a DGAT1 inhibitor potently increased GLP-1 release in HFD-fed rats *in vivo* [73]. Further, the inhibition of DGAT-1 was associated with an increased mitochondrial FAO [76] indicating a possible role of intestinal FAO in the release of GLP-1 from ECs [73].

Among all FA, especially monounsaturated FA are potent stimuli of GLP-1 release [78]. Diets rich in olive oil, as they are abundant in the Mediterranean diet, are associated with beneficial effects for cardiovascular diseases and metabolic syndrome [131 - 135]. The positive effect of diets rich in monounsaturated FA on the development of obesity has been suggested to involve the secretion of GLP-1 [78]. By far the most abundant monounsaturated fatty acid in food is oleic acid (OA), which has been shown to stimulate the release of GLP-1 via activation of GPR120, a G_{αq}/G_{α11} GPR [124], as well as through GPR40, a G_{αq}/G_{αs} GPR [121, 136, 137]. In addition to stimulating the L-cell via membrane bound GPR, OA is also actively taken up by the GLUTag L-cell line model [138] via the free fatty acid transport protein (FATP) 4 as well as the class B scavenger receptor cluster-of-differentiation 36 (CD₃₆)/fatty acid translocase (CD₃₆) [139]. An inhibition of OA uptake through specific inhibition of CD₃₆ decreased the OA-induced release of GLP-1 *in vitro*. It has thus been hypothesized that OA directly activates the isoenzyme PKCζ [119, 139, 140]. In mice lacking FATP4 OA had a significantly weaker effect on GLP-1 release [138], suggesting that OA uptake into L-cells via the FATP4 somehow contributes to OA-induced GLP-1 release. All in all, although many pathways have been proposed to contribute to the fatty acid-induced release of GLP-1, the complexity of the intracellular pathways, the morphology of the intestine and the challenge to isolate these cells, make it still difficult to identify a generally accepted pathway.

1.3 References

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Chapter 2:

Objectives of the thesis

2 Objectives of the thesis

The ability of the epithelial monolayer to sense and adapt to changes in the diet is an attractive feature of this organ that could be useful for the early prevention and treatment of obesity. It has been reported that consumption of a high-fat-diet (HFD) potentially changes the metabolic properties of the small intestine. Moreover, a pharmacological modulation of intestinal fatty acid metabolism was associated with an enhancement of the metabolic effects of HFD in the jejunum, but not in the duodenum or the liver. Also, it has been reported that an inhibition of the re-esterification of absorbed fat in the enterocyte was associated with an upregulation of enzymes involved in fatty acid oxidation (FAO) and with an increased level of circulating GLP-1 in HFD-fed rats.

Therefore, in the first part of the study we wanted to assess the early adaptation of the different sections of the intestine to HFD and to compare it to hepatocytes. We therefore assessed the HFD-induced changes in gene and protein expression in primary epithelial cells from the duodenum and the jejunum and compared them to the corresponding changes in hepatocytes of mice fed a HFD for 3 (short-term) or 14 (medium-term) days.

Given the ability of HFD to modulate the metabolic properties of the jejunum rather than the duodenum or the liver, the next aim of this work was to assess the metabolic properties of immortalized cell lines from the duodenum, the jejunum, and the liver. We assessed the glycolytic flux and the oxidative phosphorylation with the Extracellular Flux Analyzer (XF24) as well as the production of lactate at baseline conditions, as well as in response to nutrients and pharmacological metabolic modulators.

Finally, we tested the influence of mitochondrial FAO on the OA-induced release of GLP-1 from enteroendocrine cells.. To that end we assessed cellular bioenergetics as well as GLP-1 release in response to OA and different pharmacological metabolic modulators in an immortalized EC line model as well as in rats.

Chapter 3

Metabolic adaptation of the small intestine to short- and medium-term high-fat exposure

Based on Clara *et al.*, Journal of Cellular Physiology, *Submitted*.

3 Metabolic adaptation of the small intestine to short- and medium-term high-fat exposure.

3.1 Introduction

Whole body energy homeostasis is regulated via complex mechanisms involving continuous communication between the brain and peripheral organs [1]. During food ingestion, absorption, metabolism and storage of nutrients, information about the energy status of the body is conveyed to the brain through metabolites and hormones circulating in the blood as well as through afferent nerve signals from peripheral organs [2 - 4].

The small intestine is one of the peripheral organs involved in the regulation of energy homeostasis. It provides a large surface for the breakdown and absorption of the ingested food [5 , 6] and has been shown to efficiently adapt to the composition of the diet. Thus, diet composition modulates the digestive and absorptive capacities of the epithelium [7 - 11]. The interaction of luminal food components with the vast monolayer of absorptive, goblet, enteroendocrine and Paneth cells [12, 13] makes the small intestine an important player in the physiological regulation of energy homeostasis [9, 11, 14 - 17].

High-fat diet (HFD) feeding changes the functional properties of the small intestinal epithelium significantly. In particular enzymes involved in the fatty acid metabolism and oxidative phosphorylation pathways are substantially increased after 4 weeks of HFD exposure [10, 18]. Also, we and others have shown that the systemic administration of fatty acid metabolism modulators induced an acute stimulation of fatty acid oxidation (FAO) and ketogenesis in the jejunum, but not in the liver [19 - 23]. These studies highlight the prominent role of the small intestine in the metabolic response to a dietary fat load and indicate that the different small intestinal sections might respond differently to dietary fat. Up to date, however, little is known about the metabolic characteristics of the enterocytes in the different small intestinal sections and how rapidly these cells respond to dietary fat.

Therefore, we investigated the metabolic response of duodenal, jejunal and hepatic cell lines to glucose and oleic acid (OA) by assessing cellular bioenergetics using the Seahorse Extracellular Flux Analyzer (XF24). In addition, we investigated the HFD-induced changes in gene and protein expression of FAO-related enzymes in the intestine and the liver following short- (3 days) and medium-term (14 days) HFD feeding in mice.

3.2 Experimental procedures

3.2.1 Compounds and concentrations

Dulbecco's modified eagle medium (DMEM 41965-039), fetal bovine serum (FBS, 10500-064), penicillin streptomycin (PenStrep, 15140-122) were purchased from GIBCO, Life Technologies Corporation, Carlsbad, California. Oligomycin (Oligo, O4876), carbonyl cyanide 4-trifluoromethoxy-phenylhydrazone (FCCP, C2920), rotenone (Rot, R8875), antimycin (Anti, A8674), dimethyl sulfoxide (DMSO, D8418), OA (O7501) and fatty acid-free bovine serum albumin (BSA, 7030) were purchased from Sigma Aldrich (St. Louis, MO)

3.2.2 Cell culture

We used human duodenal adenocarcinoma (HuTu-80, 300218, Cell Lines Services GmbH, Eppelheim; Germany), porcine normal intestinal jejunal epithelial (IPEC-J2, ACC 701, DSMZ, Braunschweig, Germany) and human liver hepatocellular carcinoma cell lines (HepG2, ATCC HB-8065 DSMZ, Braunschweig, Germany). All cell lines were cultured at 37°C, 95% relative humidity (rHu) and 5% CO₂ in DMEM supplemented with 10% of FBS and 1% of PenStrep. No PenStrep was added to the IPEC-J2 growth medium [24].

3.2.3 Metabolic characterization of the cell lines

All cell lines were subjected to oxygen consumption (OCR) and media acidification (ECAR) measurements using the XF24 (Seahorse Bioscience, Copenhagen, Denmark). To do so, 10×10^4 cells were seeded into each well of the customized XF24 cell culture plate and incubated at 37 °C, 95% relative humidity (rHu) and 5% CO₂ overnight. One hour prior to the experiment, all cells were incubated at 37 °C, 95% rHu and 0% CO₂ in pre-warmed non buffered DMEM (XFDMEM, 102352-000, Seahorse Bioscience, Copenhagen, Denmark) or unbuffered Krebs-Henseleit buffer (XFKHB, (mmol/l) 111 NaCl, 4.7 KCl, 2 MgSO₄, 1.2 Na₂HPO₄ (pH 7.4,37°C)) as described in the figure legend. After the start of the experiment and 15 min equilibration time, OCR and ECAR were assessed in HuTu-80 and in IPEC-J2 cells every 8 min (4 min mix, 2 min wait, 2 min measure) and in HepG2 every 9 min (5 min mix, 2 min wait, 2 min measure). OCR and ECAR were monitored both under basal conditions and after addition of different metabolic modulators to the medium. OCR and ECAR values are expressed as absolute values/number of cells.

First, to assess the basal metabolism of the three cell lines, the basal OCR and ECAR were measured for 24 - 27 min after 1 h incubation in XFKHB or XFKHB containing 2.5 mM glucose. Second, to determine the activity of the oxidative phosphorylation pathway (OXPHOS) of the different cell lines, the mitochondrial stress test was performed. To do so, 1 µg/mL Oligo, different concentrations of FCCP, 2 µg/mL Anti and 1.5 µM Rot, were consecutively (every 24 - 27 min) added to the cells incubated in XFDMEM. To determine the minimal dose of FCCP needed to maximally increase the OCR, FCCP was titrated for every cell line.

The cellular response to metabolites was assessed by adding 100 µM OA, or 20 mM glucose to the cells incubated in XFKHB. In case of HepG2 cells, OA was bound to 1% BSA. XFKHB or XFKHB containing 1% BSA was used as control.

3.2.4 Lactate analysis

Confluent layers of HuTu-80, IPEC-J2 or HepG2 in P60 cell plates were washed 2x with PBS and growth medium was replaced with 1 mL of XFKHB. After 1 h incubation at 37 °C, 95% rHu, and 0% CO₂, 100 µM OA, 20 mM glucose or KHB were added to the cells. After 20 min incubation with the substrates, the medium was taken for analysis of lactate using standard colorimetric and enzymatic methods adapted for the Cobas MIRA® autoanalyzer (Hoffman LaRoche). The cells were washed 2x with PBS, scraped from the bottom of the cell plate, centrifuged at 1000 rpm and 4 °C for 2 min, and the obtained cell pellet was subjected to protein extraction using RIPA lysis buffer containing (mM) 100 Tris pH 7.4, 400 NaCl, 10 EDTA and 1% Triton X-100. The total protein content was determined using the DC Biorad Protein Assay (BioRad DC Protein Assay).

3.2.5 Housing conditions

C57/Bl6N mice (6 - 9 weeks old) were group-housed (2 - 3 mice per cage) under standard conditions, in an air-conditioned room (22 ± 2°C and 60% relative humidity) kept on a 12 h light/12 h dark cycle (lights off at 09:00 AM). During two weeks of adaptation to the housing conditions, mice were fed *ad libitum* a control diet (CtD, SNIFF, D12450B mod., Pork lard 0%, Soybean oil 4.3%). After the adaptation time, half of the mice were switched to HFD (SNIFF diet D12492 60 kJ% fat, E15742: 60% of energy from fat) for 3 or 14 days, respectively. The remaining animals were kept on CtD. All animals had continuous *ad libitum* access to tap water, and body weight (BW) was assessed every 3 days. All experiments were approved by the Cantonal Veterinary Office of Zurich.

3.2.6 Tissue collection

After 3 or 14 days of diet exposure, mice were fasted for 8 hours during the dark phase, re-fed for 30 min, and then sacrificed by decapitation. The liver was removed and immediately stored in liquid nitrogen. The small intestine was

removed and divided into duodenum (5 cm of the proximal part of the small intestine (starting 1.5 cm from the pylorus) and jejunum (next 8 cm) and flushed with ice-cold PBS.

Then the intestine was inverted and enterocytes were isolated as described elsewhere [25]. The collected enterocytes were immediately transferred into cryo tubes (Nunc Cryotube 1.8 mL) and placed in liquid nitrogen before being stored together with the liver samples at -80 °C until further analysis.

3.2.7 Gene expression studies

RNA was extracted from all tissues using the Trizol method. Following DNase treatment (Quiagen, 79254), cDNA was synthesized with the High Capacity cDNA RT Kit (Applied Biosystems, 4368814) from 1µg RNA; then all samples were analyzed by qPCR. The following genes were analyzed: *CPT1* (5'GACGAATCGGAACAGGGA3' and 5'TGGACTTGTCAAACCCACCTG3'), *HMGCS2* (5'AATGTCACCACAGACCAC3' and 5'GGAGCAGGAGGGATTGTA3'), *SLC5A1* (*SGLT1*) (5'GAAGCATCCTTTCTTCTTCTG3' and 5'AAGAGCGAATCGACCTGGA3') and *Hexokinase (HK)* (5'GTGGACGGGACGCTCTAC3' and 5'TTCACTGTTTGGTGCATGATT3'). Each sample was run in duplicate, analyzed with the ddCT method and normalized against *GAPDH* (*GAPDH*: 5'ATGGTGAAGGTCCGGTGTGA3' and 5' AATCTCCACTTTGCCACTGC3') as reference gene.

3.2.8 Western Blot analysis

Proteins were isolated from phenol-ethanol supernatant layer left over after the RNA extraction. The total protein content was estimated using the DC Biorad Protein Assay (BioRad DC Protein Assay). Thirty µL of the protein mixture (1 µg protein/µL) dissolved in Laemli Buffer (100 mM TRIS pH 6.8; 4% (w/v) SDS; 0.2% (w/v) Bromophenol Blue, 20% (W/v) glycerol and 200 mM DTT) were loaded into an electrophoresis gel (10% SDS and 12% polyacrylamide). The separated proteins were then transferred on a 0.2 µm

nitrocellulose membrane and blocked with 5% milk in TBS-T for 1 h. Thereafter, the membrane was incubated over night with the primary antibody (Rabbit anti-HMGC_oAS2 (mitochondrial), dilution 1 : 200, Aviva System Biology, ARP41562, T100) of the target protein. Then the membrane was washed with TBS-T and incubated for 1 h with the appropriate secondary antibody (Anti-rabbit IgG, HRP-linked, dilution 1 : 2000, Cell Signaling Technology, 7074). β -actin was used as reference protein using β -actin antibody (Mouse monoclonal Anti- β -actin, dilution 1 : 3000, Sigma-Aldrich, A-2228) and secondary antibody (Anti-mouse IgG, HRP-linked, dilution 1 : 2000, Cell Signaling Technology, 7076). The bands of the target proteins were detected with enhanced chemiluminescence using the Image Quant detector (ImageQuant, GE Health Care).

3.2.9 Data presentation and statistics

All results are expressed as mean \pm SEM of absolute values, % changes or fold changes, as specified in the figure legends. Statistical significance was tested using Student's *t* test, One-Way or Two-Way ANOVA followed by Tukey's multiple comparison post-hoc test as indicated in the figure legends. In all experiments $p < .05$ was used as threshold for significance. The gene expression data were expressed as fold changes against CtD.

3.3 Results

3.3.1 The jejunal cell line had a higher basal oxygen consumption rate than the duodenal or the liver cell lines

The assessment of basal bioenergetics of different cell lines was performed with the XF24. Basal metabolism was measured in cells incubated for one hour in the modified XFKHB. Cellular respiration, an indicator for the activation of the oxidative phosphorylation pathway was higher in the jejunal (IPEC-J2 331.6 \pm 14.6 mmol/min) than in the duodenal (HuTu-80 66.6 \pm 9.2 mmol/min) or hepatic (HepG2 107.8 \pm 19.7 mmol/min) cell lines (Figure 1 A). In

contrast, medium acidification, the indicator for the glycolytic pathway, was greater in the hepatic cell line (12.0 ± 1.5 mpH) than in the intestinal cell lines (Figure 1, B).

To test the effect of glucose on basal cellular metabolism we added 25 mM glucose to the XFKHB. Again, the consumption of oxygen by the jejunal cells was higher than the one of duodenal or hepatic cells (Figure 1 C). Jejunal cells also displayed the lowest ECAR after addition of glucose to the medium (Figure 1 D).

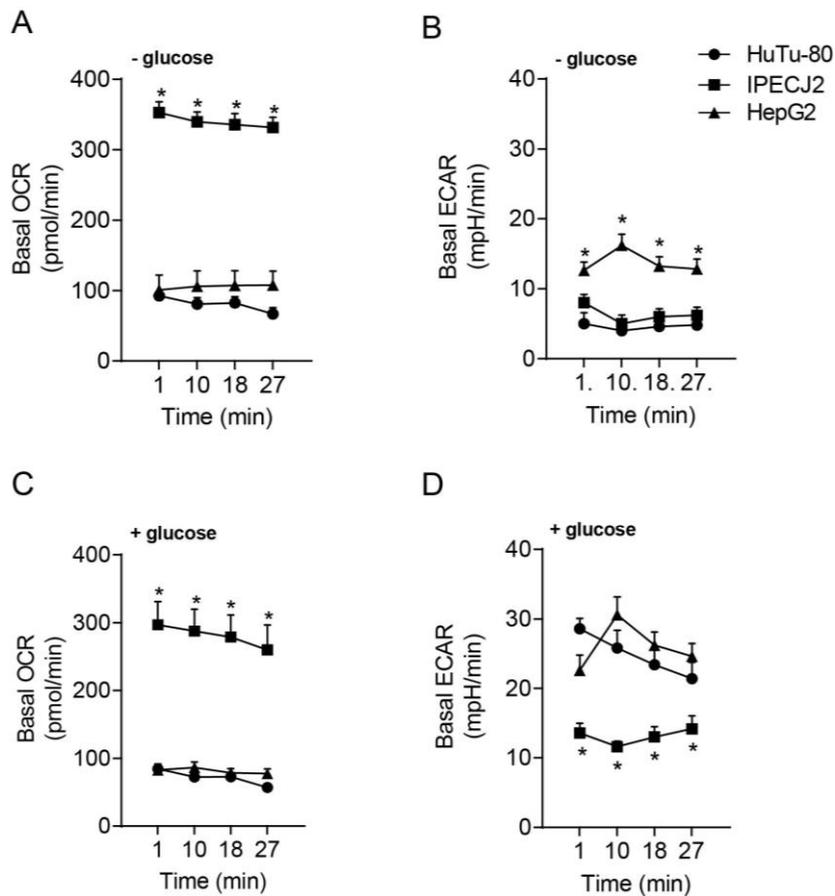


Figure 1. The jejunal cell line had a higher basal oxygen consumption rate than the duodenal or the liver cell lines. Basal oxygen consumption rate (OCR) (pmol/min) of HuTu-80, IPEC-J2 and HepG2 cells incubated in (A) XFKHB or (B) XFKHB containing 25 mM glucose (for each group $n = 5$). Basal extracellular acidification rate (ECAR) (mpH/min) of HuTu-80, IPEC-J2 and HepG2 cells incubated in (C) XFKHB or (D) XFKHB containing 25 mM glucose (for each group $n = 5$). * $p < .05$, One way ANOVA followed by Tukey's multiple comparisons post hoc test.

3.3.2 The jejunal cell line had a higher metabolic spare capacity than the duodenal cell line

In order to test the mitochondrial capacities of the different cell lines and to assess the potential of the different cell lines to oxidize fat, we consecutively challenged the mitochondrial respiratory chain with Oligo, the inhibitor of the ATP synthase, FCCP, a mobile ionophore, Anti, the inhibitor of Coenzym Q (Complex III) and Rot, the inhibitor of Complex I. As expected, Oligo reduced cellular respiration by 65.5 ± 6.4 , 48.1 ± 6.3 and $62.3 \pm 1.2\%$ of basal respiration in the HuTu-80, IPEC-J2 and HepG2 cell line, respectively (Figure 2 A - C). To test the maximal capacity of each cell line to activate the respiratory chain, we injected the minimal dose of FCCP needed to maximally increase cellular respiration. FCCP increased respiration in all cell lines. The spare respiratory capacity, defined as the difference between basal and maximal respiration rate, was 20.2 ± 11.5 and $113.8 \pm 13.4\%$ of the basal respiration in IPEC-J2 and HepG2 cells, respectively. Despite titration of FCCP, the maximal FCCP-induced respiration in HuTu-80 cells was lower than respiration at basal levels. Non-mitochondrial respiration was for all cell lines lower than basal respiration. Indeed, after inhibition of complex I and III with Rot and Anti, only 24.0 ± 9.8 , 44.7 ± 8.6 and $31.8 \pm 1.8\%$ of the basal respiration of HuTu-80, IPEC-J2 and Hep2, respectively, were not due to the respiratory chain.

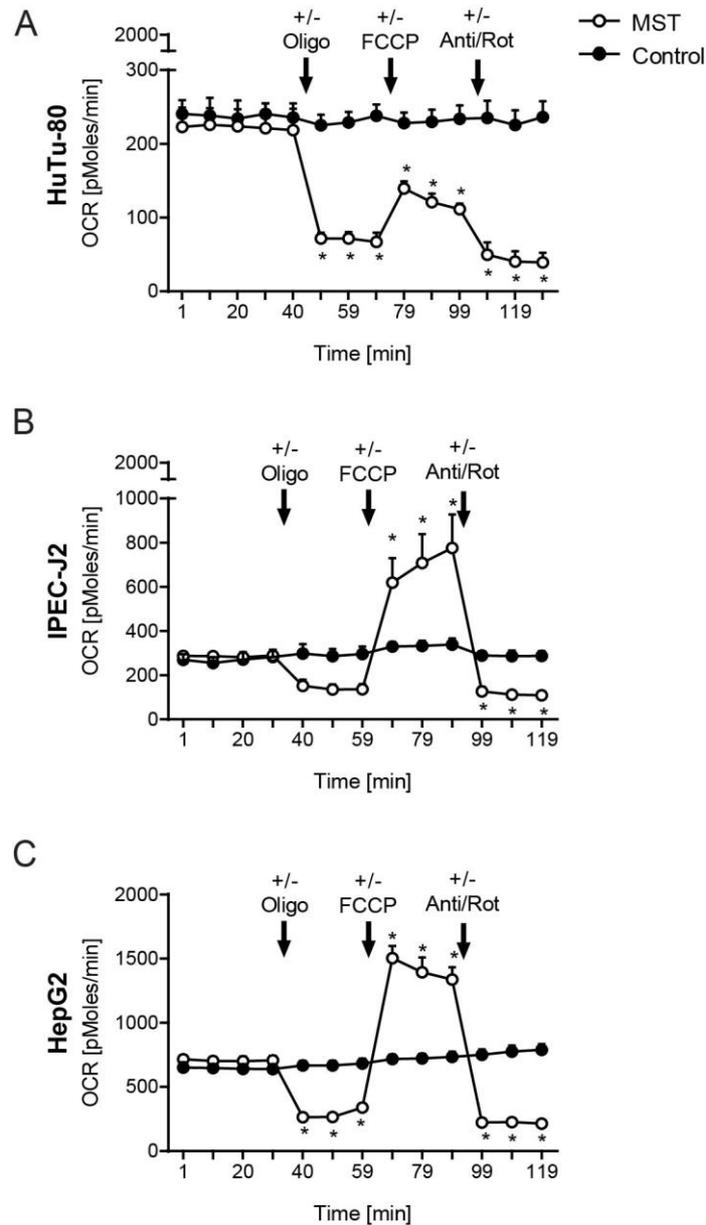


Figure 2. The jejunal cell line had a greater maximal respiratory capacity than the duodenal or the liver cell lines. Oxygen consumption rate (OCR) (pmol/min) of (A) HuTu-80, (B) IPEC-J2 and (C) HepG2 cells incubated in XFDMEM (for each group n = 5). The mitochondrial stress test was performed via consecutive injection of 1 μ g/mL oligomycin (Oligo), (A) 0.25 μ M, (B) 1.0 μ M, (C) 0.5 μ M FCCP and 2 μ g/mL antimycin + 1.5 μ M rotenone (Anti/Rot). XFDMEM was injected as control. * $p < .05$ to the control, Two way ANOVA followed by followed by Tukey's multiple comparisons post hoc test.

3.3.3 None of the substrates significantly increased cellular respiration, but glucose increased medium acidification in all three cell lines

To test the acute response of the cell lines to energy yielding substrates, we injected glucose or OA and measured OCR and ECAR with the XF24. We

did not see an increase in OCR after addition of OA in any of the tested cell lines (Figure 3 A, C and E). Interestingly, glucose decreased the OCR of all cell lines and potently increased their ECAR (Figure 3 B, D and F).

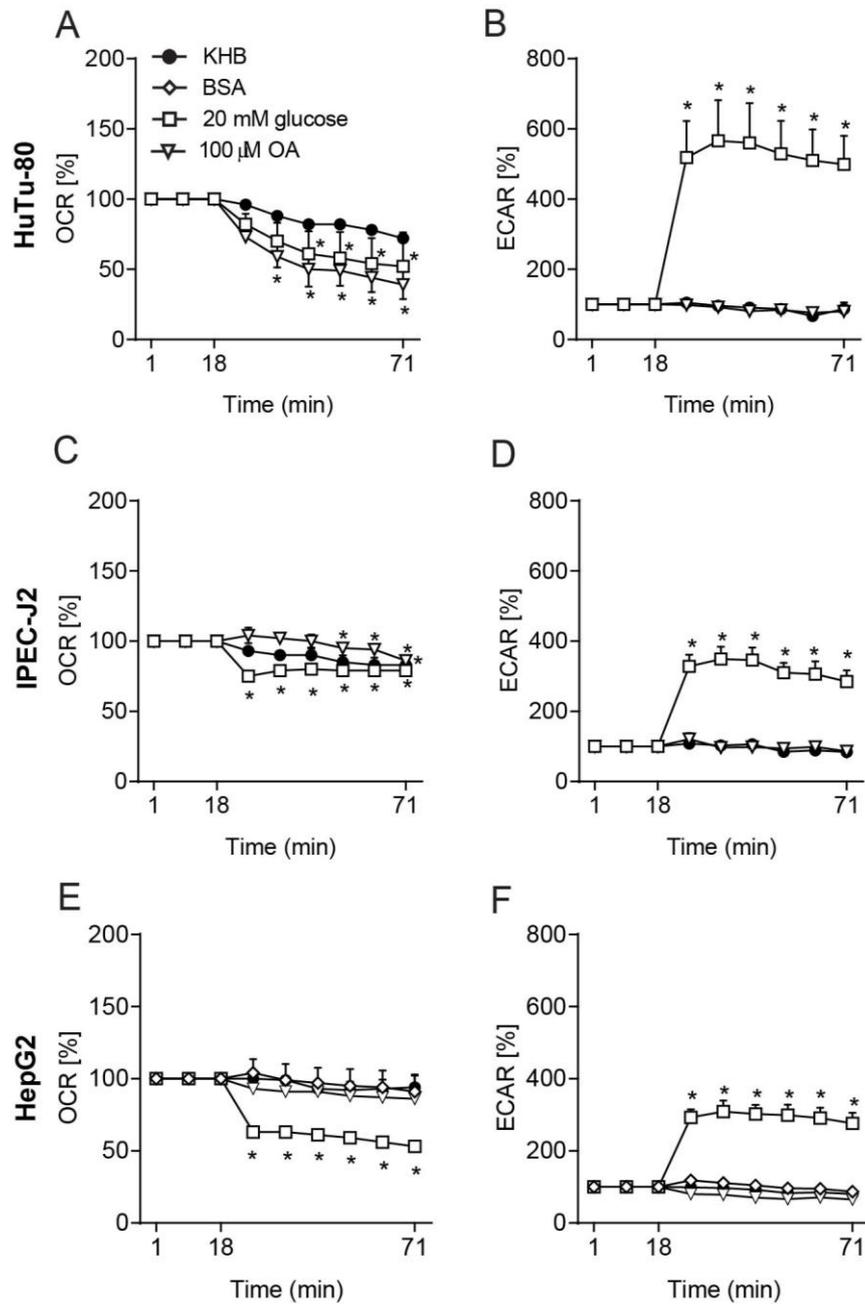


Figure 3. None of the energy yielding substrates stimulated mitochondrial respiration, whereas glucose potently increased medium acidification in all cell lines. Oxygen consumption rate (OCR) (%) and extracellular acidification rate (ECAR) (%) of (A-B) HuTu-80 and (C-D) IPEC-J2 line incubated in XFKHB and stimulated with 20 mM glucose, 100 μM OA or as XFKHB as control. (E-F) HepG2 cell line incubated in XFKHB and stimulated with 20 mM glucose, 100 μM OA bound to 1% BSA, XFKHB or XFKHB containing 1% BSA as control. * $p < .05$ to measurement point before injection of the compound, Two way ANOVA followed by Dunnett's multiple comparisons post hoc test.

3.3.4 Glucose induced the production of lactate in all cell lines

To test whether the potent glucose-induced increase in ECAR was due to lactate production, we measured the lactate concentration in the cellular medium after addition of glucose. As expected, glucose induced a robust increase in lactate concentration in all cell lines (HuTu-80: 18.4 ± 0.4 , IPEC-J2: 3.7 ± 0.2 and HepG2: 5.3 ± 0.2 fold change of lactate production in KHB buffer) (Figure 4 A, B and C).

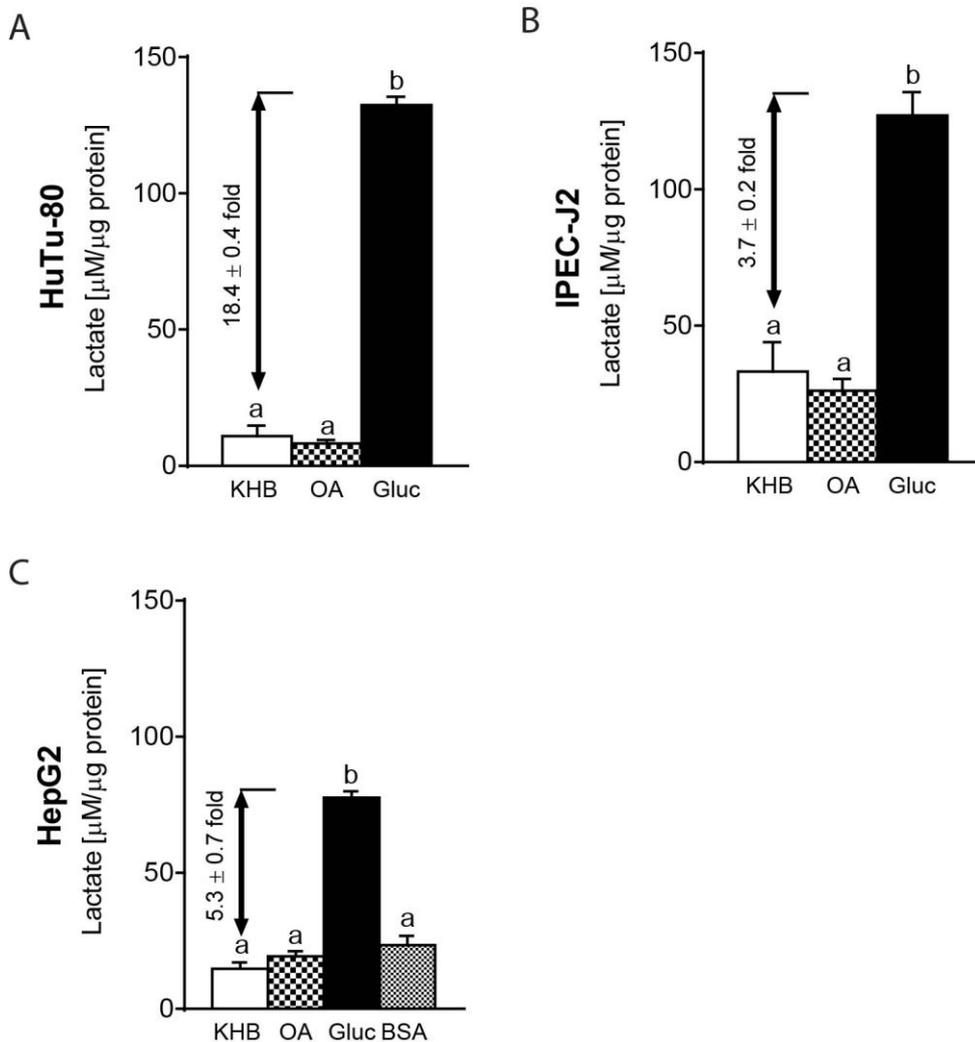


Figure 4. Glucose potently induced the production of lactate in all cell lines. Lactate concentration ($\mu\text{mol}/\mu\text{g protein}$) in the cellular medium of (A) HuTu-80 and (B) IPEC-J2, and in response to KHB, 100 μM oleic acid (OA), or 20 mM glucose (Gluc) and (C) HepG2 in response to KHB, 100 μM OA bound to 1% BSA (OA-BSA), 20 mM glucose (Gluc) or 1% BSA (BSA) (for each group $n = 5$). * $p < .05$, One-way ANOVA followed by Tukey's multiple comparisons post hoc test.

3.3.5 Medium-term, but not short-term high fat diet exposure increased body weight in mice

To test the effect of HFD on the BW of the mice, we first fed all animals with CtD as a run-in for 14 days. Thereafter, half of the animals continued on CtD whereas the others were switched to HFD for 3 or 14 days. Three days of HFD did not induce an increase in BW compared to CtD-fed animals (Figure 5 A), whereas 14 days of HFD did (Figure 5 B).

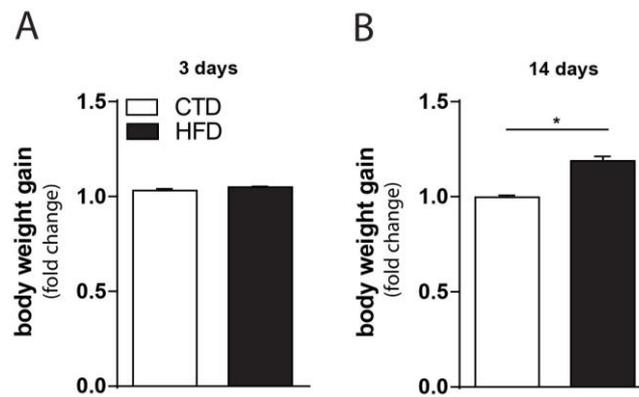


Figure 5. Exposure to high fat diet for 3 days did not influence the body weight gain of the mice. Relative body weight gain (fold change) of mice fed control (CtD) or high fat diet (HFD) for (A) 3 or (B) 14 days (for each group $n = 7 - 9$). * $p < .05$, Unpaired t-test.

3.3.6 Short- and medium-term high fat diet exposure had different effects on the expression of genes related to fatty acid and glucose metabolism in the duodenum, jejunum and liver

To test whether HFD exposure induced different metabolic changes in the duodenum, jejunum or liver, we isolated the enterocytes and the liver of the mice fed CtD or HFD for 3 or 14 days. The 3-day HFD exposure did not affect the gene expression of *CPT1* (carnitine palmitoyltransferase I), *HMGCS2*, *SLC5A1* or *HK* in the liver (Figure 6 C). In the jejunum, however, 3-day HFD exposure increased the expression of *CPT1*. Interestingly, 3-day HFD exposure also reduced the expression of genes related to glucose metabolism in both intestinal sections. Fourteen day HFD exposure did not alter the expression of *CPT1* in the intestine (Figure 6 A and B). It did, however, increase the expression of *HMGCS2*, in the duodenum, but not in the liver or the jejunum.

Interestingly, 14 day HFD exposure induced an increase in the expression of genes involved in glucose metabolism in the liver (Figure 6C).

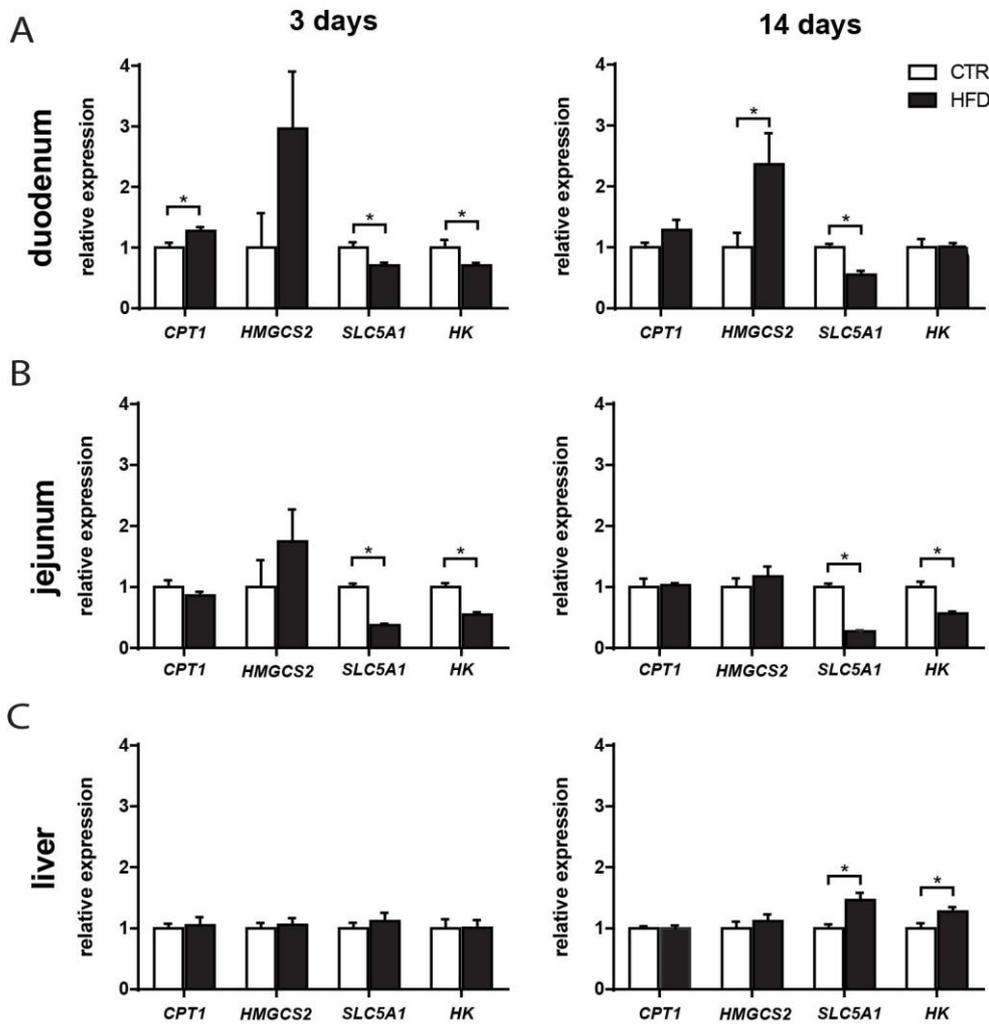


Figure 6. Exposure to high fat diet for 3 days influenced the gene expression in the intestine but not in the liver. Relative expression of *CPT1*, *HMGCS2*, *SLC5A1* and *HK* in the (A-B) duodenum, (C-D) jejunum or (E-F) liver of mice exposed for (left) 3 days or (right) 14 days to control (CtD) or high fat diet (HFD) (for each group n = 7 - 9). The data are expressed as relative changes to the expression in animals fed CtD. * $p < .05$, unpaired *t*-test.

3.3.7 Short and medium-term high fat diet exposure induced an upregulation of HMG-CoAS2 in the duodenum and jejunum, but not in the liver

Whereas it is known that HFD feeding influences the expression of fatty acid metabolism related genes, we wanted to test whether short or medium-

term HFD exposure also induces a change in the protein levels of HMG-CoAS2, the key enzyme of ketogenesis. Three and 14 day HFD exposures clearly upregulated the level of mitochondrial HMG-CoAS2 in the intestine, but not in the liver (Figure 7).

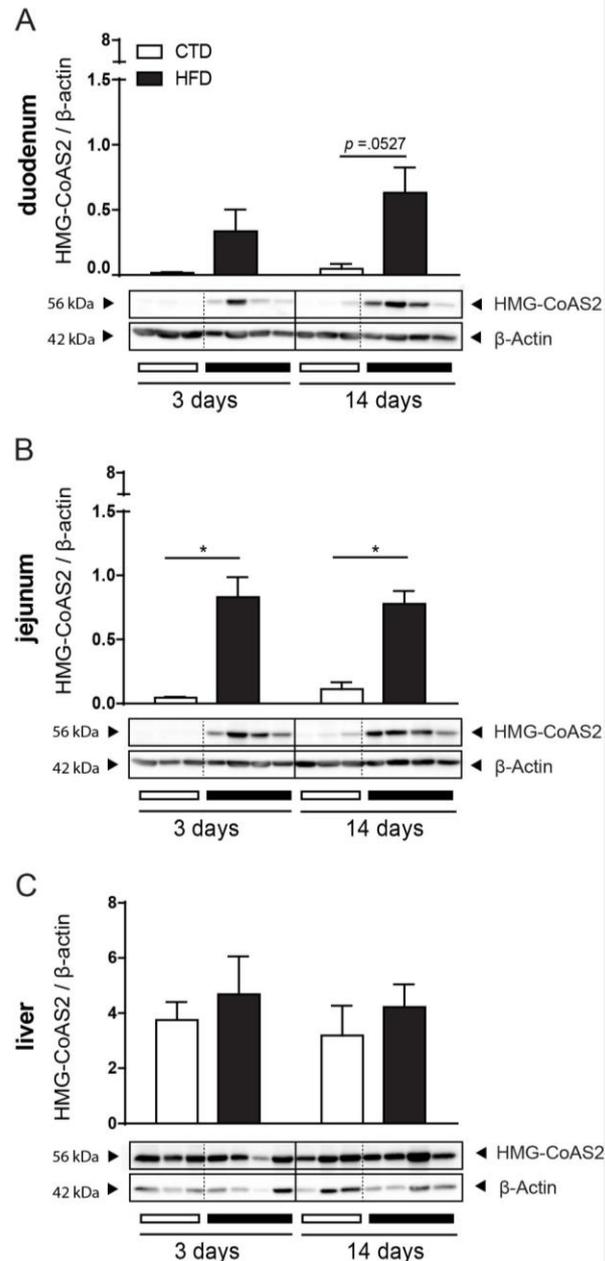


Figure 7. High fat diet exposure potentially upregulated HMG-CoAS2 protein expression in the intestine, but not in the liver. Relative quantification and Western Blots of HMG-CoAS2 from the (A) duodenum (B) jejunum and (C) liver of mice exposed for 3 days or 14 days to control (CtD) or high fat diet (HFD) (for each group $n = 3 - 4$). * $p < .05$, unpaired t -test.

3.4 Discussion

We characterized the metabolic features of cell lines derived from the proximal and more distal parts of the small intestine regarding lipid and glucose metabolism *in vitro* and *in vivo* and compared them to liver-derived cells. *In vitro*, we noticed a clear difference between the duodenal and jejunal cell lines in that the duodenal cells displayed more anaerobic glycolysis whereas the jejunal cells favored oxidative metabolism in response to glucose. These results emphasize the higher potential of jejunal compared to duodenal cells to activate mitochondrial metabolism when energy yielding substrates are available. When the enterocytes were isolated from mice fed HFD or CtD for 3 or 14 days, the HFD feeding induced an early ketogenesis in the small intestine but not in the liver.

Ingested foods go through different digestion steps. Lipid mixtures are hydrolyzed in the stomach, then digested in the duodenum by the pancreatic lipases, emulsified with bile acids and finally absorbed in the jejunum [26 - 28]. Recently we and others reported that HFD feeding has a direct influence on the catabolism of fatty acids (FA) in the small intestinal epithelium [19, 20, 22, 29 - 31]. Interestingly, reducing fatty acid re-esterification through inhibition of the enzyme diacylglycerol O-acyltransferase-1 (DGAT1) [20] or stimulating FAO via a pharmacological activation of the peroxisome proliferator-activated receptor alpha (PPAR α) enhanced the HFD-induced upregulation of ketogenesis in the jejunum, but not in the duodenum or in the liver [32].

Based on these findings, we hypothesized that enterocytes derived from the jejunum are metabolically more active than those of the duodenum. *In vitro*, we could clearly show a higher metabolic activity as well as flexibility of the cell line derived from the jejunum (IPEC-J2) compared to the one from the duodenum (HuTu-80 cells). The potent mitochondrial uncoupler, FCCP, did not induce maximum basal respiration in HuTu-80 cells, indicative of a weak mitochondrial oxidative flexibility of these cells. In contrast, FCCP potently increased OCR in the jejunal and hepatic (HepG2 cells) cell lines. The maximal respiratory capacity is defined as the additional capacity of a cell to activate mitochondrial respiration under conditions of increasing energy demand, increased energy availability, or under stress conditions [33, 34]. The fact that

jejunal and hepatic cells showed a higher maximal respiratory capacity than duodenal cells indicates that these cells can more flexibly activate mitochondrial bioenergetics in response to energy availability. Thus, the fact that the jejunal cell line showed a higher metabolic oxidative capacity than the duodenal cell line is consistent with the result of other studies indicating that HFD exposure or an additional acute modulation of fatty acid metabolism affects the metabolic characteristics of the jejunal rather than the duodenal epithelial cells [10, 19, 20]. In general, the greater metabolic flexibility of jejunal compared to duodenal cells may be related to the energy demands of the jejunum's absorptive function.

The two main energy-yielding pathways in the cell are glycolysis and oxidative phosphorylation. The parameter we used to quantify the glycolytic flux is the ECAR, which is commonly used to quantify the anaerobic glycolytic production of lactate. An increase in ECAR therefore suggests an increase in anaerobic glycolysis [35]. In line with the increase in medium acidification after addition of glucose, the oxygen consumption rate in all cell lines decreased, indicating that the presence of glucose reduced oxidative metabolism and enhanced glycolysis in all cell lines. Indeed, pyruvate, the last product of cytosolic glycolysis, is either transported into the mitochondria for aerobic oxidation or converted into lactate via lactate dehydrogenase [36]. The use of the ECAR as indicator for only anaerobic glycolysis has, however, been questioned recently [37]. Therefore, we also measured the cellular production of lactate in response to glucose. Similar to the observation in the XF analysis, we noticed that the lactate production in response to glucose was much greater in the duodenal than in the jejunal or hepatic cell lines. Physiologically, glucose is rapidly absorbed predominantly via active uptake in the proximal part of the small intestine [38 - 40]. After the enterocyte passage, glucose is released into the circulation as glucose, lactate or, in a small amount, as pyruvate and carbon dioxide [39]. Despite all the limitations of the use of cell lines as model for metabolic studies [41], our results clearly indicate a more active metabolism of glucose in the duodenal than in the jejunal or the hepatic cell line models.

The intestine is the first organ exposed to the energy derived from food. Early studies showed that the intestine is metabolically very flexible and that the

metabolic features change dependent on the composition of the diet and the substrate load [7, 8]. Indeed, animals exposed to HFD (20% w/w fat content) had increased lipid absorption and reesterification in comparison to animals fed chow diet (4% w/w fat content) [7]. Since then, there is growing evidence that the intestine plays a role in the etiology of obesity and the metabolic syndrome [10, 11, 18, 42]. Previous studies reported an effect of HFD exposure for at least one week on the metabolic characteristics of the intestine [10, 18]. We here show that changes in the gene and protein expression in the intestine are already detectable after only 3 days of exposure to HFD, i.e., at a time before we could detect any changes in the liver or in BW. To test whether a 3-day HFD exposure differentially regulated the gene and protein expression of key metabolic enzymes, we isolated the enterocytes with a novel and very specific method. This method allows for a fast and specific isolation of epithelial cells without the use of enzymes or EDTA and can be performed in ice cold conditions [25]. We consistently found that 3 and 14 days of HFD feeding reduced the expression of genes related to glucose metabolism. This is in line with earlier reports [8, 18, 43] and underlines the plasticity of the intestine to specifically adapt to changes in diet conditions. In relation to fatty acid metabolism, we observed that 14 days of HFD exposure potently upregulated protein levels of HMG-CoAS2 in the jejunum, whereas there was only a tendency towards an upregulation in the duodenum and no effect in the liver. The fact, however, that 3 days of HFD exposure were enough to potently upregulate the protein levels of HMG-CoAS2 in the jejunum emphasizes the flexibility of this section in sensing the luminal nutrient content and adapting to it rapidly. HFD exposure clearly induced a metabolic re-programming of these cells. The duodenum seems to be less reactive to a change in the diet than the jejunum, although after 14 days of HFD exposure it also adapted in terms of gene and protein expression. Our study revealed that HFD feeding for 3 or 14 days induced the upregulation of enzymes involved in jejunal ketogenesis. This is in line with previous reports showing that 2 weeks of HFD exposure increased the activity of CPT-1, the key enzyme of FAO [44].

In sum, our results suggest the existence of a metabolic gradient for glucose and lipid metabolism from the duodenum to the jejunum. This assumption is supported by the fact that the metabolic flexibility of the intestinal

epithelial cells is diet- and segment-dependent [8] and that glucose and fatty acid metabolism-related enzymes are differentially regulated along the intestine. Moreover, the *in vitro* studies showed that the jejunal cells had a higher metabolic capacity than duodenal cells with a trend towards oxidative metabolism, suggesting a more efficient and faster adaptation to changes in diet conditions than duodenal cells. This could also explain the faster HFD-induced upregulation of HMG-CoAS2 protein in the jejunum than in the duodenum. Short-term HFD exposure already upregulated FAO in the small intestine without affecting it in the liver. This emphasizes the possible importance of the small intestine in sensing food-derived energy and thus influencing whole body energy homeostasis [15, 17].

During the pre-agricultural time, the plasticity of the intestine to metabolically adapt to the diet could have been a crucial evolutionary feature to efficiently take up nutrients from the often limited food supply [8, 45]. Nowadays, the almost unlimited availability of high-density food, challenges this important evolutionary feature of the small intestine, but studying diet-induced metabolic changes in the small intestine may show ways to help counteract obesity by nutritional interventions.

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Chapter 4

Oleic acid stimulates glucagon-like peptide-1 release from enteroendocrine cells by modulating cell respiration and glycolysis

Based on Clara *et al.*, *Metabolism-Clinical and Experimental*, *In Press*.

4 Oleic acid stimulates glucagon-like peptide-1 release from enteroendocrine cells by modulating cell respiration and glycolysis

4.1 Introduction

GLP-1 is a potent satiating and incretin hormone synthesized and released by enteroendocrine cells (ECs) in response to eating [1]. Different mechanisms have been proposed for the stimulatory effect of macronutrients and their digestion products on GLP-1 release [2 - 5]. Most of the studies investigating the mechanisms of GLP-1 secretion from ECs focused on the role of different receptors or transporters located on the luminal or basolateral membrane of EC [6 - 8]. Only few studies addressed a possible effect of intracellular metabolism in this context [9 - 11], and there is no consensus about the metabolic pathways possibly involved.

Fatty acids (FA), especially monounsaturated FA, potently stimulate GLP-1 release *in vivo* [12, 13]. GPR, such as GPR 40 [14], GPR 43 [15] and GPR 120 [1, 16, 17] have been implicated in fatty acid-induced GLP-1 release. *In vitro*, the monounsaturated fatty acid, oleic acid (OA), potently stimulates the release of GLP-1 without impairing cell viability [2 - 5, 18, 19]. Besides stimulating the release of GLP-1 via GPR 120, free OA is also actively taken up by GLUTag cells via the FAT 1, 2 and 4 as well as via CD₃₆ [6 - 8, 20]. A specific inhibition of CD₃₆ with sulfo-N-succinimidyl oleate antagonized the uptake of OA and decreased the OA-induced release of GLP-1 *in vitro* [9 - 11, 20]. Also, mice lacking FAT4 display a decreased release of GLP-1 in response to OA suggesting a possible role of intracellular OA handling in the release of GLP-1. OA could influence exocytosis of GLP-1 vesicles by direct activation of the isoenzyme PKC ζ [11 - 13, 19] or by modulating mitochondrial energy metabolism [14, 21] but the mechanisms that link intracellular OA handling and GLP-1 release are still unclear.

We here investigated the relationship between cellular bioenergetics and GLP-1 release in the EC line model GLUTag. In a first experiment we

characterized the optimal medium for the bioenergetics measures. It is crucial for such experiments that the cells are metabolically active and respond to metabolic inhibitors and stimulators. We used the Extracellular Flux Analyzer (XF24) to simultaneously assess OCR, an indicator of respiratory chain activity, and ECAR, an indicator for the glycolytic flux [15, 22]. We monitored the metabolic response of the ECs to OA, and to different inhibitors of the potential intracellular pathway of OA metabolization, and measured GLP-1 release into the medium.

4.2 Experimental Procedures

4.2.1 Cell culture

Differentiated GLUTag cells (kindly provided by Dr. D. Drucker, University of Toronto, Toronto, Canada) were cultured in complete growth medium (CGM: Dulbecco's modified Eagle medium (DMEM) 1g/L glucose (Life Technologies 31885) supplemented with 10% FBS (Life Technologies 16000) and 1% PenStrep (Life Technologies 10378) at 37°C, 95% rHu and 5% CO₂. For all experiments GLUTag cells were used between passage numbers 15 and 25.

4.2.2 Compounds and concentrations

Oligo 1 µg/mL (Sigma O4876 from 10 mg/mL stock, dissolved in DMSO (Sigma D2438)), FCCP 0-1.25 µM (Sigma C2920 from 2.5 mM DMSO stock), Rot 1.5 µM (Sigma R8875 from 2.5 mM DMSO stock), Anti 2 µg/mL (Sigma A8674 from 2 mg/mL DMSO stock) glucose 1-40 mM (Fisher Scientific 50-99-7 from 3M NaCl stock), OA 62.5 – 1000 µM (Sigma O7501 from 5 mM NaCl stock), 2-desoxy-D-glucose 10 - 200 mM (2DG, Sigma D6134 from 2 M NaCl stock) and Etomoxir 0.5 - 4 mM (Eto, Labor Dr. Heusler GmbH HHAC-Bez. 31770/4, from 4 NaCl stock), UK 5 - 135 µM (UK - 5099, Sigma PZ0160 from 69.37 mM DMSO stock). The medium and all compounds were adjusted to pH 7.4 and 37 °C before every experiment.

4.2.3 XF24 experiments

The XF24 (Seahorse Bioscience) was used to continuously monitor oxygen consumption and media acidification. One day prior to the experiments, 60 K cells were seeded in CGM in a customized XF24 cell plate and cultured overnight at 37 °C, 95% rHu and 5% CO₂. One hour prior to the experiment, cells were washed with XF assay medium (non buffered DMEM (XFDMEM), Seahorse Bioscience, 102353) supplemented with 1 g/L glucose, 1 mM sodium pyruvate (Life Technologies 11360-088) and 1% PenStrep) and incubated with 500 µL XF assay medium for one hour at 37°C, 95% rHu and 0% CO₂. After 15 min equilibration time, OCR and ECAR were assessed every 9 min (5 min mix, 2 min wait, 2 min measure). The different compounds were added to the injection ports of the XF cartridge in 10x concentration and were all prepared in NaCl (0.9% B. Braun).

4.2.4 GLP-1 secretion experiments

To measure GLP-1 release, 150 K GLUTag cells were seeded in 24 - well plates and cultured for 1.5 days to reach 80% confluence. Then the cells were washed twice with PBS and incubated in assay medium (DMEM 1g/L glucose supplemented with 1 mM pyruvate, 1% PenStrep, 0.1 mM, DPPIV inhibitor (DPPIVi) (ANAWA Trading SA) containing the test compound dissolved in NaCl against medium with NaCl as a control. After the desired incubation period, the medium was collected for GLP-1 measurements using Active GLP-1 (ver. 2) Assay Kit (MesoScale Discovery, K150JWC-1) according to the manufacturer's instructions. All results were normalized to GLP-1 secretion in experimental medium containing saline and are expressed as % changes of the control.

4.2.5 Data analysis and statistics

All results are presented as means ± SEM of absolute values, % changes or fold changes, as specified in the figure legends. Statistical significance was tested using Student's t test or One-Way or Two-Way ANOVA

with Tukey's multiple comparison test as indicated in the figure legends. In all experiments $p < .05$ was used as threshold for significance.

4.3 Results

4.3.1 Glucose and pyruvate were necessary to keep GLUTag cells metabolically active

Bioenergetic measurements of cellular metabolism with the XF require metabolically active cells. Therefore, to determine the optimal medium we incubated cells in modified and non buffered XFDMEM basal medium supplemented with 1% PenStrep with or without 5.5 mM glucose and/or 1 mM pyruvate and measured OCR and ECAR (Figure 1 A, B). First we measured basal cellular metabolism and found that the basal respiration and medium acidification of cells incubated in a minimal medium without glucose and pyruvate were very low (70.1 ± 14.3 $\mu\text{mol}/\text{min}$ and 4.7 ± 0.4 mpH/min , respectively, Figure 1 C, D). Supplementation of the XFDMEM with glucose, pyruvate, or both, markedly increased basal respiration (glucose: 440.7 ± 11.1 , pyruvate: 478.1 ± 9.8 $\mu\text{mol}/\text{min}$, glucose and pyruvate: 355.3 ± 7.2 , respectively) (Figure 1 C). This indicates that the presence of at least one of the two metabolites activates mitochondrial energy pathways. Further, with respect to glycolytic flux or medium acidification, glucose with or without pyruvate increased ECAR (Figure 1 D), indicating that glucose is necessary for an active basal glycolytic flux in these cells. In the next step we tested the capacity of the cells to modulate their bioenergetics in response to pharmacological metabolic modulators. Thus, we sequentially added the inhibitor of the mitochondrial ATP synthase Oligo, the mobile ionophore FCCP and the inhibitors of the respiratory chain complexes III and I Anti and Rot. Again, in cells incubated XFDMEM only, we could not modulate intracellular metabolism (Figure 1 A, B). Indeed, the cells incubated in XFDMEM only, did not react to Oligo, FCCP or Anti/Rot. In contrast, cells incubated in XFDMEM supplemented with glucose, pyruvate or both, potently reduced ATP Synthase coupled respiration in response to Oligo (-167.7 ± 14.6 , -240.0 ± 14.1 , -196.3 ± 7.7 $\mu\text{mol}/\text{min}$, respectively) (Figure 1 E).

This suggests that the presence of glucose and/or pyruvate stimulates mitochondrial ATP production via the ATP Synthase. Moreover, although respiration was inhibited potently in all three groups, Oligo stimulated the glycolytic flux only in cells incubated with glucose and pyruvate (22.0 ± 4.0 mpH/min) (Figure 1 F) highlighting the essential role of both, glucose and pyruvate, in the metabolic function of GLUTag cells. Indeed, in cells incubated in XFDMEM supplemented with pyruvate or glucose only, Oligo barely increased glycolytic flux. Next, to test the maximum metabolic capacity, we stimulated the cells with the mobile ionophore FCCP. The presence of pyruvate in the medium was sufficient to increase respiration in response to FCCP (Figure 1G). An additional supplementation with glucose, however, even enhanced this effect. Based on these results we used XFDMEM supplemented with 5.5 mM glucose and 1 mM pyruvate for all subsequent XF24 experiments. Similarly, for the subsequent GLP-1 secretion experiments, we used medium containing DMEM 5.5 mM glucose (1 g/L), supplemented with 1 mM pyruvate, 1% PenStrep and 0.1 mM DPPiVi.

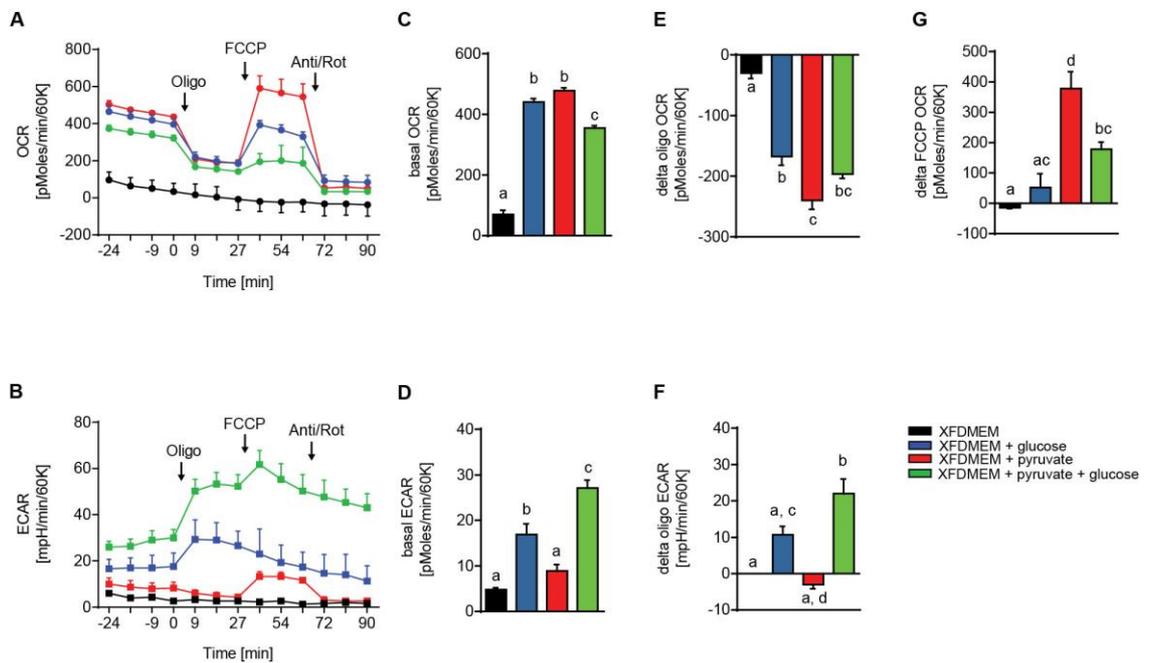


Figure 1. Optimal medium for extracellular flux experiments. (A and B) Oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) of GLUTag cells incubated one hour prior to the extracellular flux experiment in XFDMEM; XFDMEM with glucose; XFDMEM with pyruvate or XFDMEM with pyruvate and glucose (for each group $n = 3$) and consecutively injected with 1 $\mu\text{g}/\text{mL}$ oligomycin (Oligo), 0.75 μM FCCP and 2 $\mu\text{g}/\text{mL}$ antimycin + 1.5 μM rotenone (Anti/Rot). **(C and D)** Basal oxygen consumption rate

(OCR) and extracellular acidification rate (ECAR) of GLUTag cells incubated one hour prior to the XF experiment in XFDMEM; XFDMEM with glucose; XFDMEM with pyruvate or XFDMEM with pyruvate and glucose (for each group $n = 3$). Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test. **(E and F)** Delta respiration and delta medium acidification induced by injection of 1 $\mu\text{g}/\text{mL}$ Oligo on GLUTag cells incubated one hour prior to the XF experiment in XFDMEM; XFDMEM with glucose; XFDMEM with pyruvate or XFDMEM with pyruvate and glucose (for each group $n = 3$). Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test. **(G)** Delta respiration induced by injection of 0.75 μM FCCP on GLUTag cells incubated one hour prior to the XF experiment in XFDMEM; XFDMEM with glucose; XFDMEM with pyruvate or XFDMEM with pyruvate and glucose (for each group $n = 3$). Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test.

4.3.2 Oleic acid potently stimulated the release of GLP-1 and oxidative phosphorylation in GLUTag cells

Next, we measured the influence of OA on cellular bioenergetics. Addition of 250 μM OA increased cellular respiration (OCR $\approx + 26\%$) and medium acidification (ECAR $\approx + 81\%$) (Figure 2 A, B). To mimic the experimental conditions used for the XF24 experiments and to determine the potential influence of intracellular metabolism on the OA-induced release of GLP-1, we incubated GLUTag cells in experimental medium containing OA (250 μM) or saline as control. Consistent with the previous published experiments [11], [23], we found that OA potently (4.0 ± 0.3 fold change) stimulated the release of GLP-1 from GLUTag cells (Figure 2 C).

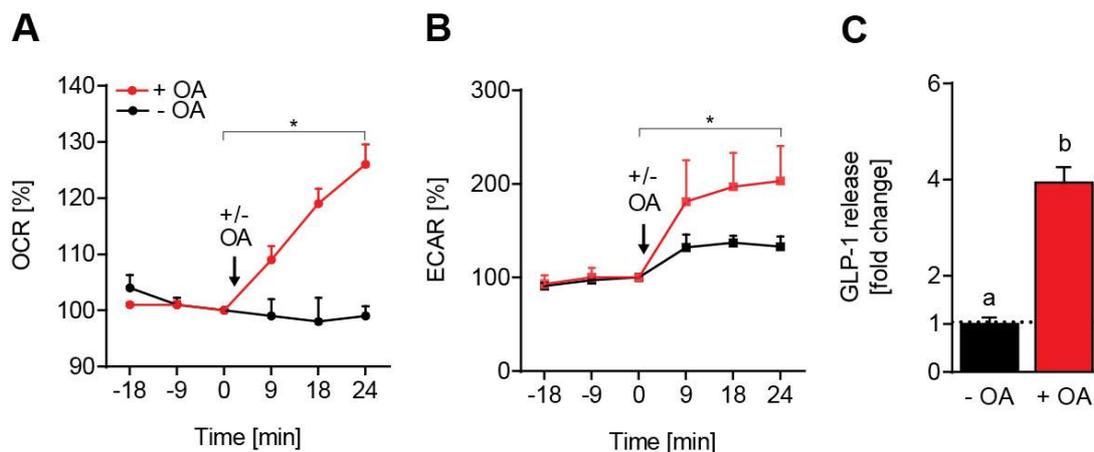


Figure 2. Oleic acid influenced GLUTag cell bioenergetics and potently stimulated the release of GLP-1. (A and B) Changes (%) in oxygen consumption rate (OCR) or extracellular acidification rate (ECAR) of GLUTag cells (27 min) induced by 250 μM OA or saline (-OA) (for each group $n = 10$). * $p < .05$, Two way ANOVA, followed by Tukey's

multiple comparisons test. **(C)** GLP-1 secretion from GLUTag cells incubated for 36 min in assay medium (- OA) or assay medium containing 250 μ M OA (+ OA). Results were normalized to secretion in assay medium (= 1). Different letters: $p < .05$, unpaired t test.

4.3.3 Etomoxir reduced cell respiration but had no effect on GLP-1 release

To determine whether it is the oxidation of OA that triggers GLP-1 release, we specifically inhibited CPT-1 using Eto and mitochondrial ATP production using Oligo (Figure 3 A). OA (250 μ M) again potently increased the OCR of the GLUTag cells ($+23.7 \pm 1.7\%$) (Figure 3 B). Eto (400 μ M) similarly decreased OCR of cells stimulated with OA or saline (Figure 3 B). Interestingly, independent of the inhibition of CPT-1, Oligo did not decrease respiration to the same extent in cells preincubated with OA or with saline. This indicates that OA activated the respiratory chain despite inhibition of CPT-1 and ATP synthase. To test the influence of CPT-1 on the OA-induced release of GLP-1, we also incubated GLUTag cells with Eto, OA or both. Eto alone had no effect on the release of GLP-1 and did not affect OA-induced GLP-1 release (Figure 3 C).

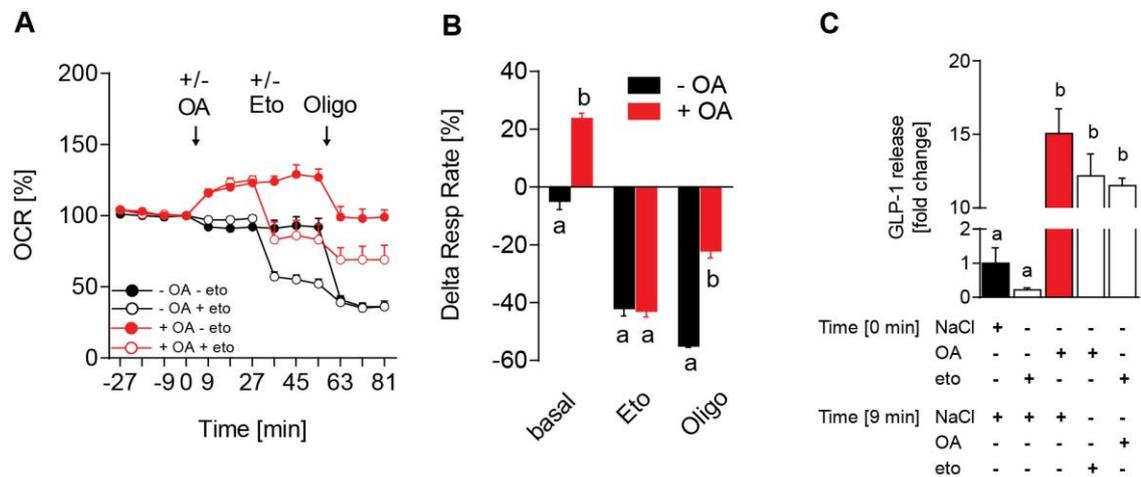


Figure 3. Inhibition of CPT-1 with etomoxir reduced the oleic acid-induced respiration to the baseline but had no effect on the oleic acid-induced GLP-1 release. **(A)** Changes (%) in oxygen consumption rate (OCR) of GLUTag cells induced by consecutive injections of 250 μ M oleic acid (+ OA) or NaCl (-OA), 400 μ M etomoxir (+ Eto) or NaCl (- Eto) and 1 μ g/mL oligomycin (Oligo) (for each group $n = 5$). **(B)** Delta Respiration (%) of GLUTag cells after injection of 250 μ M OA or NaCl, 400 μ M Eto and 1 μ g/mL Oligo (for each group $n = 5$). Measurements were normalized to the pre-injection value of each compound. Different letters: $p < .05$ to the group without OA, unpaired t test. **(C)** GLP-1 secretion experiments were performed on cells incubated for 36 min in assay medium containing NaCl, 250 μ M OA, 400 μ M Eto or both (for each group $n = 4$).

In order to compare the secretion assay to the XF experiments, compounds reported in the legend of the figure were added at minute 0 or 9 after the start of the incubation period. Results were normalized to secretion in assay medium containing NaCl (= 1). Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test.

4.3.4 Oleic acid stimulated GLP-1 release by uncoupling respiration and inhibiting mitochondrial ATP production

Given the insensitivity of OA-induced respiration to Oligo, we next investigated the effect of OA on the respiratory chain by applying different inhibitors (Figure 4 A). In brief, the OCR reduction by Oligo reflects the ATP-linked OCR, whereas the OCR reduction by Anti and Rot reflects the complex III and I respiration. Following the preincubation of the GLUTag cells with saline or OA (250 μ M), 1 μ g/mL Oligo decreased the OCR by $69.0 \pm 4.4\%$ in cells preincubated with saline, but had no effect in cells preincubated with OA (Figure 4 B). Inhibition of the respiratory chain using Anti/Rot decreased cellular respiration in both groups (Figure 4 A). The fact that in cells stimulated with OA respiration was active despite the inhibition of ATP synthase but not when complex I or III was blocked suggests, again, that OA can stimulate respiration independent of ATP synthase. We also examined the influence of these manipulations on the OA-induced GLP-1 release. Again, OA potently stimulated GLP-1 release (Figure 4 C, D). Interestingly, the addition of Oligo alone stimulated the release of GLP-1, and when given together with OA, Oligo further increased the OA-induced GLP-1 release (Figure 4 C). Inhibition of the complexes I and III of the respiratory chain markedly reduced the OA-induced GLP-1 release, whereas Anti/Rot alone had no effect (Figure 4 D).

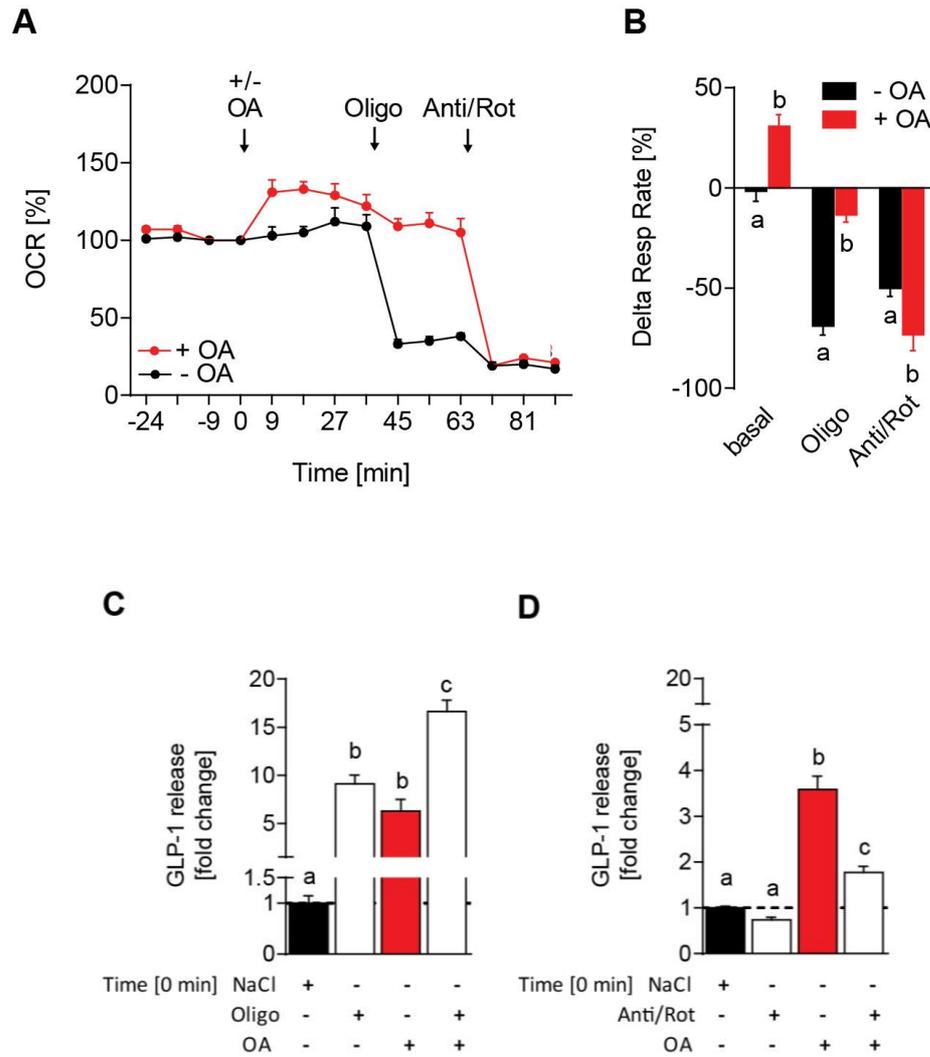


Figure 4. Oleic acid induced a proton leak in the respiratory chain of GLUTag cells. Mitochondrial respiratory chain was involved in the oleic acid-induced GLP-1 release. (A) Changes (%) in oxygen consumption Rate (OCR) of GLUTag cells induced by consecutive injection of 250 μ M oleic acid (+ OA) or NaCl (-OA), 1 μ g/mL oligomycin (Oligo), 2 μ g/mL antimycin + 1.5 μ M rotenone (Anti/Rot) (for each group $n = 6$). (B) Delta Respiration (%) of GLUTag cells after injection of 250 μ M OA or NaCl, 1 μ g/mL Oligo and 2 μ g/mL antimycin + 1.5 μ M rotenone (Anti/Rot). Measurements were normalized to the pre-injection value of each compound (for each group $n = 6$) Different letters: $p < .05$, unpaired t test. (C - D) GLP-1 secretion experiments were performed on cells incubated for 36 min in assay medium containing (C) NaCl, 1 μ g/mL Oligo, 250 μ M OA or both (or each group $n = 6$) and (D) NaCl, 2 μ g/mL Anti + 1.5 μ M Rot, 250 μ M OA or all three compounds (or each group $n = 6$). Results were normalized to secretion in assay medium (= 1). Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test.

4.3.5 Oleic acid and oligomycin induced a metabolic switch towards glycolysis and stimulated GLP-1 release whereas FCCP did not

We next tested the influence of OA on the OCR and ECAR, i.e., on the glycolytic flux of the GLUTag cells (Figure 5). To that end we used the specific inhibitor of glycolysis, 2DG. As expected, 0.75 μ M FCCP increased OCR and ECAR by $76.0 \pm 4.5\%$ and $168.0 \pm 32.7\%$ of the baseline (Figure 5 B and F). 2DG, however, did not affect the FCCP-induced increases in OCR and ECAR. Unlike the effect of 2DG after FCCP, the marked increase in ECAR after inhibition of mitochondrial ATP production with Oligo was immediately attenuated by 2DG (Figure 5 G). Similar to the inhibition of mitochondrial ATP production with Oligo, the pronounced OA-induced increase in ECAR was markedly attenuated by 2DG (Figure 5 H). FCCP alone or in combination with 2DG had no effect on the release of GLP-1 (Figure 5 I), whereas the Oligo- and OA-induced GLP-1 release were markedly reduced after addition of 2DG (Figure 5 J and K).

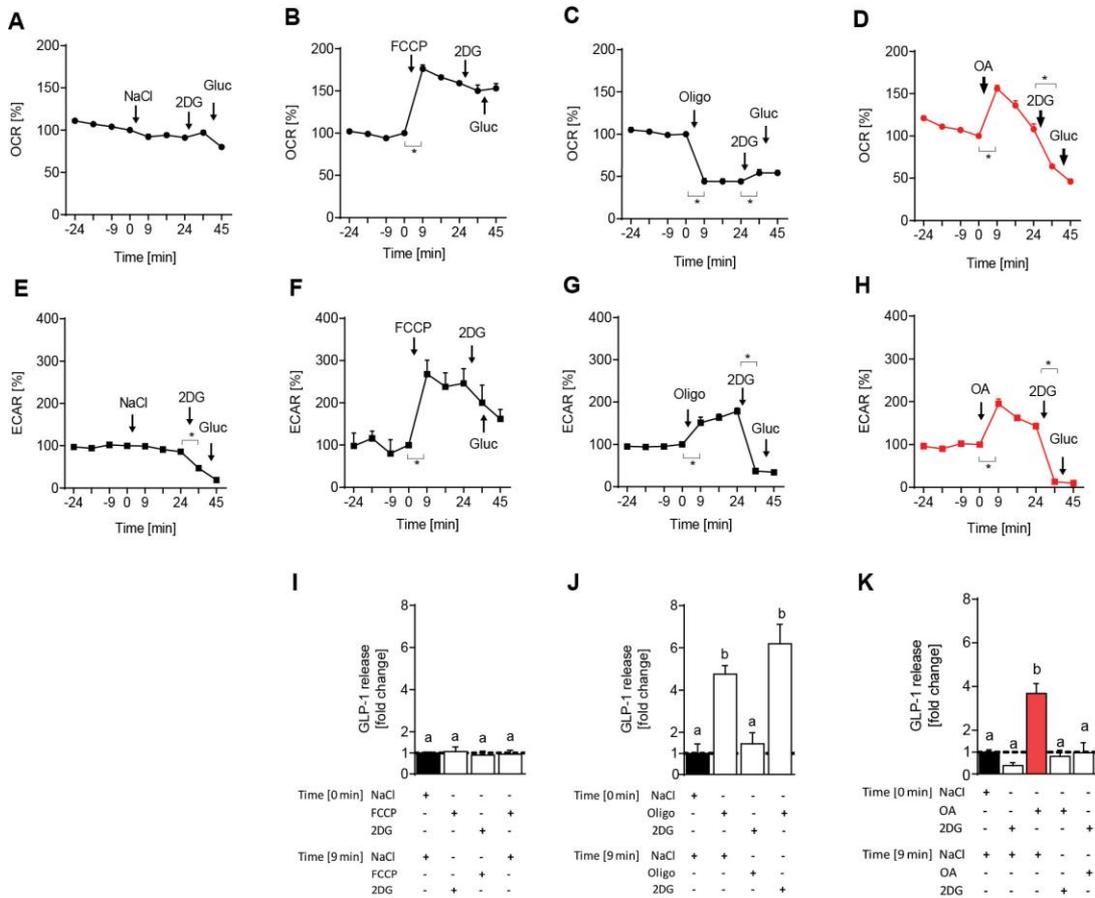


Figure 5. Mobile ionophore FCCP, ATP synthase inhibitor oligomycin and oleic acid induced an increase in medium acidification of GLUTag cells. Inhibition of glycolysis with 2DG potently decreased the oligomycin- and oleic acid-induced medium acidification and secretion of GLP-1. Changes (%) in oxygen consumption rate (OCR) or extracellular acidification rate (ECAR) of GLUTag cells induced by consecutive injections of **(A and E)** NaCl, 100 mM 2 deoxy-D-glucose (2DG) and 5.5 mM glucose (n = 5), **(B and F)** 0.75 μ M FCCP, 100 mM 2DG and 5.5 mM glucose (n = 5), **(C and G)** of 1 μ g/mL oligomycin (Oligo), 100 mM 2DG and 5.5 mM glucose (n = 5) and **(D and H)** of 250 μ M oleic acid (OA), 100 mM 2DG and 5.5 mM glucose (n = 5). * $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test. GLP-1 secretion experiments were performed on cells incubated for 36 min in assay medium containing **(I)** NaCl, 0.75 μ M FCCP or FCCP in combination with 2DG (n = 6), **(J)** NaCl, 1 μ g/mL Oligo or Oligo in combination with 2DG (n = 6), **(K)** NaCl, 100 mM 2DG, 250 μ M OA, or both (n = 6). In order to compare the secretion assay to the XF experiments, compounds reported in the legend of the figure were added at minute 0 or 9 after the start of the incubation period. Results were normalized to secretion in assay medium (= 1). Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test.

4.3.6 The oleic acid-induced increase in respiration, medium acidification and stimulation of GLP-1 release required the import of pyruvate into the mitochondria

Because Eto did not completely abolish the OA-induced respiration and failed to block the release of GLP-1, we tested the influence of aerobic glycolysis on OA-induced respiration and GLP-1 release. Thus, we inhibited the mitochondrial pyruvate carrier (MPC) with its specific inhibitor UK (15 μ M) followed by additions of Oligo and Anti/Rot (Figure 6 A). After 18 min incubation with UK, cellular respiration decreased by $18.7 \pm 1.5\%$ (Figure 6 C). OA further decreased OCR similar to when the mitochondrial ATP production was inhibited using Oligo (Figure 6 B). In contrast, cells not pre-incubated with UK, but stimulated with OA, were insensitive to Oligo. The first experiment revealed that glucose and pyruvate potently increased the basal OCR of the GLUTag cells. Here we show that the uptake of pyruvate into the mitochondria via MPC accounts for about 20% of the basal respiration of these cells as well as for the OA-induced respiration. The inhibition of MPC by 15 μ M UK also reduced OA-induced GLP-1 release, but had no effect when cells were not stimulated with OA (Figure 6 D).

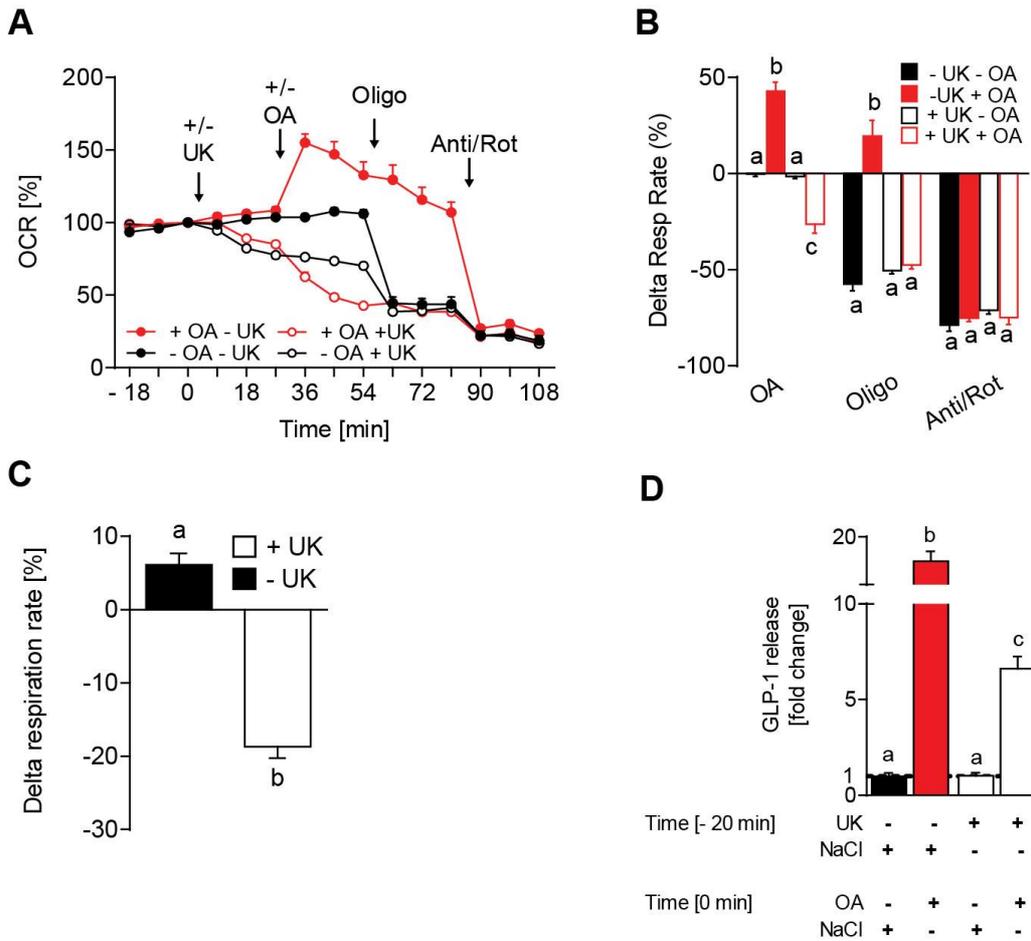


Figure 6. UK-5099 inhibited oleic acid-induced proton leak and medium acidification. UK-5099 significantly reduced the oleic acid-induced GLP-1 release. (A) Changes (%) in oxygen consumption rate (OCR) of GLUTag cells induced by consecutive injections of NaCl (-UK) or 15 μ M UK-5099 (+ UK), NaCl (- OA) or 250 μ M oleic acid (+ OA), 1 μ g/mL oligomycin (Oligo) and 2 μ g/mL antimycin + 1.5 μ M rotenone (Anti/Rot) (for each group n=5). (B) Delta Respiration (%) of GLUTag preincubated for 18 min with UK (+ UK) or NaCl (- UK) after injection of 250 μ M OA (+OA) or NaCl (-OA), 1 μ g/mL Oligo and 2 μ g/mL antimycin + 1.5 μ M rotenone. Measurement (for each group n = 5). Measurements were normalized to the measurement before injection of each compound. Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test. (C) Delta respiration (%) of GLUTag cells after 18 min incubation with assay medium containing 15 μ M UK or NaCl. Measurements were normalized to the pre-injection value of the compound (for each group n = 10). Different letters: $p < .05$, unpaired t -test. (D) GLP-1 secretion experiments were performed on cells pre - incubated for 20 minutes in assay medium containing 15 μ M UK or NaCl. After that time, cells were stimulated with 250 μ M OA or NaCl (for each group n = 6). In order to compare the secretion assay to the XF experiments, compounds reported in the legend of the figure, were added at minute 0 or 9 after the start of the incubation period. Results were normalized to secretion in assay medium (= 1). Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test.

4.4 Discussion

In this study, we used *in vitro* measurements of cellular respiration, medium acidification and GLP-1 release from GLUTag cells to demonstrate that free OA potently stimulates the release of GLP-1 via its action on intracellular metabolism.

As *in vitro* model, we used the GLUTag cells, a commonly employed EC line model for the study of the mechanisms that control the release of GLP-1. Previous GLP-1 secretion experiments with GLUTAg cells were performed in cells incubated in a minimal medium and in the absence of glucose [24, 25]. The results obtained under such conditions may not reveal all metabolic features of GLP-1 release because we observed that glucose and pyruvate in the experimental medium are crucial for the cells' basal metabolic activity as well as for their metabolic flexibility in response to pharmacological modulators. Thus, we used a medium supplemented with glucose and pyruvate for all our experiments. Addition of more than 5.5 mM glucose to the experimental medium did not further increase GLP-1 release, which is consistent with what was published before [24].

Monounsaturated FA have been shown to potently stimulate the release of GLP-1 *in vitro* and *in vivo* [12, 13, 20], but a possible role of intracellular metabolic mechanisms in the release of GLP-1 is still not well defined. With the use of the XF24 technology it is possible to assess cellular bioenergetics by measuring the extracellular flux [26, 27]. The assessment of OCR and ECAR and the parallel modulation of the respiratory chain is a widely used approach to assess mitochondrial bioenergetics [27]. We could assess the effect of OA and metabolic inhibitors alone or in combination on cellular respiration and glycolytic flux, and we measured the release of GLP-1 in parallel.

We found that OA enhanced mitochondrial activity by increasing cell respiration. Cells cover most of their ATP needs by transferring electrons from the reducing equivalents (NADH and FADH₂) generated by glycolysis, fatty acid oxidation (FAO) and/or the tricarboxylic acid (TCA) through the different complexes of the respiratory chain. The resulting proton motive force is coupled to ATP synthase (complex V) to generate ATP [28]. Schober *et al.*, showed that an inhibition of triglyceride (TG) synthesis in the intestine using a Diglyceride

acyltransferase inhibitor (DGAT1i) was associated with an increased FAO in enterocytes as well as with an increased GLP-1 level in circulation [29]. Thus, we here tested whether the OA-induced increase in cellular respiration was related to a stimulation of FAO or to another metabolic effect. The transfer of OA into the mitochondria for FAO requires the carnitine system, in which CPT-1 is the key regulatory enzyme. Treatment of the cells with Eto, a potent blocker of CPT-1 [30], reduced cellular respiration by almost 40% in control and OA-stimulated cells. The remaining respiration indicates, however, that the FAO was not the only contributor to oxygen consumption under these conditions. Also, the OA-induced GLP-1 release was not affected by Eto. This is consistent with earlier findings [31] suggesting that a different metabolic pathway is involved in the OA-induced GLP-1 release and stimulation of cell respiration.

Thus, in addition to oleic acid stimulation, we manipulated the electron transport chain at the levels of complexes I, III and V. Under basal conditions, the protons produced in the electron transport chain are mainly coupled to ATP production via ATP synthase [32]. In this case, inhibition of the ATP synthase with the specific inhibitor Oligo increases protons in the inner mitochondrial matrix, decreases oxygen consumption and renders the cell dependent on the glycolytic ATP production. An uncoupled respiration, e.g., via uncoupling proteins or other mechanisms [28, 33, 34], is characterized by a dissipation of the H⁺ gradient before it is used to produce ATP via the ATP Synthase [28]. If this situation occurs, cell respiration is active despite the fact that ATP synthase is inhibited. We found that the OA-induced OCR was insensitive to Oligo, suggesting a potent OA-induced dissipation of the H⁺ gradient.

Recently, cellular respiration has been associated with the release of GLP-1 [9]. Moreover, the inhibition of mitochondrial NADH dehydrogenase (complex I of the respiratory chain) with Indole and Rot has been shown to reduce the release of GLP-1 from ECs. In line with this study, we show that independent of the coupling state of the respiration, the inhibition of complexes I and III potently decreased respiration as well as the OA-induced GLP-1 release. Thus, we could clearly show that an active respiration is necessary for the OA-induced GLP-1 release, whereas the ensuing production of mitochondrial ATP is not.

The oligo insensitive respiration raised the question of how exactly the proton leak was induced and how it is associated to the release of GLP-1. We did not address this question in our study, but different mechanisms have been proposed for the induction of a mitochondrial proton leak by FA [33, 34]. In relation to hormone secretion, uncoupling protein 2 (UCP2) has been shown to play a role in the glucose-stimulated insulin release from β -cells [35, 36], in glucagon secretion from α -cells [37] or in GLP-1 release from ECs [21], although UCP2's role as an uncoupler has been questioned [38, 39].

The fact that OA stimulated the release of GLP-1 and activated respiration that was not coupled to mitochondrial ATP production, raised the possibility that OA induced GLP-1 release by increasing glycolytic ATP production. Indeed, recently, the importance of the glycolytic flux in the release of GLP-1 has been shown [40]. ATP can control the release of peptides through two well-known mechanisms. First, ATP has been shown to be involved in the release of GLP-1 via acting as an inhibitor of the K_{ATP} channels [25]. In the absence of cytosolic ATP, the K_{ATP} channel is spontaneously active, allowing the exchange of potassium ions with the extracellular matrix. The presence of ATP inhibits the channel, inducing a depolarization of the membrane and the subsequent release of GLP-1 [25, 41]. The crucial role of glycolysis in this context is well characterized and accepted for the glucose-induced insulin release in pancreatic β -cells. Here the ATP produced during a high glycolic flux induces the closure of this channels and the release of insulin. The second well-known ATP-dependent pathway in the release of peptides is associated to the activation of GPR present on the cell membrane. This pathway involves the adenylate cyclase, which transfers the phosphate from ATP to cAMP and stimulates the release of GLP-1 via activation of the PKA or EPAC [2, 42, 43].

Our measurement of the cellular flux clearly showed that the OA-induced increase in ECAR was due to an enhanced glycolytic flux. Indeed, the OA induced ECAR was completely inhibited with 2DG, indicating that the OA-induced increase in the ECAR was almost totally due to an increased glycolytic flux. The inhibition of glycolysis potently reduced the OA-induced GLP-1 release. The fact that FCCP, the widely used un-coupler, did not stimulate the

release of GLP-1 as well as the 2DG sensitive ECAR may be related to FCCP's activity as an unspecific proto-ionophore in the cellular membrane [44].

Recently, the use of medium acidification as an indicator for anaerobic glycolysis has been questioned because mitochondrial CO₂ production can contribute substantially to medium acidification and thus to an increase in ECAR [45]. Interpretation of the OA-induced medium acidification therefore requires a differentiation between aerobic and anaerobic glycolysis. Pyruvate is a key molecule in cellular bioenergetics and constitutes the end product of the glycolytic pathway in the cytosol [46]. During anaerobic glycolysis, pyruvate is converted to lactate via lactate dehydrogenase, whereas during aerobic glycolysis, pyruvate enters the mitochondria via the MPC and is subsequently oxidized in the TCA cycle [47]. We showed that inhibition of MPC by UK [48] decreased the basal respiration of GLUTag cells by almost 20%. This suggests that under basal conditions the import of pyruvate into the mitochondria contributes to cellular respiration. This is also in line with our first experiment where we observed that glucose and/or pyruvate were necessary for an active basal OCR.

The OA-induced increase in cell respiration and medium acidification was abolished by the inhibition of MPC, indicating that the OA-induced entrance of pyruvate into the mitochondria is crucial for the effect of OA on ECs bioenergetics. Our secretion experiments showed that at basal levels the inhibition of MPC did not influence the release of GLP-1. Inhibiting MPC did, however, decrease the OA-induced release of GLP-1 without abolishing it completely. Together these results indicate that there is a metabolic component in the OA-induced GLP-1 release. Indeed, by moderately uncoupling cellular respiration, OA indirectly activates glycolysis, the final trigger for GLP-1 release. This pathway presumably connects to the well-characterized mechanisms that govern the release of GLP-1. Also, the activation of GPR on the cell surface and the intracellular ATP providing pathways may actually interact in the metabolite-stimulated GLP-1 release. In the present study we could not mimic a physiological release of GLP-1. One limitation of the cell culture model is the density of L-cells in a small area and their comparatively long exposure to high metabolite concentrations. *In vivo* the L-cells are scattered along the whole

intestine and represent only 1% of all the intestinal cells [49 , 50]. Further, in particular small intestinal motility ensures the constant movement of a complex nutrient and digestion product mixture towards distal intestinal segments. As a result, the cell is not exposed to the nutrients and their digestion products for long [51]. The measurement of cellular bioenergetics in primary ECs is, however, still a challenge, and thus immortalized cells are a valid alternative.

Does the release of GLP-1 by OA have any practical relevance? The Mediterranean diet is characterized by a high consumption of olive oil rich in OA and has been associated with beneficial effects for cardiovascular diseases and metabolic syndrome [52 - 55]. The positive effect of this and other diets rich in monounsaturated FA on the development of obesity has been suggested to involve the secretion of GLP-1 [12]. Also, GLP-1 receptor agonists are widely used for the treatment of obesity and type-2-diabetes (T2D) [56]. Understanding the mechanisms of OA-induced GLP-1 release may therefore be useful to replace or complement pharmacological with nutritional interventions in the fight against obesity, metabolic syndrome and T2D.

4.5 Conclusion

In conclusion, our findings do not support a role of mitochondrial OA oxidation in the release of GLP-1. Rather, OA induces cellular respiration via uncoupling of the respiratory chain and, hence, indirectly increasing glycolytic flux which is the final trigger for the release of GLP-1 (Figure 7). Despite all the limitations of *in vitro* studies, this work suggests the option of nutritional interventions as a valuable alternative to the use of pharmacological GLP-1 receptor agonists. To elucidate the physiological significance of this work, further investigations in ECs primary cultures as well as in animal models are indicated

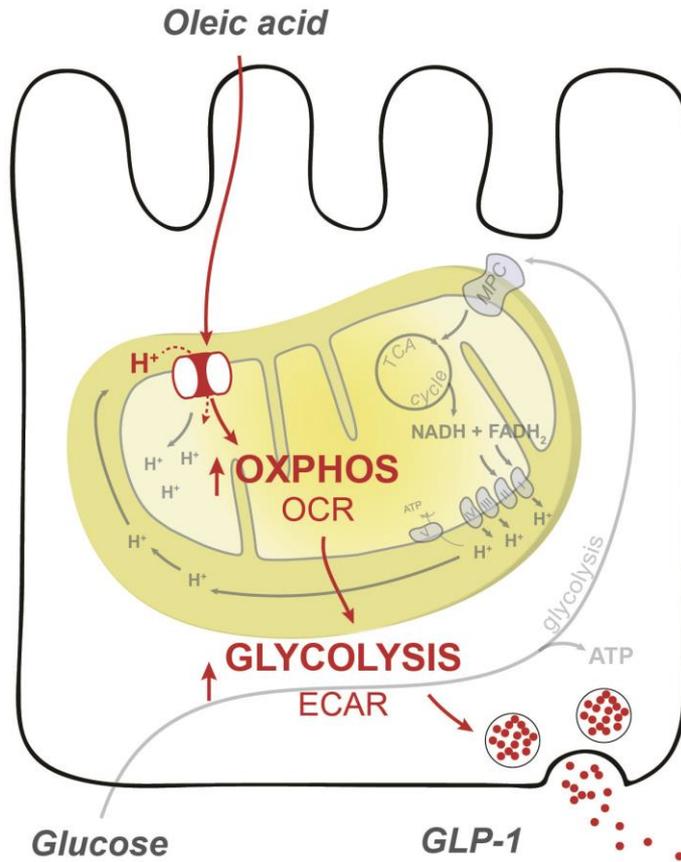
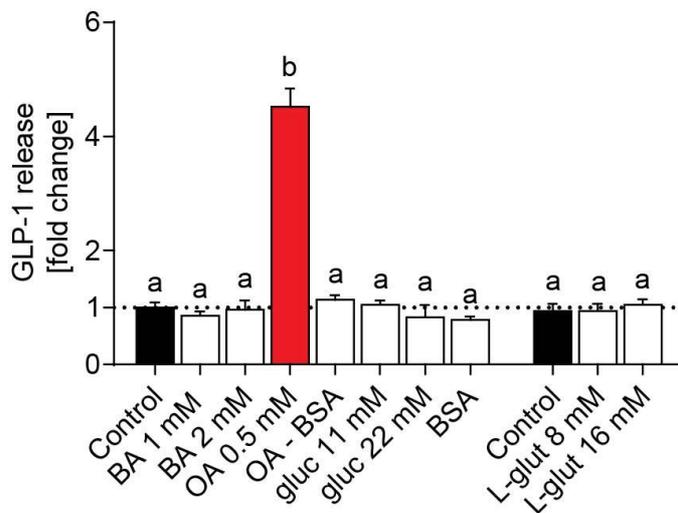


Figure 7. Proposed mechanism of oleic acid-induced GLP-1 release from the enteroendocrine cell line model GLUTag.

4.6 Supplementary figure



S1: GLP-1 secretion experiments were performed on cells incubated for 120 min in assay

medium containing NaCl (Control), 1 or 2 mM butyric acid (BA), 0.5 mM oleic acid (OA), 11 or 22 mM glucose, 0.17 mM BSA or 0.17 mM BSA bound to 0.5 mM OA or 8 or 16 mM L-glutamine (L-Glut) (for each group $n = 3 - 6$). Results were normalized to secretion in assay medium containing NaCl (= 1). Different letters: $p < .05$, one-way ANOVA followed by Tukey's multiple comparisons test.

4.7 References

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Chapter 5

Intragastric infusion of oleic acid induced a peak in hepatic portal vein GLP-1 concentration 5 to 10 minutes after infusion.

Based on Clara et al., *In preparation*.

5 Intra-gastric infusion of oleic acid induced a peak in hepatic portal vein GLP-1 concentration 5 to 10 minutes after infusion.

5.1 Introduction

GLP-1 is a peptide secreted by the enteroendocrine cells (ECs) in response to eating [1]. The diverse actions of GLP-1 on whole body energy homeostasis include the inhibition of eating, the stimulation of glucose-induced insulin secretion and the inhibition of glucagon secretion from the pancreas as well as the reduction of gastric emptying [2]. Because of these actions GLP-1 analogs are currently considered to be promising tools to counteract obesity and type-2-diabetes (T2D). Understanding the mechanisms of nutrient-induced GLP-1 release is important to pursue potentially valuable nutritional intervention therapies as alternative to the pharmacological GLP-1 receptor (GLP-1R) agonist treatment widely used [3, 4]. Different mechanisms have been proposed to explain the nutrient-induced GLP-1 release from ECs [1, 5 - 8], but there are still several open questions.

A Mediterranean diet has been associated with beneficial effects in patients with cardiovascular diseases as well as in the prevention and treatment of T2D and the metabolic syndrome [9 - 12]. The positive effect of this diet has been suggested to be related to the high consumption of monounsaturated fatty acids (FA) [12, 13] and seems to also involve an enhanced release of GLP-1 [13 - 15]. In contrast, the typical western diet is characterized by a high consumption of saturated FA [16] and has less or no protective effect in terms of whole body metabolic parameters [13, 15]. In fact, a diet rich in the monounsaturated oleic acid (OA) improved whole body glucose homeostasis and stimulated the release of GLP-1 in rats compared to a diet rich in the saturated palmitic acid [15]. Also, an intraduodenal infusion of OA stimulated the release of GLP-1, whereas an intracolonic infusion did not alter the release of this peptide in rats [17].

The stimulatory effect of OA on the release of GLP-1 from EC line models has been shown before [18 - 20], and we found that OA potently stimulated cell respiration and the release of GLP-1 in the GLUTag cell line model by uncoupling mitochondrial respiration and, hence, indirectly increasing glycolysis [44]. With this study we assessed the possible effect of OA and the OA stimulated glycolytic pathway on the release of GLP-1 in rats *in vivo*. To do so, in a first set of experiments, we tested the influence of an IG infusion of OA or glucose on the concentration of GLP-1 in the HPV of rats. In the second set of experiments, we examined the effect of an inhibition of glycolysis by 2-desoxy-D-glucose (2DG) on the OA-induced GLP-1 release in rats.

5.2 Material and methods

5.2.1 Animals and housing

Fourteen male Sprague-Dawley rats (2-4 weeks old) were housed individually under standard conditions, in an air-conditioned room ($22 \pm 2^\circ\text{C}$ and 60% relative humidity) kept on a 12 h light/12 h dark cycle (lights off at 04:00 AM). The rats were fed *ad libitum* standard chow diet (No. 3436; Provimi Kliba, Switzerland: Dry matter 88.0%, Crude protein 18.5%, Crude fat 4.5%, Crude fiber 4.5%, NFE 54.2%, Starch 35%, Crude ash 6.3%, Gross energy 16.1 MJ/kg, Metabolizable energy 13.2 MJ/kg) and had full access to tap water. All procedures were approved by the Veterinary Office of the Canton of Zürich.

5.2.2 Catheter implantation

All animals were adapted to the housing conditions and the diet for at least 2 weeks before catheter implantation. After adaptation, 14 rats of about 300 g body weight (BW) were equipped with chronic IG and hepatic portal vein (HPV) catheters for substrate administration or blood sampling as previously described ([27]-[29]). The catheter implantation was performed under aseptic conditions. All instruments were autoclaved and the catheters sterilized (Kodan, Schulke, Switzerland). Before surgery all animals were food deprived for 8

hours. For infection prophylaxis and analgesia, rats received a subcutaneous (SC) injection of antibiotics (20 mg/kg BW of Sulfadoxin and 5 mg/kg BW Trimethoprin (Borgal 24%, Intervet/Shering Plough Animal Health, NJ) two hours before anesthesia. Fifteen to 30 min prior to anesthesia, animals received an additional SC injection of 0.05 mg/kg Atropin Sulfate (Sintetica, Switzerland). Thereafter, animals were anesthetized by an IP injection of 5 mg/kg BW Xylazin (Rompun; Bayer, Germany) and 88 mg/kg BW Ketamin (Ketalar, 50mg/mL, Inresa Arzneimittel, Germany). During the whole surgery, eye ointment (Vitamin A 15000 IE/g, Bausch&Lomb, Switzerland) was applied and animals were kept on heating pads to maintain body temperature at 37 – 38 °C. During surgery, anesthesia was assessed via testing the interdigital reflexes. As post-surgery treatment, all animals were injected daily and for two consecutive days with 4 mg/kg BW Carprofen (Rimadyl; E.Gräub, Switzerland), 20 mg/kg BW of Sulfadoxin and 5 mg/kg BW Trimethoprin (Borgal 24%, Intervet/Shering Plough Animal Health, NJ). The BW of the animals was daily monitored.

To prevent the obstruction of the IG catheter as well as to adapt the animals to the IG infusions, 6 mL of sterile saline (0.9% saline, Braun, Germany) was daily injected into the stomach. Also, to avoid blood clotting in the HPV catheters as well as to adapt the rats to the blood sampling, all catheters were flushed daily with 200 uL of sterile saline. Thereafter the catheters were filled with 50% glycerol and 200 IU of heparin/mL saline.

Verification of the IG catheter position was done post mortem. In more detail, at the end of the experiment, rats were euthanized with carbon dioxide (CO₂), the abdomen was opened and the position of the catheter was verified visually (Figure 1). Verification of HPV catheter patency was done during the experiment, i.e., only data from animals with successful blood withdrawal throughout the whole experiment were included into the analyses.

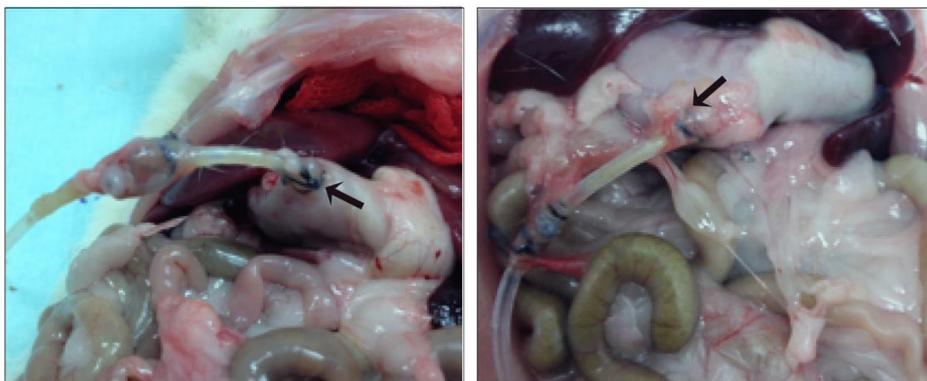


Figure 1: Visual verification of intragastric catheter. At the end of the experiment, rats were euthanized with CO₂, the abdomen was opened and the position of the IG catheter was verified visually. The two representative pictures show the position of the IG catheter in two different rats.

5.2.3 Experimental procedures

5.2.3.1 Experiment 1: Influence of IG oleic acid and glucose infusion on HPV plasma GLP-1 concentration

The experiment started approximately ten days after catheter implantation and was performed in a cross-over design with one intervening day. The animals involved in the experiment were fasted for 8 h before dark onset. At dark onset (04:00 PM) and with 15 min between animals, all animals received an IG infusion of 3 mL of vehicle (Veh: 0.9% saline containing 1% w/v Tween 80 (Sigma, P4780)), OA (139 mg sodium salt of OA (Sigma, O7501) dissolved in 3 mL Veh and dissolved at 37 °C in the water bath [17]) or glucose (1 g/kg BW dissolved in 3 mL Veh). Blood samples (approx. 150 uL) were taken from the HPV catheter into a syringe containing 500 KIU Aprotinin /mL blood (Aprotinin, Sigma A6103), 0.1 mM DPPIV inhibitor (Millipore, USA) and 1.5 mg EDTA /mL blood (EDTA, Triplex 3, Merk, Germany). Blood was sampled in the fasted state during the light phase before the IG infusion as well as 5, 10, 30 and 60 min after the start of the infusion. Immediately after aspiration, the blood was transferred into an 0.5 mL tube, stored on ice and centrifuged (10000 rpm / 8 min / 4 °C) within 20 minutes. After centrifugation, the plasma was transferred into a new 0.5 mL tube and stored at -80 °C until further analyses. GLP-1 was

analyzed using the GLP-1- PYY Assay Kit (MesoScale Discovery) according to the manufacturer's instructions. Only data from the 7 rats that had patent catheters at the end of the whole cross-over experiment were included in the analysis.

5.2.3.2 Experiment 2: Influence of 2-deoxy-d-glucose on the oleic acid-induced GLP-1 release

The experiment started approximately ten days after catheter implantation. The animals involved in the experiment were fasted for 8 h before dark onset. At dark onset (04:00 PM) and with 15 min between animals, all animals received a first IG infusion of 3 mL Veh (0.9% saline containing 1% w/v Tween 80 (Sigma, P4780)) or 2DG (200 mg/kg BW dissolved in 3 mL Veh). After 2 min all animals received a second IG infusion of 3 mL Veh or OA (139 mg sodium salt of OA (Sigma, O7501) resuspended in 3 mL Veh and dissolved at 37 °C in the water bath [17]). In a cross-over design and with one intervening day, each animal received the following combinations of IG infusions: Veh/Veh, Veh/OA, 2DG/Veh or 2DG/OA. Again, as in Experiment 1, blood samples (approx. 150 uL) were aspirated from the HPV catheter into a syringe containing 500 KIU Aprotinin /mL blood (Aprotinin, Sigma A6103), 0.1 mM DPPIV inhibitor (Millipore, USA) and 1.5 mg EDTA /mL blood (EDTA, Triplex 3, Merk, Germany). Blood was sampled in the fasted state during the light phase before the IG infusion as well as 5, 10, 30 and 60 min after the first infusion. Immediately after aspiration, the blood was transferred into an 0.5 mL tube, stored on ice and centrifuged (10000 rpm / 8 min / 4 °C) within 20 minutes. After centrifugation, the plasma was transferred into a new 0.5 mL and stored at -80 °C until further analyses. GLP-1 was analyzed using the GLP-1 - PYY Assay Kit (MesoScale Discovery) according to the manufacturer's instructions. Only data from the 5 rats that still had patent catheters at the end of the whole cross-over experiment were included in the analysis.

5.2.4 Statistical analysis

Data are presented as mean \pm SEM and were analyzed using the repeated measurements two-way ANOVA with treatment and time as within subject factors. The Grubbs test was used to identify outliers ($\alpha = .05$), and the missing values were replaced by the mean of the remaining values. Significant ANOVA results were followed by the Bonferroni-Holm post-hoc test.

5.3 Results

5.3.1 Oleic acid induced a peak of HPV plasma GLP-1 concentration 5 minutes after intragastric infusion

Fasting GLP-1 levels were not different between rats. IG infusion of OA increased the HPV plasma level of GLP-1 only 5 minutes after the start of the infusion. Interestingly, at the same time point, IG infused glucose did not induce an increase in HPV GLP-1 concentration. From 10 to 60 min after infusion, neither glucose nor OA caused any change in HPV plasma GLP-1 concentrations. As expected, Veh infusion did not change HPV GLP-1 concentration at any time (Figure 2).

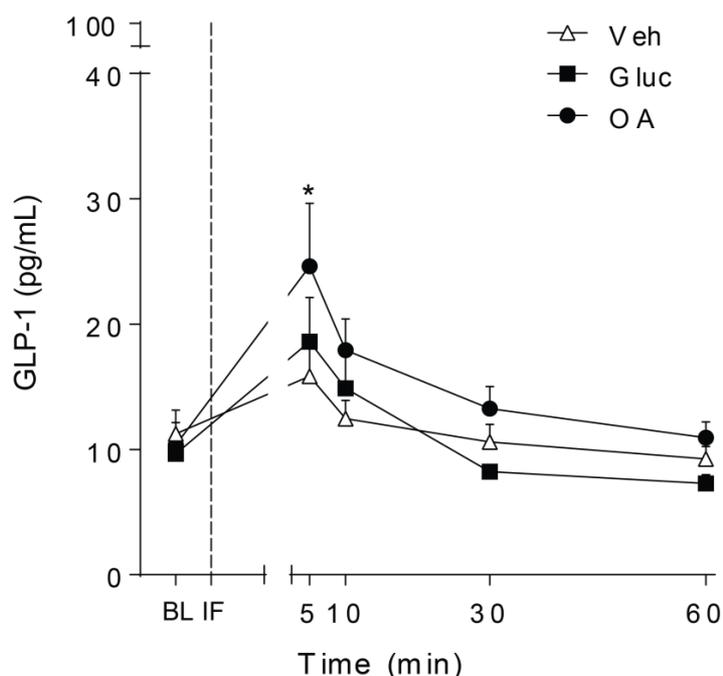


Figure 2. Oleic acid increased HPV plasma GLP-1 concentration 5 min after the start of the infusion. Rats ($n = 7$) were food deprived for 8 h in the light phase. In the light phase and shortly before IG infusion (IF), fasting blood samples (BL) were taken. At the beginning of the dark phase (04:00 PM), and with 15 min time difference between rats, all animals received an IG infusion of Veh, glucose (Gluc) or OA. Blood samples from the HPV were taken at 5, 10, 30 and 60 min after infusion onset, and GLP-1 concentration was measured. * $p > .05$ vs. Veh.

5.3.2 Oleic acid increased the concentration of GLP-1 in the HPV only 10 minutes after intragastric infusion. 2-deoxy-D-glucose did not inhibit the oleic acid-induced HPV plasma GLP-1 levels

The fasting HPV plasma GLP-1 level was not different among rats. As in Experiment 1, OA increased HPV GLP-1 concentration only at one time point, but different from the first experiment, the GLP-1 peak in HPV plasma was 10 min after the start of the infusion, which may be due to the fact that OA was infused 2 minutes after the start of the first infusion. 2DG significantly reduced the OA-induced increase in HPV GLP-1 concentration 10 min after the first injection. Neither IG infusion of 2DG nor Veh caused a change in HPV plasma GLP-1 concentration at any time point measured (Figure 3).

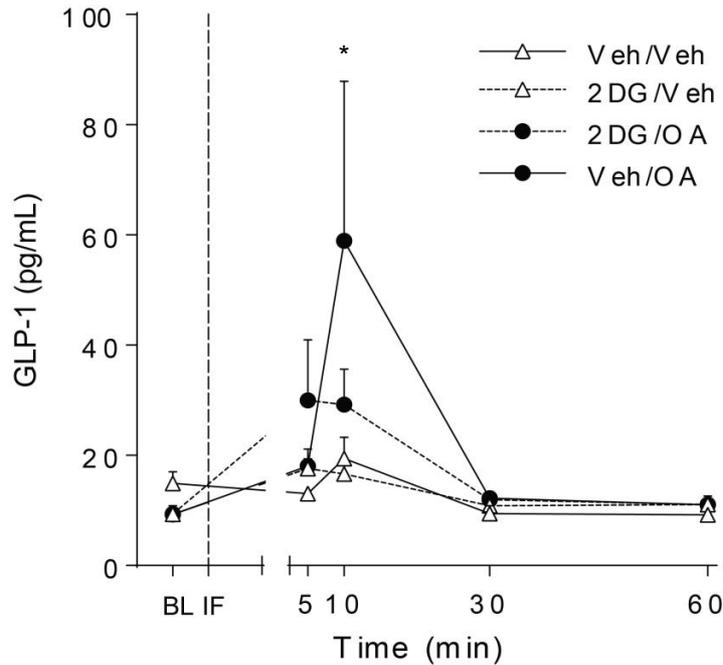


Figure 3. Oleic acid increased HPV plasma GLP-1 concentration 10 min after the start of the first infusion. 2 deoxy D glucose reduced the oleic acid-induced HPV plasma GLP-1 concentration. Rats (n = 5) were food deprived for 8h in the light phase. In the light phase and shortly before IG infusion (IF), fasting blood samples (BL) were taken. At the beginning of the dark phase (04:00 PM), and with 15 min time difference between rats, all animals received a first infusion of Veh or 2DG. After 2 min all animals received a second IG infusion of Veh or OA. In a cross-over design and with one intervening day, each animal received the following combinations of IG infusions: Veh/Veh, Veh/OA, 2DG/Veh or 2DG/OA. $p > .05$ * Veh/Veh, vs. Veh/OA.

5.4 Discussion

We here show that IG OA infusion induced a peak in HPV GLP-1 concentration 5 - 10 minutes after infusion. The increase in HPV plasma GLP-1 concentration most likely reflects the stimulating effect of OA on GLP-1 release. In a previous study we showed that OA potently induces the release of GLP-1 from the EC line model GLUTag [44]. Bioenergetic measurements with the XF24 convincingly demonstrated that OA activated glycolysis via uncoupling the mitochondrial respiration in the EC line model GLUTag. In addition, we showed that glycolysis was necessary for the OA-induced GLP-1 release because an inhibition of this pathway with 2DG markedly reduced the OA-induced GLP-1 release. In this study, however, we failed to prevent the transient increase in HPV GLP-1 concentration by inhibiting glycolysis with an IG infusion of 2DG.

The concentration of GLP-1 we measured in the HPV plasma after an IG OA or glucose infusion was characterized by a peak 5-10 min after infusion. Previous studies that reported the influence of a HFD test meal on the HPV plasma concentration of GLP-1 [28, 35] showed that HFD gradually increased the concentration of GLP-1 in the HPV showing a peak around 30 - 60 min after the start of the test meal. Further, Anini *et al.* showed that an intraduodenal infusion of the same OA solution we used in our study markedly increased GLP-1 concentration in the carotid artery of rats [17]. Interestingly, although it was consistently shown that glucose and OA both, are potent stimuli of GLP-1 release *in vitro* [19, 33, 44], we did not observe a similar gradual increase in the HPV plasma concentration of GLP-1 in rats that received a single IG infusion of glucose or OA.

Glucose as well as OA have been shown to be absorbed predominantly in the proximal intestine [38 - 40]. In this segment, GLP-1 secreting cells are scattered between enterocytes at a lower density than in the distal segments of the small intestine [41, 36]. The amount as well as the matrix of the glucose and OA solution used in our study was probably not optimal to reach enough ECs and, hence, to have a gradual effect on the HPV GLP-1 concentration. Further, 2DG is treated as glucose with respect to absorption, i.e., similar to glucose, 2DG may have reached only a small number of ECs in the proximal small intestine, which may not have been enough for 2DG to antagonize the stimulatory effect of OA on GLP-1 secretion by its inhibiting effect on glycolysis. 2DG is a glucose analogue that is also phosphorylated by hexokinase. The resulting 2DG-phosphate is, however, not further metabolized by the phosphoglucose isomerase and thus 2DG inhibits the glycolytic pathway [37].

Another possible explanation for the failure of 2DG to block the OA-induced increase in HPV plasma GLP-1 concentration could be the nutrient composition of our infusates. Indeed, in our *in vitro* studies we observed that OA stimulated the release of GLP-1 by increasing glycolysis in the EC line model GLUTag [44]. We performed all our cell line studies with incubation medium containing glucose. The presence of glucose has been shown to induce the translocation of glucose transporter 2 (GLUT2) transporters from the cytoplasm to the brush border membrane [34, 42, 43]. We can therefore

assume that glucose is taken up by the EC line model via both, SGLT1 and GLUT2. Further, based on previous reports, glycolysis stimulates the release of GLP-1 only if glucose is taken up through the GLUT2 but not through SGLT1 [24]. The presence of GLUT2 on the apical and basolateral membrane depends, however, on the amount of glucose present in the lumen and in the circulation [34, 42, 43]. Based on these pieces of evidence it is feasible that the failure of OA as well as 2DG to potentially modulate the secretion of GLP-1 into the HPV of rats over longer periods of time (> 10 min) was related to the nutritional state of the rat (fasted for 6 hours) as well as to the missing glucose in the infusate.

Previous reports emphasized the crucial role of glycolysis in the release of GLP-1 [30]. Studies performed on isolated perfused rat small intestines clearly showed that inhibiting sodium-glucose linked transporter 1 (SGLT1), the Na⁺/glucose cotransporter present on the luminal membrane of epithelial cells, with phlorizin or the basolateral passive GLUT2 transporter with phloretin markedly reduced the glucose-induced GLP-1 release [23, 24]. The same effect was observed in SGLT1 (SGLT1^(-/-)) or GLUT2-deficient mice (GLUT2^(-/-)) [23, 31, 32]. The importance of the glycolytic pathway for the release of GLP-1 was further shown in studies in which the closure of the K_{ATP} channels with sulfonylurea (gliclazide and tolbutamide) potentially stimulated the release of GLP-1, whereas the opening of the same channels inhibited it [23, 25].

In sum, additional *in vivo* experiments will be needed to examine the physiological relevance of the OA-induced GLP-1 release in the *in vitro* EC line studies. The fact, however, that this is the first study that showed the effect of a single intragastrically infused nutrient on the HPV plasma GLP-1 concentration opens an attractive novel line of research into the mechanisms that control nutrient-induced GLP-1 release.

5.5 References

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Chapter 6:

General discussion.

6 General discussion

The last decades have been characterized by profound changes towards a sedentary life style that promotes the intake of energy dense food [1, 2]. The consequential exponential increase in obesity and its associated comorbidities [2, 3] stimulates research into understanding the mechanisms that contribute to the development and progression of diet-induced metabolic disorders. The digestive tract is the first organ of the body exposed to dietary nutrients. In addition to its absorptive function, the intestine is optimally placed to sense the availability of energy in the gut lumen and to convey this information to the brain [4]. In this context, gastrointestinal peptides as well as vagal and spinal afferents play a crucial role [5, 6]. While gastrointestinal peptides are released by the enteroendocrine cells (ECs) into the circulation in response to a meal, the extrinsic nerves densely innervate the gastrointestinal tract, providing an early and fast monitoring system for the potential availability of energy coming from the ingested food [6, 7].

6.1 Metabolic adaptation of the small intestine to high fat diet

As all animals, we have genetically adapted to the environment of our ancestors [8]. During pre-agriculture times, a high plasticity of the intestine was crucial for surviving. The efficient uptake and storage of nutrients during periods in which food was scarce was necessary to not waste valuable nutrients. Nowadays, the virtually unlimited availability of food as well as the profound changes in the life style are highly associated with the development of obesity. Hence, we try to understand how the diet affects intestinal metabolic plasticity as well as how the intestinal bioenergetics affect whole body energy homeostasis.

Simple carbohydrates, such as sucrose or glucose, are rapidly absorbed in the proximal intestine [9, 10] whereas fatty acids (FA) are packed into chylomicrons, stored in triglycerides (TG) or transported into the mitochondria via CPT-1 and 2, where they undergo fatty acid oxidation (FAO). The amino

acid glutamine is the preferred nutrient for enterocytes [11], and its possible role in intestinal gluconeogenesis and in the control of food intake has also been considered [12].

The idea that the diet [13, 14] as well as the nutritional state [15, 16], can modulate intestinal bioenergetics is an attractive concept. Indeed, enterocytes, the absorptive cells of the epithelial layer, metabolically react to digested nutrients via extensive activation of intracellular metabolic pathways to finally be able to translocate the food-derived energy yielding substrates into the circulation [10]. Well-known examples in this context include the glucose-induced translocation of glucose transporter 2 (GLUT2) from the cytoplasm to the basolateral and apical membrane of the enterocyte [17] as well as the protein-induced upregulation of intestinal gluconeogenesis following consumption of a high protein diet [12, 16]. Fat in particular has been shown to modulate the expression of metabolically relevant enzymes and to change the absorptive and morphological properties of the epithelial layer [13, 14, 18 - 23]. Recent studies from our laboratory showed that a pharmacological modulation of fat metabolism in the small intestine modulated food intake and increased the high fat diet (HFD)-induced metabolic adaptation of the jejunum more than in the duodenum or the liver. This indicates an important role of the jejunum, and less so of the duodenum and the liver, in responding and adapting to diet composition.

In the enterocyte, FA are primarily reesterified, packed into chylomicrons and then transported through the lymph into the general circulation [24 - 27]. Several lines of evidence indicated, however, that enterocytes [20, 28 - 30] of animals exposed to HFD upregulated the expression of genes related to fatty acid catabolism and ketogenesis. In our study, HMG-CoAS2, a mitochondrial enzyme that catalyzes the crucial step of ketogenesis, was substantially upregulated in the jejunum of HFD-fed animals already after 3 days of exposure. Ketone bodies are synthesized from acetyl-CoA resulting from enhanced FAO if they are not converted into CO₂ and ATP through the TCA cycle and oxidative phosphorylation pathway [31]. For long time ketone bodies have been thought to be almost exclusively derived from the liver. There, under fed conditions, the production of acetoacetate and D-3-hydroxybutyrate is

minimal, whereas during starvation the depletion of the TCA cycle from the essential intermediates, such as succinyl-CoA, potently activates the HMG-CoAS2 and, thus, ketogenesis [32]. In the intestine, the accumulation of Acetyl CoA derived from FAO after a HFD meal, challenges the capacity of the TCA cycle, directing the Acetyl-CoA into ketogenesis rather than towards the TCA cycle [34, 35]. In this context, intestinal gluconeogenesis may also play a role [12, 33, 36, 37].

The influence of the diet on the expression of the enzyme HMG-CoAS2 in the jejunum and the generally proposed potential role of ketone bodies in the control of eating [38 - 40] support the hypothesis of a possible role of HFD-induced intestinal FAO and ketogenesis [4, 22, 41], as well as gluconeogenesis [12] in the control of eating.

Our findings and several other lines of evidence [13, 18 - 21, 23, 42] are consistent with the idea that afferent nerve fibers terminating in the lamina propria of the mucosa [43] could sense changes in the bioenergetically relevant pathways in the enterocyte following food intake and convey them to the brain. This may open an interesting line of research towards an understanding of the mechanisms that contribute to the obesity promoting effects of HFD. Moreover, given the fact that the diet-induced changes in the intestine are macronutrient specific [14], it would be useful to investigate the influence of different diets on gene and protein expression patterns in the intestine. Further, it would be interesting to investigate whether an isocaloric modulation of the macronutrient composition of the diet could reverse the HFD-induced changes in the intestine.

6.2 Influence of fatty acid metabolism on the release of GLP-1 from enteroendocrine cells.

It is well known that a HFD meal, especially if rich in monounsaturated FA, potently increases the levels of circulating GLP-1 in humans [44, 45]. How exactly the digested food components stimulate the release of GLP-1 into the circulation is still a topic of debate [46, 47]. Recent studies performed on cell culture models [48 - 50], transgenic mouse models [49], organoids [51] or

perfused intestines [52] revealed some potential pathways that might link the stimulation of the ECs by nutrients to the release of GLP-1 [46, 53].

Recently, Schober and others showed that an IG infusion of an inhibitor of DGAT1, one of the two enzymes known to catalyze the final step of triacylglycerol (TG) synthesis, was associated with an upregulation of enzymes involved in FAO in the small intestine as well as with an increased level of circulating GLP-1 in HFD-fed rats [23, 54, 55].

Thus, we hypothesized that the increased level of GLP-1 observed in the plasma of these animals could be related to an increased FAO in the ECs. With our study we could, however, clearly show that the FAO did not mediate the substantial oleic acid (OA)-induced GLP-1 release we observed in our GLUTag cell line study. Further, we could also not observe any effect of the DGAT1 inhibitor on the release of GLP-1 from GLUTag cells (data not shown). Despite the discrepancies between our experimental setup and the *in vivo* setup of Schober *et al.*, we can still assume that the substantial GLP-1 release observed after DGAT1 inhibition in HFD-fed rats was probably not due to an increased FAO in the ECs. This is also consistent with findings demonstrating that the enzyme DGAT1 is not co-localized with EC line markers [54], thus, in essence excluding a potential influence of TG synthesis on the release of GLP-1. The authors suggested that the inhibition of TG synthesis in enterocytes delays and therefore alters the luminal distribution of the dietary lipids, which may lead to a modulation of peptide secretion via indirect mechanisms.

The influence of fuel-coupled secretion has been extensively studied for the release of insulin from the pancreatic β cells [56]. As reviewed in this paper [56], in beta cells the carbohydrates, amino acids and FA coming from the circulation promote the secretion of insulin mainly via their metabolism and the production of signaling metabolites. Cellular metabolic pathways are, however, tightly regulated and connected with each other. Thus, glycolysis has been shown to potently influence the release of insulin via two pathways. The first one is via blocking the K_{ATP} channel [57]. This inhibition induces a change in membrane potential and the subsequent release of insulin via a stimulation of the influx of calcium into the cell. The second one is via providing phosphates for cAMP, a downstream element of the activation of G-protein coupled

receptors (GPR). Indeed, the glucose-induced insulin secretion is potentiated by the binding of GLP-1 to the G_s and G_q -coupled GLP-1 receptor [58]. To make the story more complex, it has also been shown that both a crosstalk between glucose and mitochondrial aerobic metabolism [59, 60] as well as the presence of lipids in the β cell [61, 62] are necessary for a potent effect of glucose on insulin secretion from pancreatic beta cells [63, 64]. These findings make it more likely that a combination of intracellular pathways rather than only a single pathway is involved in glucose-induced insulin release [56].

Insulin as well as GLP-1 are released into the circulation after a meal [65, 66]. Recently findings suggested that, similar to the metabolically coupled release of Insulin, also the release of GLP-1 from the EC is probably coupled to cell metabolism [49, 52]. Indeed, also ECs express K_{ATP} channels as well as GPR [67], glucose and fatty acid transporters [49, 50]. In relation to fatty acid-stimulated GLP-1 release, several basolateral [67 - 69] and apical [70] membrane bound GPR have been implicated in the release of GLP-1 via G_s , G_i and G_q -coupled pathways [71]. In addition, FA have been shown to stimulate GLP-1 release via a direct activation of the PKC [46, 72, 73] as well as via activation of the uncoupling protein-2 [74]. As for the release of insulin from the β cells, also for the release of GLP-1 from the ECs, more than one single intracellular pathway seems to be involved. We did not test the influence of OA on membrane bound GPR. With the extracellular flux (XF) measurements we could, however, convincingly demonstrate that OA acid is taken up by the cell, uncouples the mitochondrial membrane and, subsequently, increases the glycolytic flux in the cells. An inhibition of this pathway at any level of the aerobic glycolysis inhibits the oleic acid-induced GLP-1 release. An uncoupling effect of FA, especially unsaturated FA, has been postulated earlier [75, 76]. Also the increased glycolytic flux following an uncoupling of the respiratory chain has been observed before [77].

Two studies showed that decreasing mitochondrial efficiency via administering the uncouplers 2, 4 - dinitrophenol (DNP) or CZ5 to mice, reduced their BW and improved their glucose and lipid homeostasis [78, 79]. Whether the beneficial effect of the administered uncoupling agents was also due to a modulation of GLP-1 release is, however, unknown. The positive

effects of diets rich in monounsaturated FA on cardiovascular diseases and the metabolic syndrome [80 - 83] have, however, been associated with their effect on the secretion of GLP-1 [84].

In our *in vivo* study, IG infused OA potently induced a peak of GLP-1 concentration in the HPV 5-10 min after administration. The fact that we did not mix OA with a non-digestive matrix presumably promoted a fast absorption of OA in the proximal small intestine, stimulating only a peak of GLP-1 release 5-10 minutes after injection. Further, 2-desoxy-D-glucose (2DG) is a glucose analogue that is taken up via GLUT or SGLT1 transporters and is phosphorylated by hexokinase to a 2-deoxyglucose-6-phosphate, a molecule that does not further undergo glycolysis [85]. Also this molecule could have been absorbed in the proximal intestine without even reaching the area of high density GLP-1-secreting L-cells in the more distal intestine. Therefore, it would be interesting to test the influence of small doses of OA as well as 2DG in a mixed complex food that allows the two molecules to be slowly absorbed along the whole small intestine.

6.3 Technical approaches

Recently, profiling of cellular bioenergetics has been made more accessible and with higher data throughput via the Extracellular Flux Analyzer (XF24) [86]. The simultaneous quantification of two parameters, the consumption of oxygen and the acidification of the medium, in combination with metabolic modulators, makes it possible to get an insight into the current metabolic status of a defined monolayer of cells in a relatively short period of time and with a relatively low amount of biological material needed [87, 88]. The consumption of oxygen is a parameter generally used to classify the mitochondrial aerobic energy production, whereas medium acidification is generally used to quantify the anaerobic glycolytic energy production [87]. Diverse recent studies, however, challenge these simple assumptions [89, 90]. Indeed, cell surface oxygen consumption [89] as well as aerobic glycolysis [64, 90] complicates interpretation of XF data. Also, the measurement of cellular

metabolism with the XF24 requires the use of adherent cells with a stable metabolism until the end of the experiment [87].

The isolation and culture of primary enterocytes as well as ECs are still not standardized cell culture protocols. Regarding the first cell type, the major problems include 1) the fact that the epithelial layer is inhabited by many microorganisms, which substantially increases the risk of a microbial contamination of the culture, and 2) differentiated epithelial cells have a turnover of three days and are highly dependent on their extracellular matrix [91]. Nevertheless, we isolated the enterocytes with a novel method that preserves as much as possible the metabolic properties of the cell [92], but we did not succeed in measuring the bioenergetics of these primary enterocytes with the XF24. Almost the same obstacles complicate the isolation and culture of ECs. Moreover, ECs make up less than 1% of all gut epithelial cells and are scattered along the whole intestine. Based on the peptides they release, they are also categorized into different types [91, 93].

Therefore, as valuable and standardized alternative for the study of single cell molecular mechanisms, we used immortalized enterocytes and EC line models [94]. Although we standardized our experiments, the different passage numbers and the immortalization process can still affect the metabolic properties of the used cell line models [95, 96]

6.4 Conclusion

In the present study we could show that the small intestine reacts to a HFD by changing its metabolic properties. The role of these changes, or better, the role of the extent of these changes, in the development of obesity needs to be further studied. The possibility, however, that a modulation in the intestinal epithelial cell bioenergetics through pharmacological or even nutritional intervention could modulate the production of signals involved in whole body energy homeostasis renders the intestine an attractive organ for scientific research that tries to understand the influence of our life style on the development of obesity and its comorbidities at the molecular level.

6.5 References

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List of abbreviations

2DG	2-deoxy-D-glucose
2MG	sn-2-monoacylglycerol
Anti	antimycin
BA	butyric acid
BSA	bovine serum albumin
BW	body weight
cAMP	cyclic adenosine monophosphate
CCK	cholecystokinin
CD ₃₆	cluster of differentiation 36
CGM	complete growth medium
CPT-1	carnitine palmitoyltransferase I
CtD	control diet
DAG	diacylglycerol
DGAT	Di Acyl-CoA:diacylglycerol acyltransferase-1
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethyl sulfoxide
DNP	2,4-dinitrophenol
DPPIV	dipeptidyl peptidase – IV
DPPIVi	dipeptidyl peptidase – IV inhibitor
EC	endoendocrine cell
ECAR	extracellular acidification rate
EPAC	exchange protein directly activated by cAMP
Eto	etomoxir
FA	fatty acids
FADH ₂	Flavin adenine dinucleotide
FBP	fructose 1,6-bisphosphatase
FBS	fetal bovine serum
FCCP	carbonyl cyanide-p-trifluoromethoxyphenylhydrazone
FFAR 1-4	Free fatty acid receptor 1-4
G-6-Pase	glucose 6-phosphatase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
GLP-1	Glucagon - like peptide - 1
GLP-1R	Glucagon - like peptide – 1 Receptor
Glu	glucose
Gluc	glucose
GLUT2	Glucose transporter 2
GPR	G protein coupled receptor
HepG2	human liver hepatocellular carcinoma cell line
HFD	high fat diet
HK	hexokinase
HMG-CoAS2	3-hydroxy-3-methylglutaryl-CoA synthase 2
HPV	hepatic portal vein
HRP	horse radish peroxidase
HuTu-80	human duodenum adenocarcinoma cell line

IG	intra-gastric
IP3	inositol 1,4,5-trisphosphate
IPEC-J2	porcine normal intestinal jejunal epithelial cell line
K _{ATP}	ATP-sensitive K ⁺ channel
KHB	Krebs-Henseleit Buffer
L-Glut	L-glutamine
FAO	fatty acid oxidation
MGAT	Acyl CoA:monoacylglycerol acyltransferase
MPC	mitochondrial pyruvate carrier
NADH	Nicotinamide adenine dinucleotide
OA	oleic acid (sodium oleate)
OCR	oxygen consumption rate
Oligo	oligomycin
OXPPOS	oxidative phosphorylation
PBS	phosphate - buffered saline
PenStrep	penicillin streptomycin
PKC	protein kinase C
PLC	Phospholipase C
PPAR α	peroxisome proliferator-activated receptor alpha
PYY	peptide tyrosine tyrosine
rHu	relative humidity
RIPA	Radioimmunoprecipitation assay buffer
Rot	rotenone
SC	subcutaneous
SGLT1	sodium-glucose linked transporter 1
T2D	typ-2-diabetes
TBS-T	Trisaminomethane - buffered saline and tween 20
TCA	tricarboxylic acid cycle
TG	triglyceride
TRIS	Trisaminomethane
UCP2	uncoupling protein 2
UK	UK-5099
Veh	vehicle
XF	extracellular flux
XF24	extracellular flux analyser 24 wells
XFDMEM	unbuffered Dulbecco's modified Eagle medium
XFKHB	unbuffered Krebs - Henseleit Buffer

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List of Publications

Peer-reviewed publications

Clara R, Langhans W, Mansouri A. 2015. Oleic acid stimulates glucagon-like peptide-1 release from enteroendocrine cells by modulating cell respiration and glycolysis. *Metabolism –Clinical and Experimental. In Press.*

Clara R, Schumacher M, Ramachandran D, Fedele S, Krieger JP, Langhans W, Mansouri A. 2015. Metabolic adaptation of the small intestine to short- and medium-term high-fat diet exposure. *Cell Physiology. Submitted.*

Clara R, Arnold M, Fedele S, Krieger JP, Ramachandran D, Langhans W, Mansouri A. 2015. Intragastric infusion of oleic acid induced a peak in hepatic portal vein GLP-1 concentration 5 to 10 minutes after infusion. *In Preparation.*

Published abstracts

Clara R, Langhans W, Mansouri A. 2015. Enhanced glycolysis mediates the oleic acid (OA)-induced stimulation of glucagon like peptide 1 (GLP-1) secretion from enteroendocrine cells. Society for the Study of Ingestive Behavior. Denver.

Clara R, Langhans W, Mansouri A. 2014. Metabolic characterization of GLUTag cells. Society for the Study of Ingestive Behavior. Seattle.

Clara R, Langhans W, Mansouri A. 2013. Possible role of mitochondrial bioenergetics in the release of GLP-1 from enteroendocrine cells. All roads take to the brain – Workshop. Ascona 2014.

Clara R, Schumacher M, Langhans W, Mansouri A. 2014. Caco-2 and IPEC-J2 cells have a higher fatty acid oxidation (FAO) capacity than HuTu-80 or HepG2 cells. DVG Meeting. Zurich. Keystone Symposium. Vancouver.

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