Intestinal nuclear receptors in control of energy metabolism

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7 General Discussion

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The increasing prevalence of metabolic diseases

The global rise in obesity and related chronic metabolic diseases is one of the biggest health threats of this time. Today, 1 in 3 adults are overweight and it is predicted that this will increase unless effective prevention strategies are immediately implemented[1]. Our modern sedentary lifestyle and high-caloric diet consumption are the main causes for the increased prevalence of overweight and obesity, both risk factors for the development of metabolic diseases such as type 2 diabetes mellitus (T2DM), cardiovascular- and renal diseases, as well as certain types of cancer[2].

Recently, the role of the intestine in whole-body energy homeostasis was highlighted by the success of bariatric surgery including the Roux-en-Y gastric bypass (RYGB) and vertical sleeve gastrectomy (VSG) procedures, where improvements in glucose homeostasis already occur before significant post-surgery weight loss[3,4]. Importantly, gastric bypass results in long-term remission of T2DM[5,6], indicating the clinical importance of the gut as regulator of glucose homeostasis. The intestine is not only responsible for nutrient absorption and sensing but also the release of endocrine signals that govern whole-body metabolism[7]. The recently recognized importance of nutrient absorption, sensing and subsequent endocrine actions of the gut in systemic metabolism provides new opportunities to treat metabolic diseases by specifically targeting the intestine and thereby avoiding unwanted systemic adverse effects[8,9].

The role of dietary fructose in the development of metabolic imbalance

In the past decades, the consumption of fructose, mainly in the form of sweeteners like sucrose and high fructose corn syrup (HFCS), has increased dramatically. Fructose has been considered as one of the driving forces of metabolic imbalance, in particular the development of non-alcoholic fatty liver disease (NAFLD) and hepatic insulin resistance. The underlying mechanisms, however, remain incompletely understood. Several potential mechanisms have been proposed, including uncontrolled glycogenesis and de novo lipogenesis causing hepatic lipid deposition, increased uric acid production caused by ATP depletion and increased inflammation[10].

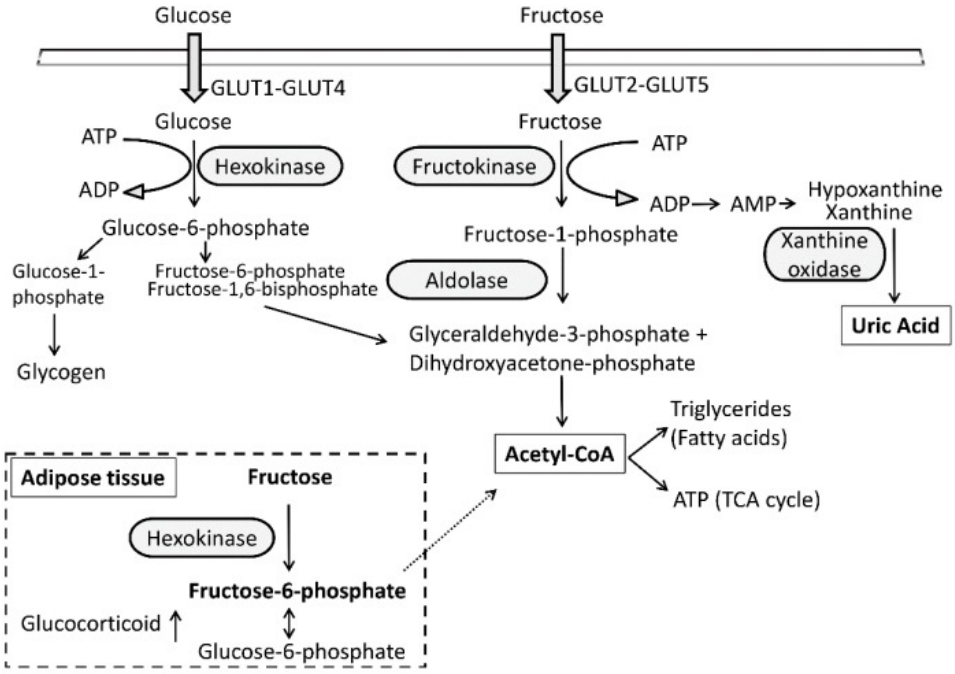

Figure 1. Intracellular metabolism of fructose and glucose in fructokinase (ketohexokinase, KHK) positive tissues like liver and small intestine. *The main fructose metabolizing enzyme is fructokinase, which uses ATP to phosphorylate fructose to fructose-1-phosphate. In adipocytes, which lack fructokinase, fructose is metabolized by hexokinase to fructose-6-phosphate*[11].

Although glucose and fructose have the same molecular formula (C6H12O6), the pathways by which they are metabolized are distinct (**Figure 1**). Glucose and fructose are not only taken up by different transporters but also metabolized by different enzymes, thereby causing distinct changes in metabolic effects. Recently, the metabolic fate of dietary fructose in mice was characterized in detail. While the small intestine was identified as the primary site for its metabolism, the detrimental problems caused by high-fructose intake were mostly located in the liver[12]. Only after intake of high amounts of fructose, the capacity of the intestine to metabolize fructose becomes limiting and excessive fructose can spill-over to the liver and colon. While previous studies have mostly focused on the effects of fructose in the liver, its direct effects on the intestine remain poorly explored.

We therefore investigated the specific effects of fructose on intestine and liver transcriptomes by feeding mice a 60% fructose, dextrose (glucose) or cornstarch (complex carbohydrate), diet for 2 weeks (**Chapter 2**). A relative short treatment time of two weeks was chosen in order to determine the primary effects of these sugars, and not the secondary responses to damage caused by a high-sugar diet. As expected, the results show that a 2-week high-sugar diet is sufficient to cause distinct metabolic disturbances by dietary fructose or glucose, yet at this time point no fatty liver was observed.

Transcriptome analysis revealed distinct responses to fructose as compared to glucose and complex carbohydrates in both the small intestine and liver. These differences were most pronounced in the intestine, indicating that this was the predominant site of metabolism which is in accordance to literature[12]. The mice received the fructose diet *ad libitum*, resulting in around 2 gram fructose intake per 24 hours. This is considered a high-fructose intake (>1 gram/kg); however, after ingestion of fructose in solid food as compared to an acute liquid fructose intake (e.g. via oral gavage) the spill-over effects to the liver are expected to be less pronounced. Although much less than the intestine, some changes in hepatic gene expression were observed after a fructose diet, indicating that indeed there was spill-over of fructose towards the liver in our study. Using this unbiased approach, we identified several novel genes of interest for fructose metabolism which were upregulated solely after fructose feeding. For example, *Pnpla3,* a gene encoding Adiponutrin, was specifically upregulated in the liver after a high-fructose diet. Interestingly, mutations in *Pnpla3* are associated with an increased risk to develop NAFLD[13,14], similar to a high-fructose intake[15]. We also found increased expression of the bile acid (BA)-sensing nuclear receptor Farnesoid X receptor (FXR) and its target small heterodimer partner (SHP) specifically after fructose feeding in the small intestine. The changes in expression levels of BA transporters and FXR signaling can potentially be explained by altered gut microbiota[16]; however, additional experiments are required to establish this. In line with our observations, it has been demonstrated previously that a high-fructose diet reduces the biodiversity of the gut microbiota[17] as well as changes the production of short-chain fatty acids (SCFAs)[12,18]. Gut microbiota have a major impact on body weight and energy homeostasis via various mechanisms, including secretion of SCFAs, affecting the inflammatory response and inducing intestinal permeability[19–21]. Taken together, the FXR signaling pathway is a promising novel lead for further research and indicates that microbiota composition and BA homeostasis play a role in the metabolic outcomes of high fructose consumption.

Carbohydrate response element binding protein (ChREBP) is a sugar-sensing transcription factor that induces the expression of glycolytic and lipogenic genes[22]. Intestinal ChREBP is essential for fructose absorption and metabolism, as its deficiency in the intestine, but not in the liver, results in fructose intolerance in mice[23]. In our study, we observed the most effective activation of both ChREBP and FXR by dietary fructose. Interestingly, synergistic actions of ChREBP and FXR via O-GlcNAcylation have been described previously[24]. So, upon sugar sensing, ChREBP target gene activation can be

enhanced by FXR actions, ameliorating the catabolic response to carbohydrate intake. Besides, FXR is known to increase the gene expression of glycogenic genes and induce glycogen storage rather than lipogenic gene expression. Possibly, this protects the hepatocytes against the development of NAFLD.

Despite numerous studies on fructose intake in human and rodents, no consensus has been reached about the potential detrimental metabolic effects of its consumption in an energy-balanced diet. This can partly be explained by the large differences in experimental designs, resulting in a broad spectrum of outcomes. For example, intervention dose and time, administration via liquids or solid food, energy-balanced or surplus diets, and the choice of a proper control diet containing no fructose and sucrose, all influence the measured metabolic impact of dietary fructose (reviewed in[10]). Moreover, the composition of a diet also affects fructose metabolism. Simultaneous ingestion of glucose for example improves the ability of the small intestine to absorb fructose[25–27] while adding fat to the diet worsens the metabolic impact of fructose[28]. Overall, a large, long-term (epidemiological) human study is needed to provide more insight into the extent of potential harmful effects caused by fructose consumption.

The quest for novel regulators of the GLUT-family of sugar transporters

The facilitative sugar transporters of the GLUT-family (*SLC2A* genes) are indispensable for the cellular uptake of monosaccharides such as glucose, fructose and galactose (reviewed in[29]). The expression of GLUT-members is dependent on metabolic status, including dietary intake, physical activity and disease state[30]. Transcriptional regulators of GLUT expression are therefore important in maintaining energy homeostasis and response to signals from the microenvironment like sugar (metabolites) and hormones[31]. The levels of fructose transporter GLUT5 for instance are enhanced by its own substrate, fructose, and to a lesser extent by glucose[32,33]. GLUTs are localized on the plasma membrane and determine the influx of monosaccharides. For example, GLUT4 is mainly expressed in insulin-sensitive tissues such as heart, adipose tissue and skeletal muscle, and translocates to the plasma membrane in response to insulin to adequately supply these tissues with glucose and consequently reducing the blood glucose levels[34].

Aberrant GLUT levels have been reported in tumor cells where increased GLUT levels facilitate the elevated energy demand caused by uncontrolled proliferation[35,36]. Indeed, increased GLUT levels in tumors are associated with poor clinical outcome[37] and disruption of GLUTs in cancer cells has been reported to impair tumor growth (reviewed in[38]). The membrane expression of GLUT4 is reduced in diabetic mice[39], whereas GLUT5 and GLUT9 levels are elevated[40,41]. Taken together, GLUT levels are altered during metabolic diseases and certain types of cancer, providing putative options for treatment and diagnosis[42]. Thus far, however, it has been challenging to target a specific GLUT member due to high sequence similarity between the different GLUT proteins[43].

In **Chapter 3** we investigated the regulation of the GLUT-family by nuclear receptors (NRs), ChREBP and Peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC1α). NRs are ligand-activated transcription factors that act as sensors for a wide range of small lipophilic molecules. They bind to hormone response elements (HREs) in the promoter sequence of their target genes to regulate their transcriptional activity. Since NR ligands can be derived from the diet, they form a link between physiology and transcriptional regulation.

Previously discovered transcriptional regulators of GLUTs have been proven important, both as research tools and as putative treatment targets for GLUT-related diseases such as T2DM and certain types of cancer[44,45]. Under conditions of insulin resistance, GLUT4 translocation to the membrane is impaired, resulting in decreased glucose tolerance and hyperglycemia[39,46]. Vas et al. reported that androgens increased the proliferation rate of prostate cancer cells by inducing GLUT1 and GLUT3 expression in an androgen receptor (AR) dependent manner[47,48]. To identify novel NR-mediated regulations of human GLUT-members, we performed a high-throughput reporter screen including all 49 NRs. In addition, we examined the interaction between transcription factor ChREBP and the human GLUT-members since ChREBP has emerged as an essential regulator of carbohydrate metabolism, including the regulation of GLUT2 and GLUT5 expression in mice[23,49].

Using this screen, we uncovered several novel transcriptional regulators of GLUT-members as well as confirmed previously described regulators. We validated our findings by testing activation of endogenously present GLUTs in human Caco2 cells using selective NR ligands, and showed that our reporter screen is a robust discovery tool. It should be noted, however, that these reporter assays should be considered as *proof of principle* experiments and that some regulations will not occur *in vivo,* even though the NR can bind to the promoter. These regulations are highly dependent on the cellular microenvironment including presence of co-regulators, endogenous ligands and protein interactions (e.g. transrepression), therefore, further research is needed to uncover the physiological relevance of the discovered regulations. In **Chapters 4** and **5** we characterized two of the most interesting regulations, namely regulation of the intestinal fructose transporters GLUT5 and -7 by liver X receptor alpha (LXRα) and retinoic acid receptor-related orphan receptor alpha (RORα), respectively.

Transcriptional regulation of the intestinal fructose transporter GLUT5

The essential fructose transporter GLUT5 facilitates the uptake of fructose in the proximal part of the small intestine[32]. GLUT5 levels are induced by fructose and glucose and its regulation has been described for ChREBP and nuclear receptors THR and GR[50,51]. Dual target genes of LXR and ChREBP have been described previously and the synergistic actions of these transcription factors are probably needed to effectively induce postprandial lipogenesis and glycolysis[52]. GLUT5 levels are elevated in the small intestine and skeletal muscle of T2DM patients, allowing these tissues to maintain a sufficient supply of energy under insulin resistant conditions. In addition, certain types of cancer, including breast cancer and gliomas, exhibit GLUT5 overexpression which

promotes fructose utilization and tumor progression[36,53]. Interestingly, knockdown of GLUT5 significantly inhibited tumor proliferation both *in vitro* in glioma cells and *in vivo* in mice bearing glioma xenografts[54]. Treatment with oxysterols, the endogenous ligands of LXR, reduce tumorigenesis by inducing cell cycle arrest via LXR activation in many tumours[55]. This, however, is not the case for all types of cancer. For example LXR activation increased the metastatic potential of breast cancers cells[56]. GLUT5 is known to be crucial for breast cancer proliferation[57,58]. Whether the LXR-GLUT5 interaction is the underlying cause for adverse effects of LXR activation in specific tumors has yet to be determined. As manipulation of GLUT5 activity seems to be a potential therapy to treat certain types of fructose-dependent cancers and perhaps also metabolic disorders, we set out to gain more insight into the transcriptional regulation of GLUT5 in **Chapter 4.**

Previously, Matosin-Matekalo et al. reported a functional DR4 site in the human GLUT5 promoter sequence responsible for regulation by THR[26]. In **Chapter 4** we show that the regulation of the human *GLUT5* promoter by LXRα is dependent on promoter binding to this same DR4 site. LXRα and THR can thus competitively bind this DR4 site, revealing an interaction between these two NRs. This finding is in agreement with literature, where extensive crosstalk between LXR and THR has been described for other target genes[59–61].

Although the LXR response element that we identified in the human *GLUT5* promoter is not conserved in rodents, we did observe induced expression of Glut5 upon LXR activation in the intestine and adipose tissue of mice. This suggests that mouse Glut5 is driven by a different response element which remains unidentified or, alternatively, that this induction is indirect, for example via activation of ChREBP which is a target of LXR and also regulates Glut5[62,63]. The delayed response of Glut5 elevation in adipose tissue might indicate that an indirect regulation causes the increased levels of Glut5. On the other hand, since sterol regulatory element-binding protein 1c (Srebp1c), a direct target of LXR but not of ChREBP, showed a similar delayed induction, it is also possible that the delayed increase of GLUT5 in adipose tissue is caused by a relatively slow accumulation of LXR agonist into adipose as compared to the small intestine.

Transcriptional regulation of the elusive transporter GLUT7

Li et al. 2004 were the first to clone and identify human GLUT7 (SLC2A7) in the small intestine. They reported a high affinity but low capacity transport of glucose and fructose by GLUT7[64]. However, this was contradicted by Ebert et al., who found no glucose or fructose transport by GLUT7[65,66]. Although they both studied its putative transport substrates using *Xenopus oocytes,* differences in experimental methods are most likely the reason for these contradictory findings. Since fructose and glucose transport by GLUT7 has a low capacity, the choice of negative controls and amount of GLUT7 cDNA used in the experiments could explain why one study could not detect the transport. Therefore, the physiological substrate of GLUT7 still remains enigmatic.

In **Chapter 5** we found an increased intestinal expression of *Glut7* mRNA in mice fed with a semi-synthetic diet as compared to a chow diet. In rats, increased *Glut7* has also

been found after a high-carbohydrate diet as compared to a low-carbohydrate diet[67]. This agrees with our finding since semi-synthetic diets contain more sugars than regular chow. Upon further analysis, however, we did not observe a specific increase in *Glut7* by either a fructose or glucose diet as compared to a diet containing cornstarch which is composed of complex carbohydrates. In line with our observations, Patel et al. also reported no changes in murine *Glut7* mRNA after a short-term treatment with fructose or glucose by oral gavage in addition to *ad libitum* chow diet[33]. It thus remains unclear which dietary components regulate intestinal Glut7 expression.

Using our screen (**Chapter 3**) we discovered a strong and specific regulation of human GLUT7 by RORα. RORα, together with the NR Rev-erb is part of the peripheral circadian system. These NRs constitute a feedback loop with the core clock gene BMAL1. RORα and Rev-erb bind competitively to the same response element in the promoter region of their target genes[68]. Whereas RORα is a transcriptional activator and constitutively expressed, Rev-erb is a transcriptional repressor and displays a strong circadian rhythm. Together, these two NRs thus govern the rhythmic regulation of genes in a broad set of processes, like immunology, glucose metabolism and lipid metabolism to anticipate circadian oscillations[69–71]. As disruption of daily rhythm contributes to the development of obesity, this led to the hypothesis that small molecules that activate RORα or Rev-erb could provide a therapeutic strategy to treat obesity related metabolic disorders[72,73]. We are the first to report that Glut7 displays a strong diurnal expression with a peak expression at ZT8. Glut7 displays a similar intestinal expression pattern and circadian rhythm as was previously shown for Glut2 and 5.

Surprisingly, in Rev-erbα KO mice, this expression pattern was unaffected, indicating that *Glut7* is expressed with a circadian rhythm in a Rev-erbα independent manner. We cannot, however, exclude the involvement of Rev-erbβ in rescuing the circadian expression of Glut7. Indeed, in other studies a total disruption of the circadian rhythm was only observed in Rev-erbα/β double knockout mice[71]. We also found no promoter regulation of murine Glut7 by RORα in our reporter assays, although we cannot exclude the possibility that the regulatory site of RORα is located upstream of the promoter region that we used in our studies, or in an intron. However, both findings do not support RORα-mediated regulation of murine Glut7 levels. We therefore conclude that RORα can bind to the *GLUT7* promoter in humans, but not in mice, highlighting species differences in its transcriptional control.

The physiological function of PPARδ in the intestine.

The lipid-sensing NR PPARδ is, like the other two members of the PPAR-subfamily PPARα and -γ, an important regulator of energy homeostasis. Systemic PPARδ activation results in improved metabolic status including increased energy expenditure, protection against diet-induced obesity and improved glucose homeostasis in several mouse models (reviewed in[74]). Although PPARδ is highly expressed in the intestine, its function in this organ is still unclear. In **Chapter 6** we therefore used intestine-specific PPARδ deficient (PPAR-delta$^{IEC\text{-}KO}$) mice to get more insight into its physiological function in the small intestine. *PPAR-delta$^{IEC\text{-}KO}$* mice displayed increased weight gain and obesity-

related metabolic disturbances like insulin resistance, in response to a high-fat diet challenge as compared to wild-type mice. Further, the excretion of neutral sterols in the feces, as well as an increase in HDL-cholesterol after systemic PPARδ activation by the selective PPARδ ligand GW501516 was observed in wild-type, but not in *PPAR-delta*$^{IEC\text{-}KO}$ mice. Based on these findings we conclude that intestine-specific PPARδ plays a critical role in energy metabolism that cannot be compensated by PPARδ signaling in other tissues.

An important intestinal regulator that influences whole-body metabolism by enhancing insulin sensitivity, is the incretin GLP-1. Previously, the *proglucagon* gene, encoding various incretins, has been described as a PPARδ target gene[75] and, based on this finding, we explored the hypothesis that in the absence of intestinal PPARδ the GLP-1 production in response to food intake is reduced. In response to a high-fat diet, increased levels of *proglucagon* mRNA were observed in wild-type mice but not in *PPAR-delta*$^{IEC\text{-}KO}$ mice, suggesting that PPARδ is indeed a transcriptional regulator of this gene. Plasma GLP-1 levels, however, were low and did not change in any of the groups after a glucose bolus. In contrast, Daoudi et al. reported that PPARδ plays an important role in the intestinal GLP-1 production and, thereby, improves systemic insulin sensitivity[75]. Differences in experimental design can potentially explain these contradictory findings; for example, we activated PPARδ with a high-fat diet while Daoudi et al. used a synthetic ligand that is likely more potent than endogenous ligands derived from the diet. Alternatively, the metabolic effects could be caused by other bioactive incretins encoded by the *proglucagon* gene, such as glucagon-like peptide-2 (GLP-2), oxyntomodulin (OXM) or glicentin[76].

Together, these data highlight the importance of the intestine in the improved metabolic homeostasis seen after systemic PPARδ activation. PPARδ activation is a promising therapeutic strategy for treating metabolic derangements, and several short-term clinical studies have been performed[77,78]. Pleiotropic effects in organs and tissues not related to metabolic imbalance are always an extra risk factor in systemic NR activation. PPARδ is ubiquitously expressed and is implicated in cell differentiation with conflicting results about its role in tumorigenesis (reviewed in[79]). Intestinal NRs are promising drug targets since they can influence whole-body metabolism via regulation of gene expression in the gut. Additional studies, however, are still needed to uncover the molecular pathways by which intestine-specific PPARδ impacts whole-body metabolism.

Conclusions

In recent years the intestine has emerged as a major player in governing systemic metabolism, particularly fructose metabolism. In this thesis we identified genes that were distinctly regulated by fructose in the intestine and the liver (**Chapter 2**). These genes can help us to identify the mechanistic pathways underlying the detrimental metabolic effects of a high-sugar diet. We further identified a range of novel NR-mediated regulations of the GLUT-family of glucose transporters, using a directed high-throughput reporter screen (**Chapter 3**). Manipulation of specific GLUT-member levels through their transcriptional regulators is a promising strategy to potentially treat a wide range of diseases, including metabolic derangements and certain types of cancer. We identified the essential fructose transporter GLUT5 as a target of LXRα in both humans and mice (**Chapter 4**). We identified GLUT7 as a gene primarily expressed in the intestine, with a strong circadian rhythm in mice and regulated by RORα in humans (**Chapter 5**). Lastly, we showed that intestine-specific PPARδ activation is a putative strategy to improve whole-body metabolism without the adverse systemic effects (**Chapter 6**).

Taken together, these results reveal and underline the prominent role of the intestine in fructose metabolism and, provide valuable information about the target genes and functions of (intestinal) NRs. Ultimately, targeting NRs specifically in the intestine is a promising strategy for treatment of metabolic diseases as it avoids systemic uptake of the drug and minimizes the off-target effects.

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