Elucidating the mechanisms of action of short-chain fatty acids

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General discussion
Chapter 7
The alarming increase in the prevalence of the metabolic syndrome in the Western world, but also in developing countries, can be contributed to the change of dietary habits and lack of exercise. Dietary intervention by increasing the fiber content of the diet is a potential strategy in the prevention and treatment of the metabolic syndrome (171, 178, 179). Our understanding of how fibers regulate these beneficial effects at the molecular level is limited. The main products of intestinal fermentation of dietary fibers, the short-chain fatty acids (SCFAs), have been suggested to play a key role in the beneficial effects of dietary fibers. As discussed in chapter 1, the field is severely hampered by the lack of data on actual fluxes of SCFAs and metabolic processes regulated by SCFAs. In this thesis we tried to find the answers to the questions raised in chapter 1: what are the in vivo SCFA production and uptake fluxes under different conditions? How do SCFAs affect glucose and lipid fluxes via their dual role as substrates and regulators? At which timescales are different, apparently contradictory effects working? Can we quantify the role of different tissues and hormones?

What are the in vivo SCFA production and uptake fluxes under different conditions?

In chapter 2 we presented a novel method to determine in vivo SCFA fluxes under different dietary conditions. We showed for the first time that in vivo SCFA fluxes rather than cecal concentrations correlated in an inverse manner with biomarkers (i.e. body weight, glucose, insulin and HOMA-IR) of the metabolic syndrome. While our data provided a correlation for the effect of fiber dose on host physiology, they did not explain what caused the dose-dependent SCFA uptake fluxes in the first place. Evidence for the involvement of one or more additional molecular factors is the observation that a different fiber (fructooligosaccharide) gives rise to a less steep correlation between flux and metabolic markers than guar gum (Figures 1A-D). In addition, we cannot exclude that the concentration of another metabolite co-varies with the uptake fluxes and takes part in the actual causal mechanism. Next to SCFAs, the gut microbiota produces many metabolites, which are involved in the regulation of multiple host metabolic pathways (5). In addition, meta-analysis studies also showed a wide variety in beneficial effects of different dietary fibers (240-242). The complex structure of the different (classes of) fibers, together with the prebiotic action of fibers on the gut microbiota composition, complicates the supposedly beneficial effects of dietary fibers on host metabolism. Dietary fiber behaves within the gastrointestinal tract as a polymer matrix with variable physicochemical properties including susceptibility to bacterial fermentation, water-holding capacity, cation exchange, and adsorptive functions (295). These properties determine the physiological actions of fibers, but a clear association between the chemical composition and structure of fibers and the physiological effects is still lacking (296, 297). Changes in dietary fibers drive changes in the composition of gut microbiota. Although diet is a major determinant of the colonic microbiome, the host genetic background and the colonic milieu also exert a strong influence on the microbial composition in the large intestine (51, 53, 54, 201). The microbial activity in turn also affects the colonic milieu. Together, this causes a multifactorial steady state that result in strong variation of microbial populations between individuals. To elucidate the causal relation between dietary fiber type, SCFA fluxes and attenuation of
metabolic syndrome markers, more evidence should be gathered on the structural and chemical effects of dietary fiber on the composition and activity of the gut microbiota. By incubating defined gut microbiota with different types of fibers, or feeding mice with different types of fibers, responses of the gut microbiota composition to specific properties of dietary fibers can be studied using the Mouse Intestinal Tract Chip (MITChip) procedure (298). In addition, alterations in microbial gene expression and activity can be determined using metatranscriptomics (299). Finally, the resulting changes in SCFA production and delivery to the host due to altered gut microbiota activity, and the subsequent effects on host metabolism, should be studied for a wide range of different dietary fiber types following the method described in chapter 2.

**How do SCFAs affect glucose and lipid fluxes via their dual role as substrates and regulators?**

In chapter 3 we infused labeled SCFAs into the cecum of the mice to gain insight into the contributions of various biochemical pathways used by the different SCFAs. The results revealed that cecal acetate and butyrate are important substrates for mammalian lipid metabolism, whereas propionate strongly contributes to gluconeogenesis. Furthermore, mitochondrial metabolism of acetate appears to be minimal, in contrast to butyrate. The
regulatory role of SCFAs on glucose and lipid metabolism was studied in chapter 4 and 5. Short-term cecal infusion with acetate and butyrate simultaneously increased hepatic gluconeogenesis and decreased lipogenesis. Long-term supplementation of all three SCFAs enhanced oxidative metabolism by increasing the lipid oxidation capacity in liver and adipose tissue, while glucose metabolism was unaffected. Together these results showed that SCFAs could act simultaneously as substrate and as regulators of glucose and lipid metabolism. On the short-term the role of substrate and of regulator appeared to be important. On the long-term we only studied the regulatory role, which appeared to be dominant as indicated by the protection against the metabolic syndrome while supplying a diet with a higher caloric content.

The dual role as substrates and regulators of energy metabolism is not unique for SCFAs but known for multiple metabolites (300). Glucose signaling appears to require both extracellular sensing by transmembrane proteins and intracellular sensing by G proteins (301). Ketone bodies signal via extracellular and intracellular receptors and act as endogenous inhibitors of histone deacetylases (302, 303), while fatty acids can act as secondary messengers by modifying the activities of phospholipases, protein kinases, G-proteins, adenylate and guanylate cyclases (304-306). Altogether, the results described in this thesis add to the growing body of evidence that metabolites are not only static substrates, but also play an active role in coordinating signal transduction.

At which timescales are different, apparently contradictory effects working?

The different regulatory mechanisms for short- and long-term SCFA incubation raise questions about underlying mechanisms. The short-term effects were mediated by the phosphorylation of CREB by acetate and butyrate and resulted in an increase in gluconeogenesis via PGC-1α and a decrease in lipogenesis via PPARγ (Chapter 4). Long-term supplementation of SCFAs resulted in a decrease in lipogenesis and an increase in lipid oxidation via PPARγ, while no effect was observed on glucose metabolism and PGC-1α (Chapter 5 and Figure 2A). In the long-term supplementation studies, however, we observed no effect on the phosphorylation level of CREB (Figures 2B-C), which is in agreement with the finding that activation of phospho-CREB was transient and dampened over time (235, 307). We found in our short-term incubations with SCFAs no effect on AMPK phosphorylation (Chapter 4), whereas the AMP/ATP ratio and phosphorylation of AMPK was enhanced in a PPARγ-dependent manner during long-term SCFA incubation (Chapter 5). This time dependence of the SCFA-induced effects is supported by the results of Tang et al. (217) who showed that upon addition of SCFAs to HCT116 cells the intracellular ATP levels increased the first 15 hours, after which they started to decrease accompanied by an increase of the AMP/ATP ratio and subsequent phosphorylation of AMPK. We hypothesize that the long-term SCFA effects on PPARγ expression might be mediated by epigenetic changes through histone deacetylase (HDAC) inhibition. HDAC inhibitors selectively alter gene transcription, in part, by chromatin remodeling and by changes in the structure of proteins in transcription factor complexes (308). Recently, it was shown that PPARγ expression could also be regulated at the transcriptional level by distinct Activator Protein (AP) 1 dimers, which thereby controlled steatohepatitis (309). The AP-1 dimers
c-Jun/Fra-1 and c-Jun/Fra-2 inhibited PPARγ expression, while the c-Fos/JunB, c-Fos/JunD and c-Fos/c-Jun dimers promoted PPARγ expression. Interestingly, the HDACs have many nonhistone proteins substrates such as AP-1 (309-312). It has been suggested that HDAC inhibitors block the transcription of the AP-1 subunit c-Jun by inhibiting the recruitment of the preinitiation complex to the c-Jun promoter (312). DNA methylation can repress transcription via inhibition of transcription factor binding (313). Although we did not observe an increase in PPARγ promoter methylation in SCFA-fed mice (Figure 3A), we observed decreased mRNA expression of c-Jun and c-Fos in liver and adipose tissue of SCFA-fed mice (Figures 3B-C). It is possible that SCFAs regulate PPARγ expression via methylation of the c-Jun gene through the known SCFA-HDAC inhibition (314, 315).

The rate of accumulation of DNA methylation appeared to be slow in somatic cells (316-318), suggesting that possible SCFA-induced effects on PPARγ expression should be a long-term effect. To investigate this methylation hypothesis it would be interesting to introduce a short stimulation of SCFAs and study the long-term effects on c-Jun methylation, PPARγ expression and the down-stream effects.

It is, however, also important to note that in the short-term and long-term cell experiments different SCFA concentrations in the culture medium were used (0.5 mM and 3 mM, respectively). However, we do not expect that the concentration difference plays a major role in this range as it has been shown that SCFAs can increase the phosphorylation level of CREB up to 10 mM (218, 234, 235) and that lower SCFA concentrations down to 0.1 mM were able to

Figure 2. (A) mRNA expression of PGC-1α in livers of mice fed a high-fat diet supplemented with SCFAs for 12 weeks as described in chapter 5. Immunoblot of hepatic phospho-CREB and total-CREB for mice fed a high-fat diet supplemented with (B) SCFAs or (C) guar gum for 12 weeks as described in chapters 5 and 6, respectively. Relative phosphorylation levels were calculated by the ratio of phospho-CREB to total-CREB and normalized for control.
Figure 3. (A) PPARγ promoter methylation was assessed by pyrosequencing in liver and adipose tissue of mice fed a high-fat diet supplemented with SCFAs for 12 weeks as described in chapter 5. mRNA expression of AP1 subunits Fra-1, Fra-2, c-Jun, JunB, JunD and c-Fos in (B) liver and (C) adipose tissue of mice fed a high-fat diet supplemented with SCFAs for 12 weeks as described in chapter 5. All values throughout the figure are presented as mean ± SEM for n=6-8; *p<0.05 vs control.

induce AMPK phosphorylation (17, 137). It is also important to note that we depicted the regulatory effects of SCFAs in chapter 4 and 5 as linear pathways, which are a simplification of the cellular regulation network. CREB, PPARγ and AMPK are all involved in multiple nutrient-sensing signaling networks in multiple tissues (226, 319, 320), making the effects on energy metabolism via one of these modulators probably more a network based effect than a linear one. The microarray data that is available for the livers of the short-term and long-term studies offers a good opportunity to study the effects of SCFAs in a broader network. For example, the method of Rossell et al. (321) could be used to predict actual metabolic states in a top-down Systems Biology approach. In their method gene-expression measurements are integrated with genome-scale models of metabolism to identify reactions that are likely to be active in different metabolic states. These active reactions are then used to build condition-specific metabolic models and to predict actual metabolic states. In this way, microarray data from the short-term and long-term studies can be used to identify alternative metabolic flux distributions and to elucidate the origin of the different metabolic states that we observed.

The short-term regulatory role is physiologically relevant because in humans plasma SCFA concentrations vary significantly and peak after meal intake (219, 220). Different types of meals or various dietary fiber concentrations led to different plasma SCFAs concentrations (221). Together this implies that humans are not exposed to constantly high SCFA concentrations for a long period but rather for short-term bursts. The long-term regulatory role, however, can only be translated to humans when there is a high daily-intake of dietary fiber or when SCFAs are taken as daily dietary-supplements. We showed in chapter 5 and 6
that both ways are beneficial in protecting against the metabolic syndrome. In humans, daily dietary-fiber supplementation decreased body weight in both healthy and metabolic syndrome patients (171, 178, 179). In addition, a 12-week dietary acetate administration to obese Japanese subjects showed a decrease of body weight, body fat mass and serum triglycerides levels compared to the placebo-controlled group (124). Together this indicates that the long-term effects of SCFAs can be beneficial when taken daily as supplements or when produced from a high-fiber diet.

There are different signaling pathways involved for the short- and long-term effects of SCFAs. This time dependence results in a complex interpretation of the effects of SCFAs but also makes it a good example to solve with a Systems Biology approach. A dynamic network model combined with quantitative time-dependent data may reveal unknown network structures and clarify which molecular mediators and mechanisms contribute to the SCFA-induced effects, as has been shown previously for the mTOR signaling pathway (322, 323).

Can we quantify the role of different tissues and hormones?

SCFAs produced by the microbiota in the cecum and the colon can be found in hepatic, portal, and peripheral blood (11). This already indicates that the effects of SCFAs are not limited to the gastro-intestinal tract and liver. Indeed, we and others showed that SCFAs directly or indirectly affect metabolism in the gut, liver, adipose, muscle, heart and brain tissue (17, 132, 183, 324, 325). In our short-term experiments we focused mainly on the liver, but also enrichments in intermediates of central carbon metabolism and changes in plasma metabolites were measured. The unexpected incorporation patterns in plasma organic acids and amino acids (Chapter 3) and the transient changes in plasma metabolites (Chapter 4), suggests a complex situation in which other organs besides the liver also play a significant role. In the long-term study the contribution of liver and adipose PPARγ was quantified by using organ-specific knock-out mice. Liver PPARγ mediated the SCFA-induced effects on liver steatosis, whereas adipose PPARγ was responsible for the decrease in body weight and insulin resistance (Chapter 5). Next to liver and adipose, muscle PPARγ also plays an important role in whole-body physiology (320). Muscle-specific PPARγ-deficient mice developed increased adiposity
Figure 5. (A) Relative mRNA expression of PGC-1α and Fndc5 in differentiated C2C12 cells after 6h incubation with 0.5 mM acetate, propionate or butyrate. (B) Irisin concentration in cell culture media after 6h incubation with 0.5 mM acetate, propionate or butyrate. (C) Relative mRNA expression of PGC-1α and Fndc5 in skeletal muscle of mice 12 weeks on SCFA-supplemented diet as described in chapter 5. (D) Plasma irisin concentration of mice 12 weeks on SCFA-supplemented diet as described in chapter 5.

and insulin resistance (270, 326) and SCFAs have been shown to affect muscle metabolism (17). In the SCFA-feeding study, however, SCFAs did not affect muscle PPARγ expression and subsequently did not change lipid oxidation capacity (Figures 4A-B). Interestingly, in contrast to the decrease in PPARγ activation in liver and adipose tissue (Chapter 5), butyrate has been shown to increase PPARγ activation in human colon adenocarcinoma cells whereas no effect was observed in 3T3-L1 cells (327). In addition, PPAR-associated target genes were increased in colon tissue of mice fed the dietary-fiber inulin (327). Studies with colon-specific PPARγ-deficient mice would help to further understand the SCFA-PPARγ-axis in the colon and to elucidate the tissue-specific activation of PPARγ (320).

SCFAs have been shown to increase PGC1-α expression in muscle (17) and we showed similar results in liver (Chapter 4). In addition, we observed increased lipid oxidation capacity in WAT in SCFA-fed mice (Chapter 5), possibly indicating a brown-fat-like development in WAT (328). Enhanced PGC1-α expression in muscle stimulates an increase in expression of Fndc5, a membrane protein that is cleaved and secreted as the recently identified hormone irisin (329). Irisin acts on white adipose cells to induce a broad program of brown-fat-like development. To investigate if SCFAs increased irisin expression and thereby activated brown-fat-like development, we incubated differentiated C2C12 cells with SCFAs and studied the expression of PGC1-α and Fndc5. Propionate and butyrate significantly increased PGC1-α
expression whereas acetate had no effect (Figure 5A). In agreement, Fndc5 was slightly increased upon propionate and butyrate stimulation (Figure 5A). Although mRNA expression of Fndc5 was increased, the cleaved hormone irisin was not increased during propionate and butyrate incubation (Figure 5B). In addition, in the long-term SCFA-supplementation study where increased lipid oxidation capacity in WAT was observed (Chapter 5), there was no effect on muscle mRNA expression of PGC1-α and Fndc5, and also no effect on plasma irisin levels for all three SCFAs (Figures 5C-D). Together this indicates that SCFAs do not increase lipid oxidation capacity in WAT by inducing brown-fat-like development of WAT through the myokine irisin, but our results do not rule out irisin-independent changes in WAT metabolism.

In chapter 6 we suggested that guar gum enhanced peripheral glucose metabolism at least partly through the SCFA-Ffar2-GLP-1 axis. Intracolonic infusions of SCFAs and intake of fibers have been shown to increase plasma GLP-1 concentrations and glucose uptake by adipose tissue (122, 147, 149-151). In addition, mice lacking Ffar2 exhibited reduced SCFA-triggered GLP-1 secretion in vitro and in vivo, and a parallel impairment of glucose tolerance (145). Although we did not observe any effects of SCFAs on the gut hormone PYY, intracolonic infusions of SCFAs in rats and pigs have been shown to increase blood concentrations of PYY (146, 147). Enteroendocrine L cells containing PYY colocalized with Ffar2 and Ffar3 (83, 142, 143). In addition, Ffar2 and Ffar3 knockout mice showed reduced colonic PYY expression (145). Possibly the oral-fed and cecal-infused SCFA models discussed in chapter 5 and 6 did not deliver the SCFAs to the specific PYY-expressing L cells and thereby resulting in unaltered levels of PYY.

In conclusion, SCFA-induced effects on glucose and lipid metabolism work through hormone-dependent and independent mechanisms in a wide range of cells and organs. To increase the understanding of the contributions of different cell types to SCFA-induced effects, in vitro SCFA-stimulation studies should be performed in a wide range of cell types. Interesting results can be further studied in vivo in organ-specific knockout mice to elucidate the whole-body role of specific organs and molecular mechanisms. Hormone-dependent and independent mechanisms can be separated by using knockout mice or by using different administration routes as illustrated in chapter 6.

Outlook

The key findings described in this thesis are that (i) the SCFA uptake fluxes inversely correlate with metabolic syndrome markers, (ii) SCFAs regulate glucose and lipid metabolism in a time-dependent manner; (iii) SCFAs can protect and treat dietary-induced obesity and insulin resistance by enhancing oxidative metabolism and (iv) guar gum exerts a differential effect on glucose metabolism than orally supplemented SCFAs. Unfortunately, as also discussed in chapter 1, there is a lack of data for the effects of SCFAs in humans and not all results obtained in rodents can be directly translated to humans. Interestingly, there are currently two clinical trials initiated by the Top Institute Food and Nutrition (TiFN, project GH003) that investigate the acute effects of SCFAs on human energy metabolism (ClinicalTrials.gov Identifier NCT01826162 and NCT01983046). In these studies the acute metabolic effects of rectally infused SCFAs are studied in healthy obese volunteers. It will be especially interesting
to compare the results to our outcomes described in chapter 4, were we described strong metabolic effects in a similar acute experiment in obese mice. For the long-term effects, a 12-week dietary acetate administration to obese Japanese subjects showed a decrease of body weight, body fat mass and serum triglycerides levels compared to the placebo-controlled group (124), suggesting similar results as we showed in chapter 5. However, to use SCFAs as nutritional supplements to prevent and treat the metabolic syndrome, these results require confirmation in a large scale double blind randomized controlled clinical trial. Furthermore, it is important to be aware that SCFAs also act on processes involved in autophagy, immune response and the sympathetic nervous system (18, 245, 247, 248, 330). Our data, together with the clinical trials in humans, hopefully paves the way to use the inexpensive SCFAs as nutritional supplements to prevent and treat the metabolic syndrome in humans.