Regulators of hepatic glucose and glycogen metabolism
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CHAPTER 10

General discussion
Hepatic glucose metabolism is tightly regulated in a very complex system. This thesis evaluates the role of several regulators of hepatic glucose metabolism, addressing both "extrinsic" mediators (such as NO, IFN-γ, FFA) and "intrinsic" mediators (glucose concentration, glycogen, gluconeogenesis and glycogenolysis). The studies lead to a certain insight into regulation, but mostly to the awareness that there is not one director. The symphony orchestra involved in the regulation of glucose homeostasis may even be made up of various orchestras, set up in space in well-defined positions and playing together.

The role of paracrine mediators

One can study the potential role of a paracrine mediator on the regulation of glucose production by either adding or withdrawing the specific mediator, under basal or stimulated conditions. Paracrine mediators, such as TNF-α, IL-6, and IFN-γ, administered in pharmacological doses, were shown to affect glucose production (1-3). In vivo, prostaglandins cannot be given safely. Besides, the half-life of prostaglandins in the circulation is short: near 97% of an intravenous dose of PGE₂ is eliminated from plasma within 90 seconds (4). Therefore, metabolic effects of prostaglandins are usually studied by administration of prostaglandin synthesis inhibitors (5-7). Corssmit et al., using indomethacin, showed that prostaglandins are potential mediators in the regulation of basal glucose production in healthy subjects (6).

NO

NO is a volatile gas with a half-life of seconds, which cannot simply be administered due to its multiple effects, e.g. on neurotransmission and vascular tone. Chapter 2 describes the role of NO on intrahepatic pathways of glucose and glycogen formation in vitro. Others had previously shown that in vitro NO inhibited rate-limiting enzymes of the gluconeogenic pathway, leading to an inhibition of overall glucose production. We were the first to show that NO inhibited glycogen synthesis in vitro. With its short half-life, enabling a rapid and rapidly reversible effect, NO is an appealing potential modulator of basal glucose production in vivo. In the in vivo setting we chose to block basal and stimulated NO production by administration of L-NMMA, and studied the effect of reduces NO synthesis on glucose production in vivo (chapters 3 and 4).
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The rather spectacular inhibition of gluconeogenesis and glycogen synthesis caused by NO in vitro in hepatocytes was however not confirmed in vivo in healthy humans. The finding in our study that 50% inhibition of NO in vivo did not affect glucose production excludes an essential role for NO in basal glucose production in healthy subjects. However, it does not exclude a role for NO in the regulation of glucose production. In vivo we measured only (net) glucose production and not the different pathways of gluconeogenesis, glycogenolysis, and glycogen synthesis. NO might have an effect on one of these pathways, as it has in vitro, although this effect did not become manifest in vivo, due to autoregulation or counterregulation by another pathway. As was shown by Ahlborg et al., excessive inhibition of NO inhibited splanchnic glucose output (although we must bear in mind that inhibition is in contradiction with inhibitory effects of NO on glucose output in vitro) (8,9). Not only in healthy man, but also in the mildly septic dog in which NO production is presumably enhanced, no effect of NO inhibition was found on glucose production.

We conclude that either NO has no effect on glucose production in vivo, or the compensatory protection offered by other “players in the orchestra” prevents disruption of such a vital system as glucose production during fasting. In effect, failure of this protective mechanism may only become manifest under extreme conditions. In the case of NO, such an extreme condition could be systemic inflammation, or sepsis, i.e. hypoglycemia induced by septic shock.

IFN-γ

The same is true for IFN-γ. Despite mild metabolic effects (an increase in resting energy expenditure, IL-6 levels, ACTH and cortisol levels), IFN-γ did not alter glucose production in the post-absorptive state. Similarly, as for NO, this does not exclude an effect of IFN-γ on glucose production. De Metz et al. showed that under more extreme conditions, i.e. in surgical patients after pancreaticoduodenectomy, IFN-γ decreases glucose production, despite a rise in counterregulatory hormones (10).

Free fatty acids

FFA had already proven not to be just a metabolic substrate, but also a metabolic modulator. Randle was the first to describe an interaction between FFA and glucose uptake, known as the glucose-fatty acid cycle (11). The role of FFA in the
development of insulin resistance and type 2 diabetes, and the importance of plasma FFA mediating the response of insulin secretion to a glucose load, gave FFA the name of “second messengers” in the regulation of glucose homeostasis (12-15).

Effects of FFA on gluconeogenesis and glycogenolysis had been described before (16-20). However, the description of the effect of FFA on the differential pathways contributing to gluconeogenesis, glycogenolysis and glycogen synthesis is new. In chapter 9, we show that the stimulatory effect of FFA concerns only the direct pathway of gluconeogenesis (from G-6-P directly to plasma glucose). As for glycogenolysis, FFA reduce glycogen breakdown both from existing (stock) and from newly synthesized glycogen, during short-term starvation. We found that glycogen synthesis during fasting is constant and not influenced by (physiological) FFA levels. Endogenous (physiological) FFA levels therefore act as important regulators of hepatic glucose metabolism during short-term fasting. FFA clearly stimulate direct gluconeogenesis. The glycogen-sparing effect is also evident: FFA reduce breakdown while synthesis stays constant (the latter independently from FFA).

The physiological meaning of this glycogen sparing effect may be seen into the following perspective. First of all, during (short-term) starvation, i.e. in the absence of exogenous carbohydrate intake, FFA (or triacylglycerols) are the first alternative fuel for most tissues, although only to a very modest extent for the brain. FFA are highly concentrated stores of metabolic energy. Fatty acids are degraded by lipases by the sequential removal of two-carbon units, accompanied by the formation of a glycerol. Glycerol is phosphorylated and oxidized to dihydroxyacetone phosphate, which in turn is isomerized to glyceraldehyde-3-phosphate. This intermediate is on both the glycolytic and gluconeogenic pathways, and glyceral can therefore be converted to both pyruvate or glucose in the liver, which contains the appropriate enzymes (21). Glycerol therefore, during starvation, serves as a gluconeogenic substrate, besides the energy formed by fatty acid oxidation itself in the form of NADH and FADH₂, both sources for ATP.

There is however more: the inhibition of glycogen breakdown by FFA is a way to maintain a certain concentration of glycogen in the liver as a rapidly available source of glucose. The brain is critically dependent on glucose. Our organism was built to survive in the temporary absence of food, as proven by the fuel storage
forms of glycogen and fat, and gluconeogenesis from amino acids. In case of "emergency" needs, a certain reserve of glycogen may be of vital importance, e.g. for the most vital organ: the brain. The double role of FFA - a metabolic fuel and a regulator of other processes involved in fuel homeostasis during fasting - seems a very efficient and elegant one. In view of the other effects of FFA described recently, e.g. involvement in glucose-stimulated insulin secretion (15), FFA-mediated control of insulin on glucose production (12), and inhibition on muscle glucose uptake (14,22,23), it may be more appropriate to characterize the role of FFA as "endocrine", and not as just another substrate.

We conclude that paracrine mediators, and FFA, are important in the symphony orchestra, but not as first violinists.

Pathways of hepatic glucose and glycogen metabolism

In the last three chapters of this thesis, we mainly focussed on the role of glycogen and glycogen breakdown in glucose production, in falciparum malaria, type 2 diabetic patients, and in healthy volunteers. In the latter study, we also measured different pathways contributing to glycogenolysis, gluconeogenesis, and glycogen synthesis.

Glycogen

In chapter 7 we challenged the concept that glycogen content may dictate its own rate of breakdown, a concept put forward by Magnusson et al., implicating autoregulation in the strictest sense of the word. Magnusson et al. showed that in the fed state, the turnover (i.e. proportional degradation/synthesis) of glycogen was higher than in the fasted state, suggesting that this could be a mechanism preventing excessive glycogen accumulation (24). Following this concept, the rate of glycogen breakdown should slow down (or decrease) during fasting. We know that glycogenolysis decreases linearly during short-term fasting, both in disease and in the healthy state (see chapters 2,7,9). We challenged Magnusson's concept in malaria, a disease in which glycogenolysis rates are known to be very low, presumably reflecting glycogen depletion in the liver of malaria patients. We expected the (low) rates of glycogen breakdown to decrease more rapidly than in healthy volunteers, so that the (presumed) low glycogen content in malaria would
be spared as much as possible. We found that glycogen breakdown was not slowed down more in malaria than in healthy volunteers. In other words, glycogen content in malaria, although presumably close to being exhausted, is not spared. Whether glycogenolysis would adapt in a similar way in long-term fasted healthy humans when rates become very low is difficult to say. Glycogenolysis has never been measured long enough over time to observe a change in the rate of breakdown, either isotopically or with $^{13}$C-NMR spectroscopy due to technical limitations. Clore et al. found that in 72-86 hrs fasted volunteers, glucose production (supposed to be merely gluconeogenesis) was considerably lower at baseline than after 14 hrs of fasting, and glucose production no longer decreased after an overnight fast (as it does after 14 hrs of overnight fasting) (25). Hellerstein et al. measured gluconeogenesis and glycogenolysis at 60 hrs of fasting, and found that glycogenolysis was still 24% (0.36 mg/kg/min) of glucose production; unfortunately glycogenolysis was only measured at one time point (26).

We conclude that in healthy volunteers, despite enhanced glycogen turnover in the fed state preventing glycogen accumulation, and although glycogen breakdown decreases as liver glycogen content decreases, data in malaria show that glycogen breakdown is not further reduced as rates approach zero. Either the contribution of glycogen breakdown remains essential to maintain euglycemia - even at very low rates - or mechanisms to prevent complete glycogen depletion fail in malaria. As we cannot compare our data to data in healthy volunteers, we cannot rule out an effect of malaria on our findings. The adaptation of glycogenolysis in time, in relation to glycogen content, therefore needs further study in healthy volunteers.

**Type 2 diabetes**

In chapter 8 we measured the adaptation to short-term fasting in type 2 diabetes patients and we observed a “breaking point” at 20 hours of fasting. Glycogenolysis decreased consistently until 20 hours of fasting and then remained constant. Gluconeogenesis started to increase after 20 hours of fasting, and prevented a further decrease in glucose production. After 20 hours FFA levels started to rise and C-peptide levels decreased. This is different from the adaptation observed in healthy volunteers, in which changes in glycogenolysis follow a linear pattern during the first 24 hours of fasting (see chapter 9), as also seen in malaria patients (chapter 7). The diabetic patients were all obese, except one. In the absence of hormonal influences of importance, it would be interesting to speculate on the role
of the rise in FFA on gluconeogenesis after 20 hours of fasting. However, referring to chapter 9 and the influence of FFA, we would not have expected glycogenolysis to stop decreasing while FFA levels increased. The role of obesity per se remains to be studied, and especially the influence of obesity and type 2 diabetes on the different pathways of gluconeogenesis, glycogenolysis, and glycogen synthesis.

*Intrahepatic fluxes*

In chapter 9 we describe the fluxes contributing to hepatic glucose and glycogen production. As Hellerstein himself pointed out, the secreted glucuronate technique allows measurement of “internal dynamics that are not communicated with the surroundings”(27). Combining it with MIDA allows measurement of the contribution of the direct and indirect pathways of gluconeogenesis, and the contribution of the latter to glycogen synthesis. Generally accepted is the concept that glycogen stock becomes rapidly depleted during fasting, and that after 42 hrs-60 hrs gluconeogenesis accounts for all glucose output (26,28). Fascinatingly, Hellerstein found that glycogen synthesis did not decrease between 11 and 60 hrs of fasting, and we confirmed this finding between 14 and 22 hrs of fasting. There is evidence that simultaneous glycogen synthesis and breakdown occurs, both during fasting and (re)feeding (26,29). The indirect gluconeogenic pathway appears to be unaffected by fasting or FFA, and it imperturbably contributes to glycogen synthesis, independently from FFA.

Why would, during fasting, fluxes of glucose metabolism not simply be directed towards direct glucose output to plasma?

We must first note that, although substantial, the indirect gluconeogenic and total glycogen synthesis fluxes are quantitatively inferior to the direct gluconeogenic flux and glycogen breakdown flux to plasma. We speculate that guaranteeing a constant flux to glycogen from all available sources may be designed by nature to spare the “emergency fuel” glycogen for survival during starvation, although glycogen synthesis and breakdown may eventually reach a break-even point (as found by Hellerstein after 60 hrs of fasting) (26). At that point the glycogen cycle will be active although net glucose output from glycogen becomes zero. Youn and Bergman provide an explanation for both the constant synthesis of new glycogen, and the constant indirect gluconeogenic flux. They found that gluconeogenic substrates facilitated the incorporation of glucose-6-phosphate into glycogen, and
suppressed glycogen degradation. Blocking gluconeogenic flux in intact rats could virtually abolish hepatic glycogen accumulation (30). This provides a reason for which the indirect pathway of gluconeogenesis is essential during fasting, in view of the need of a constant reserve capacity of "emergency fuel".

**Autoregulation**

Why does gluconeogenesis increase during short-term fasting?

We witnessed an increase in gluconeogenesis that may have been an autoregulatory attempt to compensate for the decrease in glycogenolysis, as has been described by others (16,31). Autoregulation is pertinacious in maintaining glucose homeostasis, in health and disease, even despite experimental interventions meant to disrupt glucose homeostasis (31-34). Autoregulation may not only be needed to maintain glucose output, but also to guarantee a minimum amount of glycogen - for in case of emergency - both in the presence and absence of FFA.

In the acipimox experiment glycogenolysis no longer decreased, and we cannot rule out that this explains why gluconeogenesis, independently from a FFA effect, ceased to increased, lacking the autoregulatory stimulus. Youn and Bergman, as mentioned above, describe that gluconeogenic substrates are involved in the regulation of glycogen metabolism, both *in vitro* and *in vivo* in rats. Gluconeogenic substrates facilitate the incorporation of glucose-6-phosphate into glycogen, and hepatic glycogen accumulation is virtually abolished when blocking gluconeogenic flux in intact rats (30). *In vivo*, we found that indirect gluconeogenesis contributed considerably to glycogen replenishment and that this process was independent from FFA. We suggest that *in vivo* the indirect gluconeogenic pathway may be equally indispensable in humans (as in rats) to replenish glycogen stock from available gluconeogenic substrate.

Clore et al. showed that between 72 and 86 hrs of (overnight) fasting, the decrease in glucose production observed after an overnight 14 hrs fast no longer existed. Glucose production was 29% lower than after 14 hrs of fasting, and FFA levels were increased (25). We know that after 60 hours of fasting glycogen synthesis persists, but its rate equals that of glycogen breakdown (26). It is tempting to postulate that autoregulation of glucose production depends on the presence of glycogen stock in the liver.
**Glucose-6-phosphate (G-6-P)**

In view of our findings in chapter 9, we may address another potential regulator of hepatic glucose and glycogen production. Our results support Hellerstein's finding that - at least between 11 and 60 hours of fasting - the indirect gluconeogenic flux (G-6-P via UDP-glucose and glycogen) is constant. The breakdown of glycogen synthesized from various sources tightly regulates glucose production (glycogen - G-6-P - glucose). It seems that a central position in all these fluxes is taken in by G-6-P. G-6-P may well function as a “clearing house” for gluconeogenic flux and glycogenolytic flux. Recent studies support this thought. Van Dijk et al. measured intrahepatic fluxes using the secreted glucuronate technique and MIDA during acute inhibition of G-6-Pase in rats in vivo, followed by liver excision. They found that inhibition of G-6-Pase strongly increased G-6-P concentrations, caused massive glycogen accumulation, and diminished glucose production by 50%. The gluconeogenic flux to G-6-P itself was unaffected. Newly synthesized G-6-P was redistributed from glucose production to glycogen synthesis. The flux through glycogen synthase was doubled, glycogen cycling increased 6-fold, but the flux through glycogen phosphorylase (breakdown) was not affected. Van Dijk et al. conclude that the changes in hepatic glucose metabolism induced by G-6-Pase inhibition are aimed at maintenance of the hepatocellular G-6-P concentration (35).

**Final conclusion**

This aim of this thesis was to unravel certain aspects of the regulators of intrahepatic glucose and glycogen metabolism, leading to the conclusion that there is not one regulator but many, playing masterly together in a symphony orchestra. For as far as we can measure, not one pathway is the main director, and not one mediator explains physiological or pathological changes in glucose homeostasis. All changes observed, with or without pharmacological manipulation, are modest. Hepatic glucose production is a vital, complex and very well safeguarded process. In a healthy organism, auto- or counterregulation seems ready to compensate for the absence of a player. The development of highly sophisticated isotopic techniques allowing to study the kinetics of complex metabolic systems in man, both in physiology and pathophysiology, invite to a lot more study needed to gain further insight.
References


Chapter 10


