The metabolic response to fasting in humans: physiological studies

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1 GENERAL INTRODUCTION

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General considerations

Fasting has been part of human's nature since the very beginning of mankind: the adaptations that occur in the fasting state serve to protect the organism from substrate depletion. During fasting the plasma glucose concentration needs to be kept in narrow limits to fuel the central nervous system. In addition, adaptations to preserve protein mass will increase our chances of survival. The human organism has extensive fuel reserves, mainly represented by adipose tissue and muscle, as shown in table 1 (1).

Table 1 Fuel reserves in a typical 70-kg man

	Available energy in kcal (kJ)					
Organ	Glucose/glycogen		Triacylglycerols		Mobilizable protein	
Blood	60	(250)	45	(200)	0	(0)
Liver	4	(1700)	450	(2000)	400	(1700)
Brain	8	(30)	0	(0)	0	(0)
Muscle	1200	(5000)	450	(2000)	2400	(100000)
Adipose tissue	80	(330)	135000	(560000)	40	(170)

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Fasting can be defined as a lower energy intake than needed to maintain body weight, however in this thesis, the term fasting is reserved for the complete withdrawal of food except for water.

Tight definitions for different durations of fasting are lacking, however after critical appraisal of the literature some statements can be made regarding this topic.

Most known is *overnight fasting* (defined as 14 h fasting or post-absorptive state) which is being used extensively to prepare patients for interventions or diagnostic procedures. Surgery is most often scheduled after an overnight fast to prevent pulmonary aspiration although the evidence for this practice may be challenged (2). Screening for diabetes mellitus with a fasting plasma glucose level or oral glucose tolerance test typically occurs after an overnight fast (3), in order to compare the well defined response to 75 gram of glucose without fluctuations in plasma insulin and glucose caused by intestinal food absorption.

When fasting is continued after an overnight fast, this is named *short-term fasting*. Most studies that have examined the physiological adaptations up to 85 h of fasting, define such fasting periods as short-term (4-8). Although fasting for 85 hours unequivocally is

a very long time, it is actually quite understandable why this is called short-term fasting. Short-term fasting encomprises the first adaptive changes that occur in the adaptation in energy metabolism (hypoinsulinemia, decreased glucose oxidation, increased fatty acid oxidation (FAO), increased lipolysis, ketogenesis, decreased insulin mediated glucose uptake, glycogenolysis (and subsequent depletion of glycogen stores), gluconeogenesis and proteolysis (5;7-17). These adaptations are active in the post-absorptive state but become maximal after approximately 3 days of fasting (5)

The total energy reserves in a typical 70 kg man represent 161000 kcal, as shown in table 1 (1). The resting energy expenditure (REE) then may be approximately 1600 to 1700 kcal (18). This means that energy stores will suffice to meet caloric needs for 95 to 101 days (19). However this is a simplified example because it does not take into account activity, changes in REE and critical protein mass for survival (20).

Intermittent fasting is another way of fasting that is most easily exemplified by the way Muslims fast during the Ramadan (21). Intermittent fasting is characterized by refraining form food intake in certain intervals and was studied because of the thrifty gene concept (22). Although the thrifty gene concept has been debated (23;24), animal and human studies have shown effects of IF on energy metabolism (25). Previous human studies have not been numerous and did not yield equivocal data (22;26;27). However, the increase of insulin sensitivity after two weeks of IF shown by Halberg et al is of interest since it may provide in a simple tool to improve insulin sensitivity (22).

However it is unknown whether eucaloric IF affects basal endogenous glucose production (EGP), hepatic insulin sensitivity or protein breakdown in healthy lean volunteers.

Metabolic adaptation to short-term fasting

Fatty acid metabolism during short-term fasting

During short-term fasting, the orchestrated interplay of low insulin levels and increased plasma concentrations of catecholamines and growth hormone (GH) will increase lipolysis and thus plasma free fatty acid (FFA) concentrations (5-9;14;28). Lipolysis is activated by protein kinase A (PKA) and inhibited by insulin (29). PKA phosphorylates hormone sensitive lipase (HSL) and perilipin A, leading to translocation of HSL to lipid droplets (30-32). Adipose triglyceride lipase (ATGL) is another lipase involved in hydrolysis of triglycerides (33).

During short-term fasting, fatty acid oxidation (FAO) increases with a concomitant decrease of glucose oxidation (CHO) (6;34). Fasting increases the intramyocellular AMP/ATP ratio that activates AMP-activated protein kinase (AMPK) by AMP binding

and phosphorylation (35). Phosphorylated AMPK then phosphorylates and inhibits ACC activity, thereby inhibiting malonyl-CoA synthesis. This results in decreased inhibition of carnitine-palmitoyltransferase 1 (CPT-1) activity, thereby increasing mitochondrial import of fatty acids and FAO in muscle. The increase in FAO will yield acetyl-CoA which inhibits glycolysis and subsequent glucose oxidation via the pyruvate dehydrogenase complex (PDH_c). In a way, this has been described by Randle in his renowned paper on the glucose fatty-acid cycle in 1963 (13). Randle's paper was concluded as follows:

"Evidence is presented that a higher rate of release of fatty acids and ketone bodies for oxidation is responsible for abnormalities of carbohydrate metabolism in muscle in diabetes, starvation, and carbohydrate deprivation, and in animals treated with, or exhibiting hypersecretion of, growth hormone or corticosteroids. We suggest that there is a distinct biochemical syndrome, common to these disorders, and due to breakdown of glycerides in adipose tissue and muscle, the symptoms of which are a high concentration of plasma non-esterified fatty acids, impaired sensitivity to insulin, impaired pyruvate tolerance, emphasis in muscle on metabolism of glucose to glycogen rather than to pyruvate, and, frequently, impaired glucose tolerance. We propose that the interactions between glucose and fatty-acid metabolism in muscle and adipose tissue take the form of a cycle, the glucose fatty-acid cycle, which is fundamental to the control of blood glucose and fatty-acid concentrations and insulin sensitivity."(13)

This assumption seems more or less correct in view of changes in substrate oxidation since glucose oxidation is inhibited via PDH_c in both starvation and obesity induced insulin resistance/diabetes mellitus type 2 (34;36). In contrast, the Randle cycle was revisited by Boden and Shulman by argumenting that in peripheral insulin resistance, the decreased transmembrane glucose transport is rate limiting and not accumulation of glucose-6-phosphate as he suggested in his original paper (37).

During fasting, myocellular lipid supply exceeds FAO(16;38). This means that the excess muscle lipid must be stored in some way. It was shown by an elegant tracer study that the higher muscle lipid uptake (compared to FAO) is accompanied by increased reesterification of FFA to triglycerides (38). This is supported by a proton magnetic resonance spectroscopy (MRS) study which showed accumulation of IMCL in the vastus lateralis muscle of healthy men during short-term fasting (16).

Long-chain fatty acid transport across the plasma membrane involves a protein-mediated process by the fatty acid transporters (FAT)/CD36 and fatty acid binding protein ($FABP_{pm}$) that are regulated by stimuli like insulin and AMPK (39). After cellular uptake of plasma FFA, fatty acids are activated to (fatty) acyl-CoA. Long-chain acyl-CoAs

can only transverse the mitochondrial membrane as acylcarnitines (ACs) to be oxidized (40). The coupling of an activated long-chain fatty acid to carnitine (3-hydroxy-4-*N,N,N*-trimethylaminobutyric acid) is catalyzed by CPT1 on the outer mitochondrial leaflet. Inside the mitochondrion, the fatty acid moiety is activated to acyl-CoA again by CPT2. The released carnitine is transported to the cytosol again in exchange for a new incoming AC (by the mitochondrial membrane protein carnitine-acylcarnitine-translocase (CACT)). Measurement of the ACs in plasma is the golden standard for the diagnosis of FAO disorders at the metabolite level ((41;42). However, little is known about intramyocellular AC profiles and their role in local fatty acid and glucose metabolism. Long-chain ACs are most of interest since metabolites of long chain fatty acids are suggested to interfere with insulin sensitivity (43-46).

The accumulation of long-chain ACs in plasma may be accompanied by a concomitant accumulation of muscle ACs levels that could be related to changes in peripheral insulin sensitivity during fasting (47-49).

Ketone body metabolism during short-term fasting

Plasma ketone body (KB) levels are an important source of energy and their levels and turnover increase during fasting (1;50). It is somewhat surprising that, until 1967, KBs have been regarded as "metabolic garbage" with no beneficial physiological role (51). However, the central nervous system requires approximately 140 g of glucose per day (equivalent to almost 600 kcal) in which must be foreseen during fasting. Here, KBs are an excellent respiratory fuel: 100 g of *D*-3-hydroxybutyrate (one of the KBs) yields 10.5 kg of ATP whereas 100 g of glucose only yields 8.7 kg of ATP.

Ketogenesis (production of the KBs *D*-3-hydroxybutyrate, acetoacetate and acetone) occurs in the liver. Here fatty acids undergo beta-oxidation to form acetyl CoA which enters ketogenesis as depicted in figure 1 (19). Mitochondrial 3-hydroxy-3-methylglutaryl-CoA synthase (mHMG-CoA synthase) is the major enzyme involved in ketogenesis and is inhibited by insulin (52). It has been suggested that insulin resistance on ketogenesis, i.e. less insulin-mediated suppression of ketogenesis, is present in type 2 diabetes mellitus patients (53).

Whether the KB production rate is different under equal plasma insulin levels in lean and obese ketotic men is currently unknown.

During ketolysis (KB oxidation) in target organs, the ketogenesis pathway is reversed as depicted in figure 2. 3-ketoacyl-CoA transferase is not found in the liver; hence hepatic ketolysis does not exist.

Figure 1, Ketogenesis

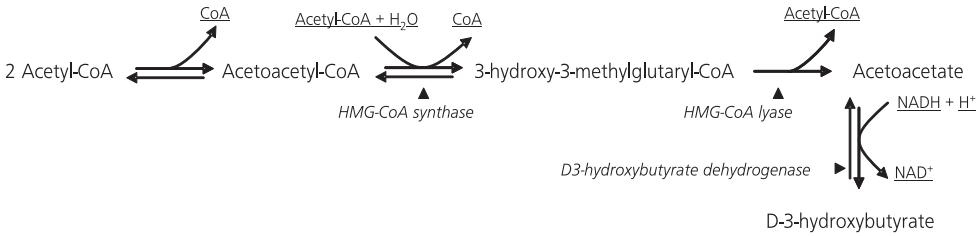
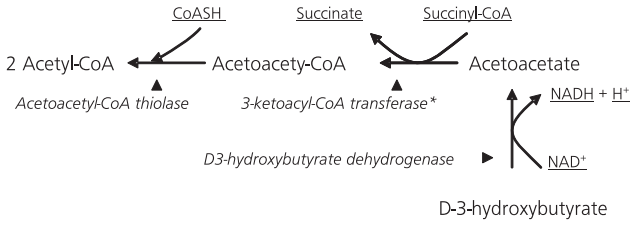


Figure 2, Ketolysis



* Note that *3-ketoacyl-CoA transferase* is absent in liver.

It has been suggested by at least two separate studies that 3-hydroxybutyrylcarnitine (3HB-carnitine) is the product of the coupling of *D*-3HB and carnitine (44;54). Although it has been described that *D*-3HB can be activated to *D*-3HB-CoA in rat liver and hepatoma cells, this has not been established in humans (55-57). Furthermore, it is unknown whether and how *D*-3HB-CoA can be coupled to carnitine resulting in *D*-3HB-carnitine.

This is in contrast to its generally known stereo isomer *L*-hydroxybutyryl-CoA (*L*-3HB-CoA), formed via beta-oxidation of butyryl-CoA (58). Although it was proposed that the total amount of tissue hydroxybutyrylcarnitine was derived from *D*-3HB and carnitine, the quantative contributions of the *L* and *D* stereo isomers were not accounted for in these studies (44;54).

Therefore it is unraveled which stereo isomers are present in vivo during short-term fasting in skeletal muscle and whether coupling of activated D-3HB to carnitine occurs.

Glucose metabolism during short-term fasting

Endogenous glucose production

It is generally known that plasma glucose levels decrease during fasting (4;8;9). This is explained by the slowly decreasing EGP (4) which is the greatest denominator of the fasting plasma glucose level (59). In resting circumstances glycogen stores will be reduced to a minimum after approximately 40h of fasting after which endogenous glucose production (EGP) primarily relies on GNG (12;60). Furthermore 80-90% of EGP is covered

by hepatic glucose production whereas 10-20% is provided by renal glucose production (mainly from lactate) (61). It is interesting that women display lower plasma glucose levels during short-term fasting compared to matched men (8;9). EGP has not shown to be different between men and women after short-term fasting (4;8;9;62), but one study demonstrated a steeper decline in time of EGP in women compared to men (4). In general, healthy humans do *not* become clinically hypoglycemic (i.e. neuroglycopenic) because of the contraregulatory response (63;64) and subsequent changes in glucose and fat oxidation as discussed above. When fasting-induced hypoglycaemia occurs, most patients undergo a supervised fasting period to exclude hyperinsulinemic hypoglycaemia. Some subjects develop a fasting-induced hypoglycaemia without neuroglycopenic symptoms in the presence of low insulin levels. This is a challenging clinical problem.

It is unknown whether patients with fasting-induced plasma glucose levels that fall well below the threshold value for hypoglycemia (i.e. 2.8 mmol/liter)(63) in the absence of hyperinsulinemia and signs of neurohypoglycemia have a metabolic disorder (FAO disorder, low EGP etc.) which could explain the lower tolerability to fasting

The hormonal contraregulatory response is characterized by stimulating effects of glucagon, growth hormone, cortisol and catecholamines on the key gluconeogenic enzymes that have been reviewed earlier (65).

The influencing role of FFA on EGP in fasting humans remains enigmatic (66;67). Despite the reciprocal changes in GGL and GNG during fasting, manipulation of plasma FFA had little or no effect on the EGP (68). Collected data in fasting humans (up to 40 h) suggest that plasma FFA increase, thereby leaving absolute GNG unchanged (66). In contrast, Féry et al demonstrated that FFA lowering by acipimox after 104 h of fasting increased GNG (69) which may reflect the need for oxidative fuel during FFA depletion. In this respect it is of interest that women have higher plasma FFA compared to men during fasting (4;8;9).

The explanation for lower plasma glucose levels with higher plasma FFA in fasting women is still lacking.

Peripheral insulin stimulated glucose uptake during fasting

Under insulin stimulated circumstances, glucose disposal occurs mainly in skeletal muscle (70) via the glucose transporter (GLUT) 4, which is activated via the insulin signaling pathway (71). Here insulin binds to the insulin receptor to activate its tyrosine kinase activity. This phosphorylates IRS-1 on tyrosine residues allowing for the recruitment of the p85/p110 PI3K to the plasma membrane. This generates $PI_{(3,4,5)}P3$ from $PI_{(4,5)}P2$, thereby

recruiting the 3' phosphoinositide-dependent kinase-1 (PDK-1). PDK-1 phosphorylates and activates both protein kinase B (AKT) and the atypical PKC λ/ζ (aPKCs) (71). PKCζ docks to munc18c, resulting in enhanced GLUT4 translocation (72). Additionally, phosphorylation of AS160, a protein containing a Rab GTPase-activating protein domain, is required for the insulin induced translocation of GLUT4 to the plasma membrane (73). AS160 is a downstream substrate of AKT.

It has been well established that peripheral (muscle) insulin mediated glucose uptake decreases during short-term fasting (17;74-78). Interestingly, in animal studies it has been shown that GLUT4 transcription decreases during fasting in adipose tissue (79;80). However in skeletal muscle, GLUT4 mRNA is *not* altered after fasting in both animals and humans (79;81). This indicates that during fasting posttranslational processes seem to dictate the amount of GLUT4 recruitment (e.g. amount or phosphorylation of AKT). The studies cited have not tried to explain lower insulin mediated glucose uptake after short-term fasting, although Bergman et al suggest that the increased plasma FFA levels will interfere with insulin mediated glucose uptake during fasting (82).

Fatty acids and insulin mediated glucose uptake

High plasma FFA are suggested to interfere with peripheral insulin mediated glucose uptake, but the exact mechanisms are still not fully elucidated (83). It was demonstrated in 1994 that increasing plasma FFA by an infusion of a lipid emulsion decreased insulin mediated glucose uptake in healthy volunteers in a dose dependent fashion (84). Interestingly, using the same study design, women were protected from FFA induced insulin resistance compared to matched men, but again the exact mechanisms have not been elucidated yet (85).

Various metabolites of FFA have been suggested to decrease insulin sensitivity in skeletal muscle (43). The sphingolipid ceramide is one of these mediators. Increased muscle ceramide concentrations have been reported in skeletal muscle of obese insulin resistant subjects (86;87), while a negative correlation of muscle ceramide with insulin sensitivity was found (87). Recently is shown that ceramide accumulates in muscle of men at risk of developing type 2 diabetes (88). However, skeletal muscle ceramide levels did not seem to play an important role in FFA associated insulin resistance in other studies (89;90).

Intramyocellular ceramide content is mainly dependent on de novo synthesis from fatty acids (91). *In vitro* studies showed that intracellular ceramide synthesis from palmitate is one of the mechanisms by which palmitate interferes negatively with insulin-stimulated phosphorylation of protein kinase B/AKT (AKT) (92;93). Furthermore, metabolites of

ceramide like complex glycosphingolipids (i.e. ganglioside GM3) may be involved in the induction of insulin resistance (43;94;95).

One can hypothesize that fasting increases muscle ceramide levels, thereby decreasing AKT phosphorylation and peripheral insulin mediated glucose uptake.

Additionally it is unknown whether the protection from FFA induced peripheral insulin resistance in women can be explained by differences in muscle ceramide or FAT/CD36 levels.

In this thesis, we aimed to shed more light on the metabolic adaptation to fasting since we are convinced that getting inside in the pathways which are involved in protecting the body from energy depletion will provide us with answers on the metabolic adaptation to overfeeding, i.e. the metabolic consequences of obesity.

Thesis Outline

Since women have higher circulating FFA and lower plasma glucose levels after short term fasting we investigated **in chapter 2** whether women would be relatively protected from FFA induced insulin resistance due to lower ceramide levels in skeletal muscle. We combined these measurements with analyses of the major FFA transporter FAT/CD36 in skeletal muscle.

In **chapter 3** we investigated whether muscle ceramide levels in skeletal muscle increase during fasting. Hyperinsulinemic euglycemic clamps and muscle biopsies were performed. We hypothesized muscle ceramide to increase during fasting, thereby reducing insulin stimulated glucose uptake, possibly via decreased AKT phosphorylation.

In **chapter 4** we explored the association of muscle long chain acylcarnitines with respect to fatty acid metabolism and peripheral insulin sensitivity. We expected long-chain ACs to increase during fasting and to correlate with whole body lipolysis, FAO rates and peripheral insulin sensitivity after short-term fasting.

In **chapter 5** we examined the quantative contributions of the *L* and *D*-hydroxybutyryl-carnitine stereo isomers after short term fasting in lean healthy subjects. Additionally we explored which biochemical synthetic route could be responsible; therefore additional studies in liver en muscle homogenates of mice were performed.

In **chapter 6** we explored the proposed insulin resistance in ketogenesis by studying ketone body metabolism using stable isotopes in lean and obese subjects under equal plasma insulin levels after short-term fasting. We expected ketogenesis to be equally sensitive to insulin in obese versus lean subjects.

Intermittent fasting has been suggested to increase insulin stimulated glucose uptake and thus insulin sensitivity although its mechanisms have not been elucidated. Therefore in **chapter 7** we tried to unravel some aspects of these beneficial effects by performing hyperinsulinemic euglycemic clamps and muscle biopsies after eucaloric intermittent fasting and a standard diet in crossover design in healthy volunteers. We focussed on the effects of intermittent fasting on glucose, fat and protein metabolism using stable isotopes.

Hypoinsulinemic hypoglycemia during fasting does usually not occur. However clinicians sometimes encounter these cases. In **chapter 8** we investigated 10 cases of hypoinsulinemic hypoglycemia. EGP was measured as well as plasma acylcarnitines and other intermediates of metabolism to rule out fatty acid oxidation disorders and disorders in organic and amino acid metabolism.

Chapter 9 is a perspective describing the integrated metabolic response to fasting regarding adaptive changes in lipid and glucose metabolism. The relevance and physiological aspects of a mechanism that is needed for survival of the organism will be discussed.

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