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The Metabolic Response to Fasting in Humans: a Perspective.

Introduction

The metabolic response to fasting consists of integrated adaptations that prevent energy depletion. Such adaptations protect the organism from hypoglycemia and protein losses to increase the chances of survival during starvation (1-3). Short-term fasting can be defined as the first 3 days of starvation in which progressive alterations in lipid and glucose metabolism occur (4-9). Although this definition may be arbitrary and debated, it is based on earlier observations that the adaptation to fasting is more or less maximal within about three days without further changes afterwards.

Fasting has been studied as a model of semi-acute lipid exposure (8;10-14), but also, perhaps as a consequence, as a model to study the interference of lipids with glucose metabolism, with special reference to the decrease of insulin mediated glucose uptake (4;15-21).

In the perspective of this thesis, the metabolic muscle adaptation to short-term fasting is discussed for its appropriateness as model for free fatty acid (FFA) induced insulin resistance *(chapter 2, 3 and 4)*. In addition, turnover and oxidation of substrates like lipid and glucose in short-term fasting are discussed in relation to several mechanisms (*chapter 2, 3 and 4)*. Although the thrifty gene concept has been debated (22-24), the adaptation to intermittent fasting is addressed since it may provide in a simple tool to increase peripheral insulin sensitivity (25) *(chapter 7)*. Then, the adaptive changes in ketone body (KB) metabolism are outlined, with special reference to a newly identified alternative KB pathway *(chapter 5 and 6)*. Finally, directions for future research on the metabolic response to fasting in humans are contemplated.

The Metabolic Response to Fasting: free fatty acid induced insulin resistance?

The only situation that causes "natural" insulin resistance in healthy lean subjects is starvation (26). Earlier studies showed decreased insulin sensitivity (i.e. decreased peripheral insulin mediated glucose uptake) after short-term fasting (4;15-21). The decreased glucose disposal was poorly explained by the idea that fasting induced a metabolic milieu favoring glucose production and the use of other fuels than glucose: it would then take a considerable time after refeeding to reverse this adaptation (4;16;17;19;21). More recently it has been tried to elucidate the fasting induced peripheral insulin resistance by suggesting that the increased plasma FFA and intramyocellular

lipid (IMCL) during short-term fasting may interfere with peripheral insulin sensitivity (15;26;27), analogous to the proposed accumulation of lipid metabolites in obesity induced insulin resistance (OIIR) and type 2 diabetes mellitus (DM) (28;29). High plasma FFA are suggested to interfere with peripheral insulin mediated glucose uptake, but the exact mechanisms are still not fully elucidated (30;31). Interestingly women are protected from FFA induced insulin resistance compared to matched men (32;33).

Various metabolites of FFA have been suggested to decrease insulin sensitivity in skeletal muscle (34). The sphingolipid ceramide is one of these mediators. Increased muscle ceramide concentrations have been reported in skeletal muscle of obese insulin resistant subjects (28;35), while a negative correlation of muscle ceramide with insulin sensitivity was found (35). However, skeletal muscle ceramide levels did not seem to play an important role in FFA associated insulin resistance in other studies (36;37).

Intramyocellular ceramide content is mainly dependent on de novo synthesis from fatty acids (38). *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) (39;40). Ceramide was shown to increase in rodents during fasting (41).

Therefore we examined the metabolic adaptation to fasting in relation to muscle ceramide levels. We found that after fasting for 38 h in lean healthy men and women no correlation exists between muscle ceramide and insulin sensitivity (42). Despite higher plasma FFA levels in the basal state, women were equally insulin-sensitive compared to men, suggesting a relative protection from FFA-induced insulin resistance in women as in other reports (32;33). To detect potential differences in FFA-uptake and fatty acid handling we assessed muscle concentrations of ceramide and the fatty acid transporter (FAT)/CD36 during the clamp. The muscle ceramide levels did not differ between females and males. Additionally, there were no differences in total FAT/CD36 amount between women and men in muscle, which is remarkable since it has been described earlier that women have higher levels of this lipid-binding protein (in muscle) after an overnight fast (8). Our data suggest that the initial gender differences in intramyocellular lipids (16) and fatty acid transporters after an overnight fast (8) are abolished after 38 h of fasting. We concluded that women are less sensitive to FFA-induced insulin resistance which is not explained by differences in muscle ceramide or FAT/CD36. Since we did find higher lipolysis rates in women without increased FAO, it is puzzling how the plasma FFA in women are disposed. Uptake of plasma FFA predominantly occurs in liver, adipocytes and muscle. FFA recycling occurs in the post-absorptive state and women display greater efficiency in direct FFA uptake in subcutaneous tissue (femoral area) (43). The liver may

be another site of increased non-oxidative FFA disposal. Although livers of females do not contain more fat than male livers (44), it is known that female livers contain more FAT/CD36 and have a greater capacity for FFA uptake and synthesis of ketone bodies and very low-density lipoproteins-triglycerides (45-47).

Since gender seems to fundamentally influence the lipid-glucose interplay (13;47-49) future studies are needed to explain how women are relatively protected from FFA-induced insulin resistance. This mechanistic insight could provide us a fundamental basis to develop strategies to reduce obesity-induced insulin resistance. Moreover these gender differences show that men and women should be examined separately in these studies.

Because the above mentioned study did not investigate the change in muscle ceramide in time during fasting, we investigated lean healthy men after 14 and 62 h of fasting focusing on muscle ceramide and the insulin signaling cascade because muscle ceramide has been shown to induce insulin resistance by interfering directly with the insulin signaling cascade (38) with most of the attention focusing on AKT (38;40;50-54). AKT is a 56 kD serine/threonine kinase and a central mediator of many insulin effects with complex regulation (52). Ceramide has been shown to interfere with the two phosphorylation steps on the serine^{473}- and threonine^{308}-residues of AKT (38;52;52-54).

We found that fasting for 62 h tends to increase muscle ceramide in young healthy volunteers though no correlation between muscle ceramide and insulin sensitivity was found (55). However we *did* find a trend towards lower pAKT-ser^{473} and a significant decrease of the ratio pAKT-ser^{473}/tAKT after 62 h of fasting. This is in contrast with the study of Bergman et al, who reported no differences of AKT between 12 and 48 h of fasting. Whether the difference in fasting duration of the two studies may explain these differences is not known at present. Whether the decrease in pAKT-ser^{473} during hyperinsulinemia after 62 hours of fasting can be attributed to the trend to the higher muscle ceramide levels in the basal state remains unanswered, since we found no correlation of ceramide with peripheral insulin sensitivity in line with previous observations (36;37;42). Since studies in OIIR and type 2 DM have shown effects on pAKT-thr^{308} (56) but not on pAKT-ser^{473} (56;57), it is possible that pAKT-ser^{473} is only involved in the physiological adaptation to fasting, inducing a reduction in peripheral glucose uptake and thereby protecting the body from hypoglycemia. Further studies are needed no investigate upstream mediators of AKT.

To be oxidized, activated long-chain fatty acids can only transverse the mitochondrial membranes as acylcarnitines (ACs, an activated long-chain fatty acid that is coupled to carnitine) (58). Recently however, muscle ACs have been implicated in insulin resistance

via a currently unknown mechanism (59-61). These studies suggested that increased beta-oxidation disrupts the tricarboxylic acid cycle (TCA) with subsequent inhibition of complete fatty acid oxidation via a high energy redox state (rising NADH/NAD$^+$ and acetyl-CoA/free CoA ratios). The accumulation of metabolic by-products (e.g. acylcarnitines) would then activate stress kinases or other signals, interfering with insulin action (60). However, how and whether ACs directly affect insulin mediated glucose uptake is currently unraveled.

To explore the acylcarnitine profiles in skeletal muscle and their possible role in the induction of insulin resistance, we investigated whether fasting elevates *muscle* long-chain ACs, since this is true for *plasma* long-chain ACs in humans (62-64), and whether this would correlate with peripheral insulin sensitivity. Such an increase of ACs during fasting could match increased lipid oxidation and possibly decreased peripheral insulin sensitivity (6;15;55). However, we demonstrated that muscle long-chain ACs do not increase during short-term fasting despite an increase in whole body fatty acid oxidation (FAO). Surprisingly, under hyperinsulinemic circumstances muscle long-chain ACs decreased after 14 h of fasting, but not after 62 h of fasting. However we could not detect a correlation of muscle long-chain ACs and peripheral insulin sensitivity. Besides the consideration that the muscle concentration of long-chain ACs during fasting in humans does not reflect the fatty acid oxidation flux, muscle long-chain ACs are unlikely to play a causal role in peripheral insulin resistance during fasting.

The Metabolic Response to Fasting: tenacious changes in substrate oxidation?

During short-term fasting, FAO increases with a concomitant decrease of glucose oxidation (CHO) (7;65). Fasting increases the intramyocellular AMP/ATP ratio that activates AMP-activated protein kinase (AMPK) by AMP binding and phosphorylation (66). Phosphorylated AMPK then phosphorylates and inhibits ACC activity, thereby inhibiting malonyl-CoA synthesis. This results in decreased inhibition of CPT-1 activity, thereby increasing mitochondrial import 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$_c$). These changes of FAO and CHO may be an example of Randle's glucose - fatty acid cycle (67). FAO yields a substantial amount of acetyl-CoA that accumulates in order to be oxidized. The accumulated acetyl-CoA increases pyruvate dehydrogenase kinase (PDK4) that results in decreased pyruvate dehydrogenase (PDH) thereby lowering glucose oxidation (65). In contrast, the Randle cycle was revisited by

Boden and Shulman by argumenting that in peripheral insulin resistance, the decreased transmembrane glucose transport via GLUT 4 is rate limiting and not accumulation of glucose-6-phosphate as originally suggested by Randle (68). How this reduction in GLUT 4 translocation to the plasma membrane occurs is still matter of debate and probably multifactorial in a setting of lipid overload.

The metabolic changes that occur after short-term fasting during hyperinsulinemic conditions are of interest since these reflect the ability of the organism to change substrate oxidation in response to insulin. The hyperinsulinemic euglycemic clamp increases CHO, and it has been shown that the CHO during hyperinsulinemia is lower after short-term fasting compared to an overnight fast (15;69). More over it was suggested that the hyperinsulinemia induced increase in CHO was lower after short-term fasting (15;69), which is called metabolic inflexibility.

However, we show that the increase in CHO during the hyperinsulinemic euglycemic clamp is not different after short-term fasting compared to an overnight fast although the point of departure is lower after short-term fasting. Also, non oxidative glucose disposal (NOGD) expressed as percentage of insulin mediated glucose uptake is not different before and after short-term fasting. NOGD expressed as part of glucose uptake takes into account the changes in glucose uptake as reviewed by Boden (68). These data are strengthened by our finding that phosphorylation of glycogen synthase kinase (GSK)-3 was not lower during hyperinsulinemia after short-term fasting compared with an overnight fast. Phosphorylation of GSK by AKT stimulates glycogen synthesis (20).

Analogous to the changes in CHO, the decrease in FAO during hyperinsulinemia is not different after short-term fasting compared to an overnight fast although the point of departure now is higher after short-term fasting. This argues with the previous suggestion that fasting induces metabolic inflexibility (15). These findings bring up the question whether metabolic inflexibility as defined by a switch in substrate oxidation upon stimulation truly exists and is not just a matter of expression of the data as absolute flux rates instead of taking the basal levels into account.

The Metabolic Response to Fasting: a newly identified alternative pathway in ketone body metabolism

Besides increased lipid oxidation, the KBs, D-3-hydroxybutyrate (D-3HB) and acetoacetate (AcAc), are an important fuel during fasting (70;71). Ketogenesis in lean healthy subjects only takes place during fasting when fatty acids reach the liver and are degraded within liver mitochondria. Acetyl-CoA then is channeled into the ketogenesis pathway (70). The transport of newly synthesized KBs from the hepatocytes to the circulation and the subsequent uptake in extrahepatic tissues occurs via diffusion and transporters (71;72).

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 (61;73). 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 (74-76). 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 (77). 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 (61;73).

In one of the studies presented in this thesis, we found a 32 fold increase of muscle 3HB-carnitine after 62 h of fasting (unpublished data). Therefore we explored KB metabolism in relation to the different stereo isomers of 3HB-carnitine. We found that the bulk (88%) of muscle 3HB-carnitine during fasting in lean healthy men consists of D-3HB-carnitine (78). Moreover muscle D-3HB-carnitine correlates well with the turnover of D-3HB. In addition, we confirmed that succinyl 3-ketoacyl-CoA transferase (SCOT) may be the responsible enzyme for the activation of D-3HB in muscle and liver homogenate studies (78). Subsequent exchange of CoA for carnitine then would be the following step and we have shown that this likely occurs via CAT (or another acylcarnitine transferase like CPT2) (79).

Currently it is unknown why D-3HB is activated and subsequently coupled to carnitine. Several explanations might be plausible: 1. reflection of true limitation of KB oxidation with the coupling to carnitine preventing mitochondrial accumulation (79;80), 2. transport needs between compartments and a reservoir of energy (79) or 3. preventing ketoacidosis during fasting. Finally D-3HB may be used for lipogenesis in muscle as it is

in liver (75;76). This would be in agreement with the increase in IMCL during fasting (27;81;82).

The Metabolic Response to Intermittent Fasting: always thrifty and beneficial?

Intermittent fasting (IF) is characterized by refraining form food intake in certain intervals and was studied because it was postulated to have beneficial effects on energy metabolism in light of the thrifty gene concept (25). The "thrifty" genotype was defined as "being exceptionally efficient in the intake and/or utilization of food" by Neel (24). Animal and human studies have shown effects of IF on energy metabolism (83). But the latter have not been numerous and did not yield equivocal data (25;84;85). 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 (25).

IF consists of repetitive bouts of short-term fasting that may alter peripheral insulin sensitivity as well as hepatic insulin sensitivity (25) and proteolysis (86;87), although protein stores are protected from excessive degradation (2;88). We found that intermittent fasting only increases peripheral insulin sensitivity with regard to glucose uptake and not hepatic insulin sensitivity after two weeks of eucaloric intermittent fasting. This effect is overcome by prolonged hyperinsulinemia. Also IF increased the insulin mediated inhibition of proteolysis. Insulin sensitivity of adipose tissue tends to be augmented where little effect was observed on proteolysis. More over resting energy expenditure (REE) decreased after two weeks of intermittent fasting.

Besides this effect on whole body kinetics, we showed that IF alters insulin and mammalian target of rapamycine (mTOR) signaling. We found a trend towards higher phosphorylation of AKT after IF and higher phosphorylation of GSK in the basal state and clamp. Such adaptive response may aid in replenishing glycogen levels. Additionally mTOR phosphorylation was decreased, suggesting that the inhibition of proteolysis is accompanied by a decrease in protein synthesis (89). Whether IF would affect skeletal muscle mass on the long run remains elusive.

These metabolic adaptations are of interest since they show that the body adapts to this situation in a thrifty way. However, the data also imply that intermittent fasting (e.g. "De Balansdag" in the Netherlands) may be disadvantageous because the decrease in REE may eventually lead to an increase in weight *even* under seemingly eucaloric conditions. To what extend IF can be beneficial in obese and diabetic patients remains elusive. The

mechanism that decreases REE during intermittent fasting is of great importance in the design of future studies on this matter.

The Metabolic Response to Fasting: What is the purpose?

The answer to this question may be simple: to safe energy. Although some of the adaptations to fasting may be known, the exact reason for these adaptations is unknown and may only be understood once refeeding is studied.

Increased lipolysis consequently increases plasma FFA, but also intracellular lipids on distant sites in both liver and muscle (27;81;90). In muscle this reesterification of FFA to yield intramyocellular triglycerides (IMCL) exceeds the rate of simultaneous intracellular triglyceride fatty acid oxidation (82). The synthesis of D-3HB-carnitine, as shown in this thesis, may be a comparable phenomenon.

The reasons for this discrepancy between muscle FAO and lipid supply during short-term fasting remain to be elucidated, though Stannard and Johnson speculated that storage of lipid would afford a survival advantage during periods of starvation by ensuring adequate fuel for necessary muscle function and protecting remaining blood glucose for the brain (26). The IMCL function as fuel for muscle during fasting may be challenged. Muscle glycogen stores are not broken into during 84 of fasting and remain available for activity opposed to liver glycogen that is depleted after 40 h of fasting (91;92). Also exercise during fasting induced higher levels of plasma FFA and KB compared to exercise after an overnight fast indicating that increased fuel supply is needed for activity (91). Data on IMCL during exercise after short-term fasting are lacking. IMCL also seem to be a marker for lipid overload in states of insulin resistance, i.e. obesity (93). Therefore, perhaps the discrepancy between muscle FAO and lipid supply during fasting plays a role in peripheral insulin resistance.

Decreased insulin sensitivity during fasting is highly regulated as shown in this thesis (55) and possibly mediated via FFA metabolites in muscle (15;26;27;55;81). It has been suggested by others, and ourselves, that this insulin resistance may prevent hypoglycemia. This may be a feeble explanation. During fasting insulin levels are typically very low with reciprocal changes in other glucoregulatory hormones (94) inducing EGP, lipolysis and the adaptations in substrate oxidation. Decreased insulin sensitivity makes sense in the presence of plasma insulin levels that stimulate glucose uptake via the insulin signaling cascade translocating the insulin specific glucose transporter GLUT4 (95). Decreased

insulin sensitivity may play a role during refeeding for two reasons. First, it may prevent massive uptake of glucose thereby preventing refeeding hypoglycemia. The other option is that decreased insulin sensitivity during refeeding lowers accumulated IMCL by, as we showed, ongoing FAO. Such mechanism could prevent the noxious effects of chronic lipid accumulation. Although it is the believe of the author that the latter mechanism is present, studies should be aimed on how the increased IMCL are lowered again during refeeding after short-term fasting and what biochemical pathways are responsible. Such knowledge may aid to increase peripheral insulin sensitivity in OIIR and type 2 DM.

As discussed, other area's of interest include the gender difference in lipid induced peripheral insulin resistance, detailed effects of fasting on insulin signaling and peripheral glucose uptake, *D*-3HB-carnitine and its routes of composition and degradation. It should be stressed in a thesis with studies on human physiology that one cannot comprehend the pathophysiology of insulin resistance, when the physiological sense of the metabolic adaptations to fasting is not understood.

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