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Endocrine effects of free fatty acids: Relevance in the pathogenesis of complex metabolic disorders

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Abbreviations

ACTH	Adrenocorticotropic hormone
AUC	Area under the curve
CBG	Cortisol binding globulin
DAG	Diacyl glycerol
DHEA	Dehydroepiandrosterone
DHEAS	Dehydroepiandrosterone sulphate
FFA	Free fatty acid
GC	Glucocorticoids
GH	Growth hormone
G6Pase	Glucose-6-phosphatase
HDL	High density lipoprotein
HPA	Hypothalamic-pituitary-adrenal
11ß-HSD	11ß-hydroxysteroid dehydrogenase
IL	Interleukin
ISI	Insulin sensitivity index
IRS-1	Insulin receptor substrate-1
LDL	Low density lipoprotein
LHI	Lipid/heparin infusion
NFκB	Nuclear Factor κB
ß-OHB	ß-hydroxybutyrate
PAI-1	Plasminogen activator inhibitor-1
PCO	Polycystic ovary syndrome
PPAR	Peroxisome proliferator-activated receptor
PEPCK	Phosphoenolpyruvate carboxykinase
SHI	Saline/heparin infusion
TGF-ß	Transforming growth factor ß
THE	Tetrahydrocortisone
THF	5ß-tetrahydrocortisol
αTHF	5α-tetrahydrocortisol

TNFα	Tumor necrosis factor α
TZD	Thiazolidinediones
UFF	Urinary free cortisol
UFE	Urinary free cortisone

1. Introduction

1.1. The burden of obesity

The increased prevalence of obesity and its co-morbidities results predominantly from multifactorial changes in lifestyle (1). In that context, low physical activity and hyperalimentation especially with energy-dense food are the two major environmental factors currently leading to the epidemic of obesity. Specifically, social and environmental circuits have a fundamental impact on the obesity risk, although a genetic susceptibility of this phenomenon is also well known.

Obesity is regarded as one of the main factors causing metabolic disorders like metabolic syndrome, insulin resistance and type 2 diabetes. The impact of obesity on insulin resistance, type 2 diabetes and cardiovascular diseases was demonstrated in several epidemiological trials (2-5). The growing incidence of obesity over the last decades is alarming (6) given that overweight and obesity increase mortality by its numerous co-morbidities. Consequently a substantial increase in the number of individuals diagnosed with diabetes worldwide was observed in the last years (1).

Apart from the increased risk of type 2 diabetes numerous further disorders are associated with obesity (2). Thus hypertension is up to three times more frequently observed in obese subjects (7). The obesity associated lipid phenotype with increased levels of triacylglycerols and low density lipoprotein (LDL), with particularly a high portion of small close LDL particles, as well as decreased high density lipoprotein (HDL) levels is characterized by a huge atherogenic potential (8).

In summary obesity is a substantial health problem worldwide. However despite more than millions of patients affected worldwide, the link between obesity and type 2 diabetes is not yet completely understood.

1.2. The impact of adipose tissue as hormonal active organ

Insulin resistance, which leads together with impaired beta-cell function to the onset of type 2 diabetes (9), is a common feature of obesity (10). Even if it is not completely understood, how obesity is linked to insulin resistance, several mechanisms have been suggested. Adipose tissue has been acknowledged to be an active participant in energy homeostasis and other physiological functions rather than simply being a fat storage organ. It is recognized as a highly active secretory organ secreting a large number of hormonally active factors (11). These factors mediate several effects on whole body metabolism. Thus, adipocyte-derived factors have been shown to be involved in the regulation of inflammatory processes, arterial blood pressure and fibrinolysis. In that context adipose tissue is well known to express and secrete a variety of novel adipocytokines and metabolic products which have been implicated in the development of insulin resistance and atherosclerosis (12,13). Dysregulation of adipocytokine production has been shown to cause the development of specific features of the metabolic syndrome and vice-versa normalization or elevation of plasma concentrations of some adipocytokines reversed characteristics of the metabolic syndrome (14,15).

A remarkably high portion of these secretion products belong to the class of cytokines such as tumor necrosis factor α (TNFα), plasminogen activator inhibitor-1 (PAI-1), interleukin-1 (IL-1), IL-4, IL-6, IL-8, IL-10 and transforming growth factor ß (TGF-ß). Some of these cytokines are also involved in the impairment of local and systemic insulin sensitivity. In that context TNFα is a potent inhibitor of the insulin signaling (16) whereas IL-6 blunted insulin's ability to suppress hepatic glucose production as well as reduced insulin-stimulated glucose uptake in skeletal muscle (17). IL-6 also appears to suppress the expression of the insulin sensitizing cytokine adiponectin in adipose tissue (18). Adiponectin inhibits hepatic glucose output and suppresses the inflammatory Nuclear Factor κ B (NFκB) pathway (19). In addition to adiponectin, several other adipokines like leptin and resistin originate predominantly from the adipocytes within the adipose tissue. These adipokines play a crucial role in the regulation of food intake (20) as well as glucose and lipid metabolism (21-23).

1.3. Metabolic effects of FFAs

Although it is well known, that these cytokines may link obesity and insulin resistance, it has become increasingly clear that metabolites such as free fatty acids (FFAs) may also play a crucial role with respect to insulin signaling (24). FFAs are released from adipose tissue and are increased in states of obesity (25,26) and diabetes mellitus (27). Especially abdominal adipose tissue secretes a high amount of FFAs due to the higher lipolytic activity compared to other fat depots. Elevation of FFAs induces peripheral and hepatic insulin resistance (28-31). The effect on hepatic insulin resistance is mediated at least in part via an increased hepatic gluconeogenesis (28,31-34) as phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) are up-regulated by FFA (31,35-37). Peripheral effects of FFAs are induced by an increase of intramyocellular lipid content and inhibition of glucose uptake in skeletal muscle (38). FFA-based metabolites like diacyl glycerol (DAG) activate protein kinase C, which has been shown to inhibit the insulin action via phosphorylation of the insulin receptor and of the insulin receptor substrate-1 (IRS-1) (24). FFAs also inhibit the insulin mediated stimulation of glycogen synthase activity in skeletal muscle (28) leading to a decreased glycogen synthesis. Physiologically the increase of hepatic and peripheral insulin resistance is partially neutralized by FFAs mediated elevation of insulin secretion (29,33,38-46). Taken together the metabolic effects of FFAs are investigated in numerous studies.

Notably, various data support that metabolic parameters themselves modulate the circulating levels of hormones and adipokines (47-49). Thus we aimed to analyze, whether apart from the known metabolic actions, FFAs have additional effects on several circulating hormones, which may play a role in obesity, insulin resistance and type 2 diabetes. Although this manuscript will primarily focus on previously published data of the applicant, some yet unpublished results will also be presented.

2. Impact of FFAs on hyperandrogenemia

2.1. Impact of androgens on insulin resistance and metabolic changes in Polycystic Ovary Syndrome

Polycystic ovary syndrome (PCOS) is one of the most common endocrine disorders in women. PCOS is characterized by hyperandrogenism, chronic anovulation and polycystic ovaries (50). About 40% of women with PCOS have been described to have a metabolic syndrome (51). Insulin resistance, a central feature of the metabolic syndrome, has been suggested to be a major driver of hyperandrogenemia in these subjects (52,53). Thus, androgen levels are positively correlated with hyperinsulinemia (53). In addition, basal and ACTH-stimulated adrenal androgens are elevated in patients with PCOS and type 2 diabetes mellitus compared to PCOS women with normal glucose tolerance and controls (54). Treatment with insulin-sensitizing agents improved androgenic features in obese and non obese PCOS patients (55-58). A potential mechanism was suggested by in vitro studies demonstrating that insulin increases the adrenal sulfotransferase activity, and thereby stimulating dehydroepiandrosterone sulphate (DHEAS) secretion (59). However, although there is no doubt that a considerable amount of data link insulin resistance and androgen production, other mechanisms affecting circulating androgen levels may exist.

2.2. Effects of FFAs on androgens and androgen precursors

Elevated FFAs, which are also associated with insulin resistance, have been described in women with PCOS (60). Interestingly, a cumulating set of data suggests a regulation of androgens by dietary fat (61-63). Black South African men with a customary low-fat diet had lower levels of urinary androgens than North American black or white men on high-fat diets (64). An elevation of testosterone levels was described during a high-fat, low-fiber diet in healthy men (65). Vice versa, a decrease of DHEAS, androstenedione, testosterone and 5α-dihydrotestosterone was demonstrated during a low-fat, high-fiber diet (62). In contrast to long-term effects of a diet, a single complex meal results in an acute post-prandial reduction of FFA levels.

Interestingly, this was associated with reduced testosterone levels, while LH secretion was not modified. These data suggest that changes in fatty acids may also modulate androgens (61). Thus, it is reasonable to speculate whether primarily FFAs itself may induce hyperandrogenemia. Therefore we aimed to analyze the effect of FFAs on androgen levels.

Making thinks more complex, regulation of androgen production may be tissue specific and FFAs might have different effects on adrenal or ovarian androgen production. It is well accepted, that both ovary and adrenal androgens contribute to hyperandrogenemia in PCOS (66). Although the adrenal cortex is the primary source of androgens in women, dehydroepiandrosterone (DHEA), DHEAS and androstenedione are also produced in relevant amounts in the ovaries (67,68). In contrast, in men at least the androgen precursors DHEA, DHEAS and androstenedione are nearly exclusively (if not completely) produced in the adrenal gland (69,70). To exclude potentially opposed effects of FFAs in ovary and adrenal gland and to exclusively investigate the effects of FFAs on the adrenal androgen production, we decided to investigate healthy men in a first study, while women were analysed in a separate second study.

2.2.3. Effects of FFAs on androgens and androgen precursor in men

8 healthy male volunteers were investigated in this randomised controlled cross-over trial analyzing the effects of a 4h-lipid/heparin infusion (LHI) and a 4h-saline/heparin infusion (SHI) on sexual steroid hormones. After 4 hours an euglycemic hyperinsulinemic clamp was performed in 6 of these men to estimate the impairment of insulin sensitivity during LHI.

There was an increase of FFA levels during LHI (Figure 1). This induced an increase of androstenedione, which can be synthesized from 17-hydroxyprogesterone or from DHEA (Figure 2 and 3). 17-hydroxyprogesterone levels were not differently influenced by LHI or SHI, suggesting that androstenedione production was not increased via this pathway.

Figure 1: FFAs during LHI (filled squares) vs. SHI (open squares); *p < 0.005 vs. saline/heparin infusion. Results are expressed as means ± S.E.M..

Figure 2: Androstenedione during LHI (filled squares) vs. SHI (open squares); *p < 0.05 vs. SHI. AUC was 479±33 ng/ml*min during LHI vs. 397±23 ng/ml*min during SHI (p<0.05). Results are expressed as means ± S.E.M..

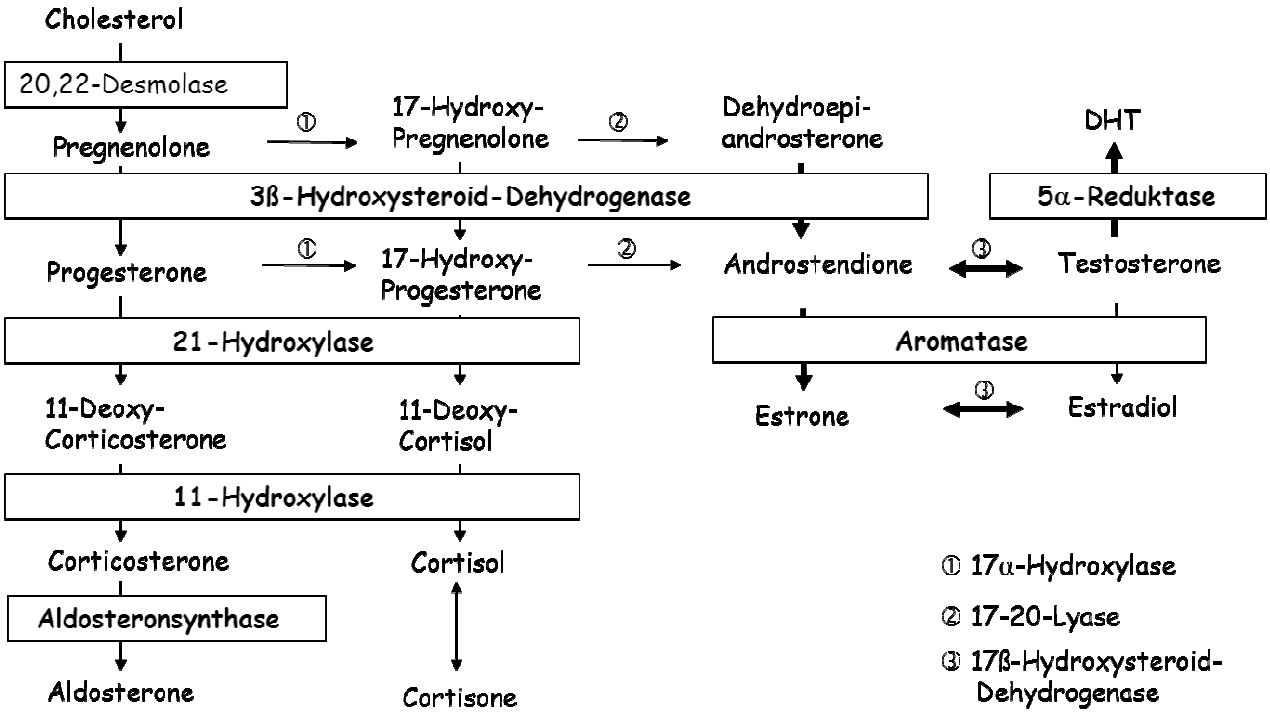

Figure 3: Metabolic pathway of sexual steroid hormones.

We observed increased DHEA levels during lipid infusion, which suggests that elevation of androstenedione is induced by increased levels of its precursor DHEA (Figure 4).

In summary, this was the first study presenting reasonable evidence that elevation of FFAs increases adrenal androgen precursor production in vivo in men (71). Given that PCOS is a female disease, we speculated that comparable mechanisms might modulate androgen levels in women and contribute to the development of PCOS.

Figure 4: 17-hydroxyprogesterone (A) and DHEA (B) during LHI (filled squares) vs. SHI (open squares). $p < 0.05$ for LHI and SHI. AUCs were 619±85 and 4263±461 ng/ml*min during LHI vs. 526±39 and 3459±380 ng/ml*min during SHI for 17-hydroxyprogesterone and DHEA, respectively (p=n.s. and $p<0.05$, respectively). Results are expressed as means ± S.E.M..

2.2.2. Effects of FFAs on androgens and androgen precursor in women

We enrolled 12 healthy young women with regular menstrual cycles and no signs of hirsutism (72) in a randomized controlled trial (73). The LHI and SHI were performed in the early follicular phase of two subsequent menstrual cycles (d 4–6). Briefly, women were 25.5 ± 1.0 years old, BMI was 21.9 ± 0.8 kg/m2, had a WHR of 0.76 ± 0.01 and the body surface area (74) was 1.71 ± 0.04 m^2.

In analogy to the findings in men an elevation of the adrenal androgen precursors DHEA, DHEAS and androstenedione could be detected during LHI, whereas progesterone and 17-hydroxyprogesterone did not differ between LHI and SHI. Androstenedione is known to be the precursor of both testosterone and estrone in women. Indeed, the elevated androstenedione levels during lipid/heparin infusion resulted in an increase of testosterone and DHT. As expected, elevated levels of estrone (AUC: 27074±1819 vs. 12869±1527 pg/ml*min; $p<0.05$) and 17β-estradiol were also detected (Figure 5).

In contrast to the findings in women, estrone but not testosterone was enhanced by LHI in men (71). This is in agreement with the well described sexually dimorphic conversion pattern of DHEA in humans, with predominant conversion into estrogens in men (71,75) and conversion into estrogens and androgens in women (76).

These data strongly suggest that FFAs con modify androgens and androgen precursors in men as well as in women. However, although the here studied intervention is a well described model to investigate a FFA induced effects (77-81), FFAs and triglycerides are both increased by LHI (82-84). Thus, the respective effects are difficult to separate. Given that triglycerides and FFAs are elevated in patients with PCOS, both, a FFA or a triglyceride induced effect would be comparably relevant within the pathogenesis of elevated androgens in those women.

Figure 5: DHEA, DHEAS, androstenedione, testosterone, DHT and 17β-estradiol during LHI (filled squares) vs. SHI (open squares); *p<0.05; **p<0.01; ^{x}p=0.117; $^{\#}$p=0.136 vs. SHI. The AUCs were DHEA: 3631±610 vs. 2341±284 ng/ml*min; p<0.05; DHEAS: 538275±100611 vs. 420300±63471 ng/nl*min; p<0.01; androstenedione: 546±44 ng/ml*min vs. 471±44 ng/ml*min; p<0.05; testosterone: 1030±112 vs. 412±62 nmol/l*min, p<0.005; DHT: 57771±5640 vs. 46636±3974 pg/ml*min, p<0.005; 17β-estradiol: 23505±2840 vs. 11462±2811 pg/ml*min, p<0.05. Results are expressed as means ± S.E.M..

2.2.3. Mechanisms of hyperandrogenemia during LHI

These data imply a novel physiological mechanism linking fat metabolism and regulation of circulating androgens, although the detailed mechanism is not yet clear. Generally elevated circulating androgen levels can result from increased synthesis or reduced excretion of androgens and its precursors. Modification of central control mechanisms might be a relevant aspect. However ACTH levels were not affected by hyperlipidemia in men and women, suggesting no ACTH mediated effect on adrenal androgen secretion (73). In accordance with these findings reduced androgens after a low-fat diet were not mediated by inhibition of ACTH (62).

While FSH and LH were unchanged in men, both gonatropines were reduced in female subjects (85). Actually, the decrease of gonadotrophine levels during LHI may be the result of the elevated estrogen levels rather than a primary lipid induced effect, although the nature of this study does not allow separation of these effects (86,87). Anyway, reduced FSH and LH concentrations would result in lower levels of sexual hormones, thus suggesting that the effects of intravenous LHI on circulating androgens may have been underestimated.

However, the urinary excretion of DHEA, DHEAS and androstenediol were decreased during LHI. Androstenediol represents a direct metabolite of DHEA, which can be considered as a marker of the urinary excretion of the adrenal secreted DHEA. Interestingly, the urinary excretion of androstenedione and its metabolites, which were strongly affected by gonadal androgen secretion (88), was not substantially decreased. This suggests that the effect predominantly exists for adrenal androgen precursors (Figure 6) (85).

Figure 6: Urinary excretion rates of DHEA, DHEAS, androstenediol and all metabolites of DHEA during LHI and SHI. Results are expressed as means ± S.E.M..

The mechanism linking elevated FFAs (and triglycerides) and urinary excretion of androgen precursors is unclear yet. As discussed by Remer (89) DHEA, DHEAS and androstenedione are largely bound to albumin. Therefore interaction of FFAs (and triglycerides) with the binding of circulating androgens to albumin may substantially modulate the free hormone concentration and thereby the metabolic clearance rate of these hormones. Future studies are desirable to investigate this question in more detail.

In addition to the reduced excretion of androgen precursors, changes in the hepatic DHEA sulfotransferase activity may also increase serum DHEA levels. However, the activity of hepatic DHEA sulfotransferase was not affected in the here investigated subjects, as demonstrated by an unchanged DHEAS to DHEA ratio. As the calculated 5α-reductase activity was also not different, we did not found any reasonable evidence for a modification of hepatic androgen metabolism.

Elevation of circulating FFAs is well known to induce insulin resistance and hyperinsulinemia (28,29,45). Thus, FFA induced hyperinsulinemia and insulin resistance and not FFAs itself, might be responsible for the observed changes during LHI. Even if no association between physiological androgen concentration and insulin sensitivity was found in healthy men (90), the findings on insulin sensitivity and androgen production are somewhat controversial. Given the used study design, we were unable to definitely differentiate the effect of hyperinsulinemia / insulin resistance and the effect of FFAs on androgens. However, although an elevation of FFAs is able to induce insulin resistance, this effect usually occurs not earlier than about 210 minutes after lipid infusion (38) (Figure 7).

Figure 7: Peripheral insulin sensitivity (GIR) in healthy men during LHI (filled circles), SHI (open triangles) and during combined LHI (0-2h)/SHI (2-4h) (open circles); *$p < 0.05$ (38).

In our studies elevated androgens and androgen precursors were already found after 1-2 hours of lipid infusion. Therefore any effect of LHI seems to appear before the FFA induced decrease in insulin sensitivity occurred. This suggests an insulin sensitivity-independent effect of the FFAs on adrenal androgen production. In accordance with these findings, the elevation of insulin by LHI was detectable after the elevation of androgen levels. Although this time course also suggests that LHI induced effects on androgens may be independent of a subsequently induced hyperinsulinemia or peripheral insulin resistance, this interpretation should not be overemphasized. There is no doubt that a considerable amount of data suggests that insulin sensitivity directly affects synthesis of androgen precursors. Thus, future studies on this interrelation between hyperlipidemia, insulin sensitivity, and androgens are desirable.

Nevertheless these data present reasonable evidence that LHI induced elevation in FFAs and triglycerides increases adrenal androgen precursors and circulating androgens due to lowering their urinary excretion in vivo in healthy young women. Increased levels of DHEA subsequently result in hyperandrogenemia with elevated levels of testosterone and DHT. This novel mechanism linking fat metabolism and androgens might contribute to the development of hyperandrogenism in women with PCOS.

Clearly it is tempting to speculate that the here described mechanism might be of therapeutic relevance in women with PCOS. However, the implied therapeutic option cannot be directly transferred, on the basis of these results, to the treatment of patients with PCOS. Therefore future intervention studies investigating this question are desirable. However, PCOS might be a heterogenous disorder and given this scenario the here presented mechanism of FFAs and triglycerides induced hyperandrogenemia may be relevant at least in a subcohort of women with PCOS (60).

3. Effects of FFAs on glucocorticoid metabolism

3.1. Metabolic impact of the HPA axis

Hypertension, central obesity, dyslipidaemia, glucose intolerance and insulin resistance are symptoms of the „metabolic syndrome“ (91,92). These features are also typical for Cushing‘s syndrome (93-96), which is characterized by high circulating glucocorticoid (GC) levels. There is considerable evidence that glucocorticoids decrease insulin sensitivity (97-99), suggesting a pathogenic role of cortisol in insulin resistance too. Two underlying mechanisms are currently discussed. First, gluconeogenesis is enhanced by glucocorticoids by transactivation of genes of key enzymes for gluconeogenesis, including PEPCK and G6Pase, leading to increased hepatic glucose output (100-102). Otherwise glucocorticoid excess causes also peripheral insulin resistance (103).

Adrenal GC secretion is regulated by the pituitary hormone ACTH. Free fatty acids (FFAs) are known to modulate pituitary function. An example for such a regulation is growth hormone (GH). GH secretion is reduced by FFAs in men and women (104,105). Given that GH has direct lipolytic effects on adipose tissue (106), this might be a potential negative feedback mechanism on somatotropic function. Comparably a lipolytic function of glucocorticoids is also well established (107). Furthermore several data suggest an increased activity of the hypothalamic-pituitary-adrenal (HPA) axis particularly in central obesity. Both, increased plasma ACTH and cortisol concentrations were observed in response to a corticotropin-releasing hormone (CRH) stimulation in women with abdominal obesity compared to normal-weight controls (108,109). Equally, 24 h urinary cortisol levels were found to be increased in obese women and were associated with visceral adiposity (108,110). Such a higher sensitivity of the HPA axis was associated with estimates of insulin resistance (111).

Human studies have demonstrated hyperlipidemia with enhanced triglyceride and FFA levels particularly in abdominal obesity and diabetes mellitus (112-116), suggesting that hyperlipidemia could link dysregulation of the HPA activity with central obesity or diabetes mellitus (108,115,117,118). Accordingly a hyperactivity of the

hypothalamic-pituitary-adrenal (HPA) axis is associated with an elevation of circulating FFA levels (119,120).

One action of FFAs in rats seems to be at the hypothalamic or pituitary level of the HPA axis (121). In addition, FFA´s, especially long-chain unsaturated FFAs, directly stimulate the steroidogenesis from cultured rat adrenocortical cells (122). Comparably, lipids seem to modulate HPA activity in humans. Kok and co-workers demonstrated a blunted ACTH release in obese women after reduction of circulating FFAs by acipimox treatment (118). Thus, one might assume that FFAs may induce the activation of the HPA axis in the metabolic syndrome. However, FFA lowering treatment with nicotinic acid did not decrease circulating cortisol levels in men compared to controls (123), suggesting a potential gender specific regulation. Such a gender-specific aspect was further supported by a study demonstrating higher cortisol levels after CRH-stimulation in women compared to men (124), suggesting a higher stimulatory sensibility of the HPA axis only in women.

In contrast, studies evaluating the effects of meal composition on the HPA axis showed that oral fat load did not modify the cortisol response to stress in normal subjects (125). Some data even suggest an inhibitory action of FFAs on the adrenocortical steroidogenic response to ACTH stimulation in cell culture (122,126). Lanfranco and co-workers demonstrated decreased ACTH and cortisol levels in young lean female volunteers (127) and in women with anorexia nervosa (128) during lipid infusion compared to saline infusion. A hypothalamic down-regulation of the HPA axis by FFAs was suggested, given that the lipids did not affect the ACTH and cortisol responses to hCRH in those healthy women (127).

Given the controversial data regarding the effect of FFA on HPA axis activity in humans, we aimed to evaluate lipid-induced effects on corticotrope function. We investigated, whether FFAs modify ACTH and cortisol secretion or metabolism in-vivo in both, men and women.

3.2. Effects of FFAs on glucocorticoid metabolism in men and women

We performed two randomized controlled trials in 8 male (129) and 13 female (130) healthy normal weight subjects undergoing LHI or SHI to investigate, whether hyperlipidemia has any effect on ACTH secretion and glucocorticoid metabolism. We included lean subjects in this study to estimate the effect of an isolated hyperlipidemia without further confounding of central obesity associated phenotypes. As the precise mechanism linking hyperlipidemia and the HPA axis is not clear, we aimed to estimate the changes in metabolic glucocorticoid pattern and several steroid generating enzymes, including activities of whole body 11β-hydroxysteroid-dehydrogenase (11β-HSD) type 1 and 2.

In men, the levels of ACTH and cortisol declined during both lipid and saline infusion, which reflects the circadian rhythm in HPA activity. However, there was no difference between saline and lipid infusion (Figure 8 A and B) (129).

In contrast to these findings in men, we demonstrated that a lipid and heparin induced increase of triacylglycerols and FFAs modifies cortisol levels in lean and apparently healthy women. Actually, while ACTH declined during both LHI and SHI, cortisol declined only during SHI. Substantial higher circulating cortisol levels were detectable during LHI (Figure 8 C and D). These data were independent of any change of circulating insulin and were also confirmed by calculating the area under the curve and the free cortisol index (cortisol-to-CBG ratio (131)) (130).

This observation suggests a gender specific effect of FFAs, which is supported by previous findings of Kok and co-workers, who demonstrated, that a drug induced reduction of FFAs is associated with an impaired ACTH secretion in women only (118).

Figure 8: ACTH and Cortisol in men (A and B) and women (C and D) during lipid/heparin infusion (filled squares) vs. saline/heparin infusion (open squares). Results are expressed as means ± S.E.M..

Most interestingly, the high cortisol levels during LHI were correlated with the parallel elevation of circulating FFAs (Figure 9). This was not observed for any other metabolic parameter such as insulin, triglycerides or glucose. We therefore assume that the cortisol changes during LHI were most likely a result of FFA changes rather than by LHI associated modulation of insulin or triglycerides. The fact that cortisol did not correlate to the FFAs during SHI otherwise indicates that alterations of the HPA

activity are particularly important in states of elevated FFAs, such as in individuals with abdominal obesity.

It should be mentioned, that our findings were in some contrast to the inhibitory effect of LHI on ACTH and cortisol secretion in six young lean women, as shown by Lanfranco and colleagues (127). However, Lanfranco and co-workers did add heparin only to the lipid but not to the saline infusion, which makes results difficult to compare. Thus, heparin per se might have an inhibitory effect on adrenal function (132,133), which may have affected the non-controlled findings of Lanfranco and colleagues.

Figure 9: Correlation between the individuals AUCs for FFAs and cortisol levels during LHI.

In general, elevated cortisol levels within the here proposed setting may be either the result of an increased central HPA stimulation, an improved sensitivity of the adrenal glands to central stimulation or a changed cortisol metabolism during LHI. Due to the comparable decline in ACTH during LHI and SHI in both men and women (Figure 8),

an effect on central stimulatory activity of HPA axis seems rather unlikely. We analysed, whether changes of cortisol metabolism might explain the observed effect. Daily urinary excretion rates were determined for the major cortisone metabolites (urinary free cortisone (UFE), tetrahydrocortisone (THE), ß-cortolone and α-cortolone) and the major cortisol metabolites (urinary free cortisol (UFF), α-tetrahydrocortisol (αTHF), tetrahydrocortisol (THF), ß-cortol and α-cortol). Furthermore, the seven quantitatively most important urinary glucocorticoid metabolites (THE, THF, αTHF, α-cortol, ß-cortol, α-cortolone and ß-cortolone) were summed to the major glucocorticoid metabolites (C21) as previously described (134). The findings were not suggestive for any change of cortisol metabolism, as urinary excretion of glucocorticoid metabolites and the 5α-reductase activity were not modified (Table 1) (130). Remarkably, the urinary secretion of all glucocorticoids and glucocorticoid metabolites tended to be decreased by LHI. Although those trends slightly failed statistical significance, we can not entirely exclude, that the increase of serum cortisol was at least in part the result of a decreased urinary glucocorticoid excretion. This would be comparable to our data on the regulation of adrenal androgens (85).

Theoretically, a hyperlipidemia induced increase of 3β-HSD, 11-, 17- or 21-hydroxylase activity might also result in elevated adrenal cortisol generation (Figure 3). Given that the enzyme activities (calculated by urinary excretion of GC metabolites) were not modified during LHI, such mechanism is unlikely to exist (Table 1).

Table 1: Urinary glucocorticoid metabolites during SHI and LHI. Results are expressed as means ± S.E.M..

Given that neither the central stimulation nor peripheral metabolism or urinary excretion of cortisol was substantially modified, our results indicate an increased sensitivity of the adrenal glands to ACTH stimulation during LHI in women. The existence of a primary adrenal hypersensitivity to ACTH has been previously hypothesized by some authors in the context of abdominal obesity and elevated

triacylglycerols and FFAs (110,112-114). Indeed, the cortisol increase after ACTH stimulation was found to be higher in women with abdominal obesity compared to women with predominantly subcutaneous fat depots or lean controls (108,110,135,136). Cortisol levels were normal in individuals with abdominal obesity despite lower ACTH levels (114). These data support a higher sensitivity of the adrenals to ACTH stimulation in addition to the well known central modulation of the HPA activity in abdominal obesity. Our findings support a comparable effect of hyperlipidemia independent of abdominal obesity and suggest that hyperlipidemia may represent at least in part a link between abdominal obesity and increased adrenal sensitivity. The mechanism of a lipid induced sensitization of the adrenal gland to ACTH is completely unclear. Some authors observed an increased response of the adrenal gland to stimulation by ACTH during testosterone treatment in female-to-male transsexual patients (137). Therefore the improved adrenal sensitivity during LHI might be mediated by a hyperlipidemia induced increase in testosterone levels. As already mentioned above, we indeed observed such a lipid induced elevation of testosterone in women, but not in men (71,85).

Taken together, the current data support a gender-specific effect of hyperlipidemia on adrenal ACTH-sensitivity. Given that hyperlipidemia and increased cortisol levels are both associated with abdominal obesity, hyperlipidemia might represent at least in part a link between abdominal obesity and the increased cortisol levels in women.

3.3. Effects of FFAs on 11ß-HSD1

Circulating glucocorticoids do not reflect the concentration of cortisol in the target tissues (e.g. liver, adipose tissue and muscle). The tissue-concentration of cortisol is controlled by two enzymes: 11ß-hydroxysteroid dehydrogenase type 1 (11ß-HSD1) acts in vivo predominantly as an oxo-reductase. It catalyzes the conversion of inactive cortisone to active cortisol (138), thereby significantly regulating intracellular levels of active glucocorticoids. 11ß-hydroxsteroid dehydrogenase type 2 (11ß-HSD2) inactivates cortisol to cortisone in the human kidney, colon and placenta [for review see (139)]. It was hypothesized, that a tissue-specific elevation of cortisol concentrations in target tissues, due to locally increased activity of the 11ß-HSD1 in these tissues, may play a pathogenic role in the metabolic syndrome (139-142). This was supported by Kotelevtsev and Alberts, who demonstrated reduced hepatic transcription of hepatic gluconeogenic enzymes, accompanied by an increased hepatic insulin sensitivity after treatment with a selective 11ß-HSD1-inhibitor (143,144) and in 11ß-HSD1 gene knock-out mice (140).

As already mentioned key enzymes of the gluconeogenesis are up-regulated by increased FFAs (31,35-37). Similarly, enhanced hepatic 11ß-HSD1 activity might increase gluconeogenesis by induction of the gluconeogenic enzymes (102). Studies in 11ß-HSD1 gene knock-out mice or treatment with a selective 11ß-HSD1-inhibitor in models of type 2 diabetes, demonstrated a reduced hepatic transcription of PEPCK and G6Pase mRNA and an improvement in hepatic insulin sensitivity (140,143-145). Concordant results were found in humans treated with carbenoxolone, an unselective inhibitor of both 11ß-HSDs (146). These data imply that FFA induced hepatic insulin resistance in humans could be mediated by local elevated cortisol levels due to increased hepatic or whole body 11ß-HSD1 activity.

Examples for such a local regulation of 11ß-HSD1 are obesity and treatment with thiazolidinediones (TZD). Human studies in obesity, which is often associated with insulin resistance, have shown an increased expression of 11β-HSD1 in subcutaneous

(147-149) and visceral adipose tissue (147). In contrast hepatic 11β-HSD1 activity was found to be decreased in obese patients (142,150).

Figure 10: Hepatic 11ß-HSD1 activity expressed as cortisol/cortisone ratio (A), 11ß-HSD1 expression in skeletal muscle (B) and adipose tissue (C), whole body 11β-HSD1 activity (D) and whole body 11ß-HSD2 activity (E)

before (open symbols) and after (filled symbols) rosiglitazone treatment in 7 male and 9 female subjects with impaired glucose tolerance. Results are expressed as means ± S.E.M..

We demonstrated in 7 male (151) and 9 female (unpublished data) volunteers with impaired glucose tolerance, that an amelioration of total body (HOMA-IR; 3.10±0.48 vs. 2.30±0.48; $p<0.05$) and muscular (ISI_{clamp}; 0.05±0.01 vs. 0.09±0.01; $p<0.001$) insulin sensitivity by the PPARγ agonist rosiglitazone is accompanied by an improvement of 11ß-HSD1 in human skeletal muscle and subcutaneous adipose tissue, while hepatic 11ß-HSD1 is enhanced by rosiglitazone treatment (Figure 10).

This opposed regulation of 11ß-HSD1 in different insulin target tissues suggests a tissue specific effect of rosiglitazone. This might at least in part explain the unchanged whole body 11ß-HSDactivity (Figure 10). Even if this was no placebo-controlled randomized cross-over trial, these data suggest, that 11β-HSD1 may be regulated by activation of PPARγ. Thus some of the insulin sensitizing effects of rosiglitazone may be partially caused by inhibition of the local cortisone-cortisol shuttle 11β-HSD1.

FFAs activate PPARα as well as PPARγ (152). Given the mentioned data concerning the PPARγ agonist rosiglitazone, a possible role of FFAs in the regulation of 11ß-HSD1 in the liver and throughout the whole body was hypothesized. This might eventually explain the observed elevation of cortisol during LHI in women.

The metabolic effects of the LHI in our participants were in agreement with other reports showing that FFAs induce peripheral and hepatic insulin resistance (28-31). However, we did not find any modulation of whole body activities of 11ß-HSDs, neither in men (153) nor in women (130). This was meanwhile partly confirmed by another group (154). Despite the comparable effects on hepatic glucose metabolism, hepatic 11ß-HSD1 was not modulated by LHI in the smaller cohort of 6 men (Table 1 and Figure 11). Accordingly, both short-term and chronic high fat diet, which increases FFAs (155), did not change the hepatic 11ß-HSD1 activity in mice (156). Even if some data suggest an inhibitory (156) or stimulatory effect of FFAs on adipose 11ß-HSD1 (154), an alteration of only adipose but not whole body 11ß-HSD1 is

unlikely to induce a change of circulating cortisol levels, as those are predominantly determined by the sum of the 11ß-HSD1 activities of all human tissues (=whole body 11ß-HSD1 activity). This may be most adequately estimated by the urinary ratio of steroid ring A-reduced metabolites of cortisol (THF and α-THF) and of cortisone (THE) (157,158). We suggest that changes in 11ß-HSD1 are unlikely to be responsible for the elevation of cortisol by FFAs.

Figure 11: Whole body 11β-HSD1 (A) and 11ß-HSD2 activity (B) in n 6 men and 13 women. Hepatic 11ß-HSD1 activity expressed as cortisol/cortisone ratio (C) during lipid/heparin infusion (filled squares) and during saline/heparin infusion (open squares) in 6 men. Results are expressed as means ± S.E.M..

4. Effects of FFAs on Fibroblastic Growth Factor 21

4.1. Metabolic characteristics of Fibroblastic Growth Factor 21

Fibroblastic Growth Factors (FGFs) are known to be involved in the regulation of cell differentiation, cell growth and angiogenesis. Some of these FGFs play a crucial role in bone, liver and adipose tissue metabolism. This includes FGF-19 which regulates energy expenditure, FGF-23 which is involved in phosphate metabolism and FGF-21. FGF-21 is a recently discovered metabolic regulator of fasting metabolism. FGF-21 activates glucose uptake in adipocytes and skeletal muscle cells, protects animals from diet-induced obesity, lowers blood glucose and triglyceride levels, and increases energy expenditure (159-163). Comparably, glucose and triglyceride lowering effects were found in diabetic rhesus monkeys during chronic FGF-21 treatment over a period of 6 weeks (164). Therefore FGF-21 was assumed to be a novel target with potential anti-diabetic properties, which might be useful in the treatment of hyperglycemia, insulin resistance and hyperlipidemia. Furthermore FGF-21 was recently described to be involved in lipolysis (165) and increased hepatic ketone body production (160).

However, human data did not directly support these assumptions, since serum FGF-21 levels were found to be increased in obesity, type 2 diabetes mellitus and metabolic syndrome (166-169). Circulating FGF-21 levels correlated positively with estimates of adiposity but also with several parameters of the metabolic syndrome like fasting insulin and triacylglycerol levels (166). Furthermore increased FGF-21 mRNA expression was found in obese individuals, at least in visceral adipose tissue (168). The effects and regulation of FGF-21 appeared to differ between animal models and humans. A recent study reported a significant increase of FGF-21 levels during prolonged fasting (161). This process was suggested to be PPARα-dependent (160,161) although exact mechanisms of the fasting induced FGF-21 elevations remained unclear. As both obesity and starvation are characterized by elevated FFAs, which activate PPARα, we speculated, that FFAs might regulate FGF-21. This hypothesis is supported by recent data demonstrating that FGF-21 levels are positively associated with FFAs in humans (170). Although the direction of that relation is

unclear in such a cross-sectional study, it offers a potential mechanism linking starvation and obesity to the increased levels of FGF-21.

4.2. Fatty acid dependent regulation of FGF-21 in-vitro

We investigated the effect of several fatty acids on FGF-21 secretion in HepG2 cells in vitro. Incubation of HepG2 cells with a mixture of palmitic, linoleic or oleic acid induced an increase of FGF-21 expression and secretion compared to BSA control (171).

The analysis of the individual fatty acids revealed that this increase was not detectable for palmitic acid, while stimulation with oleic or linoleic acid resulted in a significant elevation of the FGF-21 secretion. Within those experiments linoleic acid had the strongest effect at about 4 to 8 hours (Figure 12 A-D). The dose response experiments using different oleate to BSA ratios suggested an increase in FGF-21 secretion in a dose dependent manner (171).

We also observed an increase of FGF-21 mRNA expression (Figure 13). The maximum of these effects was observed after 2 to 4 hours for FGF-21 expression and after 4 to 8 hours for protein secretion.

Figure 12: Protein levels of FGF-21 in the supernatant of HepG2 cells after stimulation with palmitate (A), oleate (B), linoleate (C) and a FFA mixture (D) for 1, 2, 4, 8 and 24 h. Results are expressed as means ± S.E.M., *$p<0.05$ compared to BSA at the same time point, $p=0.072$ for FGF-21 protein levels during linoleate stimulation at 2 h compared to BSA, $p=0.069$ for FGF-21 protein levels during linoleate stimulation at 8 h compared to BSA, respectively.

Figure 13: Expression of FGF-21 in HepG2 cells after stimulation with linoleate for 1, 2, 4 and 8 h. Results are expressed as means ± S.E.M., *$p<0.05$ compared to BSA at the same time point.

A PPARα-specific siRNA induced knock-down of PPARα (about 60% knock-down of PPARα) abolished the increase in FGF-21 secretion and mRNA expression during stimulation with linoleate and FFA mixture (Figure 14). These data indicate that the FFA-induced effect depends on PPARα stimulation.

Figure 14: (A) FGF-21 mRNA expression in HepG2 cells during linoleate or FFA mixture stimulation after siRNA knockdown of PPARα (compared to control siRNA and linoleate or FFA mixture stimulation (100%)). Results are expressed as means ± S.E.M., *$p<0.05$ compared to expression during control siRNA, respectively. (B) Concentration of FGF-21 in the supernatant of HepG2 cells after siRNA knockdown of PPARα and subsequent stimulation by linoleate or FFA mixture compared to control siRNA and BSA medium. Results are expressed as means ± S.E.M., *$p<0.05$ compared to control siRNA and BSA medium.

4.3. Effects of FFAs on FGF-21 in-vivo

Based on these in-vitro data we performed a randomized controlled trial to explore whether an increase in circulating free fatty acids and triacylglycerols modifies FGF-21 levels in humans.

Comparable to our in-vitro findings a substantial increase of FGF-21 was found during LHI induced increase of FFAs (Figure 15 A). This effect was significant after about 4 hours of hyperlipidemia, a time course being in line with our in vitro experiments demonstrating a maximum of the effects at 2 to 4 hours for FGF-21 expression and 4

to 8 hours for protein secretion. Interestingly, the change of FGF-21 levels was positively correlated to the change of FFA levels (Figure 15 B), even after adjustment for additional confounders. Taken together, our experimental data and the results of this controlled, randomised cross-over human trial strongly characterized free fatty acids as stimulators of FGF-21 secretion in humans, an effect which was mediated via PPARα. Physiologically, such a FFA-induced increase of FGF-21 might contribute to the observed adaption of the organism to starvation or prolonged fasting.

Figure 15: (A) Concentrations of FGF-21 during SHI (open circles) and LHI (filled circles). Results are expressed as means ± S.E.M., *$p<0.05$ (treatment-vs.-time interaction: $p<0.05$.; AUC: 1191±91 vs. 1090±86 ng/ml*min, $p<0.05$). (B) Correlation between changes of FGF-21 and FFAs. The saline group (open circles) and the lipid group (filled circles) were included in the correlation analysis. Correlations after adjustment for sex, age, BMI and change in insulin levels ($r=0.474$, $p<0.005$).

Although our findings were supported by various recent cross-sectional studies describing a positive correlation between FGF-21 levels and parameters of lipid metabolism, specifically FFAs and triacylglycerols (166,167,170), it is noteworthy that FFA levels used in this study were in a marked supra-physiological range. Thus, it was

still unclear, whether physiological levels of FFAs also regulate FGF-21 in humans. We therefore aimed to investigate the effect of physiological enhanced FFA levels on circulating FGF-21 levels in 14 healthy humans in a novel randomized controlled trial. Metabolic changes during LHI and SHI are presented in table 2. As expected, the increase of FFAs and triacylglycerol levels during this modified LHI containing a lower heparin concentration was comparable to a physiological FFA and triacylglycerol elevation as observed during starvation (172,173) and postprandially in obesity (174).

Table 2: Hormonal and metabolic changes during LHI and SHI.

This more physiological elevation of FFAs during LHI also resulted in the characteristic decline of insulin sensitivity compared to SHI (ISI_{Clamp}: 0.14±0.02 vs. 0.10±0.09 $(mg*kg^{-1}*min^{-1})/(mU*L^{-1})$; $p<0.005$). In accordance with our previous findings under supra-physiological FFA levels (171), a significant increase of FGF-21 was also detected during physiologically elevated FFAs (Figure 16), although the relative increase of FGF-21 was smaller. We were not able to confirm our previously described relation between the relative change of FFAs and FGF-21 within this “low FFA” trial. Given that the only difference between those two studies was the range of FFAs, which was clearly higher in the initial study (171), we speculated that a linear

relation between FFAs and FGF-21 may be observed only above a certain threshold of FFAs, while changes below that threshold may have no effect. Indeed after exclusion of the individuals within the lowest quartile of FFAs a strong correlation between changes of FFAs and FGF-21 was found in the remaining individuals ($r=0.608$; $p<0.05$), supporting that a linear relation between FFAs and FGF-21 exists only above a certain threshold, which appears to be biologically plausible.

Figure 16: FGF-21 levels in healthy subjects at baseline and after 3 hours of either LHI or SHI.

In fact the FFA-induced effects on FGF-21 are moderate within the low FFA trial (about 10 to 12 %). Although other studies demonstrated, that FGF-21 levels might be up to 50 % higher in obese compared to lean subjects (166,168), the here observed elevation of FGF-21 levels may still have biological relevance. Comparable moderate differences of FGF-21 levels were found between different stages of insulin resistance (167,170,175). Thus FGF-21 levels were elevated by approximately 13 % in subjects with impaired fasting glucose (175) and 19 to 23 % in diabetics compared to healthy

subjects (167,170). Moreover FGF-21 levels declined by approximately 20% during fenofibrate treatment in hypertriglycerimic subjects (161). Although all those studies demonstrate that moderate differences of circulating FGF-21 exist and may be the consequence of different metabolic phenotypes, those studies and our data cannot finally prove that these difference have biological consequences. This issue clearly requires future studies with detailed dose-response analyses.

Although the here performed intervention is a well established model investigating metabolic effects of FFAs (77-81), FFAs and triacylglycerols are both increased by LHI (82-84) and the respective effects are difficult to dissociate. Recent human data indicate a specific role of FFAs on FGF-21 secretion by showing a positive correlation between both parameters in healthy subjects within cross sectional studies (167). Dostalova and coworkers observed not only reduced FGF-21 levels but also lower FFAs in anorectic women compared to controls (176), while no difference in insulin, triacylglycerols and fasting glucose was detected between anorectic and healthy women in this study. Those data suggest FFAs to be an important regulator of FGF-21 levels, which is supported by our in-vitro data and the correlation between changes in FFAs and FGF-21 found during our experiments. Although FFAs modify FGF-21, we cannot entirely exclude a role of triacylglycerols or other compounds within the applied infusions. In that context we also analysed the effect of lecithin and glycerol on FGF-21 secretion in HepG2 cells, as lipid infusion contains a mixture of different fatty acids, glycerol and lecithin. Compared to controls no significant effect of both substances on FGF-21 was detectable in HepG2 cells.

FFAs are known to activate PPARα as well as PPARγ (152). We aimed to analyse, whether PPARγ is involved in the regulation of FGF-21. Therefore we evaluated the effect of the PPARγ agonist rosiglitazone on FGF-21 in the already mentioned human trial including male and female overweight subjects with impaired glucose tolerance (151,171). Notably recent animal data suggested a regulation of FGF-21 by PPARγ (177). However, no change of FGF-21 levels was detected during treatment with the PPARγ agonist in humans (Figure 17). Although PPARγ agonists may recruit different nuclear co-factors, which may explain different biological effects, our data do not

support (but also not entirely exclude) that the observed effects on FGF-21 levels during LHI were mediated by PPARγ stimulation.

Figure 17: Effect of PPARγ stimulation by rosiglitazone treatment on FGF-21 levels in subjects with impaired glucose tolerance. Results are expressed as means ± S.E.M.

4.4. Effects of insulin on FGF-21

Lipid infusion is usually accompanied by a mild elevation of insulin. The effects of FFAs and insulin on FGF-21 are difficult to separate. Cross sectional studies demonstrated a negative correlation between fasting insulin and FGF-21 in diabetic subjects (167,170), while this association was reported to be positive in obese individuals (166). Given this discrepancy, the relationship between insulin and FGF-21 is unclear. To delineate a potential interaction of lipid- and insulin-induced effects on FGF-21, we analysed the effect of an euglycemic hyperinsulinemia in 17 subjects with impaired glucose tolerance (171). An about 700% increase of insulin levels was

observed during this protocol ($p<0.005$). Under those conditions, a small, but significant increase in FGF-21 levels was observed (Figure 18).

Figure 18: Effect of insulin on FGF-21 levels in subjects with impaired glucose tolerance. Results are expressed as means ± S.E.M.

This indicates that insulin increases circulating FGF-21 (171). However these data were not based on a controlled randomized trial and therefore it was unclear, whether these findings were the direct result of insulin or any other confounders, i.e. circadian effects. Furthermore this protocol induced supraphysiological levels of insulin. Notably, insulin levels did not differ between SHI and LHI within the study using the "low FFA" protocol (Table 2) and no correlation was found between the change of FGF-21 levels and the change of insulin levels within all lipid trials. Even if these findings argue against an indirect effect of LHI (e.g. resulting from hyperinsulinemia), an additional role of insulin on FGF-21 could not be entirely excluded. We therefore performed another randomized controlled trial analyzing FGF-21 levels in patients with type 1 diabetes after withdrawal of their insulin treatment or during ongoing

insulin supply in an attempt to dissect these two potential mechanisms. The metabolic changes during hypoinsulinemia are presented in table 3.

Table 3: Hormonal and metabolic changes during insulin withdrawal and continued subcutaneous (s.c.) insulin infusion in type 1 diabetics.

Notably, a significant increase in FGF-21 was detected during hypoinsulinemia (Figure 19), while no change was detected during continuous insulin infusion. These data argue against a stimulating effect of insulin on FGF-21 under physiologic conditions.

However hypoinsulinemia itself is known to change several other metabolites, including FFAs and ketone bodies. The rise in FFAs seen under hypoinsulinemia was comparable to the increase during the “low FFA” protocol (Table 2). Remarkably, FGF-21 levels were also enhanced to a similar extent during both experiments (about 10 to 12 %). This suggests that the elevation of FFAs (and not insulin or triglycerides) might be responsible for the up-regulation of FGF-21, independent whether the increase of FFAs is induced by LHI with relative hyperinsulinemia or by hypoinsulinemia.

Changes of FFAs failed to be significantly correlated to changes of FGF-21 during insulin withdrawal, which may be due to the complex metabolic changes during fasting under absolute insulin deficiency.

Figure 19: FGF-21 levels in subjects with type 1 diabetes mellitus after insulin withdrawal.

The studies do not exclude that supra-physiological insulin levels, as observed during a hyperinsulinemic clamp, may affect FGF-21 levels, as discussed above and also observed by others (178). Notably, only obese subjects with impaired glucose tolerance were investigated in our study with supra-physiological insulin levels (BMI 32.8±2.2 kg/m^2), whereas the investigated individuals with type 1 diabetes were lean (BMI 24.6 ± 0.6 kg/m^2). Differences in body weight might also account for the observed difference. Such a variable effect of insulin on FGF-21 levels in obese and lean subjects was suggested by Mraz and colleagues (168), who observed unchanged FGF-21 levels during hyperinsulinemic clamp in lean subjects, whereas an insulin induced increase in FGF-21 was found in obese subjects. Even if our lean subjects

were no healthy volunteers, the effect of insulin on FGF-21 might depend on body weight. On the other hand we detected an increase during insulin withdrawal, suggesting that insulin might even suppress FGF-21 levels in lean individuals. Even if those data suggest in summary that the FGF-21 response on insulin may depend on confounders such as existing obesity or existing insulin resistance, our experiments indicate a direct effect of FFAs on FGF-21, which is independent of insulin. As mentioned, this offers a potential mechanism linking regulation of FGF-21 to the switch of metabolism during starvation.

4.5. Effects of weight loss on FGF-21

The function of FGF-21 in lipid metabolism and energy balance was underlined by Potthoff and co-workers, who described the effect of FGF-21 on fatty acid oxidation and on peroxisome proliferator-activated receptor gamma coactivator protein-1alpha, a key regulator of energy homeostasis (179). Transgenic mice overexpressing FGF-21 gained significant less weight under a high fat diet (159) and FGF-21 administration resulted in a slight reduction in body weight as well as an improved metabolic pattern in monkeys (164). Given the existing data, it seems reasonable to hypothesize that FGF-21 might influence body fat stores or vice versa might itself be affected by the degree of obesity, which is closely related to lipid metabolism. Therefore human data regarding the relationship between body weight and FGF-21 would also improve the knowledge of the FFA-FGF-21-interaction. Despite several studies suggesting that FGF-21 may contribute to energy balance and body weight at least in animals, data in humans were basically missing and specifically regarding the relationship of FGF-21 and weight reduction no data existed in humans.

An association of FGF-21 levels and BMI was found in some (166,169,175) but not all (167,170) human studies. Even if such a relation exists, the direction of that relation would be unclear. Thus, circulating FGF-21 might affect body weight, but vice versa, body weight might also affect circulating FGF-21. Given this partially unclear situation in humans, we evaluated the effects of moderate weight reduction by a life-style intervention program on FGF-21 levels. A total of 30 obese individuals (24 female, 6 male) participated in a weight reduction program for 6 months. As expected, the weight loss was accompanied by an improvement of several metabolic and anthropometric parameters including lipid metabolism (Table 4), which was comparable to previous findings (180,181). Despite previous animal and human data, which suggested a strong correlation between BMI and FGF-21, FGF-21 levels were not modified by a moderate weight reduction. This was more recently confirmed in a randomized controlled trial investigating hormonal effects of a more pronounced weight loss induced by different bariatric surgery methods (182).

Table 4: Baseline characteristics of the 30 participants. Results are expressed as mean ± S.E.M.

One study demonstrated a decrease in FGF-21 of 42% during a ketogenic diet which was accompanied by weight loss (183). However the diet used in our intervention was not designed to induce pronounced ketosis, a fact that might also contribute to the differences between ours and those data, as FGF-21 seems to be regulated by ketosis (184). Otherwise the study of Christodoulides was small and therefore type 1 error may have been a problem. Considering the sample size of our study, theoretically a difference of 12 % of FGF-21 levels was detectable. Therefore the 42 % difference of FGF-21 observed by Christodoulides and co-workers should have been detectable, which was not observed. Notably, a trial analyzing the effects of a ketogenic diet in children did not induce changes in FGF-21 levels (161).

Mraz and colleagues observed increased circulating FGF-21 levels after 3 weeks of very low calorie diet (VLCD), an intervention which was also accompanied by a moderate weight loss (168). However VLCD usually leads to a pronounced catabolism which may be responsible for the observed changes in FGF-21. Due to those catabolic conditions the results might not be directly comparable to our data.

In summary current data indicate that circulating FGF-21 levels are unchanged after a moderate weight reduction without periods of excessive negative energy balance. FGF-21 may not be directly regulated by a moderate change in fat mass and body weight and otherwise FGF-21 does not substantially contribute to the endocrine response counterbalancing a reduction of fat mass. In contrast, mechanisms related to the degree of negative energy balance may contribute to the regulation of FGF-21 rather than fat mass per se. Accordingly multiple linear regression analysis suggests, that FGF-21 levels at baseline did not predict weight loss.

Even if FFAs were also reduced by weight reduction, the unchanged FGF-21 levels did not contradict the model, that FFAs regulate FGF-21 levels. A significant correlation was found at baseline to several metabolic and anthropometric parameters, including FFAs. Eventually the slightly decreased FFAs in our subjects do account for the unchanged FGF-21 levels after weight loss. This is supported by the already mentioned fact that an effect of FFAs on FGF-21 may only exist above a certain threshold of FFAs, while changes below that threshold may have no effect.

4.6. Physiological impact of the relationship between FFAs and FGF-21

Recently FGF-21 was characterized as a novel metabolically active protein, which improves glucose uptake, increases energy expenditure and inhibits lipolysis. FGF-21 is predominately secreted by the human liver. The secretion is directly stimulated by FFAs. Especially unsaturated FFAs have a substantial impact on FGF-21 expression and secretion. This effect is mediated by FFA induced activation of the nuclear receptor PPARα, especially in hepatocytes. In addition, circulating FGF-21 levels are responsible for the inhibition of lipolysis (Figure 20). This seems to be mediated by attenuating the hormone-stimulated lipolysis in human adipocytes probably due to reduced expression of perilipin, a phosphoprotein that is thought to recruit several lipases to the surface of the lipid droplets for subsequent triglyceride hydrolysis (165). Physiologically, the FFA-induced increase of FGF-21 might counterbalance a permanent FFA release by inhibition of lipolysis.

Insulin has antilipolytic properties and impaired inhibition of lipolysis is one common feature of insulin resistance. In that context the effects of FFAs on FGF-21 may counter-regulate the impaired inhibition of lipolysis during insulin resistance. This mechanism may partially explain the elevated levels of FGF-21 in obese or diabetic patients and could represent a physiological attempt to diminish lipid induced insulin resistance in obesity and type 2 diabetes. Such an insulin sensitizing effect of FGF-21 has been convincingly demonstrated within several animal experiments.

As current data suggest differential effects of saturated and unsaturated fatty acids, and especially polyunsaturated fatty acids, on glucose metabolism (185), it is also tempting to speculate that some of those effects might by mediated by FGF-21. Various studies suggested that the FFA-induced activation of PPARα depends on the degree of saturation of those FFAs (186). Therefore the different effects on FGF-21 secretion may be caused by different PPARα binding of saturated and unsaturated fatty acids.

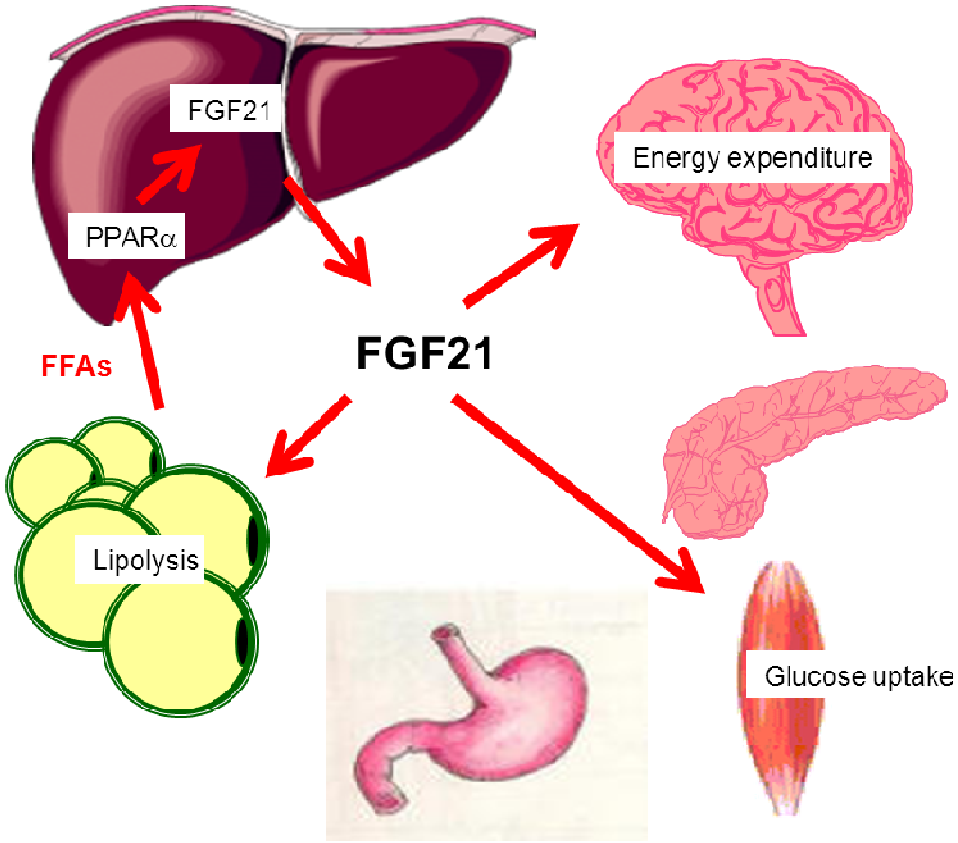

Figure 20: Physiological impact of FFAs and FGF-21

FGF-21 is also thought to induce hepatic ketogenesis (179), even if this was not consistently found in all studies (187). These biological properties suggest that FGF-21 may also contribute to the anabolic switch of the organism under conditions of starvation, a situation also associated with a moderate increase in FFA levels.

Together these mechanisms may cause the induction of FGF-21 seen in the fasting situation, but also in type 2 diabetes and obesity.

5. Summary

The metabolic effects of FFAs on peripheral and hepatic insulin resistance were well demonstrated in numerous studies (28-31). FFAs additionally act as endocrine mediators regulating on the levels of several circulating hormones. Potential mechanisms include different expression, but also modified excretion of affected hormones. These effects might play an important role in obesity, insulin resistance and type 2 diabetes. The work of the applicant focused on the role of FFAs in the regulation of androgens, glucocorticoids and FGF-21.

The effects of FFAs on both adrenal steroids were investigated using randomized controlled lipid infusion trials in men and in women. FFAs were found to regulate androgens and androgen precursors. This effect was induced by a reduced urinary excretion of androgens and was observed predominantly for secreted adrenal androgen precursors. The effect was independent of FFA induced hyperinsulinemia and insulin resistance.

Hyperandrogenemia in women with PCOS is usually thought to result from impaired insulin sensitivity and subsequent hyperinsulinemia. The here presented data indicate a novel physiological mechanism linking fat metabolism and regulation of circulating androgens, which might be relevant in the pathogenesis PCOS in women. This mechanism might have therapeutic relevance in women with PCOS. Antilipolytic nicotinic acid analogues decrease FFAs (188,189) and such drugs might lead to an improvement of hyperandrogenism. However, the implied therapeutic option cannot be directly transferred to the treatment of PCOS. PCOS is a heterogeneous disorder. Thus, the link of FFAs and androgens might be relevant in a subcohort of women with PCOS (60), although future intervention studies investigating this question are required.

The clinical features of the metabolic syndrome are comparable to those observed in patients with hypercortisolism (91-96). Accordingly, several data support an involvement of HPA axis particularly in insulin resistance and other features of the metabolic syndrome. We here investigated the effects of FFAs, which play a crucial

role in several metabolic disorders, on HPA axis. The here presented data support a pathogenic role of FFAs on adrenal ACTH-sensitivity leading to increased cortisol levels. Such a primary adrenal hypersensitivity to ACTH in the context of abdominal obesity and elevated FFAs and triacylglycerols is supported by several studies (110,112-114), even if the specific effect of FFAs was not demonstrated so far. Notably this effect is apparently gender-specific and was observed in women only. The gender-specific regulation of HPA activity is also supported by previous studies (118,123,124). Thus hyperlipidemia with elevated FFAs might represent a link between the increased cortisol levels and abdominal obesity in women. In contrast to these effects on circulating cortisol levels, FFAs did not modulate hepatic 11ß-HSD1 activity, which was found to play a role in obesity and type 2 diabetes (140,142-145,150). FFAs therefore may not act via changes in 11ß-HSD1 activity, even if the results regarding adipose 11ß-HSD1 are conflicting yet (154,156).

FGF-21 was assumed to be a novel target with potential anti-diabetic properties. FGF-21 was also characterized as an important regulator of fasting metabolism. However the exact regulation was not known in humans, as elevated serum FGF-21 levels were detected in obesity, type 2 diabetes mellitus and metabolic syndrome (166-169). We aimed to evaluate the regulation of FGF-21 in humans and investigated the modulation of FGF-21 by FFAs *in- vivo* and *in-vitro*. Free fatty acids increased the expression and secretion of FGF-21 in HepG2 cell cultures, an effect which was found to depend on the degree of saturation of FFAs. This effect was PPARα dependent, as demonstrated in knock-down experiments. This PPARα dependent regulation of FGF-21 is also supported by others (160,161). The *in-vitro* effects were confirmed in human trials. Supraphysiological as well as physiological FFA levels were investigated in two human trials. A randomized insulin withdrawal was performed in subjects with type 1 diabetes mellitus to separate insulin and FFA mediated effects. According to those trials, insulin is unlikely to directly regulate FGF-21.

A human trial using the PPARγ agonist rosiglitazone suggested that any effects of FFAs on FGF-21 do not depend on PPARγ. Physiologically, an FFA-induced increase of FGF-21 might contribute to the adaption of the organism to starvation or prolonged

fasting. Furthermore the stimulation of FGF-21 by FFAs might counter-balance the impaired inhibition of lipolysis during insulin resistance. This mechanism may partially explain the elevated levels of FGF-21 in obese or diabetic patients and could represent a physiological mechanism to diminish lipid induced insulin resistance in obesity and type 2 diabetes.

In summary, the applicant has demonstrated that FFAs regulate several endocrine circuits involved in energy homeostasis, but also glucose and lipid metabolism. Those endocrine systems being modified by FFAs include adrenal steroids, but also proteins such as FGF-21. Other examples such as the reduction of circulating adiponectin levels (190) and the inhibition of growth hormone secretion (104,105) have been demonstrated previously, supporting the complex interplay of FFAs and other hormonal factors in several metabolic states.

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Declaration

Erklärung

§ 4 Abs. 3 (k) der HabOMed der Charite

Hiermit erkläre ich, daß

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