Physiological modulation of circulating FGF21: relevance of free fatty acids and insulin

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Mai K, Bobbert T, Groth C, Assmann A, Meinus S, Kraatz J, Andres J, Arafat AM, Pfeiffer AF, Möhlig M, Spranger J. Physiological modulation of circulating FGF21: relevance of free fatty acids and insulin. Am J Physiol Endocrinol Metab 299: E126–E130, 2010. First published April 27, 2010; doi:10.1152/ajpendo.00020.2010.—Fibroblast growth factor 21 (FGF-21), a novel metabolic factor in obesity and fasting metabolism, has been shown to be regulated by supraphysiological levels of free fatty acids (FFAs) under hyperinsulinemic conditions. Interestingly, it is still unclear whether the observed effects of FFAs on FGF-21 are relevant under physiological conditions, and the relative functions of FFAs and insulin within this context also need to be determined. Fourteen healthy men were studied in a randomized controlled crossover trial (RCT) using lipid heparin infusion (LHI) at a dose inducing physiological elevations of FFAs vs. saline heparin infusion. In a second randomized controlled trial, FGF-21 was analyzed in 14 patients with type 1 diabetes (6 men, 8 women) during continuous insulin supply vs. discontinued insulin infusion and subsequently increased lipolysis and ketosis. Circulating FGF-21 increased during physiologically elevated FFAs induced by LHI, which was accompanied by mild hyperinsulinemia. Interestingly, a mild elevation of FFAs resulting from complete insulin deficiency also increased FGF-21 levels. These results from two independent human RCTs suggest that FFAs increase circulating FGF-21, while insulin is only of minor importance under physiological conditions. This mechanism might explain the apparent paradox of increased FGF-21 levels in obesity, insulin resistance, and starvation.

free fatty acid; ketosis; insulin; fibroblast growth factor 21

FIBROBLAST GROWTH FACTOR 21 (FGF-21) was recently described as a novel metabolic regulator of lipid and glucose metabolism. Several animal and in vitro studies indicated that FGF-21 improves glucose uptake in adipocytes and lowers blood glucose and triacylglycerol levels. Transgenic mice overexpressing FGF-21 were protected from diet-induced obesity (7, 11–13). FGF-21 also stimulated lipolysis in adipose tissue (11) and might be required for activation of hepatic lipid oxidation and ketogenesis during fasting (2). However, a recent report suggested that FGF-21 may not be essential for hepatic triacylglycerol clearance and ketogenesis (10).

The effects and regulation of FGF-21 in humans remain to be elucidated. Existing human data suggest that circulating FGF-21 levels are increased in obesity (21). Prolonged fasting over 7 days also led to an FGF-21 increase in a peroxisome proliferator-activator receptor α (PPARα)-dependent fashion. We therefore investigated the effect of a lipid infusion that recapitulates a physiological rise in FFAs comparable to levels during fasting or obesity (5, 8) on circulating FGF-21 levels in humans. In a novel randomized controlled trial. Additionally, lipid infusions are accompanied by a mild elevation of insulin. Given that a slight increase of FGF-21 was also seen during a pronounced, supraphysiological hyperinsulinemia (17), the effects of FFAs and insulin on FGF-21 are difficult to separate yet. We therefore performed another randomized controlled trial measuring 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 possible mechanisms.

RESEARCH DESIGN AND METHODS

Setting and Participants

Lipid infusion trial. Fourteen healthy men were studied using lipid heparin infusion (LHI) or saline heparin infusion (SHI). Metabolic and anthropometric parameters of these 14 men are presented in Table 1.

An oral glucose tolerance test was performed with a load of 75 g glucose to exclude any alterations in glucose metabolism. Therefore, blood glucose levels were measured at baseline and after 2 h. None of the subjects had any clinical or laboratory evidence of any disease of the cardiovascular system, carbohydrate metabolism, or inflammation. None of the participants had taken any medication for at least 6 mo before the study. All participants were initially screened for any systemic disease or biochemical evidence of impaired hepatic or renal function. Subjects with a history of hypertension, type 2 diabetes, renal or liver disease, dyslipidemia, heart failure, or a family history of diabetes or any other endocrine disorder were excluded from this study. Body weights were stable for at least 2 mo before the study. Informed written consent was obtained from each subject.

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In this study, 14 persons with type 1 diabetes and continuous subcutaneous insulin infusion were included. All volunteers had their insulin pump for at least 5 yr and did not have any major events of hyper- or hypoglycemia during the last 6 mo. Metabolic and anthropometric parameters of these 14 subjects (6 men, 8 women) are presented in Table 1. The experimental protocol of the study was approved by the Institutional Review Board, and all subjects gave written informed consent.

**Design Overview**

**Lipid trial.** Eligible participants were randomly assigned to receive either LHI or SHI in a cross-over design. To avoid interactions between the study procedures, the study was performed at intervals of at least 7 days. Following a 10-h overnight fast, a short polyethylene catheter was inserted in an antecubital vein for infusion of test substances at 0800. Another catheter was placed in the contralateral forearm vein for blood sampling. Within this trial, either a 0.9% saline infusion containing heparin (0.0012 IE·kg\(^{-1}\)·min\(^{-1}\)) or 20% Lipovenös ( Fresenius Kabi, Bad Homburg, Germany; contents in 1,000 ml: 200 g soybean oil, 25. g glycerol, 12.0 g egg phospholipids, and 0.3 g oleate) plus heparin (0.0012 IE·kg\(^{-1}\)·min\(^{-1}\)) (LHI) was infused at a constant rate of 1.5 ml/min for 180 min. Subsequently, a euglycemic-hyperinsulinemic clamp was performed according to the method of De Fronzo et al. (6). The LHI or SHI was performed up to 180 min after the start of LHI or SHI, before insulin withdrawal, and when urinary ketone bodies were positive (grade 2 or higher). Comparable blood samples were collected during continuous insulin infusion and evaluated at baseline and at a time point comparable to the time of ketosis after insulin withdrawal (fasting ketosis). The blood pressure, capillary blood glucose, and urine tests for ketone bodies as well as subjective parameters of well being were evaluated every 30 min to avoid a full ketotic hyperglycemic state. After detection of ketosis (grade 2 or higher), an insulin bolus calculated from individual blood glucose levels was given subcutaneously, and the continuous insulin infusion was restarted using an insulin pump. Blood glucose and blood ketone levels were subsequently evaluated every 30 min until normal levels were achieved.

**Insulin withdrawal trial.** In this study, 14 persons with type 1 diabetes and continuous subcutaneous insulin infusion were included. All volunteers had their insulin pump for at least 5 yr and did not have any major events of hyper- or hypoglycemia during the last 6 mo. Metabolic and anthropometric parameters of these 14 subjects (6 men, 8 women) are presented in Table 1. The experimental protocol of the study was approved by the Institutional Review Board, and all subjects gave written informed consent.

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**Measurement and laboratory parameters.** Capillary blood glucose was measured by the glucose oxidase method (Dr. Müller Gerätebau; Super GL, Freital, Germany), and urine ketone bodies were determined by commercial test strips (Medi-Test, Macherey-Nagel, Germany). Blood ketone bodies (β-OHB) were also measured by a commercial device (Precision Xceed; Abbott/Medisense Laboratories). Oral glucose tolerance test was performed as described previously (18). After being sampled in ETDA or serum tubes, blood was immediately chilled on ice and centrifuged, and aliquots were immediately frozen at −80°C until assayed. Metabolic parameters, including insulin, FFAs, total cholesterol, low density lipoprotein and high density lipoprotein cholesterol, and triacylglycerols were determined as described previously (17). Serum glucose was detected by the hexokinase method using ABX Pentra 400 (ABX Diagnostics, Montpellier, France).

**Statistical Analysis**

All statistical procedures were performed using SPSS version 15.0 (SPSS, Chicago, IL). The individual relative change of FGF-21 and FFAs between baseline and LHI or SHI, respectively, was calculated in percent. In analogy, the relative change of FGF-21 and FFAs was calculated during insulin withdrawal and continuous insulin infusion. Data were compared by paired Student’s t-test for normally distributed data and Wilcoxon test for skewed data. Results were considered to be significant if the two-sided α was below 0.05. Data are presented as means ± SE unless otherwise stated.
RESULTS

Lipid Infusion Trial

Metabolic parameters are presented in Tables 1 and 2. No baseline differences in FFA levels, glucose, insulin, triacylglycerol, and HOMA-IR levels were observed before LHI and SHI (P = not significant (NS)) (Table 2). As expected, insulin sensitivity declined after LHI compared with SHI infusion (M value: 6.14 ± 0.59 vs. 3.99 ± 0.43 mg·kg⁻¹·min⁻¹; P < 0.005; ISIclamp: 0.14 ± 0.02 vs. 0.10 ± 0.09 (mg·kg⁻¹·min⁻¹)/(mU/l) P < 0.005). Both FFA and triacylglycerol levels increased during LHI (P < 0.001), and, most importantly, the magnitude of change was comparable to physiological FFA and triacylglycerol elevation as observed during starvation (8, 14) and postprandial in obesity (5). Furthermore, a moderate decrease in triacylglycerol was observed during SHI (P < 0.01), whereas the FFA levels did not change during SHI. This resulted in significantly higher FFA and triacylglycerol levels during LHI compared with SHI (P < 0.001). Glucose levels remained unchanged during both LHI and SHI. No change of insulin levels was observed during LHI. Although insulin levels moderately declined during SHI, insulin levels remained comparable between SHI and LHI at all times (Table 2). FGF-21 levels were not different between SHI and LHI at baseline (P = NS). Although no changes of FGF-21 levels were detected during SHI (P = NS), an increase in FGF-21 levels was observed during LHI (P < 0.05). Accordingly, FGF-21 levels were significantly higher during LHI compared with SHI (P < 0.05) (Fig. 1). Interestingly, no correlation between the relative change of FFAs and the relative change of FGF-21 was observed in the crude analysis. Given our previous data from supraphysiological elevation of FFAs (17), which suggested a linear relationship between FFAs and FGF-21, we hypothesized that such a relation may exist only above a certain threshold of FFAs. Therefore, we excluded all individuals within the lowest quartile of FFAs. Indeed, a strong correlation between the relative changes of FFAs and FGF-21 was found in the remaining individuals (r= 0.608; P < 0.05).

Insulin Withdrawal Trial

After discontinuing subcutaneous insulin infusion, blood glucose levels increased significantly as expected, leading to higher glucose levels compared with the continuous insulin infusion day (P < 0.001). This was accompanied by a significant increase of β-OHB levels compared with baseline on the day of insulin withdrawal (P < 0.001), whereas β-OHB values increased only slightly during the continuous insulin infusion (P < 0.05 vs. baseline). Thus we detected substantially lower β-OHB values during continuous insulin infusion compared with the expected ketosis during the insulin withdrawal (P < 0.001). FFA levels increased during insulin withdrawal (P < 0.001), whereas no change was observed during continuous insulin infusion, resulting in significantly higher FFA values under conditions of insulin withdrawal (P < 0.001).

Although no change of FGF-21 levels was detected during continuous insulin withdrawal (0.46 ± 0.03 vs. 0.47 ± 0.04 ng/ml; P = NS), an increase in FGF-21 levels was found during insulin withdrawal (0.40 ± 0.03 vs. 0.44 ± 0.03 ng/ml; P < 0.05). Details are presented in Table 3 and Fig. 2. During insulin withdrawal, the relative changes of FFAs failed to be significantly correlated to the relative changes of FGF-21.

DISCUSSION

FGF-21 was recently described to be involved in several metabolic processes, including glucose uptake (12), lipolysis (1), and increased hepatic ketone body production (11). Recent in vitro and in vivo data suggested that FGF-21 expression and secretion is regulated by FFAs in a PPARα-dependent fashion (17). This mechanism may at least in part explain the elevated levels of FGF-21 in obese or diabetic patients and may also contribute to the anabolic switch of metabolism during starvation. Fasting and postprandial FFA levels usually range from 0.5 to 1.6 mmol/l in starvation and obesity (5, 8). In the aforementioned study, we used supraphysiological FFA levels to stimulate FGF-21 expression. Thus it was still unclear whether physiologically elevated FFAs can also regulate FGF-21 in humans. In addition, the role of hyperinsulinemia, which occurs when FFAs are raised, was unclear. We therefore performed two additional randomized controlled trials, one addressing the question of a

Table 2. Hormonal and metabolic changes during LHI and SHI

<table>
<thead>
<tr>
<th></th>
<th>LHI</th>
<th>SHI</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>180 min</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>0.55 ± 0.07</td>
<td>1.49 ± 0.13*</td>
</tr>
<tr>
<td>Triacylglycerols, mmol/l</td>
<td>1.03 ± 0.17</td>
<td>8.06 ± 0.93*</td>
</tr>
<tr>
<td>Insulin, mU/l</td>
<td>6.08 ± 0.85</td>
<td>6.39 ± 1.09</td>
</tr>
<tr>
<td>Glucose, mmol/l</td>
<td>4.84 ± 0.19</td>
<td>4.55 ± 0.05</td>
</tr>
<tr>
<td>HOMA-IR</td>
<td>1.33 ± 0.20</td>
<td>ND</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE. LHI, lipid heparin infusion; SHI, saline heparin infusion; FFA, free fatty acid; HOMA-IR, homeostasis model of assessment-insulin resistance. *P < 0.01 vs. baseline; †P < 0.001 vs. baseline; ‡P < 0.001 vs. SHI; ND, not determined.
Table 3. Hormonal and metabolic changes during insulin withdrawal and continued subcutaneous insulin infusion in type 1 diabetic

<table>
<thead>
<tr>
<th>Glucose, mmol/l</th>
<th>Insulin Withdrawal</th>
<th>Ketonosis</th>
<th>Baseline</th>
<th>Ketosis</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.94 ± 0.57</td>
<td>11.15 ± 0.98*</td>
<td>6.79 ± 0.56</td>
<td>6.73 ± 0.48†</td>
<td></td>
</tr>
<tr>
<td>Insulin, mIU/l</td>
<td>0.15 ± 0.08</td>
<td>0.12 ± 0.06</td>
<td>0.18 ± 0.07</td>
<td>0.16 ± 0.06</td>
</tr>
<tr>
<td>β-OHB, mmol/l</td>
<td>0.16 ± 0.06</td>
<td>1.07 ± 0.11*</td>
<td>0.13 ± 0.04</td>
<td>0.26 ± 0.06†‡</td>
</tr>
<tr>
<td>Time, min</td>
<td>0</td>
<td>173.2 ± 15.0</td>
<td>0</td>
<td>173.2 ± 15.0</td>
</tr>
<tr>
<td>C-peptide, ng/ml</td>
<td>0.016 ± 0.007</td>
<td>0.018 ± 0.007</td>
<td>0.044 ± 0.018</td>
<td>0.064 ± 0.027</td>
</tr>
<tr>
<td>FFA, mmol/l</td>
<td>0.64 ± 0.05</td>
<td>1.65 ± 0.15*</td>
<td>0.60 ± 0.05</td>
<td>0.71 ± 0.05†</td>
</tr>
<tr>
<td>Triglycerides, mmol/l</td>
<td>0.84 ± 0.08</td>
<td>0.81 ± 0.07</td>
<td>0.94 ± 0.13</td>
<td>0.91 ± 0.11</td>
</tr>
</tbody>
</table>

Results are expressed as means ± SE. β-OHB, β-hydroxybutyrate. ‡P < 0.05 vs. baseline; *P < 0.001 vs. baseline; and †P < 0.001 vs. ketosis.

possible dose-response relationship between FFAs and FGF-21 and the second study directly investigating the possible role of insulin in the regulation of FGF-21.

The used modified experimental design resulted in a substantial but mild increase of FFAs, which was comparable to physiological FFA elevation as observed during starvation and obesity. In accordance with the findings of our previous study (17), a significant increase in FGF-21 was also detected during those physiologically elevated FFAs. However, the relative increase of FGF-21 was lower than the FGF-21 elevation observed during the high FFA protocol (17). Surprisingly, we were not able to confirm the recently described relation between the relative change of FFAs and FGF-21 in a crude analysis. Given that the only difference between those two studies was the range of FFAs, which was clearly higher in the recently published study (17), 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, supporting that a linear relation between FFAs and FGF-21 exists only above a certain threshold, which appears to be biologically plausible.

However, as expected, the LHI tended to be accompanied by moderate hyperinsulinemia, although the difference slightly failed to be significant in our cohort. Thus potentially independent effects of insulin and FFAs were difficult to separate. Indeed, previous findings suggested that supraphysiological levels of insulin might also increase circulating FGF-21 (17). However, these data were not based on a controlled randomized trial; therefore, it was unclear whether these findings were the direct result of insulin or any other confounders, i.e., circadian effects. We therefore performed a second randomized crossover trial in patients with type 1 diabetes under continuous subcutaneous insulin infusion, and the effects of insulin were analyzed during insulin withdrawal. Notably, a significant increase in FGF-21 was detected during hypoinsulinemia. These data argue against a stimulating effect of insulin on FGF-21 under physiological conditions, which was suggested by previous data of our group (17). However, it should be mentioned that we still cannot exclude that supraphysiological insulin levels during a hyperinsulinemic clamp may affect FGF-21 levels, as recently observed by others and our group (9, 17). Notably, only obese subjects with impaired glucose tolerance were investigated in our previous study [body mass index (BMI) 32.8 ± 2.2 kg/m²], whereas the individuals investigated here with type 1 diabetes were lean (BMI 24.6 ± 0.6 kg/m²). Differences in body weight might therefore also account for the observed difference. Such a variable effect of insulin on FGF-21 levels in obese and lean subjects was recently suggested by Mraz and colleagues (20), 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. Thus, even if our lean subjects were no healthy volunteers, the effect of insulin on FGF-21 levels in obese and lean subjects was recently suggested by Mraz and colleagues (20), 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. Thus, even if our lean subjects were no healthy volunteers, the effect of insulin on FGF-21 levels in obese and lean subjects was recently suggested by Mraz and colleagues (20), 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. Thus, even if our lean subjects were no healthy volunteers, the effect of insulin on FGF-21 levels in obese and lean subjects was recently suggested by Mraz and colleagues (20), 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. Thus, even if our lean subjects were no healthy volunteers, the effect of insulin on FGF-21 levels in obese and lean subjects was recently suggested by Mraz and colleagues (20), 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.

Furthermore, 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 LHI experiments presented here. FGF-21 levels were also enhanced to a similar extent during LHI and hypoinsulinemia (∼10–12%). This suggests that the elevation of FGF-21, independent whether the increase of FFAs is induced by LHI (with relative hyperinsulinemia) or by hypoinsulinemia. However, during insulin withdrawal, changes of FFAs failed to be significantly correlated to changes of FGF-21, which may be because of the complex metabolic changes during fasting under absolute insulin deficiency. Thus the relevance of FFAs with respect to FGF-21 remains somewhat unclear under conditions of absolute insulin deficiency, although the primary hypothesis of that insulin withdrawal study (i.e., that insulin is not required for the FFA-induced
increase of FGF-21) is clearly supported by the presented data. Insulin is known to affect numerous metabolic pathways, and it should be mentioned that other, not yet identified, metabolites may also contribute to the changes observed here in FGF-21 under conditions of absolute insulin deficiency. The two randomized controlled trials presented in this study were performed to analyze the effects of physiological elevations of FFAs and to analyze the role of insulin in that context. Although recent data did not directly support a regulatory effect of ketones on FGF-21 secretion (4), further studies are highly desirable to investigate the role of other potentially relevant metabolites in the regulation of FGF-21.

The FFA-induced effects on FGF-21 are of moderate magnitude. Although other studies demonstrated that FGF-21 levels might be up to 50% higher in obese compared with lean subjects (20, 21), the elevation of FGF-21 levels observed in this study may still have biological relevance. Comparably moderate differences of FGF-21 levels were found between different stages of insulin resistance (3, 15, 16). Thus FGF-21 levels were elevated by ~13% in subjects with impaired fasting glucose (15) and 19–23% in diabetic compared with healthy subjects (3, 16). Moreover, FGF-21 levels declined by ~20% during fenofibrate treatment in hypertriglyceremic subjects (7). Although all of 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.

In summary, the results presented in this study support that FFAs regulate FGF-21 under physiological conditions associated with a moderate increase in FFAs, like obesity, insulin resistance, and starvation. Insulin appears to have no substantial impact on FGF-21 levels and starvation. Insulin appears to have no substantial impact on FFAs under physiological conditions associated with a moderate increase in FFAs, like obesity, insulin resistance, and starvation. Therefore, FFAs, which increase with obesity, may also contribute to the changes observed here in FGF-21 under conditions of absolute insulin deficiency.

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DISCLOSURES
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