FGF21 is an insulin-dependent postprandial hormone in adult humans

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Abstract

Context: Fibroblast growth factor 21 (FGF21) secretion has been shown to respond directly to carbohydrate consumption, with glucose, fructose and sucrose all reported to increase plasma levels of FGF21 in rodents and humans. However, carbohydrate consumption also results in secretion of insulin.

Objective: The aim of this study was to examine the combined and independent effects of hyperglycemia and hyperinsulinemia on total and bioactive FGF21 in the postprandial period in humans, and determine whether this effect is attenuated in conditions of altered insulin secretion and action.

Methods: Circulating glucose, insulin, total and bioactive FGF21 and fibroblast activation protein (FAPα) were measured in adults with and without type 2 diabetes (T2D) following an oral glucose tolerance test (OGTT), and under a series of insulin and glucose clamp conditions and following high fat diet in healthy adults.

Results: Circulating total and bioactive FGF21 levels responded acutely to OGTT, and their ratio was attenuated in T2D patients with reduced postprandial insulin response. The clamp studies revealed that insulin but not glucose accounts for the postprandial rise in FGF21. Finally, there was an attenuated rise in FGF21 in response to a high fat dietary intervention that is known to alter insulin-stimulated substrate utilization in metabolically active tissues.

Conclusions: Insulin rather than glucose *per se* increases total and bioactive FGF21 in the postprandial period in adult humans. Understanding the impact of T2D on bioactive FGF21 will have a significant effect upon the efficacy of therapeutic agents designed to target the FGF21 pathway.

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Keywords: FGF21, FAPα, glucose, insulin, type 2 diabetes, high fat diet

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51 ***Precis***

52 Circulating FGF21 levels respond acutely to insulin but not glucose**.** A high fat diet impairs this

53 response. The postprandial rise in the ratio of bioactive to total FGF21 is attenuated in T2D.

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Introduction

Fibroblast growth factor 21 (FGF21) is a 181 amino acid (aa) hormone with significant potential for the treatment of type 2 diabetes (T2D)[1,2]. The therapeutic actions of FGF21 require binding of its N-terminus to the tyrosine kinase receptor, FGF receptor 1 (FGFR1) and its C-terminus to the co-receptor βklotho (KLB), forming the FGF21 receptor complex (FGFR1-KLB)[3-5]. Circulating FGF21 levels are driven primarily by hepatic production[6,7], while other tissues including skeletal muscle and brown fat are able to contribute in response to specific stimuli[8]. In the obese and T2D states, FGF21 is elevated in the plasma, albeit demonstrating significant inter-individual variation[9-12]. Furthermore, increased circulating concentrations of FGF21 are associated with conditions characterized by increased circulating lipids and abnormal hepatic metabolism[13-15].

Recently, FGF21 secretion has been shown to respond directly to carbohydrate consumption, with glucose, fructose and sucrose all reported to increase plasma levels of FGF21 in rodents and humans[6,16-18]. Studies in rodents suggested increased hepatic expression of carbohydrate responsive-element binding protein (ChREBP) as a mechanism for the increased plasma levels of FGF21 following fructose ingestion[19]. However, the mechanism by which glucose regulates FGF21 levels in the postprandial period in adult humans has not been explored. Interestingly, in subjects diagnosed with metabolic syndrome (MetS), a greater increase in circulating FGF21 was reported when compared to healthy controls following oral glucose consumption. However, as this was accompanied with a greater blood glucose and insulin response in these subjects[14], it is difficult to disentangle whether the induction of FGF21 following a glucose load is a result of the associated hyperglycemia and/or hyperinsulinemia. Furthermore, FGF21 has a relatively short

half-life (1-2h) and circulates in inactive [3-181 aa (16-30%), 5-181 aa (10-25%)] and bioactive [1-171 aa (10-34%)] forms in healthy participants[3,20-22]. The inactive form of FGF21 is generated via proteolytic cleavage of the C-terminus by fibroblast activation protein (FAPα), a serine dipeptidase and member of the S9 family proteases[23]. Numerous studies have examined the impact of T2D on total FGF21 levels[10,24], although it is not clear what effect T2D has on the bioactive form of FGF21. Understanding the impact of T2D on bioactive rather than total FGF21 will have a significant effect upon the efficacy of therapeutic agents designed to target the FGF21 pathway.

Therefore, the aim of the current study was to investigate the combined and independent effects of hyperglycemia and hyperinsulinemia on FGF21 in the postprandial period in adult humans, and to determine whether this effect is attenuated in conditions of altered insulin secretion and action. Furthermore, we assessed the levels of total and bioactive FGF21, in addition to FAPα in these states. Our findings suggest that FGF21 is an insulin-dependent postprandially regulated hormone in adult humans.

Research Design and Methods

Subjects

Diabetes study. Seven control subjects (age 41.9 ± 4.0 yrs, BMI 31.2 ± 1.5 kg/m^2) and 7 patients with T2D (age 48.3 ± 2.3 yrs and BMI 28.5 ± 1.3 kg/m^2) controlling their T2D with diet alone (n = 2) or metformin (n = 5; dose 1300 ± 300 mg/d) participated. Patients were excluded if they were taking anti-hyperglycaemic medication other than metformin or presented with any secondary complications of T2D. Control subjects were excluded at a screening oral glucose tolerance test (OGTT) visit if their fasting blood glucose was >5.6 mmol/l or 2 h blood glucose was >7.0 mmol/l.

Clamp studies. Six healthy, non-obese male individuals [age 23.2 ± 2.4 yrs, BMI 23.9 ± 1.0 kg/m^2] participated in the ***hyperglycemic/hyperinsulinemic studies***, whereas 9 healthy males (age 26.1 ± 2.8 yrs, and BMI 23.4 ± 1.1 kg/m^2) were recruited for the ***high fat study*** (HF).

In all studies, subjects were informed of all procedures and risks associated with the experiments prior to obtaining written informed consent. All procedures were performed according to the Declaration of Helsinki and approved by the University of Nottingham Medical School Ethics Committee (Clamps studies) and the local NHS Research Ethics committee (Diabetes study).

Experimental protocols

Diabetes study. All subjects underwent an OGTT performed after consumption of an isocaloric diet for 72 h. They also refrained from strenuous exercise for 48 h before the visit. Patients with T2D that were using metformin did not take metformin on the morning of the trial. Subjects attended the laboratory after an overnight fast having consumed a standardized meal the evening before,

111 comprising of 55% carbohydrate, 30% fat, and 15% protein. At the start of the 2 h OGTT, subjects
112 consumed 75 g dextrose dissolved in 300 ml water prepared on the morning of the trial.
113 Arterialised blood samples were obtained from a dorsal hand vein of one arm (placed in a hot-air
114 box maintained at 50-55^{O}C) at baseline and every 30 min during the OGTT.

115 *Clamp studies.* On three randomised occasions, 2 weeks apart, after an overnight fast all 6 subjects
116 underwent the following 4 h clamps: ***(i)*** hyperinsulinemic (78 ± 3 mU/l)-hyperglycemic (10.1 ± 0.1
117 mmol/l) clamp (HIHG trial); ***(ii***) euinsulinaemic (7.3 ± 1.1 mU/l)-hyperglycemic (10.4 ± 0.1 mmol/l)
118 clamp (EIHG trial); ***(iii***) hyperinsulinemic (76 ± 2 mU/l)-euglycaemic (4.4 ± 0.1 mmol/l) clamp (HIEG
119 trial). On 2 occasions, infusion of human soluble insulin (Actrapid, Novo, Copenhagen, Denmark)
120 into an antecubital vein on one arm commenced at a rate of 50 mU m^{-2} min^{-1} and continued
121 throughout each clamp, with 20% dextrose infused at a variable rate to maintain blood glucose
122 concentrations at either euglycaemic (HIEG trial) or hyperglycaemic (HIHG trial) levels, respectively.
123 On both occasions, infusion of somatostatin at 500 mg/h (to inhibit endogenous insulin secretion),
124 and replacement infusion of glucagon (0.7 ng kg^{-1} min^{-1}) started 30 min before dextrose infusion.
125 On a third occasion, 20% dextrose was infused at a variable rate to maintain blood glucose
126 concentration at the designated level (EIHG trial). Infusion of somatostatin at 500 mg/h and basal
127 replacement infusions of glucagon (0.7 ng kg^{-1} min^{-1}) and insulin (5 mU m^{-2} min^{-1}) started 30 min
128 before dextrose infusion. On all occasions, arterialised blood samples were obtained from a dorsal
129 vein from the non-dominant hand at baseline and every 5 min for the determination of blood
130 glucose concentrations, and every 60 min for hormone concentrations.

High fat study. All subjects underwent two 7-day trials, at least 2 weeks apart, in a randomised cross-over design. On each occasion, subjects consumed for 6 days either a high fat [(HF) 76.7 ± 0.4% Energy as Fat] or normal diet [(CON) 32.3 ± 0.7% Fat]. On day 7, after an overnight fast, subjects underwent a 4 h hyperinsulinemic (CON: 71.8 ± 3.5 and HF: 70.0 ± 3.5 mU/L)-euglycemic (4.5 ± 0.2 mmol/l) clamp as described above. Arterialised blood samples were obtained at baseline and every 5 min for the determination of blood glucose concentration, and before and after each clamp for the determination of hormone concentrations.

Blood analysis

In all studies, blood glucose concentrations were determined using a Yellow Springs Instrument Analyzer (YSI, 2300 STAT PLUS). Serum were separated by centrifugation (15 min at 3,000 g) and analyzed for insulin concentrations by radioimmunoassay (Diagnostics Products Corporation, Llanberis, Wales, UK), and total FGF21 (Biovendor, Research and Diagnostics products, Czech Republic), bioactive FGF21 (Eagle Biosciences, USA) and FAPα (Abcam, USA) concentrations by enzyme-linked immunoassays (ELISA).

Statistics

All data are expressed as means ± SEM. Data from each study were analyzed via two-way ANOVA and Tukey's post-hoc test. $P < 0.05$ was considered significant.

Results

Baseline blood measurements

T2D subjects had higher fasting blood glucose concentrations when compared to non-diabetic controls (6.7 ± 0.4 vs. 4.4 ± 0.2 mmol/l; $P < 0.01$) but similar fasting insulin levels (12.0 ± 1.0 vs. 13.9 ± 2.3 mU/L) (Table 1). Despite higher fasting levels of FAPα in T2D patients compared with controls (168.4 ± 12.1 vs. 134.3 ± 11.8 ng/ml; $P < 0.05$), there was no difference in fasting levels of total or bioactive FGF21 (Table 1). No correlation was observed between baseline levels of FAPα and FGF21 (bioactive, total or their ratio).

Blood glucose and insulin responses to OGTT

In non-diabetic and T2D subjects, oral administration of a 75 g dextrose solution increased both circulating glucose (Fig 1A) and insulin (Fig 1B). Despite a higher increase in circulating glucose, the effect on insulin secretion was significantly attenuated in the T2D group (Fig 1A and B). To determine whether a postprandial rise of FGF21 occurred in response to OGTT, we measured circulating FGF21 levels (total and bioactive) in both groups.

FGF21 is a postprandial hormone that is impaired in T2D

In contrast to the rapid elevation of circulating glucose and insulin, total FGF21 was not increased ($P < 0.01$) until 120 min (Fig 2A). Notably, this late induction of total FGF21 was mirrored by an increase ($P < 0.01$) in the bioactive form of FGF21 after 90 and 120 min (Fig 2B). There was no effect of dextrose ingestion on circulating FAPα throughout the OGTT (Fig 2D). Taken together, these data indicated that the postprandial rise in total FGF21 following dextrose consumption is

accompanied by a proportional increase in bioactive FGF21 and elevated glucose and insulin levels.

Although there was no significant difference in the postprandial rise in total and bioactive FGF21 between the non-diabetic (control) and T2D groups (Fig 2A and B), the ratio of bioactive to total FGF21 responded differently over time ($P < 0.05$) with a significant increase observed in the control subjects that was impaired in patients with T2D (Fig 2C). In line with the lower ratio of bioactive to total FGF21, circulating FAPα concentrations remained higher ($P < 0.05$) in T2D subjects throughout the OGTT (Fig 2D). Thus, here we report that the normal postprandial rise in the ratio of bioactive to total FGF21 is attenuated in T2D patients and this effect is associated with both reduced postprandial insulin concentrations and increased circulating levels of the protease FAPα. However, based on our OGTT data, we could not distinguish whether the induction of FGF21 following dextrose consumption was a result of increased levels of circulating glucose and/or insulin.

FGF21 is an insulin-dependent postprandial hormone

To determine directly whether glucose and/or insulin account for the postprandial rise in circulating FGF21 following dextrose consumption, we assessed FGF21 in blood from healthy volunteers collected during 4 h of HIHG, HIEG and EIHG clamps. The magnitude of hyperinsulinemia and hyperglycemia achieved in those clamps was within the range of blood glucose and insulin levels typically seen after oral carbohydrate administration. Consistent with the OGTT data in non-diabetic volunteers, there was a clear time-dependent effect of the HIHG clamp on FGF21. Total circulating levels of FGF21 and its bioactive form were increased ($P < 0.01$)

after 3 h of infusion (Fig 3A and D). There was also an increase ($P < 0.01$) in total and bioactive FGF21 following the HIEG clamps (Fig 3B and E). In contrast, there was no effect of the EIHG clamps on total or bioactive FGF21 levels (Fig 3C and F). There was no difference between trials in the bioactive to total FGF21 ratio or circulating FAPα (Fig. 3G-I). Taken together with results collected in non-diabetic and diabetic patients during the OGTT, these data suggest that the rise in total and bioactive FGF21 that occurs following dextrose ingestion is facilitated by the corresponding increase in circulating insulin levels.

Insulin-mediated secretion of FGF21 is impaired following high fat feeding

Short-term HF in humans has been shown to alter inulin-stimulated substrate utilization in metabolically active tissues without inducing peripheral insulin resistance[25,26]. Thus, to investigate whether the rise in circulating FGF21 in response to insulin was altered under such conditions, we analyzed the levels of total and bioactive FGF21, and FAPα, before and after a 4 h hyperinsulinemic-euglycemic clamp in human subjects following a normal or HF for 6 consecutive days. As expected, insulin-stimulated CHO oxidation rate was 20% lower and fat oxidation 60% higher in HF compared with Control, and there was no effect of treatment on peripheral glucose uptake. High fat feeding *per se* (prior to performing the insulin clamps) did not alter fasting levels of either total or bioactive FGF21 (Fig 4A and B) nor FAPα (data not shown). In line with the clamp studies described above, subjects receiving the control diet demonstrated a robust induction of FGF21 in response to insulin ($P < 0.01$). However, following the HF diet, the response to insulin clamp was impaired resulting in lower ($P < 0.05$) total FGF21 levels (Fig 4A) and a tendency ($P =$

210 0.09) for lower bioactive form (Fig. 4B) when compared with the control diet. The ratio of
211 bioactive to total FGF21 was unaffected.

212

Discussion

This study demonstrates, for the first time, that circulating bioactive FGF21 levels respond acutely to changes in insulin *per se*, rather than glycaemia, in the postprandial period. In particular, there were 4 main findings. Firstly, we found that fasting total and bioactive FGF21 levels are similar in non-diabetic and T2D subjects, despite higher fasting levels of FAPα in T2D patients. Secondly, the normal postprandial increase in the ratio of bioactive to total FGF21 is impaired in T2D patients with attenuated insulin response to OGTT when compared to non-diabetic individuals. Thirdly, we demonstrate, in a series of clamp studies that are consistent with the OGTT data, that there is a clear time-dependent effect of the hyperinsulinemic-euglycemic clamp on FGF21 (both total and bioactive). This effect is lost in the euinsulinemic-hyperglycemic clamp. These data suggest the rise in total and bioactive FGF21 that occurs following dextrose ingestion is a consequence of the increase in insulin secretion. Finally, in response to a high fat dietary intervention that is known to alter insulin-stimulated substrate utilization, the response in circulating FGF21 is attenuated. Confirming the mechanism by which insulin regulates secretion of FGF21 and the subsequent tissue specific actions of FGF21 will require further investigation.

Recently, excess dietary carbohydrate had been shown to increase FGF21 secretion in healthy humans[18]. Furthermore, fructose ingestion briefly increased plasma total FGF21 concentration at 2 h, returning to baseline within 5 h[6]. This increase was correlated with elevated levels of circulating glucose and insulin. Baseline levels of total FGF21 have been previously shown to be elevated in subjects with MetS that also exhibit exaggerated glucose and insulin responses to an oral glucose load[24].

234 Studies in rodents have also implicated carbohydrates in FGF21 induction; 12 h sucrose feeding
235 (following a 24 h fast) was shown to induce insulin and FGF21 mRNA in rat liver[27]. Whilst glucose-
236 induced increases in FGF21 gene expression in hepatocytes and mouse liver were mediated via
237 the transcription factor ChREBP (a notion supported by no change in FGF21 content in plasma of
238 ChREBP KO mice in response to various sugars), it was suggested that glucose-stimulated FGF21
239 mRNA expression may require insulin action[28]. In support of this notion, FGF21 expression was
240 upregulated by insulin in a PI3-kinase-dependent manner in cultured C2C12 myocytes and 3T3-
241 L1 adipocytes[29]. Our data demonstrates the importance of insulin, rather than glucose *per se*, in
242 regulating secretion of FGF21 in adult humans. Interestingly, the normal postprandial increase in
243 the ratio of bioactive to total FGF21 is impaired in T2D patients with attenuated insulin response
244 to OGTT when compared to non-diabetic individuals. Whether this is a consequence of reduced
245 insulin levels *per se* or resistance to the action of insulin requires further investigation. Although
246 FGF21 is synthesized in multiple organs and can act on multiple tissues in either a paracrine or
247 endocrine fashion, the major site of FGF21 production is the liver[7]. Therefore, it is possible that
248 hepatic insulin resistance in subjects with MetS, non-alcoholic fatty liver disease or diabetes may
249 play an important role in the regulation of FGF21 by insulin, which may also explain the significant
250 inter-individual variation in its levels in those populations.

251 FAP has an extensive tissue expression profile in addition to circulating in the blood of mice, non-
252 human primates and humans[22,23,30,31]. The importance of FAP's actions were demonstrated *in*
253 *vitro*, where deletion of more than 4 amino acids from the C-terminus of FGF21 significantly
254 attenuates KLB binding affinity, and *in vivo* where the metabolic actions of FGF21 are diminished
255 in the absence of KLB binding[7,16,32-35]. Interestingly, FAP is homologous (48% sequence identity)

to dipeptidyl peptidase-4[36] (DPP4), the therapeutic target of the antidiabetic DPP4 inhibitor[37]. However, while FAPα activity has been shown to increase in liver and plasma from patients with liver disease[23], the impact of MetS on FAPα activity, and hence FGF21 biology, remains largely unknown. Here we demonstrate for the first time that in T2D, levels of FAPα are increased when compared to non-diabetic controls. Interestingly, Talabostat (TB), a known FAP inhibitor, reduced body weight and food intake, increased energy expenditure, and improved glucose tolerance and insulin sensitivity in diet-induced obese mice where total and bioactive plasma FGF21 were observed to be elevated. Interestingly, these effects were attenuated in FGF21 knockout animals[38]. TB was previously pursued as an anti-cancer treatment and was found to be safe to support repeated dosing in human clinical trials[39]. Whilst TB is not selective to FAP, further studies in humans are required to evaluate the use of FAP inhibitors as relevant treatment strategy in T2D patients, particularly as FGF21 improves MetS in humans[1,40].

In summary, we employed a physiological and dietary strategy in human subjects which revealed a stimulatory effect of insulin on FGF21 secretion. We demonstrate that dextrose ingestion acutely and robustly increases total and bioactive FGF21 in humans. The normal postprandial rise in the ratio of bioactive to total FGF21 is impaired in T2D patients that have attenuated insulin response to OGTT. The effect of insulin, rather than glucose *per se*, on FGF21 was confirmed in a series of insulin and glucose clamp experiments, in addition to the HF study, suggesting that FGF21 secretion is regulated by insulin and is therefore a postprandial hormone in adult humans.

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Author Contributions

RJS, JEL, LN, CJG, FBS and KT designed the study, carried out the experiments and researched data (with additional input by TB, DS, CC and JWP). JEL, RJS, FJPE and KT wrote the manuscript. All of the authors reviewed and edited the manuscript.

Conflicts of interest

RJS, ACA, CC, TB, DS and JWPII are employees of Eli Lilly and Company.

Table 1: Anthropometric characteristics and baseline blood biochemistry data

	Control	T2D
Subjects	n = 7	n = 7
Age (years)	41.9 ± 4.0	48.3 ± 2.3
Body mass index (kg/m^2)	31.2 ± 1.5	28.5 ± 1.3
Fasting plasma glucose (mmol/l)	4.4 ± 0.2	6.7 ± 0.4[a]
Fasting plasma insulin (mU/L)	13.9 ± 2.3	12.0 ± 1.0
Fasting total FGF21 (pg/ml)	99.7 ± 16.2	117.0 ± 28.1
Fasting bioactive FGF21 (pg/ml)	68.3 ± 18.5	53.5 ± 23.1
Fasting FAPα (ng/ml)	134.3 ± 11.8	168.4 ± 12.1[#]

Values are means ± SEM obtained in the fasted state; [a]P < 0.01 & [#]P < 0.05 from Con (n = 7)

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Figure legends

Figure 1. Effect of oral administration of dextrose on circulating glucose (A) and insulin (B). Data are means ± SEM; $^{\#}P < 0.05$ from control (n = 7).

Figure 2. Effect of OGTT on circulating total (A) and bioactive (B) FGF21, the ratio of bioactive to total FGF21 (C) and FAPα (D). Data are means ± SEM; *P < 0.05 from 0 min; **P < 0.01 from 0 min; $^{\#}P < 0.05$ from T2D (n = 7).

Figure 3. Effect of hyperinsulinemic–hyperglycemic, hyperinsulinemic-euglycemic and euinsulinemic-hyperglycemic clamps on circulating total (A, B and C) and bioactive FGF21 (D, E and F), and FAPα (G, H and I). Data are means ± SEM; *P < 0.05 from Pre; **P < 0.01 from Pre; (n = 6).

Figure 4. Circulating total (A) and bioactive (B) FGF21 before (Pre Diet) and after (Post Diet) 6 days of either a High Fat or Control diet. The Post Diet values were obtained in the fasting state on Day 7 immediately before a 4 h insulin clamp and were also used as baseline values (Pre clamp) to assess the effect of treatment on insulin-stimulated secretion of total and bioactive FGF21 (Post Clamp data). Data are means ± SEM; **P < 0.01 from Pre; $^{\#}P < 0.05$ from Control (n = 9).

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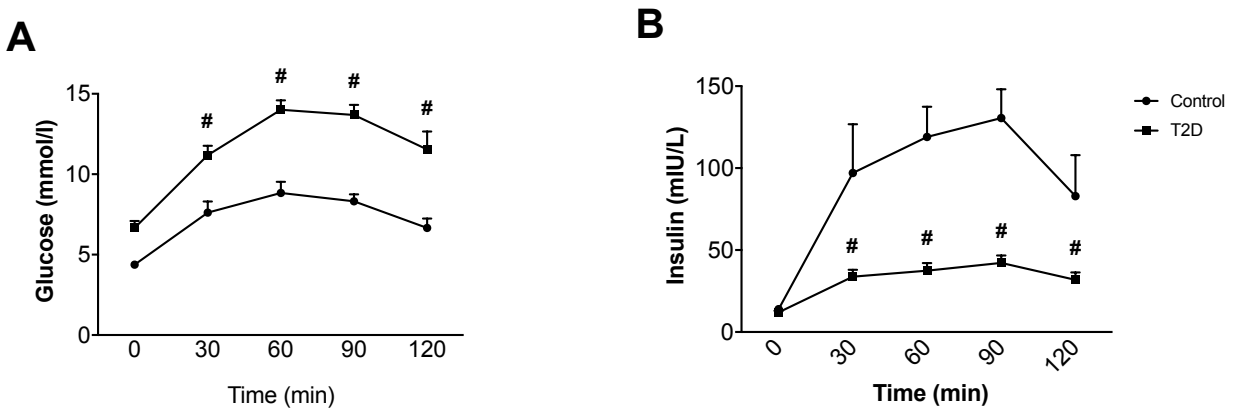
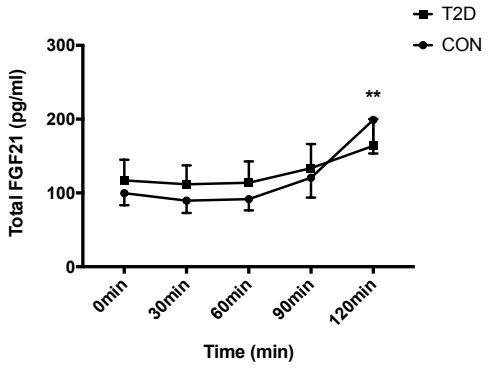
Fig. 1


A
Glucose (mmol/l)
15
10
5
0
0
30
60
90
120
Time (min)
#
#
#
B
Insulin (mIU/L)
150
100
50
0
0
30
60
90
120
Time (min)
#
#
#
#
Control
T2D

421

Fig. 2

A



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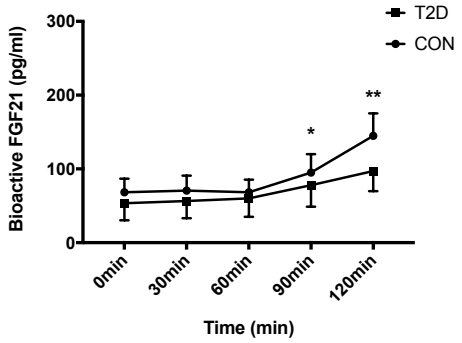

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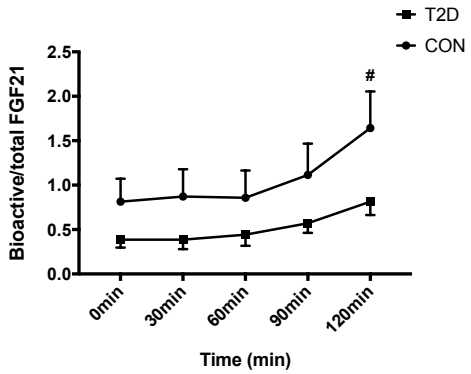
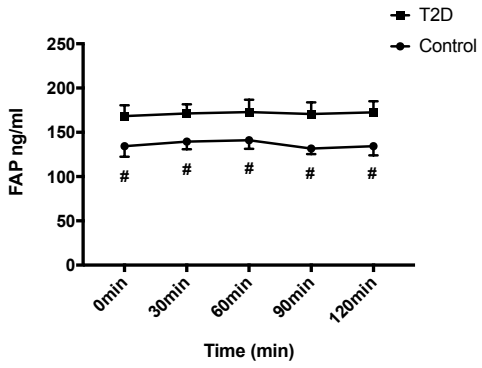
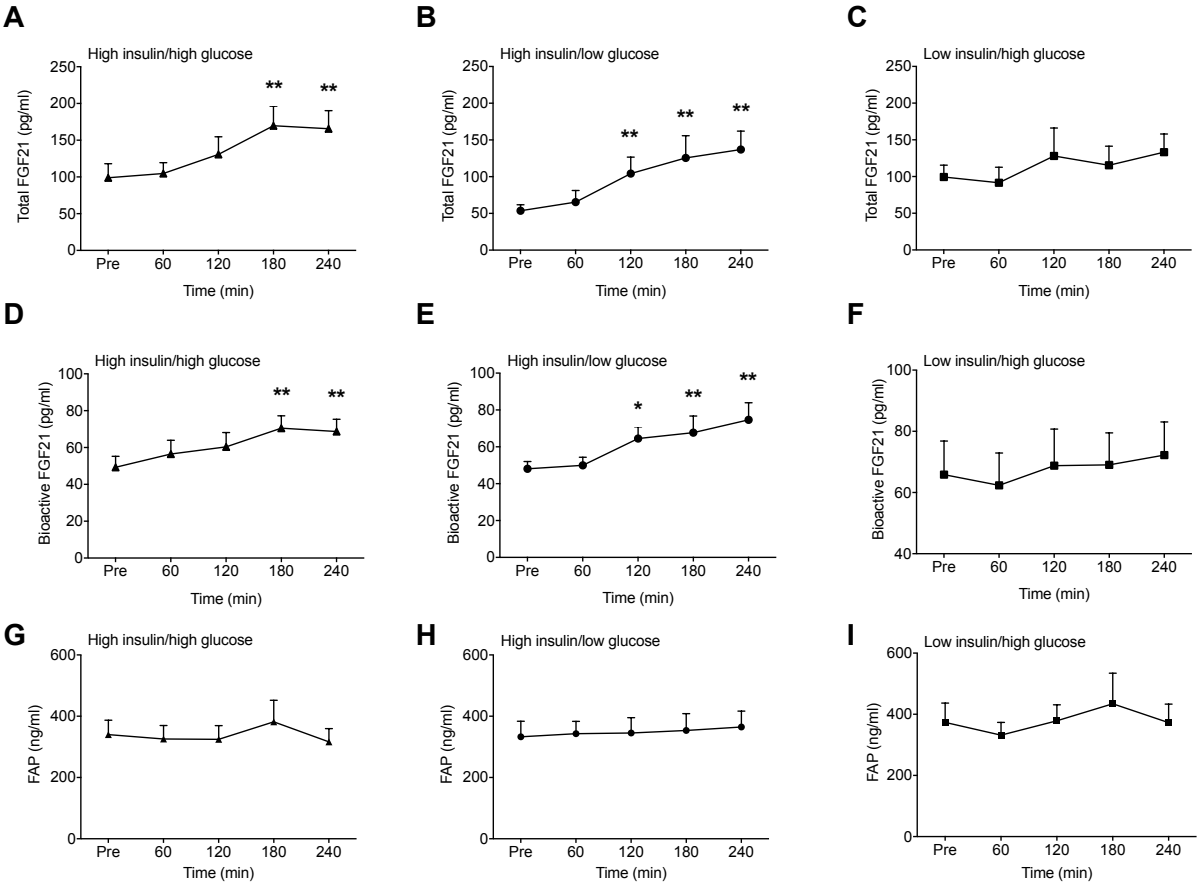


D



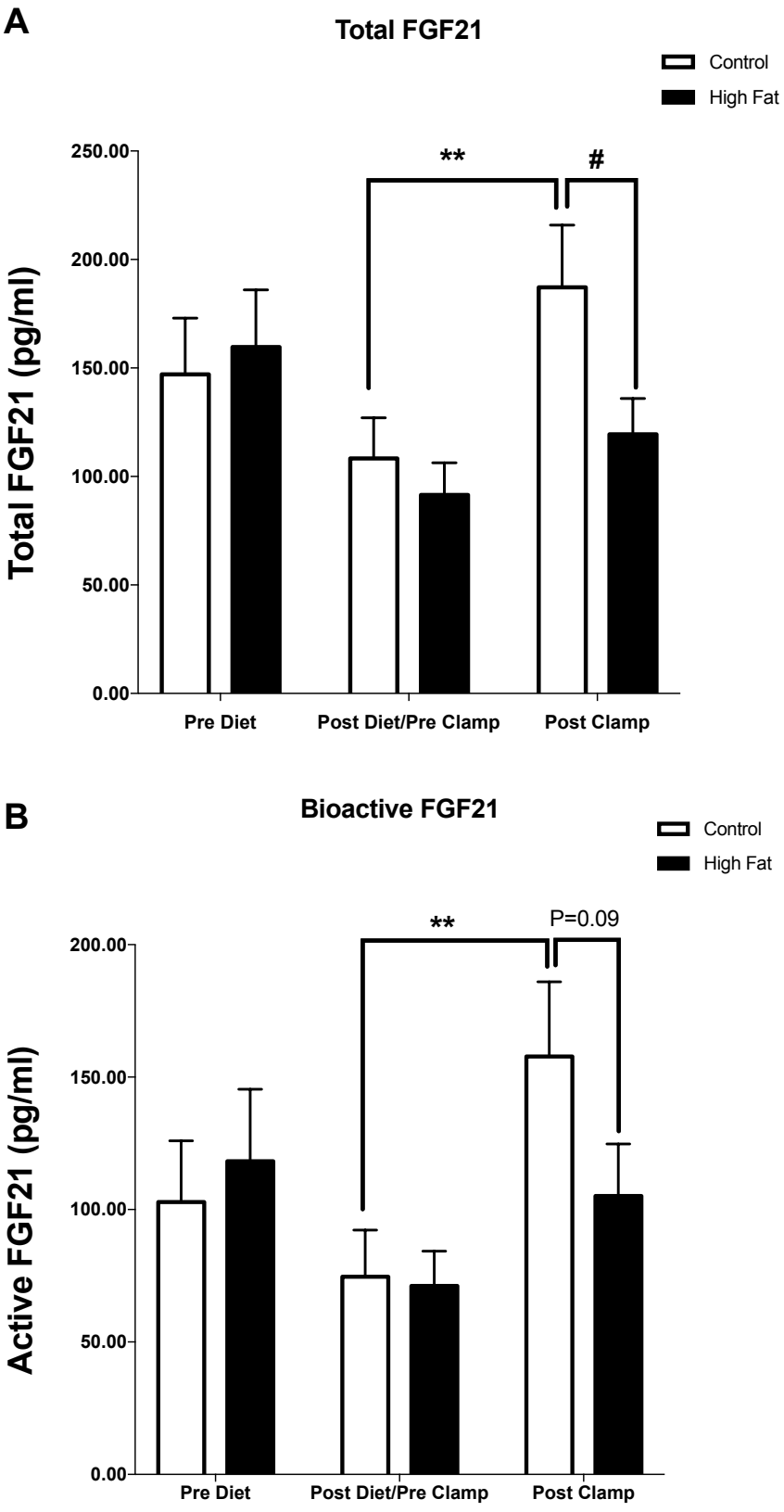
422

Fig 3


A
High insulin/high glucose
Total FGF21 (pg/ml)
Time (min)
B
High insulin/low glucose
Total FGF21 (pg/ml)
Time (min)
C
Low insulin/high glucose
Total FGF21 (pg/ml)
Time (min)
D
High insulin/high glucose
Bioactive FGF21 (pg/ml)
Time (min)
E
High insulin/low glucose
Bioactive FGF21 (pg/ml)
Time (min)
F
Low insulin/high glucose
Bioactive FGF21 (pg/ml)
Time (min)
G
High insulin/high glucose
FAP (ng/ml)
Time (min)
H
High insulin/low glucose
FAP (ng/ml)
Time (min)
I
Low insulin/high glucose
FAP (ng/ml)
Time (min)
Pre
60
120
180
240

423

Fig. 4


A
Total FGF21
Control
High Fat
Total FGF21 (pg/ml)
250.00
200.00
150.00
100.00
50.00
0.00
**
#
Pre Diet
Post Diet/Pre Clamp
Post Clamp
B
Bioactive FGF21
Control
High Fat
Active FGF21 (pg/ml)
200.00
150.00
100.00
50.00
0.00
**
P=0.09
Pre Diet
Post Diet/Pre Clamp
Post Clamp

424