

Original Paper

## Free Fatty Acids Impair FGF21 Action in HepG2 Cells

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### Key Words

FGF21 • Type 2 Diabetes • NAFLD • Free fatty acids

### Abstract

**Background/Aims:** Fibroblast growth factor 21 (FGF21) is a key mediator of glucose and lipid metabolism. However, the beneficial effects of exogenous FGF21 administration are attenuated in obese animals and humans with elevated levels of circulating free fatty acids (FFA). **Methods:** We investigated *in vitro* how FFA impact FGF21 effects on hepatic lipid metabolism. **Results:** In the absence of FFA, FGF21 reduced lipogenesis and increased lipid oxidation in HepG2 cells. Inhibition of lipogenesis was associated with a down regulation of SREBP-1c, FAS and SCD1. The lipid-lowering effect was associated with AMPK and ACC phosphorylation, and up regulation of CPT-1 $\alpha$  expression. Further, FGF21 treatment reduced TNF $\alpha$  gene expression, suggesting a beneficial action of FGF21 on inflammation. In contrast, the addition of FFA abolished the positive effects of FGF21 on lipid metabolism. **Conclusion:** In the absence of FFA, FGF21 improves lipid metabolism in HepG2 cells and reduces the inflammatory cytokine TNF $\alpha$ . However, under high levels of FFA, FGF21 action on lipid metabolism and TNF $\alpha$  gene expression is impaired. Therefore, FFA impair FGF21 action in HepG2 cells potentially through TNF $\alpha$ .

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### Introduction

The prevalence of type 2 diabetes mellitus (T2DM) and obesity is rapidly growing in western countries. T2DM ensues in 20% of obese subjects when insulin resistance develops, which highly increases the risk for other disorders such as nonalcoholic fatty liver disease

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(NAFLD) [1]. Emerging data suggest that dysfunction of the liver is one of the hallmarks of T2DM and obesity [2-5]. Disturbed hepatic lipid metabolism is associated with abnormal elevation of circulating FFA, which is observed in both T2DM and obesity. Indeed, this elevation of FFA levels has been identified as a main alteration related to insulin resistance, which represents a risk to subsequently develop T2DM and obesity [5, 6]. Nonetheless, under physiological conditions, lipid metabolism is under precise molecular regulation in the liver [7, 8]. Fibroblast growth factor 21 (FGF21) has been recently identified as a potent metabolic regulator [9]. FGF21 is a member of the FGF family mainly expressed in the liver and white adipose tissue, but also in the pancreas [10, 11]. It acts through a canonical FGF receptor (FGFR). There are four isoforms of FGFR associated with the cofactor  $\beta$ -klotho. Binding of FGF21 to FGFRs- $\beta$ -klotho complex leads to dimerization and auto phosphorylation of the receptor, which recruits downstream proteins and initiates signal transduction [9, 12].

Pharmacological doses of FGF21 have been shown to improve glucose clearance and insulin sensitivity as well as to reduce plasma and hepatic triglycerides in *ob/ob* mice, *db/db* mice, and wild-type mice fed a high-fat diet [9, 13-15]. Moreover, mice overexpressing FGF21 exhibit similar improved lipid metabolic profile and are protected against diet-induced obesity and insulin resistance [9]. In contrast, mice lacking FGF21 develop obesity, hepatic steatosis and insulin resistance when fed a ketogenic diet [16]. In addition to these investigations, a recent study conducted *in vitro* has confirmed the protective role of FGF21. Indeed, Wang and coworkers showed that FGF21 protects against high glucose induced cell damage and eNOS dysfunction in endothelial cells from human umbilical vein [17]. Therefore, FGF21 has been proposed as a potential therapeutic target for the treatment of T2DM, obesity, and their associated complications, such as NAFLD.

However, some evidence suggests that FGF21 may fail to exert its full beneficial effects in obesity due to some degree of resistance to FGF21 action. Indeed, Fisher et al. reported attenuated signaling response as well as impaired induction of FGF21 target genes in a mouse model of diet-induced obesity [18]. In line with these results, others have found that an FGF21 analog could reduce body weight and improve dyslipidemia in obese human subjects with T2DM, but only at high concentrations, but could only modestly reduce plasma glucose levels, suggesting some state of resistance to FGF21 action [19].

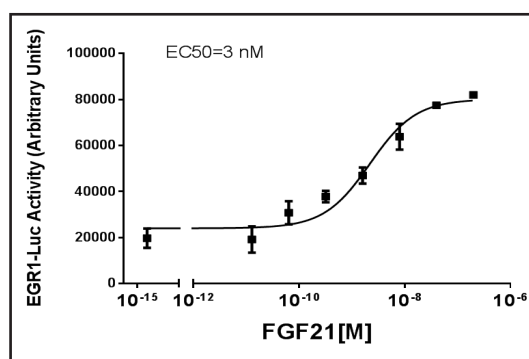
Nevertheless, the mechanisms governing impairment in FGF21 action, notably in the liver, are not fully understood. Given that FFA are increased and deleterious in situations of obesity and T2DM, we hypothesized that FFA may alter FGF21 action in human liver-derived HepG2 cells. However it should be noticed that elevated FFA is not the only factor that could induce adverse effects on FGF21 action. Indeed, it is possible that inflammation, hyperglycemia and other factors related to obesity could contribute to obesity-associated FGF21 impairment. However, the aim of this study was mainly to focus on the role of FFA in obesity-related impairment of FGF21 action. To assess the effect of FFA on FGF21 action, we used HepG2 cells for different reasons: 1) this cell line represents the liver as an ectopic site for lipid deposition; 2) HepG2 cells are derived from human liver tissue and represent a valuable cell line of physiological relevance; 3) FGF21 is mainly produced by hepatic cells and the liver is also an important target of FGF21.

## Materials and Methods

### Cell culture

Human derived HepG2 cells (ATTC, Manassas, USA) were cultured in DMEM (Life Technology, Zug, Switzerland) supplemented with 10% calf bovine serum at 37°C with 5% CO<sub>2</sub>. For treatment of HepG2 cells with FFAs, a mixture consisting of a ratio of 1:sodium-palmitic acid and 4:sodium-oleic acid (Sigma-Aldrich, Switzerland) bound to 1:fatty-acid free Bovine serum albumin (BSA) at a final concentration of 0.2 mM was used as previously described [20, 21]. Recombinant human FGF21 (Novo Nordisk, Måløv, Denmark) was added to NaCl 0.9% to get a final concentration of 3 nM. Briefly, cells were incubated overnight in serum-free medium. Thereafter, the medium was subsequently replaced with DMEM supplemented with NaCl 0.9%,

**Fig. 1.** Luciferase assay using EGR1 as a reporter. The dose-response curve shows FGF21 concentrations on the X axis.



FGF21, FFA or both FGF21 and FFA and the cells were cultured for an additional 48 h, and harvested for RNA or protein isolation. The 3 nM concentration of FGF21 was chosen after performing a luciferase assay. To this end, HepG2 cells were maintained in DMEM containing penicillin (100 U/liter), streptomycin (100 µg/liter), and 10% fetal calf serum (Invitrogen). Transient transfections were performed at 60% cell confluence in 6-well plates with 5 ng of EGR1 response element-luciferase reporter, and 295 ng of empty vector DNA, using FuGene6 reagent (Roche Diagnostics, Indianapolis, IN). After overnight serum starvation, cells were treated for 48 h with increasing doses of FGF21 (from 0 to 2000 pm) in DMEM. The cells were lysed with passive lysis buffer (Promega, Madison, WI), and assayed for luciferase activity using Promega luciferase assay system. Results were plotted and fitted with four-parameter sigmoidal dose response curves using Prism4 software (GraphPad Software Inc., San Diego, CA). Experiments were performed in triplicate and repeated at least three times. The results of this assay are displayed in Fig. 1.

#### Oil Red O staining

After treatment, HepG2 cells were stained with Oil Red O (Sigma-Aldrich, Buchs, Switzerland) as described previously [20]. Briefly, cells were washed twice with PBS, fixed in 4 % paraformaldehyde for 20 min and stained for 1 h with a 0.3% (v/v) Oil Red O solution in 60% (v/v) isopropanol. The stain was removed and cells were washed with PBS. Thereafter, optical density was computed and normalized using a routine written for the MetaMorph software (Molecular Devices, Sunnyvale, CA, USA) provided by the Bioimaging Facility of Geneva University School of Medicine.

#### RNA isolation and quantitative real-time RT-PCR (qRT-PCR)

Total RNA was isolated from HepG2 cells using Trizol (Invitrogen, Lucerne, Switzerland). All RNA samples were treated with RNase-free DNase to remove genomic DNA and stored at -80°C before use. Total RNA was reverse transcribed according to manufacturer instructions with the Reverse Transcriptase kit Takara RR064A (Takara, Saint-Germain-en-Laye, France); the cDNA product was then amplified by real-time PCR using gene-specific primers (Table 1) with SYBR Green mix (Roche, Rotkreuz, France) on an ABI StepOne Plus Sequence Detection System (Life Technologies, Zug, Switzerland). Cycle threshold values were collected and normalized to that of EE1FA1, and relative gene expression was calculated by 2DDCt method.

**Table 1.** Used primer sequences to amplify genes of interest

Gene	Forward primer	Reverse primer
PPAR $\gamma$	GGGATCAGCTCCGTGGATCT	TGCACTTTGGTACTCTTGAAGTT
TNF $\alpha$	CCCAGGGACCTCTCTCTAATCA	AGCTGCCCCCTCAGCTTGAG
Adiponectin	AACATGCCCATTCGCTTTACC	TAGGCAAAGTAGTACAGCCCA
SCD1	CTCCACTGCTGGACATGAGA	AATGAGTGAAGGGGCACAAC
SREBP1c	CACCGTTTCTTCGTGGATGG	CCCGCAGCATCAGAACAGC
FAS	TATGCTTCTTCGTGCAGCAGTT	GCTGCCACACGCTCCTCTAG
CPT1	ATCAATCGACTCTGAAACGG	TCAGGGAGTAGCGCATGGT
EEF1A1	AGCAAAAATGACCCACCAATG	GGCCTGGATGGTTCAGGATA

### Western blotting

Cells were dissolved in lysis buffer containing 150 mM NaCl 0.9%, 1% (v/v) Triton X-100, 0.1% (w/v) SDS, 0.5% (w/v) Na Deoxycholate, 50 mM Tris-HCl with phosphatase and protease inhibitors (Pierce, Lausanne, Switzerland). After incubation for 15 min at 4°C, the lysate was centrifuged at 12,000 rpm for 30 min and the supernatant collected. Protein concentration was determined by BCA protein quantitative kit according to the manufacturer's protocol (Pierce, Lausanne, Switzerland). 30 µg of total proteins were solved on 4-12% SDS-PAGE and transferred to polyvinylidene fluoride (PVDF) membranes. Membranes were blocked with TBS containing 0.1% Tween 20 and 3% BSA, then incubated overnight with primary antibody specific for phospho-AMPK (Thr<sup>172</sup>), phospho-ACC (Ser<sup>79</sup>), phospho-ERK1/2 (Thr<sup>202</sup>/Tyr<sup>204</sup>), AMPK, ACC, ERK1/2, EGR1 and IκB (dilution 1:1000, Cell signaling, Allschwil, Switzerland), Tubulin (1:4000, Sigma-Aldrich, Buchs, Switzerland). Bound antibodies were detected using HRP-labeled secondary IgG (dilution 1:1000) and a chemiluminescence kit (Amersham, Glattbrugg, Switzerland). The blots were imaged by chemiluminescence (Supersignal West Dura substrate; Pierce, Lausanne, Switzerland) on a detection system (GE Healthcare Europe GmbH, Glattbrugg, Switzerland). Densitometric analysis of chemiluminescent signals captured on a camera was performed using the Image J software (National Institutes of Health, <http://rsb.info.nih.gov/ij>).

### Palmitate oxidation

Palmitate oxidation was estimated on the basis of the release of <sup>14</sup>CO<sub>2</sub> from [1-<sup>14</sup>C] palmitate (Amersham, Glattbrugg, Switzerland) as previously described [22]. Briefly, cells were incubated for 60 min in a medium containing palmitate (0.05 mM), oleate (0.05 mM), and 1 µCi/ml [1-<sup>14</sup>C] palmitate complexed to BSA (0.2 mM) in sealed flasks containing a suspended filter paper soaked with an organic base (NCS-II; Amersham, Glattbrugg, Switzerland). The reaction was stopped by the addition of 2 N perchloric acid. <sup>14</sup>CO<sub>2</sub> produced by [1-<sup>14</sup>C] palmitate metabolism was collected overnight on the filter paper and quantified by scintillation counting.

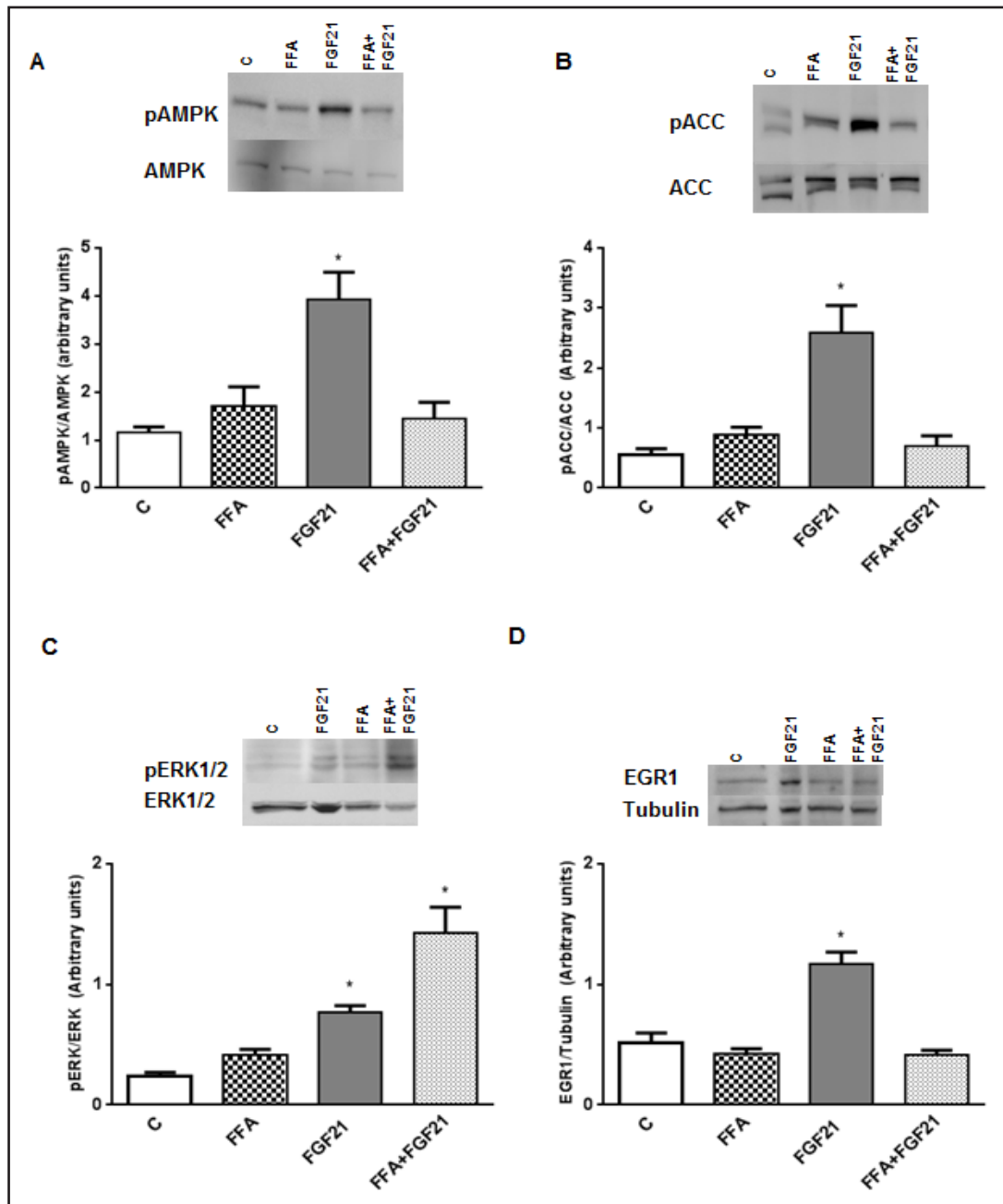
### Statistical analysis

All experiments were repeated at least three times. Data are given as means ± SEM. One-way ANOVA was used to statistically compare FFA-FGF21, FGF21, FFA to the control group (Prism 6; GraphPad Software, Ink, La Jolla, USA). A p value < 0.05 was considered as statistically significant.

## Results

### Effect of FFA on FGF21 signaling in HepG2 cells

FGF21 ameliorates hyperglycemia, reduces triglycerides levels and improves lipid metabolism notably through activation of AMPK [23]. However, FGF21 partly fails to exert its beneficial metabolic effects in obese humans. Therefore, to gain insight into the mechanism by which FGF21 action is attenuated, we hypothesized that high levels of FFA would prevent FGF21 from stimulating its downstream targets. To test this hypothesis, we exposed HepG2 cells to either FGF21 or FFA or both. We found that FGF21 strongly enhanced phosphorylation levels of key enzymes involved in lipid metabolism. As expected, FGF21 increased phosphorylation of AMPK on Thr<sup>172</sup> (Fig. 2A). Consequently, phosphorylation of the AMPK target Acetyl-CoA carboxylase (ACC) on Ser<sup>79</sup> increased as well, indicating inactivation of this rate-limiting enzyme of fatty acid synthesis (Fig. 2B). However, treatment of HepG2 cells with FGF21 in the presence of FFA blunted the effects of FGF21 on AMPK and ACC (Fig. 2A and Fig. 2B respectively). We further investigated FGF21 effects by assessing ERK1/2 phosphorylation and EGR1 expression as reporters of FGF21 signaling. By contrast to AMPK and ACC, FGF21 was able to activate the ERK1/2 signaling pathway under either basal or high FFA levels conditions (Fig. 2C). These data suggest that FGF21 at 3 nM is active and that FFA do not interfere with this pathway to inhibit FGF21 effect. However, FGF21 could not increase EGR1 expression in the presence of high levels of FFA (Fig. 2D).

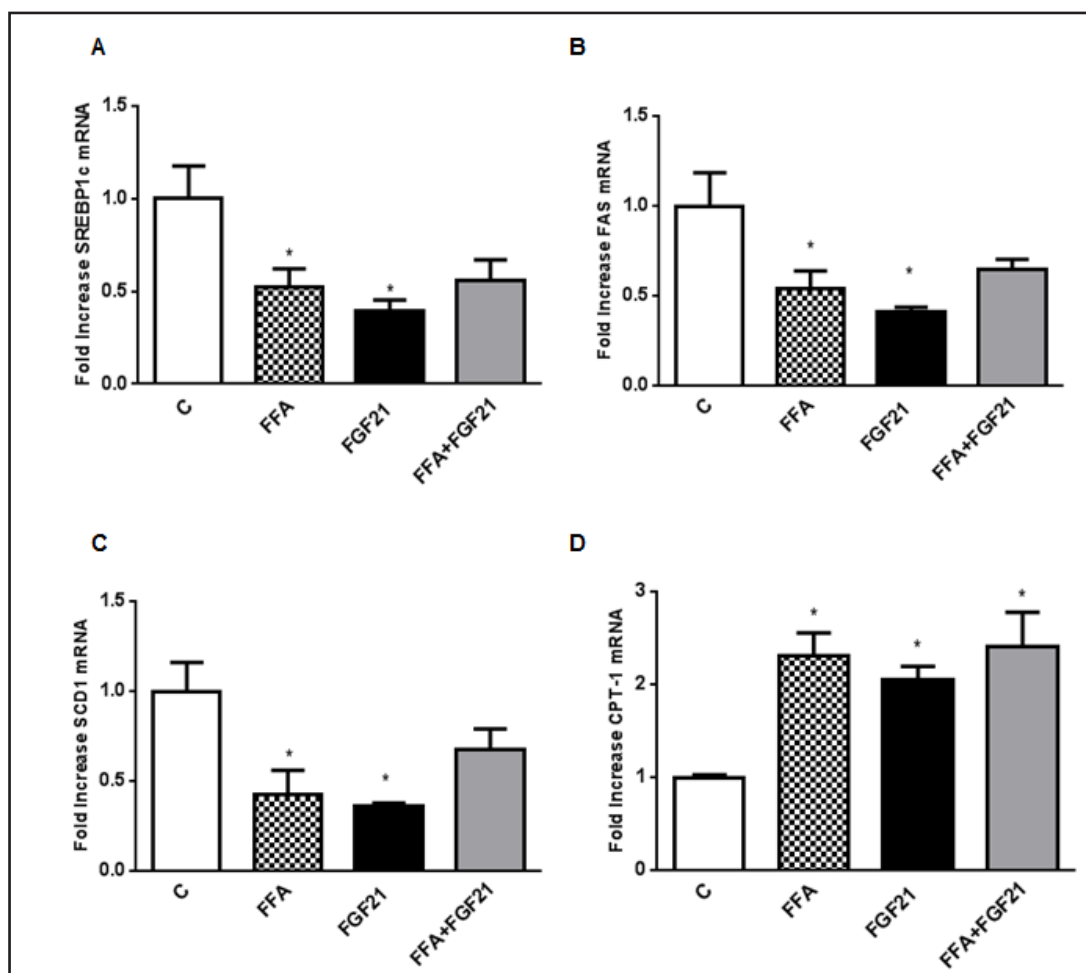


**Fig. 2.** Free fatty acids (FFA) impair FGF21 action on intracellular signaling in HepG2 cells. HepG2 cells were cultured for 48 h in the presence of FFA (0.2 mM), FGF21 (3 nM), both, or the vehicle. Cell extracts were analyzed by Western blot using antibodies specific for phosphorylated (A) pThr<sup>172</sup>-AMP-activated protein kinase (AMPK) and total AMPK; (B) pSer<sup>79</sup>-ACC and total ACC; (C) pThr<sup>202</sup>/Tyr<sup>204</sup> ERK1/2 and total ERK1/2; (D) EGR1 and tubulin. Panels on the bottom show mean data of at least three independent experiments. Results are expressed as means  $\pm$  SEM. \*Significant effect of FGF21 or FFA vs. untreated HepG2 cells.

#### Effect of FFA on FGF21 regulated genes in HepG2 cells

Since we observed inhibiting effects of FFA on FGF21-stimulated AMPK and ACC, we further investigated whether FFA could affect FGF21-induced expression of genes related to lipid metabolism. Real-time PCR analysis revealed that FGF21 significantly reduced lipogenesis through downregulation of sterol regulatory element-binding protein 1c (SREBP-1c) (Fig. 3A) and its downstream target genes fatty acid synthase (FAS) (Fig. 3B) and



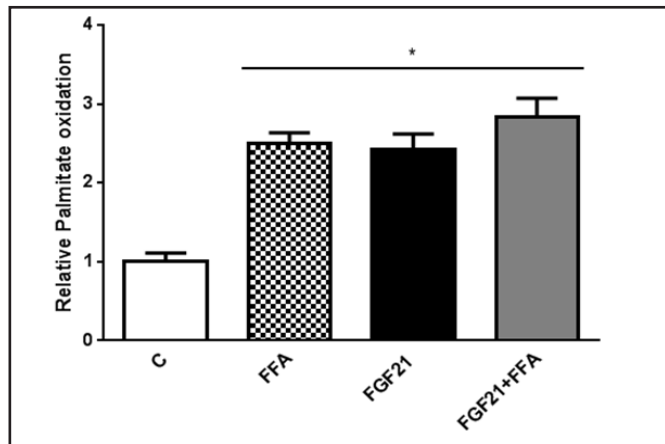


**Fig. 3.** FGF21 downregulates genes involved in lipogenesis and upregulates lipid oxidation in HepG2 cells, but not under high FFA levels. HepG2 cells were cultured for 48 h in the presence of FFA (0.2 mM), FGF21 (3 nM), both, or the vehicle and the mRNA levels of SREBP1c (A), FAS (B), SCD1 (C), and CPT-1 $\alpha$  (D) were evaluated by real-time PCR. Data are represented as means  $\pm$  SEM of at least three independent experiments. \*Significant effect of FGF21, FFA or both vs. untreated HepG2 cells.

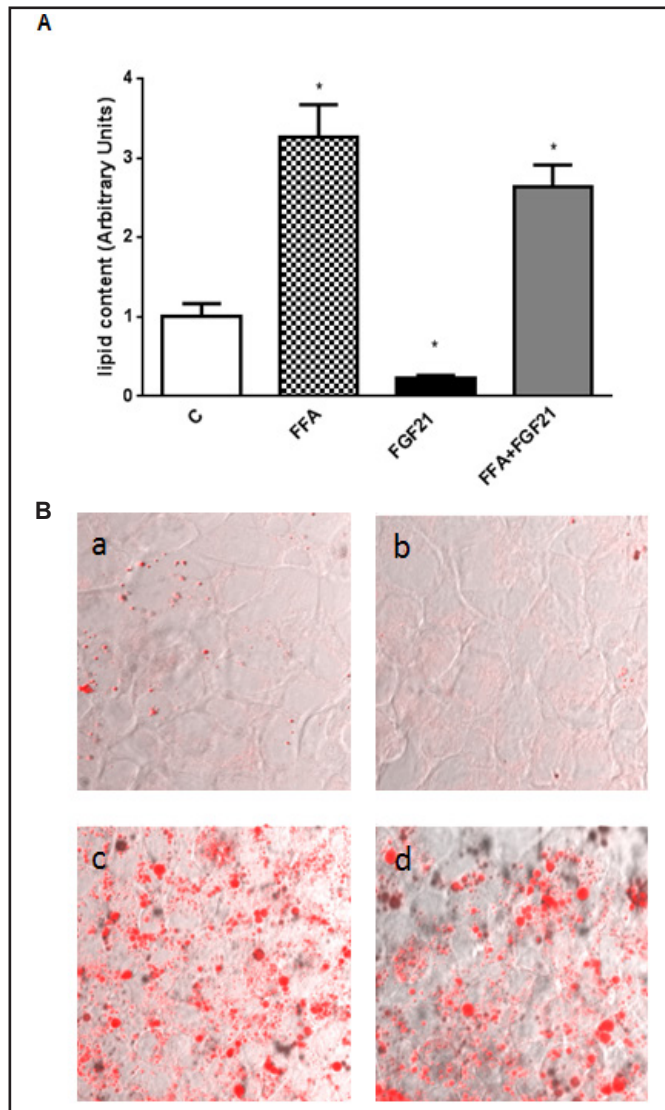
stearoyl-CoA desaturase 1 (SCD1) (Fig. 3C) by respectively 60, 65 and 60%. Moreover, FGF21 significantly upregulated mRNA levels of carnitine palmitoyltransferase-1 (CPT-1 $\alpha$ ) by 104%, an enzyme involved in lipid oxidation (Fig. 3D). Similarly to FGF21 alone, we observed that the addition of FFA to HepG2 cells not only induced lipid oxidation by significantly increasing CPT-1 $\alpha$  mRNA levels by 130% (Fig. 3D), but also reduced the expression of genes involved in lipid synthesis such as FAS and SCD1, respectively by 48% and 58% (Fig. 3B, 3C). However, when cells were treated with both FGF21 and FFA, the individual effects of both FGF21 and FFA were lost, suggesting that FGF21 is not able to initiate its effects in the presence of FFA.

Based on this latter result, we assessed whether FGF21 could increase fatty acids oxidation. To this end, HepG2 cells were incubated for 48 hours with FGF21, after what fatty acids oxidation was evaluated. FFA were used as a positive control. We found that FGF21 increased fatty acids oxidation by about 140%. This response was similarly induced by FFA which increased lipid oxidation by 140%. However, when concomitantly treated with FFA and FGF21, HepG2 cells exhibited similar response to FGF21 treatment alone regarding the mRNA level of CPT-1 and fatty acid oxidation (Fig. 4). This could be due to a loss of FGF21 action in the presence of high levels of FFA.

**Fig. 4.** FGF21 and FFA individually stimulate palmitate oxidation but do not induce an additive effect. HepG2 cells were exposed to BSA, FGF21, FFA, or both FGF21 and FFA, for 48h. Palmitate oxidation was subsequently measured. Results are expressed as means  $\pm$  SEM of four independent experiments. \*Significant effect of FGF21, FFA or both vs. corresponding stimulation in untreated HepG2 cells.

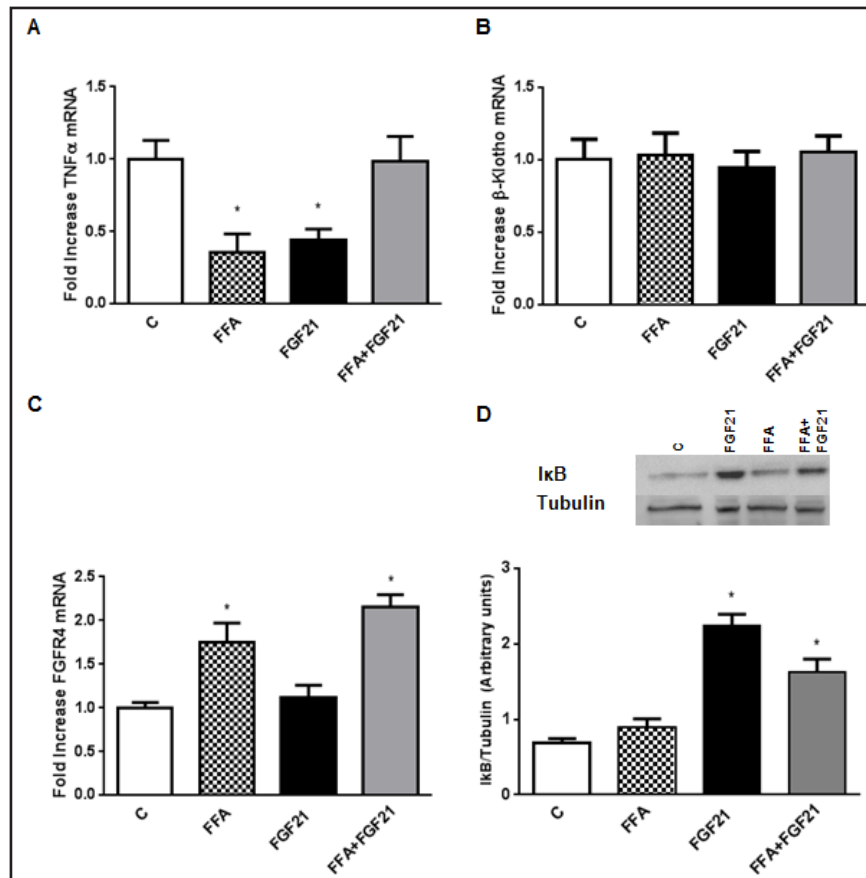


**Fig. 5.** Lipid accumulation in HepG2 cells by Oil-Red O staining after treating cells with FGF21, FFA, or both, for 48h. (A) Representative photomicrographs of HepG2 cells ( $\times 40$ ) (B). (a) Control cells; (b) Cells treated with 3 nM FGF21; (c) Cells treated with 0.2 mM FFA; (d) Cells treated with both FFA and FGF21. Results are expressed as means  $\pm$  SEM of four independent experiments. \*Significant effect of FGF21 vs. untreated HepG2 cells.



*FGF21 reduces triglycerides accumulation in HepG2 cells, but not under high FFA conditions*  
As FGF21 reduced mRNA levels of genes involved in lipid synthesis and increased fatty acids oxidation, we determined whether this was associated with an inhibition of triglycerides accumulation in HepG2 cells. To this end, HepG2 cells were incubated either

**Fig. 6.** FFA prevent the inhibiting action of FGF21 on TNF $\alpha$ . HepG2 cells were cultured for 48 h in the presence of FFA (0.2 mM), FGF21 (3 nM), both, or the vehicle and the mRNA levels of TNF $\alpha$  (A),  $\beta$ -klotho (B) and FGFR4 (C) were evaluated by real-time PCR. To confirm FGF21 effects on TNF $\alpha$ , I $\kappa$ B was evaluated by Western blot (D). Data are represented as means  $\pm$  SEM of at least three independent experiments. \*Significant effect of FGF21, FFA or both vs. corresponding stimulation in untreated HepG2 cells.



with FGF21, FFA, or both FGF21 and FFA, and visualized by Oil Red O staining. The results showed that basal triglycerides accumulation level was low but could be further decreased by 77% by FGF21 treatment in HepG2 cells. When HepG2 cells were exposed to FFA, we observed a high accumulation of triglycerides that could not be lowered by FGF21 treatment, suggesting that FGF21 could not initiate its actions under high levels of FFA (Fig. 5A).

#### Effect of FGF21 and FFA on TNF $\alpha$ expression in HepG2 cells

FFA are known to increase tumor necrosis factor  $\alpha$  (TNF $\alpha$ ) expression [24], which in turn represses  $\beta$ -klotho expression and impairs FGF21 action in adipose cells [25]. Thus, we assessed whether FFA could impair FGF21 action through an increased TNF $\alpha$  expression and down regulation of  $\beta$ -klotho. Although TNF $\alpha$  is slightly expressed in HepG2 cells, we found that its expression was intriguingly reduced by both FGF21 and FFA treatment, respectively by 57% and 65% (Fig. 6A). However, when exposed concomitantly to FFA and FGF21, TNF $\alpha$  expression levels were similar to that of the untreated cells. Thus, individually, FGF21 and FFA reduced TNF $\alpha$  mRNA levels, but the addition of both FGF21 and FFA did not affect TNF $\alpha$  expression (Fig 6A). Therefore, it is likely that FFA interfere with FGF21 signaling to abolish the down regulation of TNF $\alpha$  induced by FGF21 treatment. Additionally FFA alone reduced TNF $\alpha$  mRNA expression probably due to the presence of oleic acid in the FFA mixture, which is recognized as a protective fatty acid [26].

Because the FGF21 receptor complex is composed of four FGFR isoforms (FGFR4 being the predominant isoform in the liver) and  $\beta$ -klotho, we also evaluated  $\beta$ -klotho and FGFR4 expressions in HepG2 cells treated with FGF21, FFA, both FGF21 and FFA, or the vehicle. Intriguingly, we found that none of the treatment affected  $\beta$ -klotho expression levels (Fig. 6B). However, FFA alone or in association with FGF21 significantly increased mRNA expression levels of FGFR4 (Fig. 6C). This could represent a compensatory mechanism when impairment of FGF21 signaling develops.



To further support our results, suggesting that FGF21 is likely improving lipid metabolism through a reduction in TNF $\alpha$  expression, we assessed the expression levels of I $\kappa$ B, a known readout of TNF $\alpha$ . We found that FGF21 reduced the degradation of I $\kappa$ B, as revealed by I $\kappa$ B increased protein levels (Fig. 6D). This is concordant with an FGF21-induced TNF $\alpha$  down regulation. On the other side, when HepG2 cells were treated with both FGF21 and FFA, we observed that FGF21-induced up regulation of I $\kappa$ B was blunted, suggesting that FFA inhibit the beneficial effect of FGF21 on I $\kappa$ B (Fig 6D), and therefore on TNF $\alpha$  down regulation.

## Discussion

Several studies have reported elevated FGF21 plasma levels in situations of insulin resistance in humans. Notably, plasma FGF21 levels are increased in patients with T2DM and obesity and are independently associated with the metabolic syndrome [27, 28]. In line with this, animal models of obesity and insulin resistance also exhibit increased endogenous FGF21 levels [15, 18, 29, 30]. Thus, one would expect that exogenous administration of FGF21 would not prompt any effect. Nevertheless, at pharmacologic doses, FGF21 improves metabolic parameters and induces weight loss [13, 14, 31]. In contrast, Fisher and coworkers have reported inefficient effect of endogenous FGF21 in obese mice, demonstrating that obesity is an FGF21-resistant state [18]. Indeed, they found that diet-induced obese mice present an important attenuation of FGF21 signaling, leading to resistance toward the physiological action of FGF21. However, the mechanisms by which obesity induces FGF21 resistance remain unidentified. This hormone resistance could be compared to the concept of insulin resistance described more than a century ago in diabetic patients. To date, the exact mechanisms of insulin resistance are not fully elucidated but numerous investigations suggest that insulin resistance is a multifactorial disorder [32-34]. Amongst these factors, FFA are recognized as one of the main factors responsible for the development of insulin resistance, notably in the liver [35], and have been shown to inhibit the action of insulin [36]. Based on this, we hypothesized that high levels of FFA may also impair FGF21 signaling in HepG2 cells. Nevertheless, it should be noticed that other factors involved in insulin resistance such as hyperglycemia or inflammation could also contribute to obesity-related FGF21 altered responsiveness. For instance, it has been shown that cultured adipocytes treated with the cytokine TNF $\alpha$  exhibit a down regulation of the FGF21 receptor cofactor  $\beta$ -klotho, leading to an FGF21-resistance state [25]. Importantly, in this study, we mainly focused on the role of FFA on FGF21 signaling because FFA are considered to play a major role in both NAFLD and T2DM.

Here, we show that in the presence of FFA, FGF21 could not initiate its oxidative effect in HepG2 cells, as revealed by a lower AMPK activation and ACC inactivation. Indeed, in the absence of FFA, treatment of HepG2 cells with FGF21 reduced lipogenesis and increased palmitate oxidation. Inhibition of lipogenesis was associated with down regulation of SREBP-1c and its downstream target genes FAS and SCD1. This lipid lowering effect was associated with phosphorylation of AMPK and its downstream target ACC, and up regulation of CPT-1 $\alpha$ . These results are consistent with previous findings in white adipose tissue, which is also a major target of FGF21, suggesting that FGF21 acts through AMPK to control energy balance [23]. Moreover, we show for the first time that high levels of FFA, a pivotal component in T2DM, impair FGF21 action in HepG2 cells. Our results notably demonstrate that under basal conditions, when FFA levels are low, FGF21 further reduces fatty acids accumulation in HepG2 cells. However, in contrast to Zhang and coworkers [37], we found that FGF21 was unable to exert its lipid-lowering effect when a high concentration of FFA was added to the culture medium of HepG2 cells, suggesting adverse effects of high FFA on FGF21 action. The discrepancy between our results and the study by Zhang et al. is likely due to the methods. Indeed, Zhang et al. transfected HepG2 cells with an expression plasmid encoding FGF21, leading to an endogenous overexpression of FGF21, while we investigated the exogenous action of pharmacological doses of FGF21. Consistently, others have discussed the divergence between endogenous and exogenous roles of FGF21 [38]. It should be noticed that our study

is more relevant to the pharmacological approach in which diabetic and obese subjects with high circulating levels of FFA are treated with exogenous FGF21 [19].

To further investigate the effect of FFA on FGF21 action, we used ERK1/2 and EGR1 as readouts of FGF21 signaling and action. Surprisingly, we found that ERK1/2 was not down regulated when FFA were added to FGF21. Instead, ERK1/2 was further activated when FGF21 and FFA were concomitantly added to HepG2 cells, indicating that, depending on the targets, FGF21 and FFA lead to different consequences. This could be due to the fact that ERK is not a specific target of FGF21. Together, these data suggest that the inhibitory effect of FFA on FGF21 is probably not mediated through the ERK signaling pathway. Nevertheless, EGR1 was activated by FGF21 alone but down regulated when FFA were added to FGF21, suggesting that FFA impair FGF21 action, leading to impaired EGR1 overexpression. These results are in part in contrast with Fisher and coworkers, who reported a down regulation of ERK1/2 phosphorylation and EGR1 when FGF21 resistance developed [18]. This discrepancy between the latter study and our study could be due to the model used. Indeed, we used an *in vitro* model while Fisher et al. performed their experiments *in vivo* in mice.

Another notable observation of our study is that FGF21 reduced TNF $\alpha$  gene expression. Recently, it has been demonstrated that TNF $\alpha$  could impair FGF21 action in cultured adipocytes [25]. Thus, a negative loop between FGF21 and TNF $\alpha$  is likely. TNF $\alpha$  could therefore represent a potential culprit, inhibiting FGF21 beneficial action on lipid metabolism. Additionally, this inhibitory effect of TNF $\alpha$  on FGF21 action involves a down regulation of  $\beta$ -klotho. In contrast, we did not observe any effect of FGF21 and/or FFA on  $\beta$ -klotho expression levels in HepG2 cells. Such a discrepancy could be due to the approach used by Delfin and coworkers. Indeed, they treated adipose cells with TNF $\alpha$ , which inhibits  $\beta$ -klotho, while we observed a down regulation of TNF $\alpha$  in the presence of FGF21 that did not affect  $\beta$ -klotho expression. Interestingly, we observed that FFA increased FGFR4 mRNA expression levels, which suggests a compensatory mechanism to overcome the impairment of FGF21 action. Finally, to reinforce the potential interaction between FGF21 and TNF $\alpha$ , we assessed the protein levels of I $\kappa$ B as a readout of TNF $\alpha$ . We found that FGF21 reduced the degradation of I $\kappa$ B and this was in line with a role for FGF21 in inducing TNF $\alpha$  down regulation. However, treatment of HepG2 cells with both FGF21 and FFA blunted the FGF21-induced up regulation of I $\kappa$ B, suggesting again that FFA impair the beneficial effect of FGF21 in reducing TNF $\alpha$ -mediated inflammation. In line with these results, we have recently shown that a ketogenic diet, which increases plasma levels of FFA in wild-type mice, impairs FGF21 signaling in liver and white adipose tissue [39]. This was associated with increased cytokines expression levels in the liver. Taken together, these data suggest that inflammation is likely deleterious for FGF21 action, as it is for insulin action [40].

In conclusion, HepG2 cells both adapt to FFA and respond to FGF21 by reducing fatty acids synthesis and increasing fatty acids oxidation. However, these mechanisms cannot cope with high levels of FFA even in the presence of FGF21, possibly because of a modulation of the inflammatory cytokine TNF $\alpha$ . However, further investigations are required notably in *in vivo* models to confirm the adverse effects of FFA on FGF21 action, which could lead to a new therapeutic approach to treat diseases associated with insulin resistance and FGF21 resistance such as T2DM, obesity and NAFLD. Indeed, modulation of lipid levels with pharmacological doses of FGF21 in tissues such as the liver could induce beneficial effects in these ever growing metabolic diseases.

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## Disclosure Statement

The authors have nothing to disclose.

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