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Original Paper

Leptin as a Potential Regulator of FGF21

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Key Words
FGF21 • Leptin • Insulin resistance • Obesity

Abstract
Background/Aims: Fibroblast growth factor 21 (FGF21), a potent metabolic regulator, has been shown to improve insulin sensitivity in animal models of insulin resistance. Several studies have focused on identifying mediators of FGF21 effects. However, the identification of factors involved in FGF21 regulation is far from complete. As leptin is a potent metabolic modulator as well, we aimed at characterizing whether leptin may regulate FGF21. ***Methods:*** We investigated a potential regulation of FGF21 by leptin *in vivo* in Wistar rats and *in vitro* using human derived hepatocarcinoma HepG2 cells. This model was chosen as the liver is considered the main FGF21 expression site. ***Results:*** We found that leptin injections increased plasma FGF21 levels in adult Wistar rats. This was confirmed *in vitro*, as leptin increased FGF21 expression in HepG2 cells. We also showed that the leptin effect on FGF21 expression was mediated by STAT3 activation in HepG2 cells. ***Conclusion:*** New findings regarding a leptin-STAT3-FGF21 axis were provided in this study, although investigating the exact mechanisms linking leptin and FGF21 are still needed. These results are of great interest in the context of identifying potential new clinical approaches to treat metabolic diseases associated with insulin resistance, such as obesity and type 2 diabetes.

Introduction

Fibroblast growth factor 21 (FGF21) is a potent metabolic regulator, predominantly expressed in the liver and white adipose tissue, but also in skeletal muscle and pancreas [1, 2]. Pharmacological doses of FGF21 have been shown to exert notable anti-diabetic effects. In particular, FGF21 improves glucose tolerance and insulin sensitivity, and reduces plasma and hepatic triglycerides in ob/ob, db/db, and wild-type mice fed a high-fat diet [3-6]. It also protects against high glucose induced cellular damage in endothelial cells, thus potentially promoting vascular health in diabetes [7]. Several studies focused on the identification

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of potential FGF21 mediators [8-10]. Among these mediators, adiponectin was shown to mediate part of FGF21 effects [8, 9]. Recent evidence also suggests that other factors, such as dual specificity phosphatase 4 or uncoupling protein-1 (UCP1) may mediate FGF21 actions [11, 12]. With regard to the regulation of FGF21 expression, very limited information is available as yet [13]. Interestingly, FGF21 expression was shown to be under the control of the stress responsive transcription factor, Signal Transducers and Activators of Transcription 3 (STAT3) [14], which is also known to mediate leptin effects [15]. Subsequently, other studies demonstrated that FGF21 is modulated by key metabolic transcription factors such as PPARα and PPARγ [16-18] which are also induced by leptin [19]. Leptin is also known to promote metabolic health by notably increasing fat oxidation in muscle cells [20] and decreasing inflammation [21]. In the present study, we first investigated whether leptin may regulate FGF21 expression *in vivo* in Wistar rats. Since FGF21 is predominantly produced by the liver [22], potential leptin effects on FGF21 were also investigated *in vitro*, in human derived hepatocarcinoma HepG2 cells, a cell line known to produce FGF21 although at very low concentrations under basal conditions [23]. This allowed us to investigate the potential signaling pathways involved in the regulation of FGF21 by leptin.

Materials and Methods

Animals

All procedures were performed in accordance with and approved by the Institutional Ethical Committee of Animal Care in Geneva and Cantonal Veterinary Office. Four month-old male Wistar rats were obtained from Charles River (L'Arbresle, France). They were housed under controlled temperature (22°C) and lighting (lights on: 7 AM to 7 PM) with free access to water and food (standard diet, laboratory diet RM3, SDS, Essex, UK). To test the effect of leptin on FGF21 secretion, a cohort of rats was euthanized 30 minutes after a single intraperitoneal injection of human recombinant leptin (2 mg/kg) (PeproTech, Rocky Hill, NJ) or vehicle (saline). This leptin dose was chosen because previous experiments were performed in similar conditions with reliable results. Notably, this dose of leptin was able to decrease food intake in Wistar rats, confirming a response to leptin, which was not the case with lower doses of leptin [24]. Blood and tissues were sampled and stored at –80°C for further analysis.

Plasma measurements

A commercial ELISA kit was used for the measurement of plasma FGF21 (R&D systems Europe Ltd, Oxon, UK).

Cell culture

Human derived hepatocarcinoma HepG2 cells (American Type Culture Collection, Manassas, VA) were cultured in Dulbecco modified Eagle medium (Gibco®, Life Technologies, Zug, Switzerland) supplemented with 10% fetal bovine serum (FBS, Sigma-Aldrich, Buchs, Switzerland), 1% (v/v) penicillin streptomycin (Sigma-Aldrich,) at 37 °C with 5% CO_2. Equivalent numbers of HepG2 cells starved in DMEM basal serum-free media were treated with 200 ng/mL recombinant leptin (R&D Systems, Abingdon, UK), PBS (control group), and/or a STAT3 inhibitor (10 μmol/L) (Stattic, Sigma-Aldrich) for 1h.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

Total RNA from HepG2 cells was extracted using a single-step extraction with Trizol reagent (Sigma-Aldrich). RNA integrity was assessed by electrophoresis on a 1% agarose gel and concentration was determined by spectrophotometry. A quantity of 1 μg of total RNA was used for RT, using a commercial kit (Takara Bio Europe, St Germain-en-Laye, France). For quantitative PCR, amplification of genes was performed from 50 ng cDNA using the SYBR® green PCR Master Mix (Roche, Rotkreuz, France) and a StepOne™ Real-Time PCR system (Life Technologies), as previously described [25]. Oligonucleotides were used at 300 nmol/L and results were normalized to the expression levels of housekeeping genes (Table 1).

Table 1. Sequences of oligonucleotides used

Name	Gene symbol	Position	Primer
Rat Fibroblast growth factor 21	*mFgf21*	Forward	CTGGGGGTCTACCAAGCATA
		Reverse	CACCCAGGATTTGAATGACC
Rat Peptidylprolyl isomerase A (also known as Cyclophilin A)	*mPpia*	Forward	GGCTCCGTCGTCTTCCTTTT
		Reverse	ACTCGTCCTACAGATTCATCTCC
Human Peroxisome proliferator-activated receptor- alpha	*hPparα*	Forward	GGGATCAGCTCCGTGGATCT
		Reverse	TGCACTTTGGTACTCTTGAAGTT
Human Suppressor of cytokine signaling 3	*hSOCS3*	Forward	CCTGCGCCTCAAGACCTTC
		Reverse	GTCACTGCGCTCCAGTAGAA
Human Fibroblast growth factor 21	*hFgf21*	Forward	GCCTTGAAGCCGGGAGTTATT
		Reverse	GTGGAGCGATCCATACAGGG
Human Eukaryotic translation elongation factor 1 Alpha 1	*hEef1a1*	Forward	AGCAAAAATGACCCACCAATG
		Reverse	GGCCTGGATGGTTCAGGATA

Western blot

HepG2 cells lysates were prepared in RIPA buffer (150 mmol/L NaCl, 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 (Halt protease and phosphatase inhibitor cocktail, Pierce, Lausanne, Switzerland) as previously described [26]. Samples were resolved on a 4–12% Bis-Tris gel and transferred onto polyvinylidene difluoride membranes. Thereafter, proteins were detected using the following antibodies: anti-pSTAT3 (1:2000, Cell signaling, Allschwil, Switzerland), anti-STAT3 (1:2000, Cell signaling), anti-PPARα (1:2000, Cell signaling) and tubulin (1:4000, Sigma-Aldrich). The bands were visualized by chemiluminescence (Supersignal West Dura substrate, Pierce) on a detection system (GE Healthcare Europe GmbH, Glattbrugg, Switzerland). Densitometric analysis of chemiluminescent signals captured on camera was performed using the Image J software (National Institutes of Health).

Statistical analyses

Results are expressed as Mean ± SEM. Statistical calculations were carried out with GraphPad Prism 6 (GraphPad Prism, La Jolla, CA). Statistical significance was established at $p<0.05$ and determined by unpaired Student's *t*-test or one-way ANOVA.

Results

Leptin increases plasma FGF21 levels in rats

To study the link between leptin and FGF21, adult male Wistar rats were challenged with an intraperitoneal leptin injection (2 mg/kg). Plasma FGF21 levels were then evaluated 30 minutes later. Leptin treatment significantly increased plasma FGF21 levels compared to the injection of vehicle (saline), confirming a stimulatory effect of leptin on FGF21 release in this model (Fig. 1). The mRNA expression of *Fgf21* was thereafter evaluated in different tissues. The liver was the tissue predominantly expressing *Fgf21*, compared to brown (BAT) and inguinal white (WATi) adipose tissue (Fig. 2). However, under our experimental conditions (i.e. 30 minutes after injection), leptin was unable to increase *Fgf21* mRNA levels in the liver or in the other tissues (Fig. 2). This suggests a potential time-dependant effect, or an effect of leptin on FGF21 release rather than expression.

Leptin induces Fgf21 expression in HepG2 cells

To strengthen our *in vivo* results, we used an *in vitro* model, focusing on the liver, which is considered the main site of FGF21 production. HepG2 cells were therefore chosen and treated for 1 h with leptin (200 ng/mL). Leptin treatment enhanced *Fgf21* expression under these experimental conditions (Fig. 3A). Interestingly, an almost significant ($p=0.07$) stimulation of the mRNA expression of *Socs3*, a direct target of STAT3, was observed (Fig. 3A). As leptin is known to promote activation of STAT3 [27], we assessed whether the leptin-induced increase in *Fgf21* expression could be mediated by the STAT3 signaling pathway.

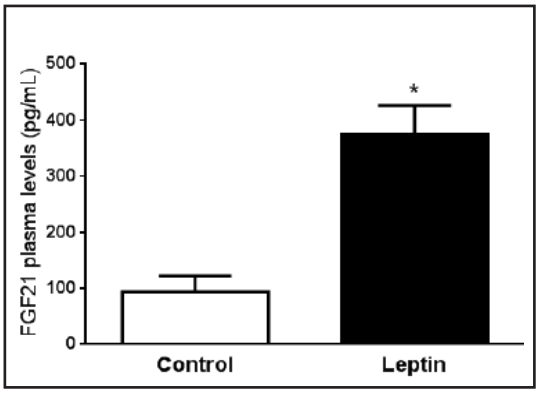

Fig. 1. Leptin increases plasma FGF21 levels in Wistar rats. Plasma FGF21 levels in control (saline) or leptin-treated (2 mg/kg, i.p.) Wistar rats. Mean ± SEM. *p<0.05 using Student's *t*-test.

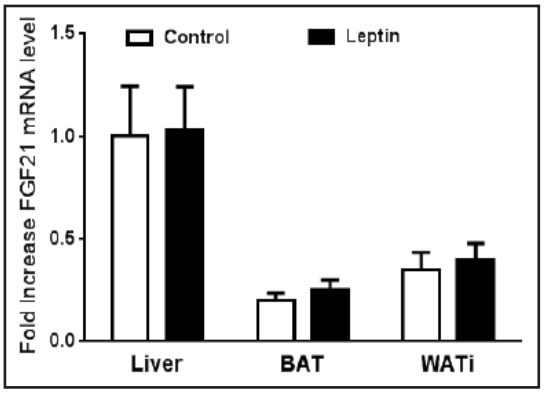

Fig. 2. Acute leptin treatment does not affect Fgf21 mRNA levels in the liver, brown adipose tissue (BAT), or inguinal white adipose tissue (WATi) of Wistar rats. mRNA expression of *Fgf21* in control (saline) or leptin-treated (2 mg/kg, i.p.) Wistar rats. Mean ± SEM.

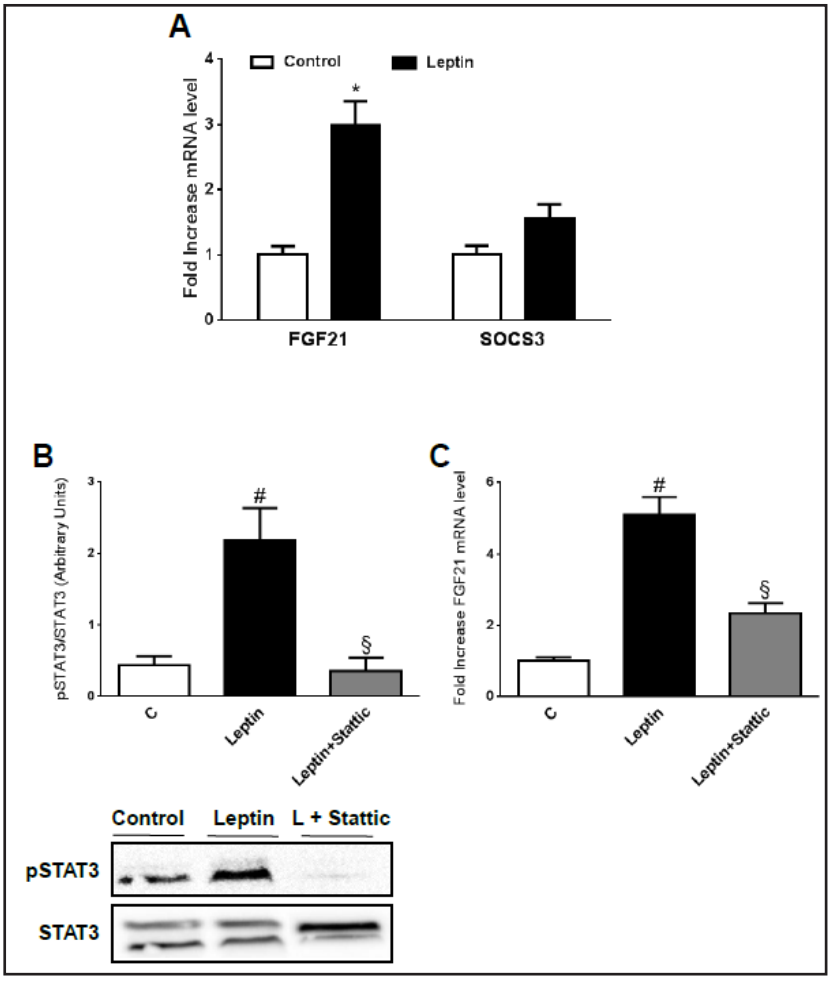

Fig. 3. Leptin regulation of FGF21 in HepG2 cells. A) mRNA expression of *Fgf21* and *Socs3* in untreated (Control) or leptin-treated (Leptin, 200 ng/mL) HepG2 cells. B) Quantification and representative Western blots of phosphorylated STAT3 (pSTAT3) and STAT3 in the presence (Leptin + Stattic, 1 h) or absence (Leptin) of the STAT3 inhibitor, Stattic, compared to untreated cells (C). C) mRNA expression of *Fgf21* in untreated (C) or leptin-treated HepG2 cells in the presence, or the absence of Stattic. Mean ± SEM of n=4 experiments for qPCR using Student's *t*-test or one-way ANOVA. *p<0.05 leptin *versus* control, #p<0.05 leptin *versus* control, §p<0.05 leptin + stattic *versus* leptin.

To this end, HepG2 cells were treated with leptin (200 ng/mL) in the presence, or in the absence, of a STAT3 inhibitor (Stattic, 10 µmol/L, 1 h). As expected, leptin increased STAT3 phosphorylation whereas the presence of the STAT3 inhibitor in the medium resulted in a significant inhibition of phosphorylation (Fig. 3B). Importantly, the presence of the

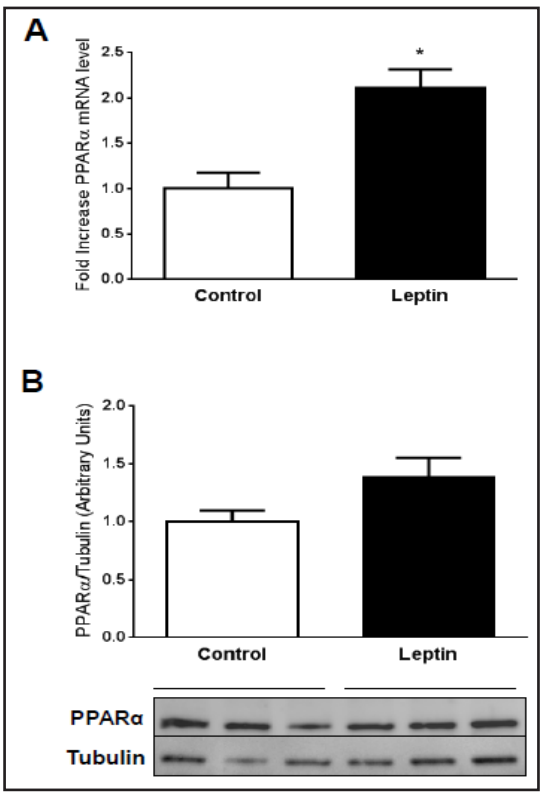

Fig. 4. Effect of leptin on PPARα in HepG2 cells. A) mRNA expression of *Pparα*; B) Quantification and representative Western blots of PPARα and Tubulin in untreated (Control) or leptin-treated (Leptin) HepG2 cells. Mean ± SEM of n=4 experiments for qPCR. *p<0.05 using Student's *t*-test.

inhibitor also prevented the leptin-induced *Fgf21* expression (Fig. 3C), confirming that the effect of leptin on FGF21 is mediated by STAT3 activation.

As PPARα is also known to regulate *Fgf21* gene expression [28], we then tested whether leptin could stimulate *Fgf21* expression through the PPARα pathway. We observed that 1 h of leptin treatment increased P*parα* mRNA expression in HepG2 cells (Fig. 4A). However, as determined using Western blotting, PPARα protein levels tended to be increased by leptin (Fig. 4B), but this was not significant (p=0.1). These results could potentially suggest a post-transcriptional regulation of PPARα by leptin, which will require further investigations in future studies. Alternatively, it is conceivable that 1 h of leptin treatment was not long enough to observe any significant difference in protein synthesis.

Discussion

FGF21, a potent metabolic regulator, has been shown to improve glucose and lipid metabolism, as well as to reduce body weight and adipose tissue mass [3-6, 29-31]. These favorable effects seem to be mainly mediated by white adipose tissue, since lack of FGF receptor 1 (FGFR1) in this tissue abolishes the metabolic actions of FGF21 [32, 33]. FGF21 has also been shown to improve insulin sensitivity and hepatic steatosis in high-fat fed mice [4]. On the opposite, mice lacking *Fgf21* develop hepatic steatosis and hepatic insulin resistance when fed a high-fat ketogenic diet [23]. Several studies focused on the identification of FGF21 mediators [8-10]. Although adiponectin was described as one of the main mediators of FGF21 action, recent evidence suggests that other factors may be involved as well [11, 12]. Nevertheless, limited information is available regarding the regulation of *Fgf21* expression itself.

Here, we report that leptin is a potential new regulator of FGF21. Indeed, we observed that leptin administration acutely increased plasma FGF21 plasma levels in Wistar rats. This likely resulted from a release of FGF21 from the liver, as suggested by the literature [3], although we could not find any difference in *Fgf21* expression in the liver or other tissues. However, skeletal muscle has also been suggested as a site of FGF21 production [34, 35] and could have revealed increased *Fgf21* expression after leptin administration. However, we did not sample any skeletal muscle in our rats. Nonetheless, it is also possible that timing of tissue harvesting (30 minutes in our *in vivo* experimental conditions) might have accounted for our findings, suggesting that a faster or slower regulation by leptin on tissue *Fgf21* expression might occur. The rapid effect of leptin on FGF21 levels however suggests an effect on secretion. Notably, leptin is known to rapidly (within minutes) and directly inhibit insulin secretion by the pancreas [36]. FGF21 is also rapidly (within 15 minutes) expressed by the pancreas after acute pancreatic injury [37]. Therefore, it could be hypothesized that leptin

might stimulate rapid pancreatic FGF21 expression and release. Unfortunately, we did not sample the pancreas of our leptin-treated rats. Altogether, these considerations therefore warrant further *in vivo* research.

Nevertheless, our *in vitro* results obtained in HepG2 cells clearly show an effect of leptin on *Fgf21* expression. Moreover, we show that this leptin effect is mediated by STAT3 activation, as not only STAT3 phosphorylation, but also *Fgf21* expression, were prevented in the presence of a STAT3 inhibitor. These findings therefore provide a potential signaling pathway by which leptin regulates FGF21. A possible involvement of PPARα activation remains to be elucidated in future studies, but is likely to play a role in the crosstalk between leptin and FGF21. As *in vitro* studies do not take into account potential interactions between tissues, for example between liver and white adipose tissue, further studies are required *in vivo* to better clarify the potential regulation of FGF21 by leptin.

The role of FGF21 as a mediator of leptin action may be of significant relevance in various metabolic regulation processes. As an example, impaired leptin signaling in the liver, known to promote hepatic steatosis [38], may be due to impaired hepatic *Fgf21* expression. Nevertheless, the peripheral effect of leptin is a subject of debate. Notably, mice with specific leptin receptor deletion in the liver do not recapitulate the metabolic phenotype of *db/db* mice. Indeed, metabolic effects of leptin and notably its weight-reducing effects are more likely to be the results of the central leptin action on the brain [39]. However, obesity is a leptin resistance state [40]. Moreover, FGF21 levels are also increased in obesity, which also suggest a resistant state [41, 42]. Therefore, it could be speculated that co-administration of leptin and FGF21 could reveal a synergistic effect in obesity to overcome this state of hormonal resistance. This may also be true in other situations of hormone resistance, notably insulin resistance, encountered not only in obesity, but also in conditions associated with the metabolic syndrome, such as type 2 diabetes and nonalcoholic fatty liver disease [43]. These considerations warrant further *in vivo* studies.

Altogether, our results provide new findings regarding a leptin-STAT3-FGF21 axis that may lead to beneficial metabolic effects. These findings warrant further *in vivo* work to assess whether leptin-induced FGF21 could improve diseases associated with insulin resistance such as obesity, type 2 diabetes and nonalcoholic fatty liver disease.

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Disclosure Statement

The authors have nothing to disclose.

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