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**PPARα is a Key regulator of Hepatic FGF21**

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PPARα is a key regulator of hepatic FGF21

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Running head: FGF21 is a PPARα target gene.

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Abstract

The metabolic regulator fibroblast growth factor 21 (FGF21) has antidiabetic properties in animal models of diabetes and obesity. Using quantitative RT-PCR, we here show that the hepatic gene expression of FGF21 is regulated by the peroxisome proliferator-activated receptor alpha (PPARα). Fasting or treatment of mice with the PPARα agonist Wy-14,643 induced FGF21 mRNA by 10-fold and 8-fold respectively. In contrast, FGF21 mRNA was low in PPARα deficient mice, and fasting or treatment with Wy-14,643 did not induce FGF21. Obese ob/ob mice known to have increased PPARα levels, displayed 12-fold increased hepatic FGF21 mRNA levels. The potential importance of PPARα for FGF21 expression also in human liver was shown by Wy-14,643 induction of FGF21 mRNA in human primary hepatocytes and PPARα response elements have been identified in both the human and mouse FGF21 promoters. Further studies on the mechanisms of regulation of FGF21 by PPARα in humans is of great interest.

Key words: diabetes, fasting, fibrates, fibroblast growth factor 21, FGF21, ob/ob mice, peroxisome proliferator-activated receptor α, PPARα, Wy-14,643
Introduction

The recently discovered metabolic regulator fibroblast growth factor 21 (FGF21), preferentially expressed in the liver [1], has been shown to exert strong antidiabetic and triglyceride-lowering effects and also to reduce body weight, when given to animals with diet-induced or genetic obesity and diabetes, but not when administered to normoglycemic animals [2-4]. In spite of these dramatic responses little is yet known about the mechanism of action of FGF21 or its regulation apart from a recently described synergistic effect with PPARγ in adipose tissue [5]. We recently found that the hepatic gene expression of FGF21 is induced 2-4 fold following pharmacological or genetic interruption of bile acid circulation, and that feeding a sucrose-rich diet induces hepatic FGF21 expression remarkably by 25-fold [6] The highest FGF21 mRNA levels were seen in mice deficient in the apical sodium dependent transporter (ASBT) receiving a sucrose-rich diet. Interestingly, this diet also strongly reduced the high hepatic bile acid synthesis present in ASBT deficient animals, down to levels seen in normal wild type (wt) mice. Furthermore, the increase in hepatic triglycerides following the sucrose-rich diet in wt animals was attenuated in ASBT knock out mice [6].

The PPARα is a nuclear receptor that has been shown to be a key transcriptional regulator of numerous genes involved in lipid metabolism (for review see [7]). Targeted disruption of the PPARα by Lee et al substantiated the role of this receptor in lipid homeostasis [8]. The PPARα was shown to be involved in the adaptive response of mice to fasting, since fasted PPARα null mice show enhanced lipid accumulation in liver, are hypoglycaemic and have an impaired ketone body formation [9, 10]. Since PPARα stimulation also reduces bile acid synthesis [11, 12] and plasma triglycerides [11, 13, 14] we speculated that PPARα may be involved in the regulation of the expression of hepatic FGF21. To test this hypothesis we determined the FGF21 gene expression in liver from PPARα deficient mice and wild-type
controls during two different conditions known to stimulate PPARα activity, i.e. treatment with the agonist Wy-14,643 and acute fasting. Our results show that the activation of a PPARα response strongly induces the expression of FGF21 in normal mouse liver whereas in PPARα deficient mice, having very low basal expression of FGF21, these responses were absent. In ob/ob mice that are known to have an increased PPARα activity [15], hepatic basal FGF21 mRNA levels were increased 12-fold. Tentative PPARα responsive elements (PPREs) were present in the promoter regions of both mouse and human FGF21 genes, and treatment of human primary hepatocytes with Wy-14,643 stimulated FGF21 mRNA expression. Our results suggest that FGF21 may be a direct transcriptional target of PPARα.
Material and methods

Animals and treatment. Ten to twelve week old wild-type or PPARα-null male mice on a pure Sv/129 background (derived from the original colony of mixed background mice [8]) and male ob/ob mice obtained from Taconic, Ry, Denmark, were housed in a temperature and light-controlled environment. Mice were fed either a control chow diet (Lactamin R36, Vadstena, Sweden) or the chow diet containing 0.1% w/w Wy-14,643 (Wyeth14,643, Calbiochem-Novabiochem International) for one week. In the fasting experiment, mice were fasted for 24 hours and sacrificed at 9.00 am. All mice had access to water ad libitum. Animals were sacrificed by CO2 asphyxiation, followed by cervical dislocation and livers were excised and snap frozen in liquid nitrogen and subsequently stored at –70°C. The studies were approved by the Institutional Animal Care and Use Committee.

Preparation of Total RNA from mouse liver and Quantitative Real Time PCR. Total RNA was prepared from mouse liver using Trizol or QuickPrep® Total RNA extraction kit and DNase treated using DNasel (Promega). cDNA was synthesized from 1µg total RNA using SuperscriptIII according to the Manufacturer’s instructions (Invitrogen). Quantitative Real time PCR was performed in the ABI 7500 FAST Real-Time PCR System and relative mRNA expression was calculated according to the Manufacturer’s instructions (Applied Biosystems). TaqMan probes/primers were Assay on Demand; mouse FGF21 Assay ID: Mm00840165_g1, human FGF21 Assay ID: Hs00173927_m1. 18S and human acidic ribosomal phosphoprotein PO (RPLPO) were used as endogenous controls for mouse and human, respectively (Applied Biosystems).

Culture and Treatment of Human Primary Hepatocytes. Histologically normal liver tissue was obtained from a male subject following surgery for liver cancer at GastroCentrum, Karolinska University Hospital at Huddinge as part of studies approved by the Ethics
Committee at the Karolinska Institutet. Hepatocytes were isolated by a two-step perfusion technique, utilizing EGTA and collagenase as described [16]. Cells were cultured in 60 mm Petri dishes coated with 200 µL Engelbreth-Holm-Swarm (EHS) matrigel in 3 mL William’s E medium with glutamax, supplemented with insulin (2 IU/L), penicillin (100 U/mL), streptomycin (100 µg/mL) and gentamycin (85 µg/mL). The medium was changed daily, and following four days in culture hepatocytes were incubated with 50 µM Wy-14,643 or vehicle (dimethylsulfoxide, DMSO) for 48 hours. The medium was replaced with fresh medium containing 50 µM Wy-14,643 after 24 hours. The hepatocytes were collected in Trizol and total RNA was isolated, treated with DNase1 (Promega Corp) and stored at −70°C. cDNA was synthesized as described above for mouse liver.

Statistics. The significance of differences between groups in Figures 1 A and B was tested by 1-way ANOVA followed by post-hoc comparisons according to Tukey using GraphPad Prism version 4.03 (GraphPad Software, San Diego California USA). For the experiments in Figure 2 and 4, statistical significance was tested by unpaired Student’s t-test (GraphPad Software, SanDiego)
Results and Discussion

We first evaluated whether the basal expression of liver FGF21 is reduced in animals devoid of PPARα and whether there may be any differences between wt mice and mice devoid of PPARα upon treatment with a selective PPARα agonist (Figure 1A). FGF21 expression was strongly reduced in PPARα deficient animals and treatment with Wy-14,643 was without effect in PPARα-null mice. This was in strong contrast to wt animals where there was a clear 8-fold increase in the expression of FGF21. Thus, in line with our hypothesis, the expression of FGF21 in this experiment appeared to be completely dependent on the presence of PPARα indicating that FGF21 could be a target of PPARα. Based on the results obtained in this pharmacological model, we next explored if FGF21 could be influenced by PPARα under more physiological conditions.

For this purpose, we studied the expression of hepatic FGF21 following 24 hours fasting. Previous experiments have shown that PPARα deficient mice have a clearly reduced capacity to withstand food deprivation compared to wt mice that appears in part to be due to an impaired ability to form glucose and ketone bodies which is vital during prolonged fasting [9, 10]. We found that the FGF21 mRNA was strongly induced (10-fold) in livers of wt mice after 24 hours fasting, whereas in PPARα deficient mice, again expressing very low basal levels of FGF21, there was no induction of FGF21 mRNA (Figure 1B). Therefore under physiological conditions that activate PPARα, FGF21 is induced, lending further support to the possibility that FGF21 is actually a target gene of PPARα.

Leptin deficient mice (ob/ob) are known to have activated PPARα function, presumably due to increased levels of hepatic fatty acids [15]. We therefore hypothesized that ob/ob mice would have an increased hepatic expression of FGF21. mRNA levels of FGF21 were therefore measured in the livers of male ob/ob mice and indeed were shown to be elevated 12-fold (Figure 2).
The FGF21 gene is positioned on chromosome 7 and 19 in mouse and human, respectively. This region appears well conserved between the species and both the human and the mouse FGF21 genes are located in close proximity to the fucosyltransferase 1 (FUT1) gene. As shown in figure 3, we identified two putative PPREs in the mouse as well as human FGF21 promoter regions. These regions are located at -1143 and -2895 upstream of the translation start site (ATG) in the mouse gene and at -684 and -2454 in the human gene. Interestingly the direct repeat 1 (DR1) site located at -2895 in mouse is in fact a direct repeat 2 (DR2) in humans, with only 9 of the 13 base pairs conserved between species.

The finding of putative PPREs also in the human FGF21 gene prompted us to evaluate whether the increased gene expression of FGF21 elicited by Wy-14,643 treatment in mice may also occur in human hepatocytes. When human primary hepatocytes were treated with Wy-14,643, FGF21 gene expression was clearly induced 3-fold (Figure 4). Although these results indicate that PPARα is important also for the hepatic expression of FGF21 in humans, further studies remain to be performed to further explore this regulation. It could be speculated that this lack of conservation between these sites in human and mouse could account for the weaker regulation evident by Wy-14,643 in human hepatocytes compared to the regulation of FGF21 in mouse liver and could be due to interaction of other transcription factors on the same regulatory element. A DR2 flanked by an AT rich 5’ region in the human Rev-erbα promoter was shown to bind the PPARα/RXR heterodimer and was responsible for fibrate activation of the human Rev-erbα [17]. Further experiments are therefore required to examine if these DR1 or DR2 elements can in fact mediate the PPARα activation of the FGF21 genes in mouse or human.

Important questions to be answered are whether the gene expression of FGF21 is influenced by the feeding-fasting transition in humans, as recently reported for FGF19 [18]. It will be of particular interest to find out whether pharmacological treatment with PPARα agonists such
as fibrates may induce hepatic FGF21 expression and possible secretion of the protein into the blood in humans. Such mechanisms may explain some of the positive effects on triglyceride and glucose metabolism reported in response to fibrate therapy in humans [14]. Future studies are thus needed to establish whether pharmacological treatment with FGF21 protein in humans has similar metabolic effects to those previously shown in rodents and monkeys.

Acknowledgements

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References


Figure legends

Figure 1. PPARα regulates FGF21 in mouse liver.

A, Male wild type or PPARα-null mice were treated for one week with 0.1% Wy-14,643. mRNA levels of FGF21 were measured using real time PCR as described in Materials and Methods. mRNA expression is displayed as fold change where the expression of the wt group is normalized to 1. Data show mean +/- SEM. n=3.

B, Mice were fasted for 24 hours and mRNA levels of FGF21 were measured using real time PCR as described in Materials and Methods. mRNA expression is displayed as fold change where the expression of the wt group is normalized to 1. Data show mean +/- SEM. n=3.

Figure 2. Comparison of hepatic expression of FGF21 mRNA in ob/ob mice and lean controls. mRNA was measured in the livers of male ob/ob and lean mice. Hepatic mRNA expression is displayed as fold change where the expression of the lean group is normalized to 1. Data show mean +/- SEM. n=5-6.

Figure 3. The promoter regions of mouse and human FGF21 genes contain putative peroxisome proliferator-response elements (PPREs). The mouse and human FGF21 promoter regions were aligned using ClustalW. Two putative peroxisome proliferator-response elements are shown in boldface and underlined. The numbering shown is the position upstream of the translation start site ATG.

Figure 4. FGF21 expression is increased in human primary hepatocytes by the PPARα agonist Wy-14,643 (Wy). Human primary hepatocytes were treated with 50 µM Wy-14,643 for 48 hours. mRNA expression is displayed as fold change where the expression of vehicle-treated cells is normalized to 1. Data show mean +/- SEM. n=6.
**Figure 1**

**A**

Relative mRNA levels for wild type and PPARα KO mice on chow and Wy diets.

- **p<0.01** indicates statistical significance.
- **n.s.** indicates no significant difference.

**B**

Relative mRNA levels for wild type and PPARα KO mice on fed and fasted conditions.

- **p<0.05** indicates statistical significance.
- **p<0.01** indicates statistical significance.
- **n.s.** indicates no significant difference.
Figure 2

Comparison of relative mRNA levels between lean controls and ob/ob mice. The bar chart shows a significant difference with a p-value of <0.0001.
Putative conserved PPReS in human and mouse FGF21 promoters

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<td>mouse</td>
<td>-2916</td>
<td>TAAAAAGCTACTGCAAGTA---CAGGTGA-GAGCTCAAAAGATTTGCTGCCAGGTCCCAGGGGAG - 2878</td>
<td></td>
</tr>
<tr>
<td>human</td>
<td>-2490</td>
<td>CCAAATGACTGATAAGGTCAAGCAGTCACGTGATCAAGTCCTCCCTCCTTT - 2431</td>
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mouse - 1188 CCGAATG-CTAAGCAGGGTTGGTGAGG--CTGGGCTGTTGAGCTGGGAGACT - 1132
human - 732 TCTGACATCTGACAGCGACAGATGAGTTGAGGTGTTGCCCACGGCCAAGTGAAGGCT - 673

* * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * * *
Figure 4

Relative mRNA level

Vehicle  Wy

p<0.05