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Metformin-induced inhibition of the mitochondrial respiratory chain increases FGF21 expression via ATF4 activation

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ABSTRACT

Fibroblast growth factor 21 (FGF21) is an endocrine hormone that exhibits anti-obesity and anti-diabetes effects. Because metformin is widely used as a glucose-lowering agent in patients with type 2 diabetes (T2D), we investigated whether metformin modulates FGF21 expression in cell lines, and in mice or human subjects. We found that metformin increased the expression and release of FGF21 in a diverse set of cell types, including rat hepatoma FaO, primary mouse hepatocytes, and mouse embryonic fibroblasts (MEFs). Intriguingly, AMP-activated protein kinase (AMPK) was dispensable for the induction of *FGF21* by metformin. Mammalian target of rapamycin complex 1 (mTORC1) and peroxisome proliferator-activated receptor α (PPARα), which are additional targets of metformin, were not involved in metformin-induced *FGF21* expression. Importantly, inhibition of mitochondrial complex I activity by metformin resulted in *FGF21* induction through PKR-like ER kinase (PERK)-eukaryotic translation factor 2α (eIF2α)-activating transcription factor 4 (ATF4). We showed that metformin activated ATF4 and increased *FGF21* expression in the livers of mice, which led to increased serum levels of FGF21. We also found that serum FGF21 level was increased in human subjects with T2D after metformin therapy for 6 months. In conclusion, our results indicate that metformin induced expression of FGF21 through an ATF4-dependent mechanism by inhibiting mitochondrial respiration independently of AMPK. Therefore, FGF21 induction by metformin might explain a portion of the beneficial metabolic effects of metformin.

1. Introduction

FGF21 functions as an endocrine hormone and is produced in peripheral tissues such as liver, white/brown adipose, pancreas, and skeletal muscle [1] During starvation, PPARα-induced FGF21 expression is required for the activation of hepatic lipid oxidation, lipolysis, and ketogenesis, suggesting that FGF21 plays a key role in the adaptive response to fasting [2,3]. In addition, accumulating evidence suggests that FGF21 is an excellent therapeutic molecule for treatment of T2D. Specifically, in diabetic or obese animals, overexpression of FGF21 or administration of recombinant FGF21 ameliorates obesity, glucose clearance and insulin sensitivity [4,5].

Metformin is one of the most widely used anti-hyperglycemic agents for treating patients with T2D and is currently recommended as the drug of first choice according to the treatment guidelines of the American Diabetes Association and the European Association for the Study of Diabetes [6]. The glucose-lowering effect of metformin is attributed to the suppression of hepatic glucose production, decreased intestinal glucose absorption, enhancement of peripheral glucose utilization and abundance of *Akkermansia muciniphila*, a mucin-degrading member of the intestinal microbiota [7,8]. Although metformin has been used clinically for several years, the molecular mechanisms by which it exerts its glucose-lowering effects are still unclear. Previous studies have demonstrated that the beneficial effects of metformin are mediated by activation of AMPK [9,10]. AMPK acts as a cellular energy and nutrient sensor and is activated by an increased AMP:ATP ratio or decreased ATP production in response to a variety of metabolic stressors, including hypoxia and glucose deprivation [11]. However, recent studies have reported that metformin reduces hepatic glucose production by reducing hepatic energy charge [12] and increases glucose uptake by inhibiting mTORC1 in an AMPK-independent manner [13]. Thus, the molecular mechanisms underlying the beneficial metabolic effects of metformin have not yet been fully elucidated.

Several recent papers have reported that anti-diabetic drugs belonging to the thiazolidinedione class, such as rosiglitazone

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and pioglitazone, induce *FGF21* expression via PPARγ in mice [14]. Furthermore, the anti-dyslipidaemic drug fenofibrate also increases *FGF21* expression through a PPARα-dependent pathway [2]. Thus, FGF21 might be involved in the mechanisms of metabolic improvement of several anti-diabetic agents. Metformin, a well-known anti-diabetic drug, has been reported to exert beneficial metabolic effects through inhibition of mitochondrial complex I [12]. However, the molecular mechanism by which inhibition of the mitochondrial respiratory chain leads to beneficial metabolic effects is poorly understood.

We recently showed that FGF21 is induced by mitochondrial stress and functions as a 'mitokine' [15], and we thus investigated the role of FGF21 in metabolic improvement induced by metformin. We found that metformin-induced inhibition of mitochondrial complex I activity led to increased *FGF21* gene expression and secretion through a PERK-eIF2α-ATF4 pathway and that serum FGF21 level was increased in mice and human subjects after metformin treatment, suggesting the possibility that FGF21 induction is one of the important mechanisms linking mitochondrial complex I inhibition and metformin to metabolic improvement.

2. Material and methods

2.1. Animal experiments

Male C57BL/6 mice were purchased from Orient-Bio Laboratory (Sungnam, Korea). All animals were maintained in a facility accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International. All animal experiments were conducted in accordance with a protocol approved by the Institutional Animal Care and Use Committee of Samsung Biomedical Research Institute.

2.2. Human subjects

Seven patients with T2D were recruited to receive metformin therapy (1000 mg/day) for 6 months. The enrollment criteria were as follows: age between 20 and 70 years, HbA_{1c} > 8% (67 mmol/mol) on glimepiride 4 mg/day or equivalent dose of other sulfonylureas, no other severe illnesses including liver failure, renal failure, heart failure, etc. Baseline characteristics of human subjects are summarized in Supplementary Table 1. The study protocol was approved by the institutional review board of the Clinical Research Institute of the Seoul National University Hospital, and informed consent was obtained from the subjects. The laboratory studies were performed in the morning at 8 a.m. after an overnight fast. Subjects were asked not to take medication in the morning.

2.3. Cell culture

FaO cells were obtained from the American Type Culture Collection. AMPK MEFs were provided by Benoit Viollet [16]. PERK, general control nonderepressible 2 (GCN2), and ATF4 MEFs were generous gifts from David Ron. dsRNA-dependent protein kinase (PKR) or eIF2α MEFs were gifted from Randal Kaufman. Primary mouse hepatocytes were isolated by previously described protocol [15]. All cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2.

2.4. RNA isolation and real-time reverse transcription (RT)-PCR

Total RNA from various cells or tissues was prepared using TRIzol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. cDNA was synthesized from 2 μg of total RNA using MMLV (Moloney murine leukemia virus)-reverse transcriptase (Promega, Madison, WI, USA) and oligo (T) primer and then was subjected to PCR amplification using mouse (m), rat (r), and human (h) gene-specific primers (5′–3′): hATF4, GGAGTTCGACTTGGATGCCC (forward, F) and GCCAATTGGGTTCACCGTCT (reverse, R); m,rFGF21, TACACAGATGACGACCAAGA (F) and GGC TTCAGACTGGTACACAT (R); hFGF21, GCGGTACCTCTACACAGATG (F) and ACATTGTATCCGTCCTCAAG (R); m,rL32, CAGTCAGACCGATATGTG AA (F) and TAGAGGACACATTGTGAGCA (R); hL32, CATCCGG CACCAGTCAGACC (F) and TGTGAGCGATCTCGGCACAG (R); m,rPPARα, GGATGTCACACAATGCAATTCG (F) and TCACAGAA CGGCTTCCTCAGGT (R). Real-time RT-PCR was performed using SYBR Green I (Takara, Otsu, Japan) with an ABI Prism 7000 instrument (Applied Biosystems, Foster City, CA, USA). All expression values were normalized to *L32* mRNA levels.

2.5. Statistical analysis

All values were expressed as mean ± SEM. Statistical analyses were performed using GraphPad Prism Version 5.02 Software. Statistical significance was tested by unpaired Student's *t*-test or Wilcoxon matched pairs test (for comparison of metabolites in diabetic subjects before and after metformin therapy). *p* values less than 0.05 were considered to represent statistically significant differences.

2.6. Supplementary materials and methods

Supplementary data includes experimental information for plasmid constructs and reagents, construction of adenovirus, luciferase assay, immunoblot analysis, measurement of metabolites and FGF21, glucose tolerance test (GTT) and measurement of mitochondrial complex I activity.

3. Results

3.1. Metformin induces FGF21 expression and secretion in an AMPK independent manner

Because FGF21 is predominantly expressed in the liver, we used rat hepatoma FaO cells and primary mouse hepatocytes to investigate the effect of metformin on FGF21 expression. Importantly, metformin significantly increased *FGF21* gene expression in both a time and dose-dependent manner in FaO cells (Fig. 1A and B). We also observed increased gene expression of *FGF21* in primary mouse hepatocytes treated with metformin (Fig. 1C). Consistently, metformin significantly enhanced secretion of FGF21 in the culture supernatants of mouse hepatocytes (Fig. 1D). Together, these data suggest that metformin induces *FGF21* gene expression in hepatocytes.

We next studied the molecular mechanisms by which metformin induces *FGF21* gene expression. Because AMPK has been reported to play a crucial role in the beneficial effects of metformin [9,10], we utilized AMPKα null MEFs deficient in both AMPKα1 and AMPKα2. Intriguingly, metformin continued to have an effect on *FGF21* expression in AMPKα null MEFs compared to AMPKα WT MEFs (Fig. 1E). To further study the role of AMPK, we generated adenoviruses expressing either constitutively active (CA)-AMPKα1 or dominant negative (DN)-AMPKα1 (Fig. 1F). We observed that *FGF21* gene expression was not affected by adenoviral expression of CA-AMPKα1 or DN-AMPKα1 (Fig. 1G and H), suggesting that AMPK is not required for FGF21 induction by metformin. Furthermore, aminoimidazole carboxamide ribonucleotide (AICAR), an AMPK activator, did not increase the expression of *FGF21* gene or the secretion of FGF21 in FaO cells or mouse hepatocytes (Fig. S1A–C). Unexpectedly, *FGF21* expression was significantly

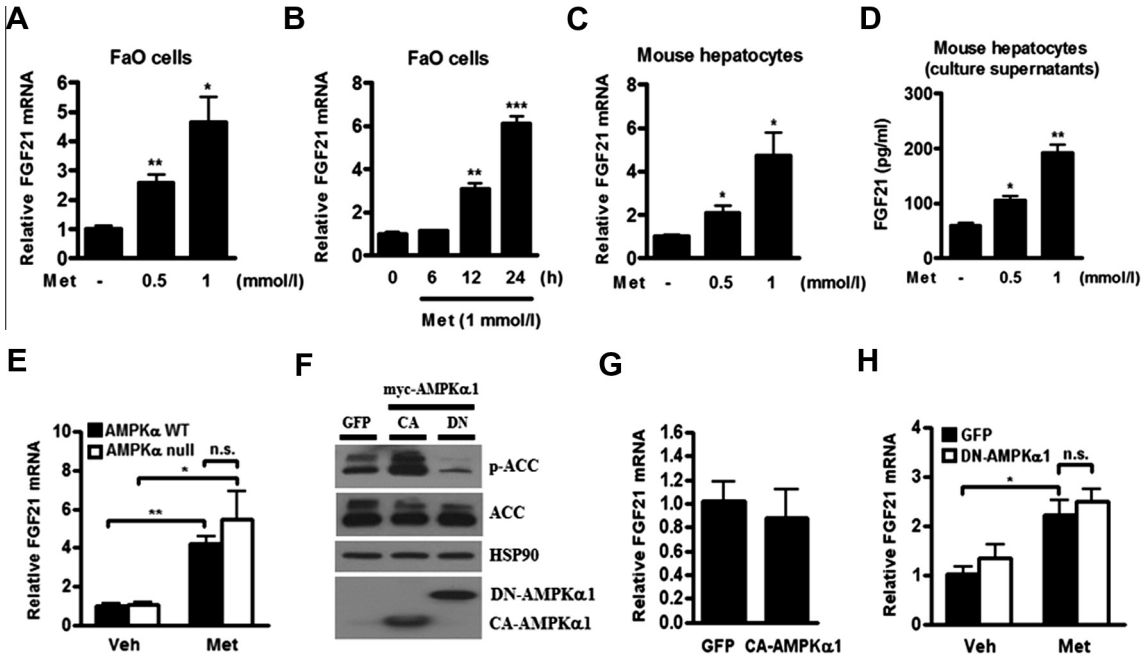

Fig. 1. AMPK-independent induction of FGF21 by metformin. (A, B) Relative *FGF21* mRNA expression in FaO cells treated with metformin (Met) at the indicated concentrations for 24 h ($n = 3$) (A) and at 1 mmol/l metformin for the indicated times ($n = 3$) (B). (C) Relative *FGF21* mRNA expression in primary mouse hepatocytes treated with metformin for 24 h ($n = 3$). (D) FGF21 concentration in culture supernatants of mouse hepatocytes treated with metformin for 24 h ($n = 3$). FGF21 protein levels were measured by ELISA. (E) Relative *FGF21* mRNA expression in AMPKα WT or null MEFs treated with 2 mmol/l metformin or vehicle for 24 h ($n = 3$). (F) Immunoblotting for p-ACC, ACC, CA-AMPKα1 and DN-AMPKα1 in primary mouse hepatocytes infected with adenovirus expressing GFP, CA-AMPKα1, or DN-AMPKα1 (Hsp90, loading control). (G) Relative *FGF21* mRNA expression in mouse hepatocytes infected with adenovirus expressing GFP or CA-AMPKα1 for 48 h ($n = 3$). (H) Relative *FGF21* mRNA expression in mouse hepatocytes treated with 1 mmol/l metformin for 24 h after infection with adenovirus expressing GFP or DN-AMPKα1 ($n = 3$). Data are mean ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n.s. indicates not significant).

decreased in cells treated with AICAR. Furthermore, AICAR-mediated *FGF21* expression was repressed to a similar degree in both AMPK WT and AMPK null MEFs (Fig. S1D), implying that AICAR influences *FGF21* gene expression in an AMPK-independent manner. Taken together, these results indicate that AMPK is not necessary for the induction of FGF21 by metformin.

3.2. Metformin enhances FGF21 expression by inhibiting the activity of mitochondrial complex I

It has recently been shown that metformin stimulates glucose uptake and the incretin axis via AMPK-independent mTORC1 inhibition and PPARα activation, respectively [13,17]. Thus, we next investigated if either mTORC1 or PPARα could affect *FGF21* gene expression. To examine the relationship between mTORC1 inhibition and FGF21 induction, we treated FaO cells and mouse hepatocytes with either rapamycin or Torin1, which are inhibitors of mTORC1. Neither rapamycin nor Torin1 had an effect on *FGF21* induction in these cells (Fig. S2A and B). We next studied the effects of metformin on the gene expression and transcriptional activity of PPARα, which is a positive regulator of *FGF21* expression [2,3], and found that metformin did not increase promoter activity or gene expression of PPARα (Fig. S2C and D). Moreover, we found no evidence of a positive effect of metformin on PPARα transcriptional activity, whereas metformin suppressed the increased transcriptional activity of PPARα by WY-14643, a specific PPARα agonist (Fig. S2E). These data suggest that mTORC1 and PPARα, two AMPK-independent targets of metformin, are not associated with FGF21 induction by metformin.

Because previous reports showed that metformin inhibits mitochondrial complex I activity and mitochondrial stress leads to FGF21 induction as a 'mitokine' [12,15,18], we studied whether FGF21 induction by metformin is associated with repression of respiratory chain complex I activity. Consistent with previous reports, we found that metformin inhibited mitochondrial complex I activity of FaO cells (Fig. 2A). Consistently, intracellular ATP content was decreased in FaO cells treated with metformin compared

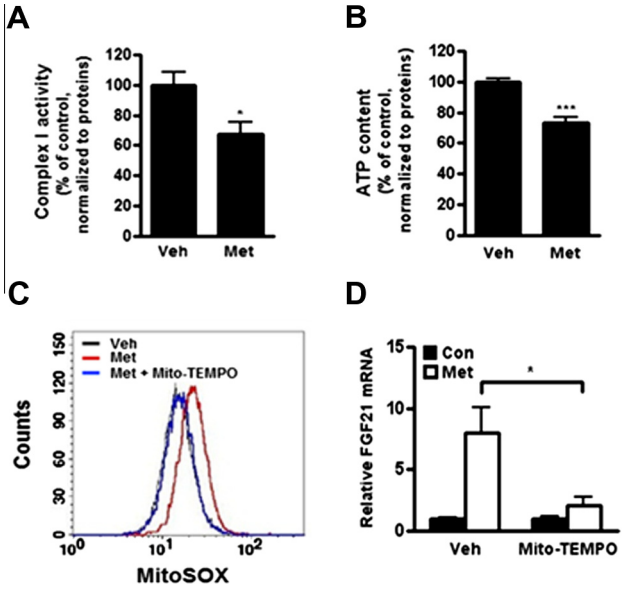

Fig. 2. Metformin-induced FGF21 expression via inhibition of the mitochondrial respiratory chain. (A, B) Mitochondrial complex I activity ($n = 4$) (A) and ATP content ($n = 3$) (B) in FaO cells treated with 1 mmol/l metformin or vehicle for 24 h. (C, D) MitoSOX staining (C) and relative *FGF21* mRNA expression (D) in FaO cells treated with 1 mmol/l metformin or 50 μmol/l Mito-TEMPO for 24 h ($n = 3$). Cells were pretreated with Mito-TEMPO for 1 h prior to metformin treatment. Data are mean ± SEM ($^{*}p < 0.05$, $^{***}p < 0.001$).

to that of vehicle-treated cells, which was probably due to reduced production of ATP by inhibition of mitochondrial electron transport (Fig. 2B). In addition, metformin caused a mild increase of mitochondrial superoxide levels via partial inhibition of complex I activity (Fig. 2C). Because our previous report showed that FGF21 expression is induced by impairment of mitochondrial oxidative phosphorylation [15], we next studied whether metformin-induced mild oxidative stress is involved in FGF21 induction. Interestingly, metformin-induced *FGF21* expression was significantly alleviated by treatment with Mito-TEMPO, a mitochondria-targeted superoxide scavenger (Fig. 2C & D). Together, these results indicate that metformin induces a mild increase in mitochondrial redox oxidative stress (ROS), which enhances the expression of FGF21.

3.3. The PERK-eIF2α-ATF4 axis mediates metformin-induced FGF21 expression

We next investigated the mechanism by which mild mitochondrial oxidative stress induced by metformin increases *FGF21* gene expression. ROS generated by mitochondrial stress has been reported to activate the PERK-eIF2α-ATF4 signaling pathway [19]. Likewise, we previously showed that FGF21 induction by mitochondrial stress is mediated by ATF4 [15]. Thus, we asked whether the PERK-eIF2α-ATF4 axis mediates FGF21 induction by metformin. Importantly, PERK deletion attenuated the induction of *FGF21* in MEFs treated with metformin (Fig. 3A). In contrast, deletion of PKR or GCN2, other eIF2α upstream kinases, had no effect on metformin-induced *FGF21* expression (Fig. 3A), despite previous suggestions that PKR or GCN2 is involved in the adaptive response to mitochondrial stress [20,21]. Intriguingly, *FGF21* induction by rotenone, a known complex I inhibitor, was also attenuated by a deficiency of PERK, but not PKR and GCN2, implying that metformin induces FGF21 expression in a PERK-dependent manner by inhibiting the activity of complex I (Fig. S3). We also showed that the induction of *FGF21* by metformin was remarkably reduced in eIF2α$^{A/A}$ and ATF4 null MEFs compared to eIF2αS$^{/S}$ and ATF4 WT MEFs, respectively (Fig. 3B and C). In addition, transfection of mutant *FGF21* reporter harboring mutations in ATF4 binding sites confirmed the importance of ATF4 for metformin-mediated induction of *FGF21* expression (Fig. 3D). Consistent with these results, we observed increased expression levels of ATF4 protein in mouse hepatocytes or FaO cells treated with metformin (Fig. 3E). Furthermore, Mito-TEMPO treatment alleviated metformin-induced ATF4 activation (Fig. 3F), consistent with the attenuation of metformin-mediated *FGF21* induction by Mito-TEMPO (Fig. 2D). In parallel, hydrogen peroxide increased *FGF21* gene expression, which was partially mediated by ATF4 (Fig. S4). Taken together, these data suggest that ATF4 plays a crucial role in mediating metformin-induced FGF21 expression.

3.4. Metformin activates the ATF4-FGF21 axis in mouse liver and increases serum FGF21 level in diabetic human subjects

Metformin improves glucose tolerance and insulin resistance in high-fat diet (HFD)-fed mice [7,12]. To investigate the *in vivo* effects of metformin in FGF21 induction, we challenged HFD-fed mice with metformin for 2 weeks. Consistent with previous reports [7,12], metformin attenuated glucose intolerance in HFD-fed mice (Fig. 4A). Importantly, serum FGF21 levels were higher in mice treated with metformin compared to vehicle-treated mice (Fig. 4B). Because FGF21 is predominantly expressed in the liver, we next investigated hepatic FGF21 expression in mice treated with metformin. We found that *FGF21* gene expression as well as ATF4 protein levels were increased in the livers of mice treated with metformin (Fig. 4C and D). Therefore, our findings indicate that metformin is able to induce FGF21 expression in mouse liver *in vivo* as well as in hepatocytes *in vitro*.

To investigate whether metformin affects FGF21 level in human subjects, we analyzed serum FGF21 level in subjects with T2D before and after metformin therapy for 6 months. Body weight and BMI were not changed in diabetic subjects after metformin therapy

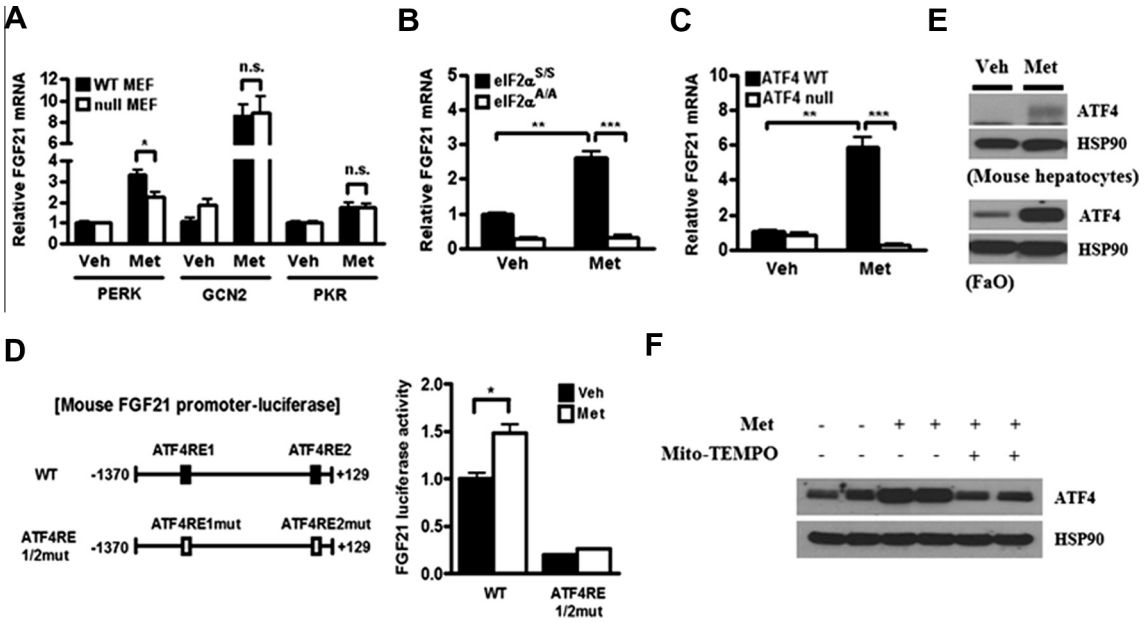

Fig. 3. Role of the PERK-eIF2α-ATF4 axis in metformin-induced FGF21 expression. (A) Relative *FGF21* mRNA expression in PERK, GCN2 or PKR MEFs after treatment with 2 mmol/l metformin for 24 h ($n = 3$). (B, C) Relative *FGF21* mRNA expression in eIF2α$^{S/S}$ (WT) or eIF2α$^{A/A}$ MEFs (mutant) (B) and ATF4 WT or null MEFs (C) after treatment with 2 mmol/l metformin for 24 h ($n = 3$). (D) Luciferase activity of WT and ATF4RE1/2mut *FGF21* promoter in FaO cells after treatment with 1 mmol/l metformin for 24 h ($n = 3$). (E, F) Immunoblotting for ATF4 and HSP90 in mouse hepatocytes or FaO cells treated with 1 mmol/l metformin for 24 h (E) and in FaO cells treated with 1 mmol/l metformin or 50 μmol/l Mito-TEMPO for 24 h (F). Cells were pretreated with Mito-TEMPO for 1 h prior to metformin treatment. Data are mean ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n.s. indicates not significant).

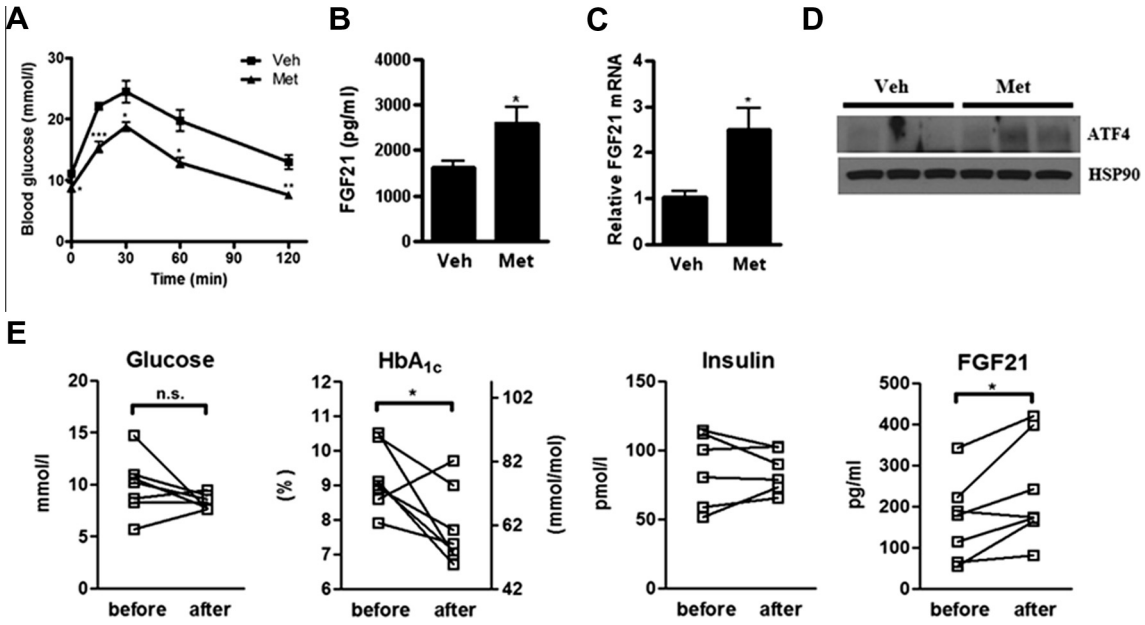

Fig. 4. Activation of the hepatic ATF4-FGF21 axis in metformin-treated mice and increased serum FGF21 level in diabetic human subjects after metformin therapy. (A) GTT in HFD-fed male C57BL/6 mice treated with metformin for 2 weeks ($n = 4$). (B–D) Fasting serum FGF21 concentrations ($n = 9$–10) (B), relative *FGF21* mRNA expression ($n = 3$) (C) and ATF4 protein levels in the livers ($n = 3$) (D) of HFD-fed male C57BL/6 mice treated with metformin for 2 weeks. (E) Fasting glucose ($n = 7$), HbA_{1c} ($n = 7$), insulin ($n = 6$) and FGF21 ($n = 7$) levels in human subjects with T2D before and after metformin therapy for 6 months. Data are mean ± SEM ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$; n.s. indicates not significant).

(data not shown). Fasting glucose and HbA_{1c} levels were lower after metformin treatment, when compared to those before metformin therapy, although the difference in fasting glucose level was not statistically significant (Fig. 4E). There was no difference in serum insulin level between before and after metformin treatment (Fig. 4E). Intriguingly, after metformin treatment, serum FGF21 level was significantly increased in diabetic subjects (Fig. 4E). Together, these findings suggest that metformin treatment is able to induce FGF21 in human subjects as well.

4. Discussion

Although metformin has a glucose-lowering effect by suppressing hepatic glucose output and enhancing peripheral glucose utilization, the molecular mechanism underlying such metabolic effects of metformin have not been clearly elucidated. In the present study, we showed that metformin induced FGF21 expression in hepatocytes *in vitro* and in the liver of mice *in vivo*. Intriguingly, FGF21 induction by metformin was due to ATF4 activation via inhibition of mitochondria complex I activity. In addition, we found that serum FGF21 level was increased in diabetic subjects after metformin therapy.

Although the AMPK has been reported to play a crucial role in metformin-induced beneficial metabolic effects [9,10], recent studies have suggested the potential role of AMPK-independent pathways such as the inhibition of mitochondrial respiratory chain activity [12,22]. However, the mechanisms by which inhibition of mitochondrial electron transport can lead to beneficial metabolic effects are poorly understood. Here, we demonstrated that metformin induced expression of FGF21 through an ATF4-dependent mechanism by inhibiting mitochondrial respiration independently of AMPK. Thus, our findings suggest the possibility that ATF4 is a downstream mediator responsible for the beneficial metabolic effects of metformin.

Consistent with our results, a recent paper has been reported that metformin induces *FGF21* gene expression in hepatocytes [23]. However, this paper studied only *in vitro* effects of metformin on FGF21 induction. In the present study, we found that metformin was able to induce FGF21 expression in mice and human subjects. These data, to our knowledge, provide the first evidence for *in vivo* effects of metformin on FGF21 induction. Thus, our findings suggest a possibility that FGF21 induction is one of the mechanisms by which metformin exerts its beneficial metabolic effects.

In contrast to previous results showing the importance of AMPK on metformin- or AICAR-induced FGF21 expression in primary rat and human hepatocytes [23], our present data suggest that metformin induced FGF21 induction independent of AMPK. Likewise, we also showed that AICAR suppressed FGF21 expression in an AMPK-independent manner, although further studies are necessary to determine the molecular mechanism underlying AICAR-mediated repression of FGF21 expression. Our experiments using AMPK null MEFs and CA- or DN-AMPK adenovirus indicated that AMPK was not related to metformin-mediated FGF21 induction. Although the exact mechanism responsible for this discrepancy is unknown, differences in experimental procedures and cell lines may provide an explanation.

In conclusion, the data in the present study show that metformin induces inhibition of mitochondrial respiration, leading to FGF21 induction via an ATF4-dependent mechanism. FGF21 has been reported to have a glucose-lowing effect by enhancing glucose uptake in skeletal muscle and adipose tissue, suppressing glucose production in liver and increasing insulin secretion in islet β-cells. Thus, our data showing FGF21 induction by metformin *in vitro* and *in vivo* provides new insights regarding the role of FGF21 on the beneficial metabolic effects induced by metformin.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2013.09.026.

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