Diet Polyphenol Curcumin Stimulates Hepatic Fgf21 Production and Restores Its Sensitivity in High-Fat-Diet–Fed Male Mice

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We found previously that short-term curcumin gavage stimulated mouse hepatic fibroblast growth factor 21 (Fgf21) expression. Here we conducted mechanistic exploration and investigated the potential pathophysiological relevance on this regulation. Fgf21 stimulation was observed at messenger RNA and protein levels in mice with daily curcumin gavage for 4 or 8 days and in primary hepatocytes with curcumin treatment. Using peroxisome proliferator-activated receptor α (PPARα) agonist and antagonist, along with luciferase reporter and chromatin immune-precipitation approaches, we determined that curcumin stimulates Fgf21 transcription in a mechanism involving PPARα activation. High-fat diet (HFD) feeding also increased mouse hepatic and serum Fgf21 levels, whereas dietary curcumin intervention attenuated these increases. We found that HFD feeding reduced hepatic expression levels of genes that encode FGFR1 and βKlotho, PGC1α, and the targets of the PPARα–PGC1α axis, whereas concomitant curcumin intervention restored or partially restored their expression levels. Importantly, hepatocytes from HFD-fed mice showed a loss of response to FGF21 treatment on Erk phosphorylation and the expression of Egr1 and cFos; this response was restored in hepatocytes from HFD-fed mice with curcumin intervention. This investigation expanded our mechanistic understanding of the metabolic beneficial effects of dietary curcumin intervention involving the regulation of Fgf21 production and the attenuation of HFD-induced Fgf21 resistance. (Endocrinology 158: 277–292, 2017)

Fibroblast growth factor 21 (Fgf21) is mainly produced in and released by the liver in response to fasting (1–4). The lack of the conventional heparin binding domain of this unique FGF family member allows this hepatokine to diffuse away from its site of origin and function as a hormone (4–6). Intensive investigations have revealed that the elevation of Fgf21 production during fasting occurs via a mechanism involving activation of peroxisome proliferator–activated receptor α (PPARα) (1, 2, 7, 8). During the adaptive starvation response, Fgf21 may induce hepatic expression or activation of peroxisome proliferator-activated receptor γ coactivator 1-α (PGC1α), which facilitates fatty acid oxidation, tricarboxylic acid cycle flux, and gluconeogenesis (9).

Over the past a few years, we have seen growing evidence for the therapeutic potential of Fgf21 in metabolic disorders, including diabetes and its complications,
obesity, dyslipidemia, and liver steatosis, in humans and in animal models (4, 10–15); however, animals or human subjects with obesity or diabetes were paradoxically found to have elevated plasma Fgf21 levels, raising the hypothesis that obesity is an Fgf21-resistant state (16–21). Indeed, Fisher et al. (16) have shown that high-fat/high-sucrose diet–induced obese mice displayed an attenuated response to Fgf21 treatment, associated with reduced messenger RNA (mRNA) expression of FGF receptors in the liver and white adipose tissue. In a mouse model with mild nonalcoholic fatty liver disease, Rusli et al. (22) demonstrated that an elevated plasma Fgf21 level was associated with decreased expression of the gene that encodes the Fgf21 co-receptor βKlotho.

Curcumin, anthocyanin, and resveratrol are the most studied dietary plant polyphenols (23, 24). As a traditional medicine and the major component of turmeric, curcumin possesses antimicrobial, insecticidal, cardioprotective, radioprotective, and anticancer properties (24). Importantly, several preclinical investigations have shown the effect of curcumin in improving insulin signaling and reducing body weight gain in high-fat diet (HFD)-fed mice (25–27) and have attributed such effects to the anti-inflammation and antioxidative stress functions (25, 26, 28, 29). A recent small-scale clinical trial indicated that curcumin intervention in prediabetic subjects lowered the number of individuals who eventually developed type 2 diabetes (T2D) (30). Thus, the nutraceutical curcumin possesses great potential in the prevention and treatment of obesity, diabetes, and other metabolic disorders in which insulin resistance plays a fundamental role.

We demonstrated very recently that daily curcumin gavage for 6 days improved insulin signaling in mice that received dexamethasone injections (31). In that study, we also noticed the stimulation of hepatic Fgf21 protein expression by curcumin gavage (31). Here we conducted a mechanistic exploration examining Fgf21 induction by curcumin treatment in vivo and in vitro and investigated the potential pathophysiological relevance of the role of curcumin on Fgf21 production and function in the HFD-fed mouse model. Our findings suggest the existence of both a short-term stimulatory effect of curcumin on Fgf21 production and a long-term effect of dietary curcumin intervention on attenuating Fgf21 resistance in mice consuming a HFD.

Materials and Methods

Reagents

Curcumin was purchased either from Sigma-Aldrich (St. Louis, MO) for in vitro cell culture experiments or from Organika Health Products (Richmond, BC, Canada; a 95% standardized curcumin extract) for short-term gavage or long-term dietary intervention in mice. The PPARα antagonist GW6471 (G5045) and the agonist Wy14643 (C7081) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Actinomycin D was purchased from Calbiochem (La Jolla, CA). Human recombinant Fgf21 was from Novoprotein Scientific Inc. (Summit, NJ). The cyclic adenosine monophosphate-promoting agents forskolin and 3-isobutyl-1-methylxanthine (IBMX) were from Sigma-Aldrich.

Animals and animal experimental design

Eight-week-old, male C57BL/6J mice purchased from Charles River Laboratories (St. Laurent, QC, Canada) were housed at constant temperature (22°C) under a 12-hour light/dark cycle with free access to food and water. The animal experiments and protocols were approved by the University Health Network Animal Care Committee and performed in accordance with the guidelines of the Canadian Council of Animal Care.

Experiment 1

The protocol for 6 days curcumin gavage was previously presented (31). Briefly, male mice received either sesame oil (solvent) or curcumin (500 mg/kg body weight/d) gavage every day for 5 consecutive days. After 1 day of rest, an intraperitoneal insulin tolerance test was performed at day 7. A booster curcumin (or solvent) gavage was given on day 8. The mice were killed for liver tissue and serum collection on day 10. This experiment is defined as 6-day curcumin gavage.

Experiment 2

The same experiment as experiment 1 was performed, except the dosage of curcumin was reduced to 100 mg/kg body weight/d for 4 or 8 days before animals were killed for liver tissue and serum collection.

Experiment 3

Eight-week-old male mice were fed a low-fat diet (LFD) or an HFD (60% Kcal from soybean fat) with or without curcumin (4 g/kg diet) for 12 weeks (26). An intraperitoneal glucose tolerance test (IPGTT) was performed as previously described (26).

Cell culture

The human HepG2 and mouse Hepa1-6 cell lines were cultured in Dulbecco’s modified Eagle medium supplemented with 10% fetal bovine serum (32). C57BL/6J male mice (8–12 weeks old) were used for primary hepatocyte isolation as described previously (33, 34). Human hepatocytes were isolated from a 16-year-old male donor without a known hepatic disease, with the 2-step collagenase isolation method in the laboratory approved with Good Laboratory Practice. The study was approved by the Research Ethics Board of the University Health Network.

Fgf21, triglyceride, and free fatty acid measurements

The quantitative measurement of Fgf21 in mouse serum was performed with the Fgf21 Immunoassay Kit (catalogue number 32180) provided by Antibody and Immunoassay Services (The University of Hong Kong). Liver triglyceride contents were determined using the Serum Triglyceride Determination Kit.
Preparation of cell lysates and Western blotting

Whole-cell lysates from mouse liver tissue, primary hepatocytes, or cultured cell lines were prepared and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis followed by Western blotting as previously described (35). Antibodies for Western blotting are listed in Table 1.

RNA extraction, real-time reverse transcription polymerase chain reaction, and the assessment of the effect of curcumin treatment on Fgf21 mRNA stability

TRI reagent (Sigma-Aldrich) was used for RNA extraction. Real-time polymerase chain reaction (PCR) was performed as previously described (36). PCR primers are listed in Supplemental Table 1. The method for assessing the effect of curcumin on Fgf21 mRNA stability is detailed in Supplemental Methods.

Luciferase assay, chromatin immunoprecipitation, and GAL4 nuclear receptor assay

The construction of the fusion gene constructs in which the expression of the luciferase (LUC) reporter is driven by an ~1.5-kb or ~3.1-kb mouse Fgf21 5' flanking sequence is detailed in the Supplemental Methods. The LUC assay was conducted as previously described (33). The procedures for chromatin immunoprecipitation (ChIP) and quantitative ChIP (qChIP), as well as GAL4 nuclear receptor assay, are detailed in the Supplemental Methods. Supplemental Table 1 also contains the primers used in ChIP and qChIP.

Statistics

Quantitative results are expressed as a mean ± standard deviation. For the comparison of 2 groups, the 2-tailed, unpaired Student t test was used in the statistical analysis, with significance at P < 0.05. Comparisons between groups with 1 treatment were determined by one-way analysis of variance with a post hoc Bonferroni test.

Results

Curcumin treatment increases hepatic Fgf21 expression

We found previously that 6-day curcumin gavage in dexamethasone-injected mice improved insulin signaling and increased hepatic Fgf21 protein levels (31). Because dexamethasone can stimulate Fgf21 expression as well (37), we tested whether short-term curcumin gavage in mice on chow diet increases Fgf21 expression at both mRNA and protein levels in the absence of dexamethasone injection. As shown in Fig. 1(A), 6-day curcumin

Table 1. Antibodies Used

<table>
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<tr>
<th>Peptide/Protein Target</th>
<th>Antigen Sequence (if known)</th>
<th>Name of Antibody</th>
<th>Manufacturer, Catalog No. and/or Name of Individual Providing the Antibody</th>
<th>Species Raised in; Monoclonal or Polyclonal</th>
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<td>Anti-Heme oxygenase 1</td>
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GAPDH, glyceraldehyde 3-phosphate dehydrogenase; NF, nuclear factor; RRID, Research Resource Identifier.

* Diluted 1:25 for ChIP assay.
Figure 1. Curcumin increases hepatic Fgf21 expression in mice and mouse primary hepatocytes. Hepatic Fgf21 (A) and PPARα (B) mRNA levels in mice receiving daily curcumin (500 mg/kg body weight) or control vehicle gavage for 6 days, assessed by quantitative reverse transcription PCR. (C) Random serum Fgf21 levels of the mice depicted in A. Hepatic Fgf21 (D) and PPARα (E) mRNA levels in mice receiving curcumin (100 mg/kg body weight) or control vehicle gavage for 4 or 8 days. (F) Western blotting of hepatic Fgf21 protein expression in aforementioned mice depicted in panel D. (G) Quantification of panel F. (H) Random serum Fgf21 levels of the mice depicted in panel D. (I) Mouse primary hepatocytes were treated with the indicated doses of curcumin for 6 hours followed by cell harvesting and Western blotting with the indicated antibodies. (J–L) Mouse primary hepatocytes were treated with the indicated doses of curcumin for 6 hours (J and L) or with 2 μM curcumin for the indicated time intervals followed by cell harvesting and quantitative reverse transcription PCR against indicated genes. Values are means ± SD (n = 6 for A–C, n = 5 for D–H, and n ≥ 3 for J–L). Panel I is a represented blot of 3 independent experiments. *P < 0.05 vs the corresponding control. CUR, curcumin; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; V, control vehicle.
gavage increased hepatic *Fgf21* mRNA level, associated with PPARα elevation [Fig. 1(B)]. Plasma *Fgf21* levels in mice in this set of experiments showed a trend toward an increased response to curcumin gavage, but the difference did not reach statistical significance [Fig. 1(C)]. To confirm the in vivo stimulatory effect, we repeated the curcumin gavage experiment in C57BL/6 mice and reduced the dosage from 500 to 100 mg/kg body weight/d. Figure 1(D) and 1(E) shows that curcumin gavage for either 4 or 8 days increased hepatic *Fgf21* and PPARα mRNA levels. We also observed an increase of *Fgf21* expression at the protein level [Fig. 1(F) and 1(G)]. In this experiment, the effect on random plasma *Fgf21* level elevation was observed for mice receiving either 4- or 8-day curcumin gavage (Fig. 1H).

We then conducted in vitro experiments. As shown in Fig. 1(I) and 1(J) and in Supplemental Fig. 1A, *Fgf21* mRNA and protein expression in mouse primary hepatocytes can be stimulated by 12 hours of curcumin treatment at dosages varying from 0.5 to 10 μM but not at 20 μM, although the activation on *Fgf21* protein expression at 0.5 μM was not always observable. Figure 1(K) shows that *Fgf21* mRNA expression can be stimulated by 2 μM curcumin at time courses from 4 to 24 hours. Stimulated *Fgf21* mRNA expression is associated with elevated expression of PPARα [Fig. 1(L)]. The stimulatory effect of curcumin on *Fgf21* was also observed in human hepatocytes [Supplemental Fig. 1(B) and 1(C)].

The *Fgf21* protein level in the human HepG2 cell line was very low in our study, and in the mouse Hepa1-6 cell line it was below the detection limit by Western blotting [Supplemental Fig. 2(A) and 2(B)]. Indeed, we could not detect *Fgf21* mRNA expression in the Hepa1-6 cell line by reverse transcription PCR [Supplemental Fig. 2(C)].

Because curcumin was shown to exert anti-inflammatory and antioxidative stress effects in hepatocytes and elsewhere (25, 26), we also tested its effect on reducing nuclear factor κB and increasing heme oxygenase-1 expression in mouse hepatocytes (Supplemental Fig. 3). Because these effects were achieved only at concentrations higher than 2 μM, we suggest that curcumin-stimulated *Fgf21* expression is likely independent of its anti-inflammatory and antioxidative stress effects.

PPARα is among mediators that convey the stimulatory effect of curcumin on *Fgf21* transcription

PPARα positively regulates *Fgf21* transcription, and 2 evolutionarily conserved PPAR response elements (PPREs) were located on the *Fgf21* gene promoter regions (1, 8). We have hence constructed the *Fgf21*-LUC reporter gene constructs in which the expression of the LUC reporter is driven by either -1.5 kb or -3.1 kb mouse *Fgf21* gene 5′ flanking region [Fig. 2(A)]. Figure 2(B) and 2(C) shows that when either the 1.5 kb or the 3.1 kb *Fgf21*-LUC reporter plasmid was transiently transfected into HepG2 cells, 4 hours of curcumin treatment increased the LUC activity. The stimulatory effect of the cyclic adenosine monophosphate/protein kinase A activation agents [forskolin and IBMX (F/I)] on *Fgf21*-LUC reporter expression was also shown in Fig. 3(B) and 3(C), and Supplemental Fig. 4 shows that in the baby hamster kidney fibroblast naive cell system, curcumin, or F/I also stimulated the activity of the 1.5 kb *Fgf21*-LUC.

To determine the involvement of PPARα on curcumin-stimulated *Fgf21* expression, PPARα antagonist GW6471 and agonist Wy14643 were used. Figure 2(D) and 2(E) shows that although GW6471 did not inhibit *Fgf21* expression on its own, it blocked the stimulatory effect of curcumin treatment. Figure 2(F) and 2(G) shows that both curcumin and Wy14643 increased *Fgf21* expression, and there was no appreciable synergistic effect when both curcumin and Wy14643 were used. We were unable to detect appreciable stimulation on PPARα expression at the protein level by curcumin treatment [Fig. 2(E) and 2(G)]. However, curcumin treatment (at both 1.5 and 2 μM) reduced PPARα (S12) phosphorylation, which has been suggested to be inversely associated with its activity (38) [Fig. 2(H)]. Figure 2(I) shows that curcumin treatment generated no appreciable effect on attenuating the degradation of *Fgf21* mRNA in the presence of actinomycin D. These observations collectively suggest that curcumin stimulates *Fgf21* expression via a mechanism involving PPARα activation.

**Four-hour curcumin treatment increases the binding of PPARα to the *Fgf21* gene promoter**

ChIP was then used to test whether curcumin treatment increases binding of PPARα to *Fgf21* promoter. Figure 3(A) shows the overall organization of mouse *Fgf21* intron–exon organization and the 5′ flanking region, including the locations of two PPREs. Figure 3(B) and 3(C) shows that the anti-PPARα antibody, but not the control rabbit IgG, pulled down DNAs that contain PPRE1 and PPRE2. We were also able to pull down PPRE1 and PPRE2 with the RNA polymerase II antibody [Fig. 3(B) and 3(C)]. None of the 3 antibodies pulled down the intron II fragment that contains no PPRE [Fig. 3(D)]. Figure 3(E) and 3(F) shows the qChIP results, which indicate that treatment of mouse hepatocytes with the PPARα agonist Wy14643 for 0.5 hours enriched the binding of PPARα to both PPRE1 and PPRE2. The enriched binding of PPARα to PPRE1 and PPRE2 was
Figure 2. PPARα is among the mediators that convey the stimulatory effect of curcumin on Fgf21 expression. (A) Illustration of Fgf21-LUC reporter gene constructs including the positions of PPRE1 and PPRE2. (B, C) Effect of 4-hour treatment with curcumin (at the indicated dosages) or F/I (10 μM each) on the activity of indicated Fgf21-LUC reporter in HepG2 cells. Values were presented as fold change with untreated sample defined as 1-fold. Effect of curcumin (2 μM) on Fgf21 mRNA (D) or Fgf21 and PPARα protein (E) expression in mouse primary hepatocytes in the absence and presence of the indicated dosages of the PPARα antagonist GW6471. Effect of curcumin (2 μM) on Fgf21 mRNA (F) or Fgf21 and PPARα protein (G) expression in mouse primary hepatocytes in the absence and presence of GW6471 (2 μM) or the PPARα agonist Wy14643 (10 μM). For panels D–G, cells were pretreated with GW6471 or Wy14643 for 1 hour, followed by 4 hours of curcumin treatment. (H) Effect of 4 hours of curcumin treatment (at the indicated dosage) on PPARα S12 phosphorylation. (I) Effect of curcumin (2 μM) on Fgf21 mRNA expression...
also observed in mouse hepatocytes treated with curcumin for 4 hours but not for 0.5 hours [Fig. 3(E) and 3(F)], suggesting that curcumin may not serve as a direct ligand of PPARα. Indeed, we conducted a GAL4 nuclear receptor/upstream activation sequence–luciferase reporter assay in HEK293 cells. Curcumin did not activate PPARα, although the vitamin D receptor could be activated with 10 μM curcumin treatment. Furthermore, conditioned medium of mouse primary hepatocytes treated with curcumin cannot stimulate the GAL4 nuclear receptor/upstream activation sequence–luciferase reporter (detailed in Supplemental Fig. 5).

Figure 3. Curcumin treatment increases the binding of PPARα to the mouse Fgf21 gene promoter. (A) Illustration of the organization of the mouse Fgf21 gene and its proximal 5' flanking region (not to scale), including the positions of primers used for ChIP. The detailed positions and nucleotide sequences of PPRE1 and PPRE2 are shown. (B–D) Agarose gel electrophoreses (2%) show the detection of PPRE1, PPRE2, or Intron II containing DNA fragments by PCR after ChIP with the indicated antibody in mouse primary hepatocytes with the indicated treatment of the indicated time. qChIP shows the effect of Wy14643 (10 μM) or curcumin (2 μM) treatment on the occupancy of PPARα on PPRE1 (E) and PPRE2 (F). Values are means ± SD (n ≥ 3). * P < 0.05 vs the corresponding control treatment. CUR, curcumin; DMSO, dimethyl sulfoxide; IgG, immunoglobulin G; IP, immunoprecipitation; NTC, no template control; Poly II, RNA polymerase II.
HFD feeding increases hepatic Fgf21, whereas curcumin intervention attenuates the increase

Beneficial metabolic effects of curcumin intervention in mice were reported previously (25, 26). We repeated the mouse study with 12 weeks of dietary curcumin intervention. Figure 4(A–C) shows the attenuation of curcumin intervention on HFD-induced body weight increase. Figure 4(D) and 4(F) shows that there was no appreciable difference in food intake among the three groups of mice during the experimental period. Figure 4(G) and 4(H) shows our IPGTT results, indicating that curcumin intervention attenuated the impairment on glucose disposal induced by HFD consumption.

The increases in serum and hepatic triglyceride and serum free fatty acid content with HFD feeding were attenuated with curcumin intervention [Fig. 5(A–C)]. In contrast to what we observed with short-term curcumin gavage, long-term curcumin intervention attenuated the increase of serum Fgf21 level induced by HFD feeding [Fig. 5(D)]. Therefore, we examined hepatic Fgf21 levels in these mice. As shown in Fig. 5(E) and 5(F), HFD feeding increased hepatic Fgf21 protein levels, and this increase was completely blocked with curcumin intervention. Figure 5(G) shows the stimulation of HFD feeding on hepatic Fgf21 mRNA expression, and this stimulation was also blocked with dietary curcumin intervention. Figure 5(H) shows that, when compared with LFD, 12 weeks of HFD feeding reduced the PPARα mRNA level, whereas curcumin intervention partially restored the PPARα mRNA level.

Curcumin intervention restores or partially restores the expression of genes that are altered by HFD feeding

Elevated plasma Fgf21 levels in obese mice were shown to be associated with reduced Fgfr1 or βKlotho expression (16, 22). In our HFD-fed mouse model, hepatic Fgfr1 and βKlotho levels were also reduced, whereas curcumin intervention partially restored their expression levels [Fig. 6(A) and 6(B)]. Figure 6(C) shows that HFD feeding also reduced hepatic levels of PGC1α, a key mediator of Fgf21 hepatic function (9), and this reduction was completely blocked by curcumin intervention.

Acox1, Pdk4, and Ehhadh are suggested downstream targets of the PPARα/Fgf21/PGC1α axis, importantly involved in lipid β-oxidation (7). The hepatic expression levels of these three genes were reduced in HFD-fed mice, whereas 12-week dietary curcumin intervention restored the levels of Acox1 and Ehhadh but not Pdk4 [Fig. 6(D)].

Pnpla2, Lipe, and Mgll encode adipose triglyceride lipase, hormone-sensitive lipase, and monoacylglycerol lipase. Figure 6(E–G) shows the repressive effect of HFD on their expression. Curcumin intervention at least partially restored their expression levels. The stimulation on hepatic PGC1α/Pparc1α, Acox1, Pdk4, and Ehhadh expression was also observed in LFD-fed mice with 6-day curcumin gavage [Fig. 6(H) and 6(I)].

Curcumin intervention likely attenuates Fgf21 resistance

The fact that long-term curcumin intervention attenuates hepatic Fgf21 production in HFD-fed mice prompted us to wonder whether curcumin attenuates Fgf21 resistance. To prove that Fgf21 resistance can be assessed in vitro, we treated mouse hepatocytes with 50 nM FGF21 overnight. Figure 7(A) shows that the treatment reduced Fgfr1 and βKlotho levels. Overnight high-dose FGF21 treatment also attenuated the response of cells to further insulin treatment on Akt phosphorylation (Supplemental Fig. 6).

We then tested the in vitro effect of FGF21 in mouse hepatocytes on Erk phosphorylation. Supplemental Fig. 7 shows that cells treated with 1, 2, or 10 nM FGF21 for 60 minutes (but not for 5 or 30 minutes) exhibited elevated Erk phosphorylation. Figure 7(C) and 7(D) show that the activation was absent in hepatocytes isolated from HFD-fed mice and was restored in hepatocytes isolated from HFD-fed mice with curcumin intervention. Figure 7(E–J) shows that the stimulatory effect of 1 and 10 nM FGF21 on the expression of Egr1 and cFos was absent in hepatocytes isolated from HFD-fed mice and restored in hepatocytes isolated from HFD-fed mice with curcumin intervention.

Discussion

After the recognition of Fgf21 as a novel hepatic hormone with potential in the treatment of T2D, obesity, liver steatosis, and other metabolic disorders (4, 10, 13, 14, 39, 40), great efforts have been made to explore the mechanisms underlying its production under physiological and pathophysiological conditions (16, 41–44). Despite its therapeutic potential, elevated plasma Fgf21 levels were reproducibly demonstrated in subjects with T2D, coronary heart disease, and nonalcoholic fatty liver disease (20, 45–49) and in various animal models of diabetes or obesity (16, 20, 50, 51). In the current study, we have demonstrated the acute stimulatory effect of the dietary polyphenol compound curcumin on hepatic Fgf21 production in mice on chow diet or in hepatocytes in vitro, and our mechanistic exploration suggests that the stimulation of Fgf21 production involves the activation of the nuclear receptor PPARα, a key stimulator for hepatic Fgf21 gene transcription (1, 2, 8). More importantly, we
Figure 4. Twelve-week dietary curcumin intervention improves metabolic profiles of mice consuming HFD. (A) Body weight measurement during the 12-week experimental period. (B) Body weight at the end of the 12-week experimental period. (C) Area under the curve (AU) of panel A. (D) Food intake during the 12-week experimental period. (E) Average of food intake in 3 groups of mice. (F) AU of panel D. (G) IPGTT performed at week 8. (H) AU for panel G. n = 5 per group. CUR, curcumin. * or **, HFD vs LFD; § or §§, LFD vs HFD+CUR; # or ##, HFD vs HFD+CUR. CUR, curcumin.
have reproduced the observation that in the HFD-induced obese mouse model, hepatic Fgf21 mRNA and protein expression, as well as circulating Fgf21 level, were elevated. Our findings also revealed that long-term curcumin intervention attenuated the effect of HFD feeding, likely through the restoration of Fgf21 sensitivity.

The findings of our current study were initiated from a serendipitous observation. To determine whether curcumin administration could sensitize insulin signaling via a mechanism that is independent of its anti-inflammation and antioxidative stress properties, we tested the effect of short-term curcumin gavage in an intraperitoneal dexamethasone-injection–induced insulin-resistant mouse model (52). We found that in this model, short-term (i.e., 6-day) curcumin gavage improved insulin signaling, and this improvement was associated with increased hepatic Fgf21 protein expression (31). Because dexamethasone was shown to induce hepatic Fgf21 expression as well by the activation of the glucocorticoid receptor (37), we conducted 2 sets of additional in vivo experiments with short-term curcumin gavage and observed that the stimulatory effect of curcumin on hepatic

Figure 5. Twelve-week HFD feeding increases hepatic Fgf21 expression and plasma Fgf21 levels, whereas concomitant dietary curcumin intervention attenuated the increase. (A, B) Serum and liver triglyceride (TG) levels in the indicated groups of mice. (C) Serum free fatty acid (FFA) levels in the indicated groups of mice. (D, E) Serum Fgf21 hormone (D) and hepatic Fgf21 protein (E) levels in the indicated groups of mice. (F) Quantification of panel E. Hepatic Fgf21 (G) and PPARα (H) mRNA levels. Values are means ± SD (n = 5 per group of mice). *P < 0.05 vs the corresponding control treatment. CUR, curcumin.
Fgf21 and plasma Fgf21 levels occurred in the absence of dexamethasone administration. When the dosage of curcumin was 500 mg/kg body weight/d for 6 days, we observed elevated hepatic Fgf21 mRNA levels associated with elevated PPARα mRNA expression but without the increase of ambient circulating Fgf21 levels. When we reduced the dosage of curcumin to 100 mg/kg body weight/d for either 4 or 8 days, elevated circulating Fgf21 became appreciable, associated with increased hepatic Fgf21 mRNA and Fgf21 protein expression. Further investigations are needed to clarify whether multiple-day curcumin administration at a relatively higher dosage

Figure 6. Twelve-week curcumin intervention attenuates the effect of HFD feeding on hepatic gene expression. Liver tissues from the indicated groups of mice were collected for detecting the expression of Fgfr1 (A), BKLklotho (B), Ppargc1α (C), as well as Acox, Pdk4, and Ehhadh (D). Expression levels of Pnpla2 (E), Lipe (F), and Mgll (G) in the indicated groups of mice. Expression levels of Ppargc1α (H) as well as Acox, Pdk4, and Ehhadh (I) in mice received with or without 6-day curcumin gavage. Values are means ± SD (n = 5 for panels A–G; n = 6 for panels H and I). *P < 0.05 vs the corresponding control treatment. CUR, curcumin; V, control vehicle.
affects Fgf21 hormone production or release or whether this is simply due to the variations among the animals. In our long-term intervention study, we prepared the diet containing 4 g curcumin per kg weight, as we had previously (26); this concentration of curcumin is much lower than the one used in a previous study by another team (25).

The fundamental role of PPAR family members, especially PPARα, in regulating hepatic Fgf21 expression has been demonstrated previously (1, 2, 8, 51, 53, 54). It is well known that during starvation PPARα controls the utilization of fat as the alternative energy source, and it serves as the molecular target for the fibrate dyslipidemia drugs. Inagaki et al. (2) found that mouse hepatic Fgf21 expression is directly induced by PPARα in response to fasting, whereas Fgf21 mediates the pleiotropic effects of PPARα, including the stimulation of lipolysis in the white adipose tissue and ketogenesis in the liver. Badman et al. reported that hepatic Fgf21 expression and plasma Fgf21 levels were increased by fasting or ketogenic diet consumption but rapidly suppressed by refeeding (1). Indeed, circulation Fgf21 levels can be elevated 5- to 10-fold after prolonged fasting (55). Elevated hepatic Fgf21 expression...
in response to ketogenic diet feeding is associated with upregulated expression of PPARα downstream targets. Badman et al. (1) have also demonstrated the in vitro stimulatory effect of the fibrate dyslipidemia drug on Fgf21 mRNA expression in mouse hepatocytes. Furthermore, PPARα−/− mice were shown to be Fgf21 deficient and compromised during ketosis. In human subjects, the induction of FGF21 expression by prolonged fasting and PPARα activation was demonstrated by Galman et al. (56). Reduced Fgf21 mRNA expression in PPARα-deficient mice was also observed by Lundasen et al. (8). These investigators have also demonstrated the in vivo stimulatory effect of Wy-14643, a potent PPARα agonist, on mouse hepatic Fgf21 mRNA expression. This stimulation was repeated in vitro in human hepatocytes (8). Oishi et al. (53) found that injection of the PPARα ligand bezafibrate during the nighttime led to elevated Fgf21 expression, whereas bezafibrate-induced circadian Fgf21 expression was abolished in PPARα-deficient mice.

Here we determined the stimulatory effect of curcumin treatment on Fgf21 transcription and suggest that it is unlikely that curcumin treatment increases Fgf21 mRNA stability because the decay of Fgf21 mRNA in the presence of actinomycin D was not affected by curcumin treatment. In addition to reproducing the observation that the PPARα agonist Wy-14643 stimulates Fgf21 mRNA transcription and Fgf21 protein production, involving the attenuation of the repression on FGFR1 and PPARα protein levels with curcumin treatment for 4 hours but not for 0.5 hours, although profound stimulation was clearly demonstrated with the PPARα agonist Wy14643 for 0.5 hours. Therefore, we suggest that either curcumin treatment decreases intracellular endogenous PPARα ligand levels or that curcumin itself can be metabolized within hepatocytes, generating a yet to be defined PPARα ligand, or that curcumin treatment affects the activity of PPARα by modifying its phosphorylation status. Intensive further investigations are required to test these potential underlying mechanisms.

Animals or human subjects with obesity or diabetes were paradoxically found to have elevated plasma Fgf21 levels, raising the suggestion that obesity is an Fgf21-resistant state (16–21). We reproduced the observation that HFD feeding reduced hepatic expression of Fgfr1 by Fisher et al. (16) and βKlotho by Rusie et al. (22). We have also demonstrated that mouse primary hepatocytes receiving overnight Fgf21 treatment showed reduced

Figure 8. Illustration of the major findings of the study. Curcumin treatment activates PPARα. This can be achieved by attenuating its S12 phosphorylation, the generation of a yet to be identified ligand of PPARα, or the increase of the intracellular ligand levels. Activated PPARα upregulates Fgf21 mRNA transcription and Fgf21 protein production, involving the elevation of binding of PPARα to PPRe1. It is likely that long-term curcumin intervention blocks HFD-induced Fgf21 resistance, involving the attenuation of the repression on FGFR1 and βKlotho expression by HFD feeding and the activation of the PPARα/Fgf21/PGC1α transcriptional axis (not detailed on the figure).
expression of these two genes and that curcumin intervention restored their expression in HFD-fed mice, along with the restoration of expression of genes that are downstream targets of the PPARα/PGC1α axis and genes that are involved in fatty acid β-oxidations. Finally, we demonstrated that the direct effects of Fgf21 treatment in mouse hepatocytes on Erk phosphorylation and the expression of Egr1 and cFos were blocked with HFD feeding and restored by curcumin intervention. These observations collectively suggest that curcumin intervention prevented the development of Fgf21 resistance in response to HFD consumption.

Profound metabolic beneficial effects of dietary curcumin intervention have been demonstrated in animal models and in clinical trials (25–27, 30). Many previous investigations have attributed the beneficial effects of dietary curcumin to its antioxidative stress and anti-inflammation properties, secondary to the body weight-lowering effect. We present here the in vitro and in vivo evidence for the role of curcumin in regulating Fgf21 production and function. Our in vitro study indicated that the dosage required for stimulating Fgf21 expression is much lower than that required for increasing HO1 and reducing nuclear factor κB (Supplemental Fig. 3). The bioavailability of curcumin and other dietary polyphenols is relatively low (59, 60), raising the possibility that the observed in vivo anti-inflammation and antioxidative stress effects of curcumin intervention may be secondary to the regulation of Fgf21 expression and function.

A very recent study revealed that, in human subjects, insulin resistance is negatively associated with plasma levels of betaine, a modified amino acid found in many foods, including whole grains and sugar beet (61). Dietary betaine supplementation was then demonstrated to increase Fgf21 levels, to improve glucose homeostasis, and to reduce hepatic steatosis in HFD-fed mice (61). In contrast to this recent betaine study, we observed the attenuation effect of long-term curcumin intervention on hepatic Fgf21 production and its plasma level elevation.

Figure 8 summarizes the major findings of this study. Our observations collectively suggest that curcumin activates PPARα indirectly. This can be achieved by blocking its S12 phosphorylation or by facilitating the generation of intracellular PPARα ligands. These mechanisms, however, are not mutually exclusive. Activated PPARα upregulates Fgf21 transcription and protein production via binding to the 2 evolutionarily conserved PPREs. Importantly, long-term curcumin intervention blocks HFD-induced Fgf21 resistance, involving the attenuation of the reduction of FGFR1 and βKlotho expression by HFD feeding and the activation of the PPARα/Fgf21//PGC1α axis. This study has expanded the mechanistic exploration of the beneficial metabolic effects of curcumin from its anti-inflammation and antioxidative stress roles to the regulation of Fgf21 production and function. It remains to be assessed whether these effects apply to other dietary polyphenols.

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