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Hepatic Aryl Hydrocarbon Receptor Attenuates Fibroblast Growth Factor 21 Expression*

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The Aryl hydrocarbon receptor (AHR) is a ligand-activated transcription factor involved in many physiological processes. Several studies indicate that AHR is also involved in energy homeostasis. Fibroblast growth factor 21 (FGF21) is an important regulator of the fasting and feeding responses. When administered to various genetic and diet-induced mouse models of obesity, FGF21 can attenuate obesity-associated morbidities. Here, we explore the role of AHR in hepatic *Fgf21* expression through the use of a conditional, hepatocyte-targeted AHR knock-out mouse model (*$Cre^{Alb}Ahr^{Fx/Fx}$*). Compared with the congenic parental strain (*$Ahr^{Fx/Fx}$*), non-fasted *$Cre^{Alb}Ahr^{Fx/Fx}$* mice exhibit a 4-fold increase in hepatic *Fgf21* expression, as well as elevated expression of the FGF21-target gene *Igfbp1*. Furthermore, *in vivo* agonist activation of AHR reduces hepatic *Fgf21* expression during a fast. The *Fgf21* promoter contains several putative dioxin response elements (DREs). Using EMSA, we demonstrate that the AHR-ARNT heterodimer binds to a specific DRE that overlaps binding sequences for peroxisome proliferator-activated receptor α (PPARα), carbohydrate response element-binding protein (ChREBP), and cAMP response element-binding protein, hepatocyte specific (CREBH). In addition, we reveal that agonist-activated AHR impairs PPARα-, ChREBP-, and CREBH-mediated promoter activity in Hepa-1 cells. Accordingly, agonist treatment in Hepa-1 cells ablates potent ER stress-driven *Fgf21* expression, and pre-treatment with AHR antagonist blocks this effect. Finally, we show that pre-treatment of primary human hepatocytes with AHR agonist diminishes PPARα-, glucose-, and ER stress-driven induction of *FGF21* expression, indicating the effect is not mouse-specific. Together, our data show that AHR contributes to hepatic energy homeostasis, partly through the regulation of *FGF21* expression and signaling.

The Aryl hydrocarbon receptor (AHR)[2] is a ligand-activated basic helix-loop-helix/Per-ARNT-Sim (bHLH-PAS) transcription factor, classically known for mediating 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD)-induced toxicity. AHR normally resides in the cytoplasm, bound to molecules of heat shock protein 90, X-associated protein 2, and p23. Upon agonist (*i.e.* TCDD) binding, AHR translocates to the nucleus and aryl hydrocarbon receptor nuclear translocator (ARNT) displaces the cytoplasmic complex to form an AHR-ARNT heterodimer. The AHR-ARNT complex is then able to bind dioxin response elements (DRE) in the promoter region of a wide array of genes, many of which are involved in endogenous and xenobiotic metabolism (*e.g. CYP1A1*, *CYP1A2*, and *CYP1B1*) (1).

Several lines of evidence indicate that AHR is involved in the regulation of metabolic homeostasis. For example, TCDD activation of AHR alters the expression of various genes involved in hepatic metabolism (2). Additionally, AHR is involved in the regulation of gluconeogenesis via the AHR-responsive gene TCDD-inducible poly(ADP-ribose) polymerase (*Tiparp*) (3). However, these studies primarily examine the role of AHR in metabolism when activated by TCDD, a xenobiotic compound. Therefore, the physiological role of AHR in metabolic homeostasis remains poorly understood.

Recently, there has been keen interest in the potential use of the metabolic hormone fibroblast growth factor 21 (FGF21) as a treatment for obesity. Administration of recombinant FGF21 in various animal models of obesity consistently results in weight loss, fat pad reduction, and improved insulin sensitivity (4, 5). Physiologically, FGF21 is induced by fasting, and acts as an endocrine hormone to induce gluconeogenesis, ketogenesis, and torpor (6). Further evidence shows that these FGF21-mediated responses depend upon direct binding of PPARα-RXRα to the *Fgf21* promoter region to activate transcription (7). PPARα, in combination with cAMP-responsive element-binding protein, hepatocyte specific (CREBH), has also been implicated in the activation of *Fgf21* expression (8). Alternatively, carbohydrate response element-binding protein (ChREBP) is known to activate *Fgf21* expression under hyperglycemic conditions (9). Recent studies suggest that ChREBP-dependent transcription might also be directly involved in the FGF21-mediated control of sweet taste preference and sugar intake (10).

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[2] The abbreviations used are: AHR, aryl hydrocarbon receptor; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin; ARNT, aryl hydrocarbon receptor nuclear translocater; DRE, dioxin response element; PPAR, peroxisome proliferator-activated receptor; ChREBP, carbohydrate response element-binding protein; MLX, MAX-like protein X; CREBH, cAMP-responsive element-binding protein hepatocyte specific; ER, endoplasmic reticulum; ICZ, indolo[3,2-*b*]carbazole; GW7647, 2-methyl-2-[[4-[2-[[(cyclohexylamino)-carbonyl](4-cyclohexylbutyl)amino]ethyl]phenyl]thio]-propanoic acid; GNF351, *N*-(2-(1*H*-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9*H*-purin-6-amine; ANOVA, analysis of variance; ChoRE, carbohydrate response element; PPRE, peroxisome proliferator-activated receptor response element.

Last, the unfolded protein response is also capable of regulating *Fgf21* transcription via the transcription factor X-box binding protein 1 (XBP1) (11). In summary, *Fgf21* is involved with several biological processes, and is therefore subject to complex regulatory control.

Recent studies have indicated that AHR can increase hepatic *Fgf21* expression in the presence of TCDD (12). Similarly, a separate study revealed elevated *Fgf21* expression in mice expressing a constitutively active form of AHR in the liver (13). However, data from the former study indicate that hepatic *Fgf21* expression is also greater in $Ahr^{-/-}$ mice compared with $Ahr^{+/+}$ mice. Furthermore, their data demonstrate that activation of AHR with relatively low doses of TCDD represses hepatic *Fgf21* expression over time (12). Such contradicting results warrant further investigation into the role of AHR in regulating *Fgf21* mRNA.

In this study, we examined the physiological role of AHR in hepatic *Fgf21* expression using a mouse model that lacks functional AHR protein in hepatocytes ($Cre^{Alb}Ahr^{Fx/Fx}$) (14). Compared with the parental strain ($Ahr^{Fx/Fx}$), $Cre^{Alb}Ahr^{Fx/Fx}$ mice exhibit increased hepatic expression of *Fgf21* during a non-fasting state, along with elevated serum FGF21 levels. Therefore, we hypothesize that AHR may constitutively, or through endogenous ligand binding, interfere with the activation of hepatic *Fgf21* expression.

The *Fgf21* promoter region contains several putative DREs, one of which overlaps a peroxisome proliferator-activated receptor response element (PPRE) and a carbohydrate response element (ChoRE). Furthermore, this DRE is found adjacent to a cAMP response element (CRE). Using EMSA, we demonstrate that AHR is able to bind to this specific DRE within the *Fgf21* promoter region, while ligand-activated AHR *in vitro* impairs PPARα-, ChREBP-, and CREBH-mediated increases in promoter activity. In addition, AHR agonist treatment in Hepa-1 cells ablates potent, endoplasmic reticulum (ER) stress-driven activation of *Fgf21* expression. Finally, we present evidence that ligand activation of AHR in human primary hepatocytes similarly attenuates PPARα-, glucose-, and ER stress-driven *FGF21* expression.

Results

$Cre^{Alb}Ahr^{Fx/Fx}$ Mice Exhibit Increased Expression of the Fasting-induced Hormone Fgf21 during a Non-fasted State—FGF21 is a key regulator of the fasting response; therefore, hepatic *Fgf21* expression occurs at a low basal level during a non-fasting state. However, non-fasting $Cre^{Alb}Ahr^{Fx/Fx}$ mice exhibit a significant 4-fold increase in hepatic *Fgf21* expression compared with $Ahr^{Fx/Fx}$ mice (Fig. 1*A*). In addition, circulating FGF21 concentrations in non-fasted $Cre^{Alb}Ahr^{Fx/Fx}$ mice are 2-fold higher than the levels observed in $Ahr^{Fx/Fx}$ mice. Similarly, non-fasting $Ahr^{-/-}$ mice exhibit a significant 3.8-fold increase in hepatic *Fgf21* expression compared with wild-type mice (Fig. 1*B*). Consistent with the known effects of elevated *Fgf21* expression, we observe increased expression of the downstream target gene, insulin-like growth factor-binding protein 1 (*Igfbp1*), in conjunction with the down-regulation of genes involved in fatty acid synthesis (Fig. 1, *C* and *D*). Specifically, the expression of fatty acid synthase (*Fasn*) and sterol response element-binding protein 1c (*Srebp1c*) are reduced ~2-fold in $Cre^{Alb}Ahr^{Fx/Fx}$ mice. In contrast, the *Fgf21* expression levels in adipose tissue are comparable between $Ahr^{Fx/Fx}$ and $Cre^{Alb}Ahr^{Fx/Fx}$ mice (Fig. 1*E*). These results are consistent with the targeted deletion of AHR to hepatocytes, and not adipocytes. Interestingly, the repression of genes associated with fatty acid synthesis still occurs within white adipose tissue, despite no differences in *Fgf21* expression (Fig. 1*F*). Whereas hepatic *Fasn* expression was reduced without a significant reduction in stearoyl-CoA desaturase 1 (*Scd1*) expression, we observe the opposite in white adipose tissue.

Next, we investigated the effects of ligand-mediated AHR activation in fasting mice, given that FGF21 regulates the fasting response. For this experiment, 6-week-old male $Ahr^{Fx/Fx}$ mice were exposed for 24 h to 10 μg/kg of TCDD or vehicle (corn oil) by gavage, then fasted overnight. We observe that TCDD treatment significantly reduces hepatic *Fgf21* expression by 50% compared with vehicle-treated mice (Fig. 1*G*). However, circulating levels of FGF21 are not altered with TCDD treatment.

The ChREBP-MLX and AHR-ARNT Heterodimer Complexes Bind to the Fgf21 Promoter at a Composite DRE-PPRE-CRE-ChoRE Regulatory Region—As depicted in Fig. 2*A*, the mouse *Fgf21* promoter region contains four overlapping response elements (designated DRE, PPRE, CRE, and ChoRE). Previous studies have already shown direct binding of PPARα to the *Fgf21* promoter at this site of DRE-PPRE-CRE-ChoRE overlap (7). However, direct binding of ChREBP and its heterodimeric partner, MLX, to this region has not been demonstrated. Utilizing EMSA, we show that the ChREBP-MLX complex does bind to the proximal *Fgf21* ChoRE (Fig. 2*B*, *lanes 5–8*). Also shown is ChREBP-MLX complex formation with a positive control oligonucleotide containing a consensus ChoRE (Fig. 2*B*, *lanes 1–4*). Next, to demonstrate that the AHR-ARNT heterodimer binds to the putative DRE in this region, we incubated *in vitro* translated AHR and ARNT proteins in the presence of a ^{32}P-labeled oligonucleotide that contains the region of response element overlap (−88 to −54), with or without the addition of AHR ligand. For a positive control, we utilized ^{32}P-labeled *Cyp1a1* oligonucleotide. As shown in Fig. 2*C*, TCDD results in AHR-ARNT heterodimerization and subsequent binding to the positive control oligonucleotide. We demonstrate that AHR agonists TCDD (Fig. 2*D*, *lanes 1–5*) and indolo[3,2*b*]carbazole (ICZ) (Fig. 2*D*, *lanes 6–10*) also result in AHR-ARNT heterodimerization and DRE binding to the ^{32}P-labeled *Fgf21* oligonucleotide. To confirm that this binding is specific, we incubated AHR and ARNT in an excess of unlabeled competitor *Fgf21* oligonucleotide, with or without a mutation in the DRE, followed by incubation in the presence of labeled *Fgf21* oligonucleotide. As shown in Fig. 2*E*, we observed that preincubating in an excess of unlabeled, non-mutated oligonucleotide prevents AHR-ARNT binding to the labeled *Fgf21* oligonucleotide (*lanes 1* and *2*), whereas addition of an unlabeled *Fgf21* mutant oligonucleotide does not (*lanes 3* and *4*).

Ligand-activated AHR Attenuates PPARα-, ChREBP-, and ER Stress-dependent Fgf21 Expression in Hepa-1 Cells—Having demonstrated that the AHR-ARNT heterodimer can bind to

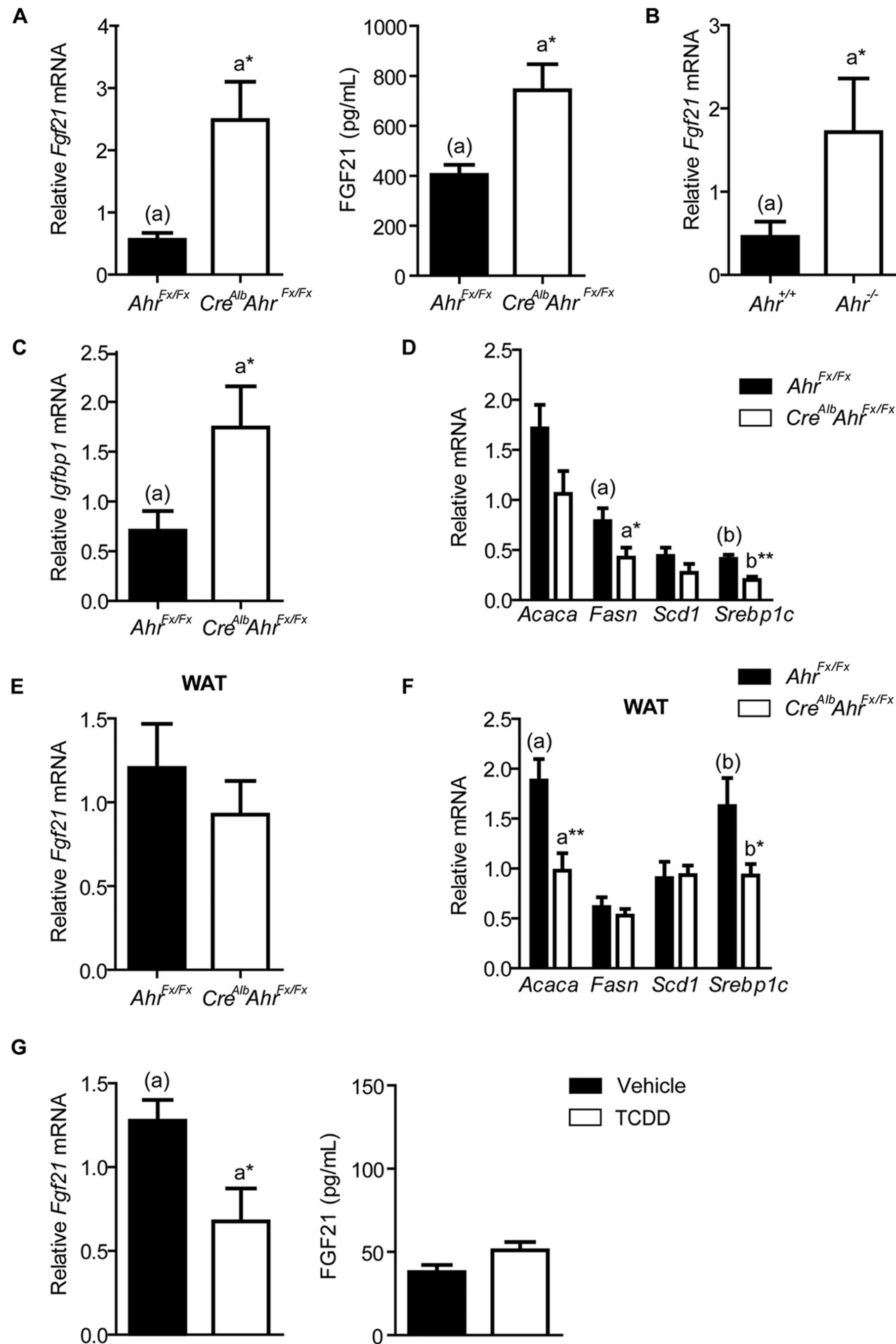

FIGURE 1. *A,* hepatic *Fgf21* expression and serum concentrations in $Ahr^{Fx/Fx}$ and $Cre^{Alb}Ahr^{Fx/Fx}$ mice. *B,* hepatic *Fgf21* expression in C57BL6/J and *Ahr*-null mice. *C,* expression of *Fgf21* target gene *Igfbp1*. *D,* hepatic expression of genes involved in *de novo* lipogenesis. *E, Fgf21* expression and *F, de novo* lipogenesis gene expression in adipose tissue from $Ahr^{Fx/Fx}$ and $Cre^{Alb}Ahr^{Fx/Fx}$ mice. *G,* hepatic *Fgf21* mRNA and serum FGF21 levels in fasted $Ahr^{Fx/Fx}$ mice, exposed to vehicle or 10 μg/kg of TCDD by gavage. *Fasn*, fatty acid synthase; *Scd1*, stearoyl-CoA desaturase 1; *Srebp1c*, sterol response element-binding protein 1c. All data are presented as mean $\pm$ S.E. from three or more mice. Statistical analyses were performed using either a two-tailed Student's *t* test or one-way ANOVA. The latter analysis was performed when there were more than two treatment groups; *, $p < 0.05$; **, $p < 0.01$. These experiments have been repeated twice.

the composite DRE-PPRE-CRE-ChoRE, we next explored the possibility of cross-talk between AHR and promoter-driven PPARα, ChREBP, or CREBH signaling. To do so, we transfected a luciferase reporter construct containing the -1906 to $+52$ *Fgf21* promoter region into Hepa-1 cells, along with expression vectors for the different transcription factors of interest. In the absence of AHR ligands, ectopic PPARα, ChREBP, and CREBH expression results in significant *Fgf21* promoter-dependent expression. However, treatment with AHR agonist ICZ ablates PPARα-, ChREBP-, and CREBH-dependent induction (Fig. 3*A*), suggesting that ligand-activated AHR can interfere with the promoter-driven activation of *Fgf21* expression by these transcription factors. We chose to utilize ICZ in our cell culture experiments as it represents a common dietary AHR ligand. ICZ is formed from the acid condensation of its parent compound, indole-3-carbinol, a breakdown product of glucobrassi-

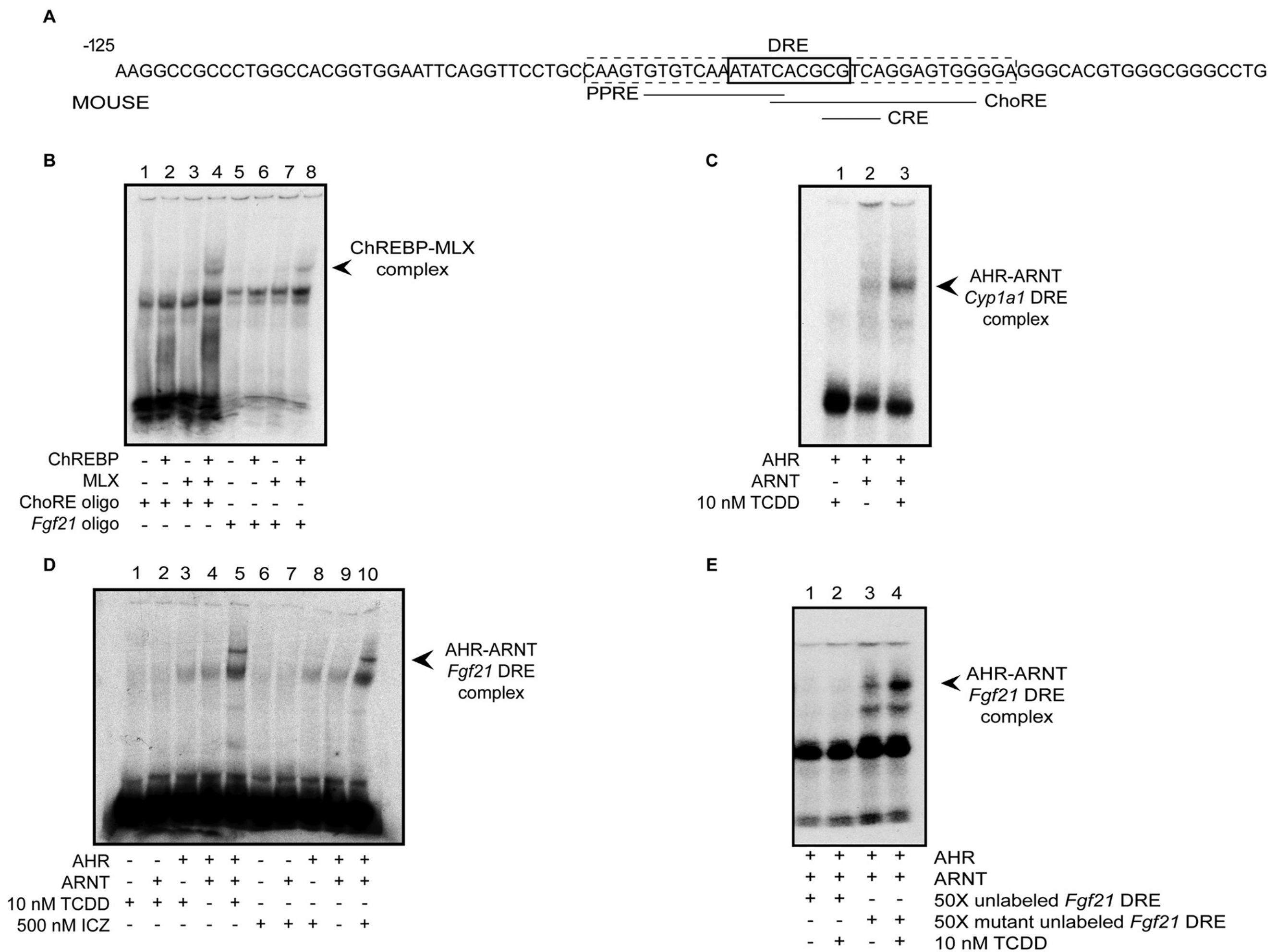

FIGURE 2. *A,* diagram of overlapping response elements within the *Fgf21* promoter region. The *boxed* (*dashed line*) region of the promoter was utilized for EMSA. *B,* formation of the ChREBP-MLX complex with a control labeled oligonucleotide (*lanes 1–4*) and at the *Fgf21* ChoRE (*lanes 5–8*). *C*, TCDD induces AHR-ARNT heterodimerization and binding to a control ^{32}P-labeled oligonucleotide. *D,* TCDD and ICZ treatment results in formation of the AHR-ARNT complex at the *Fgf21* DRE. *E,* addition of unlabeled oligonucleotide interrupts AHR-ARNT binding to the *Fgf21* DRE (*lanes 1* and *2*). Introducing a mutation into the DRE of the unlabeled oligonucleotide eliminates this competitive binding (*lanes 3* and *4*). All data are representative of at least two independent experiments.

cin, which naturally occurs at high concentrations within vegetables of the *Brassica* genus (15).

To further investigate AHR-mediated repression of *Fgf21* expression in the presence of ligand, we examined the ability of AHR to attenuate ER stress-mediated activation of *Fg21* in Hepa-1 cells. To stimulate ER stress, we incubated cells overnight in serum-free medium supplemented with 0.2% BSA, then refreshed the medium and added 1 mM dithiothreitol (DTT) or 1 μM thapsigargin for 4 h. Confirming activation of ER stress, we observe significantly elevated expression of *Ddit3* and *Atf4* in DTT- and thapsigargin-treated cells (Fig. 3*B*). In the absence of AHR ligand, DTT and thapsigargin both markedly increase *Fgf21* expression >150-fold. However, 1 h pre-treatment with 500 nM ICZ reduces thapsigargin- and DTT-driven expression by 74 and 75%, respectively (Fig. 3*C*). Notably, significant ICZ-mediated repression is evident at a 5-fold lower ICZ concentration (Fig. 3*D*). Consistent with AHR activation, we also observe a statistically significant increase in *Cyp1a1* transcriptional levels in ICZ-treated cells (Fig. 3*E*). To confirm that the repressive action of AHR agonists occurs specifically through AHR, we attempted to suppress agonist-mediated activity by pretreating cells with 1 μM *N*-(2-(1*H*-indol-3-yl)ethyl)-9-isopropyl-2-(5-methylpyridin-3-yl)-9*H*-purin-6-amine (GNF351), a known AHR antagonist (16). Surprisingly, GNF351 was unable to antagonize 100 nM ICZ-mediated AHR activity (Fig. 3*F*). In contrast, GNF351 does successfully antagonize 2 nM TCDD-mediated AHR activity, as evidenced by the significant suppression of TCDD-driven *Cyp1a1* expression (Fig. 3*G*). Shown in Fig. 3*H*, treatment with 1 μM thapsigargin results in a 32.6-fold induction of *Fgf21* mRNA. Similar to pretreatment with ICZ, 1 h pretreatment with 2 nM TCDD reduces this effect by ~70%. However, the addition of 1 μM GNF351 for 1 h prior to TCDD treatment reverses TCDD suppression of *Fgf21* expression (Fig. 3*H*).

Comparable with treatment using DTT or thapsigargin, incubating Hepa-1 cells for 24 h in glucose-free medium also activates ER stress and *Fgf21* expression (Fig. 4*A*). Exposing glucose-starved cells to various doses of ICZ, ranging from 250 to 1000 nM, results in a dose-dependent increase in the suppres-

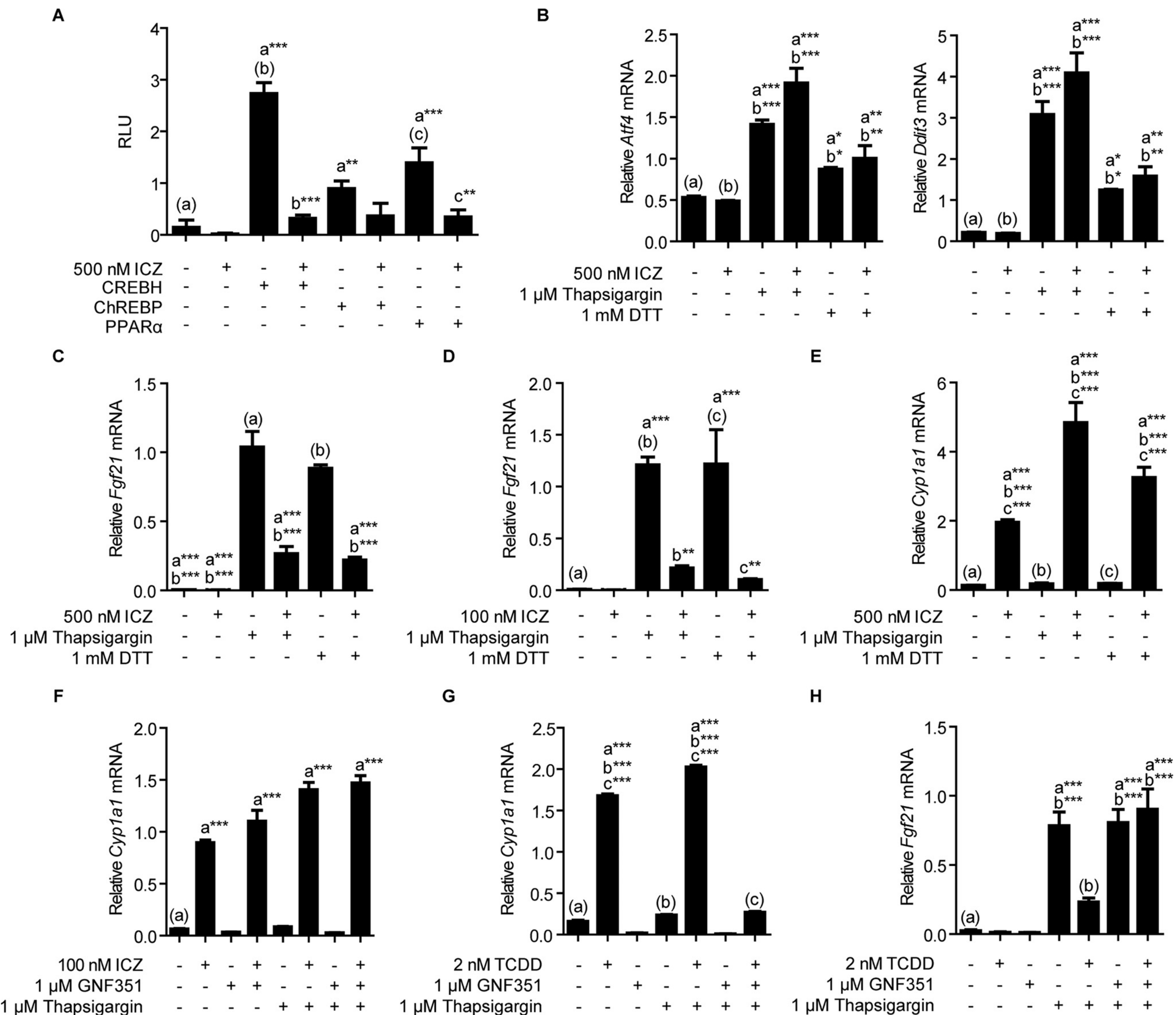

FIGURE 3. *A,* ICZ treatment of Hepa-1 cells that transiently express PPARα, ChREBP, or CREBH suppresses promoter-driven expression. *B,* treatment of Hepa-1 cells with 1 mM DTT or 1 μM thapsigargin leads to the activation of ER stress. *C,* ligand activation of AHR in Hepa-1 cells diminishes thapsigargin and DTT-mediated induction of *Fgf21*. *D,* 1 h preincubation with only 100 nM ICZ ablates thapsigargin- and DTT-mediated induction of *Fgf21*. *E,* ICZ activates *Cyp1a1* expression in Hepa-1 cells. *F,* GNF351 fails to antagonize ICZ-mediated activation of AHR. *G,* GNF351 successfully antagonizes TCDD-dependent *Cyp1a1* expression. *H,* activation of AHR with 2 nM TCDD inhibits thapsigargin-driven *Fgf21* expression. However, a 1-h preincubation with AHR antagonist GNF351 blocks this effect. All data are representative of two or more experiments. Statistical analyses were performed using one-way ANOVA; significance *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

sion of *Fgf21* expression (Fig. 4*B*). Furthermore, we observe the same dose-dependent suppression of *Fgf21* expression upon exposure to various doses of TCDD, ranging from 0.1 to 5 nM (Fig. 4*C*).

AHR Activation Suppresses PPARα-, ChREBP-, and ER Stress-mediated Induction of FGF21 Expression in Primary Human Hepatocytes—To further demonstrate that AHR activity attenuates PPARα-agonist, glucose-mediated, and ER stress-driven *FGF21* expression, we examined the effects of AHR activation in primary human hepatocytes. As shown in Fig. 5*A*, treatment with PPARα ligand GW7647 activates *CPT1A* expression and significantly increases *FGF21* expression >3-fold, whereas pretreatment of cells with AHR agonist significantly inhibits this response. Similarly, incubation of primary human hepatocytes in medium supplemented with 30 mM glucose activates *FGF21* expression after 6 and 24 h (Fig. 5, *B* and *C*). However, 10 nM TCDD or 500 nM ICZ treatment significantly suppresses *FGF21* expression by 58 and 46%, respectively. Confirming ChREBP activation, we observe that incubating primary cells in 30 mM glucose increases the expression of liver and RBC pyruvate kinase (*PKLR*) (Fig. 5*D*). Finally, to activate ER stress, we incubated primary human hepatocytes in the presence of 1 mM DTT for 4 h, with or without a 500 nM ICZ pretreatment for 1 h. In the presence of DTT, we observe an increase in *ATF4* and *DDIT3* gene expression, thereby confirming the onset of ER stress (Fig. 5*E*). Consistent with previous experiments, DTT increases *FGF21* expression 69-fold, whereas a 1-h pretreatment with 500 nM ICZ significantly impairs DTT-induced *FGF21* expression by 46% (Fig. 5*F*).

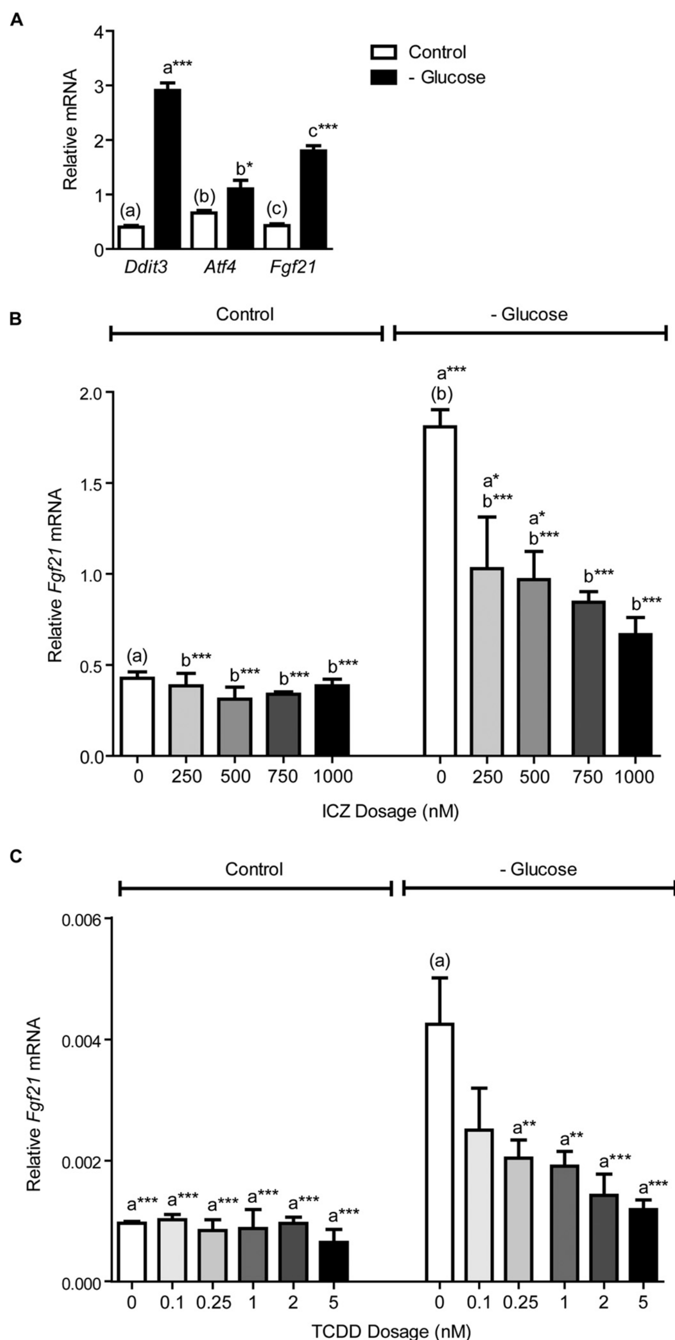

FIGURE 4. *A,* 24 h glucose starvation activates the expression of ER stress-response genes *Ddit3* and *Atf4*, and increases *Fgf21* mRNA in Hepa-1 cells. *B,* ICZ exposure results in a dose-dependent suppression of *Fgf21* expression in glucose-starved Hepa-1 cells. For this experiment, cells were re-treated with ICZ after 12 h. *C,* in glucose-starved Hepa-1 cells, 24 h TCDD exposure also suppresses *Fgf21* expression in a dose-dependent manner. Treatment groups were performed in triplicate, and the data presented are representative of data from at least two independent experiments. Statistical analyses were performed using one-way ANOVA; significance *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Discussion

To date, pharmacological administration of FGF21 is well characterized and known to exert beneficial effects in various animal models of obesity (4, 5). Additionally, FGF21 overexpression is linked to increased longevity in mice (17). However, the regulation of this metabolic hormone remains poorly understood. Our data reveal that AHR is an important regulator of *Fgf21* expression during the non-fasting state. When fed a semi-purified diet, $Cre^{Alb}Ahr^{Fx/Fx}$ mice display elevated hepatic *Fgf21* expression and serum FGF21 concentrations. Importantly, the $Ahr^{Fx/Fx}$ background used to generate $Cre^{Alb}Ahr^{Fx/Fx}$ mice carries the Ahr^{d} allele, which encodes a form of AHR exhibiting reduced ligand affinity relative to the Ahr^{b} allele (18). Therefore, a study that utilized Ahr^{b} conditional knock-out mice (which are not currently available) would likely yield a greater increase of *Fgf21* expression relative to hepatocyte-targeted AHR knock-out mice. Consistent with elevated hepatic *Fgf21* expression, we observed a repression of fatty acid synthesis genes in the liver. Interestingly, we observed a similar repression of fatty acid synthesis gene expression in adipose tissue collected from $Cre^{Alb}Ahr^{Fx/Fx}$ mice, without any increase in adipose *Fgf21* expression. Such results are consistent with the ongoing hypothesis in the literature that liver-excreted FGF21 acts in an endocrine fashion (6).

In agreement with recent data (12), our results suggest that AHR can bind directly to the *Fgf21* promoter at the same location to which the PPARα-RXRα and PPARα-CREBH heterodimers bind (7, 8). Evidence implicates the PPARα-RXRα heterodimer in regulating lipid metabolism (19), whereas CREBH, independent of PPARα, induces a systemic inflammatory response upon ER stress (20). CREBH also modulates lipid metabolism in response to metabolic stress (21). However, the exact function of the PPARα-CREBH complex is not known. To date, formation of this complex has only been observed at the -62 to -93 region of the *Fgf21* promoter (8). Furthermore, whether CREBH can participate with other transcription factors (*e.g.* activating transcription factor 6) to activate *Fgf21* expression upon ER stress remains unknown. Also unclear is whether AHR can compete with the PPARα-CREBH complex for DNA binding at these sites.

Our study presents novel data indicating the ChREBP-MLX heterodimer, which mediates the insulin-independent response to glucose (22), is able to bind a ChoRE that overlaps the binding sites for the AHR-ARNT, PPARα-RXRα, and PPARα-CREBH heterodimers. Recently, investigators determined that ChREBP plays a crucial role in the FGF21-dependent control of simple sugar intake and sweet taste preference (10). By extension, the ability of AHR to attenuate ChREBP-dependent *Fgf21* expression therefore presents the possibility that dietary AHR ligands, or the direct administration of AHR ligands can influence simple sugar intake and/or sweet taste preference. We are currently investigating the validity of this hypothesis through the modulation of AHR activity with different classes of ligand.

Importantly, 31 bp upstream from the site of overlapping response elements lies a characterized ER stress-response element. XBP1, which facilitates the mammalian unfolded protein response (23), binds to this site to activate *Fgf21* expression in response to ER stress (11). Given the close proximity of this site to the DRE, this element likely competes with AHR for binding to the *Fgf21* promoter. In fact, we hypothesize that mutually antagonistic interactions must inherently exist between all the transcription factors that bind to the proximate binding elements (*i.e.* DRE, ChoRE, PPRE, CRE, ER stress-response element) in this region of the *Fgf21* promoter.

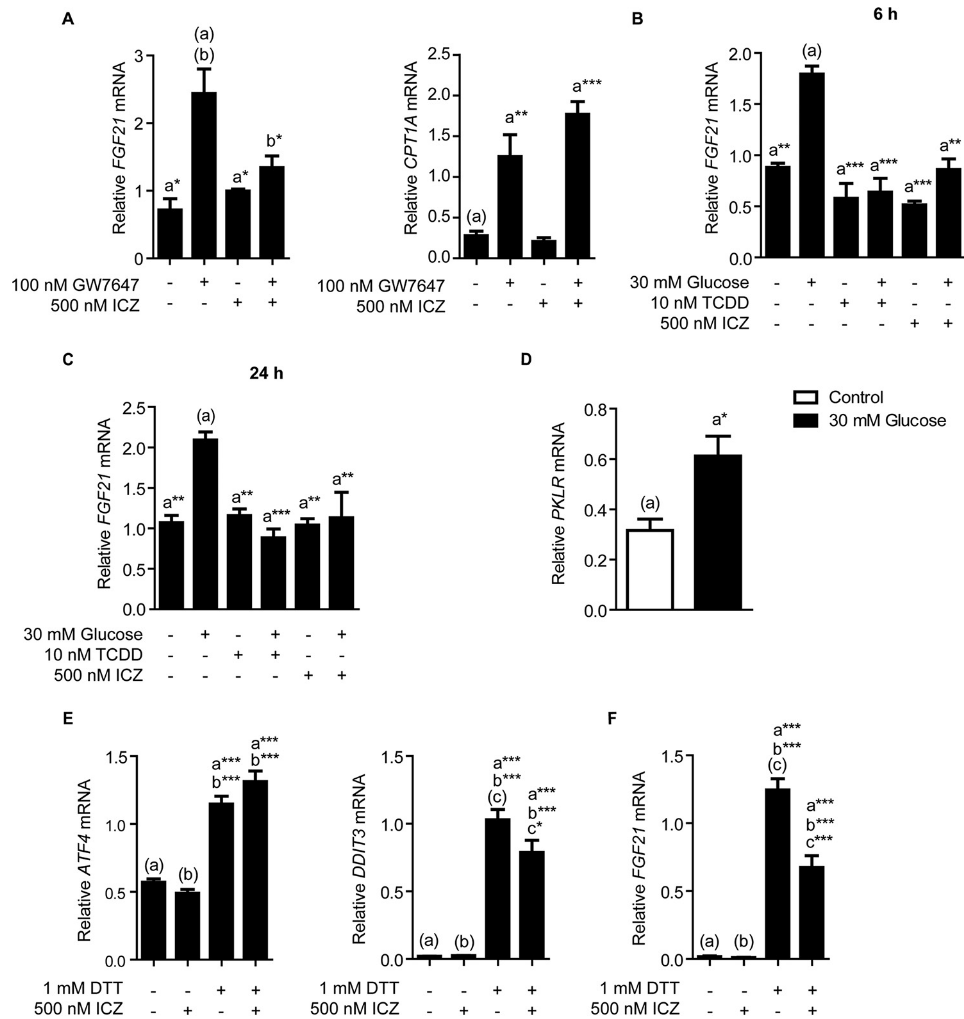

FIGURE 5. *A,* ICZ-mediated AHR activation attenuates GW7647-driven *FGF21* expression after 24 h co-incubation, without any effect on the expression of PPARα target gene *CPT1*. *B,* liganded AHR diminishes glucose activation of *FGF21* expression in primary human hepatocytes at 6 h and (*C*) 24 h. *D,* incubation of primary human hepatocytes in medium containing 30 mM glucose activates ChREBP-target gene *PKLR*. *E,* treating primary human hepatocytes with 1 mM DTT for 4 h activates gene pathways involved in ER stress. *F,* DTT treatment induces *FGF21* expression in primary human hepatocytes, whereas, 1 h preincubation with 500 nM ICZ attenuates this effect. Treatment groups were performed in triplicate and the data presented are representative of experiments from at least two individual donors. Statistical analyses were performed using one-way ANOVA; significance *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$.

Our data indicate that ligand-activated AHR successfully ablates *Fgf21* induction within Hepa-1 cells. Specifically, our reporter transfection experiments demonstrate that ICZ-stimulated AHR can compete with transcriptional activators of *Fgf21* to repress promoter-driven activity. Using a Hepa-1 cell line, we demonstrate that ICZ-mediated AHR activation ablates potent, ER stress-mediated *Fgf21* induction. Importantly, this effect is specific to AHR because AHR antagonists successfully block agonist-driven repression. Unexpectedly, our data indicate that thapsigargin, in combination with AHR agonist, synergistically increases *Cyp1a1* expression. We hypothesize that this increase likely represents a cellular mechanism for preventing further toxicity, after the onset of ER stress. Last, we demonstrate that ligand activation of AHR in primary human hepatocytes suppresses PPARα-ligand, glucose-, or ER stress-mediated induction of *Fgf21* expression, indicating that the ability of the AHR to attenuate *Fgf21* expression is conserved between mice and humans.

Throughout all of our cell culture experiments, AHR activation solely modulated *Fgf21* expression, and not the expression of known PPARα, ChREBP, or XBP1 target genes. This indicates that the AHR does not affect the underlying pathways that each transcription factor regulates, and instead impacts *Fgf21* expression directly. However, whether AHR-mediated repression of *Fgf21* expression occurs directly through the interruption of transcription factor binding to the proximal *Fgf21* promoter *in vivo* will require additional studies.

We provide *in vitro* and *in vivo* data that suggest that the AHR plays a role in the constitutive repression of hepatic *Fgf21* expression. Data presented in a recent publication (12) implicating *Fgf21* as an AHR target gene supports our conclusions. The authors observed long-term, time-dependent repression of hepatic *Fgf21* expression at 24 h and beyond with low dose (0.1, 1, and 10 μg/kg) TCDD treatments in C57BL6/J mice. Also consistent with our results, their data demonstrate elevated hepatic *Fgf21* expression in *Ahr*-null mice. However, the authors demonstrated a marked increase in hepatic *Fgf21* expression in C57BL6/J mice given daily i.p. injections of TCDD (40 μg/kg) or β-naphthoflavone (100 mg/kg) for 4 days. In addition, they reported >100-fold increase of *Fgf21* expres-

sion in human and mouse hepatoma cell lines treated with 10 nM TCDD. Although we repeated their mouse hepatoma cell line experiment, we failed to observe this TCDD-mediated induction of *Fgf21*.[3] In a related, separate study involving mice that express a constitutively active form of AHR in the liver, investigators also observed increased hepatic *Fgf21* expression (13). However, this constitutively active form of AHR was previously determined to exhibit AHR activity similar to high-dose TCDD exposure (24). Data obtained using high-dose treatments are often hard to interpret due to acute toxicity and AHR-independent effects on metabolism (1, 25). In addition, ER stress resulting from high-dose TCDD treatments might also contribute to the activation of *Fgf21*, further complicating data interpretation (26). Despite these complications, we maintain that high-dose TCDD-mediated induction of *Fgf21* represents a new marker for TCDD exposure.

We believe our data illustrates a homeostatic role of AHR in the liver, when activated by dietary ligands. We speculate that ligand-activated AHR may play a previously unrecognized role in maintaining low basal *Fgf21* expression, given its ability to attenuate *Fgf21* activation by PPARα, a key regulator of the fasting response, and ChREBP, a glucose-sensing transcription factor. We further speculate that AHR may modulate the cellular response to ER stress, given the ability of agonist-stimulated AHR to attenuate potent ER stress-driven *Fgf21* expression in cell culture experiments. Ultimately, the physiological role of AHR in attenuating *Fgf21* expression presented here contrasts with the ability of high-dose TCDD exposure to increase *Fgf21*, as others have observed (12, 13). Therefore, additional research is required to fully understand the intricate role of AHR in *Fgf21* expression. Nonetheless, our data firmly supports a physiological role for AHR in metabolic homeostasis.

To conclude, we show that AHR is involved in the homeostatic regulation of *Fgf21*, possibly through attenuation of ChREBP-, PPARα-, and/or ER stress-dependent activation of *FGF21* expression. Although we demonstrate that AHR binds to the *Fgf21* promoter at a DRE that overlaps other response elements, the exact mechanism of AHR repression remains unclear. Current work in our laboratory aims to identify whether or not AHR impairs *Fgf21* activation via direct binding to its core promoter region, or through an alternative DRE-dependent or -independent (*e.g.* sequestration of transcription factors in the cytoplasm) mechanism. Most importantly, the AHR may represent a useful target to modulate *FGF21* expression levels. The fact that many non-nutritive dietary components, such as the flavonoids present in a variety of plants, can repress or activate AHR highlights the potential for regulating *FGF21* expression through dietary modulation of AHR activity.

Experimental Procedures

Animal Experiments—6-Week-old, age-matched male C57BL6/J, congenic C57BL6/J-$Ahr^{-/-}$, C57BL6/J-$Ahr^{Fx/Fx}$, and C57BL6/J-$Cre^{Alb}Ahr^{Fx/Fx}$ mice ($n = 3$–6 per group) were maintained on semi-purified diet AIN-93G (Dyets, NJ) for 3 weeks. $Ahr^{Fx/Fx}$ and $Cre^{Alb}Ahr^{Fx/Fx}$ mice were kindly provided by Chris Bradfield (University of Wisconsin). Mice were housed on corncob bedding in a pathogen-free, temperature- and light-controlled facility, and were given access to food *ad libitum*. Upon sacrifice, serum samples were collected and FGF21 concentration measured using an ELISA kit, according to manufacturer's instructions (R&D Systems). All mouse experiments were carried out humanely with approval and in accordance to the Animal Care and Use Committee of the Pennsylvania State University guidelines.

TABLE 1
Primers used for RT-qPCR

Gene name		Sequence (5′ → 3′)
Acaca	F[a]	TAACAGAATCGACACTGGCTGGCT
	R	ATGCTGTTCCTCAGGCTCACATCT
Fasn	F	GGTGTGGTGGGTTTGGTGAATTGT
	R	TCACGAGGTCATGCTTTAGCACCT
Fgf21	F	GACTGCTGCTGGCTGTCTTC
	R	AGGAGACTTTCTGGACTGCG
FGF21	F	CAGAGCCCCGAAAGTCTCC
	R	GTGGGCTTCGGACTGGTAAA
Igfbp1	F	AGATCGCCGACCTCAAGAAATGGA
	R	TGTTGGGCTGCAGCTAATCTCTCT
Rpl13a	F	TTCGGCTGAAGCCTACCAGAAAGT
	R	GCATCTTGGCCTTTTTCCGTT
RPL13A	F	CCTGGAGGAGAAGAGGAAAGAGA
	R	GAGGACCTCTGTGTATTTGTCAA
Scd1	F	TCCCTCCGGAAATGAACGAGAGAA
	R	AGTGCAGCAGGACCATGAGAATGA
Srebp1c	F	TATGGAGGGCATGAAACCCGAAGT
	R	TTGACCTGGCTATCCTCAAAGGCT

[a] F, forward; R, reverse.

Cell Culture—Hepa-1 cells were obtained from the American Type Culture Collection and maintained as previously described (27). Enriched normal primary human hepatocytes were obtained through the Liver Tissue Cell Distribution System (University of Pittsburgh, PA). The isolated hepatocytes were seeded at >90% confluence in 24-well collagen-coated dishes and cultured as previously described, with minor modifications (28). Briefly, the cells were overlaid with 225 μg/ml of BD Matrigel™ Basement Membrane Matrix (Corning, NY) and cultured in serum-free Leibovitz's L-15 media (Life Technologies), supplemented with 10 mM HEPES, 100 units/ml of penicillin, 100 μg/ml of streptomycin, 25 nM dexamethasone, 10 nM insulin, 5 ng/ml of selenium, 5 μg/ml of transferrin, 1% linoleic acid, 1% albumin, and 1% sodium bicarbonate. For ER stress experiments, we replaced the above stated medium with Williams E Medium containing GlutaMAX™ (Thermo Fisher Scientific) and 5 mg/ml of BSA, immediately prior to addition of compounds. Due to possible human variability, primary hepatocyte experiments were repeated using two or more donors.

RNA Extraction and Quantitative RT-PCR—RNA was isolated as previously described (29). Gene expression was measured using quantitative RT-PCR as previously described (29), with the primers described in Table 1. The relative level of expression was normalized to ribosomal protein L13a mRNA (*Rpl13a*).

Plasmid Constructs—For the luciferase reporter assay, the −1906 to +52 upstream regulatory region of *Fgf21* was cloned into pGL3-basic vector (Promega). The resulting plasmid is referred to as pGL3-FGF21.

Transfections—Hepa-1 cells were seeded onto 6-well plates and grown overnight, then transfected using Lipo-

[3] N. G. Girer, I. A. Murray, C. J. Omiecinski, and G. H. Perdew, unpublished results.

fectAMINE3000 reagent (Promega) with pGL3-FGF21, pSV-β-galactosidase, and pcDNA3, along with pCMV-TNT-PPARα, pChREBP, or pcDNA3-hCREBH(N), according to the manufacturer's instructions. pChREBP was a gift from Isabelle Leclerc (Addgene plasmid number 39235) (30). pcDNA3-hCREBH(N) was a gift from Dr. Laurie Glimcher at Weill Cornell Medical College. After a 24-h recovery, cells were treated overnight with dimethyl sulfoxide vehicle or 500 nM ICZ (Sigma).

Electromobility Shift Assay—EMSA was performed as previously described (31), using *in vitro* translated (Promega) mouse AHR, ARNT, ChREBP, and MLX. As a positive control for AHR, we used a ^{32}P-labeled *Cyp1a1* oligonucleotide as previously described (32). In addition to the oligonucleotide described in Fig. 2*A*, the following ^{32}P-labeled oligonucleotides were used: *ChoRE*, 5′-GCGACATGTGATCAAGCCATGAACCC; competitor *Fgf21*, 5′-ACTCCTGACGCGTGATATTTGACACACTTG; mutated competitor *Fgf21*, 5′-ACTCCTGACGCGCAATATTTGACACACTTG.

Statistical Analysis—All experiments were performed at least twice. All data are presented as mean ± S.E. Statistical analyses were performed using a two-tailed Student's *t* test or one-way ANOVA; *, $p < 0.05$; **, $p < 0.01$; or ***, $p < 0.001$.

Author Contributions—N. G. G. wrote the manuscript and conducted the experiments, with technical assistance from I. A. M. C. J. O. provided the primary human hepatocytes used in this study. G. H. P. is responsible for conceiving and coordinating the project, in addition to assisting with data analysis and manuscript preparation. All authors reviewed the results and approved the final version of the manuscript.

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