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The Cholesterol-Raising Factor from Coffee Beans, Cafestol, as an Agonist Ligand for the Farnesoid and Pregnane X Receptors

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Cafestol, a diterpene present in unfiltered coffee brews such as Scandinavian boiled, Turkish, and cafetière coffee, is the most potent cholesterol-elevating compound known in the human diet. Several genes involved in cholesterol homeostasis have previously been shown to be targets of cafestol, including cholesterol 7α-hydroxylase (CYP7A1), the rate-limiting enzyme in bile acid biosynthesis. We have examined the mechanism by which cafestol elevates serum lipid levels. Changes in several lipid parameters were observed in cafestol-treated APOE3Leiden mice, including a significant increase in serum triglyceride levels. Microarray analysis of these mice identified alterations in hepatic expression of genes involved in lipid metabolism and detoxification, many of which are regulated by the nuclear hormone receptors farnesoid X receptor (FXR) and pregnane X receptor (PXR). Further studies demonstrate that cafestol is an agonist ligand for FXR and PXR, and that cafestol down-regulates expression of the bile acid homeostatic genes CYP7A1, sterol 12α-hydroxylase, and Na^+-taurocholate cotransporting polypeptide in the liver of wild-type but not FXR null mice. Cafestol did not affect genes known to be up-regulated by FXR in the liver of wild-type mice, but did increase expression of the positive FXR-target genes intestinal bile acid-binding protein and fibroblast growth factor 15 (FGF15) in the intestine. Because FGF15 has recently been shown to function in an enterohepatic regulatory pathway to repress liver expression of bile acid homeostatic genes, its direct induction in the gut may account for indirect effects of cafestol on liver gene expression. PXR-dependent gene regulation of cytochrome P450 3A11 and other targets by cafestol was also only seen in the intestine. Using a double FXR/PXR knockout mouse model, we found that both receptors contribute to the cafestol-dependent induction of intestinal FGF15 gene expression. In conclusion, cafestol acts as an agonist ligand for both FXR and PXR, and this may contribute to its impact on cholesterol homeostasis. (*Molecular Endocrinology* 21: 1603–1616, 2007)

CONSUMPTION OF UNFILTERED coffee brews such as the Scandinavian-type boiled, cafetière (French-press), and Turkish coffee raises triacylglycerol and low-density lipoprotein (LDL)-cholesterol concentrations in humans (1–3). A high intake of boiled coffee was associated with hypercholesterolemia and risk of coronary heart disease in Norway and Finland (1, 4, 5), and cafestol was later identified as the factor responsible (6–8). Scandinavian and other unfiltered coffee brews contain 3–6 mg cafestol per cup, with variable but smaller amounts present in espresso coffee (3). Cafestol may also act as an anticarcinogen, with some studies suggesting that there is an inverse association between coffee consumption and the development of colorectal cancer (9, 10).

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Abbreviations: ABCA1, ATP-binding cassette transporter type A1; APOA5, apolipoprotein A5; BSEP, bile salt export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; CYP3A4, cytochrome P450 3A4; CYP3A11, cytochrome P450 3A11; CYP7A1, cholesterol-7α-hydroxylase; CYP8B1, sterol 12α-hydroxylase; CYP27A1, mitochondrial sterol 27-hydroxylase; DKO, double knockout; FGF15, fibroblast growth factor 15; FXR, farnesoid X receptor; GST, glutathione-*S*-transferase; GST-α1, GST subclass α1; GSTμ1, glutathione-s-transferase, subclass μ1; IBABP, intestinal bile acid-binding protein; LDL, low-density lipoprotein; LXR, liver X receptor; NTCP, Na^+-taurocholate co-transporting polypeptide; PCN, pregnenolone-16α-carbonitrile; PXR, pregnane X receptor; RXR, retinoid X receptor; SHP, small heterodimer partner; SRC-1, steroid receptor coactivator 1; VLDL, very low-density lipoprotein.

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Cholesterol homeostasis is achieved through the coordinate regulation of its dietary uptake, endogenous biosynthesis, and disposal in the form of bile acids. Bile acids are not only metabolic byproducts, but are essential for the appropriate absorption of dietary lipids and fat-soluble vitamins. Approximately 95% of bile acids are recycled in the small intestine by enterocytes and returned to the liver via the enterohepatic circulation. Bile acids have been shown to be physiological ligands for the farnesoid X receptor (FXR). FXR regulates transcription by binding as a heterodimer with retinoid X receptors (RXRs) to DNA response elements in the regulatory regions of target genes. When activated by bile acids, FXR induces expression of small heterodimer partner (SHP), which potently inhibits the activity of another orphan receptor, liver receptor homolog 1. Liver receptor homolog 1 is required for cholesterol 7α-hydroxylase (CYP7A1) promoter activity (11, 12), and this inhibition results in transcriptional repression of the gene encoding CYP7A1, the rate-limiting enzyme in bile acid biosynthesis (13). The involvement of the FXR-SHP pathway in the repression of CYP7A1 has been well characterized in rodents, but the relevance to humans remains to be established. FXR also activates the gene encoding intestinal bile acid-binding protein (IBABP), a bile acid transporter (14), thereby demonstrating that bile acids can transcriptionally regulate their own biosynthesis and enterohepatic transport. The pregnane X receptor (PXR) can also inhibit CYP7A1 expression (15). PXR is activated by a variety of xenobiotics, and it protects the liver from toxic compounds (15, 16). Certain bile acids inhibit CYP7A1 expression independently of SHP, and this process is thought to involve PXR (17, 18).

Recent studies have provided an additional mechanism for the repression of bile acid synthesis that involves communication between the intestine and liver. Fibroblast growth factor 15 (FGF15) is selectively induced by bile acids and the synthetic FXR agonist GW4064 in the small intestine and then acting through FGFR4, in hepatocytes, represses CYP7A1 expression in the liver (19).

APOE3Leiden transgenic mice are an established mouse model in which to study hyperlipidemia and atherosclerosis (20, 21). Due to the concomitant expression of APOE3Leiden and APOC1 in these mice, they have an attenuated clearance of apoB-containing lipoproteins (20). Therefore, APOE3Leiden mice display a lipoprotein profile comparable to that of patients with dysbetalipoproteinemia, *i.e*. plasma cholesterol and triglyceride levels are increased, which is mainly confined to the very low-density lipoprotein/low density lipoprotein (VLDL/LDL) fraction, and they respond to hypolipidemic drugs and dietary compounds such as statins (22), fibrates (23), fish oil (24), stanol esters (25), and cafestol (26) in a similar way to humans.

Cafestol has been shown to suppress bile acid synthesis in APOE3Leiden mice by down-regulation of CYP7A1 (26), with a concomitant increase in serum lipids similar to that observed in humans (26). In humans, a disabling mutation in the CYP7A1 gene is associated with increased plasma triglycerides and LDL cholesterol levels (27).

The objective of this study was to elucidate the molecular mechanism underlying the ability of cafestol to suppress CYP7A1 and increase serum lipids. Here we report the effect of cafestol on the expression of FXR target genes in pathways of bile acid biosynthesis both in the liver and intestine and show that cafestol has a tissue-specific effect upon PXR-target genes in the intestine, including FGF15, further supporting the recent finding that the intestine plays a major role in regulating bile acid biosynthesis in the liver. Our findings offer an explanation for the hyperlipidemic action of this widely consumed dietary component.

RESULTS

Cafestol Regulates Metabolic and Detoxification Genes in Mice

Our first aim was to identify novel genes and regulatory pathways associated with the cholesterol-raising effect of cafestol by genome-wide expression analysis. ApoE3Leiden transgenic mice were used to identify genes differentially regulated by a 30-d cafestol-supplemented diet compared with control diet. APOE3Leiden transgenic mice are a frequently used model to study diet-induced hyperlipidemia (20), and consistent with this the cafestol-fed mice had a 40% increase in serum cholesterol levels compared with the control group ($P < 0.05$) (Table 1), in accordance with a previous report (26). This increase in serum cholesterol is of the same order of magnitude as that observed in humans after cafestol consumption (8). An increase of 62% was observed in serum triglyceride levels ($P < 0.05$) (Table 1), which is

Table 1. The Effect of Cafestol Consumption on Lipid Parameters in ApoE3Leiden Mice

Treatment	Weight (g)	Total Cholesterol (mM)	Total Triglyceride (mM)	Free Fatty Acid (FFA) (mM)	Lipoprotein Lipase Activity (μmol/FFA/ml/h)	Hepatic Lipase Activity (μmol/FFA/ml/h)
Control	23.4 ± 1.4	14.9 ± 3.4	1.3 ± 0.5	0.84 ± 0.12	14.5 ± 3.7	9.9 ± 1.3
Cafestol	22.7 ± 1.4	20.8 ± 4.3[a]	2.11 ± 0.6[a]	0.93 ± 0.16	9.5 ± 1.3[a]	7.8 ± 0.9[a]

APOE3Leiden mice were fed a control diet or diet supplemented with cafestol for 30 d. Values are the mean ± SD of eight mice per group. Significant differences between the treatment groups were calculated using the nonparametric Mann-Whitney test.
[a] $P < 0.05$.

also the same order of magnitude observed in humans (28). In addition, lipoprotein lipase activity was decreased by 34% in the cafestol-treated group compared with the control group ($P < 0.05$) (Table 1), and hepatic lipase activity was decreased by 21% by cafestol treatment ($P < 0.05$) (Table 1).

Hepatic mRNA from four individual cafestol-fed mice was subjected to microarray analysis using mRNA from the control group (n = 7) as a reference. A total of 648 genes showed a significant difference in expression in the cafestol-fed group compared with the control group ($P < 1 \times 10^{-6}$, z-test). The data show that cafestol alters the expression of a large number of genes, including many involved in metabolism and detoxification processes, as defined in the Gene Ontology database (Table 2; and supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). The GEO data deposition number is GSE 3809.

Cafestol Activates FXR and PXR *in Vitro*

Based on the observed effect on potential FXR and PXR target genes in the microarray, we investigated the effect of cafestol on several nuclear receptors. In the Gal4-based transactivation assay, ligand-binding domains of different nuclear receptors are fused to the Gal4 DNA-binding domain, and effects on expression directed by a Gal4-dependent reporter plasmid are tested. Cafestol was found to activate the ligand-binding domains of FXR and PXR, as compared with their established ligands (Fig. 1). Additionally, human constitutive androstane receptor, ROR, retinoic acid receptor-related orphan receptor-α, retinoic acid receptor-related orphan receptor-β, and mouse SHP chimeras were not activated by cafestol (data not shown).

To confirm the activation of FXR, the effect of cafestol on full-length receptor for human and mouse FXR was tested. Cafestol and the bile acid chenodeoxycholic acid (CDCA) activate wild-type human FXR but not two different mutant forms of the receptor, a FXR Δ*AF2 and a* W469A mutant (29) (Fig. 2A). With respect to mouse FXR, both compounds showed dose-dependent activation, with a greater response observed with the bile acid (Fig. 2B). These responses were dependent on FXR activation, because they were not observed with the FXR Δ9C mutant, lacking the terminal amino acids 476–484 corresponding to helix 12 (Fig. 2B). A mammalian two-hybrid assay was used to test the ability of cafestol to induce coactivator recruitment to mouse FXR. In this assay, Gal4 was fused with the coactivator SRC-1 (steroid receptor coactivator 1), and mouse FXR was fused with the transactivator VP16. The ability of both cafestol and CDCA to induce high levels of luciferase expression in a dose-dependent manner indicates that both compounds induced interaction of FXR with the coactivator SRC-1 (Fig. 2C).

Next we analyzed the cafestol responsiveness of the promoter of a known FXR target gene. Both cafestol and CDCA induced the activity of the human bile salt

Table 2. Microarray Data of Selected Genes in APOE3Leiden Mice after Cafestol Treatment

GenBank Accession No.	Gene Name	Function	Fold Change	Potential Target Gene	Reference
Genes up-regulated by cafestol					
AA067003	GSTμ1	Detoxification	5.9	PXR	48
W34862	GSTμ6	Detoxification	4.3		
AA437941	GST pi2	Detoxification	3.8		
AI892747	GSTα2	Detoxification	2.5		
AA105866	GSTα4	Detoxification	1.9		
AA106125	Cytochrome P450, 2A4/5	Detoxification	1.8		
AA822098	APOA1	Cholesterol metabolism	1.5	PXR	73
AA666595	Phospholipid transfer protein	Cholesterol metabolism	1.5	FXR	50
AA739040	Lipoprotein lipase	Fatty acid/triglyceride metabolism	1.5		
Genes down-regulated by cafestol					
AI464796	Cytochrome P450, 7α1	Bile acid metabolism	−3.1	FXR and PXR	3, 15, 51
AA822113	Cytochrome P450, 17	Steroid metabolism	−1.8		
AA245848	3β-Hydroxysteroid dehydrogenase type 3	Steroid metabolism	−1.8		
AI597312	3β-Hydroxysteroid dehydrogenase type 1	Steroid metabolism	−1.5		
AA674450	APOA5	Triglyceride metabolism	−1.2		

APOE3Leiden mice were fed a control diet (n = 7) or diet supplemented with cafestol (n = 4) for 30 d. Poly(A)$^+$ RNA was isolated from the livers and microarray analysis was performed. −, Indicates a down-regulation in gene expression.

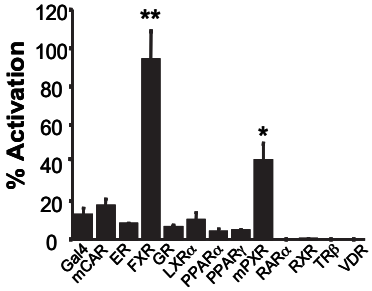

Fig. 1. Determination of the Ability of Cafestol to Interact with a Range of Nuclear Receptors *in Vitro*

HepG2 cells were cotransfected with the Gal4 luciferase reporter and a series of chimeras in which the Gal4 DNA-binding domain is fused to the indicated nuclear hormone receptor ligand-binding domain. The cells were treated with a known receptor-specific agonist or 20 μM cafestol. Results are expressed as normalized luciferase activity relative to the known ligand control (set at 100%) (mean $\pm$ SEM). Gal4 with cafestol was normalized to the transactivation value obtained with the Gal4-receptor chimera with ligand, which was set at 100%. The ligands used were as follows. Mouse constitutive androstane receptor (mCAR): 250 nM 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP); estrogen receptor-α (ERα): 1 μM estradiol (E_2); FXR: 100 μM CDCA; glucocorticoid receptor (GR): 100 nM dexamethasone; LXRα: 1 μM LG101268; peroxisome proliferator-activated receptor (PPAR)α: 300 nM clofibrate; PPARγ: 1 μM roziglitazone; mPXR: 10 μM PCN; RARα: 1 μM all-*trans* retinoic acid; RXR: 1 μM 9-*cis*-retinoic acid; thyroid hormone receptor (TR)β: 1 μM T_3; vitamin D receptor (VDR): 100 nM 1α,25-dihydroxyvitamin D_3.

export pump (BSEP) promoter in HepG2 cells cotransfected with expression plasmids for FXR and RXR (Fig. 2D). Both cafestol and CDCA also induced activity of the IBABP promoter similarly in cotransfected HepG2 cells (Fig. 2E). Overall, these results demonstrate that cafestol, like CDCA, activates FXR.

The capability of cafestol to activate PXR was also further examined using the full-length receptor. Because mouse and human PXR differ in their responses to xenobiotics, both the full-length mouse and human PXR were tested. Cafestol activated both mouse (Fig. 3A) and human PXR (data not shown), and the observed responses were dependent on PXR activation, because they were not observed with PXR mutants, E424K (mouse) (Fig. 3A) and E427K (human) (not shown) lacking the activation function 2 (AF-2) transactivation function. Cafestol also induced coactivator recruitment to mouse PXR (Fig. 3B) and induced cytochrome P450 3A4 (CYP3A4) promoter activity via human PXR, although to a lesser extent than the known ligand, rifampicin (Fig. 3C).

FXR Is Required for Cafestol Regulation of Target Genes in Mice

$FXR^{-/-}$ and $PXR^{-/-}$ mice were used to analyze the role of these receptors for the *in vivo* responses to cafestol. As expected, cafestol feeding for 7 d did not alter serum cholesterol and triglyceride levels in these mice (data not shown). Also as expected, dietary administration of cholic acid (CA) strongly repressed expression of CYP7A1, CYP8B1, and Na^+-taurocholate cotransporting polypeptide (NTCP) in wild-type mice. Cafestol feeding resulted in a more modest but reproducible inhibition of these three well-known negatively regulated targets of FXR (Fig. 4A). In $FXR^{-/-}$ mice, basal expression of all three genes was increased, as expected, and the expression was completely unresponsive to either CA or cafestol feeding, demonstrating that FXR is required for the repression of these genes by CA and cafestol *in vivo*.

In contrast to the effects on genes down-regulated by FXR, no consistent effects of cafestol feeding were observed on genes known to be up-regulated by FXR in the liver. For example, cafestol feeding did not affect BSEP or SHP expression in either wild-type or $FXR^{-/-}$ mice, although dietary CA induced both target genes in wild-type mice (Fig. 4A).

However, cafestol did induce the expression of the FXR-target gene IBABP in the intestine, albeit to a more modest level than the induction seen with CA after 7 d feeding (Fig. 4B). A recent publication showed that FGF15 is also induced by CA and the synthetic FXR agonist GW4064 in the intestine, and that the FGF15 released from the gut signals to the liver to repress CYP7A1 (19). Due to the induction of IBABP in the intestine, but not other positively regulated FXR target genes in the liver, we hypothesized that induction of FGF15 in the gut could account for the repression of CYP7A1 observed in the liver. To test this hypothesis we performed a 14-h oral gavage feeding study in wild-type and $FXR^{-/-}$ mice. Figure 5A shows that cafestol does indeed induce FGF15 in the intestine of wild-type mice. However, the induction in the intestine appears to be partially via FXR because the expression is reduced only 50% in the FXR knockout mice. As predicted, there was a concomitant dramatic repression in hepatic CYP7A1 mRNA expression (Fig 5B), but there was no induction in expression of SHP or other positively regulated target genes in the liver (data not shown).

Cafestol Regulates PXR-Target Gene Expression in a Tissue-Specific Manner

Next we wanted to determine the *in vivo* role of PXR in response to cafestol. Surprisingly, we did not observe cafestol-induced activation of cytochrome P450 3A11 (CYP3A11) gene expression in the liver after feeding for 14 h (data not shown), 3 d (Fig. 6A), or 7 d (data not shown). However, cafestol did induce CYP3A11 expression in the intestine in a PXR-dependent manner after 3 d (Fig. 6B) and 7 d feeding (data not shown).

Cafestol is known to induce the activity of several glutathione-*S*-transferase (GST) enzymes (30, 31), and, consistent with this, we observed induction in gene expression of various GST isoforms in both the livers of APOE3Leiden mice (Table 1) and in the wild-

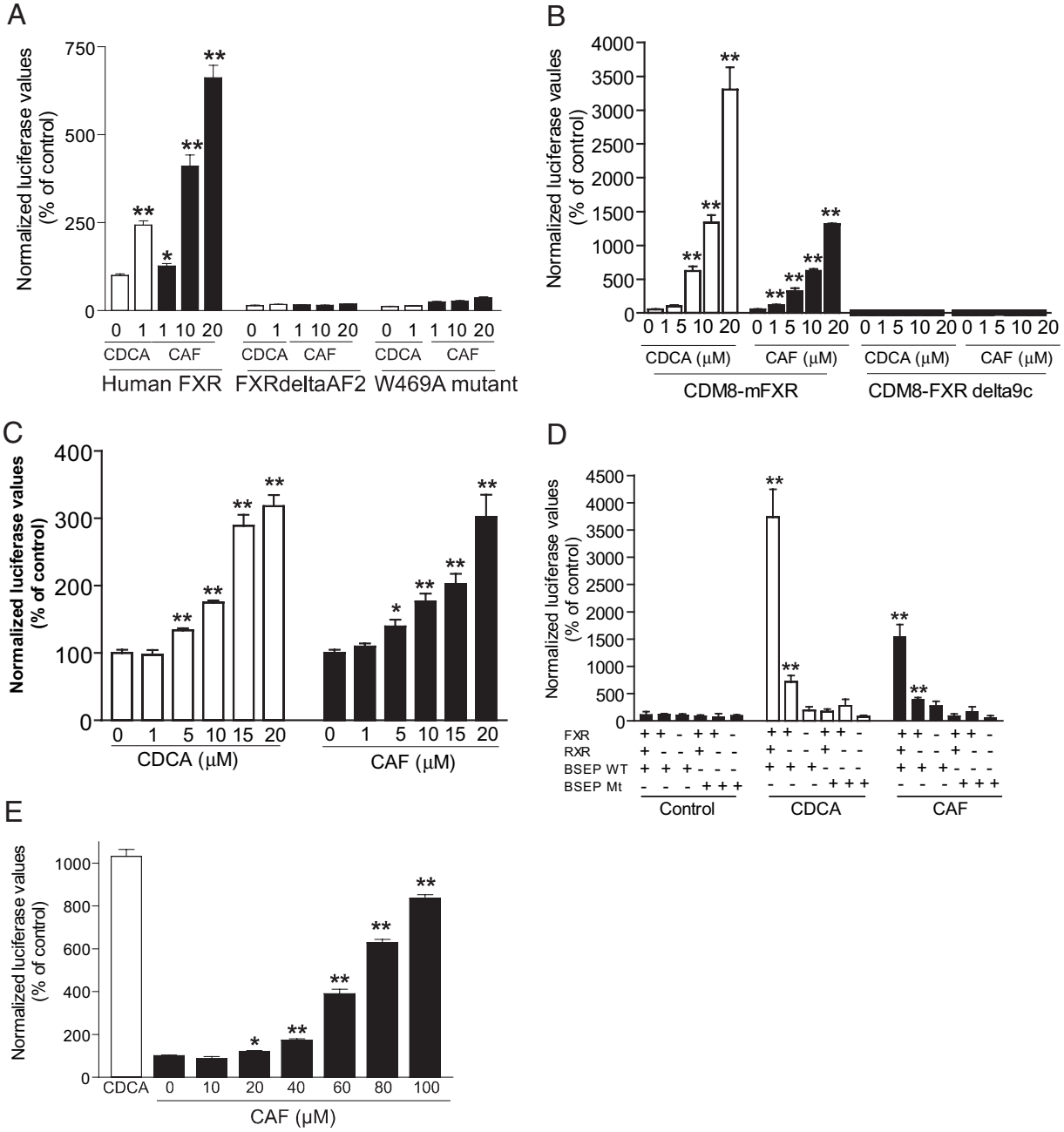

Fig. 2. Cafestol Transactivates FXR

A, Cafestol transactivates human FXR. CV-1 cells were cotransfected with a luciferase reporter construct plus expression vectors as indicated and treated with vehicle (dimethylsulfoxide), 1 μM CDCA (*open bars*) or 1, 10, 20 μM cafestol (*black bars*). B, Cafestol transactivates mouse FXR. CV-1 cells were cotransfected with a luciferase reporter construct plus expression vectors as indicated and treated with vehicle (dimethylsulfoxide), 1, 5, 10, 20 μM CDCA (*open bars*), or cafestol (*black bars*). C, Cafestol enhances FXR interaction with the coactivator SRC-1. CV-1 cells were cotransfected with a luciferase reporter construct plus expression vectors as indicated and treated with vehicle (dimethylsulfoxide), 1, 5, 10, 15, 20 μM CDCA (*open bars*), or cafestol (*black bars*). Results are expressed as percent of the control, normalized to the GH internal control (mean ± SEM). D, Cafestol induces activity of the native BSEP promoter via FXR. HepG2 cells were transfected with a BSEP-promoter-luciferase construct plus expression vectors and treated with vehicle (dimethylsulfoxide), 100 μM CDCA, or 56 μM cafestol. E, Cafestol induces activity of the native IBABP promoter. HepG2 cells were transfected with an IBABP-promoter-luciferase construct plus expression vectors and treated with 100 μM CDCA (*open bar*), vehicle (dimethylsulfoxide), or 10, 20, 40, 60, 80, 100 μM cafestol (*black bars*). Results for each graph are expressed as normalized luciferase values relative to the GH or β-gal internal control (mean ± SEM). CAF, Cafestol; Mt, mutant; WT, wild type.

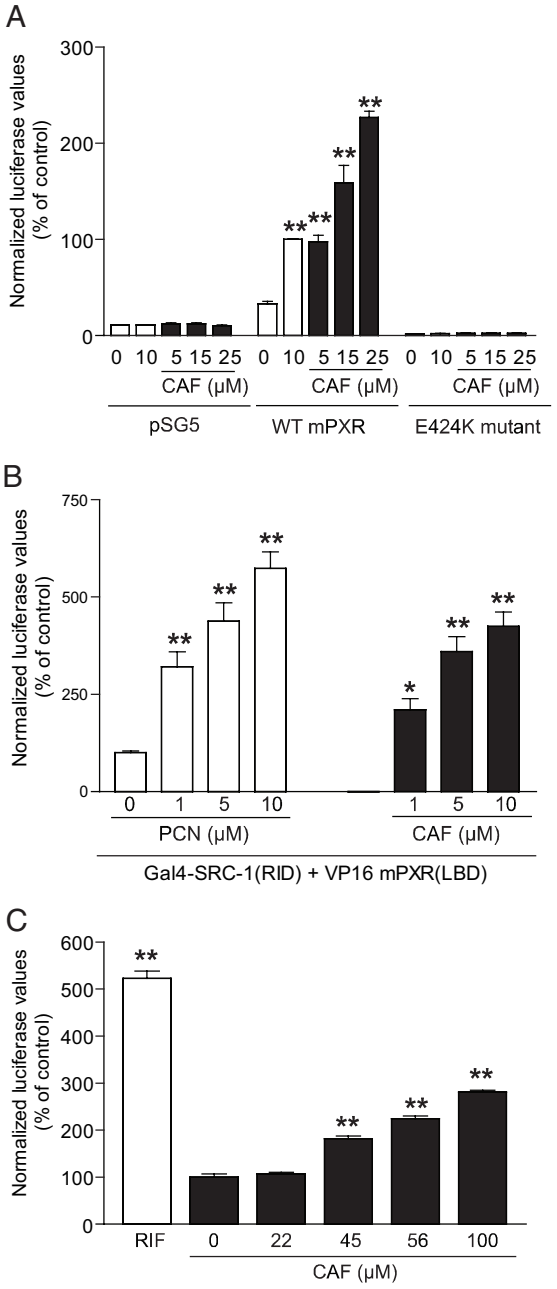

Fig. 3. Cafestol Transactivates PXR

A, HepG2 cells were cotransfected with a luciferase reporter construct plus expression vectors and treated with vehicle (dimethylsulfoxide), 10 μM PCN (*open bar*), or 5, 15, 25 μM cafestol (*black bars*). B, Cafestol enhances PXR interaction with the coactivator SRC-1. HepG2 cells were cotransfected with a luciferase reporter construct plus expression vectors and treated with vehicle (dimethylsulfoxide), 1, 5, or 10 μM PCN (*open bars*), or 1, 5, or 10 μM cafestol. C, Cafestol induces activity of the native CYP3A4 promoter. HepG2 cells were transfected with a CYP3A4-promoter-luciferase construct plus expression vectors and treated with 10 μM rifampicin (*open bar*), vehicle (dimethylsulfoxide), 22, 45, 56, 80, or 100 μM cafestol (*black bars*). Results are expressed as percent of the control normalized to the GH or β-gal control (mean $\pm$ SEM). CAF, Cafestol; LBD, ligand-binding domain; RID, receptor interaction domain; WT, wild type.

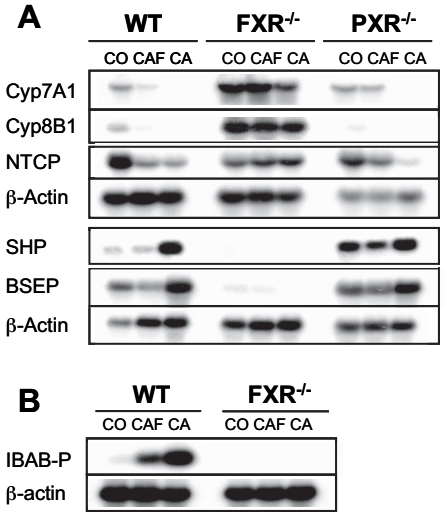

Fig. 4. Regulation of FXR Target Gene Expression *in Vivo* by Cafestol in Wild-Type *vs.* Knockout Mice

A, Groups of male wild type (WT), FXR$^{-/-}$, or PXR$^{-/-}$ mice were fed with control diet (CO), control diet supplemented with 0.25% (wt/wt) cafestol (CAF), or 1% (wt/wt) CA for 7 d. Total RNA was prepared from the liver of each individual mouse, and equivalent amounts of RNA were pooled together for each treatment group, respectively. Twenty micrograms of each RNA sample were used for Northern hybridization with different probes as indicated, normalized to β-actin as internal control, and presented as n = 6 mice per treatment, per group. B, The mice are the same as those used in panel A. Total RNA was prepared from the intestine of each individual mouse, and equivalent amounts of RNA were pooled together for each treatment group, respectively. RNA (20 μg) was loaded per lane for Northern hybridization and probed with IBABP. Data were normalized to β-actin as the internal control and are presented as n = 6 mice per treatment, per group.

type mice used for the 7-d study (data not shown). The xenobiotic receptor PXR has been previously shown to regulate several GST isoforms, including GST subclass α1 (GSTμ1) and GST subclass α1 (GSTα1) in the liver. In the 3-d study we observed induction in the expression of GSTμ1 in the liver by both pregnenolone-16α-carbonitrile (PCN) and cafestol (Fig. 6C). However, in contrast to PCN, the hepatic induction by cafestol was not PXR dependent. On the other hand, in the intestine we observed a robust induction of GSTμ1 expression by cafestol, which was blunted in the PXR knockout mice (Fig. 6D). GSTα1 was found to be induced by both PCN and cafestol in the liver, and by cafestol in the intestine; however, cafestol does not regulate this gene via PXR in either the liver or the intestine (data not shown).

The ATP-binding cassette transporter type A1 (ABCA1) regulates the basolateral transport of cholesterol and phospholipids from enterocytes into the circulation. It has been reported that ABCA1 knockout mice have reduced intestinal cholesterol absorption (32). A recent study showed that ABCA1 is induced in the intestine by PCN in wild-type mice, but not in PXR$^{-/-}$ mice

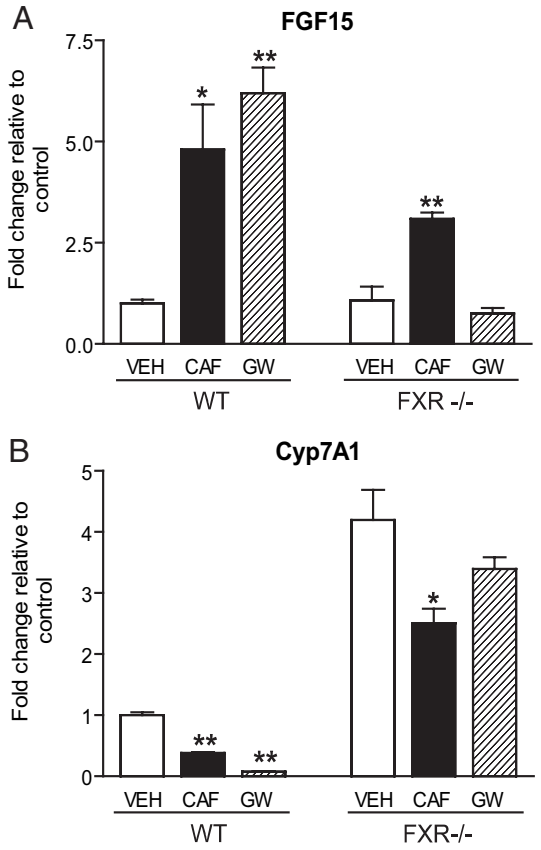

Fig. 5. The Effect on FGF15 and CYP7A1 mRNA Levels after Treatment with Cafestol for 14 h in Wild-Type *vs.* $FXR^{-/-}$ Mice

A, FGF15 expression in the ileum after 14-h treatment with cafestol. B, CYP7A1 expression in the liver after treatment with cafestol. Groups of male wild type (WT) and $FXR^{-/-}$ mice (n = 10 per group) were treated for 14 h with vehicle (VEH) (*open bar*), cafestol (CAF, 400 mg/kg) (*black bar*), or GW4064 (GW, 30 mg/kg) (*hatched bar*). Total mRNA was prepared from the intestine and liver of each individual mouse, and gene expression was measured by quantitative RT-PCR using glyceraldehyde-3-phosphate dehydrogenase as the internal control. Each individual sample was analyzed separately, and the results were pooled together. Data represent the mean ± SEM and are plotted as fold change relative to the vehicle control.

(33). Consistent with this we observed induction of ABCA1 in the intestine by PCN and cafestol in wild-type mice, and the response was lost in the $PXR^{-/-}$ mice (Fig. 7A). It appears that analogous to PCN, cafestol may enhance the efflux of cholesterol back to the liver via a PXR-dependent induction of ABCA1.

Mitochondrial sterol 27-hydroxylase (CYP27A1) is expressed in liver, peripheral tissues, and macrophages. In the liver, CYP27A1 catalyzes the oxidative cleavage of the steroid side chain in the classic bile acid biosynthesis pathway and hydroxylation of cholesterol to 27-hydroxycholesterol and 3β-hydroxy-5-cholestenoic acid in most tissues (34, 35). A recent study showed that rifampicin, the human PXR ligand, could induce CYP27A1 expression in Caco2 cells, but not in liver cells, revealing an intestine-specific regulation of human CYP27A1 by PXR (36). The authors suggested that PXR may play an important role in regulating HDL metabolism in the intestine. Thus, we also assessed the effect of cafestol on CYP27A1 expression in the intestine and observed a slight induction in response to PCN as well as a more robust response to cafestol (Fig. 7B). Both responses were lost in the $PXR^{-/-}$ mice (Fig. 7B), and no effect was seen on CYP27A1 expression in the liver (data not shown).

Due to the observation that FXR was not the sole factor regulating the induction of FGF15 by cafestol (Fig. 5), and that cafestol has an intestine-specific effect upon PXR-target genes (Figs. 6 and 7), we wondered whether FGF15 could also be regulated via PXR and generated a FXR/PXR double-knockout (DKO) mouse model. As shown in Fig. 8, indeed FGF15 is induced by PCN (2.5-fold) and GW4064 (15-fold) in wild-type mice after 14-h oral gavage, and the response is lost in the double FXR and PXR null (DKO) mice. Cafestol also induces FGF15 in the wild-type mice, as shown previously, and this induction is reduced in the DKO mice, indicating that, indeed, PXR does play a role in the regulation of FGF15 by cafestol.

DISCUSSION

Cafestol is the most potent cholesterol-elevating compound identified in the human diet and, although several human and animal studies have investigated its effect on lipoprotein metabolism, its precise molecular mechanism has remained unclear (37, 38). Mechanistic studies have been hampered because cafestol does not raise serum cholesterol levels in wild-type mice or several other animal species (39–42), but it is effective in APOE3Leiden mice, a well-established model in which to study diet-induced hyperlipidemia (20). In these transgenic mice the LDL-receptor-mediated clearance of apoB-containing lipoproteins is hampered, leading to accumulation of VLDL remnants and low-density lipoprotein (LDL). Previous studies showed that in both rat hepatocytes and livers of APOE3Leiden mice, cafestol repressed the expression and activity of CYP7A1 (26, 43). Down-regulation of CYP7A1 in the APOE3Leiden mice increases intracellular cholesterol, thereby down-regulating the LDL receptor and increasing accumulation of VLDL and LDL in the plasma.

In the present study, we found that cafestol-treated APOE3Leiden mice had alterations in lipid parameters similar to those observed in humans after cafestol consumption. For example, serum cholesterol levels were increased by 40%; serum triglycerides were increased by 62%; and the total lipolytic activity in plasma (both lipoprotein lipase and hepatic lipase activity) was significantly decreased by cafestol treat-

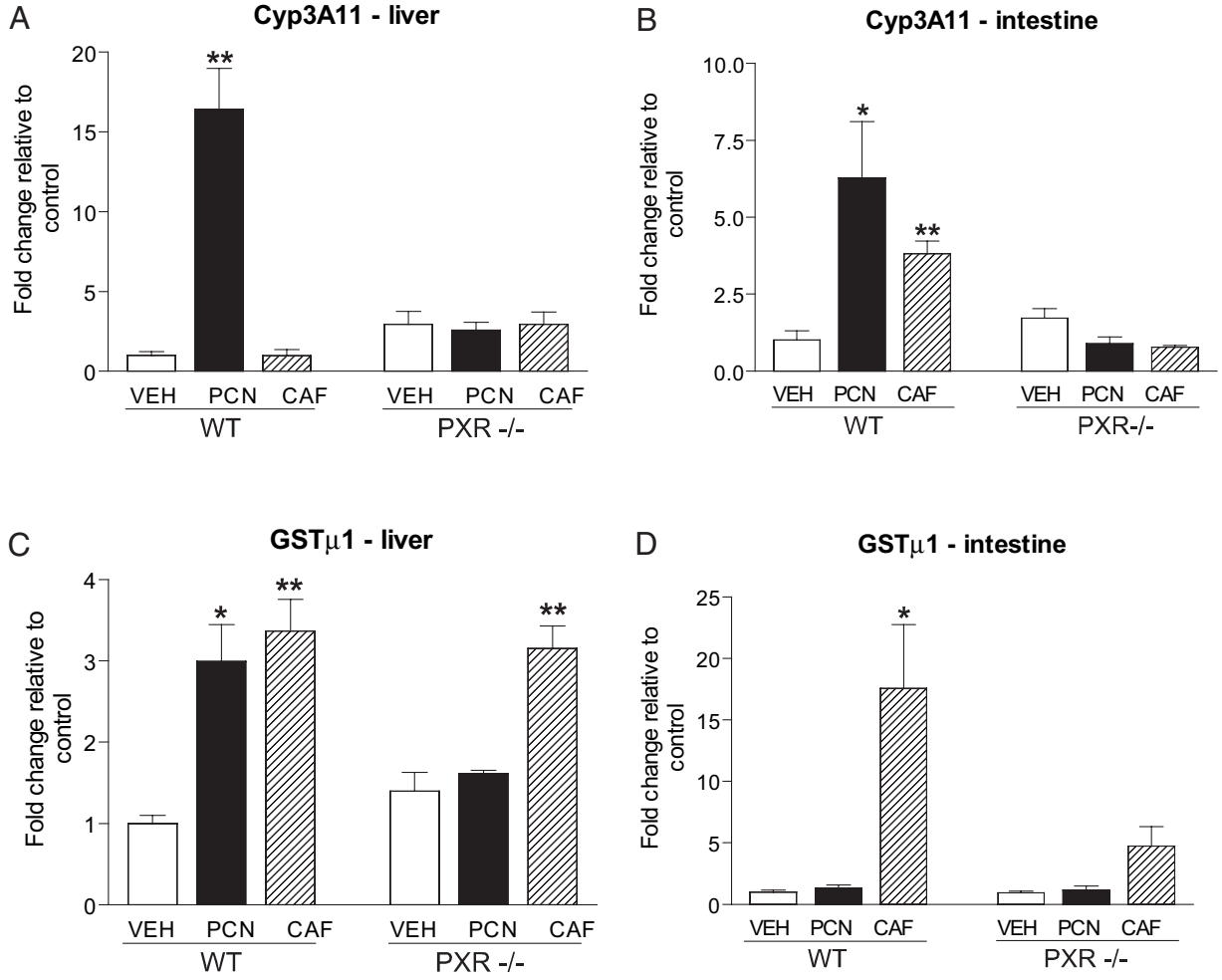

Fig. 6. Tissue-Specific Regulation of PXR Target Gene Expression by Cafestol *in Vivo*

A, CYP3A11 expression in the liver. B, CYP3A11 expression in the intestine. C, GSTμ1 expression in the liver. D, GSTμ1 expression in the intestine. Groups of male wild type (WT) and PXR$^{-/-}$ mice (n = 4–9 per group) were treated for 3 d with vehicle (VEH) (*open bars*), pregnane-16α-carbonitrile (PCN, 200 mg/kg) (*black bars*), or cafestol (CAF, 400 mg/kg) (*hatched bars*). Total mRNA was prepared from the intestine and liver of each individual mouse, and gene expression was measured by quantitative RT-PCR using glyceraldehyde-3-phosphate dehydrogenase as the internal control. Each individual sample was analyzed separately and the results were pooled together. Data represent the mean ± SEM and are plotted as fold change relative to the vehicle control.

ment (34% and 21%, respectively). In addition, microarray analysis of cafestol-treated APOE3Leiden mice identified alterations in hepatic expression of genes involved in lipid metabolism and detoxification. Functional studies have shown that Apolipoprotein A5 (APOA5) regulates plasma triglyceride levels, whereby mice that overexpress human APOA5 display significantly reduced triglyceride levels, whereas mice that lack APOA5 have a large increase in this lipid parameter (44). Consequently, the decreased expression in APOA5 mRNA, together with the observed effects on total lipolytic activity in plasma, correlates with the increase in plasma triglyceride levels observed in the APOE3Leiden mice after cafestol treatment.

Many of the genes identified from the microarray analysis that are altered by cafestol treatment are known to be regulated by the nuclear hormone receptors FXR and PXR (see Table 2). Consistent with this, we found that cafestol specifically activates FXR both *in vitro* and *in vivo* and observed suppression of CYP7A1, CYP8B1, and NTCP expression in the livers of wild-type, but not FXR$^{-/-}$ mice after cafestol feeding. The down-regulation of CYP7A1 expression by specific FXR activators is well described (11, 12) and is thought to be mediated by the induction of the negative regulator SHP (17, 18). Unexpectedly, we did not observe induction of SHP or other positive FXR target genes in the liver by cafestol in either the 14-h (data not shown) or 7-d studies (Fig. 4A). However, several studies have demonstrated that SHP induction is not an absolute requirement for CYP7A1 repression (17–19, 45), indicating the existence of additional pathways.

Of particular importance for the current results is the recent demonstration that FXR-dependent expression of

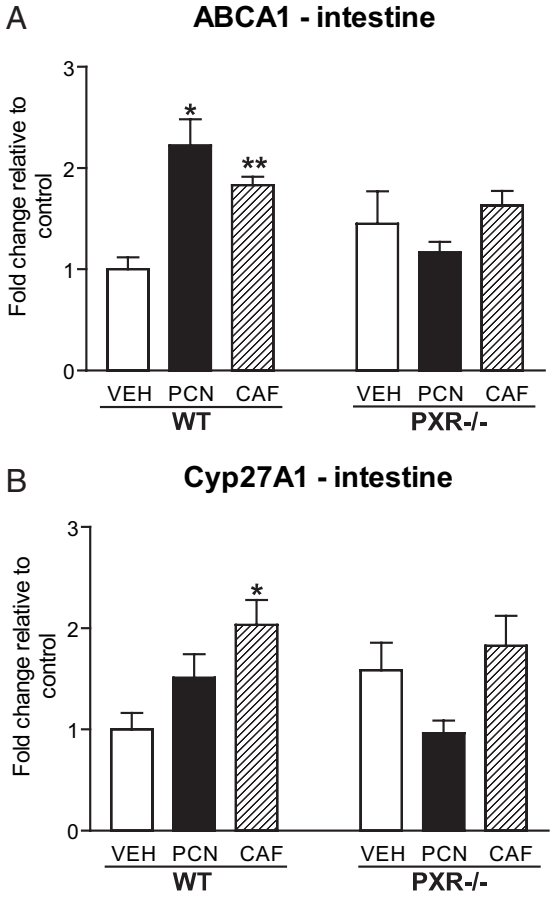

Fig. 7. Cafestol Regulates Genes Involved in Cholesterol Efflux from the Intestine via PXR

A, ABCA1 expression in the intestine. B, Cyp27A1 expression in the intestine. The samples are the same as those used in Fig. 6. Groups of male wild type (WT) and PXR$^{-/-}$ mice (n = 4–9 per group) were treated for 3 d with vehicle (VEH) (*open bars*), PCN (200 mg/kg) (*black bars*), or cafestol (CAF, 400 mg/kg) (*hatched bars*). Total mRNA was prepared from the intestine and liver of each individual mouse, and gene expression was measured by quantitative RT-PCR using glyceraldehyde-3-phosphate dehydrogenase as the internal control. Each individual sample was analyzed separately, and the results were pooled together. Data represent the mean ± SEM and are plotted as fold change relative to the vehicle control.

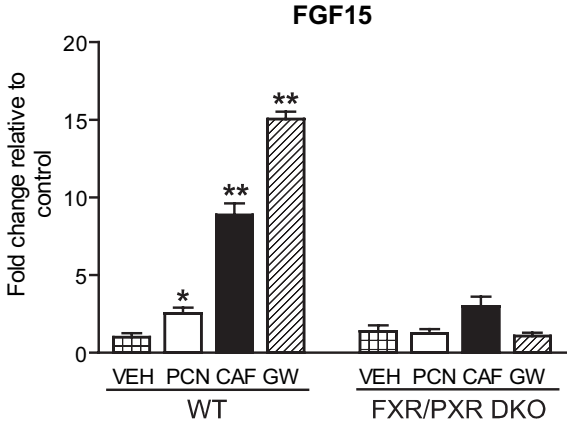

Fig. 8. FGF15 Gene Expression Is Regulated by Cafestol *in Vivo* via Both FXR and PXR

Groups of male wild-type (WT) and FXR/PXR DKO mice (n = 4–10 per group) were treated for 14 h with vehicle (VEH) (*crossed bars*), PCN (200 mg/kg) (*open bars*), cafestol (CAF, 400 mg/kg) (*black bars*), or GW4064 (GW, 30 mg/kg) (*hatched bars*). Total mRNA was prepared from the intestine of each individual mouse, and gene expression was measured by quantitative RT-PCR using glyceraldehyde-3-phosphate dehydrogenase as the internal control. Each individual sample was analyzed separately, and the results were pooled together. Data represent the mean ± SEM and are plotted as fold change relative to the vehicle control.

FGF15 in the small intestine is a key component of an enterohepatic negative feedback pathway for bile acid production. In this intriguing pathway the growth factor released from the gut activates its FGFR4 receptor in hepatocytes, which represses CYP7A1, via still poorly understood mechanisms dependent on both kinase activation and SHP expression (19). This provides an elegant explanation for the seemingly paradoxical observations that cafestol results in FXR-dependent repression of CYP7A1 and other negative target genes in the liver, but fails to induce expression of positive FXR targets. Interestingly, from the results presented herein, it appears that NTCP may also be repressed by the FGF15 signaling pathway. In this study we have also shown that FGF15 can be regulated via PXR, in response to either PCN or cafestol. Thus, we propose that the FXR/PXR-mediated cafestol induction of FGF15 expression that we observe in the intestine accounts for the negative effects in the liver, and that the absence of positive FXR hepatic responses is likely due to a failure of cafestol to achieve a concentration sufficient to increase FXR activation above the basal levels associated with the relatively high endogenous levels of bile acids in the liver.

The failure of cafestol to directly modulate FXR activation in the liver likely also accounts for the discrepancy between the transient elevation of serum triglycerides observed in humans after cafestol consumption (46) and the strong triglyceride-lowering effects observed in rodents after treatment with potent synthetic FXR agonists, which directly alter the expression of hepatic FXR targets such as SREBP1c (47).

It is well established that PCN induces CYP3A11 expression in mouse liver and intestine by a PXR-dependent mechanism (15, 48). We also observed induction of CYP3A11 expression; however, this appears to be in a tissue-specific manner because cafestol-induced CYP3A11 expression was seen in the intestine only and not in the liver. The observed induction by cafestol in the intestine was dependent upon PXR because the effect was lost in the PXR$^{-/-}$ mice. This is not the first demonstration that there is a difference in the regulation of PXR target genes in different tissues. For example, Mrp2 mRNA expression is not altered by PCN in mouse liver, but is induced in the intestine by PCN in a PXR-dependent manner (33).

A key role in the detoxifying mechanisms of xenobiotic metabolism is played by glutathione *S*-transferase (GST), a system of phase II enzymes that catalyzes the conjugation of a great variety of electrophilic compounds with the tripeptide glutathione (49). Several studies have demonstrated that cafestol induces the expression of various GST isoforms (30, 31, 52–55), and this has been postulated to be how cafestol exerts an anticarcinogenic effect (10, 56). The xenobiotic receptor PXR has also been shown to regulate several GST isoforms including GSTα1 and GSTμ1 (48). In this study we observed induction of GSTμ1 in the liver of the wild-type mice by both cafestol and PCN. The effect by PCN was abolished in the PXR$^{-/-}$ mice, whereas the induction by cafestol remained. In the intestine we saw an induction in GSTμ1 expression by cafestol, which was partially lost in the PXR$^{-/-}$ mice. However, we did not see an induction in the expression of GSTμ1 in the intestine as previously reported by Maglich and colleagues (48) in response to ip injected PCN. From the results obtained in this study we conclude that cafestol can regulate PXR-target gene expression in a tissue-specific manner.

The remaining induction in GSTμ1 expression seen in the intestine of the PXR$^{-/-}$ mice treated with cafestol may be due to regulation by Nrf2, a basic leucine zipper transcription factor (NF-E2 p45-related factor). Evaluation of the role of Nrf2 in GST enzyme induction by chemoprotective agents, including cafestol, was studied using a gene knockout mouse model (57). GST activity was found to be lower in the Nrf2$^{-/-}$ mouse than in the wild type, suggesting that Nrf2 regulates the constitutive activity of the GST enzymes. Additionally, cafestol treatment for 14 d induced GST enzyme activity and protein expression in the small intestine of wild-type but not Nrf2$^{-/-}$ mice. The authors also reported Nrf2-dependent regulation of GSTα1 by cafestol, and this may explain why we did not see any PXR-dependent regulation in our study. It is intriguing as to why cafestol potently induces the expression of GSTs in the liver whereas no such induction was seen on positive FXR target genes in the liver. At the present time we do not know whether the observed effects upon GSTs in the liver are caused by cafestol itself or a metabolite, and studies are currently underway to clarify this issue.

In summary, we conclude that cafestol, the cholesterol-raising factor from coffee beans, can directly regulate expression of genes involved in cholesterol metabolism by activating the nuclear receptors FXR and PXR (Fig. 9). We have demonstrated that cafestol is an intestine-specific activator of PXR. We also show that FGF15 is a PXR target gene and that both FXR and PXR contribute to the cafestol-mediated induction of this gene. We propose that direct regulation of such FXR and PXR target genes in the intestine combines with indirect effects in the liver to contribute to the cholesterol-raising effect of cafestol in humans. Our results provide new insights into the complexity of the actions of cafestol and suggest specific directions for the elucidation of the detailed biochemical mechanisms that account for its diverse biological effects.

MATERIALS AND METHODS

Materials

Cafestol was purchased from LKT Laboratories, Inc. (St. Paul, MN). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO). Media and supplements for cell culture were purchased from Invitrogen Corp. (Carlsbad, CA) or Cambrex Bioscience (Verviers, Belgium). GW4064 was a gift from Tim Willson at GlaxoSmithKline (Research Triangle Park, NC).

Plasmids

The various Gal4 DNA-binding domain-receptor ligand-binding domain chimeras (58), full-length human FXR (14), hFXRδAF2 mutant, hW469A mutant (29), full-length murine FXR, the Δ9C FXR mutant (59), full-length murine (60) and human PXR (61), wild-type and mutant human BSEP (62), IBABP, and CYP3A4 (63) promoter constructs have been reported previously. The PXR AF2 mutants for mouse (E424K) and human (E427K) were created by site-directed mutagenesis using the QuikChange Site-Directed Mutagenesis kit from Stratagene (La Jolla, CA), and were confirmed by sequencing.

Animal Feeding Study for the Microarray

Hyperlipidemic APOE3Leiden mice (line no. 2, N21st generation, >99% C57BL/6Jico genetic background) (20) were housed under standard conditions in individually vented cages. Experimental procedures were approved by the local Committee for Care and Use of Laboratory Animals at Leiden University Medical Center. Female APOE3Leiden mice, 10 wk of age received either the control diet [a semisynthetic diet, diet W (18.2 MJ/kg (Hope Farms, Woerden, The Netherlands) enriched with saturated fat (15 g/100 g) and cholesterol (0.25 g/100 g)] (n = 7) or the same diet supplemented with 0.04% cafestol (wt/wt) (n = 4). After 30 d, mice were bled and killed, and livers were harvested.

Measurement of Serum Lipids

Blood (200 μl) was collected from each mouse after a 4-h fast at time points 0 and 4 wk of dietary treatment. Total serum cholesterol (kit no. 236691; Roche Nederland, Woerden, The Netherlands), triglyceride without free glycerol (kit no. 905321; Roche), and free fatty acids (NEFA-C kit; Wako Chemicals Neuss, Germany) were measured enzymatically according to the manufacturer's protocol.

Determination of Lipoprotein Lipase and Hepatic Lipase Enzyme Activity in Plasma

Plasma from mice fasted for 4 h was collected from the tail vein 10 min after injection of heparin (0.1 U/g body weight). The assay of postheparin plasma-lipolytic activity was performed in the absence or presence of 1 M NaCl to estimate both the hepatic lipase activity and the lipoprotein lipase activity. The lipoprotein lipase activity was calculated as the portion of total lipase activity inhibited by 1 M NaCl, as previously reported (64).

Microarray Analysis: Gene Expression Profiling and Functional Annotation of Genes

Total RNA from the caudate liver lobe of APOE3Leiden mice was isolated using RNA-STAT 60 (Tel-Test, Friendswood, TX). Poly (A) RNA was then separated from the total RNA using a poly (A) Tract mRNA systems kit (Promega Corp.,

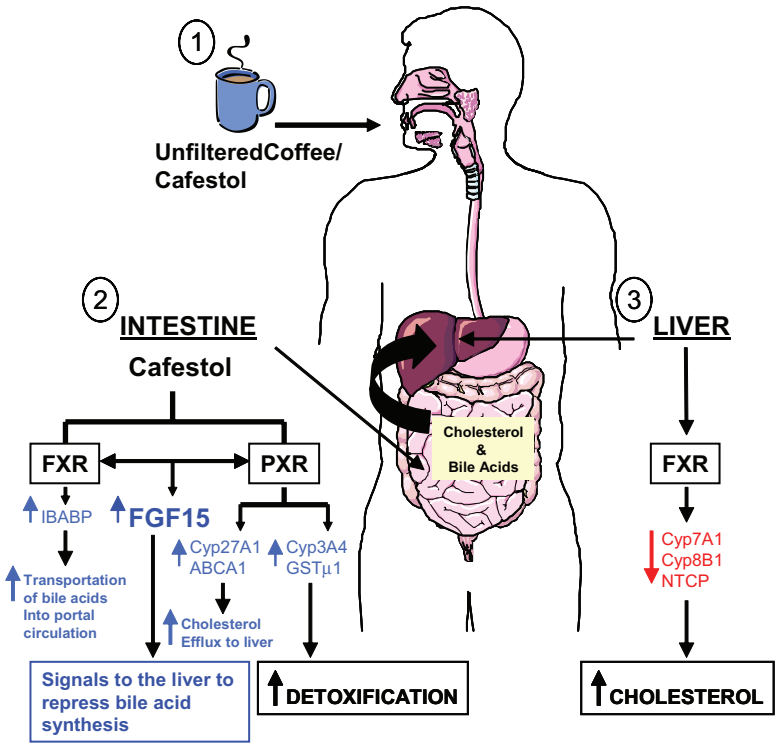

Fig. 9. Schematic Representation of the Proposed Mechanisms by Which Cafestol Increases Cholesterol Levels *in Vivo*

1) Cafestol is consumed in the form of unfiltered coffee and passes into the stomach and then into the small intestine. 2) In the small intestine cafestol activates FXR and PXR. IBABP is induced by cafestol in a FXR-dependent manner, further increasing the transportation of bile acids into the portal circulation. Upon activation of PXR, cafestol induces the expression of Cyp27A1 and ABCA1, resulting in an increase in the efflux of cholesterol into the portal circulation. Cafestol also induces Cyp3A11 and GSTμ1 gene expression via PXR, leading to an increase in detoxification. Cafestol acts via both FXR and PXR to induce FGF15, which signals to the liver to repress bile acid synthesis. 3) In the liver, Cyp7A1, Cyp8B1, and NTCP expression is repressed via FXR, thereby reducing the synthesis of bile acids. The direct regulation of such FXR and PXR target genes in the intestine combines with indirect effects in the liver to contribute to the cholesterol-raising effect of cafestol in humans.

Madison, WI) and labeled with Cy5 and Cy3 fluorescent dyes for hybridization, as previously described (65). Labeled RNA was hybridized in duplicate on Gem 2.03 microarrays containing 9552 genes and expressed sequence tags (Incyte Genomics, Palo Alto, CA). Arrays were scanned on a GenePix 4000A scanner. Data were normalized and analyzed using the GEMTools software version 2.5.1 (Incyte Genomics), as previously described (66).

Statistical Analysis for the Microarray

Significant differential gene expression was determined for each individual mouse in the microarray using the Z-test on the log-transformed expression ratios of the duplicate arrays, using all (7816) valid measurements (67). Based on the Z-test, the overall minimal significant difference in expression was determined at fold change = 1.4 with a power $(1 - \beta)$ of 0.8. ANOVA was performed using MA ANOVA software to account for differential variations between samples to obtain unbiased estimates of expression ratios (68). Hierarchical clustering was performed by average linkage clustering using euclidean distance in Spotfire (Spotfire Inc., Somerville, MA). GeneHopper was used to retrieve information from the Gene Ontology database (December 2003 version) (69).

Cell Culture

HepG2 and CV-1 cells were maintained in DMEM supplemented with 10% FBS. Transient transfection was performed using the calcium phosphate precipitation method. Cells were assayed for luciferase (Promega) activities 24 h after addition of ligands, and reporter expression was normalized to GH activity (Quest Diagnostics, Chicago, IL) or β-gal (Applied Biosystems, Chicago, IL). Similar results were obtained from at least three independent experiments, performed in triplicate.

Animal 14-h, 3-d, and 7-d Feeding Studies and Diets

Mice were housed under standard conditions. Experimental procedures were approved by the local Committee for Care and Use of Laboratory Animals at Baylor College of Medicine. $FXR^{-/-}$ mice (70) and $PXR^{-/-}$ mice (71) were described previously. The $FXR^{-/-}$ mice were backcrossed with C57BL6 mice to the fifth, and the $PXR^{-/-}$ mice were maintained on a mixed C57BL6/129 background. The double FXR/PXR null mice were generated by cross-breeding the FXR and PXR single-knockout mice and were maintained on a mixed C57BL6/129 background. The correct genotype was verified for all strains using previously reported primer sequences and reaction conditions (70, 71). Age-matched groups of 8- to 10-wk-old male mice were used in all experiments (n = 6 per experimental group). Experiments were performed with a control diet (rodent diet 5001; LabDiet, Brentwood, MO) supplemented with 0.25% cafestol, or 1% CA for 7 d (all wt/wt). On the last day the mice were fasted for 4 h and tissues were harvested and mRNA isolated as described below. For the 14-h studies, mice were fed either vehicle (polyethylene glycol 400-Tween 80; 4:1), cafestol (400 mg/kg), or GW4064 (30

mg/kg) by oral gavage (n = 10 per experimental group). For the 3-d study (wild type *vs.* $PXR^{-/-}$) mice were fed either vehicle (polyethylene glycol 400-Tween 80, 4:1), PCN (200 mg/kg), or cafestol (400 mg/kg) by oral gavage (n = 4–9 per experimental group).

RNA Isolation and mRNA Quantification

Total RNA was isolated from livers or intestines using Trizol reagent (Invitrogen). Gene expression was analyzed by Northern blot analysis (72), or by quantitative PCR using Taqman one-step RT-PCR Master mix reagents (Applied Biosystems). Primer and probe sequences can be provided upon request.

Statistical Analysis

An independent sample two-sided *t* test was performed to determine statistically significant differences between samples. Statistical significance is displayed as * ($P < 0.05$) or ** ($P < 0.01$).

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