Regulation of Complement C3 Expression by the Bile Acid Receptor FXR*

Received for publication, October 7, 2004, and in revised form, December 2, 2004
Published, JBC Papers in Press, December 7, 2004, DOI 10.1074/jbc.M411473200

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The farnesoid X receptor (FXR; NR1H4) is an intracellular bile acid-sensing transcription factor that plays a critical role in the regulation of synthesis and transport of bile acids as well as lipid metabolism. Although the reciprocal relationship between bile acid and triglyceride levels is well known, the mechanism underlying this link is not clearly defined. In this study, we demonstrate that FXR regulates the expression of at least two secreted factors, complement component C3 and FGF15, the rat ortholog of FGF19, known to influence lipid metabolism. The analysis of the human complement C3 gene reveals the presence of functional FXR response elements in the proximal promoter of C3. Furthermore, rats given a single dose of an FXR agonist exhibit an increase in the plasma concentration of complement C3 protein. These studies demonstrate a mechanism by which FXR, a nuclear receptor with a limited tissue expression pattern, regulates secretion of factors that ultimately can affect lipid metabolism in an endocrine or paracrine manner.

Nuclear hormone receptors comprise a superfamily of transcription factors that is usually activated by small lipophilic ligands (1). These receptors bind to specific cis-acting elements in their target genes influencing development, differentiation, and physiological homeostasis (2). The nuclear receptor superfamily can be divided into three categories. The first category includes the classical steroid hormone receptors activated by high affinity ligands. The second group includes true orphan receptors for which no physiological ligand has been discovered. The third category corresponds to receptors such as peroxisome proliferator-activated receptors, liver X receptors, farnesoid X receptor (FXR), steroid xenobiotic receptor/pregnane X receptor, and constitutive androstane receptor. These receptors are activated by dietary low-affinity ligands and have recently been defined as metabolic sensors (3).

FXR (NR1H4) is a nuclear receptor that is bound and activated by specific bile acids at physiological concentrations (4–6). FXR controls bile acid concentrations in the liver by inhibiting the expression of CYP7A1 encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid synthesis, and Na(+)/taurocholate cotransporting peptide, a transporter responsible for bile acid uptake. Additionally, FXR induces the expression of the bile salt export pump (BSEP). Together these changes in gene expression result in a net efflux of bile acids from the hepatocyte into bile (7–9). Furthermore, FXR controls the accumulation of toxic bile acids by inducing bile-acid-amino acid conjugation and the conversion of hydrophobic bile acids into more hydrophilic less toxic glucuronide derivatives (10, 11).

The recent identification of bile acids as physiological ligands for FXR may explain studies performed more than 20 years ago in which chenodeoxycholate (3α,7α-dihydroxy-5β-cholanic acid) (CDCA) given to patients with gallstones lowered plasma triglycerides (12, 13). In addition, bile acid-binding resins are known to induce the production of very low density lipoprotein triglycerides underscoring the importance of bile acids in maintaining triglyceride homeostasis (14). The demonstration that plasma triglycerides are elevated in FXR knock-out mice and that a synthetic FXR agonist causes a 50% reduction in triglycerides in rats clearly establish a role for FXR in lipid metabolism (15–17). Furthermore, the induction of apolipoprotein CII and the simultaneous repression of apolipoprotein CIII in the liver by FXR ligands represent an important pathway for the clearance of plasma triglycerides (18, 19). Nevertheless, the expression of apoCII and apoCIII is not significantly altered in FXR knock-out mice, suggesting that FXR may utilize multiple mechanisms to regulate plasma triglyceride levels. For instance, recent evidence suggests that FXR can regulate fatty-acid synthesis through an indirect mechanism involving SHP, liver X receptor, and sterol regulatory element-binding protein-1C (20). Similarly, the identification of FGF19, a known regulator of metabolic rate, as an FXR target gene suggests that FXR may indirectly regulate lipid metabolism by controlling the expression of secreted factors (21). Interestingly, lipids in the form of polyunsaturated fatty acids may in fact be endogenous FXR ligands and thus may act to limit their own synthesis (22).

Here we report that complement C3 (C3) and fgf15, the mouse ortholog of FGF19 (23), are direct FXR target genes. C3 in addition to its role in innate immunity is a precursor for acylation stimulating protein (ASP) (24). Produced by adipocytes in response to a fat-containing meal, ASP acts in a paracrine fashion to increase fatty acid uptake and triglyceride synthesis. The identification of C3 as an FXR target gene suggests a previously unknown link between FXR and complement activation and further supports a described previously link among complement activation, lipid metabolism, and atherosclerosis.

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1 The abbreviations used are: FXR, farnesoid X receptor; BSEP, bile salt export pump; CDCA, 3α,7α-dihydroxy-5β-cholanic acid; C3, complement C3; ASP, acylation stimulating protein; WAT, white adipocyte tissue; RT, reverse transcription; SHP, small heterodimer partner; IB-ABP, ileal bile acid-binding protein; 6FAM, 6-carboxyfluorescein; TAMRA, carboxytetramethylrhodamine; FXRE, farnesoid X receptor element; LDLR, low density lipoprotein receptor.
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EXPERIMENTAL PROCEDURES

Cell Cultures—Fao (a designated rat hepatoma cell derived from rat H4IE cells), Caco-2, and CV-1 cells were obtained from ATCC and maintained according to its depository. Cells were treated with vehicle (MeSO) or the following FXR ligands: 100 nM CDCA or 1 μM GW4064. After 20 h, cells were lysed and assayed for luciferase and β-galactosidase activities. The normalized luciferase units were determined by dividing the luciferase activity by the β-galactosidase activity.

Electrophoretic Mobility Shift Assays—Core sequences of C3 and fgf15 IR-1s are shown in Fig. 2A. Annealed double-stranded oligonucleotides were radiolabeled with [α-32P]dCTP using the Klenow fragment of DNA polymerase II. hFXR and hRXR were synthesized from pCMX-Flag and pCMX-RXRα expression vectors using the T7 coupled reticulocyte system (Promega, Madison WI). Binding reactions were done in 3% (wt/vol) polyacrylamide gel, transferred to nylon membrane, and cross-linked to the membrane with UV-light. cDNA probes were radiolabeled with [α-32P]dCTP using the Rediprime II labeling kit (Amersham Biosciences). Membranes were hybridized using the Rapid hybridization buffer (Amersham Biosciences), washed according to the manufacturer, and quantified using a Molecular Imager (Bio-Rad).

TagMan Primers and Probes—Oligonucleotide primers and the probes for mouse complement C3, fgf15, SHP, IBABP, FXR, rat SHP and IBABP and human FGFR9 were designed using the Primer Express program and synthesized by Integrated DNA Technologies Inc. The sequences (5'-3') were as follows: C3 (forward primer, AAGGCGCAACGGCTATACATC; probe, 6FAM-ACTGCTTGGACTGAGATGGCTTGGCT-TAMRA; and reverse primer, ACTGCTTGGACTGAGATGGCTTGGCT-TAMRA); FGFR9 (forward primer, GGAGAGAAGTTCTGCCTAC; probe, 6FAM-GGCATGGACTTCTGACAGC- TAMRA; and reverse primer, CCTCGGAGGCTACATATC); mouse SHP (forward primer, CAGGAGACGTGATTGAAAGGG; probe, 6FAM-CCAGCAGGACTTCACC-TAMRA; and reverse primer, CGTCCCCTTTCAATCACATCT); mouse IBABP (forward primer, CGGAGACAGGACTTCACC-TAMRA; and reverse primer, CGTCCCCTTTCAATCACATCT); rat SHP and IBABP (forward primer, CAGGAGACGTGATTGAAAGGG; probe, 6FAM-CCAGCAGGACTTCACC-TAMRA; and reverse primer, CGTCCCCTTTCAATCACATCT); mouse IBABP (forward primer, CAGGAGACGTGATTGAAAGGG; probe, 6FAM-CCAGCAGGACTTCACC-TAMRA; and reverse primer, CGTCCCCTTTCAATCACATCT); mouse IBABP (forward primer, CAGGAGACGTGATTGAAAGGG; probe, 6FAM-CCAGCAGGACTTCACC-TAMRA; and reverse primer, CGTCCCCTTTCAATCACATCT). M 3-(2,6-dichlorophenyl)-4-(3-carboxy-2-chloro-stilben-4-yl)oxymethyl-5-isopropyl-isoaxazole (GW4064) (17). After 20 h, total RNA was isolated from the FAO and Caco-2 cells.

RNA Isolation and mRNA Quantitation—Total RNA was isolated from rat and mouse liver, intestinal mucosa, mouse white adipocyte tissue (WAT), FAO, and Caco-2 cells using TRizol reagent (Invitrogen) and further purified by using an RNAeasy kit (Qiagen, Valencia, CA). For Northern analysis, total RNA (20 μg) was resolved on a 1% agarose/formaldehyde gel, transferred to nylon membrane, and cross-linked to the membrane with UV-light. cDNA probes were radiolabeled with [α-32P]dCTP using the Rediprime II labeling kit (Amersham Biosciences). Membranes were hybridized using the Rapid hybridization buffer (Amersham Biosciences), washed according to the manufacturer, and quantified using a Molecular Imager (Bio-Rad).

Western Blots—Plasma from rats (1 μg) was denatured by boiling in SDS-loading dye and run on a SDS-acrylamide gel, and proteins were blotted to a nylon membrane. The blot was incubated with a polyclonal antibody made against the C3a protein (Santa Cruz Biotechnology, Inc.) and then developed using a secondary antibody conjugated with horseradish peroxidase.

Animal Studies—Animal care and use was conducted according to the National Institutes of Health Guide for the Care and Use of Laboratory Animals. Male F344/NHsd rats and male C57BL/6 mice were fed ad libitum and were kept under standard light/dark cycle. The GW4064 compound was suspended in 0.5% carboxymethyl cellulose vehicle for a dosing volume of 1 ml for rats and 0.1 ml for mice. Animals were given a single dose per mouse with a feeding needle to give a final concentration of 100 mg/kg. The animals were sacrificed 8 h following the dose, and liver, spleen, small intestinal, and epididymal fat tissues were excised. The intestinal mucosa scraped from the lower 30% small intestine were frozen in liquid nitrogen and kept at −80 °C until RNA preparation. Liver and intestinal mucosa collected from Fxr knockout mice (3) backcrossed more than four generations into C57BL/6 mice, and age-matched wild type C57BL/6 mice was a kind gift from Dr. David Moore.

RESULTS

Induction of Complement C3 and FGFR19 by FXR Agonists—An analysis of microarray data from rat primary hepatocytes indicates that the gene encoding C3 is induced by both natural (CDCA) and synthetic FXR (GW4064) agonists (10). The induction of C3 was confirmed by Northern analysis in Fao, a rat hepatoma cell line (Fig. 1A), and in Caco-2, a human colorectal adenocarcinoma expressing Fxr (Fig. 1B). The regulation of C3 in intestinal cells was unexpected, because the liver is considered the predominant site of expression. Therefore, we examined whether FGFR19, another secreted factor previously identified to be a FXR target gene in hepatocytes (21), is also regulated in Caco-2 cells. As shown in Fig. 1C, FGFR19 is significantly induced by FXR ligands in these cells, suggesting that FXR activity in the intestine may influence lipid metabolism by promoting the synthesis and secretion of these factors.

Identification of FXREs in Human Complement C3 and Rodent fgf15 Genes—The strong induction of C3 mRNA by GW4064 and CDCA suggests that this gene is a direct target of FXR activation. A search for FXR-responsive elements similar to an inverted repeat of the consensus sequence AGGTCA spaced by one nucleotide (IR-1) revealed a potential element located ~150 base pairs upstream of the transcriptional start site in the human C3 gene (Fig. 2A). This IR-1 sequence, as well as the location, is conserved in rat and mouse C3 genes. fgf15 has been suggested to be the mouse ortholog of human FGFR19 despite the relatively low homology (51% amino acid identity). Nonetheless, rat and mouse fgf15 contain IR-1 sites in intron 2,
a similar location that was reported for human FGF19 (Fig. 2A).

To demonstrate that FXR-RXR heterodimers directly bind the IR-1s identified in the proximal human C3 and in the rodent fgf15 genes, electrophoretic mobility shift assays were performed. Radiolabeled DNA fragments corresponding to the putative IR-1s from the human C3 and fgf15 (Fig. 2A) genes were prepared and examined for ability to bind FXR-RXR heterodimers. When added together, a significant FXR-RXR complex is formed on the C3 and fgf15 IR-1s that is not detected with either receptor alone. Additionally, binding is competed by an excess of their respective unlabeled IR-1 oligonucleotides (Fig. 2B). More detailed binding affinity studies revealed that FXR-RXR heterodimers bind to the SHP IR-1 with ~4–5-fold higher affinity compared with the C3 IR-1 as evidenced by a lower IC50 value of the SHP IR-1 competing for binding to the C3 IR-1 (Fig. 2C). On the other hand, FXR-RXR binding affinities are similar on the IR-1s for C3 and BSEP (Fig. 2, D and E).

Chimeric human C3-luciferase reporter gene constructs were made, and the ability of FXR to transactivate luciferase expression was tested by transient transfections into CV-1 cells. The 450-base pair upstream sequences from the human C3 gene conferred FXR responsiveness indicated by the increase in luciferase activity when cells are treated with CDCA and GW4064 ligands in the presence of co-transfected FXR and RXR (Fig. 3A). The maximal transcriptional activity mediated by FXR appears equal when comparing C3 to the known FXR target genes BSEP and SHP despite a higher FXR-RXR binding affinity on the SHP IR-1. However, the fold induction of the C3 promoter by FXR ligands is lower compared with the fold activation of BSEP and SHP because of higher basal activity of the C3 promoter. Mutation of the putative FXRE abolishes FXR activity but not the basal activity of the C3 promoter (Fig. 3B).

Regulation of Complement C3 and fgf15 Levels in Vivo—To determine whether FXR regulates C3 expression in vivo, C3 mRNA levels were compared in FXR+/− and FXR−/− mice. In the intestine, deletion of FXR resulted in decreased levels of C3 mRNA (Fig. 4A). Although there is a trend for reduced levels of C3 mRNA in the liver of FXR−/− mice, expression levels were not significantly different from FXR+/− mice (Fig. 4B). The relatively high levels of C3 expression in the liver, >100 times the level in the intestine, may make it difficult to detect an effect of deleting FXR. In C57Bl/6 mice treated with a single dose of GW4064, an ~2-fold induction of C3 mRNA was observed in intestinal mucosa, whereas a small but significant induction was again observed in the liver of these animals (Fig. 4, C and D). The basal expression of C3 mRNA in WAT, the target tissue for ASP (the cleaved product of C3), was also high compared with the intestine, but a significant increase in C3 mRNA expression was seen in response to GW4064 (Fig. 4E). The relatively low expression level of FXR mRNA in WAT compared with the liver and ileum may account for the modest increase in this tissue (Fig. 4F). Thus, a combination of genetic deletion and pharmacological activation of FXR identifies C3 as a target gene in vivo. Although expression levels did not differ significantly between FXR+/− and FXR−/− mice (data not shown), fgf15 mRNA levels were dramatically increased by GW4064 in the intestine (Fig. 4G). Even though mouse fgf15 was suggested to be the ortholog of FGF19, it was not expressed in the liver (data not shown). The two established target genes SHP and IBABP were induced in liver and intestine, respectively, confirming significant exposure of GW4064 in these tissues (Fig. 4, H and I).

Northern analysis also shows a robust induction of C3 and fgf15 mRNA expression in the intestine of rats that was given a single dose of GW4064 (Fig. 5A). The major source of C3 in plasma is traditionally believed to be the liver, although secre-
tion from other tissues has been implicated (25). As was seen in mice, rat liver C3 levels were only modestly increased by an FXR ligand, presumably because of already high basal mRNA expression levels in this tissue (data not shown). Regardless of the source of C3, plasma levels were dramatically increased in rats treated with the FXR agonist GW4064 (Fig. 5B). As seen in

| Binding Site | Core Sequence |
|--------------|---------------|
| hC3 IR-1: (-168, -156) | AGGTCA a TGACC |
| rC3 IR-1: (-173, -161) | AGGTCA a TGACC |
| mC3 IR-1: (-148, -136) | AGGTCA a TGACC |
| hFGF19 IR-1: (+3322, +3334) | AGGTCA g TGACC |
| rFGF15 IR-1: (+1948, +1960) | AGGTCA g TGACC |
| mFGF15 IR-1: (+1963, +1975) | AGGTGA a TGACC |
| IBABP IR-1 | AGGTGA a TACC |
| IR-1 (consensus) | AGGTCA a TGACC |

**Fig. 2. Identification of FXRE in the human C3 and rodent fgf15 genes.** A, comparison of FXR response elements from human, rat, and mouse C3 and FGF19/15 genes to an IR-1 found in the mouse IBABP gene and an idealized IR-1 consensus sequence. B, putative FXREs from the C3 and fgf15 genes bind FXR-RXR heterodimers. C, affinity comparison of FXR-RXR binding to C3 and SHP IR-1s. Only the C3 IR-1 electrophoretic mobility shift assay is shown on the left. Calculated IC50 for C3 IR-1 competed with unlabeled C3 (square) or SHP (triangle) or SHP IR-1 competed with unlabeled C3 (circle) are shown below graph to the right. D, EMSA comparing binding of FXR-RXR binding to BSEP and C3 IR-1s. E, calculated IC50 values for competition with unlabeled IR-1s from BSEP (square) or C3 (triangle). Electrophoretic mobility shift assays were performed using in vitro translated FXR-RXR proteins and radiolabeled IR-1s as outlined under "Experimental Procedures." Competitions were done using unlabeled oligonucleotides at 2.5–100-fold molar excess as indicated.
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Fig. 3. FXR-response is mediated through putative IR-1 elements in the human C3 gene. A, the C3 IR-1 is a functional FXRE in the context of the native C3 gene. Comparison of FXR transcriptional activity on C3, BSEP, and SHP promoters. B, mutation of putative IR-1 in the human C3 promoter abolishes FXR activity. CV-1 cells were transfected with wild type or mutated C3, BSEP, or SHP reporter genes with or without pCMX-RXRα, pCMX-FXR, and pCMV-β-galactosidase and treated with FXR ligands GW4064 (1 μM) and CDCA (100 μM) for 20 h. The results were normalized to β-galactosidase activity and expressed as relative light units (RLU) and represent the mean ± S.D. of triplicate determinations. Transfections were performed at least three times, and one representative experiment is shown.

mice, GW4064 increased SHP and IBABP mRNA levels in the liver and intestine, respectively (Fig. 5, C and D). Taken together, these data suggest that regulation of C3 in the intestine may indirectly influence lipid metabolism at other peripheral sites.

**DISCUSSION**

In this study, we have identified complement C3 as a novel FXR target gene. Our results showed that C3 is induced in cell lines of hepatic and intestinal origin and that regulation of C3 by FXR occurs predominantly in the small intestine in vitro. Regulation of C3 was also observed in WAT; however, the observation that FXR mRNA levels are 50–100-fold less in this tissue relative to liver and intestine (Fig. 4F) (26) may contribute to the relatively modest change seen with GW4064. High basal levels of hepatic and adipocyte C3 expression, perhaps resulting from tissue specific factors and/or endogenous fatty acid-derived FXR ligands (22), may also make it difficult to detect an effect of synthetic ligands in these tissues. We showed that the human and rodent C3 genes contain FXREs reminiscent of an IR-1 sequence, that FXR-RXR heterodimers bind to this sequence, and that mutating the binding site abolishes FXR responsiveness of a C3 promoter-reporter construct. Likewise, the genes encoding mouse and rat fgf15 hold IR-1 elements at an almost identical position as the human FGF19 gene. Mice disrupted for FXR showed decreased expression of C3 in the intestine, confirming the importance of this nuclear receptor for regulation of C3. Finally, C3 and fgf15 mRNA levels were increased in response to the FXR agonist GW4064 in intestinal mucosa of mice and rats and C3 protein levels were increased in plasma of rats. The antibody to C3 used in these studies cannot unambiguously detect mouse C3; therefore, additional studies will be needed to determine whether FXR agonists increase plasma C3 levels in mice and whether FGF15 is secreted into plasma.

Taken together, our results establish that FXR directly regulates the genes encoding for C3 and fgf15 and also point to an important function of the intestine as a site of expression. Furthermore, the induction of human FGF19 in the colon-derived Caco-2 cell line similarly suggests the intestine as a site of expression for this factor. The question arising from these studies is whether activation of target genes and ultimately secretion of factors from the intestine play a role in the triglyceride-lowering activity of FXR ligands. FGF19 transgenic mice displayed increased metabolic rate and decreased adiposity (27). Importantly, although a muscle specific promoter (myosin light chain) was used to create the FGF19 transgenic animals, the major target tissue for FGF19 is believed to be the liver. This finding suggests that, in the transgenic mice, FGF19 is produced in muscle and secreted to its target tissue. The mouse counterpart of FGF19, fgf15, was induced by FXR in the intestine rather than in the liver. Although further studies are needed to establish whether fgf15 exerts similar functions as FGF19, our studies suggest that, in addition to C3, FXR regulates production of fgf15.

Studies have shown that, in adipose tissue, C3 is converted to C3a by the action of factors B and D (adipsin) and subsequently is des-arginated by carboxypeptidase N to yield C3desArg, which is identical to ASP. ASP acts in a paracrine fashion to increase free fatty acid uptake and triglyceride synthesis in the adipocyte (24). Although C3 mRNA and protein levels are robustly increased by FXR, we found no evidence of an increase in plasma ASP after administration of the FXR ligand GW4064 in either mice or rats. The reason for this discrepancy may be that ASP is produced by cleavage of C3 locally in the adipose tissue, making it difficult to detect an increase in plasma ASP. Furthermore, in humans, the concentration of ASP in plasma is over 200-fold less than C3 (24). Apparently, only a relatively small fraction of C3 is converted to ASP in circulation and may be difficult to detect. On the same note, whereas the primary site of ASP production is believed to be in adipose tissue, the source of cleaved ASP may be derived from C3 synthesis in other tissues. Although it has traditionally been assumed that the liver is the primary site of synthesis for C3 (28), our results show FXR-dependent induction of C3 mRNA predominantly in the small intestine. Although an increase in plasma C3 protein and mRNA does not necessarily reflect the functional activity of ASP, it is tempting to speculate that an up-regulation of C3 expression leads to increased fatty acid uptake in the adipocyte. Hence, the reduction of plasma triglyceride levels by FXR ligands may in part be the consequence of increased fatty acid uptake and triglyceride storage in adipose tissue.

Mice lacking C3, thus also deficient in ASP, have delayed postprandial triglyceride clearance that can be normalized by administration of recombinant ASP (29). Although these mice lack both C3 and ASP, there is no evidence to suggest that intact C3 plays a direct role in lipid metabolism or fat storage. Nevertheless, serum concentrations of C3 have been associated...
with serum lipid and insulin levels (30–32). Patients with familial combined hyperlipidemia have elevated serum C3 but not ASP (33). These patients have, in addition to hyperlipidemia, metabolic abnormalities including prolonged postprandial lipemia with an excess of free fatty acids in serum. Although still controversial, increased C3 levels in familial

Fig. 4. FXR-dependent regulation of complement C3 and fgf15 in FXR knock-out and wild type mice. Total RNA was prepared from intestinal mucosa (A) and liver of FXR null mice (B), and mRNA levels of C3 were determined by quantitative RT-PCR. C57Bl/6 mice were given a single dose of GW4064 as described under “Experimental Procedures,” and mRNA levels of C3 in intestinal mucosa (C), C3 in liver (D), C3 in WAT (E), FXR in liver, ileum, and WAT (F), fgf15 in intestinal mucosa (G), IBABP in intestinal mucosa (H), and SHP in the liver (I) were determined by quantitative RT-PCR. Data are the mean ± S.E. of 3–4 mice/group. *, p < 0.05 (n = 3); **, p < 0.05 (n = 4); and ***, p < 0.001 in comparison with vehicle-treated animals.
combined hyperlipidemia may indicate a dysfunctional ASP signaling pathway in these patients. A blunted response due to impaired C3 cleavage and consequently impaired peripheral fatty acid uptake may explain the increased hepatic free fatty-acid flux and very low density lipoprotein overproduction in familial combined hyperlipidemia.

The influence of complement activation on the development of atherosclerosis has also been explored. It is well known that atherosclerosis is associated with an inflammatory response to the accumulation of subendothelial lipids, and there is evidence that complement activation is involved in atheroma formation (34). Complement components have been identified in the walls of diseased vessels, and mRNAs of complement factors are expressed in atherosclerotic plaques (35–38). When C3 deficient mice were crossed with apoe-deficient mice, and challenged with a high fat diet, a greater lesion size in the apoe-deficient mice was measured (39).

The activation of C3, a precursor of ASP, and fgf15 in the intestine may have profound effects on lipid metabolism and may in part explain the hypolipidemic effects of FXR ligands.

Acknowledgments—We thank Drs. Richard Martin and Jeff Kahl for the chemical synthesis of GW4064, Mary Petrowski and Amy Liu for Q-PCR assays, and Eric Bischoff, Chris Daige, and Calvin Vu for help with animal studies.

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