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# Suppression of interleukin-6-induced C-reactive protein expression by FXR agonists

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ABSTRACT

C-reactive protein (CRP), a human acute-phase protein, is a risk factor for future cardiovascular events and exerts direct pro-inflammatory and pro-atherogenic properties. The farnesoid X receptor (FXR), a member of the nuclear hormone receptor superfamily, plays an essential role in the regulation of enterohepatic circulation and lipid homeostasis. In this study, we report that two synthetic FXR agonists, WAY-362450 and GW4064, suppressed interleukin-6-induced CRP expression in human Hep3B hepatoma cells. Knockdown of FXR by short interfering RNA attenuated the inhibitory effect of the FXR agonists and also increased the ability of interleukin-6 to induce CRP production. Furthermore, treatment of wild type C57BL/6 mice with the FXR agonist, WAY-362450, attenuated lipopolysaccharide-induced serum amyloid P component and serum amyloid A3 mRNA levels in the liver, whereas no effect was observed in FXR knockout mice. These data provide new evidence for direct anti-inflammatory properties of FXR.


C-reactive protein (CRP) is a major acute-phase protein (APP) in humans. Elevated plasma CRP levels are an independent risk predictor for future cardiovascular events [1,2]. Moreover, many studies have shown that CRP plays direct role in inflammation and pathogenesis of atherosclerosis, including vascular endothelial cell dysfunction, vascular smooth muscle cells migration and proliferation, foam cell formation and inflammatory cell recruitment [3–7]. CRP is synthesized and secreted primarily in human hepatocytes and is regulated mainly by interleukin-6 (IL-6) and interleukin-1 (IL-1) [8]. Fibrates, through activation of the nuclear receptor peroxisome proliferator-activated receptor-α (PPAR-α), lowers plasma levels of triglycerides and cholesterol in humans [9]. Fibrates have also been shown to reduce elevated CRP levels in humans, and strongly suppress IL-1-induced, but not IL-6-induced, CRP expression in human hepatocytes [10,11]. Furthermore, agonist for the nuclear receptor, liver X receptor, also inhibited IL-6/IL-1β-induced CRP expression in human hepatocytes. This was shown to be mediated through inhibition of cytokine-induced NCoR clearance from the CRP promoter [12].

The farnesoid X receptor (FXR; NR1H4) is a member of the nuclear hormone receptor superfamily that functions as a ligand-activated transcription factor and is highly expressed in the liver, intestine, kidney and adrenal glands [13]. FXR regulates many genes involved in bile acid synthesis, lipid and lipoprotein metabolism [14,15]. FXR can be activated by physiological concentrations of bile acids [16–18], or by potent synthetic FXR ligands including GW4064, 6α-ethyl-chenodeoxycholic acid (6ECDCA) and WAY-362450 [19,20,30]. Activation of FXR has been shown to induce eNOS expression in vascular endothelial cells and inhibit vascular smooth muscle cell inflammation by down-regulation inducible nitric oxide synthase and cyclooxygenase-2 expression [21,22].

In this study, we demonstrate that FXR agonists suppress IL-6-induced CRP expression in human Hep3B cells. FXR short interfering RNA (siRNA) abrogated the inhibitory effect of FXR agonists and enhanced the ability of IL-6 to induce CRP production. Finally, FXR agonist attenuated lipopolysaccharide (LPS)-induced serum amyloid P component (SAP) and serum amyloid A3 (SAA3) mRNA levels in the livers of C57BL/6 mice, whereas no effect was observed in FXR knockout (FXR/KO) mice. Thus, inhibition of CRP expression by FXR agonists may provide a promising approach to impact initiation and progression of atherosclerosis.

## Materials and methods

*Cell culture, treatment and siRNA transfection.* Human hepatoma Hep3B cells (ATCC, Rockville, MD) were cultured in MEM supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/strep-

*Abbreviations:* FXR, farnesoid X receptor; SHP, small heterodimer partner; CRP, C-reactive protein; SAP, serum amyloid P component; SAA, serum amyloid A; APP, acute-phase protein; IL-6, interleukin-6; IL-1, interleukin-1; PPAR-α, peroxisome proliferator-activated receptor-α; LXR, liver X receptor; 6ECDCA, 6α-ethyl-chenodeoxycholic acid; FXR/KO, FXR knockout; LPS, lipopolysaccharide; siRNA, short interfering RNA.

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tomycin. For treatment, cells were washed, incubated in serum-free medium, and stimulated with 50 ng/mL IL-6 in the presence of DMSO, 1 μmol/L WAY-362450 or 1 μmol/L GW4064. After 24 h of treatment, the supernatants were collected for CRP measurement with a commercial ELISA kit from Meso scale (K151EPC-3), and the cells were stored in −80 °C for RNA analyses. FXR siRNA and nonsilencing control siRNA were purchased from Dharmacon. Transfections were performed using siRNAs (40 nmol/L) with the transfection reagent Dharma*FECT* Reagent 4. After 48 h, cells were treated as indicated.

*Animal studies.* All procedures involving animals were reviewed and approved by the Wyeth Institutional Animal Care and Use Committee. Male 10–14 week old wild type (WT) C57BL/6 mice and FXR/KO mice on a C57BL/6 background (#004144) were obtained from Jackson Laboratories. C57BL/6 and FXR/KO mice were orally administered with FXR agonist WAY-362450 (30 mg/kg/day) or vehicle (corn oil/ethanol, 9/1) daily for 4 days. At day 4, bacterial LPS (10 μg from Sigma) was injected intraperitoneally. After 4 h, animals were euthanized, and liver samples were harvested for RNA analyses.

*Quantitative RT-PCR and statistical analysis.* Gene expression analysis was performed with quantitative RT-PCR as described previously [23]. Sequences of gene-specific primer and probe sets were designed with Primer Express Software (Applied Biosystems) and available upon request. All results were normalized to GAPDH (4308313; PE Applied Biosystems, Foster City, CA) and are means ± SEM. Statistical significance was determined by ANOVA and *t* test.

## Results

### *FXR agonists suppress IL-6-induced CRP expression in Hep3B cells*

Since human hepatoma cell line Hep3B is a superior model to investigate CRP acute phase response in human hepatocytes [12,24,25], we chose the Hep3B cells to investigate whether FXR agonists modulate IL-6-induced CRP production. Hep3B cells were stimulated with 50 ng/mL IL-6 in the presence or absence of WAY-362450 or GW4064. After 24 h of treatment, CRP levels were determined in the cell supernatants by ELISA. No CRP protein was detected in unstimulated Hep3B cell supernatants, whereas IL-6 treatment resulted in a significant release of CRP (157.8 ± 8.7 pg/ml). The two synthetic FXR agonists were both able significantly inhibit the IL-6-induced CRP release (Fig. 1A, 74.3% inhibition for WAY-362450 at 1 μmol/L and 93.0% inhibition for GW4064 at 1 μmol/L vs vehicle DMSO, $P < 0.01$). The reduction in the media expressed CRP by FXR agonists correlated with a reduction in its mRNA levels. As shown in Fig. 1B, FXR agonists significantly inhibited IL-6-induced CRP mRNA expression (87.8% inhibition for WAY-362450 at 1 μmol/L and 98.1% inhibition for GW4064 at 1 μmol/L vs vehicle DMSO, $P < 0.01$). Importantly, both FXR agonists induced the mRNA levels of the FXR target gene, SHP in Hep3B cells demonstrating the presence of functional FXR in these cells (Fig. 1C). Finally, the suppression of IL-6-induced CRP protein release in Hep 3B cells was dose-dependent with an $IC_{50}$ of 220 nmol/L for WAY-362450 and 90 nmol/L for GW4064 (Fig. 2). Taken together, these data demonstrate that FXR agonists suppress IL-6-induced CRP mRNA and protein expression in Hep3B cells.

### *FXR siRNA reduces the inhibitory effect of FXR agonists and increases the ability of IL-6 to induce CRP production in Hep3B cells*

To confirm that the inhibitory effect of WAY-362450 and GW4064 was mediated through FXR, we used siRNAs to knock-down FXR expression in Hep3B cells. The FXR siRNA transfection resulted in an 85% suppression of endogenous FXR mRNA levels while no reduction was observed with the control siRNA transfection (Fig. 3). The knockdown of FXR mRNA levels correlated with a dramatic loss in FXR agonist inhibition of IL-6 mediated induction of CRP protein and mRNA levels (Fig. 3). There was only a ≈15%

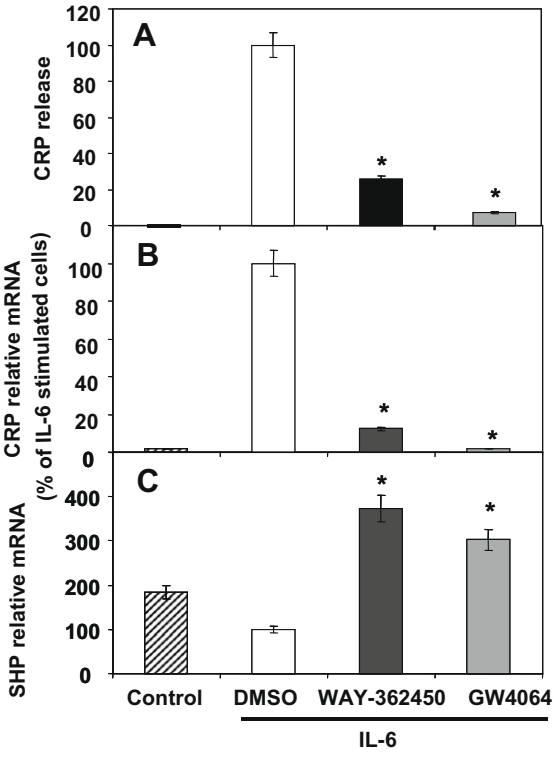


**Fig. 1.** FXR agonists WAY-362450 and GW4064 suppress IL-6-induced CRP protein and mRNA expression in Hep3B cells. Hep3B cells, incubated in serum-free medium, were stimulated with 50 ng/mL IL-6 in the presence of vehicle/DMSO, 1 μmol/L WAY-362450 or 1 μmol/L GW4064 for 24 h. (A) CRP protein levels in cell supernatants were determined by ELISA. (B) CRP mRNA levels in Hep3B cells were determined by quantitative RT-PCR. (C) SHP mRNA levels were determined by quantitative RT-PCR. The results ($n = 8$, in triplicate) are expressed as percentage of levels in IL-6 stimulated cells (mean ± SEM, $^{*}P < 0.01$ vs DMSO). Control represents Hep3B cells in serum-free medium with no treatment.

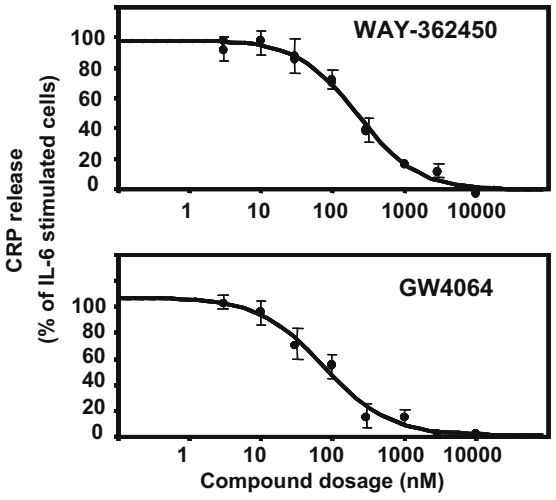


**Fig. 2.** Titration of WAY-362450 and GW4064 in inhibition of IL-6-induced CRP protein release assay in Hep3B cells. The data are plotted as means ± SEM ($n = 4$).