Published in final edited form as:

J Pharmacol Exp Ther. 2007 March ; 320(3): 1153–1162. doi:10.1124/jpet.106.113837.

The Hypolipidemic Agent Guggulsterone Regulates the Expression of Human Bile Salt Export Pump: Dominance of Transactivation over Farnenoid X Receptor-Mediated Antagonism

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Abstract

Conversion of cholesterol to bile acids in the liver is initiated by the rate-limiting enzyme cholesterol 7 α -hydroxylase (CYP7A1) and excretion of bile acids from the liver is mediated by the bile salt export pump (BSEP). The expression of CYP7A1 and BSEP is coordinately regulated by a negative feedback and positive feed-forward mechanism, respectively, through bile acid-mediated activation of farnenoid X receptor (FXR). It is well established that hypolipidemic agent guggulsterone is an FXR antagonist and down-regulates FXR target genes. In this study, however, we have demonstrated that guggulsterone synergistically induced the expression of BSEP in cells treated with FXR agonist bile acids. A dissection study located in the BSEP promoter an activating protein (AP)-1 site supporting the action of guggulsterone. Deletion or mutation of the AP-1 element was diminished, whereas insertion of the AP-1 element into a heterologous promoter enhanced activation of the promoter by guggulsterone. Selective c-Jun N-terminal kinase and extracellular signal-regulated kinase inhibitors markedly decreased the transactivation, suggesting an involvement of AP-1 activation pathway in the up-regulation of BSEP by guggulsterone. Consistent with its FXR antagonism, guggulsterone antagonized bile acid-mediated transactivation of BSEP promoter when the AP-1 element was disrupted. In conclusion, guggulsterone regulates BSEP expression through composite mechanisms, and the transactivation through the AP-1 element is dominant over the FXR-mediated antagonism. The up-regulation of BSEP expression by guggulsterone without activating FXR pathway as an FXR agonist to suppress CYP7A1 expression represents a possible mechanism for guggulsterone-mediated hypolipidemic effect.

Cholesterol is a building block for cell membranes and precursor for the synthesis of steroid hormones. However, excessive cholesterol is associated with the development of many cardiovascular diseases (Kratz, 2005). Conversion of cholesterol to bile acids is initiated by the rate-limiting enzyme cholesterol 7 α -hydroxylase (CYP7A1) in the liver and represents a major pathway for cholesterol elimination (Russell, 2003). Bile acids are physiological

detergents and play important roles in transport, absorption, and elimination of cholesterol and lipids (Fuchs, 2003). However, excessive intracellular accumulation of bile acids is toxic to hepatocytes and causes liver injury (Maher, 2004; Pauli-Magnus and Meier, 2005). Bile acid homeostasis is maintained through the bile acid enterohepatic circulation with canalicular secretion being the rate-limiting step (Fuchs, 2003; Kullak-Ublick et al., 2004). Studies have established that the bile salt export pump (BSEP) is responsible for the canalicular secretion of bile acids from the liver (Strautnieks et al., 1998; Jansen et al., 1999; Noe et al., 2002). Lack of functional BSEP is linked directly to the progressive familial intrahepatic cholestasis type II (Strautnieks et al., 1998; Jansen et al., 1999).

The expression of BSEP and CYP7A1 is coordinately regulated by bile acids through activation of nuclear receptor farnesoid X receptor (FXR) (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999; Goodwin et al., 2000; Lu et al., 2000; Ananthanarayanan et al., 2001; Davis et al., 2002; Gerloff et al., 2002; Plass et al., 2002). Bile acids, such as chenodeoxycholic acid (CDCA), function as FXR agonists and markedly induce BSEP expression (Ananthanarayanan et al., 2001; Gerloff et al., 2002; Plass et al., 2002). On the other hand, the expression of CYP7A1 is negatively regulated by bile acids through a negative feedback mechanism (Goodwin et al., 2000; Lu et al., 2000; Davis et al., 2002). The negative feedback regulation of CYP7A1 is achieved by induction of a nuclear receptor small heterodimer partner (SHP) through bile acid-activated FXR pathway (Goodwin et al., 2000; Lu et al., 2000). SHP forms heterodimers with liver receptor homolog 1 (LRH-1) required for CYP7A1 transactivation, thereby diminishing LRH-1-mediated transactivation of CYP7A1 (Nitta et al., 1999; Goodwin et al., 2000; Lu et al., 2000). This coordinated positive feed-forward regulation of BSEP and negative feedback regulation of CYP7A1 through bile acid/FXR pathway represents an elegant protective mechanism against excessive accumulation of toxic bile acids in hepatocytes.

Guggulsterone is a natural product from the *Commiphora mukul* tree and has been used for lowering cholesterol in human for over several thousands of years (Urizar and Moore, 2003; Ulbricht et al., 2005). Studies in animal models and human trials have demonstrated that guggulsterone improves serum lipoprotein profiles, although there are inconsistent reports (Nityanand et al., 1989; Urizar and Moore, 2003; Ulbricht et al., 2005). Several studies have demonstrated that guggulsterone is an FXR antagonist and down-regulates FXR target genes (Urizar et al., 2002; Wu et al., 2002). FXR antagonism of guggulsterone reportedly accounts for its cholesterol-lowering effect (Urizar et al., 2002). However, as an FXR antagonist, guggulsterone presumably down-regulates BSEP and decreases the secretion of bile acids. As a result, the intrahepatic concentrations of bile acids would increase and in turn trigger the negative feedback suppression on CYP7A1. Consistent with its function as an FXR antagonist, guggulsterone reportedly antagonizes bile acid-mediated activation of the BSEP promoter in a reporter assay (Owsley and Chiang, 2003). However, guggulsterone has been shown to enhance bile acid-mediated induction of BSEP in HepG2 cells (Cui et al., 2003).

In this study, we demonstrated that guggulsterone regulated BSEP expression through composite mechanisms. Overall, guggulsterone alone induced the expression of BSEP. The induction was independent of FXR activation and mediated through an activating protein (AP)-1 site in the BSEP promoter. Indeed, guggulsterone antagonized bile acid-mediated

transactivation of BSEP promoter. However, the antagonistic effect was apparent only when the AP-1 site was disrupted. Therefore, guggulsterone has two distinct functions with an opposite effect on the regulated expression of BSEP: transactivation through the AP-1 element and trans-repression through FXR antagonism with the transactivation being dominant. The up-regulation of BSEP expression by guggulsterone without activating the FXR pathway as an FXR agonist does to suppress CYP7A1 expression thus represents a possible mechanism for guggulsterone-mediated hypolipidemic effect.

Materials and Methods

Chemicals and Supplies

CDCA, U0126, SP600125, SB203580, dimethyl sulfoxide (DMSO), and Williams' E medium E were purchased from Sigma (St. Louis, MO). Guggulsterone was from Steraloids Inc. (Newport, RI). DMEM, Lipofectamine, and Plus Reagent were from Invitrogen (Carlsbad, CA). Kits for luciferase detection and the null-*Renilla* luciferase plasmid were from Promega (Madison, WI). Fetal bovine serum and 100× nonessential amino acids were from HyClone (Logan, UT). Unless otherwise specified all other reagents were purchased from Fisher Scientific Co. (Suwanee, GA). Oligonucleotides for PCR amplification, site-directed mutagenesis, and cloning were chemically synthesized by Invitrogen.

Plasmid Constructs

The preparation of the human BSEP promoter reporter pBSEP(−2.6 kb) was described elsewhere (Deng et al., 2006). This reporter was used as the template to prepare reporters with shorter genomic sequences including pBSEP(−405 bp), pBSEP(−205 bp), pBSEP(−165 bp), and pBSEP(−125 bp). All reporters were prepared with the pGL4.10 vector at the XhoI and NheI sites. The resulting reporter constructs were sequence-verified. The sequences of the corresponding oligonucleotides were listed in Table 1. To construct pBSEP(−205 bp) +1xGuRE and pBSEP(−205 bp)+2xGuRE, the sense and antisense oligonucleotides containing one or two guggulsterone-responsive element (GuRE) repeats were synthesized, annealed, and inserted into the pBSEP(−205 bp) at the NheI site. To construct pGL3/p-3xGuRE, -3RxGuRE, -3xAP-1 BSEP, -3xAP-1 BSEP Mut, -3xAP-1 BSEP5, -3xAP-1 Cons, and -3xAP-1 Cons Mut, the sense and antisense oligonucleotides consisting of three copies of the element were chemically synthesized, annealed, and cloned into a luciferase reporter vector pGL3 promoter (pGL3/p) (Promega) at the XhoI and NheI sites followed by sequencing verification. The pBSEP(−2.6 kb)-IR1 Mut was made as described previously (Deng et al., 2006). Expression plasmids for human nuclear receptors FXR was kindly provided by Dr. D. Mangelsdorf (University of Texas Southwestern Medical Center, Dallas, TX).

Site-Directed Mutagenesis

Mutagenesis was performed using QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Four nucleotide substitutions in the AP-1 site were introduced into pBSEP(−2.6 kb) and pBSEP(−205 bp), resulting in mutant pBSEP(−2.6 kb)-AP-1 Mut and pBSEP(−205 bp)-AP-1 Mut, respectively. Two putative LRH-1-responsive elements (LRHREs) in the GuRE were mutated by substituting four nucleotides of the core sequences, resulting in

mutant pBSEP(–2.6 kb) LRHRE1 Mut and pBSEP(–2.6 kb) LRHRE2 Mut. Two amino acid residues, Tyr361 and His447, in the ligand-binding domain of human FXR were substituted by leucine (FXR-Y361L) and phenylalanine (FXR-H447F), respectively. The sequences of the mutagenic oligonucleotides were listed in Table 1. The mutagenesis reactions were performed essentially according to the manufacturer's manual (Stratagene, La Jolla, CA). The resulting mutants were subjected to sequence analysis to confirm the desired substitutions being made without introducing errors.

Human Primary Hepatocyte and Hepatoma Cell Culture and Treatments

Plated human primary hepatocytes (in six-well plate) were obtained from the Liver Tissues Procurement and Distribution System (University of Minnesota, Minneapolis, MN). Upon arrival, cell supernatants were replaced with rich Williams' medium E containing 1% penicillin/streptomycin. After incubation at 37°C with 5% CO₂ for 6 h, cells were treated with 0.1% DMSO, 10 μM guggulsterone, and 10 μM CDCA or a combination of 10 μM guggulsterone and CDCA. The treatment was continued for 30 h with a change of fresh treatment medium at 24 h. Hepatoma Huh7 cells purchased from the American Type Culture Collection (Manassas, VA) were maintained in DMEM containing 10% fetal bovine serum, 1% penicillin/streptomycin, and 1× nonessential amino acids. Cells were seeded at a density of 2×10^5 cells/well (12-well plates) and cultured overnight. The cells were then treated with appropriate chemicals at the concentrations specified above. Unless specified, all the treatments lasted for 30 h.

Reverse Transcription Coupled Real-Time PCR

Total RNA was isolated from treated cells as described previously (Deng et al., 2006). cDNA was synthesized with 1 μg of total RNA and random primers in a total volume of 25 μl. The reactions were incubated initially at 25°C for 10 min and then at 50°C for 50 min, followed by inactivation of the reaction at 70°C for 10 min. The cDNAs were then diluted five times with water and subjected to real-time PCR using TaqMan Gene Expression Assay (Applied Biosystems, Foster City, CA). The TaqMan assay mixtures for BSEP (assay identification, Hs00184824_m1), NAD(P)H:quinine oxidoreductase 1 (NQO1) (assay identification, Hs00168547_m1), and GAPDH (assay identification, 4352934E) were purchased from Applied Biosystems. The real-time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems) in a total volume of 20 μl containing 10 μl of universal PCR master mix, 1 μl of gene-specific TaqMan assay mixture, and 5 μl of cDNA templates. Cycling profile was 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 15 s at 95°C and 1 min at 60 °C, as recommended by the manufacturer. Amplification and quantification were done with the Applied Biosystems 7500 Real-Time PCR System.

Reporter Luciferase Assay

Huh7 cells were plated in 24-well plates in DMEM supplemented with 10% fetal bovine serum at a density of 8×10^4 cells/well and cultured for overnight. Transient transfection was conducted by lipofection with Lipofectamine and Plus Reagent (Invitrogen) as described previously (Song et al., 2004). For all the transfections, standard amounts of

plasmid DNA used per well were 100 ng for BSEP promoter construct, 100 ng for nuclear receptor expression plasmids, and 10 ng for the null-*Renilla* luciferase plasmid as the internal control. After cells were transfected for 3 h, 1 ml of fresh medium was added into each well, and cells were incubated overnight. Then cell supernatants were replaced with treatment medium containing appropriate chemicals at a concentration specified in the figure legends. The treatment lasted for 30 h unless specified. The luciferase activities were assayed with a Dual-Luciferase Reporter Assay System as described previously (Song et al., 2004). Treated Huh7 cells were washed once with PBS and lysed by adding 100 μ l of passive lysis buffer with gentle rocking for 30 min. Cell lysates (10 μ l) were transferred to a 96-well reader plate, and luciferase activities were measured by EG and G Berthold Microplate Luminometer (PerkinElmer Life and Analytical Sciences, Boston, MA). The firefly luminescence was normalized based on the *Renilla* luminescence signal, and the ratio of treatment over control served as -fold activation. Data are presented as mean $\pm$ S.D. of at least three separate experiments.

Results

Guggulsterone Induces BSEP Expression and Synergistically Up-Regulates BSEP with Bile Acids

Several studies have established that guggulsterone is an FXR antagonist and down-regulates FXR target genes (Urizar et al., 2002; Wu et al., 2002). However, its function in regulating BSEP remains unclear (Cui et al., 2003; Owsley and Chiang, 2003). To determine whether guggulsterone functions as an activator or antagonist for BSEP expression, human primary hepatocytes derived from three donors were treated with guggulsterone, CDCA, or both for 30 h, and the level of BSEP mRNA was determined by real-time PCR. As shown in Fig. 1, marked increase in BSEP mRNA levels was detected in hepatocytes treated with guggulsterone (2.8–3.6-fold) or CDCA (4–6.7-fold) compared with vehicle-treated hepatocytes. In addition, the level of BSEP mRNA was synergistically increased in hepatocytes treated with both guggulsterone and CDCA (~13-fold). Similar induction of BSEP expression by guggulsterone was also detected in hepatoma Huh7 cells (Fig. 1). It should be mentioned that, as expected, the expression of SHP, an FXR target gene, was decreased in hepatocytes treated with a combination of guggulsterone and CDCA compared with cells treated with CDCA alone (data not show).

Guggulsterone-Mediated Up-Regulation of BSEP Expression Is Achieved through Activation of BSEP Promoter

We have demonstrated that guggulsterone induces BSEP expression and synergistically up-regulates BSEP with bile acids. To determine whether the action of guggulsterone is achieved through activation of the BSEP promoter, a BSEP promoter reporter, pBSEP(–2.6 kb), was transfected into Huh7 cells. The transfected cells were then treated with increasing concentrations of guggulsterone in the presence or absence of 10 μ M CDCA. As shown in Fig. 2, guggulsterone activated the BSEP promoter in a dose-dependent manner, and significant activation was detected when guggulsterone was assayed as low as 2 μ M. Consistent with the real-time PCR analysis, guggulsterone synergistically enhanced CDCA-mediated activation of the BSEP promoter. At 10 μ M, guggulsterone and CDCA caused an

activation of 3.5- and 4.7-fold, respectively, whereas over 11-fold induction was detected in cells treated with a combination of guggulsterone and CDCA. Taken together, the data suggested that guggulsterone-mediated induction of BSEP expression was achieved through activation of the BSEP promoter.

Guggulsterone-Mediated Up-Regulation of BSEP Is Independent of FXR Activation through FXR Element

To determine whether guggulsterone-mediated activation is achieved through the activation of FXR via its binding to FXR element (FXRE) in the BSEP promoter, mutational analyses of both FXR and FXRE were performed, and the resultant mutants were tested for their ability to respond to guggulsterone treatment.

According to the crystal structure of FXR ligand binding domain (Downes et al., 2003; Mi et al., 2003), the two residues, Tyr361 and His447, that serve as the receptor's activation trigger through direct interaction with FXR ligand and were chosen for substitution with leucine and phenylalanine, respectively. Considering functional and structural importance of the two residues, we expected that substitution of either residue would result in loss of its ability to activate BSEP promoter. Consistent with the expectation, substitution of Y361L or H447F almost completely abolished the activation of BSEP promoter by CDCA (Fig. 3A). However, in contrast, guggulsterone-mediated activation was not affected by either substitution. In addition, guggulsterone but not CDCA markedly activated the BSEP promoter in the absence of cotransfected FXR (Fig. 3A), although the basal activity was decreased as well. The finding strongly indicated that guggulsterone induced BSEP expression through a mechanism independent of FXR activation.

To further support the finding, an FXRE mutant, pBSEP-IR1-Mut (Deng et al., 2006), was tested for the ability to respond to guggulsterone. As showed in Fig. 3B, disruption of the FXRE resulted in marked decrease in activation by CDCA. However, guggulsterone-mediated activation remained unchanged. This finding further suggests that activation of the BSEP promoter by guggulsterone is achieved through a mechanism rather than FXR activation.

Guggulsterone Activates the BSEP Promoter through a Guggulsterone-Responsive Element

We have demonstrated that guggulsterone-mediated activation of the BSEP promoter is independent of the FXR/FXRE pathway. To localize the putative GuRE, a series of BSEP promoter reporter constructs with various lengths were prepared (Fig. 4A) and tested for the ability to respond to guggulsterone. As shown in Fig. 4B, no activation was detected with pBSEP(-125 b) and pBSEP(-165 b) in response to guggulsterone. However, when the promoter was extended by another 40 bp in pBSEP(-205 bp), the activation was comparable as those of the promoter constructs containing far upstream sequences pBSEP(-405 bp) and pBSEP(-2.6 kb) (Fig. 4B). On the other hand, the activation of the BSEP promoter by CDCA remained similar among all the promoter constructs (Fig. 4B). Thus, the results localized the GuRE in a region from -165 to -205 bp. It should be mentioned that the basal activity of the pBSEP(-125 bp) and pBSEP(-165 bp) promoter constructs was significantly

decreased compared with the pBSEP(−205 bp) construct, underscoring the importance of the GuRE in the basal activity of the BSEP promoter.

To determine whether the GuRE is sufficient to support the activation by guggulsterone, various copies of GuRE were inserted into either homologous or heterologous promoter reporters, and the resultant constructs were tested for the ability to respond to guggulsterone. As shown in Fig. 5A, pBSEP(−205 bp)+1xGuRE or pBSEP(−205 bp)+2xGuRE exhibited enhanced activation by guggulsterone (4.6- and 6.9-fold increase, respectively) compared with parental construct pBSEP(−205 bp) (2.5-fold increase). As expected, CDCA-mediated activation remained unchanged (Fig. 5A). In addition, insertion of three copies of the GuRE into a heterologous enhancer reporter vector pGL3/p in either orientation resulted in marked increase not only in basal activity but also in guggulsterone-specific activation of the promoter, whereas no activation was detected by CDCA.

The AP-1 Binding Site in the GuRE Is Responsible for Mediating Guggulsterone-Specific Transactivation of BSEP

Bioinformatic analysis of the GuRE revealed one AP-1 binding site and two putative LRHREs (Fig. 6A). To determine whether those elements are involved in guggulsterone activation, mutational analyses of those elements were performed. Using pBSEP(−2.6 kb) as template, four nucleotide substitutions were introduced into each of the three sites (Fig. 6A). The resulting mutants, pBSEP(−2.6 kb)-AP-1 Mut, pBSEP(−2.6 kb)-LRHRE1 Mut, and pBSEP(−2.6 kb)-LRHRE2 Mut, were transfected into Huh7 cells and tested for the ability to respond to guggulsterone. As shown in Fig. 6A, disruption of the AP-1 site not only reduced basal activity but also completely abolished guggulsterone-mediated activation. On the other hand, mutation of the two LRHREs had no significant effect on guggulsterone activation, although slight increase or decrease in activation was detected in mutant LRHRE1 and LRHRE2, respectively (Fig. 6A). Taken together, the data indicated that the AP-1 site, but not the LRHREs in the GuRE, was responsible for guggulsterone activation.

To further confirm the importance of the AP-1 element for the transactivation, three copies of the wild-type and mutant AP-1 element were cloned into the pGL3/p vector, and the corresponding reporters pGL3/p-3xAP-1 BSEP and pGL3/p-3xAP-1 BSEP Mut were tested for the ability to respond to guggulsterone. As shown in Fig. 6B, guggulsterone activated the reporter containing wild-type AP-1 element but not the mutant reporter, further establishing that the AP-1 site supports guggulsterone activation of the BSEP promoter. Furthermore, removal of the 5′-flanking sequence containing the LRHRE1 had no effect on guggulsterone-mediated activation (Fig. 6B), consistent with the results from the mutational analysis of the LRHRE1 (Fig. 6A). Guggulsterone also activated the reporter containing an AP-1 consensus motif, further establishing the role of the AP-1 pathway in supporting the action of guggulsterone (Fig. 6B).

Guggulsterone Acts as an Antagonist of FXR in the AP-1-Less BSEP Promoter

We have demonstrated that the activation of the BSEP promoter by guggulsterone is mediated through the AP-1 element in the BSEP promoter. On the other hand, it has been well established that guggulsterone is an antagonist of FXR (Urizar et al., 2002; Wu et al.,

2002). We hypothesized that guggulsterone acted as an FXR antagonist when the AP-1 site in the BSEP promoter was disrupted. To directly test the hypothesis, various BSEP promoters containing deletions or mutations in the AP-1 site were evaluated for the ability to respond to guggulsterone, CDCA, or both. As shown in Fig. 4B, BSEP promoters carrying an intact AP-1 site, including pBSEP(–2.6 kb), pBSEP(–405 bp), and pBSEP(–205 bp), exhibited synergistic activation of BSEP promoter in response to combined treatment of both guggulsterone and CDCA, whereas BSEP promoters without the AP-1 element, including pBSEP(–165 bp) and pBSEP(–125 bp), showed decreased activation by guggulsterone and CDCA compared with cells treated with CDCA alone, suggesting that guggulsterone antagonized CDCA-mediated activation of BSEP promoter in the absence of the AP-1 element. To further support the finding, two AP-1 mutants, pBSEP(–2.6 kb)-AP-1 Mut and pBSEP(–205 bp)-AP-1 Mut, were constructed and tested for the ability to respond to CDCA or a combination of CDCA and guggulsterone. Both mutants exhibited a decreased activation of BSEP promoter in cells treated with a combination of guggulsterone and CDCA in comparison with cells treated with CDCA alone (Fig. 7), suggesting an antagonistic effect of guggulsterone on CDCA-mediated activation of BSEP promoter. It should be mentioned that the antagonistic effect of guggulsterone was minimal at the concentration of 10 μ M, indicating that guggulsterone is a weak FXR antagonist, consistent with its weak binding affinity with FXR (Burris et al., 2005). Taken together, the data demonstrated that guggulsterone was indeed an FXR antagonist, and the antagonistic effect was detectable only with reporters containing the disrupted AP-1 element. Therefore, for the regulation of BSEP expression, guggulsterone has two distinct functions with opposite effect: its transactivation through the AP-1 element and repression through FXR antagonism with the transactivation being dominant.

AP-1 Activation Pathway Is Involved in the Guggulsterone-Induced Expression of BSEP

We have demonstrated that the AP-1 binding site is required for the activation of the BSEP promoter in response to guggulsterone. AP-1 proteins are a large group of transcription factors, including members from the Jun and Fos family. Activation of the Jun family is achieved mainly by the c-Jun N-terminal kinase (JNK), whereas extracellular signal-regulated kinase (ERK) mediates activation of Fos family (Whitmarsh and Davis, 1996; Dhanasekaran and Premkumar Reddy, 1998). To determine whether activation of the Jun and Fos family of proteins is involved in the activation of the BSEP promoter by guggulsterone, inhibitors of both kinases were tested for the ability to decrease guggulsterone-mediated activation of BSEP. Huh7 cells were transfected with pBSEP(–2.6 kb) reporter and treated with either DMSO or guggulsterone with JNK inhibitor SP600125 or ERK inhibitor U0126 for 24 h, followed by detection of luciferase activity. A selective inhibitor for p38 kinase, SB203580, was also included in the treatment as control. As shown in Fig. 8A, treatment with SP600125 or especially U0126 resulted in marked decreases in guggulsterone-mediated activation, whereas treatment with SB203580 had little effect, suggesting that both the JNK and ERK signaling pathways, which potentially lead to AP-1 activation, are involved in guggulsterone-mediated induction of BSEP expression.

Guggulsterone Induces the Expression of NQO1

We have demonstrated that guggulsterone activates the promoter reporter carrying an AP-1 consensus sequence (Fig. 6B), indicating that guggulsterone may induce the expression of other AP-1 target genes. To test this possibility, the expression of NQO1 was studied after guggulsterone treatment in both human primary hepatocytes and Huh7 cells. NQO1 is a phase II detoxification enzyme responsive for electron transfer to a wide variety of redox-cycling species, including quinones. The promoter of NQO1 contains a perfect AP-1 site, and the expression of NQO1 is regulated by xenobiotics, such as baicalin, through AP-1 transactivation (Patten and DeLong, 1999; Park et al., 2004). As shown in Fig. 8B, NQO1 mRNA was significantly induced in hepatocytes (4.3–6.1-fold) as well as Huh7 cells (8.3-fold) treated with guggulsterone compared with vehicle-treated cells. The results were consistent with the notion that AP-1 supports the action of guggulsterone.

Discussion

Conversion of cholesterol to bile acids and subsequent excretion through the enterohepatic circulation represent a major pathway to remove excessive cholesterol from the body (Russell, 2003). CYP7A1 is the rate-limiting enzyme in the classic pathway of bile acid synthesis from cholesterol in the liver (Fuchs, 2003; Russell, 2003). On the other hand, canalicular secretion of bile acids by BSEP is the rate-limiting step in the bile enterohepatic circulation (Fuchs, 2003; Kullak-Ublick et al., 2004). Both BSEP and CYP7A1 are coordinately regulated by the bile acids/FXR activation pathway through the positive feed-forward and negative feedback mechanism, respectively (Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999; Goodwin et al., 2000; Lu et al., 2000; Ananthanarayanan et al., 2001; Davis et al., 2002; Gerloff et al., 2002; Plass et al., 2002). Such coordinated regulation represents an elegant protective mechanism against hepatic injury by excessive accumulation of bile acids. However, this regulatory mechanism is not effective in removing excessive cholesterol from the body by either FXR agonist or antagonist. FXR agonist up-regulates BSEP expression but suppresses CYP7A1 transcription, which decrease the conversion of cholesterol into bile acids. On the other hand, the FXR antagonist enhances CYP7A1 but suppresses BSEP expression, which results in accumulation of bile acids in hepatocytes and subsequently suppresses CYP7A1. Therefore, it remains unclear whether an FXR agonist or antagonist is beneficial for treating hypercholesterolemia.

Guggulsterone is an active compound from guggulipid, an extract from the guggul tree. It has been widely used for treatment of obesity, arthritis, inflammation, and hypercholesterolemia (Sinal and Gonzalez, 2002; Urizar and Moore, 2003). Studies have demonstrated that guggulipid had beneficial effect on improving cholesterol and lipid profile (Nityanand et al., 1989; Urizar and Moore, 2003; Ulbricht et al., 2005) in animal models and human, although there are inconsistent reports (Nityanand et al., 1989; Szapary et al., 2003; Urizar and Moore, 2003; Ulbricht et al., 2005). However, the molecular mechanism for cholesterol-lowering effect of guggulsterone remains elusive. Studies have established that guggulsterone is an antagonist of FXR and down-regulates FXR target genes (Urizar et al., 2002; Wu et al., 2002). Such FXR antagonism by guggulsterone is suggested as a mechanism for its hypolipidemic effect (Urizar et al., 2002). However, inconsistent with this

notion is the finding that hypolipidemic effects are observed in rats administrated with a selective synthetic FXR agonist (Willson et al., 2001; Maloney et al., 2000). It was also speculated that inhibition of CYP7A1 by guggulsterone through activating pregnane X receptor is a possible mechanism for its cholesterol-lowering effect (Owsley and Chiang, 2003). In addition to acting as an FXR antagonist and pregnane X receptor agonist, guggulsterone also functions as an antagonist of androgen receptor glucocorticoid receptor and mineralocorticoid receptor but as an agonist of progesterone receptors (Burris et al., 2005). Taken together, those data suggest that, in addition to its FXR antagonism, there are other possible mechanisms by which guggulsterone exerts its hypolipidemic effect. In this study, we demonstrated that in contrast to its FXR antagonism, guggulsterone induced and synergistically up-regulated BSEP expression with bile acids in human primary hepatocytes and hepatoma Huh7 cells. The induction of BSEP by guggulsterone was independent of FXR activation and mediated through the AP-1 element in the BSEP promoter. On the other hand, guggulsterone antagonized bile acid-mediated transactivation of BSEP promoter when the AP-1 element was disrupted by deletion or mutation. The results are inconsistent with the finding that guggulsterone functions as an FXR antagonist in coactivator recruitment assays but enhances BSEP transcription (Cui et al., 2003). However, our results are clearly in conflict with a previous report showing that guggulsterone antagonized CDCA-mediated BSEP promoter activation in a reporter assay (Owsley and Chiang, 2003). Currently, we have no obvious explanation for the discrepancy. With the finding from the current study, it is concluded that guggulsterone regulates BSEP expression through composite mechanisms, and its transactivation through the AP-1 element is dominant over its FXR-mediated antagonism (Fig. 9A).

The unique properties associated with guggulsterone as FXR antagonist as well as BSEP activator separated itself from the classes of sole FXR agonists or antagonists. With the new findings from the current study, we are proposing that enhanced BSEP expression by guggulsterone without activating the FXR pathway represents a possible mechanism by which guggulsterone exerts its hypolipidemic effect (Fig. 9B). As an activator by itself and synergistic inducer with bile acids for BSEP transcription, guggulsterone up-regulates BSEP expression and consequently increases secretion of bile acids, which in turn relieves the suppression of CYP7A1 expression by bile acids and favors cholesterol metabolism (Fig. 9B). The up-regulation of BSEP expression by guggulsterone without activating FXR to suppress CYP7A1 is the driving force to promote cholesterol metabolism by removing the trigger, bile acids, for the negative feedback suppression on CYP7A1. Consistent with this notion is the observation that hepatic overexpression of BSEP in transgenic mice containing additional copy of BSEP increased hepatobiliary lipid secretion and reduced hepatic steatosis (Figge et al., 2004) and administration of FXR agonist, presumably resulting in up-regulation of BSEP expression, improves lipid profiles in rats (Willson et al., 2001; Maloney et al., 2000).

The finding that guggulsterone transactivates BSEP through the AP-1 element implies that guggulsterone may regulate a large number of AP-1 site-containing genes and have broader pharmacological and therapeutic effects, given the fact that functional AP-1 element is present in a myriad of genes involved in cell proliferation, differentiation, inflammation, and cancer cell invasion and metastasis. Consistent with this notion are the results that

guggulsterone potentially activated a promoter reporter containing the AP-1 consensus sequence (Fig. 6B) and markedly induced the expression of NQO1 (Fig. 8B), which is regulated by xenobiotics through the AP-1 transactivation (Patten and DeLong, 1999; Park et al., 2004). In addition to hypolipidemic effects, guggulsterone has beneficial effects on treating arthritis, inflammation, and obesity (Sinal and Gonzalez, 2002; Urizar and Moore, 2003), which may be related to its property to transactivate various AP-1 target genes.

AP-1 proteins consist of a group of transcription factors, including members from the Jun and Fos families. Activation of the Jun family members through their phosphorylation is achieved by JNK, whereas ERK mediates activation of the Fos family members. Both JNK and ERK selective inhibitors markedly suppress guggulsterone-mediated induction of BSEP expression, suggesting that activations of the Jun and Fos family members are involved in the induction. However, the identity of the AP-1 proteins or other closely related transcription factors involved in the binding and activating BSEP promoter remains to be determined.

We have demonstrated that guggulsterone synergistically induces BSEP expression with bile acids in both human hepatocytes and Huh7 cells. Bile acid/FXR-mediated trans-activation of BSEP is achieved through the FXRE, whereas guggulsterone-specific induction of BSEP expression is accomplished through the AP-1 element. The observed synergistic effect suggested a cross-talking between the FXR signaling and AP-1 activation pathway triggered by bile acids and guggulsterone, respectively. However, the mechanistic insights into the synergistic transactivation of BSEP by bile acids and guggulsterone remain to be revealed.

In summary, guggulsterone regulates BSEP expression through composite mechanisms and its transactivation through the AP-1 element is dominant over its FXR antagonism. The up-regulation of BSEP by guggulsterone without activating FXR pathway as an FXR agonist does to suppress CYP7A1 represents a possible mechanism for its cholesterol-lowering effect.

Acknowledgments

This work was partially supported by the New Investigator Program for pharmacy faculty, American Association of Colleges of Pharmacy; by the Rhode Island Foundation; by the Rhode Island-IDEA Network of Biomedical Research Excellence (Grant P20 RR016457 from National Center for Research Resources/National Institutes of Health); and by the National Institutes of Health (Grants R01GM61988, R01ES07965, and F05AT003019).

We thank David Mangelsdorf for providing human FXR expression plasmid. The contributions to the project by Xiulong Song are gratefully acknowledged.

ABBREVIATIONS

CYP7A1	cholesterol 7 α -hydroxylase
BSEP	bile salt export pump
FXR	farnesoid X receptor
CDCA	chenodeoxycholic acid

SHP	small heterodimer partner
LRH	liver receptor homolog
AP	activating protein
U0126	1,4-diamino-2,3-dicyano-1,4-bis(methylthio)butadiene
SB203580	4-(4-fluorophenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1 <i>H</i> -imidazole
DMSO	dimethyl sulfoxide
PCR	polymerase chain reaction
GuRE	guggulsterone-responsive element
LRHRE	LRH-1-responsive element
NQO1	NAD(P)H quinine oxidoreductase 1
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
FXRE	FXR element
JNK	c-Jun N-terminal kinase
ERK	extracellular signal-regulated kinase
SP600125	1,9-pyrazoloanthrone anthrapyrazolone
U0126	1,4-diamino-2,3-dicyano-1,4-bis[2-aminophenylthio]butadiene
SB203580	4-(4-fluoro-phenyl)-2-(4-methylsulfinylphenyl)-5-(4-pyridyl)-1 <i>H</i> -imidazole

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Fig. 1.

Guggulsterone induces BSEP expression and synergistically up-regulates BSEP with bile acids. Human primary hepatocytes derived from three individual donors and hepatoma Huh7 cells were treated with 0.1% DMSO, 10 μ M guggulsterone (Z form), 10 μ M CDCA, or a combination of 10 μ M guggulsterone and 10 μ M CDCA for 30 h. Total RNA was isolated from treated cells and subjected to cDNA synthesis as described previously (Deng et al., 2006), followed by detection and quantitative analysis of BSEP mRNA by TaqMan real-time PCR with GAPDH mRNA level as the internal control. The real-time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems), following the instruction of the manufacturer in a total volume of 20 μ l. Cycling profile was: 50 $^{\circ}$ C for 2 min, 95 $^{\circ}$ C for 10 min, followed by 40 cycles of 15 s at 95 $^{\circ}$ C and 1 min at 60 $^{\circ}$ C, as recommended by the manufacturer. Amplification and quantification were done with the Applied Biosystems 7500 Real-Time PCR System. The BSEP mRNA levels were normalized with GAPDH mRNA levels. Data are presented as mean $\pm$ S.D. of two repeated experiments.

Fig. 2.

Guggulsterone-mediated induction of BSEP is achieved through activation of the BSEP promoter. Huh7 cells seeded in 24-well plates were transfected with 100 ng of BSEP promoter reporter pBSEP(−2.6 kb), 100 ng of human FXR expression plasmid, and 10 ng of the null-*Renilla* luciferase plasmid (as the internal control) and incubated overnight. Cells were treated with increasing concentrations of guggulsterone (0–20 μM) in the absence or presence of 10 μM CDCA for 30 h, followed by a luciferase assay. Luciferase activities were measured by a Dual-Luciferase Reporter Assay System according to the instruction of the manufacturer (Promega). The firefly luminescence was normalized based on the *Renilla* luminescence signal. The data are presented as mean ± S.D. of at least three separate experiments.

Fig. 3.

Guggulsterone-mediated activation of the BSEP promoter is independent of FXR activation through FXRE. A, two amino acid residues, Tyr361 and His447, in the FXR ligand binding domain were mutated to leucine and phenylalanine, respectively. Huh7 cells were transfected with 100 ng of BSEP promoter reporter pBSEP(-2.6 kb), 10 ng of the null-*Renilla* luciferase plasmid, and 100 ng of wild-type FXR, mutant FXR(Y361L), mutant FXR(H447F), or empty vector (no FXR). B, FXRE, known as inverted repeat with one nucleotide space (IR-1) in the BSEP promoter, was disrupted as described previously (Deng et al., 2006). The mutant reporter pBSEP(-2.6 kb)-IR1 Mut (100 ng) was transfected into Huh7 cells with 100 ng of FXR expression plasmid and 10 ng of the null-*Renilla* luciferase plasmid. Sixteen hours post-transfection, cells were treated with 0.1% DMSO, 10 μ M guggulsterone, or 10 μ M CDCA for 30 h, followed by detection of luciferase activities with the Dual-Luciferase Reporter Assay System. The data are presented as mean $\pm$ S.D. of at least three separate experiments.

Fig. 4.

Guggulsterone activates BSEP promoter through a distinct GuRE. A, series of BSEP promoter reporters, including pBSEP(-405 bp), pBSEP(-205 bp), pBSEP(-165 bp), and pBSEP(-125 bp), were prepared by PCR amplification using pBSEP(-2.6 kb) as the template. The oligonucleotides used for PCR amplifications were listed in Table 1. The resulting reporter constructs were sequence-verified. B, Huh7 cells were transfected with 100 ng each of the promoter reporters, 100 ng of FXR expression plasmid, and 10 ng of the null-*Renilla* luciferase plasmid, followed by treatment of the transfected cells with 0.1% DMSO, 10 μ M guggulsterone, 10 μ M CDCA, or a combination of 10 μ M guggulsterone and 10 μ M CDCA for 30 h. Luciferase activities were measured by a Dual-Luciferase Reporter Assay System according to the instruction of the manufacturer (Promega). The data are presented as mean $\pm$ S.D. of at least three separate experiments.

Fig. 5.

The GuRE functions as an independent element to mediate guggulsterone-specific activation in homologous and heterologous promoters. A, one or two additional copies of the GuRE were added to the homologous BSEP promoter reporter, pBSEP(−205 bp), resulting in two constructs, pBSEP(−205 bp)+1xGuRE and pBSEP(−205 bp)+2xGuRE. Each of the reporter constructs (100 ng) was cotransfected into Huh7 cells with 100 ng of FXR expression plasmid and 10 ng of the null-*Renilla* luciferase plasmid. B, three copies of the GuRE in both orientations were inserted into a heterologous promoter reporter pGL3/p, resulting in two corresponding constructs, pGL3/p-3xGuRE and pGL3/p-3RxGuRE. Each of the reporter constructs (100 ng) was cotransfected into Huh7 cells with 10 ng of the null-*Renilla* luciferase plasmid as the internal control. Sixteen hours post-transfection, the transfected cells were treated with 0.1% DMSO, 10 μ M guggulsterone, or 10 μ M CDCA for 30 h. Reporter activities were detected by the Dual-Luciferase Reporter Assay System. The data are presented as mean $\pm$ S.D. of at least three separate experiments.

Fig. 6.

The AP-1 binding site in the GuRE is responsible for mediating guggulsterone-specific transactivation of BSEP. A, AP-1 binding site and two putative LRHREs in the GuRE were individually mutated by substituting four nucleotides in their core sequences, resulting in three mutants, pBSEP(−2.6 kb)-AP-1 Mut, pBSEP(−2.6 kb)-LRHER1 Mut, and pBSEP(−2.6 kb)-LRHER2 Mut. The sequences of the wild type and mutants were shown on the top panel. B, reporter constructs, pGL3/p-3xAP-1 BSEP, -3xAP-1 BSEP Mut, -3xAP-1 BSEP5, -3xAP-1 Cons, and -3xAP-1 Cons Mut, were prepared by cloning the synthetic fragments containing three copies of the element into the luciferase reporter vector pGL3/p, followed by sequencing verification. The sequences of each element were presented on the top panel. To characterize all those mutants, Huh7 cells were cotransfected with each mutant (100 ng) and internal control plasmid null (10 ng). Sixteen hours post-transfection, the cells were treated with 0.1% DMSO or 10 μ M guggulsterone for 30 h, followed by detection of luciferase activity. The data are presented as mean $\pm$ S.D. of at least three separate experiments.

Fig. 7.

Guggulsterone acts as an FXR antagonist when the AP-1 element in BSEP promoter is disrupted by mutation. Using pBSEP(−2.6 kb) and pBSEP(−205 bp) as parental template, the AP-1 element was mutated by site-directed mutagenesis, resulting in two corresponding mutants, pBSEP(−2.6 kb)-AP-1 Mut and pBSEP(−205 bp)-AP-1 Mut. Each of the four reporter constructs (100 ng) was cotransfected into Huh7 cells with 100 ng of FXR expression plasmid and 10 ng of the null-*Renilla* luciferase plasmid, followed by treatment of transfected cells with 0.1% DMSO, 10 μ M guggulsterone, 10 μ M CDCA, or a combination of 10 μ M CDCA and 10 μ M guggulsterone for 30 h. Reporter activities were detected by the Dual-Luciferase Reporter Assay System. The data are presented as mean $\pm$ S.D. of at least three separate experiments. *, $p < 0.05$ by Student's *t* test between the two treatments: CDCA and CDCA+guggulsterone.

Fig. 8.

A, AP-1 activation pathway is involved in the guggulsterone-mediated transactivation of BSEP. Huh7 cells were cotransfected with pBSEP(-2.6 kb) reporter (100 ng) and internal control null plasmid (10 ng), followed by treatment of transfected cells with guggulsterone (10 μ M) in the absence or presence of the JNK inhibitor SP600125 (20 μ M), ERK inhibitor U0126 (10 μ M), or P38 inhibitor SB203580 (5 μ M) for 30 h, followed by detection of luciferase activities with the Dual-Luciferase Reporter Assay System. The data are presented as mean $\pm$ S.D. of at least three separate experiments. B, guggulsterone induces the expression of NQO1. Human primary hepatocytes derived from three individual donors and hepatoma Huh7 cells were treated with 0.1% DMSO or 10 μ M guggulsterone (Z form) for 30 h. Total RNA was isolated from treated cells and subjected to first strand cDNA synthesis as described previously (Deng et al., 2006), followed by detection and quantitative analysis of NQO1 mRNA by TaqMan real-time PCR with GAPDH mRNA level as the internal control. The real-time PCR was performed using TaqMan Universal PCR Master Mix (Applied Biosystems), following the instruction of the manufacturer in a total volume of 20 μ l. Cycling profile was: 50 $^{\circ}$ C for 2 min, 95 $^{\circ}$ C for 10 min, followed by 40 cycles of 15 s at 95 $^{\circ}$ C and 1 min at 60 $^{\circ}$ C, as recommended by the manufacturer. Amplification and quantification were done with the Applied Biosystems 7500 Real-Time PCR System. The NQO1 mRNA levels were normalized with internal control GAPDH mRNA levels. The data are presented as mean $\pm$ S.D. of two repeated experiments.

Fig. 9.

The dual functions of guggulsterone in the regulation of BSEP and possible mechanism for its cholesterol-lowering effect. A, guggulsterone regulates BSEP expression through its dual functions: suppressing BSEP expression through its function as an FXR antagonist and transactivating BSEP through the AP-1 site. However, the trans-activation through the AP-1 element is dominant over the FXR-mediated antagonism. B, possible mechanism for guggulsterone-mediated hypolipidemic effect. CYP7A1 is the rate-limiting enzyme catalyzing the conversion of cholesterol into bile acids in the liver, whereas BSEP is responsible for canalicular secretion of bile acids, a rate-limiting step in the bile acid enterohepatic circulation. Both BSEP and CYP7A1 are coordinately regulated by the bile acid/FXR activation pathway through the positive feed-forward and negative feedback mechanism, respectively. Such coordinated regulation represents a protective mechanism to prevent hepatic injury by excessive accumulation of bile acids. However, this regulatory mechanism is not effective in removing excessive cholesterol from the body by either FXR agonist or antagonist. FXR agonist up-regulates BSEP expression but suppresses CYP7A1 transcription, which decrease the conversion of cholesterol into bile acids. On the other hand, FXR antagonist enhances CYP7A1 expression but suppresses BSEP expression, which results in accumulation of bile acids in hepatocytes and subsequently suppresses CYP7A1. The unique properties associated with guggulsterone as FXR antagonist as well as BSEP activator separated itself from the classes of sole FXR agonists or antagonists. Enhanced BSEP expression by guggulsterone without activating FXR pathway to suppress

CYP7A1 is a possible mechanism by which guggulsterone exerts its hypolipidemic effect. As an activator by itself and synergistic inducer with bile acids, guggulsterone up-regulates BSEP expression and consequently increases secretion of bile acids, which in turn relieves the suppression of CYP7A1 expression by bile acids and favors cholesterol metabolism. On the other hand, as an FXR antagonist, guggulsterone presumably decreases SHP and consequently increases CYP7A1 expression, also favoring cholesterol metabolism. However, it should be emphasized that its FXR antagonistic effect is rather weak. Therefore, the enhanced expression of BSEP without activating FXR to suppress CYP7A1 by guggulsterone is the driving force to promote cholesterol metabolism by removing the trigger, bile acids, for the negative feedback suppression on CYP7A1. Solid arrows, activation or positive regulation; brakes, antagonism or negative regulation.

TABLE 1

Sequences of PCR and mutagenesis oligonucleotides

Oligos	Sequences (5'-3')
BSEP(+85) antisense	TTGAGGAAGCCAGAGGAAATAATGGACTCC
BSEP(-2.6 kb) sense	AGGAGTGGTCCTCAAGCTTCAGCTCATTGG
BSEP(-405) sense	TGGGCCAGGAGCATCTGGATCCTGCAACC
BSEP(-205) sense	CCTCTCGTTCCAAGGTGAATCAGCAATTTC
BSEP(-165) sense	TGACACCCTCAGAGGGTTCCCAAGCACAC
BSEP(-125) sense	GGGTTATTGCTCTGAGTATGTTTCTCGTATG
FXR Y361L-sense ^a	CGAAATAGTGGTATCTCTGATGAA <u>CTT</u> ATAACACCTATGTTTAGTTTTATA
FXR Y361L-antisense	TATAAAACTAAACATAGGTGTTAT <u>AAG</u> TTCATCAGAGATACCACTATTTTCG
FXR H447F-sense	GAATTACGGACATTCAATCATCAC <u>TTC</u> GCTGAGATGCTGATGTCATGGAGAG
FXR H447F-antisense	CTCTCCATGACATCAGCATCTCAGC <u>GAA</u> GTGATGATTGAATGTCCGTAATTC

^aThe mutated nucleotides are underlined and in boldface.