Rexinoids Modulate Steroid and Xenobiotic Receptor Activity by Increasing Its Protein Turnover in a Calpain-dependent Manner*

The steroid and xenobiotic receptor SXR (human pregnane X receptor) is a nuclear receptor that plays a key role in the body’s detoxification response by regulating genes involved in drug metabolism and transport. SXR ligands include a wide range of compounds, which induce transcription of SXR target genes via activation of a heterodimeric transcription factor consisting of SXR and the related nuclear receptor retinoid X receptor (RXR). We investigated the effect of RXR-selective ligands, rexinoids, on SXR/RXR activity. In agreement with previous reports, we found that rexinoids are weak activators of SXR, but we also found that they can antagonize SXR activation by the potent SXR agonist rifampicin. This antagonism included suppression of rifampicin-induced expression of SXR target genes, as well as reduced binding of SXR/RXR to SXR response elements both in vivo and in vitro. Interestingly, two rexinoids, beaxarotene (LG1069/Targetretin) and LG100268, caused a rapid and sustained decrease in the protein levels of both SXR and RXR. The decrease in SXR level was due to an enhanced rate of protein degradation and was dependent on calpain activity, as opposed to rexinoid-induced RXR degradation, which is mediated via the proteasome. Thus, we have demonstrated a novel, rexinoid-modulated mechanism regulating SXR protein stability, which may explain why rexinoids are only weak activators of SXR/RXR-mediated transcription, despite reports that they bind to SXR with high affinity. We suggest that the ability of rexinoids to induce degradation of both SXR and RXR, in combination with competition for binding to SXR, can also explain why rexinoids antagonize the activation of SXR by drugs like rifampicin.

The steroid and xenobiotic receptor SXR2 (human PXR) plays a key role in the body’s detoxification response as a regulator of several genes involved in drug metabolism and transport (1–5). SXR ligands comprise a structurally diverse group of compounds, including natural and synthetic steroids and many xenobiotics, such as the antibiotic rifampicin, the insulin-sensitizing drug troglitazone, the cholesterol-lowering drug SR12813, and the anti-cancer drugs paclitaxel, cisplatin, and tamoxifen (2, 6–11). Because activation of SXR by these drugs induces transcription of genes like CYP3A4 and MDRI, SXR is a central regulator of drug/drug interactions and may also play a role in the development of resistance to chemotherapy in tumor cells (2). SXR is a member of the nuclear receptor superfamily (subfamily 1, group I, member 2) and as such is a ligand-inducible transcription factor for which transcriptional activity requires heterodimerization with the retinoid X receptor, RXR (7). The SXR/RXR heterodimer binds to a variety of response elements in target gene promoters, including direct repeats (DR3–5) and inverted repeats (IR6), and is involved in the regulation of SXR target genes predominantly via these RXR/RXR dimers. However, in the context of different heterodimers, RXR can function either as a silent receptor that does not bind rexinoids (non-permissive dimers), as an active partner that binds rexinoids and activates transcription (permissive dimers), or even as an antagonistic partner that binds rexinoids and thus inhibits activation of transcription. For example, rexinoids stimulate transcription of peroxisome proliferator-activated receptor and LXR target genes through activation of peroxisome proliferator-activated receptor/RXR and LXR/RXR (15). In contrast, binding of several rexinoids to farnesoid X receptor/RXR antagonizes its activation by farnesoid X receptor ligands via reduced DNA binding and coactivator recruitment (16). In the case of the SXR/RXR heterodimer, rexinoids have been shown to bind to SXR itself in vitro but only weakly activate transcription of SXR target genes in vivo (2, 8).

Nuclear receptors are targets of many post-translational modifications, including phosphorylation, sumoylation, ubiq-

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2 The abbreviations used are: SXR, steroid and xenobiotic receptor; SXRE, SXR response element; PXR, pregnane X receptor; RXR, retinoid X receptor; RAR, retinoic acid receptor; LXR, liver X receptor; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay.
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uitation, and acetylation, which critically affect their activity and often protein stability. For example, all-trans-retinoic acid triggers ubiquitination and proteasome-dependent degradation of its cognate receptors RARα and γ (17–19). Degradation of RARγ is dependent on its phosphorylation by p38 mitogen-activated protein kinase and is essential for transactivation of this receptor, whereas phosphorylation of RARα inhibits its degradation (17, 18). Of note, all-trans-retinoic acid also triggers RXR degradation, and likewise, rexinoids stimulate degradation of both RARα and RXR via the proteasome (19, 20). Activation of the estrogen receptor ERα by estradiol has also been convincingly shown to involve its ubiquitin-dependent degradation, further demonstrating that regulation of protein stability can be a critical factor controlling receptor activity. Murine PXR has been reported to be a target of proteasomal degradation as well, but in contrast to RAR, RXR, and estrogen receptor, its turnover rate was shown to be significantly slower in the presence of several chemicals that bind and activate murine PXR, indicating that stabilization of this receptor may promote its activation (21).

In addition to proteasome-mediated degradation, nuclear receptors are targets of other proteases, including calcium-dependent cysteine proteases, calpains, which tend to cleave their substrates in a limited manner and thereby modify their activity (22). Accordingly, cleavage of the androgen receptor by calpain produces a truncated protein that acts as a ligand-insensitive, constitutively active transcription factor that may play a role in androgen-independent prostate cancer (23, 24). Calpain cleavage of RXR has been demonstrated in liver, and the resulting 44-kDa protein has been shown to localize to the mitochondrial matrix, where it forms a heterodimer with the thyroid hormone receptor and increases mitochondrial mRNA in response to the endogenous rexinoid 9-cis-retinoic acid and the thyroid hormone T3 (25, 26).

In the present study, we have investigated the effect of two rexinoids, bexarotene (LGD1069/Targretin®) and LG100268, on SXR/RXR activity and levels. Bexarotene is currently used as an effective treatment of early and advanced stage cutaneous T-cell lymphoma in patients who have failed on other therapies. Bexarotene has been tested in several clinical trials in patients with other types of cancer, including non-small cell lung cancer (27), and preclinical studies have demonstrated antiproliferative activity in breast cancer cells as well as in vivo models of mammary carcinoma (28–31). Interestingly, bexarotene has also been shown to significantly delay the development of resistance to several chemotherapeutic drugs in lung, breast, and prostate cancer cells (32–35). LG100268 is a newer generation rexinoid with high specificity for RXR (36) but is not potent activators of transcription. Rifampicin, on the other hand, which is a potent activator of SXR, did not significantly affect SXR stability. We thus propose that rexinoids may antagonize rifampicin-induced SXR activation by competing for binding to SXR and by triggering degradation of the SXR/RXR transcription factor.

EXPERIMENTAL PROCEDURES

Drugs and Reagents—Rexinoids and TTNPB were supplied by Ligand Pharmaceuticals Inc. (San Diego, CA). Rifampicin and SR122813 were purchased from Sigma and Biomol, respectively, and all compounds were dissolved in DMSO and stored in the dark at −70 °C. Monoclonal and polyclonal antibodies for SXR (H-11 and H-160), RXRα (ΔN 197), c-Jun, and lamin B were purchased from Santa Cruz Biotechnology, and monoclonal anti-Pgp (C-19) was from Calbiochem. Antibodies for FLAG and actin were from Sigma. MG132 in solution (10−2 M), lactacystin, and calpeptin were purchased from Calbiochem, and cycloheximide was purchased from Sigma.

Cell Culture—LS180, HepG2, and MCF-7 cells (ATCC) were routinely maintained in α-modified Eagle’s medium (Invitrogen) supplemented with 5–10% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO2. During all treatment experiments, cells were cultured in phenol red-free α-modified Eagle’s medium with 5% charcoal-stripped serum or in serum-free medium supplemented with bovine serum albumin and holo-transferrin.

Transient Transfections—Cells were seeded into 6-well plates at a density of 3 × 105 cells/well. The next day, fresh medium containing 5% charcoal-stripped serum was added, and the cells were cotransfected with pCMX-SXR (a kind gift from Dr. W. W. Lamph, Ligand Pharmaceuticals), reporter plasmid BD4R(LXR)3-tk-luc (a gift from R. M. Evans, The Salk Institute for Biological Studies), and control plasmid pRL-tk (Promega), using FuGENE 6 transfection reagent (Roche Applied Science) as specified by the manufacturer. 4–5 h post-transfection, SXR and/or RXR agonists were added, and the cells were harvested 24 h post-transfection. Luciferase activity was measured using the Promega Dual-Luciferase assay kit.

Quantitative PCR—RNA was prepared using TRIzol (Invitrogen). CDNA was prepared from 5 μg of total RNA, using SuperScript II reverse transcriptase (Invitrogen). Quantification of MDR1 and CYP3A4 gene expression was performed using the Applied Biosystems 7500 fast real-time PCR system with TaqMan® gene expression assays Hs00184500_m1 and Hs00430021_m1, respectively. Glyceraldehyde-3-phosphate dehydrogenase was used as the endogenous control. Quantification of SXR mRNA was done using the following primers: 5′-GAAGTGGAGGCTCTCCAAA-3′ (forward) and 5′-TGGAAAAGCCCTTGTGAT-3′ (reverse) with SYBR® Green-based detection. Primers for amplification of RXR mRNA were 5′-GGCTTCTCCGGATCTTCTCT-3′ (forward) and 5′-CTCCCTGCGTGTTCCTTT-3′ (reverse). Amplifications were performed with 40 cycles of 95 °C for 15 s and 60 °C for 60 s.

Western Blotting—Whole cell lysates were prepared by washing the cells in ice-cold phosphate-buffered saline, resuspending them in lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, and
1% Triton X-100 plus protease and phosphatase inhibitors) followed by incubation on ice, and microcentrifugation at maximum speed for 10 min to exclude insoluble material. To prepare nuclear extracts, plasma membranes were lysed on ice for 10 min in 10 mM Hepes-KOH, pH 7.9, 1.5 mM MgCl₂, 10 mM KCl, and 2% Nonidet P-40 plus protease inhibitors, followed by gentle vortexing for 10 s. Intact nuclei were pelleted by quick centrifugation and resuspended in 10 mM Hepes-KOH, pH 7.9, 25% glycerol, 420 mM NaCl, 1.5 mM MgCl₂, and 0.2 mM EDTA in immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1 mM dithiothreitol, and 100 ng/ml protease inhibitors), and proteins were extracted on ice for 20 min. Insoluble material was excluded by microcentrifugation at maximum speed for 15 min. Protein concentrations were determined by the Bradford assay (Bio-Rad). Equal amounts of protein were electrophoretically separated in 8–10% SDS-polyacrylamide gels, and proteins were immobilized by transfer onto nitrocellulose membranes. Membranes were immunopробed with primary antibody followed by a secondary, peroxidase-labeled antibody. The proteins were visualized by enhanced chemiluminescence (GE Healthcare).

Chromatin Immunoprecipitation (ChIP)—ChIP was performed using the Upstate Biotechnology protocol with some modifications. 2–3 × 10⁶ LS180 cells were used per immunoprecipitation. Protein-DNA cross-linking was achieved by incubation in formaldehyde (1%) at room temperature for 10 min, followed by glycine (0.125 M) for 10 min to stop the reaction. The cells were lysed in SDS lysis buffer (50 mM Tris, pH 8.1, 10 mM EDTA, and 1% SDS + protease inhibitors), followed by sonication (5 × 15 at 20% amplitude using a Sonic disemnibrator Model 500, Fisher) to shear DNA into fragments of 200–1000 base pairs. Insoluble material was pelleted by microcentrifugation at 4 °C for 15 min, and the supernatant was diluted in immunoprecipitation buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.6 mM Tris-HCl, pH 8.1, and 16.7 mM NaCl + protease inhibitors). Samples were precleared with 60 μl of protein A-agarose/salmon sperm DNA (Upstate Biotechnology) and subsequently incubated with two different anti-SXR antibodies (3 μg of mouse monoclonal antibody, Santa Cruz Biotechnology clone H-11, plus 3 μg of rabbit polyclonal, H-160) or 4 μg of anti-RXRα antibody (Santa Cruz Biotechnology, D6N197) or 4 μg RARα antibody (Santa Cruz Biotechnology, C-20) at 4 °C overnight. DNA–protein complexes were pulled down for 4 h at 4 °C with a mixture of protein A-Sepharose and protein G-agarose beads (GE Healthcare and Sigma, respectively) for SXR or protein A-agarose/salmon sperm DNA (Upstate Biotechnology) for RXR and RARα. The beads were sequentially washed with wash buffer one to three as described in the protocol and twice with Tris/EDTA (pH 8). DNA/proteins were eluted in 120 μl of elution buffer (1% SDS, 50 mM Tris-HCl, pH 8, 10 mM EDTA, and 200 mM NaCl), and cross-links were reversed by incubation at 65 °C overnight. DNA was purified using the QiaQuick PCR purification kit (Qiagen). PCR amplification was then performed using the Applied Biosystems 7500 fast real-time PCR system with SYBR® Green-based detection. Primers used to amplify the region around the SXRE (DR4) in the MDR1 promoter (1) were 5’-GGGAGGTGAACG-3’ (forward) and 5’-CGGAGCTCATTAGCCAAATG-3’ (reverse). Amplification was performed with 45 cycles of 95 °C for 15 s and 60 °C for 60 s.

Electrophoretic Mobility Shift Assay (EMSA)—Analysis of in vitro binding of nuclear proteins (isolated as described above) or in vitro translated proteins (prepared from expression plasmids using the TNT® coupled reticulocyte lysate system from Promega) to the 32P end-labeled, double-stranded probe 5’-gtcagAGTTAGCTCAGTCTC-3’ (sense), containing a βDR4 SXRE (7, 37) was assessed. 10 μg of nuclear extract was used for each binding reaction, which was carried out for 30 min at room temperature in a 20-μl reaction mixture containing 10 mM Tris (pH 8), 40 mM KCl, 6% glycerol, 0.05% Nonidet P-40, 1 mM dithiothreitol, and 100 ng/μl poly(dI-dC). Protein-DNA complexes were separated on 5% polyacrylamide gels and visualized by autoradiography.

In Vivo 35S-Labeling Experiments—MC/7 cells were seeded in 6-well plates at a density of 3 × 10⁵ cells/well and transfected with FLAG-SXR (constructed by insertion of the complete SXR cDNA sequence into 3xFLAG-pCMV14) the following day. 1 day later, the cells were labeled by addition of Expre35S in vivo labeling mixture (PerkinElmer Life Sciences, 100 μCi/well) to culture medium containing no methionine or cysteine. To measure protein degradation by pulse-chase analysis, cells were labeled for 3 h and then rinsed three times with phosphate-buffered saline. Complete culture medium was added, and cells were incubated in the presence or absence of reinoid (10⁻⁶ M) for up to 3 h. For the analysis of protein synthesis, cells were pulse-labeled for up to 3 h in the absence or presence of bexarotene (10⁻⁶ M). Whole cell extracts were prepared by 15–30-min incubation in lysis buffer without SDS (50 mM Tris, pH 8.0, 150 mM NaCl, and 1% Triton X-100 plus protease and phosphatase inhibitors). Equal amounts of protein (~200 μg) were pre-cleared with protein A-agarose for at least 1 h at 4 °C, and the cleared lysates were subjected to immunoprecipitation with anti-FLAG-agarose beads (Sigma) overnight at 4 °C. The beads were washed three times with Tris-buffered saline/Tween at 4 °C, followed by elution of bound proteins with SDS loading buffer. Immunoprecipitated proteins were separated in 10% SDS-polyacrylamide gels, and labeled proteins were visualized and quantified using a Cyclone phosphorimaging system (PerkinElmer Life Sciences) with Optiquant 4.0 software (Packard Instruments Co.).

In Vitro Calpain Cleavage Assay—Nuclear extracts (60 μg), prepared as above and diluted in phosphate-buffered saline to a final volume of 30 μl, were incubated for 90 min at 30 °C in the presence of calpain-1 (1 unit; purchased from Sigma) and CaCl₂ (10⁻³ M). The reaction was stopped by addition of an equal volume of 2× SDS loading buffer, the proteins were separated in a 10% SDS-polyacrylamide gel, and SXR was visualized by Western blotting.

In Vivo Calpain Activity Assay—Cells were seeded on coverslips and allowed to adhere overnight. The cells were then treated as indicated, with addition of the fluorogenic calpain substrate t-Boc-LM-CMAC (10⁻⁵ M; purchased from Molecular Probes) during the last hour. At the end of 3 h, fluorescence was immediately visualized using a Zeiss microscope, and images were captured using equal exposure times.
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**Statistics**—Experiments were performed at least three times, and results from representative experiments are shown as means ± S.E. of the number of replicates indicated in each figure. Unpaired Student’s *t* tests (comparing two groups, see Fig. 5) or one-way analysis of variance with Tukey’s post test (comparing more than two groups, see Figs. 1 and 2) were performed using GraphPad InStat 3.0. Significant differences compared with control (untreated cells) are indicated by *(p < 0.05)* or ***(p < 0.01)***, and significant differences compared with rifampicin or SR12813 are indicated by # *(p < 0.05)* or ## *(p < 0.01)*.

**RESULTS**

**Rexinoids Suppress Activation of Rifampicin-induced SXR-dependent Transcription**—To assess the effect of rexinoids on SXR transcriptional activity, LS180 cells were transiently cotransfected with pCMX-SXR and a reporter plasmid containing the βDR4 SXRE (7, 37), and luciferase activity was assessed after treatment with rifampicin ± bexarotene (Fig. 1A). A similar pattern was seen in MCF-7 cells (Fig. 1C) and in HepG2 cells (Fig. 1D). Additionally, rifampicin-induced expression of another SXR target gene, CYP3A4, was seen to be reduced in the presence of bexarotene (Fig. 1D). Of note, the three cell lines expressed endogenous SXR at low (MCF-7) or high (LS180 and HepG2) levels. Furthermore, none of the drugs or drug combination used in these experiments had a significant effect on cell viability at the time points studied (data not shown).

**Bexarotene Alters Binding of SXR/RXR to the MDR1 SXR Response Element**—We performed ChIP analysis to assess binding of the SXR/RXR heterodimer to the MDR1 promoter in vivo. Induction of *MDR1* by rifampicin has been shown to be mediated by a DR4 element located 8 kb upstream of the transcription initiation site (1), so we designed primers to selectively amplify this region, as well as primers to amplify the proximal promoter region. Using either anti-SXR (Fig. 2A) or anti-RXRα (Fig. 2B), a basal level of receptor association with the DR4 region in untreated cells was demonstrated. This association was significantly strengthened upon treatment with rifampicin alone, but not after cotreatment with bexarotene plus rifampicin. Importantly, amplification of either the proximal promoter (Fig. 2, A and B) or a region 4 kb downstream (data not shown) indicated no binding of either SXR or RXR to these regions of the *MDR1* promoter. To confirm specificity of the immunoprecipitations, ChIP analysis was also performed using an RARα-specific antibody, which showed no binding to the *MDR1* promoter (Fig. 2C). EMSA assessment of binding of nuclear proteins isolated from cells treated with rifampicin, bexarotene, or both was also performed and showed strongly enhanced binding by rifampicin, which was decreased with the addition of bexarotene, despite the fact that bexarotene alone also caused enhanced binding (Fig. 2D). On the contrary, EMSA assessment of the ability of *in vitro* translated SXR/RXR to bind to the synthetic SXRE in the presence or absence of rifampicin and/or bexarotene showed no difference in binding affinity regardless of the drugs present (Fig. 2E), indicating that additional factors, present in nuclear extracts, are needed to alter receptor DNA binding.

**Rexinoids Decrease Protein Levels of Both SXR and RXR by Enhancing Protein Turnover**—It is known that rexinoids, including bexarotene and LG100268, stimulate degradation of the RXR protein, as well as the RXR heterodimer partner RAR and the thyroid hormone receptor (20). We thus examined the effect of...
these drugs on the levels of SXR and found a remarkable decrease in total SXR protein levels in cells treated with either bexarotene or LG100268 for 24 h (Fig. 3A). In contrast, rifampicin did not significantly alter the level of either SXR or RXR (Fig. 3A).

A more detailed time course study showed that the decrease in SXR was observed as early as 1 h after addition of bexarotene and was sustained for at least 48 h (Fig. 3B). This was similar to the effects of bexarotene on the levels of RXR (Fig. 3B). Furthermore, no decrease of either SXR or RXR was observed on the mRNA level (Fig. 3C). In fact, bexarotene appears to have a positive effect on the transcription of SXR.

To test whether the rexinoids affected synthesis or degradation of the SXR protein, we then performed in vivo labeling experiments. Pulse-chase analysis of the degradation rate of 35S-labeled FLAG-SXR, transiently transfected into MCF-7 cells, was performed in the presence of DMSO, bexarotene, or LG100268, and we found that both rexinoids triggered a faster degradation rate (Fig. 4A). Of note, the degradation rate of FLAG-SXR in LS180 was faster than in MCF-7 both in the absence and presence of rexinoids, and the labeled protein was undetectable after <1 h (data not shown). We then analyzed the rate of synthesis of FLAG-SXR in MCF-7 cells by pulse labeling for up to 3 h in the absence or presence of bexarotene. In contrast to its effects on protein degradation, bexarotene did not significantly alter the synthesis rate (Fig. 4B).

We went on to examine how bexarotene affects endogenous SXR levels in cells where de novo protein synthesis has been blocked. The cells were pretreated with cycloheximide for 30 min, followed by DMSO or bexarotene. Nuclear extracts were prepared after 4 and 8 h, and the levels of SXR and RXR were examined. The basal turnover rate of SXR was seen to be higher than that of RXR, which remained stable for the 8-h period. However, in the presence of bexarotene, both proteins were degraded at a faster rate, indicating that induction of rexinoid target genes is not required for stimulation of receptor degradation (Fig. 5A). We also assessed SXR degradation in cycloheximide-pretreated cells in the presence of rifampicin, but the SXR agonist did not significantly affect the turnover rate (Fig. 5B), consistent with the fact that up to 24 h of treatment with rifampicin did not significantly alter the level of SXR protein (Fig. 3A).

SXR Is Degraded in Response to Bexarotene in a Calpain-dependent Manner—To begin to examine the mechanism of SXR degradation stimulated by rexinoid treatment, we used selective protease inhibitors. The cells were pretreated with cycloheximide for 30 min to ensure specific assessment of protein degradation. First, a selective inhibitor of the proteasome, lactacystin, was tested. Cotreatment of the cells with lactacystin, a selective inhibitor of the proteasome, lac-

FIGURE 2. Bexarotene reduces receptor occupancy at the MDR1 promoter. A, ChIP analysis of SXR occupancy on the MDR1 promoter. LS180 cells were treated with bexarotene (BEX; 10−8 M) and/or rifampicin (RIF; 5 × 10−6 M) for 18 h. Bexarotene plus rifampicin; IgG, immunoglobulin G; CTL, control. Primers were designed to amplify the DR4 region known to mediate SXR regulation of RXR binding to the MDR1 promoter. Treatments and amplifications were performed as in A. C, ChIP analysis of RXR binding to the MDR1 promoter. LS180 cells were treated with bexarotene (Bex) or LG100268 (10−8 M) or rifampicin (5 × 10−6 M) for 24 h. LS180 cells were treated with bexarotene plus rifampicin; IgG, immunoglobulin G; CTL, control. D, EMSA showing regulation of binding of nuclear proteins from LS180 and MCF-7 to a synthetic SXRE. Nuclear extracts were isolated from cells treated as indicated for 24 h, and EMSA was performed as described under “Experimental Procedures.” E, EMSA showing no regulation by rifampicin or bexarotene of the binding of in vitro translated RXR/SXR to the synthetic SXRE. *, p < 0.05; **, p < 0.01; #, p < 0.05; ##, p < 0.01.

FIGURE 3. Rexinoids down-regulate SXR at the protein level. A, LS180 cells were treated with bexarotene (Bex) or LG100268 (10−8 M) or rifampicin (5 × 10−6 M) for 24 h, and SXR and RXR protein levels were analyzed by Western blotting (WB). Lamin B was used as a control for equal loading of nuclear extracts. B, LS180 cells were treated with bexarotene for the times indicated, and SXR, RXR, and actin protein levels were assessed. C, RNA was isolated from LS180 cells treated with 10−8 M bexarotene as indicated, and SXR and RXR mRNA was quantified by real-time PCR. RQ, relative quantity. Numbers in A and B indicate optical density measured by densitometry and normalized to lamin B or actin. CTL, control.
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FIGURE 4. Bexarotene enhances the degradation rate of SXR. A, upper left panel, FLAG-SXR, transiently transfected into MCF-7 cells, is down-regulated by LG100268 (LG; 10^{-6} M, 24 h). WB, Western blotting; Ctl, control. Lower left panel, pulse-chase analysis measuring the degradation rate of FLAG-SXR in the absence or presence of rexinoids (10^{-6} M) is shown. Time shown refers to chase time, from the removal of radioactive labeling mixture. Bex, bexarotene; Neg, negative. Right panel, densitometry analysis performed on blots obtained from three separate experiments. As a negative control (Neg), immunoprecipitation was performed with mouse IgG.

FIGURE 5. Bexarotene enhances degradation of endogenous SXR independent of de novo protein synthesis. LS180 cells were pretreated with cycloheximide (CHX; 10 \mu g/ml) for 30 min before addition of the other treatments. A, bexarotene (Bex; 10^{-6} M) or vehicle (DMSO) was added at time 0, cell extracts were prepared at the times indicated, and SXR and RXR levels were assessed by Western blotting (WB). Graph shows densitometry analysis performed on blots from three separate experiments, normalized to actin. Ctl, control. B, rifampicin (Rif; 5 \times 10^{-6} M) or DMSO was added at time 0, and SXR levels were assessed and quantified by densitometry as in A.

some inhibitor, MG132, which also inhibits calpains (39), blocked degradation of both SXR and RXR (Fig. 6B). The cells were then treated with a selective calpain inhibitor, calpeptin, in the absence or presence of bexarotene. Calpeptin was found to rescue bexarotene-induced degradation of SXR, but not RXR (Fig. 6C), indicating that the two receptors are in fact degraded by two independent mechanisms. To confirm that SXR can be a target of calpain degradation, nuclear extracts from LS180 cells were incubated with calpain-1 (also known as \mu-calpain) in vitro, in the presence or absence of calcium chloride. Indeed, in the presence of both calpain and calcium, full-length SXR was undetectable, and a band appeared around 30 kDa (Fig. 6D). Total calpain activity in the cells was then assessed by incubation of cells treated with bexarotene ± calpeptin with the fluorogenic calpain substrate t-Boc-LM-CMAC. This synthetic substrate is designed so that fluorescent quenching is removed upon calpain cleavage, and increased fluorescence thus represents increased calpain activity. We found that calpain activity was indeed increased in cells treated with bexarotene for 3 h (Fig. 6E), providing a mechanism by which the rexinoid may stimulate degradation of SXR. As further evidence of bexarotene-induced calpain activation, bexarotene was also shown to induce a calpain-dependent decrease of c-Jun (Fig. 6F), a known nuclear calpain target (40).

DISCUSSION

SXR is an important regulator of metabolism and transport of a wide range of drugs. As such, it is bound and activated by vastly different ligands, ranging from bulky, macrocyclic drugs such as rifampicin to small, metal-containing compounds such as cisplatin. Rexinoids have been shown to bind to SXR with high affinity in competition binding assays, where bexarotene and LG100268 in fact competed with radiolabeled SR12813 for binding to immobilized SXR more efficiently than rifampicin (8). However, the same study showed that the rexinoids are much less potent in inducing the transcriptional activity of SXR (8). In the present study, we confirmed that bexarotene and LG100268 are weak activators of SXR transcription, and we also showed that the rexinoids significantly reduce transcriptional activation by both rifampicin and SR12813, indicating an antagonistic effect (Fig. 1). Reduced transcription of the MDR1 gene in the presence of rifampicin plus rexinoid was associated with a lower level of receptor occupancy at the previously published response element mediating MDR1 regulation by rifampicin (1) (Fig. 2). Such altered association of SXR/RXR with promoter response elements in the presence of the different drugs may indicate changes in affinity brought on by conformational changes upon ligand binding or may reflect changes in receptor levels and/or turnover at the promoter. Based on our finding that binding of in vitro translated SXR/RXR to an SXR response element is unaffected by the addition of ligand, we reasoned that direct conformational changes cannot be responsible, but additional factors present in the cell must be involved. We established that the rexinoids increase the SXR protein degradation rate in a manner independent of the induction of transcription, which appears similar to the known effect of rexinoids on RXR protein turnover (19, 20). It thus seemed logical to postulate that the whole SXR/RXR heterodimer is brought to the proteasome for degradation upon binding of an RXR ligand. However, we found that the proteasome inhibitor lactacystin was unable to block degradation of SXR, whereas it efficiently blocked RXR-degradation (Fig. 6A). In contrast, calpeptin, which selectively inhibits calpains, blocked the decrease in SXR but did not affect the rexinoid effect on RXR (Fig. 6C), indicat-
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A inhibiting a selective targeting of SXR by cellular calpains. We confirmed that SXR can act as a substrate for calpain cleavage, which produces a protein fragment of ~30 kDa (Fig. 6D). Notably, the 30-kDa fragment was never observed on Western blots of SXR after rexinoid treatment or in pulse-chase experiments, suggesting that this fragment is subject to subsequent, rapid degradation and presumably does not play a physiological role in the cells. To begin to investigate how rexinoids may mediate calpain-dependent SXR degradation, total calpain activity in cells treated with bexarotene was assessed, and in fact, the calpain activity was found to increase (Fig. 6E). This was further supported by the fact that bexarotene was shown to decrease the protein level of another nuclear calpain substrate, c-Jun, in a manner inhibited by calpeptin (Fig. 6F).

Thus, we have shown for the first time that the SXR protein can be degraded via an increase in calpain activity, adding a new level of complexity to the turnover of SXR. Murine PXR was previously shown to be degraded via the proteasome, and interestingly, several ligands causing murine PXR activation were found to slow down this process (21). We did not observe any significant effect on SXR levels or stability in the presence of rexinoids and rifampicin, showing that increased stability is not required for efficient activation of transcription. However, we postulate that the rexinoid-induced de-stabilization of both SXR and RXR may be important for the inability of these agents to potently activate SXR-driven transcription. Competition between the rexinoids and rifampicin (or other agonists such as SR12813) for binding to SXR may then explain why transcription is less efficiently activated in the presence of the two drugs than in the presence of rifampicin alone.

The calpains are ubiquitously expressed proteases that catalyze the limited cleavage of target proteins in response to calcium signaling. In general, the calpains cleave target proteins at a limited number of sites and produce large polypeptide fragments rather than small peptides or amino acids (22). Calpain activity is tightly regulated at the post-transcriptional level by intracellular calcium level, the presence of an endogenous inhibitor, calpastatin, as well as by phosphorylation events triggered by growth factor signaling (41). The mechanism of increased calpain activity in response to bexarotene is unknown, but our data indicate that induction of rexinoid target genes is not necessary because SXR is degraded in a calpain-dependent manner even in the presence of cycloheximide (Fig. 6C). We suggest that bexarotene may trigger activation of a kinase that directly activates calpains or may negatively modulate the level of calpastatin. Alternatively, bexarotene may trigger calcium release from intracellular stores.

Several transcription factors, in addition to SXR, are targets of calpain cleavage. These include p53, c-Myc, c-Jun, and, as mentioned above, the nuclear receptors androgen receptor and RXR (24, 26, 40, 42, 43). However, as opposed to the calpain cleavage products of androgen receptor and RXR, which acquire novel functions (23–25), the cleavage product of SXR appears to be subjected to rapid further degradation in intact cells because we did not observe the 30-kDa protein, observed after in vitro cleavage, in any of our Western blot experiments. We therefore conclude that calpain-mediated cleavage is a novel mechanism of SXR protein degradation that can be stimulated by rexinoid treatment, and the fact that rexinoids induce degradation of both SXR and RXR may help explain why rexinoids do not act as potent activators of SXR-dependent transcription. Because bexarotene appears to trigger SXR degradation simply by increasing calpain activity, we postulate that the basal SXR turnover rate in cells may also be regulated by the calpain activity level, and it would be interesting to correlate SXR levels with calpain activity in different cell lines and tissue samples. A study recently showed an inverse correlation between SXR levels and expression of a microRNA in human liver samples (44), and it would be interesting to examine
whether increased calpain activity modulates the expression of this microRNA. Moreover, it would be an interesting avenue for future research to investigate whether the ability of rexinoids, such as bexarotene, to antagonize SXR activation also translates to suppression of drug transport and metabolism induced by rifampicin and other SXR-activating drugs, as this might be a way of modulating drug/drug interactions as well as development of drug resistance.

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