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Benzoate X Receptors α and β Are Pharmacologically Distinct and Do Not Function as Xenobiotic Receptors*

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The *Xenopus* benzoate nuclear hormone receptors, BXRα and BXRβ, share 82% identity within their ligand-binding domains and are classified as members of the NR1I2 subfamily that includes the mammalian steroid and xenobiotic receptor, SXR/PXR. Although alkyl benzoates have been identified as endogenous ligands, the exact role of the benzoate receptors in amphibian physiology has not been established. In this report, we show that BXRα and BXRβ are pharmacologically distinct from each other: BXRα is more promiscuous than BXRβ with respect to both ligand specificity and co-activator recruitment. BXRα can be transactivated by a number of benzoate derivatives including 4-amino-butylbenzoate (4-ABB), 4-hydroxy-butylbenzoate (4-HBB), 3-hydroxy ethyl benzoate (3-HEB), and benzyl benzoate, but only 4-HBB acts as an agonist for both receptors. Furthermore, BXRα-specific agonists such as 4-ABB, chlorpyrifos, and trifluralin act as antagonists on BXRβ. BXRs are widely distributed in adult tissues but do not show any enrichment in liver and intestine, major sites of SXR/PXR expression that are critical in xenobiotic metabolism. Neither BXR shows the broad specificity toward steroids or xenobiotics exhibited by SXR/PXR. Therefore, we conclude that the BXRs are pharmacologically distinct from each other and unlikely to serve as xenobiotic sensors.

Nuclear receptors are ligand-modulated transcription factors that respond to steroids, retinoids, and thyroid hormones to control development and body physiology. Orphan nuclear receptors possess apparent DNA and ligand-binding domains but lack identified ligands (1–3). Each orphan has the potential to regulate a distinct signaling pathway. The promise of orphan receptors is that the identification of novel and perhaps unsuspected classes of ligands may offer insight into potentially new principles of development and physiology. In recent years, a number of orphan receptors have been adopted or matched with physiological ligands (3, 4). Consequently, new insights into cholesterol and bile acid metabolism and transport (4) have been gained.

Previously, we identified a *Xenopus* orphan nuclear receptor that represented a distinct branch of the nuclear receptor superfamily and named it benzoate "X" receptor (BXR)[1] (5) (also known as xONR1 (6)). The name reflects its activation by alkyl esters of amino and hydroxyl benzoic acids, one of which is found endogenously in the *Xenopus* embryo (5). The identification of BXR as a receptor for benzoate ligands illustrates the potential of uncovering previously unsuspected signaling pathways through orphan receptor characterization. Recently, a second *Xenopus* BXR cDNA was described (7). This BXR shares only 88% nucleic acid sequence identity and 83% amino acid sequence identity (see Fig. 1 and Ref. 7) with the BXR we characterized previously (5). Therefore, the two receptors have been designated as BXRα and BXRβ (7).

BXR is most closely related to the human steroid and xenobiotic receptor, SXR (8) (also known as pregnane X receptor, PXR (9), and pregnane activated receptor, PAR (10)). SXR and its rodent ortholog PXR function as xenobiotic sensors in the liver and intestine. They mediate the breakdown and elimination of steroids, drugs, and xenobiotic compounds by activating the expression of degradative cytochrome P450 enzymes and members of the ABC family of organic molecule transporters. BXR and SXR/PXR have been assigned to the NR1I2 family by the Nuclear Receptor Nomenclature Committee (11). This indicates that these receptors are orthologous, *i.e.* the same gene from different species. During our characterization of SXR, we noted that none of the compounds that activated SXR was able to activate BXRα (8).[2] This led us to question whether BXR and SXR are functionally equivalent, *i.e.* do BXRs function as xenobiotic sensors? As described below, we found that neither BXRα nor BXRβ is activated by the types of xenobiotic compounds that activate SXR/PXR. In addition, BXRs are ubiquitously expressed at varying levels in different tissues rather than showing the high level expression only in the liver and intestine characteristic of SXR/PXR. We infer that BXRs are unlikely to be functioning as xenobiotic sensors and are therefore functionally distinct from their mammalian relatives. Lastly, we show that BXRβ is activated only by 4-hydroxyl benzoates as compared with BXRα, which can also be activated by other related compounds including amino benzoates, benzyl benzoate, chlorpyrifos, and trifluralin. Interestingly, several of the BXRα-selective activators function as antagonists for BXRβ, thus suggesting that the two BXRs are also pharmacologically distinct from each other.

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[1] The abbreviations used are: BXR, benzoate X receptor; SXR, steroid and xenobiotic receptor; PXR, pregnane X receptor; 4-ABB, 4-amino-butylbenzoate; 4-HBB, 4-hydroxy-butylbenzoate; 3-HEB, 3-hydroxy ethyl benzoate; DMEM, Dulbecco's modified Eagle's medium; RT, reverse transcription; ABC, ATB-binding cassette; PBP, peroxisome proliferator-activated receptor (PPAR)-binding protein; luc, luciferase; DBD, DNA-binding domain.

[2] B. Blumberg, unpublished observations.

EXPERIMENTAL PROCEDURES

Cell Culture and Transfection—COS-7 cells were cultured in phenol red-free DMEM supplemented with 10% FBS. For transient transfection experiments, COS-7 cells were seeded into 96-well plates at a density of 5000 cells/well. 4–5 h after seeding, the cells were transfected with CMX-GAL4-xBXRα (5), CMX-GAL4-xBXRβ, or CMX-GAL4 (control) together with tk(MH100)4-luc reporter (12) and CMX-β-galactosidase transfection control plasmids using standard calcium phosphate precipitation methodology. 22–24 h after transfection, the cells were washed twice with phosphate-buffered saline supplemented with 1 mM $MgCl_2$ or DMEM-ITLB (DMEM containing 5 μg/ml insulin, 5 μg/ml holo-transferrin, 5 μg/ml selenium, 0.5% defined lipid mix (Invitrogen), 0.12% w/v delipidated bovine serum albumin (Sigma)) (13). Ligands were next added in DMEM-ITLB, and the cells were incubated for an additional 24–48 h. Ligands were typically purchased from Sigma, ChemService (West Chester, PA), or Roche Molecular Biochemicals and made freshly from powder in Me_2SO as 0.1 M stocks, diluted in Me_2SO to appropriate concentrations and added to media with vigorous vortex mixing. The cells were incubated with ligands for 24–48 h and then lysed *in situ*. Extracts were prepared and assayed for β-galactosidase and luciferase activity as described (8). Reporter gene activity was normalized to the β-galactosidase transfection controls, and the results were expressed as normalized relative luciferase units per OD of β-galactosidase per minute to facilitate comparisons between plates. Fold induction was calculated relative to solvent controls. Each data point represents the average of triplicate experiments ± S.E. and was replicated in independent experiments.

Isolation of Total RNA—Tissues were obtained from adult *Xenopus laevis* males and females, dissected into small pieces, flash-frozen in liquid N_2, and stored at −80 °C. Total RNA was isolated from the tissues using standard guanidine thiocyanate procedure (14). Northern blots were performed using the ligand-binding domain of BXRα, BXRβ, or EF-1α according to standard methods (15). For RT-PCR analysis, 1 μg of total RNA was reverse-transcribed using Superscript II reverse transcriptase according to the manufacturer-supplied protocol (Invitrogen). Quantitative real time RT-PCR was performed using the following primer sets: BXRa (5′-CTGTCCTGGTAGGGCAATGT-3′, 5′-AATGGGACTGAAGCAACGTC-3′), BXRb (5′-CAGCCGGTGAATTGTCTTCT-3′, 5′-AGTTGTGGGGCTTGATTTTG-3′), or EF1a (5′-CCTGAATCACCCAGGCCAGATTGGTG-3′, 5′-GAGGGTAGTCTGAGAAGCTCTCCACG-3′) using the SYBR green PCR kit (Applied Biosystems) in a DNA Engine Opticon continuous fluorescence detection system (MJ Research). All samples were quantitated by the comparative cycle threshold (Ct) method for relative quantitation of gene expression, normalized to EF-1α (16).

Isolation of Xenopus BXRβ—*Xenopus* BXRβ was isolated by RT-PCR based on the published sequence (GenBank™ accession number AF305201) (7). 1 μg of *Xenopus* total RNA was primed with $oligo(dT)_{12–18}$ and reverse-transcribed with Superscript II reverse transcriptase according to the manufacturer-supplied protocol (Invitrogen). The cDNA was PCR-amplified using *Pwo* polymerase (Roche Molecular Biochemicals) and the specific primers (5′-TCCGTGCTCACCTGGTTCCGT-3′) and (5′-CCTATCCATGTAGGTATCCCAGAT-3′) that annealed in the 5′- and 3′-untranslated regions of BXRβ. The amplified product was gel-purified, and the ligand-binding domain of BXRβ (amino acids 104–388) was amplified using nested primers (5′-TCGCCGGAATTCAGGAAAGAGCTGATCATGTCA-3′) and (5′-TGGCCAGGATCCCTATCACTCATTCAGGGATCC-3′). The resulting product was purified and ligated into pCMX-GAL4 to generate a GAL4-DBD-BXRβ-ligand-binding domain fusion protein.

Similarly, GAL4 coactivator plasmids were generated by cloning the receptor interaction domains of human TIF2 (GenBank™ accession number NM006540, amino acids 563–790), human F-SRC-1 (GenBank™ accession number U59302, amino acids 600–800), or human ACTR (GenBank™ accession number AF036892, amino acids 600–788) into pCMX-GAL4. The GAL4-PBP construct was a gift from B. Forman (City of Hope Medical Center). To construct *Herpesvirus* VP16 activation domain fusion proteins, full-length BXRα and BXRβ were PCR-amplified and ligated in-frame into pCMX-VP16 vector. All constructs were sequenced to verify that no errors were introduced in the PCR.

RESULTS

Comparative Expression of BXRα and BXRβ—Recently, a second *Xenopus* BXR cDNA was described (7). This BXR shares only 88% nucleic acid sequence identity and 83% amino acid sequence identity (Fig. 1A and Ref. 7) with the BXR we characterized previously (5). This is more than would be expected for the divergence between two duplicated genes in the pseudotetraploid *X. laevis* genome (17). Accordingly, the new receptor was called BXRβ (7). To gain insight into the possible target tissues for BXR action, we examined the expression patterns of BXRα and BXRβ in adult frogs by Northern blot and quantitative real time RT-PCR analysis. Both genes encode ubiquitously expressed single transcripts of ~3.2 kb (data not shown) and are found at very high levels in the ovary (Fig. 1*B*). BXRα is expressed at high levels in the brain and skin with moderate levels in the testis, stomach, and intestines and lower levels in the lung, kidney, liver, and heart (Fig. 1*B*). BXRβ is expressed at comparable levels to BXRα in the intestines, lung and kidney with slightly higher levels in the testis and heart and lower levels in the liver, skin and brain (Fig. 1*B*). It is notable that these ubiquitous expression patterns for BXR differ considerably from those of its putative human ortholog, the steroid and xenobiotic receptor SXR (8). SXR functions as a xenobiotic sensor and is expressed primarily in the liver and intestine, where it modulates the levels of cytochrome p450 enzymes and ABC family transporters (8, 9). Since BXRs are ubiquitously expressed, they do not show the tissue distribution expected for a xenobiotic sensor.

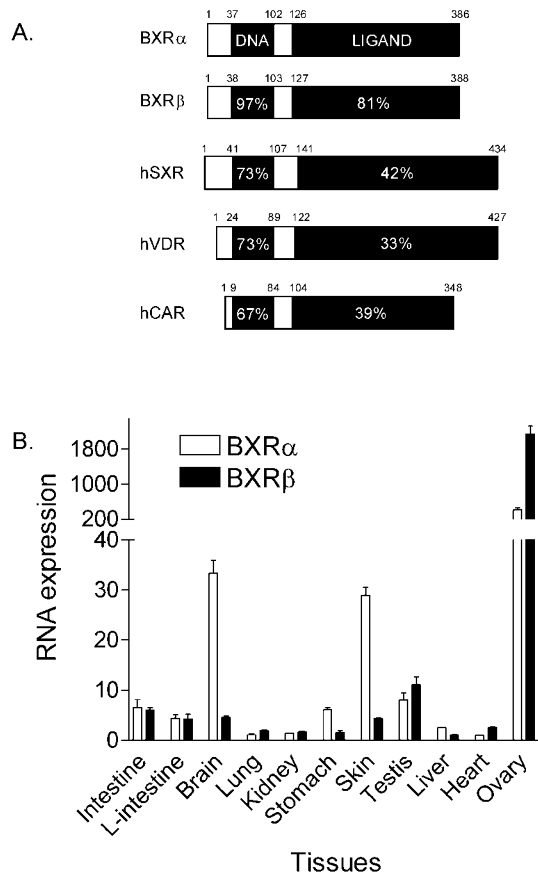

FIG. 1. **BXRs are expressed in different patterns in adult tissues**. *A*, comparison of BXR sequences to related receptors in an optimal sequence alignment. Percentages indicate the degree of homology conservation in the receptor DNA- and ligand-binding domains relative to BXRα. *hVDR*, human vitamin D receptor; *hCAR*, human constitutive androstane receptor. *B*, tissue-specific expression of BXRα and BXRβ in adult tissues as determined by real time PCR analysis of reverse-transcribed total cellular RNA. Data are shown as the RNA expression levels normalized to *X. laevis* EF1α controls. Values represent the average of duplicates ± range.

BXRα and BXRβ Are Pharmacologically Distinct—Our previous work showed that alkyl esters of amino and hydroxyl benzoic acids specifically bind to and activate BXRα (5). Therefore, it was surprising that BXRβ was reported to be activated

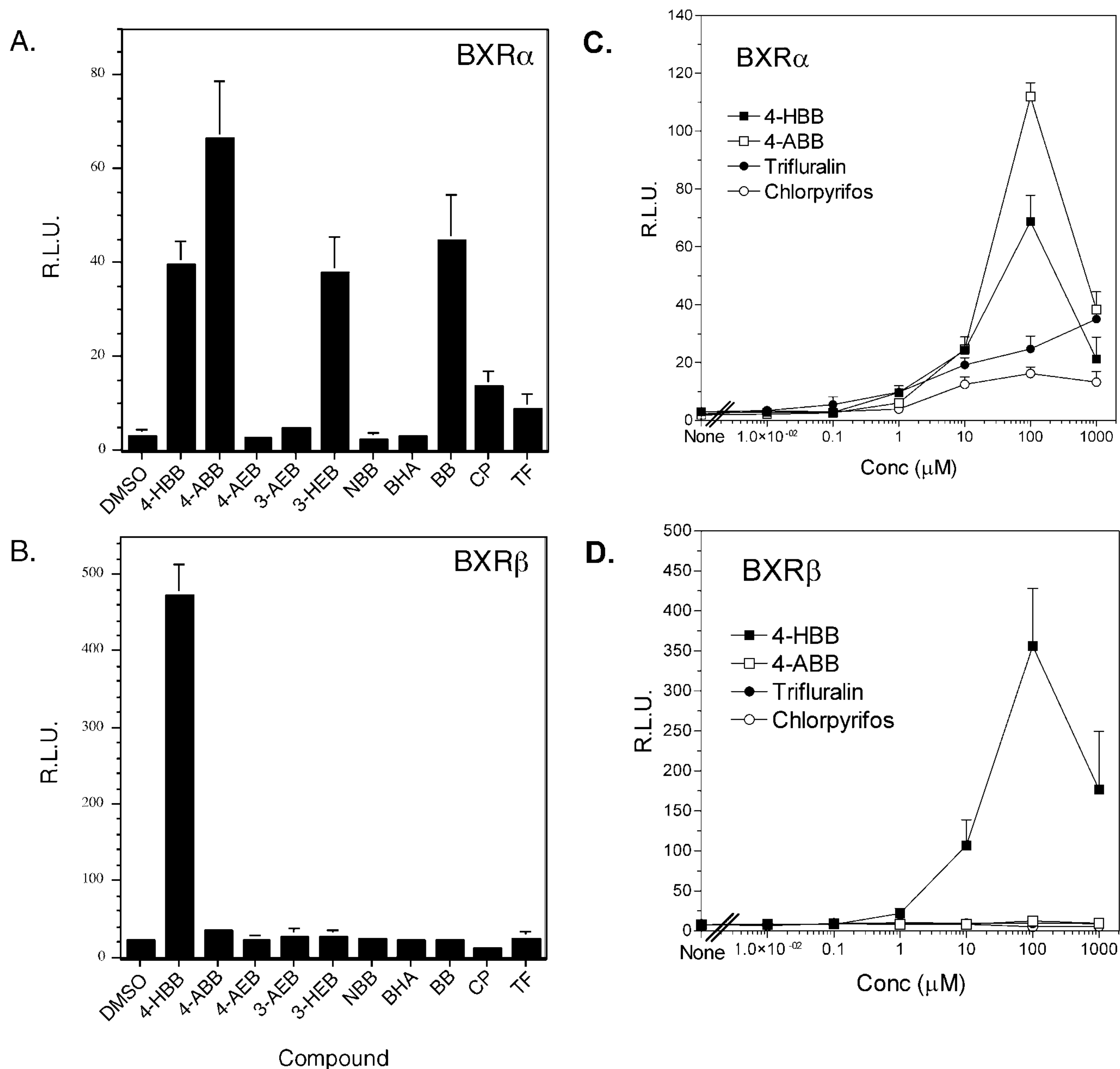

FIG. 2. **Comparative activation of BXRs by benzoates and related compounds.** Cells were transfected with GAL-BXRα (*A* and *C*) or GAL-BXRβ (*B* and *D*) reporter and control plasmids as described under "Experimental Procedures." Ligands were added at a fixed concentration of 50 μM (*A* and *B*) or as indicated (*C* and *D*). *DMSO*, Me_2SO solvent control; *4-AEB*, 4-amino-ethylbenzoate; *3-AEB*, 3-amino-ethylbenzoate; *NBB*, 4-nitro-butylbenzoate; *BHA*, butyl-hydroxyanisol; *BB*, benzylbenzoate; *CP*, chlorpyrifos; *TF*, trifluralin; *RLU*, relative luciferase units. Cells were further incubated for 24 h, harvested, and assayed for luciferase and β-galactosidase activity. Data were normalized to β-galactosidase activity and plotted as relative luciferase units (*RLU*) against concentration. Points represent the means of triplicates ± S.E. from a representative experiment. The compounds were cytotoxic at concentrations greater than 100 μM as measured by reduced activity for the β-galactosidase transfection controls.

only very weakly by 4-amino butyl benzoate (7), which strongly activates BXRα (5). We tested the activation profiles of these receptors to determine whether the two BXRs might exhibit different ligand specificity. BXRα and BXRβ were transiently transfected into COS-7 cells, and then a panel of benzoates and related compounds was tested for their ability to activate transcription of a luciferase reporter gene (Fig. 2). Activation of BXRα paralleled our published results (Fig. 2*A*) in that both hydroxyl and amino benzoates were robust activators. In addition, we observed that benzyl benzoate, trifluralin, and chlorpyrifos also activated BXRα (Fig. 2*A*). In contrast, only 4-hydroxyl butyl benzoate was able to activate BXRβ (Fig. 2*B*). It is particularly notable that 3-hydroxyl ethyl benzoate, which was identified as an endogenous embryonic activator of BXRα, could activate BXRα but was inactive on BXRβ (Fig. 2, *A* and *B*). BXRβ was considerably more active in response to ligand than was BXRα (Fig. 2, *A* and *B*). A similar trend was also observed in dose-response experiments (Fig. 2, *C* and *D*). BXRα was strongly activated by 10 μM 4-ABB, 4-HBB, chlorpyrifos, and trifluralin (Fig. 2*C*). BXRβ was only activated by 4-HBB among the many compounds tested (Fig. 2, *B* and *D*). The activation of BXRβ was very robust with 50 μM of 4-HBB yielding between 50- and 100-fold activation of the reporter gene (Fig. 2, *B* and *D*).

Co-activator Recruitment by BXRs—To confirm that BXRα and BXRβ show distinct pharmacological responses to ligand, we conducted co-activator recruitment studies to determine the ability and preferences of the various ligands to support the

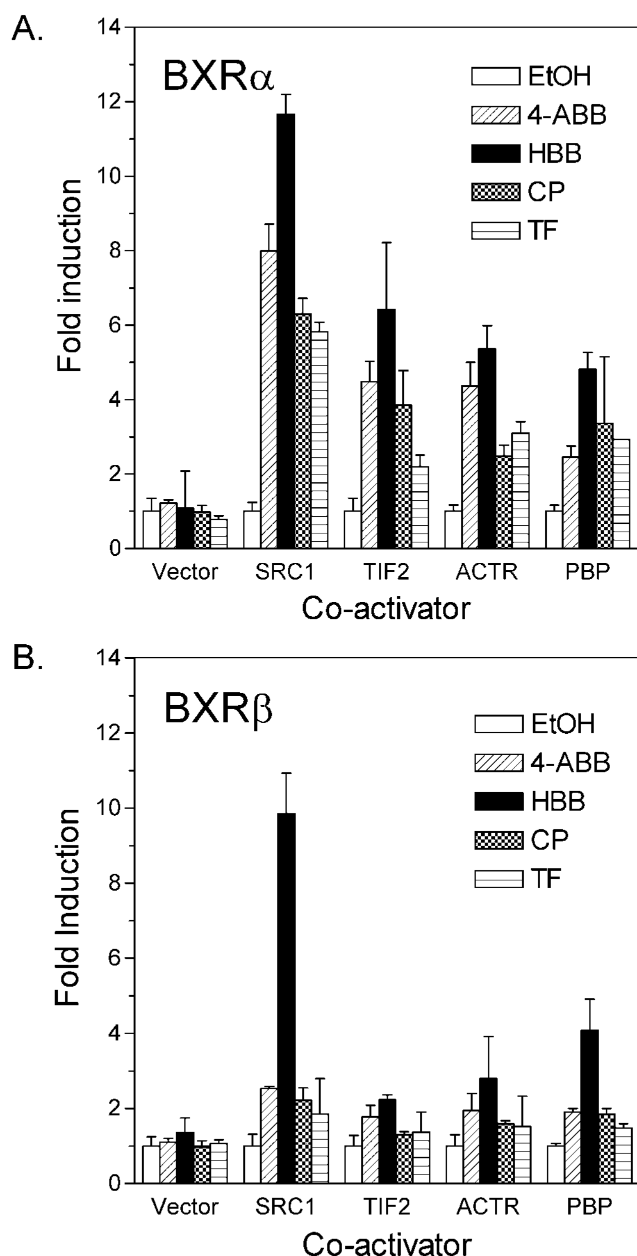

FIG. 3. **BXRα and BXRβ co-activator recruitment.** COS-7 cells were transiently transfected with a GAL4 reporter and VP16-BXRα (*A*) or VP16-BXRβ (*B*) together with expression vectors for the GAL4 DNA-binding domain (*vector*) or the GAL4 DNA-binding domain linked to the receptor interaction domains of the indicated nuclear receptor co-activators. Cells were treated with 50 μM 4-ABB, 4-HBB, chlorpyrifos (*CP*), or trifluralin (*TF*) or with vehicle (ethanol) alone.

formation of specific active transcriptional complexes. This mammalian two-hybrid assay utilized VP16-BXRα and VP16-BXRβ together with fusions between the GAL4-DNA-binding domain and the receptor-interacting domain of the nuclear hormone receptor co-activators, TIF-2, ACTR, SRC-1, and PBP (18) to investigate the ability of BXRα agonists to promote productive transcriptional interactions.

VP16-BXRα was able to interact with all four co-activators in the presence of agonistic ligands (Fig. 3A). VP16-BXRα showed the strongest interaction with SRC-1 irrespective of the ligand tested (4-HBB, 12-fold; 4-ABB, 8-fold; chlorpyrifos and trifluralin 6-fold), although, a rank order of co-activator response could be identified (SRC-1 > TIF-2 > PBP = ACTR) (Fig. 3*A*). This is notably different from the rank order of potency of SXR for the same coactivators (SRC1 > PBP > TIF-2/GRIP > ACTR) (19). Overall, the rank order of potency of compounds in the co-activator recruitment assay paralleled their potency in the activation assays (Fig. 2).

TABLE I
BXRα agonists are BXRβ antagonists

Compound	EC_{50}	K_i[a]	n
	μM	μM	
4-HBB	33 ± 3.2[b]		4
4-ABB		82 ± 9	15
Chlorpyrifos		0.5 ± 0.2	13
Trifluralin		5.2 ± 0.8	8

[a] K_i values were derived from inhibition curves at constant HBB concentrations in the range of 1–50 μM 4-HBB using the Cheng-Prusoff equation (20). Values represent the mean ± S.E. calculated from the indicated number of inhibition curves (n).

[b] EC_{50} for BXRβ activation by 4-HBB was determined from non-linear regression analysis of dose-response curves.

Co-activator interaction with BXRβ was much more restricted in response to agonist (Fig. 3*B*). Consistent with the dose-response experiments (Fig. 2*D*), only 4-HBB possessed any strong ability to promote co-activator interaction. The response observed with SRC-1 was approximately equivalent to that seen with BXRα (10- *versus* 12-fold). The level of interaction with PBP was weaker (4-fold) although comparable with that seen with BXRα (Fig. 3*A*). The activation responses with ACTR and TIF-2 were poor. The rank order was SRC-1 > PBP > ACTR = TIF-2. The data suggest that SRC-1 is a strong co-activator for both BXRα and BXRβ. BXRα is promiscuous both in its choice of ligand and in its choice of co-activator, whereas BXRβ is not promiscuous for either (Fig. 3).

BXRα-specific Activators Are Antagonists for BXRβ—The observation that most BXRα activators could not activate BXRβ leads to two possible inferences. One is that the compounds specifically bind only to BXRα. In this case, we would not expect to discern any effect of these compounds on BXRβ activation. Alternatively, the compounds might bind to both receptors but only activate BXRα. In this scenario, the BXRα activators could act as antagonists for BXRβ. Accordingly, we conducted antagonism experiments using the BXRβ agonist 4-HBB and BXRα-specific agonists. Two types of experiments were employed.

First, we tested the ability of BXRα-specific agonists to directly interfere with BXRβ-mediated activation. COS-7 cells were transfected with GAL4-BXRβ, and inhibitory dose-response curves were derived. Cells were treated with increasing doses of 4-ABB, chlorpyrifos, or trifluralin in the presence of a fixed series of agonist 4-HBB concentrations in the range of 1–50 μM. Transcriptional activation of BXRβ by 4-HBB alone gave a derived mean EC_{50} of 33 ± 3.2 μM (n = 4) (Table I). Titration with either chlorpyrifos or trifluralin resulted in a dose-dependent inhibition of 4-HBB-mediated transcriptional activation of the reporter gene (Fig. 4A). In contrast, 4-ABB showed a small additive effect up to 10 μM and was inhibitory at 100 μM under these conditions (Fig. 4*A*). Data were fitted by non-linear regression analysis, and inhibitory constants (K_i) for BXRβ-mediated transcriptional activation were calculated using the Cheng-Prusoff equation (20) and shown in Table I. Chlorpyrifos yielded a K_i of 0.5 ± 0.2 μM (n = 13), trifluralin yielded a K_i of 5.2 ± 0.8 μM (n = 8), and 4-ABB yielded a K_i of >82 ± 9 μM (n = 15). By comparison with the EC_{50} value of 33 μM for 4-HBB on BXRβ, the data suggest that both chlorpyrifos and trifluralin can act as potent competitive antagonists of BXRβ activation, whereas 4-ABB demonstrates weaker antagonism on this receptor. 3-HEB did not antagonize the activation of BXRβ (data not shown).

We next tested the ability of the BXRα-specific agonists to

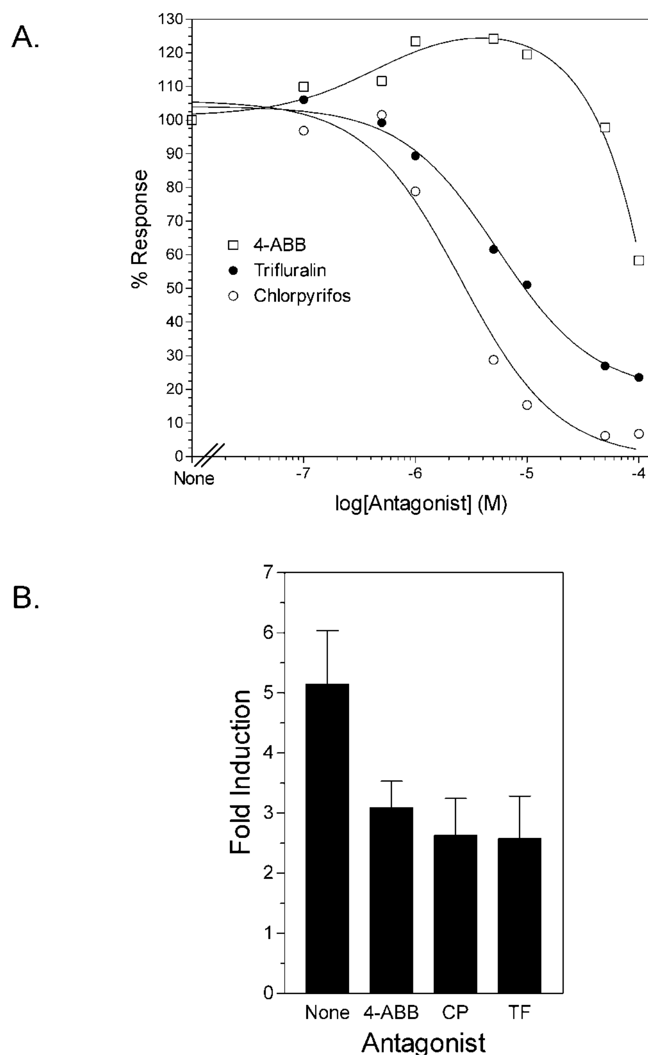

FIG. 4. **BXRα activators are BXRβ antagonists.** In *A*, COS-7 cells were transfected with GAL-BXRβ, reporter, and control plasmids as described under "Experimental Procedures." Cells were incubated at a constant concentration of 4-HBB within the range of 1–50 μM (10 μM shown in figure). The BXRα agonists 4-ABB (*white square*), trifluralin (*black circle*), or chlorpyrifos (*white circle*) were then titrated from 0.1 to 100 μM. Cells were incubated with ligands for 24 h, harvested, and assayed for luciferase and β-galactosidase activity. Data are from a typical experiment and plotted as the percent of relative luciferase units obtained with 10 μM 4-HBB alone. Data points are the means of triplicates; S.E. was less than 15%. In *B*, COS-7 cells were transfected with a GAL4 reporter together with VP16-BXRβ and GAL4-SRC1 expression vectors. Cells were then treated with 50 μM 4-HBB in the absence (*none*) or presence of 50 μM 4-ABB, 50 μM chlorpyrifos (*CP*), or 10 μM trifluralin (*TF*).

interfere with 4-HBB-mediated co-activator recruitment in the mammalian two-hybrid assay as described above. COS-7 cells were transfected with VP16-BXRβ, GAL-SRC1, and CMX-β-galactosidase and treated with 50 μM 4-HBB in combination with a dose series of 4-ABB, chlorpyrifos, trifluralin, or solvent controls. Fig. 4*B* shows that 4-ABB, chlorpyrifos, and trifluralin were each able to impair co-activator recruitment by 4-HBB. Therefore, we conclude that these BXRα-selective activators are able to act as antagonists for BXRβ, supporting the contention that BXRα and BXRβ are pharmacologically distinct from each other.

BXRs Are Not Xenobiotic Sensors—The very different tissue distributions of BXRs and SXR/PXRs led us to suspect that these receptors might be functionally different. To test this hypothesis, we examined a panel of compounds for their ability

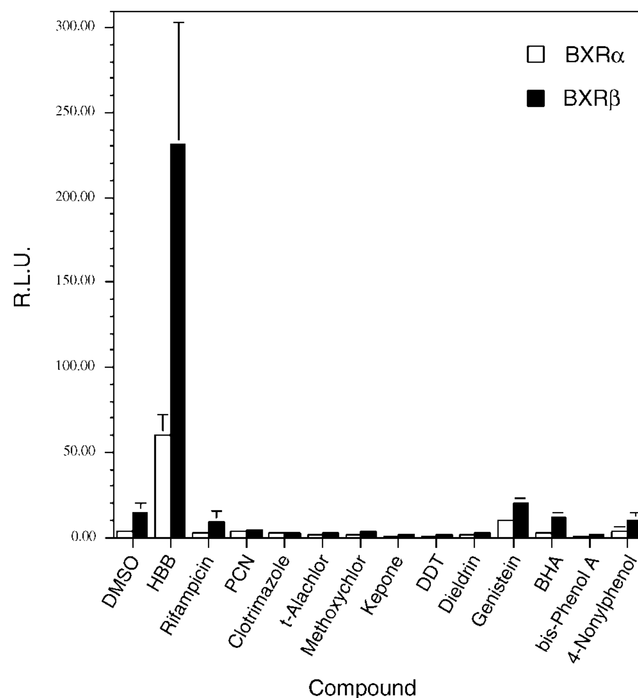

FIG. 5. **BXRs are not xenobiotic receptors.** COS-7 cells were transfected with BXRα or BXRβ, reporter, and control plasmids as described under "Experimental Procedures." Cells were treated with vehicle only (Me_2SO (*DMSO*)) or the indicated xenobiotic ligands at 50 μM for 24 h. Data represent the means of triplicates ± S.E. from a representative experiment.

to activate BXRα, BXRβ, and human SXR. We found that neither BXRα nor BXRβ was activated by any of the classic SXR/PXR activators (*e.g.* rifampicin, pregnenolone-16 α-carbonitrile, phenobarbitals, or clotrimazole) (Fig. 5). The only xenobiotic compounds that activated BXRα, chlorpyrifos and trifluralin, have chemical structures similar to benzoates. We also evaluated other known SXR activators including steroids and bile acids for their ability to activate BXR. None of these SXR activators were able to activate BXRs (data not shown). We also note that two compounds reported previously to activate BXRβ, dexamethasone and methylprednisolone (7), do not activate either BXR in our experiments. The reason for this discrepancy is unknown at present but may relate either to the different experimental systems used or to the very low levels of activation originally observed (~2-fold) (7).

DISCUSSION

Despite a flood of genomic and expressed sequence tag sequence information in recent years, there is still little information available that suggests a function for BXR or confirms its presence in animals other than *X. laevis*. The recent identification of BXRβ (7) and its activation by 4-HBB (Figs. 2 and 3) suggests that both BXRs are receptors for endogenous hydroxyl benzoates or closely related compounds. This narrows the range of compounds expected to activate both BXRα and BXRβ to a relatively small group and suggests that a detailed focus on these structures will lead to the elucidation of endogenous ligands. It is particularly interesting that BXRα and BXRβ are pharmacologically distinct and that several potent BXRα activators are BXRβ antagonists. This means that treating early embryos with compounds such as 4-ABB would simultaneously activate BXRα while inhibiting the activity of BXRβ. This could explain why such treatments do not have obvious adverse effects on the embryos at subtoxic doses.[2] To sort out the biology of the two receptor subtypes, it will be necessary to

perform targeted loss-of-function experiments in early embryos using, for example, morpholino antisense oligonucleotides (21, 22) coupled with phenotypic rescue experiments.

The results presented in Fig. 2 show that both BXRs are robustly activated only by 4-hydroxyl benzoates, whereas BXRα is promiscuously activated by other benzoates and related compounds. This is notable because one of the endogenous benzoates found in *Xenopus* embryos, 3-HEB, is a BXRα−selective activator (5) (Fig. 2). Although three endogenous benzoates remain to be identified in *Xenopus* embryos, the total concentration of benzoate BXRα activators is 10 μM in the blastula stage embryo (5), which is in the range required for receptor activation. In accord with our results, Moore *et al.* (23) recently showed that BXRβ is robustly activated by 4-hydroxyl benzoates and noted, without comment, that BXRα is activated by amino benzoates, whereas BXRβ is not. In contrast to our findings, Nishikawa *et al.* (7) report only a 1.5-fold activation of BXRβ by 4-HBB. The reason for this difference is currently unknown but could result from different cell lines utilized in the different laboratories (COS-7 *versus* HeLa). Alternatively, since *bona fide* target genes for BXR are unknown, the reporter construct used (7) might not be an effective target for BXRβ *in vivo*. We note that BXRα shows a strong preference for the sequence AGTTCAnnnnAGTTCA (5) as compared with the AGGTCAnnnnAGGTCA used by Nishikawa *et al.* (7) (where n equals any nucleotide).

BXRs are most closely related to the mammalian SXR/PXR gene family. Indeed, BXR was used as a probe to isolate human SXR (8). BXR and SXR/PXR have been assigned to the NR1I2 family by the Nuclear Receptor Nomenclature Committee (11), indicating that these receptors are orthologous, *i.e.* they represent the same gene from different species. During our original characterization of BXR (5) and SXR (8), we noted that there was little overlap in the set of compounds that activated each receptor. This raised an important issue about the relationship between BXR and SXR/PXR because the latter are known to function as xenobiotic sensors. It is well known that human SXR and mouse PXR are pharmacologically distinct in that each has species-specific activators as well as compounds that activate across species (8, 9, 24; also reviewed in Refs. 1 and 25). Therefore, one possibility is that BXRs are xenobiotic sensors but that the spectrum of compounds that activates them is species-specific.

We conducted extensive ligand screening experiments and showed that neither BXR is activated by the broad spectrum of steroids and xenobiotic compounds that are known to activate SXR (Fig. 5). Moore and *et al.* (23) have also tested a large number of steroids and xenobiotic compounds for BXR activation. In accord with our results, they show that BXRs are not activated by most steroids or xenobiotics (23), which argues that BXRs do not function as broad specificity xenobiotic sensors. The only point of disagreement between our datasets is that they show 2.6-fold induction of BXRα by rifampicin, whereas it does not activate either BXR in our experiments. However, considering that activation of BXRα by *bona fide* ligands ranges from 180- to 1200-fold, it is difficult to ascertain whether the 2.6-fold induction by rifampicin represents ligand-dependent transactivation. We infer from our data and those of Moore *et al.* (23) that BXRs are unlikely to function as xenobiotic sensors.

SXR and PXR expression are enriched in the liver and intestine, where they activate the expression of cytochrome P450 genes and ABC family organic transporters to detoxify and eliminate xenobiotic compounds (25). On the other hand, BXRs are ubiquitously expressed with no particular enrichment in liver or intestine. No known bona fide BXR targets have been identified yet although it is believed that the *Pit-1* gene contains a high affinity BXR target element (5). Taken together with their lack of activation by the types of xenobiotic compounds that activate SXR/PXR, one is forced to conclude that these receptors are functionally distinct. It is highly unusual for orthologous nuclear receptors to exhibit such different expression patterns, hence one wonders whether the evolutionary pressure operating on members of this gene family is the same in different species. One possibility is that the BXRs are not really orthologous to SXR/PXR but rather represent a distinct family of receptors with an entirely different biology. For this model to be correct, one would expect to find BXR relatives in other vertebrates and SXR/PXR homologs in *Xenopus*. We have not found sequences more closely related to BXR than SXR/PXR in the draft human and mouse genome sequences. Moreover, no sequences more closely related to SXR/PXR than BXR have appeared in the more than 200,000 *Xenopus* expressed sequence tags identified to date. We have not identified it in numerous screening experiments (data not shown). Thus, if BXR is not orthologous to SXR, it might be a receptor restricted to amphibians or lower vertebrates. This would make it the first such nuclear receptor identified.

An alternative possibility is that BXR is orthologous to SXR in an evolutionary sense but has diverged functionally. This could be due to the different constraints on poikilothermic, aquatic animals *versus* homoeothermic mammals. *Xenopus* is a carnivorous frog that likely has a very different diet from humans and rodents. Moore *et al.* (23) recently identified SXR homologs from zebrafish, pig, dog, and monkey. They showed that the zebrafish gene was activated by several steroids and xenobiotic compounds (23). Interestingly, the BXRs are about equally similar to the zebrafish (46% identity) and the mammalian sequences (~50% identity). Therefore, it appears that the functional divergence of BXRs from the SXR/PXR group of genes cannot be explained by the diet or aquatic nature of the organisms. Therefore, it will be very interesting to identify and characterize BXR and SXR homologs from other amphibians, reptiles, fishes, and lower chordates to determine at which point in evolution BXR localization and function diverged from SXR. Indeed, the question of whether BXR and SXR are true orthologs must await the identification of similar receptors from related species of fish and amphibians.

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