Author

When she became interested in performing research, Emily Grossman contacted Professor Blumberg, who put her under the guidance of Dr. Matthew Milnes, a postdoctoral researcher in his lab. Emily's work focuses on a nuclear hormone receptor that is vital in de-toxifying the body and is responsible for the regulation of the metabolism of over 60% of currently used pharmaceutical drugs. She has enjoyed working with the people in her lab and learning new techniques and protocols. Completing projects on her own has increased her confidence in her abilities. Emily will move on to graduate school in September 2007.

Key Terms

- Ligand
- Nuclear Receptor
- Single Nucleotide Polymorphisms (SNPs)
- Steroid and Xenobiotic Receptor (SXR)
- Xenobiotic Receptor

Variation in Ligand Dependent Activation of Steroid and Xenobiotic (SXR) Single Nucleotide Polymorphisms

Emily Grossman
Biological Sciences

Abstract

Homeostasis in the body is maintained through many mechanisms. The steroid and xenobiotic receptor (SXR) is a nuclear hormone receptor that regulates catabolic enzymes responsible for the metabolism of over 60% of currently used drugs. Individuals show considerable differences in their ability to metabolize drugs; one hypothesis is that single nucleotide polymorphisms in this receptor are responsible for some of this variability. In this study, we tested the ability of the SXR harboring single nucleotide polymorphisms to respond to known activators. We re-created 14 known single nucleotide polymorphisms within this receptor, and tested these mutant receptors in a cell-based assay system using a luciferase reporter for responsiveness to three known ligands. Compared with the activity of the wild type receptor, we found that most of the polymorphisms had little or no effect on the ability of this receptor to respond to the ligands. However, D163G and A370T had profound effects, differing from wild type by over 77%. Considering that SXR plays such a central role in drug metabolism, the frequency of these polymorphisms in the population has important implications for drug development and potential drug-drug interactions.

Faculty Mentor

There are striking differences in individuals' abilities to detoxify and metabolize pharmaceuticals, bioactive dietary compounds, and xenobiotic chemicals. Understanding these differences is critical for identifying individualized treatments and in assessing the real risks posed by exposure to dietary or xenobiotic chemicals. The steroid and xenobiotic receptor, SXR, is the primary regulator of enzymes that lead to the metabolism of drugs and chemicals. Emily's research tested the effects of variations in the SXR sequence on the ability of SXR to respond to a panel of xenobiotic chemicals. She found significant differences in the ability of SXR to activate gene expression in response to chemical exposure, and that the effects were specific to the tested chemical. These results have broad implications in understanding human response to drugs and chemicals.

Bruce Blumberg
School of Biological Sciences

Introduction

The steroid and xenobiotic receptor (SXR; NR1I2) is a low affinity, broad specificity nuclear hormone receptor that is a key regulator of bile acid, steroid hormone, and xenobiotic metabolism and excretion (Blumberg et al., 1998; Krasowski et al., 2005). SXR is highly expressed in the small intestinal epithelium and liver. This receptor is activated by a large number of natural and synthetic compounds. Known activators encompass bactericidal antibiotics such as rifampicin (RIF), as well as numerous steroid hormones, medicinal herbs, and xenobiotics (Tabb et al., 2004). SXR forms a heterodimer with the 9-*cis* retinoic acid receptor (RXR) and binds to specific DNA response elements located in the 5' regulatory region of genes encoding catabolic enzymes including *CYP3A4* and *CYP2B* (Xie et al., 2000). *CYP3A4* is a member of the cytochrome P450 family and is responsible for the oxidative metabolism of many endogenous substances, including steroids, fatty acids, and plant metabolites (Nebert and Gonzalez, 1987), as well as xenobiotic chemicals, including approximately 60% of currently used pharmaceuticals (Maurel, 1996).

The broad ligand specificity of SXR and the degradative action of its target genes on xenobiotics and endogenous substances presents a potential problem for patients prescribed multi-drug therapies and individuals inadvertently exposed to SXR ligands. Co-administration of drugs, one of which activates SXR, can lead to increased clearance of the other drugs and loss of therapeutic efficacy (Lamba et al., 2005). A well-characterized example of drug-drug interactions is the relationship between oral contraceptives and antibiotics. Antibiotics such as RIF are ligands that activate SXR, upregulating catabolic enzymes that degrade the steroid hormones in birth control pills, rendering them ineffective. Increasing occurrences of adverse drug events are a major concern, with 21,298 adverse drug events cases reported in the last two years alone (Budnitz et al., 2006).

Decreased activation of SXR resulting from exposure to receptor antagonists, or functional changes in the receptor can have the opposite effect on drug metabolism. Moreover, a nonfunctional receptor can be the cause of a disease, as is the case with inflammatory bowel disease. In a subset of patients with this disease, reduced expression of SXR was directly linked to the downregulation of SXR downstream target genes, such as the fatty acid synthase gene (Langmann et al., 2004). There was a strong correlation between loss of SXR expression and inflammatory bowel disease, although this connection remains to be proven.

The degree of drug sensitivity in the human population varies. One potential mechanism for this variation includes single nucleotide polymorphisms (SNPs) in the SXR protein, promoter and/or target genes. SNPs in SXR can alter how the receptor responds to exogenous ligands, which is particularly relevant to drug metabolism and drug-drug interactions. Recent studies suggest that polymorphisms in SXR or in SXR-binding sites in target genes could be responsible for variations in drug response among patients (Zhang et al., 2001).

Although several receptor variants result from alternative splicing or promoter usage, the predominant form consists of 434 amino acid residues. The first 40 constitute the N-terminal region, which may play a role in activated transcription. The DNA binding domain includes amino acids 41–107, and the ligand-binding domain contains amino acids 141–434 (Blumberg et al., 1998). The amino acids making up the space between are referred to as the "hinge" region.

Fourteen SNPs in SXR that change the amino acid sequence of the protein have been identified. The effects of these "non-synonymous" SNPs range from no change in receptor activation to nearly complete loss of activity and the inability to bind DNA (Koyano et al., 2004). Lim *et al.*, (2005) found that the Q158K polymorphism (change of amino acid 158 from glutamine (Q) to lysine (K)) caused a dramatic reduction in the response of SXR to the strong activator rifampicin (RIF). Polymorphism R98C was reported to cause a complete loss of receptor binding to the downstream DNA response element ER6 and, as a result, a nearly complete loss of the ability of SXR to regulate its target genes. Although these SNPs caused dramatic changes in the receptor activity, others such as R381W were observed only to have reduced activation and slight attenuation of promoter activity (Koyano et al., 2004). It was found that induction of the D163G SNP is dependent on the ER6 element in the proximal promoter of *CYP3A4* (Hustert et al., 2001).

We sought to examine the effects of SXR SNPs on the ability of this receptor to respond to xenobiotic ligands. Previous studies tested a small number of doses for each compound, which precluded a rigorous study of receptor activation. We conducted extensive dose-response experiments so that we could derive EC_{50} values for comparison. EC_{50} is the concentration at which 50% of the maximum activation is reached, and serves as a basis for comparing the function of receptor mutants to each other and to the wild type receptor. We hypothesized that SXR SNPs could

lead to ligand-specific changes in sensitivity and maximum activation of a reporter gene relative to the wild type receptor. Therefore, we tested ten non-synonymous SXR SNPs and wild type SXR in response to three ligands using a cell-based luciferase reporter assay.

Materials and Methods

Construction of SXR Plasmids

Site directed mutagenesis was used to create ten known SNPs. Each SXR protein coding region containing a known SNP was PCR amplified and subcloned into *Nco*I and *Bam*H1 sites of the vector pCDG1 (Blumberg et al., 1998) using exonuclease III-mediated ligation-independent cloning (Li and Evans, 1997). Each SXR SNP construct was completely sequenced and we verified that they contained only the intended SNP prior to use in transient transfection assays. As a further validation of the plasmids, we used a coupled rabbit reticulocyte transcription and translation (TnT) reaction (Promega, Madison, WI) to verify that all SNPs produced proteins of the correct molecular weight and in comparable amounts per μg of input DNA (Figure 1).

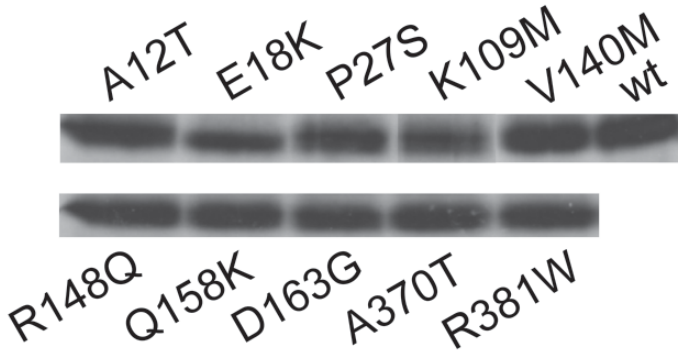

Figure 1

Western Blot analysis of recombinant SXR protein expression. All bands are ~50 kDa. Note that the expression levels of all SNPs are similar, suggesting equivalent translation efficiency.

Cell Culture

We cultured COS-7 cells ranging from passage 50 to 70 in phenol red-free Dulbecco modified Eagle's Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS). For transient transfection experiments, 96 well plates were seeded with COS-7 cells at a density of 5 x 10^5 cells/plate. Following 24 hours' incubation at 37 ºC (5% CO_2 in air), cells were transfected with SXR SNP receptor constructs, luciferase reporter constructs (pXREM-luc), and pCMX-β-galactosidase transfection control plasmids using standard calcium phosphate methods as described (Zhou et al., 2004). Cells were incubated for another 24 hours prior to ligand treatments with RIF, bis-(2-ethylhexyl)-phthalate, or dibutyl phthalate. The ligands were initially dissolved in DMSO and further diluted in DMEM supplemented with resin-charcoal stripped Fetal Bovine Serum. Cells were incubated in ligand concentrations ranging from 20 nM to 50 μM for 24 hours. Cell extracts were prepared and assayed for β-galactosidase and luciferase activity as described (Blumberg et al., 1998). β-galactosidase activity was used to normalize luciferase activity for transfection efficiency, and the results were expressed as fold-activation relative to solvent controls (0.5% DMSO). All ligand treatment experiments were performed in triplicate, and each SNP was tested a total of three times.

Data Analysis

Maximum fold-activation was determined for each SNP at the highest ligand concentration that did not result in cytotoxic effects as evidenced by reduced β-galactosidase activity. Activity as fold activation for each receptor-ligand combination was fitted to a sigmoidal dose-response curve to estimate the effective dose resulting in 50% maximum activation (EC_{50}), and the fold-activation at that concentration using GraphPad Prism software, version 4.0 (GraphPad, San Diego, CA). A non-parametric statistical significance test, the Mann-Whitney U test, was used to determine when differences between SNPs and the wild type were significant. Results were considered to be significant when p-values were less than 0.05.

Results

Maximum Fold Activation

In response to RIF, the polymorphisms occurring in the N-terminal region of the receptor (A12T, E18K, P27S, amino acids 1–40) showed slightly lower maximum activations, ranging from 63% to 75% of wild type (Figure 2). In contrast, the two polymorphisms contained in the hinge region (K109N and V140M) showed differing results in response to RIF and bis-(2-ethylhexyl)-phthalate. K109N activation was comparable to wild type, whereas V140M showed 40% lower activation than wild type. Activity of the SNPs A370T and D163G differed substantially from wild type. A370T shows a 77% increase over wild type at the 50 μM RIF dose, and is nearly double that of wild type activation in response to bisphthalate at 16.6 μM. D163G shows roughly a 78% and 85% decrease in activation in response to RIF and bisphthalate treatments respectively. Another SNP, Q158K showed a 41% reduction in activity. There was no significant variation in response to dibutyl phthalate between wild type SXR and any of the SNPs with the exception of D163G. D163G activity was reduced 91% compared to wild type.

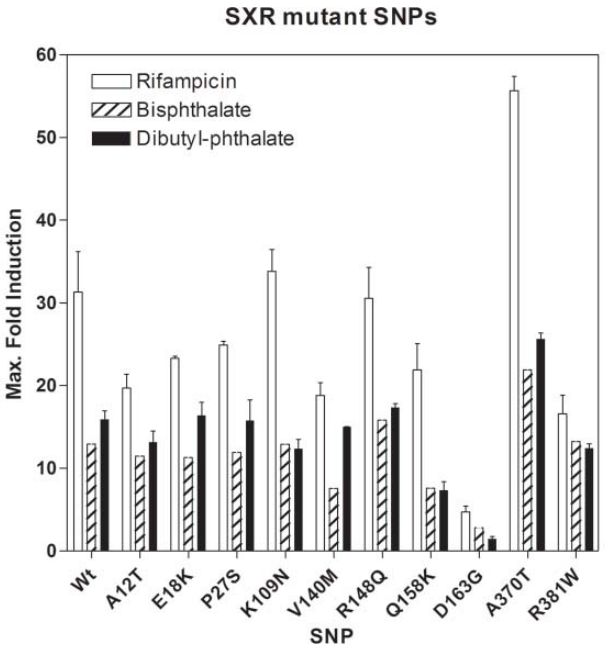

Figure 2

Maximum fold activation of wild type and SXR SNPs in response to three ligands (first run)

EC_{50} values

Next we examined average EC_{50} values for each polymorphism in response to each ligand. For RIF, all the SNPs except V140M, Q158K and D163G show similarity to wild type values (Table 1). None of the SNPs are significantly different from wild type in response to bisthphalate. Similarly, no SNPs differ significantly from wild type in response to the dibutyl phthalate. Values for K109N, Q158K, D163G, and R381W in response to this last ligand could not be determined due to a poor fit to a sigmoidal curve. The values for the Q158K mutation did not increase above baseline until a dose of 5.55 μM was applied, and the D163G mutation did not ever increase above baseline in any sort of dose dependent fashion.

Table 1

Average EC_{50} (μM) of wild type and SXR SNPs exposed to Rifampicin, Bis(2-ethylhexyl) phthalate and Dibutyl phthalate

SNP	Rifampicin	Bisphthalate	Dibutyl phthalate
Wild type	10.84	7.98	49.29
A12T	5.96	8.12	55.23
E18K	11.25	8.24	55.07
P27S	7.96	7.10	80.77
K109N	10.45	12.40	**
V140M	3.95*	4.22	29.72
R148Q	6.56	7.79	176.52
Q158K	69.60*	18.13	**
D163G	**	**	**
A370T	7.03	10.36	58.57
R381W	23.93	15.08	**

* significant deviation from wild type, $p<0.05$
** data does not fit a sigmodial curve

Fold Induction at EC_{50} values

A12T, V140M and R381W showed a 36–46% reduction in fold activation in response to RIF (Table 2). In contrast, A370T exhibited a 74% increase in induction relative to wild type in response to this ligand at its EC_{50} value. V140M and Q158K showed a 37–64% reduction in fold activation compared to wild type in response to bisphthalate. Finally, D163G showed little to no activation in response to either bisphthalate or dibutyl phthalate. EC_{50} values could not be accurately determined due to the poor fit of the data to a sigmoidal curve.

Table 2

Fold induction at the EC_{50} value of SNPs in response to ligands

SNP	Rifampicin	Bisphthalate	Dibutyl phthalate
Wild type	18.911	7.015	15.300
A12T	10.283	6.180	9.029
E18K	13.107	6.539	11.986
P27S	13.881	8.503	13.547
K109N	19.595	5.224	9.422
V140M	12.143	3.970	10.370
R148Q	16.860	6.805	12.530
Q158K	15.746	4.401	*
D163G	*	1.468	*
A370T	32.929	11.189	17.459
R381W	10.739	6.413	8.476

* extrapolation of graph gives incorrect results

Discussion

We compared the activity of 10 SXR SNPs with the wild type receptor and found that two mutations stand out as having significantly different activity than that of wild type. A370T shows a 77% increase above wild type in response to RIF and bis-(2-ethylhexyl)-phthalate. In contrast, D163G consistently shows an 80–90% reduction in receptor activity irrespective of the ligand applied. Hustert *et al.* (2001) previously showed that D163G produces similar amounts of protein to wild type, but that induction by RIF is dependent on a specific SXR response element (ER6) found in the proximal promoter of *CYP3A4*. They observed reduced basal and RIF-induced activities with this receptor while using the same plasmid construct we used, containing the *CYP3A4* proximal promoter and distal enhancer. Based on

the approximately 40-fold lower sensitivity of this polymorphism to RIF, it was concluded that this mutation impaired ligand binding. However, based on the SXR crystal structure, D163G is not found in the ligand-binding pocket and is unlikely to play a direct role in ligand binding (Watkins et al., 2001). This SNP probably disrupts the stability of the protein or its ability to recruit necessary transcriptional co-factors. It is notable that most SNPs showed relatively little difference in response to bisphthalate or dibutyl phthalate. In contrast, there was often considerable variability in the responses of mutant receptors to RIF. This suggests that the SNPs might lead to a differential response to ligands, although the mechanism underlying such a response is still unknown. Perhaps the mutation changes the shape of the ligand-binding pocket such that smaller molecules like bisphthalate and dibutyl phthalate can still bind well, but the larger RIF has more difficulty binding, which decreases the activation.

To evaluate the overall effect of the SNPs on transcriptional activity, we can broadly group them into four categories based on maximum fold induction and EC_{50} values. The first category includes the SNPs that had maximum activations as well as EC_{50} values that did not differ significantly from ($p>0.05$) wild type. A12T, E18K, P27S, K109N, R148Q, and R381W do not behave significantly different from wild type when tested with RIF. Category 2 contains the polymorphisms that have similar maximum activation, but significantly different EC_{50} values, making them more sensitive to ligand. Only V140M and Q158K fall into this group. Since a lower dose of RIF is required to achieve the same response we observed in the wild type, we infer that these mutations have increased the sensitivity of the receptor to ligand treatment. Category 3 includes polymorphisms that had different maximum activation, but similar EC_{50} values. A370T is the only SNP in this group. Since the receptor reaches half-maximal activation at the same RIF dose but has a higher overall magnitude of activation, we conclude that the SNP enhances receptor activity without affecting ligand binding. Category 4 includes SNPs that significantly differ from wild type, both in maximum activation and EC_{50} values. Only D163G fell into this category, and it was less sensitive to ligand and activated to a lesser extent. This is consistent with its performance in response to all three ligands applied, although it should be noted that the activation profile did not fit the expected sigmoidal dose response. This mutation caused little to no increase in activation values under increasing ligand concentrations, and therefore did not generate a normal sigmoidal dose response curve. It did generate an activation profile that would be expected in a non-functional receptor.

A little less than half of the SNPs (A12T, E18K, P27S, and R381W) show no significant change in response to bisphthalate. The others (K109N, V140M, R148Q, Q158K, and A370T) show a significant change in maximum activation, with decreased activation for all except A370T, but no significant difference with respect to EC_{50} values. Dibutyl phthalate elicited the least number of differences compared with wild type SXR. Seven of the ten SNPs showed no significant difference in activation or EC_{50}. Q158K showed a reduction in activation, whereas A370T exhibited a higher degree of activation, but neither SNP demonstrated a change in sensitivity.

Due to the major role played by SXR in maintaining chemical homeostasis in the body, our findings may have considerable clinical relevance, particularly for those who have SNPs in their SXR. For example, individuals carrying the D163G or A370T SNPs may face greater incidence of drug interactions, including reduced drug clearance or loss of therapeutic efficiency. SXR and its downstream target genes are responsible for metabolism of over half the current pharmaceutical drugs. Therefore, severe toxic reactions to certain drugs caused by compromised clearance could occur in patients who have SXRs such as D163G with reduced function. Patients who have the A370T mutation and higher than average activation of SXR may face a related problem. Exposure to SXR activators could lead to the increased metabolism of endogenous steroids, thereby disrupting the body's homeostatic mechanism. Further investigation involving more ligands and SNPs will more clearly illuminate the effects of mutations in SXR on human health.

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