Short Communication

LEVOTHYROXINE UP-REGULATES P-GLYCOPROTEIN INDEPENDENT OF THE PREGNANE X RECEPTOR

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ABSTRACT:

P-Glycoprotein (P-gp) and cytochrome P450 3A4 (CYP3A4) constitute a physiologic barrier in the intestine for many of the same substrates. Their expression can be influenced by nuclear receptor NR1I2 (pregnane X receptor; PXR), which acts as a receptor for various endobiotics and xenobiotics. However, P-gp and CYP3A4 are not identical in anatomic localization, suggesting unique as well as shared regulatory mechanisms of gene expression. We used established human colon carcinoma cell lines (LS180 and Caco-2) and measured mRNA and protein levels in cells after exposure to levothyroxine (L-T₄), triiodo-L-thyronine (L-T₃), and rifampin. Results indicate that L-T₄, L-T₃, and rifampin can up-regulate the expression of P-gp mRNA and protein in LS180 cells, but only L-T₄ and L-T₃ can produce the same effect in Caco-2 cells, which are relatively lacking in PXR. In addition, L-T₄ and L-T₃ did not affect the expression of CYP3A4 in either cell line. We conclude that P-gp, but not CYP3A4, can be up-regulated by thyroid hormones in vitro by a PXR-independent mechanism. Considering the widespread prescription use of L-T₄ preparations in the older adult population, these results may be important for the clinical consideration of drug-drug interactions mediated by P-gp.

Active efflux and metabolic transformation are two mechanisms that help mammalian cells control entry of a range of compounds (Nelson et al., 1996). P-gp is the product of the human ABCB1 (formerly multidrug resistance 1) gene and a member of the ATP-binding cassette transporter family. This efflux protein transports recognized hydrophobic substrates from the inside to the outside of cells and is expressed at various sites that determine drug disposition such as intestinal enterocytes, blood-brain barrier endothelia, renal proximal tubular cells, and hepatic canalicula cells (Thiebaut et al., 1987; Cordon-Cardo et al., 1990). The cytochrome P450 superfamily plays an important role in the oxidative metabolism of numerous endogenous and exogenous compounds. More than 60% of currently marketed therapeutics are reported to undergo significant metabolism by the human CYP3A4 isoform in the intestine and/or liver (Li et al., 1995).

Expression of both ABCB1 and CYP3A4 can be regulated by nuclear receptor PXR, which binds to the enhancer region located 8 kb upstream from the transcriptional initiation sites of ABCB1 (Geick et al., 2001) and CYP3A4 (Goodwin et al., 1999). PXR dimerizes with retinoid X receptor (RXR) and binds to direct repeats of AG(G/T)TC A with either a three-nucleotide gap (DR3), a four-nucleotide hexamer in a DR3 arrangement, and elimination of the enhancer site resulted in elimination of PXR-mediated induction (Geick et al., 2001). Similarly, the CYP3A4 enhancer site contains two consensus hexamers in a DR3 arrangement, and elimination of the enhancer site prevented PXR-mediated induction (Goodwin et al., 1999). Additional factors such as hepatocyte nuclear factor 4α also appear to be important in PXR-mediated CYP3A4 activation (Tirona et al., 2003).

A consensus half-site, (G/A)GGT(C/G)A, with a striking similarity to the PXR response element forms in various arrangements response elements for different members of the nuclear hormone receptor superfamily, such as thyroid hormone receptor (TR), vitamin D receptor (VDR), and retinoic acid receptor (RAR) (reviewed in Yen, 2001). Direct repeat arrangement of consensus hexamers has been described as the most common motif (Williams and Brent, 1994). In addition, VDR preferentially transactivates via reporter vectors containing DR3, TR via DR4, and RAR via DR5, according to a “3-4-5” rule (Umesono et al., 1991; Glass, 1994) (Table 1). These nuclear hormone receptors can act as monomers, or form homo- and heterodimers. In the latter scenario, they bind to RXR and form a complex, which is believed to be the most important entity (at least for some hormone receptors) in hormonal regulation of gene expression (reviewed in Yen, 2001).

In a previous study (Siegmund et al., 2002), P-glycoprotein levels were increased in duodenal biopsy specimens from healthy human volunteers after administration of levothyroxine (L-T₄). However, the increase in ABCB1 mRNA levels was not statistically significant. Results from this in vivo study cannot indicate whether the effect was due to a direct action of L-T₄ on intestinal cells, and whether this effect was mediated through PXR. The similarities between the mechanisms

ABBREVIATIONS: P-gp, p-glycoprotein; CYP3A4, cytochrome P450 3A4; PXR, pregnane X receptor (nuclear receptor NR1I2); L-T₄, levothyroxine; L-T₃, 3,3',5-triiodo-L-thyronine; TR, thyroid receptor; VDR, vitamin D receptor; RXR, retinoid X receptor, Vit D₃, 1α,25-dihydroxyvitamin D₃; DR, direct repeat; RAR, retinoic acid receptor; ABCB1, ATP-binding cassette protein B1; kb, kilobase(s); HPLC, high-performance liquid chromatography; PCR, polymerase chain reaction.
Comparison of nucleotide sequences recognized by PXR and hormonal receptors (VDR, TR, and RAR)

<table>
<thead>
<tr>
<th>Motif</th>
<th>PXR Response Element</th>
<th>Consensus Sequence</th>
<th>Hormonal Receptor Response Element</th>
<th>Receptor</th>
</tr>
</thead>
<tbody>
<tr>
<td>DR3</td>
<td>AG(G/T)TCA</td>
<td>NNN AG(G/T)TCA</td>
<td>(G/A)G(T/C)G A NNN (G/A)G(T/C)G A</td>
<td>VDR</td>
</tr>
<tr>
<td>DR4</td>
<td>AG(G/T)TCA</td>
<td>NNNN AG(G/T)TCA</td>
<td>(G/A)G(T/C)G A NNNN (G/A)G(T/C)G A</td>
<td>TR</td>
</tr>
<tr>
<td>DR5</td>
<td>AG(G/T)TCA</td>
<td>NNNNN AG(G/T)TCA</td>
<td>(G/A)G(T/C)G A NNNNN (G/A)G(T/C)G A</td>
<td>RAR</td>
</tr>
</tbody>
</table>

Results and Discussion

LS180V cells were induced for 72 h with two different concentrations of L-T4: 100 nM, which approximates the physiologic plasma levels of total L-T4 in normal individuals, and 100 μM, which approximates the intraluminal concentration in patients receiving oral levothyroxine. Rifampin (10 μM) was used as the known PXR-mediated inducer (Pfrunder et al., 2003) of both ABCB1 and CYP3A4. The LS180V cell line has been shown in our laboratory to have abundant PXR, while lacking constitutive androstane receptor (unpublished data). Stability of L-T4 in the culture medium over this period was verified by HPLC analysis.

In LS180V cells, ABCB1 was induced in a concentration-dependent manner by L-T4 (p < 0.05), with EC50 determined at 3.6 μM, and by rifampin (Fig. 1A). Levels of CYP3A4 mRNA were undetectable in all conditions except in rifampin-induced cells. Default values for these conditions were assigned by the real-time PCR software, and rifampin-treated samples were compared. Consequently, the magnitude of the actual mRNA increase with rifampin is at least as great as reported, but may be higher. L-T4 did not produce a detectable increase in CYP3A4 mRNA.

The mechanism of ABCB1 induction by L-T4 was evaluated using the Caco-2 cell line, which lacks significant amounts of PXR (Thummler et al., 2001; T. Mitin, L. van Molle, M. H. Court, and D. J. Greenblatt, unpublished data) and therefore should not demonstrate induction of PXR-regulated genes. Rifampin failed to increase levels of ABCB1 mRNA or protein, but L-T4 significantly increased ABCB1 expression in a concentration-dependent manner (p < 0.05) (Fig. 1, B and C).

These results suggest that L-T4 differs from rifampin in its ability to induce P-gp and regulates the expression of P-gp by a PXR-independent mechanism, perhaps through the TR. Both cell lines express the three major types of TR (TRα1, TRα2, and TRβ1) (Fig. 1, L-T4 may therefore regulate P-gp expression by a mechanism involving binding to its endogenous receptor. T4 accounts for most of the thyroid hormone secreted by the thyroid gland. T3 is converted to T2 by target tissues, and T2 directly interacts with TR. T2 also induced ABCB1 expression in LS180V cells in a concentration-dependent manner, with no effect on CYP3A4 expression (data not shown). Our data were generated in human carcinoma cell lines, and any extrapolation to normal cells must be made with great caution.

Our findings may relate to the effect of 1α,25-dihydroxyvitamin D3 (Vit D3) on CYP3A4 and ABCB1 induction. The regulation of intestinal CYP3A4 expression by VDR and Vit D3 was first suggested by observations that treatment of Caco-2 cells with vitamin D analogs led to significant enhancement of CYP3A4 mRNA and protein levels and increased midazolam hydroxylation activity (Schmiedlin-Ren et al., 1997). These findings were confirmed later in LS180 cells, and ABCB1 up-regulation by Vit D3 occurred in the same study (Thummler et al., 2001), as well as in subsequent studies (Pfrunder et al., 2003).

Although ABCB1 gene expression can be up-regulated by L-T4, L-T3, and Vit D3, CYP3A4 can only be up-regulated by Vit D3. The ABCB1 upstream enhancer contains a perfect DR4 and an overlapping DR3 with only one nucleotide substitution at the fifth position (C→A) (Fig. 3). The presence of both motifs could allow binding of...
TR/RXR as well as VDR/RXR to the enhancer site. The CYP3A4 enhancer region also contains a perfect DR3 and a DR4 with two nucleotide substitutions at position five (C→T), as well as at a critical position three (G>T→A). The nucleotide at the third position may be critical for transactivation of DNA by hormonal receptors (Yen, 2001). The absence of effect by L-T₄ or L-T₃ on CYP3A4 induction therefore might be the result of this nonfunctional DR4, which lacks a specific hexamer recognizable by TR/RXR. L-T₄ and L-T₃ might also lead to mRNA stabilization, resulting in the observed increase in mRNA and protein levels.

To our knowledge, this is the first report of L-T₄- and L-T₃-mediated ABCB1 gene expression up-regulation in vitro. A recent clinical study demonstrated an increase in duodenal P-gp in human volunteers after administration of L-T₄ (Siegmund et al., 2002), but with no significant pharmacokinetic changes for the P-gp substrate talinolol in individu-
als with P-gp increases. Since this study included only healthy individuals under the age of 30, the potential functional relevance of P-gp induction by thyroxine remains unclear. In clinical reports, higher doses of digitals (a prototypic P-gp substrate) were necessary to control the heart rate of patients with hyperthyroidism and atrial fibrillation (Frye and Braunwald, 1961), and this phenomenon was attributed in part to the lower plasma concentration of digoxin in hyperthyroid patients (O’Connor and Feely, 1987).

In addition, L-T4 is widely prescribed, with prescriptions for Synthroid (one commercial formulation of L-T4) alone ranking as number four among all drugs for 2002 (http://www.rxlist.com/top200.htm). Reports indicate that 6.9% of a sample U.S. population over 58 years of age are treated with L-T4 (Sawin et al., 1989).

P-gp recognizes many of the same substrates as CYP3A4 and responds to some of the same pharmacologic inducers (Schuetz et al., 1996; Dürr et al., 2000), but overlap is incomplete, and P-gp and CYP3A4 are not identical in anatomic localization. The presence of P-gp without appreciable CYP3A4 at sites such as the blood-brain barrier and in subsets of circulating lymphocytes supports our findings that P-gp is likely to have unique regulatory elements and responses that need to be elucidated.

Peroxisome proliferator-activated receptor and RXR activate estrogen-dependent genes by binding to the estrogen receptor response elements in the upstream enhancer region (Nunez et al., 1997). A similar cross talk mechanism was proposed for PXR/constitutive androstane receptor and VDR (Drocourt et al., 2002). Such regulatory overlap may have implications for treatment of conditions in which the function of a hormonal nuclear receptor is altered, such as the thyroid hormone resistance syndrome, linked to mutations in TR (Connor and Feely, 1987).