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Human PXR-mediated induction of intestinal CYP3A4 attenuates 1α,25-dihydroxyvitamin D_3 function in human colon adenocarcinoma LS180 cells

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ABSTRACT

Oxidative catabolism of 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] is mediated by either CYP24A1 or CYP3A4. In this paper, we tested whether induction of CYP3A4 in the LS180 intestinal cell model enhances clearance of 1α,25$(OH)_2D_3$ and blunts its hormonal effect on expression of the apical membrane calcium transport protein, TRPV6. Treatment with the hPXR agonist rifampin significantly increased CYP3A4 mRNA content and catalytic activity, but had no effect on CYP24A1 or TRPV6 mRNA content. Pre-treating cells with rifampin for 48 h, prior to a 24 h 1α,25$(OH)_2D_3$ treatment phase, was associated with a subsequent 48% increase in the elimination of 1α,25$(OH)_2D_3$ and a 35% reduction of peak TRPV6 mRNA. Introduction of the CYP3A4 inhibitor, 6′,7′-dihydroxybergamottin, an active inhibitor in grapefruit juice, reversed the effects of rifampin on 1α,25$(OH)_2D_3$ clearance and TRPV6 expression. Over-expression of hPXR in LS180 cells greatly enhanced the CYP3A4 responsiveness to rifampin pretreatment, and elicited a greater relative suppression of TRPV6 expression and an increase in 1α,25$(OH)_2D_3$ disappearance rate, compared to vector expressed cells, following hormone administration. Together, these results suggest that induction of CYP3A4 in the intestinal epithelium by hPXR agonists can result in a greater metabolic clearance of 1α,25$(OH)_2D_3$ and reduced effects of the hormone on the intestinal calcium absorption, which may contribute to an increased risk of drug-induced osteomalacia/osteoporosis in patients receiving chronic therapy with potent hPXR agonists. Moreover, ingestion of grapefruit juice in the at-risk patients could potentially prevent this adverse drug effect.

1. Introduction

Osteomalacia, characterized by defective bone mineralization, most commonly occurs in adults with vitamin D deficiency. It is often associated with a reduced dietary intake or synthesis of vitamin D, however osteomalacia has also been reported in patients receiving long-term therapy with certain drugs including rifampin, an antimicrobial agent [1], and the anti-epileptic drugs, carbamazepine, phenytoin and phenobarbital [2,3]. Although the clinical manifestation and histology of drug-induced osteomalacia resembles that found in other adults with vitamin D deficiency [4], the molecular mechanism of this adverse drug effect is still unclear.

A primary function of vitamin D in maintaining calcium homeostasis and bone health is accomplished by stimulation of transcellular and paracellular calcium transport in the small intestine through vitamin D receptor (VDR)-dependent pathways [5]. One protein thought to regulate the rate and extent of intestinal calcium absorption is TRPV6. TRPV6 is a calcium channel protein expressed in the intestinal luminal epithelium and its synthesis and function is regulated in part by 1α,25-dihydroxyvitamin D_3 [1α,25$(OH)_2D_3$] through a VDR signaling pathway [5–8]. The basal duodenal TRPV6 expression is correlated with serum 1α,25$(OH)_2D_3$ levels in men, as well as with VDR expression in both men and women, although TRPV6 expression in women is influenced predominantly by an age effect, not by vitamin D level [8]. In addition, TRPV6 transcription responds rapidly to 1α,25$(OH)_2D_3$ in the human duodenum [9]. Moreover, studies with TRPV6 knockout mice demonstrate that transporting calcium by TRPV6 may be the rate-limiting step and gatekeeper of transcellular calcium flux [10]. Interestingly, 1α,25$(OH)_2D_3$ might

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also mediate paracellular transport of calcium by a non-TRPV6 mediated mechanism [11].

Metabolic inactivation of $1\alpha,25(OH)_2D_3$ constitutes a negative feedback mechanism for regulating its effect on calcium absorption in the small intestine [12]. Human cytochrome P450 24A1 (CYP24A1) and P450 3A4 (CYP3A4) contribute to the inactivation of $1\alpha,25(OH)_2D_3$ [13–16]. However, the relative contributions of these two enzymes to vitamin D catabolism may be tissue-dependent. For example, in the healthy kidney, CYP24A1 activity likely dominates $1\alpha,25(OH)_2D_3$ catabolism, because the expression of CYP3A4 is very low in that organ [17–19]. In contrast, in tissues such as the liver and small intestine, CYP3A4 could play a more important role in $1\alpha,25(OH)_2D_3$ catabolism, because constitutive levels of CYP3A4 are high, whereas CYP24A1 expression is very low [17]. In addition, local production and hormonal effects of $1\alpha,25(OH)_2D_3$ in the duodenum were indicated in previous studies [9]. Thus, $1\alpha,25(OH)_2D_3$-mediated calcium absorption could be disrupted locally by drugs that alter CYP3A4 expression in the intestinal epithelium, independent of any change in the systemic vitamin D level [20].

In fact, it has been reported that treatment of healthy volunteers with the pregnane X receptor (hPXR) agonist rifampin causes preferential induction of human duodenal CYP3A4, but not CYP24A1, mRNA content [17]. Thus, acceleration of intestinal $1\alpha,25(OH)_2D_3$ catabolism by stimulation of CYP3A4 synthesis may result in down-regulation of calcium transporters, such as TRPV6, and an impairment of calcium absorption. Long-term impairment of intestinal calcium absorption by chronic drug administration and CYP3A4 induction could result in osteomalacia [17]. Those drugs associated with osteomalacia share the capacity to activate the hPXR and constitutive androstane receptor (CAR) and thus up-regulate the expression of multiple cytochrome P450 enzymes, including CYP3A4 [21–23].

In this investigation, we tested the hypothesis that induction of CYP3A4 by hPXR agonists alters the intestinal cellular clearance of $1\alpha,25(OH)_2D_3$, and thereby affects the transcription of calcium transporters such as TRPV6, LS180 cell line was used as a model for intestinal enterocytes due to the robust expression of both VDR and hPXR [24,25]. We report that hPXR agonists induce CYP3A4, but not CYP24A1 in LS180 cells, and under most conditions enhance $1\alpha,25(OH)_2D_3$ catabolism and decrease the transcriptional response of TRPV6 to $1\alpha,25(OH)_2D_3$. The findings presented here provide a viable molecular mechanism of drug-induced osteomalacia by hPXR activators.

2. Materials and methods

2.1. Chemicals and reagents

Rifampin, hyperforin, carbamazepine, and 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) were purchased from Sigma (St. Louis, MO) or Fisher Scientific (Itasca, IL). 25-Hydroxyvitamin D_3 [$25(OH)D_3$] and $1\alpha,25(OH)_2D_3$ were obtained from Calbiochem (La Jolla, CA) and 24R,25-dihydroxyvitamin D_3 [$24R,25(OH)_2D_3$] was obtained from Sigma. Deuterated standards of vitamin D_3 metabolites, d_6-25OHD$_3$ and d_6-$1\alpha,25(OH)_2D_3$ (containing six deuterium atoms at C-26 and C-27), were purchased from Medical Isotope Inc. (Pelham, NH). Levetiracetam was purchased from LKT Laboratories (St. Paul, MN). Midazolam (MDZ), 1′-hydroxymidazolam (1-OH MDZ), d_2-1′-hydroxymidazolam (d_2-1′-OH MDZ) and phenobarbital were purchased from Cerilliant (Round Rock, TX). Cell culture medium, penicillin-streptomycin and TRIzol reagent were purchased from Invitrogen (Carlsbad, CA). Fetal bovine serum (FBS) was obtained from Atlanta Biologicals (Lawrenceville, GA). Sodium pyruvate was purchased from Cellgro (Herndon, VA) and cell dissociation solution was purchased from Sigma. High capacity cDNA reverse transcription kits and PCR primers, SYBR green master mix and other PCR reagents were obtained from Applied Biosystems (ABI, Foster City, CA).

2.2. Cell culture conditions

The human intestinal epithelial cell line, LS180 and Caco-2 cell lines were obtained from ATCC (Manassas, VA). Cells were cultured in minimum essential medium (MEM) supplemented with 10% FBS, 1% penicillin-streptomycin and 1% sodium pyruvate at 37 °C in 5% CO_2. After reaching 80% confluence, the cells were de-attached by addition of dissociation solution, collected and isolated by centrifugation. The passaged cells were then seeded into 6-well plates and grown in MEM with 10% FBS until 60–70% confluence was reached. Twenty-four hours before the first experimental treatment, the medium was replaced with MEM containing 10% resin-charcoal-treated FBS. To initiate an experimental treatment, this medium was removed and the cells were washed twice with PBS and then treated with the drug or its vehicle (e.g., 0.1% DMSO for rifampin) in the same culture medium (2 mL final volume) for a specified period of time. For some experiments, sequential treatments or co-treatments were employed. Experiment-specific modifications of the general treatment regimen are described in more detail in the result sections, for clarity. At the end of each treatment period, cell culture medium was collected for quantification of 1-OH MDZ, $1\alpha,25(OH)_2D_3$, or $24R,25(OH)_2D_3$ concentrations, depending on the study design. Cells were collected for measurement of intracellular $1\alpha,25(OH)_2D_3$ and total protein, or mRNA, again depending on the study design. All samples were stored at −80 °C until analyzed.

2.3. Isolation of total cellular RNA and qRT-PCR analysis

After experimental treatment, LS180 or Caco-2 cells collected from each culture well were homogenized in 1 mL TRIzol reagent and stored at −80 °C. Total cellular RNA was isolated according to the manufacturer-supplied protocol for TRIzol reagent. The isolated RNA was dissolved in DEPC-treated water and the concentration was determined using a Nanodrop spectrophotometer (ND-1000, Thermo Scientific, Wilmington, DE).

Reverse transcription was performed according to the manufacturer's instructions for the high capacity cDNA reverse transcription kit. For each reaction, 4 μg of isolated RNA was mixed with dNTP, random hexamer primers, and MultiScribe reverse transcriptase in reaction buffer in a total volume of 40 μL. The reverse transcription condition was set as 25 °C for 10 min, 37 °C for 120 min and 85 °C for 5 s. qRT-PCR was performed using gene-specific primers and the SYBR green master mix with the ABI 7900 system (Applied Biosystems). The PCR mixture consisted of 1 μL of cDNA, gene-specific forward and reverse primers (25 pmol each), SYBR green master mix and distilled water, in a total volume of 25 μL for each reaction. The following program was used: a denaturation step at 94 °C for 10 min, 40 cycles of PCR (denaturation 94 °C for 30 s; annealing 65 °C for 30 sec; and extension 72 °C for 30 s), followed by 72 °C for 5 min and then a dissociation/melting step (95 °C for 15 s, 65 °C for 15 s, 95 °C for 15 s, 25 °C for 5 min). All tested gene products were quantified using the comparative ΔΔCt calculation for relative quantification of gene expression, normalized to GAPDH. The following primer sets (F/R) for human genes were used:

CYP3A4: (5′-GGCTTCATCCAATGGACTGCATAAAT-3′ and 5′-TCCCAAGTATAACACTCTACACAGACAA-3′);
CYP24A1: (5′-GGTGACATCTACGGCGTACAC-3′ and 5′-CTTGAGACCCCCTTTCCAGAG-3′);