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24, 25-Dihydroxycholecalciferol but not 25-hydroxycholecalciferol suppresses apolipoprotein A-I gene expression

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ABSTRACT

Aims: Ligands for the vitamin D receptor (VDR) regulate apolipoprotein A-I (apo A-I) gene expression in a tissue-specific manner. The vitamin D metabolite 24, 25-dihydroxycholecalciferol (24, 25-$(OH)_2D_3$) has been shown to possess unique biological effects. To determine if 24, 25-$(OH)_2D_3$ modulates apo A-I gene expression, HepG2 hepatocytes and Caco-2 intestinal cells were treated with 24, 25-$(OH)_2D_3$ or its precursor 25-OHD_3.

Main methods: Apo A-I protein levels and mRNA levels were measured by Western and Northern blotting, respectively. Changes in apo A-I promoter activity were measured using the chlorampenicol acetytransferase assay.

Key findings: Treatment with 24, 25-$(OH)_2D_3$, but not 25-OHD_3, inhibited apo A-I secretion in HepG2 and Caco-2 cells and apo A-I mRNA levels and apo A-I promoter activity in HepG2 cells. To determine if 24, 25-$(OH)_2D_3$ represses apo A-I gene expression through site A, the nuclear receptor binding element that is essential for VDRs effects on apo A-I gene expression, HepG2 cells were transfected with plasmids containing or lacking site A. While the site A-containing plasmid was suppressed by 24, 25-$(OH)_2D_3$, the plasmid lacking site A was not. Likewise, treatment with 24, 25-$(OH)_2D_3$ suppressed reporter gene expression in cells transfected with a plasmid containing site A in front of a heterologous promoter. Finally, antisense-mediated VDR depletion failed to reverse the silencing effects of 24, 25-$(OH)_2D_3$ on apo A-I expression.

Significance: These results suggest that the vitamin D metabolite 24, 25-$(OH)_2D_3$ is an endogenous regulator of apo A-I synthesis through a VDR-independent signaling mechanism.

Introduction

Apo A-I is the primary protein component of high-density lipoprotein (HDL) and possesses anti-atherosclerotic activity (Kawashiri et al., 2000). Understanding the hormonal and nutrient regulation of apo A-I levels may have profound clinical implications.

There has been increasing interest in the role of vitamin D in human health and disease culminating in calls for increasing the limits of minimal daily requirements and encouraging supplementation of the diet with vitamin D (Holick, 2007). Given the increasing demand for vitamin D supplementation, a thorough examination of the interaction of vitamin D with atherosclerotic risk factors including HDL is warranted.

Calcitriol as well as 25-hydroxycholecalciferol (25-OHD_3) are hydroxylated by renal 24-hydroxylase to less active metabolites destined for excretion in the bile, the primary route of excretion of vitamin D (Ohyama and Okuda, 1991; Chen et al., 1993; Makin et al., 1989). Since this initial description, the role of 24, 25-dihydroxycholecalciferol (24, 25-$(OH)_2D_3$) has been relegated to a method for elimination of excess 25-OHD_3 not needed for metabolism to 1, 25-$(OH)_2D_3$. Alternatively, 24, 25-$(OH)_2D_3$ could be further hydroxylated to form 1, 24, 25-trihydroxycholecalceferol (1, 24, 25-$(OH)_3D_3$), which exhibited some of the properties of 1, 25-$(OH)_2D_3$, leading to the conclusion that active vitamin D metabolites required hydroxylation on the 1-carbon while 24 hydroxylation is merely a method of regulating 1, 25-$(OH)_2D_3$ synthesis (Tanaka and DeLuca, 1984). However, studies using 24-hydroxylase knockout mice have shown that chondrogenesis requires this vitamin D metabolite (St-Arnaud et al., 1996; St-Arnaud, 1999). In addition, putative basal-lateral membrane receptors for 24, 25-$(OH)_2D_3$ have been identified in carp and Atlantic cod enterocytes (Larsson et al., 2001), and experiments showing that 24, 25-$(OH)_2D_3$ is involved in regulating endochondral ossification (Dickson and Maher, 1985; Lidor et al., 1987; Wientroub et al., 1987; Miyahara et al., 1994), have led to the belief that

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24-hydroxylated vitamin D metabolites can have important biologic effects. Since previously published studies have shown that vitamin D receptor (VDR) activation can alter apo A-I expression (Wehmeier et al., 2005, 2008), the effect of 25-OHD_3 and its two major metabolites, 1, 25-$(OH)_2D_3$ and 24, 25-$(OH)_2D_3$, were examined.

Materials and methods

Materials

Acetyl-coenzyme A, 24, 25-$(OH)_2D_3$, 1, 25-$(OH)_2D_3$, and 25-OHD_3 were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO) and Lipofectamine was from Invitrogen (Gaithersburg, MD). The ^{14}C-chloramphenicol and [α-^{32}P]-dCTP were purchased from Perkin Elmer-New England Nuclear (Boston, MA). Tissue culture media and fetal calf serum were purchased from BioWittaker (Walkersville, MD). All other reagents were from Sigma-Aldrich or Fisher Scientific (Pittsburgh, PA).

Cell culture

HepG2 cells were maintained in Dulbecco's Modified Essential Medium containing 10% fetal bovine serum (FBS) and penicillin and streptomycin (100-units/ml and 100-μg/ml, respectively). Caco-2 (HTB-37) cells were obtained from American Type Culture Collection (Manassas, VA) and maintained in Eagle's Minimal Essential Medium containing 10% FBS, non-essential amino acids, sodium pyruvate, and penicillin and streptomycin. Cells were maintained in a humidified environment at 37 °C and 5% CO_2. In each experiment, cells were washed extensively to remove serum components of the growth medium and the cells were switched to serum-free DMEM or EMEM containing either the indicated drug or the solvent ethanol. After 24-hours, aliquots of this conditioned medium were removed and used to measure apo A-I and albumin content. No dilution or concentrating was required. Cell viability was monitored by trypan blue exclusion (Jauregui et al., 1981) and was greater than 95% in all experiments.

Measurement of Apo A-I protein and mRNA

Apo A-I and albumin accumulation in the conditioned medium of HepG2 cells were measured by Western blot. Briefly, 5-μg of each protein sample (Bradford, 1976) was fractionated by electrophoresis on a 10% sodium dodecylsulfate polyacrylamide gel (Laemmli, 1970), transferred to nitrocellulose (Towbin et al., 1979), and incubated with antiserum to human apo A-I (diluted 1:1000) (Calbiochem, San Diego, CA) and albumin (1:5000) (Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After incubation with a goat-anti-rabbit secondary antibody (1:10,000) and enhanced chemiluminescence with reagents from Pierce Chemical Co. (Rockford, IL), the signal was detected on film and quantified with a densitometer (Gel Logic Imaging System 100, Carestream Healthcare, New Haven, CT). Optical density was expressed in arbitrary units (A.U.)

Total RNA was isolated from control or treated cells as previously described (Chomczynski and Sacchi, 1987), fractionated by electrophoresis on a 1% formaldehyde-agarose gel (15-μg/lane) and transferred to a nylon hybridization membrane. Apo A-I and glyceraldehydes-3-phosphate dehydrogenase (GAPDH) probes were labeled with ^{32}P (Feinberg and Vogelstein, 1983), and hybridized to the RNA-bound membrane in Rapid Hyb (GE Healthcare, Piscataway, NJ). The filter membranes were washed under high-stringency conditions (0.1x standard saline citrate, 0.1% sodium dodecylsulfate, 65 °C) and exposed to Hyperfilm MP autoradiography film (GE Healthcare). Band intensities on the autoradiographs were measured with a densitometer (Gel Logic Imaging System 100.) After hybridization with the apo A-I probe, the membrane was stripped following the manufacturer's instructions and hybridized a second time with the ^{32}P-labeled GAPDH cDNA. After measuring GAPDH mRNA levels, apo A-I mRNA levels were normalized to GAPDH levels.

Plasmids and transient transfection analysis

The plasmid pAI.474.CAT, containing the apo A-I promoter region from −474 to +7 bp and the vitamin D-responsive region between −214 and −190 bp, was used to assess promoter-dependent changes in apo A-I gene transcription. HepG2 cells were transfected as indicated in each figure using Lipofectamine. The plasmid pCMV.SPORT-β-gal (Invitrogen), expressing β-galactosidase under the control of the cytomegalovirus immediate-early promoter, was used to control for transfection efficiency. After 24-hours, the culture medium was replaced with serum-free medium containing either equal volumes of vehicle (0.1% ethanol) or each tested compound. After 24-hours, the cells were collected and assayed for chloramphenicol acetyltransferase (CAT) (Gorman et al., 1982) and β-galactosidase activity (Herbomel et al., 1984).

To determine if site A mediates inhibition by 24, 25-$(OH)_2D_3$, HepG2 cells were transfected with pAI.225.CAT and pAI.186.CAT. These plasmids contain 225 and 186 bp of apo A-I gene 5′ flanking sequence, respectively. Since site A is located from −190 to −214 (relative to the transcriptional start site), pAI.186.CAT lacks this region while pAI.225.CAT retains it. To assess the effect of each VDR ligand on site A activity, HepG2 cells were transfected with the plasmid p5′A-CAT, which contains two copies of site A adjacent to the SV-40 virus early region promoter, or p5′ΔA-CAT, which lacks these elements. The cells were treated with vehicle or each compound for 24-hours and assayed for CAT and β-galactosidase activity.

To determine if repression of apo A-I gene expression by 24, 25-$(OH)_2D_3$ is dependent on vitamin D receptor (VDR) production, VDR was depleted using an antisense oligodeoxynucleotide (5′-CTG GCC GCC ATT GCC TCC AT-3′) as previously described (Wehmeier et al., 2005, 2008). Specificity of antisense-mediated inhibition was assessed by treating cells with an oligodeoxynucleotide containing a scrambled sequence of similar chemical composition (5′-ATT GAC TCA AAT TCA TTC TT-3′). Cells were transfected with 0.5 μg of pAI.474.CAT and 0.5 μg of pCMV.SPORT.β-gal and 200 nM of each oligodeoxynucleotide, and after 48 h, treated with either 100 nM 24, 25-$(OH)_2D_3$ or 100 nM 1, 25-$(OH)_2D_3$ in serum-free medium. Twenty four hours later, apo A-I promoter activity was measured and normalized to β-galactosidase activity.

Statistics and data analysis

The mean ± SEM are presented. Statistical significance between mean values was analyzed using Student's *t*-test for independent variables and Statistica for Windows (Statsoft Inc, Tulsa, OK). Significance was defined as a two-tailed $p<0.05$.

Results

The effects of 24, 25-$(OH)_2D_3$ and 25-OHD_3 on apo A-I secretion

The results of Western blot analyses of apo A-I content in the conditioned medium of HepG2 cell cultures treated with vitamin D metabolites are shown in Fig. 1. In HepG2 cells treated with 24, 25-$(OH)_2D_3$ (Fig. 1A), apo A-I levels, expressed in arbitrary units (A.U.) (Fig. 1C) decreased from 786 ± 56 A.U. in vehicle-treated cells to 725 ± 66, 475 ± 109, 321 ± 89, 301 ± 33 and 380 ± 35 A.U. in cells treated with 0.1, 1.0, 10, 100, and 1000 nM 24, 25-$(OH)_2D_3$, respectively (N.S., $p<0.02$, $p<0.001$, $p<0.001$, $p<0.04$, respectively). However, apo A-I levels did not change appreciably in 25-OHD_3-treated cells (1171 ± 83 A.U. in control cells and 1097 ± 99, 1079 ± 122, 1182 ± 29, 1121 ± 67, 1105 ± 140 A.U. in cells treated with 0.1, 1.0, 10, 100, and 1000 nM