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Vitamin D Transport Proteins Megalin and Disabled-2 Are Expressed in Prostate and Colon Epithelial Cells and Are Induced and Activated by All-*Trans*-Retinoic Acid

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Abstract

Megalin and disabled-2 (Dab2) are essential for uptake of the 25-hydroxycholecalciferol (25D3)-vitamin D binding protein (DBP) complex in tissues. In the kidney, this mechanism regulates serum 25D3 levels and production of 1,25-dihydroxycholecalciferol (1,25D3) by CYP27B1 for systemic use. Previously, we showed that mammary epithelial cells expressing CYP27B1 express megalin and Dab2 and internalize DBP by endocytosis, indicating 25D3 was accessible for conversion to 1,25D3 in extra-renal tissues. Moreover, induction of megalin and Dab2 (protein and mRNA abundance) by all-*trans*-retinoic acid (RA) enhanced DBP uptake. This suggests megalin and Dab2 play a central role in uptake of vitamin D and may predict actions of vitamin D in extra-renal tissues. Here, we characterized megalin and Dab2 expression and uptake of DBP in transformed human prostate and colon epithelial cells. Megalin and Dab2 were expressed in prostate and colon epithelial cells, which was markedly enhanced following treatment with RA. Furthermore, DBP uptake was stimulated by low-dose RA supplementation in LNCaP, PC-3, and Caco-2 cells. Taken together, these are the first studies to our knowledge that have demonstrated modulated expression of megalin and Dab2, as well as an association between increased expression of endocytic proteins with DBP uptake in prostate and colon cells.

INTRODUCTION

The active derivative of vitamin D [1,25-dihydroxychole calciferol (1,25D3)] has potent antitumorigenic activities including induction of apoptosis, cell cycle arrest, and cell differentiation in human tissues (1–8). The majority (>99%) of circulating vitamin D is in a relatively inactive form as 25-hydroxycholecalciferol (25D3), virtually all of which

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circulates tightly bound to vitamin D-binding protein (DBP). Serum concentrations of 25D3 are reflective of cholecalciferol intake and sunlight exposure, and increased serum concentrations of 25D3 have been linked to reduced incidence of many chronic diseases, including numerous types of cancer, autoimmune diseases, and type 2 diabetes (9–14). However, defining the mechanism by which 25D3 becomes accessible to CYP27B1 for activation to 1,25D3 by tissues other than kidney has been problematic. In kidney, uptake of the 25D3-DBP complex by renal proximal tubules is dependent on megalin-mediated endocytosis for maintenance of serum vitamin D concentrations and activation to 1,25D3 (15,16). Once internalized by cells, DBP is degraded in lysosomes, freeing 25D3 for activation to 1,25D3 by CYP27B1 (Fig. 1). Previous work has demonstrated that megalin, cubilin, and/or Dab2 deficiency leads to marked vitamin D deficiency due to excessive urinary excretion of the 25D3-DBP complex (15–17). Thus, identifying tissues expressing CYP27B1 that also possess the machinery capable of internalizing protein-bound 25D3 could lead to better nutritional strategies for the prevention of chronic disease.

Our earlier work demonstrated that uptake of DBP was essential for the antiproliferative actions of 25D3 in mammary epithelial cells (18,19). This was supported by the inability of mammary cells not expressing all of the components of the DBP receptor complex to internalize DBP and respond to treatment with 25D3-DBP. Thus, it appears that the endocytosis of DBP may be the rate-limiting step for both cellular uptake and anti-tumorigenic effects of vitamin D. In addition to their role in 25D3-DBP uptake, megalin and its endocytic partners are expressed in a number of absorptive epithelial tissues and are responsible for the uptake of other ligands, such as thyroid hormone, albumin, and vitamin A (20–23). Despite this evidence, the characterization of 25D3-DBP uptake mechanisms in extra renal tissues is still lacking. Megalin is expressed in mammary, prostate, and colonic epithelial tissue, which all have functional CYP27B1 (24–26) and sensitivity to 25D3 and 1,25D3 (27–30). We previously reported that mammary epithelial cells express megalin, cubilin, and Dab2 and that treatment with differentiating compounds, RA or forskolin, upregulated megalin and Dab2 expression and uptake of DBP. (19). In addition, DBP uptake was blunted by inhibitors of megalin-mediated endocytosis. However, there is uncertainty with respect to whether circulating 25D3-DBP becomes accessible to prostate and colon, tissues that are among the most prone to tumorigenesis and believed to be sensitive to the antiproliferative actions of vitamin D (26,31–33).

In the present study, our objectives were to characterize megalin and Dab2 expression as well as to evaluate DBP-uptake capabilities of human prostate and colonic epithelial cells. We also explored the possibility that megalin and Dab2 expression and DBP uptake could be enhanced following treatment with RA in these cells.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

LNCaP and PC-3 human prostate cancer and Caco-2 colon cancer cells were obtained from the American Type Culture Collection (Manassas, VA). Cells were routinely grown in a humidified incubator with 5% CO₂ and a temperature of 37°C. LNCaP, PC-3, and Caco-2 cells were cultured in their appropriate media (RPMI, F-12K, and Eagle's Minimum

Essential Medium, respectively) with 10% fetal bovine serum, penicillin (100kU/L), and streptomycin (0.1 g/L).

Real-Time RT-PCR

LNCaP, PC-3, and Caco-2 cells (2×10^5) were plated in 6-well plates and treated with RA (10 $\mu\text{mol/L}$) or vehicle (dimethyl sulfoxide, DMSO) for 48 h. The dose of RA and length of treatment were chosen based on both our preliminary and published dose-response studies (19) that showed a 10 $\mu\text{mol/L}$ RA treatment for 48 h gave us the most robust and consistent induction of both megalin and Dab2 mRNA and protein expression. Total RNA was isolated from cell lines using TriReagent (Molecular Research Center, Cincinnati, OH). Total RNA was then used for first strand cDNA synthesis using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Carlsbad, CA). For each cell line, reactions were performed in duplicate, generating 2 cDNA stocks per sample. Each of the cDNA stocks generated from PC-3, LNCaP, and Caco-2 RNA were then independently analyzed in duplicate for megalin and Dab2 expression via real-time quantitative PCR using SYBR Green Detection reagents (Bio-Rad, Hercules, CA) as previously described (19) with primer sets specific for human megalin (*forward primer*: AAATTGAGCACAGCACCT-TTGA, *reverse primer*: TCTGCTTTCCTGACTCGA-ATAATG) or human Dab2 (*forward primer*: GGTGTAAGTGTACACTC-CCTCAG, *reverse primer*: TGACCACCCATCATGGTCC) and were normalized against human 18S mRNA (*forward primer*: TCAACTTTCGAT-GGTAGTCGCCGT, *reverse primer*: TCTTGGCAATGCTTTCGCTCTGG).

Western Blotting

LNCaP, PC-3, and Caco-2 cells (5.0×10^5 cell/dish) were plated in plastic dishes or flasks (surface area of 100-mm and 75 cm^2 , respectively) and treated with RA (10 $\mu\text{mol/L}$) or vehicle (DMSO). After 2 days, cell monolayers were trypsinized, sonicated in 250 μL 2X Laemmli buffer containing Halt Protease Inhibitor Cocktail and EDTA solution (ThermoFisher Scientific), and total protein was analyzed by the bicinchoninic acid protein assay (BCA, Pierce, Rockford, IL). For analysis of megalin protein abundance, 60 μg total cellular protein was separated by SDS-PAGE under nonreducing conditions using 6% polyacrylamide gels. For analysis of Dab2 protein abundance, 60 μg total cellular protein was separated by SDS-PAGE under reducing conditions using 10% polyacrylamide gels and proteins were transferred to nitrocellulose and stained with Ponceau to confirm equal loading as we have done previously when immunoblotting for detection of megalin (19). For detection of megalin, membranes were blocked overnight in 100 g/L skim milk in PBS/10 g/L Tween 20 at 4°C. Membranes were then incubated for 6 h at 4°C with a 1:50 dilution of polyclonal rabbit anti-megalin antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in 100 g/L skim milk in PBS/10 g/L Tween 20 (PBS-Tween). A goat antirabbit IgG horseradish peroxidase conjugated secondary antibody (1:5000) was then applied for 1 h at RT. For Dab2 protein analysis, membranes were blocked at room temperature (RT) for 1 h in 50 g/L skim milk in PBS-Tween. For detection of Dab2, the membrane was incubated for 2 h at RT with 1:50 (LNCaP) or 1:250 (PC-3 and Caco-2) dilutions of polyclonal mouse anti-Dab2 antibody (BD Pharmingen, San Jose, CA) in 50 g/L skim milk in PBS-Tween. Membranes were then incubated in goat antimouse IgG horseradish peroxidase secondary antibodies (1:5000 for LNCaP or 1:7500 for PC-3 and Caco-2) for 1 h at RT. For all proteins, specific

binding was detected by chemiluminescence (Pierce) and exposure to autoradiography film (Kodak Biomax, Rochester, NY).

Analysis of DBP Uptake

LNCaP, PC-3, and Caco-2 cells were plated on Lab-Tek™ II CC2 Chamber Slides and grown in 0.1 $\mu\text{mol/L}$ RA or vehicle (DMSO) for 7 days. Media was then aspirated, and cells were washed 3 $\times$ with PBS to remove residual serum and then incubated in serum free media for 1 h prior to incubation with DBP. To determine whether PC-3, LNCaP and Caco-2 cells internalize vitamin D-binding protein via receptor-mediated endocytosis, cells were incubated with 0.02 mg/mL DBP (EMD Millipore, Darmstadt, Germany) conjugated to Alexa-488 (Life Technologies, Grand Island, NY) at 37°C or 4°C (to inhibit endocytosis) in serum-free media for 30 min as we have previously described (18,19). Cells were then rapidly fixed in 10% formalin and mounted with ProLong Gold Antifade Reagents with DAPI (Invitrogen, Carlsbad, CA) for visualization of the nuclei. Fluorescent images were captured on a Zeiss Axioplan II compound research microscope with a high resolution Zeiss digital camera. Fluorescent and UV images were acquired with a constant exposure time.

Analysis of Megalin Uptake and Colocalization with DBP

PC-3 cells were plated on Lab-Tek™ II CC2 Chamber Slides and grown in 0.1 $\mu\text{mol/L}$ RA or vehicle (DMSO) for 7 days. Cells were then washed 3 $\times$ with PBS switched to serum free media for 1 h prior to incubation with DBP. Cells were incubated with 0.02 mg/mL vitamin D-binding (DBP) conjugated to Alexa-488 for 30 min. Cells were washed 5 $\times$ for 10 min in PBS then fixed in 100 g/L formalin and permeabilized in ice-cold methanol for 5 min. Cells were then washed in PBS for 5 min and incubated for 2 h at RT with a 1:50 dilution of polyclonal rabbit anti-megalin antibody in 30 g/L bovine serum albumin (BSA) in PBS-Tween or vehicle (30 g/L BSA in PBS-Tween). After three 10-min washes in PBS-Tween, cells were incubated for 1 h at RT with a 1:400 dilution (5.3 ng/L) of Alexa-Fluor 568 donkey antirabbit IgG (Invitrogen, Carlsbad, CA) in 30g/L BSA in PBS-Tween. Following three 5-min washes in PBS, slides were mounted with ProLong Gold Antifade Reagents with DAPI (Invitrogen, Carlsbad, CA) for visualization of the nuclei. Fluorescent images were captured on a Zeiss Axioplan II compound research microscope with a high resolution Zeiss digital camera. Fluorescent and UV images were acquired with a constant exposure time.

Statistical Analysis

Data were analyzed by Student's *t*-test with InStat software (version 3.0 for Windows, GraphPad Software, San Diego, CA). Differences between means were considered significant when *P* values less than 0.05 were obtained.

RESULTS

Induction of Megalin and Dab2 Expression by RA

We determined whether our observations of increased expression of Dab2 and megalin in mammary epithelial cells could be translated into prostate and colon cells. In the present study, we found that expression of megalin and Dab2 was elevated in prostate and colon

cells following treatment with 10 $\mu\text{mol/L}$ RA. Dab2 mRNA expression was elevated in PC3, LNCaP, and Caco-2 cells treated RA compared to vehicle-treated cells (Fig. 2A–2C). Moreover, immunoblotting for both Dab2 (Fig. 2D–2F) and megalin (Fig. 3) yielded consistent results that revealed both proteins were expressed and inducible in PC-3, LNCaP, and Caco-2 cells.

DBP Was Internalized in Prostate and Colon Cells Treated with RA

We previously reported that a 7 day treatment with 0.1 $\mu\text{mol/L}$, a dose of RA that reflects serum concentrations that are on the low end of those found in acute promyelocytic anemia patients receiving RA therapy (34) induced differentiation and enhanced endocytosis of DBP in mammary epithelial cells (19). Thus, we used this strategy to examine whether prostate and colon cells would similarly internalize DBP, which to our knowledge, has never been previously reported. We found similar results in multiple experiments for each cell line and as represented in Fig. 4, a 7-day treatment with 0.1 $\mu\text{mol/L}$ RA stimulated DBP uptake in LNCaP, PC-3 and Caco-2 cells, whereas vehicle-treated cells and RA-treated cells incubated at 4°C, which halts the endocytic process as we have previously demonstrated (18), internalized virtually no DBP. These results suggest that RA-mediated induction of megalin and Dab2 (illustrated in Figs. 2 and 3) resulted in increased uptake of the DBP receptor complex in all 3 cell lines with the most robust uptake occurring in PC-3 cells.

DBP and Megalin Colocalized in PC-3 Prostate Cells Treated with RA

After observing that the most robust uptake of DBP occurred in PC-3 prostate cells treated with RA, despite our immunoblotting that revealed that relatively low megalin protein induction occurred following RA treatment in these cells, we sought to confirm that 1) megalin protein expression could be better visualized by other means (i.e., immunofluorescent microscopy) and 2) binding of DBP to megalin could be visualized. Thus, we performed immunofluorescence studies using antibodies for megalin and Alexa-labeled secondary antibodies following incubation of PC-3 cells with Alexa-labeled DBP. As indicated in Fig. 5, DBP and megalin were colocalized around the perimeter of the majority of PC-3 cells.

DISCUSSION

The mechanism of binding and uptake of the major circulating form of vitamin D (25D3) bound to DBP (which binds to virtually all circulating 25D3) may dictate the sensitivity of extra-renal tissues that express CYP27B1, such as breast, prostate, and colon, to the antitumorigenic activity of vitamin D. It has been shown in in vitro studies that extra renal cells expressing CYP27B1 are able to locally activate 25D3 to 1,25D3 in the absence of DBP (24), a process that was previously thought to occur only in renal proximal tubules. Hence, researchers have postulated that autocrine or paracrine activation and use of vitamin D may be predictive of the tissues that are sensitive to its anticancer activities (1). The kidney tightly regulates serum concentrations of 1,25D3 and these concentrations do not vary without severe vitamin D status. Therefore, recent interest is focused more on serum 25D3 concentrations, which are not tightly regulated and correlate with vitamin D intake and ultraviolet radiation exposure. In addition, serum 25D3 levels have been inversely

correlated with cancers of the prostate, colon, breast, and other tissues (35,36). However, whether 25D3 bound to DBP becomes accessible to these tissues in vivo remains unclear. In the present study, we demonstrated that like renal proximal tubule cells, prostate and colon cells express megalin and Dab2 and internalize DBP.

Previously, we demonstrated that T-47D breast cancer cells expressed megalin and its endocytic partners and readily internalized the 25D3-DBP complex. Moreover, we showed that treatment with RA increased expression of megalin and Dab2 and stimulated enhanced DBP uptake. Here, we found that the ability of prostate and colon cells to internalize DBP was not present without RA treatment. Though we cannot rule out the possibility that RA-treatment triggered DBP uptake through mechanisms that are not mediated by RAR or RXR signaling, a concept we are currently investigating, the results of these studies indicate that unlike mammary cells, prostate and colon cells do not readily internalize DBP without treatment with an exogenous agent, which is somewhat surprising given that colon and breast cancer have a similar geographic mortality rate that differs from prostate cancer (37). Moreover, as in the present study, we have consistently observed that DBP uptake in RA-treated prostate and colon cells is lower than what we observe in mammary cells (18). Because we have used cancer cell lines for our experiments, the present study does not rule out the possibility that as prostate and/or colon cells become transformed they may lose the capacity to bind and internalize the DBP complex, which is a focus of our future work. In support of this concept, we visualized DBP uptake in untreated, non-transformed mammary cells (Rowling and Welsh, unpublished observations) and Dab2 expression was suppressed during tumorigenesis in absorptive epithelial tissue (38–41). Moreover, our laboratory has observed that cancer cell lines with a more differentiated phenotype have tended to be more efficient at internalizing protein-bound ligands such as retinol.

If tumorigenesis reduces the endocytic activity of megalin and Dab2, improving vitamin D status in cancer patients to improve survival may be far less effective than prevention strategies through the optimization of vitamin D status. However, several studies have shown that improved vitamin D status correlates with improved survival rates of various types of cancers (42–47). Thus, evaluating the accessibility of 25D3 to extra-renal tissues for activation to 1,25D3 could be critical when defining a role of therapeutic vitamin D for cancer patients or improved vitamin D status for cancer prevention. Administration of 1,25D3 is highly toxic and serum 25D3 levels can increase dramatically without toxicities due to hypercalcemia (48). Conversion of 25D3 to 1,25D3 in extra-renal tissues therefore appears to be a more feasible strategy for the use of supplemental vitamin D or UVB exposure for reduction of cancer risk (49). This concept is under debate however, because observational and case-control studies evaluating the benefits of increased serum 25D3 concentrations with respect to cancer risk have provided conflicting results due to several factors, which include long follow-up times after blood draw (50–55). Hence, a protective role of vitamin D against cancer cannot be accurately defined without mechanistic studies linking vitamin D's accessibility to extra-renal tissues and the autocrine/paracrine utilization of 25D3. Nor can dietary or UVB exposure recommendations for increasing vitamin D status be accurately defined until this mechanism is characterized in extra-renal tissues. This work is the first, at least to our knowledge, to suggest that the expression of megalin and

Dab2 in prostate and colon may predict their 25D3-DBP uptake capabilities and that endocytosis of DBP can be modulated in transformed prostate and colon cells.

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FIG. 1.
Vitamin D pathway in renal tissue.

FIG. 2.

All-*trans*-retinoic acid (RA) enhanced Dab2 mRNA and protein expression in PC-3, LnCap, and Caco-2 cells. Cells were treated with 10 μ mol/L RA or control (DMSO) for 48 h. mRNA abundance was determined by real-time qPCR and total cell protein was subjected to SDS-PAGE and immunoblotting with an anti-Dab2 monoclonal antibody as indicated in **Materials and Methods**. A: Dab2 mRNA abundance in PC-3 cells. B: Dab2 mRNA abundance in LnCap cells. C: Dab2 mRNA abundance in Caco-2 cells. D: Dab2 protein abundance in PC-3 cells. E: Dab2 protein abundance in LnCap cells. F: Dab2 protein abundance in Caco-2 cells. Data from bar graphs are means $\pm$ SEM, $n = 5$. The asterisk (*) indicates statistically different from untreated control cells, $P < 0.05$.

FIG. 3.

All-*trans*-retinoic acid (RA) enhanced megalin protein expression in PC-3, LnCap, and Caco-2 cells. Cells were treated with 10 $\mu\text{mol/L}$ RA or control (DMSO) for 48 h. Total cell protein was subjected to nonreducing SDS-PAGE and immunoblotting with antimegalin polyclonal antibodies as indicated in **Materials and Methods**. A: Megalin protein expression in PC-3 cells. B: Megalin protein expression in LnCap cells. C: Megalin protein expression in Caco-2 cells.

FIG. 4.

Internalization of vitamin D binding protein (DBP) in prostate and colon cells pretreated with RA. PC-3, LNCaP, and Caco-2 cells were incubated 7 days with 0.1 $\mu\text{mol/L}$ RA or vehicle (DMSO) then incubated at 37°C or 4°C with 0.02 g/L DBP conjugated to Alexa-488 as indicated in **Materials and Methods**. Cells were stained and mounted with ProLong Gold Antifade Reagents with DAPI for visualization of nuclei. A: PC-3 cells; B: LNCaP cells; and C: Caco-2 cells.

FIG. 5.

Colocalization of vitamin D binding protein (DBP) and megalin. PC-3 cells were grown in 0.1 $\mu\text{mol/L}$ RA or vehicle (DMSO) for 7 days. Cells then were switched to serum free media for 1 h prior to incubation with DBP. A: Staining of nuclei with DAPI. B: Cells incubated with DBP conjugated to Alexa-488 (green). C: Cells incubated with a polyclonal rabbit antimegalin antibody, followed by an incubation with an Alexa Fluor 568 secondary antibody (red). D: Merged image. Colocalization of DBP and megalin is indicated by the appearance of yellow.