Age-dependent regulation of rat intestinal type IIb sodium-phosphate cotransporter by 1,25-(OH)₂ vitamin D₃

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INORGANIC PHOSPHATE (Pi) plays important roles in regulating phosphate absorption across cell membranes and in maintaining serum Pi levels. The small intestine is an important site for phosphate absorption. Early studies showed that Pi transport through the apical membrane of small intestinal epithelial cells is coupled with sodium (4, 7, 12, 21, 29, 43, 47, 48). One transporter involved in intestinal Pi absorption is the type IIb sodium-coupled Pi cotransporter (NaPi-IIb), which has been cloned from rodents and humans (20, 25, 28, 55). Pi absorption is modulated by many physiological factors, including hormonal and dietary factors (2). Glucocorticoids inhibit intestinal sodium-dependent Pi (NaPi) absorption (5, 38). EGF decreases intestinal NaPi absorption at least partially by inhibiting NaPi-IIb mRNA expression (56). Estrogen also plays a possible role in regulating intestinal Pi absorption (41). Phosphate content of the diet also regulates intestinal Pi absorption, and Pi deprivation stimulates intestinal NaPi absorption (10, 37, 42, 44, 49).

Vitamin D₃, a steroid hormone, plays a central role in modulating phosphate homeostasis and Pi uptake by the small intestine (1, 19). The active form of vitamin D₃ is 1,25-(OH)₂ vitamin D₃, which is mainly synthesized in kidney from 25-(OH) vitamin D₃. 1,25-(OH)₂ vitamin D₃ binds the vitamin D receptor (VDR) to elicit its effect on regulation of gene expression. 1,25-(OH)₂ vitamin D₃ plays important roles in calcium and phosphate homeostasis, regulation of the parathyroid hormone system, inhibition of cell growth, and induction of cellular differentiation (9). Many previous studies showed that 1,25-(OH)₂ vitamin D₃ increases intestinal Pi absorption through modulation of NaPi-IIb expression (13, 16, 17, 22, 23, 27, 30, 33, 34, 39, 40, 52). In adult rodents, this increase is at least partially mediated by gene transcription in suckling rats.

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uptake in 1,25-(OH)₂ vitamin D₃-treated suckling rats. These results suggested a possible role for 1,25-(OH)₂ vitamin D₃ in transcriptional regulation of the NaPi-IIb gene in young animals. To further understand the role of 1,25-(OH)₂ vitamin D₃ in intestinal P₃ absorp-

**MATERIALS AND METHODS**

**Animals.** Sprague-Dawley rats at 2, 3, and 6 wk and 95–100 days of age were used for these studies. Two-week-old and adult (90–100 days) rats were used for 1,25-(OH)₂ vitamin D₃ studies. Animals received subcutaneous injections of 1,25-(OH)₂ vitamin D₃ (6 µg/kg body wt, 1 dose; Sigma, St. Louis, MO) or vehicle [ethanol-propylene glycol (1:4, vol/vol)]. Sixteen hours after the injection, rats were killed and jejunal mucosa was harvested and used for mRNA and brush-border membrane vesicle (BBMV) purification. All animal work was approved by the University of Arizona Institutional Animal Care and Use Committee.

**Cell culture.** RIE cells were a gift from Dr. Raymond DuBois (Dept. of Medicine; Vanderbilt University, Nashville, TN). RIE cells are nontransformed, epithelium-derived cells isolated from the small intestinal epithelium of a 20-day-old rat (3). They were cultured as previously described (57). Media and other reagents used for cell culture were purchased from Irvine Scientific (Irvine, CA). In 1,25-(OH)₂ vitamin D₃ treatment experiments, cells were incubated with 100 nM 1,25-(OH)₂ vitamin D₃ for 16 h before cells were harvested. For transcriptional assays, cells were pretreated with actinomycin D (100 nM; Calbiochem-Novabiochem; San Diego, CA) for 2 h and then treated with 100 nM 1,25-(OH)₂ vitamin D₃ for 16 h in the presence of actinomycin D before cells were harvested.

**RNA purification and Northern blot analyses.** mRNA was isolated from RIE cells and rat jejunal mucosa with the Fast-Track mRNA purification kit (Invitrogen; Carlsbad, CA). Ten micrograms of mRNA was used for Northern blot analyses with rat NaPi-IIb cDNA probes (56) under high-stringency washing conditions (several washes with a 0.1× sodium chloride-sodium citrate-0.1% SDS solution at 65°C) as described previously (11). 1B15 (encoding rat cyclophilin; Ref. 15) cDNA-specific probes were used as internal standards for quantitating NaPi-IIb gene expression. Blots were exposed to a phosphorimaging screen, and band intensities were determined with Quantity One software (FX Molecular Imager; Bio-Rad, Hercules, CA). NaPi-IIb gene expression levels were estimated by taking the ratio of hybridization intensities of NaPi-IIb mRNA over 1B15 mRNA. The experiment was repeated three times with mRNA isolation from different groups of animals.

**Na/P₃ uptake analysis in BBMVs.** BBMVs were prepared from rat jejunal mucosa, and Na/P₃ uptake was carried out as previously described (4, 24, 36). The contribution of Na/P₃ uptake was calculated by subtracting the sodium-independent uptake values observed in the absence of sodium from the uptake values in the presence of sodium. The experiment was repeated three times with BBMV isolated from different groups of animals.

**PCR analysis to detect NaPi-IIb expression in RIE cells.** mRNA was purified from RIE cells cultured in normal medium. RT-PCR conditions were identical to those described in a previous publication (56). The primers used to detect NaPi-IIb were designed from rat NaPi-IIb cDNA (GenBank accession no. AF157026). The forward primer was at 1446–1465 bp (5′-AGCCAGGCAACACATTGA-3′), and the reverse primer was at 1899–1917 bp (5′-ACACCATGCCACGACA CCG-3′). The expected amplification size from NaPi-IIb mRNA is 472 bp. The primers used to detect β-actin were purchased from Stratagene (La Jolla, CA). The size of the amplified product from the β-actin gene is 661 bp.

**Semiquantitative RT-PCR analysis of NaPi-IIb gene expression.** mRNA was purified from RIE cells treated for 16 h with vehicle (ethanol) or 1,25-(OH)₂ vitamin D₃ (100 nM). RT-PCR conditions were described previously (56). Subsaturation levels of cDNA templates needed to produce a dose-dependent amount of PCR product were defined in initial experiments by testing a range of template concentrations. Subsequent PCR was carried out with subsaturation levels of RT reactions with identical amplification parameters. PCR was performed with rat NaPi-IIb or β-actin primers in separate reactions; equal volumes of both PCR reactions were loaded on the same gel and visualized with ethidium bromide, and the optical density of each band was determined by gel-doc analysis. NaPi-IIb mRNA expression levels were estimated by taking a ratio of NaPi-IIb over β-actin ampiclon optical densities.

**Construction of reporter plasmids.** Reporter plasmids used in this study were derived from pGL3-Basic (Promega), which contains the firefly luciferase reporter gene. The human NaPi-IIb promoter-reporter constructs pGL3–2783 bp, pGL3–1103 bp, and pGL3–181 bp were made by restriction enzyme digestion and PCR (56). The 3′-end of all constructs ends at +15 bp of the human NaPi-IIb gene. All constructs were confirmed by sequencing on both strands.

**Transient transfection and functional promoter analysis.** RIE cells were cultured in 24-well plates. When cells reached 70–80% confluence, liposome-mediated transfection was performed as follows. Promoter vector DNA (0.5 µg), pRL-CMV (30 ng, Renilla luciferase reporter construct used as an internal standard; Promega), and Lipofectamine (5 µl; GIBCO BRL, Grand Island, NY) were mixed with 200 µl of OptiMEM medium (GIBCO BRL) for 30 min at room temperature. The mixture was then added to the cells, and they were incubated for 5 h, followed by the addition of an equal volume of Dulbecco's modified Eagle's medium (DMEM) containing 20% fetal bovine serum (FBS). The next day, the medium was removed and replaced with standard medium with 10% FBS. Twenty-four hours later, cells were harvested for reporter gene assays. For 1,25-(OH)₂ vitamin D₃ treatment, 100 nM 1,25-(OH)₂ vitamin D₃ or vehicle (ethanol) was added for 16 h before cells were harvested. Promoter reporter assays were performed using the Dual Luciferase assay kit according to the manufacturer's instructions (Promega). Luciferase activities were measured with a luminometer (Femtomaster FB 12; Berthold Detection System, Pforzheim, Germany), and all assays provided data that were well within the linear range of the instrument.

**Statistical analysis.** ANOVA post hoc tests (StatView 5.0.1 version; SAS Institute, Cary, NC) were used to compare values of the experimental data. P values of <0.05 were considered significant.
RESULTS

Effect of age on NaP-i-IIb gene expression in rat jejunum. Jejunal mRNAs from 2-wk, 3-wk, 6-wk, and adult rats were purified, and Northern blot analyses were performed with NaP-i-IIb- and 1B15-specific cDNA probes (Fig. 1). Expression levels of NaP-i-IIb mRNA were significantly different among age groups, with highest expression seen in 2-wk-old rats and expression levels gradually decreasing three- to fourfold into adulthood.

Effect of 1,25-(OH)₂ vitamin D₃ treatment on BBMV phosphate absorption in rat jejunum. Two-week-old and adult rats were treated with 1,25-(OH)₂ vitamin D₃, BBMVs were purified from jejunum, and Na/Pᵢ absorption was measured by a membrane filtration method. Na/Pᵢ uptake (in nmol Pᵢ/mg protein⁻¹·10⁻⁴ s⁻¹) in suckling rats was significantly higher than in adult rats (94.6 ± 4.7 in 2-wk-old rats vs. 30.4 ± 2.3 in adult rats; Fig. 2A). Vitamin D₃ treatment increased Na/Pᵢ absorption in both suckling and adult rats (94.6 ± 4.7 for control vs. 232.5 ± 45.3 for treated in 2-wk-old rats, 30.4 ± 2.3 for control vs. 67.2 ± 16.3 for treated in adult rats; Fig. 2A), with the fold inductions being similar (Fig. 2B).

Effect of 1,25-(OH)₂ vitamin D₃ treatment on NaP-i-IIb mRNA levels in rat jejunum. Two-week-old and adult rats were treated with 1,25-(OH)₂ vitamin D₃, mRNA was purified from jejunal mucosa, and Northern blots were performed with rat NaP-i-IIb and 1B15 cDNA probes. Hybridization patterns clearly showed that intestinal NaPi-IIb mRNA abundance significantly increased approximately twofold in 1,25-(OH)₂ vitamin D₃-treated 2-wk-old rats, but no change was detected in adult rats (Fig. 3). Also, there was no change in intestinal 1B15 mRNA abundance with vehicle or 1,25-(OH)₂ vitamin D₃ treatment.

1,25-(OH)₂ vitamin D₃ treatment increases NaP-i-IIb mRNA abundance in RIE cells. Preliminary results from RT-PCR indicated that RIE cells endogenously express the NaP-i-IIb gene (data not shown). Subsequently, NaP-i-IIb mRNA expression in RIE cells after exposure to vehicle or 1,25-(OH)₂ vitamin D₃ was assessed by semiquantitative RT-PCR with rat NaP-i-IIb and β-actin primers. Data showed that NaP-i-IIb gene expression was significantly increased in 1,25-(OH)₂ vitamin D₃-treated RIE cells compared with vehicle-treated cells, and the increase was approximately twofold (Fig. 4).

Actinomycin D treatment blocks NaP-i-IIb mRNA increase induced by 1,25-(OH)₂ vitamin D₃ treatment in RIE cells. To test whether the effect of 1,25-(OH)₂ vitamin D₃ on NaP-i-IIb gene expression is due to tran-
scriptional regulation, RIE cells were first treated with actinomycin D and then treated with 1,25-(OH)₂ vitamin D₃ in the presence of actinomycin D before cells were harvested. NaPi-IIb abundances were determined by semiquantitative RT-PCR with rat NaPi-IIb and /H9252-actin primers. The results showed that the increase in NaPi-IIb mRNA abundance induced by 1,25-(OH)₂ vitamin D₃ treatment was abolished by actinomycin D treatment (Fig. 5). In these experiments, actinomycin D did not alter basal expression levels of either the NaPi-IIb or /H9252-actin genes in RIE cells.

Human NaPi-IIb gene promoter analysis in RIE cells. To determine whether the 5'-flanking region of the human NaPi-IIb gene contains a functional promoter in RIE cells, three constructs (pGL3/−2783 bp, pGL3/−1103 bp, and pGL3/−181 bp) were transfected by Lipofectamine into RIE cells (57). Promoter reporter gene assays were performed 48 h after transfection. The promoter assay data showed that all promoter constructs were functional in RIE cells (Fig. 6).

To test the effect of 1,25-(OH)₂ vitamin D₃ on human NaPi-IIb gene promoter activity, RIE cells were first transfected with promoter constructs and then treated with 100 nM 1,25-(OH)₂ vitamin D₃ or vehicle for 16 h before cells were harvested. 1,25-(OH)₂ vitamin D₃ treatment of transfected RIE cells did not affect the activity of the internal control construct, Renilla luciferase driven by the CMV promoter. The data showed that human NaPi-IIb promoter activity increased ~1.6-fold with the pGL3/−2783 bp and pGL3/−1103 bp constructs in 1,25-(OH)₂ vitamin D₃-treated RIE cells compared with control cells (Fig. 6B). The pGL3/−181 bp construct showed no effect with 1,25-(OH)₂ vitamin D₃ treatment.

**DISCUSSION**

Earlier studies indicated that intestinal Na/Pi absorption declined with age in several mammalian species (4, 6, 45). These observations suggested that the expression of the transport protein(s), which is responsible for Na/Pi absorption, likely decreases with age. Our data demonstrate that NaPi-IIb gene expression decreases with age, and this observation correlates well with the functional studies. Thus it seems likely that NaPi-IIb expression contributes to the ontogenic changes seen in intestinal P₃ absorption.

Studies also showed that 1,25-(OH)₂ vitamin D₃ treatment stimulates intestinal NaPi absorption (13, 14, 16, 17, 22, 23, 27, 30, 33, 34, 39, 40, 52). More...
1,25-(OH)₂ vitamin D₃ regulates intestinal Na/Pᵢ absorption by NaPi-IIb and expression in suckling rats, which suggests that the effect of 1,25-(OH)₂ vitamin D₃ on NaPi-IIb expression is age specific. To decipher the molecular mechanism of 1,25-(OH)₂ vitamin D₃ regulation of intestinal NaPi-IIb gene expression in suckling animals, we explored the RIE cell line as an in vitro model. Our results demonstrate that the NaPi-IIb gene is endogenously expressed in RIE cells and that it is 1,25-(OH)₂ vitamin D₃ responsive. Furthermore, we performed Na-Pᵢ cotransport studies in RIE cells with 1,25-(OH)₂ vitamin D₃ treatment and found that activity was increased by ~25% and was blockable by actinomycin D treatment (data not shown). However, these data are difficult to interpret because RIE cells likely contain other endogenous Na-Pᵢ cotransporters, including ubiquitously expressed type III Na-Pᵢ cotransporters and possibly other unidentified Na-Pᵢ cotransporters. It is further possible that this other Na-Pᵢ cotransporter(s) may also be regulated by 1,25-(OH)₂ vitamin D₃ (as has been shown for the type III Na-Pᵢ cotransporters; Ref. 32), and thus it is extremely difficult to assess the single contribution of NaPi-IIb cotransporters. Moreover, we could not selectively study the activity of NaPi-IIb in RIE cells, because no specific inhibitors are available at this time. Our intention was simply to demonstrate that RIE cells are a good in vitro model to study NaPi-IIb gene regulation by 1,25-(OH)₂ vitamin D₃, as exemplified by the fact that the cells endogenously express this gene and that the gene is 1,25-(OH)₂ vitamin D₃ responsive. However, these data suggest that other in vitro models would have to be developed to study posttranscriptional regulation of the NaPi-IIb gene.

In vivo studies in suckling rats and in vitro studies in RIE cells showed that 1,25-(OH)₂ vitamin D₃ treatment increased apical NaPᵢ-IIb protein expression in adult rats but not NaPi-IIb mRNA expression, which is comparable to previous studies. The increase in Pᵢ uptake is most likely the result of increased NaPᵢ-IIb protein expression in adult animals (26). Our data also showed that 1,25-(OH)₂ vitamin D₃ treatment increased intestinal Na/Pᵢ absorption and NaPi-IIb mRNA expression in suckling rats, which suggests that the effect of 1,25-(OH)₂ vitamin D₃ on NaPi-IIb gene expression is age specific.

Fig. 5. Effect of actinomycin D (ActD) on NaPi-IIb mRNA expression in 1,25-(OH)₂ vitamin D₃-treated RIE cells. A: mRNA isolated from RIE cells treated under different conditions was used for first-strand cDNA synthesis. Subsequent PCR was performed with subsaturation levels of the RT reaction, and NaPi-IIb or β-actin primers were used in separate reactions. Equal volumes of PCR reactions for NaPi-IIb and β-actin were loaded on the same gel and visualized with ethidium bromide. B: fold induction in NaPi-IIb mRNA expression induced by 1,25-(OH)₂ vitamin D₃ treatment in RIE cells in the presence or absence of actinomycin D (100 nM for 16 h). Data were calculated by comparing the ratio of NaPi-IIb mRNA to β-actin mRNA in 1,25-(OH)₂ vitamin D₃-treated cells with the ratio of NaPi-IIb mRNA to β-actin mRNA in vehicle-treated cells. Results are means ± SE from 4 separate experiments. *P < 0.02 for absence of actinomycin D vs. presence of actinomycin D.

Fig. 6. Activity of human NaPi-IIb gene promoter constructs in transfected RIE cells. A: RIE cells were transiently transfected with 0.5 µg of pGL3 Basic (Basic) or 0.5 µg of human NaPi-IIb promoter constructs. To control for transfection efficiency, cells were cotransfected with 30 ng of pRL-CMV. Reporter gene assays were performed 48 h after transfection. Data are presented as relative luciferase activity (firefly luciferase activity driven by the human NaPi-IIb gene promoter over Renilla luciferase activity driven by the CMV promoter). Results are means ± SE from 10 separate experiments. *P < 0.003 for pGL3b vs. other constructs. B: RIE cells were cotransfected with pGL3 Basic or human NaPi-IIb promoter constructs + pRL-CMV. 1,25-(OH)₂ vitamin D₃ was applied 16 h before cells were harvested. Fold induction is shown as the ratio of luciferase activity in 1,25-(OH)₂ vitamin D₃-treated cells over luciferase activity in nontreated cells. Results are means ± SE from 10 separate experiments. *P < 0.0004 for pGL3/-1103 bp and pGL3/-2783 bp vs. pGL3b and pGL3/-181 bp.
ment increases NaPi-IIb mRNA abundance by approximately twofold. Therefore, transcriptional regulation seems likely. Further studies showed that activation of NaPi-IIb gene expression by 1,25-(OH)2 vitamin D3 in RIE cells could be abolished by 100 nM actinomycin D, a transcriptional inhibitor. These results suggest that the increase in NaPi-IIb mRNA abundance induced by 1,25-(OH)2 vitamin D3 likely involves synthesis of new NaPi-IIb mRNA. Furthermore, transfection studies with human NaPi-IIb promoter constructs showed that 1,25-(OH)2 vitamin D3 increased NaPi-IIb gene promoter activity by ~1.6-fold in transiently transfected RIE cells. Together, these data indicate that the effect of 1,25-(OH)2 vitamin D3 on intestinal NaPi-IIb gene expression can be mediated by control of transcriptional initiation.

Transfection of cells with three NaPi-IIb gene promoter constructs (pGL3/−2783 bp, pGL3/−1103 bp, and pGL3/−181 bp) resulted in significant reporter gene expression. This finding suggests that the basal promoter region of the NaPi-IIb gene is located within the −181 bp region in RIE cells, as was previously described in Caco-2 cells (57). Interestingly, the promoter construct pGL3/−2783 bp showed lower activity in transfected RIE cells compared with Caco-2 cells (56). Furthermore, the two longer promoter constructs (pGL3/−2783 bp and pGL3/−1103 bp) were responsive to 1,25-(OH)2 vitamin D3 treatment, but the smaller construct (pGL3/−181 bp) was unresponsive. This observation suggests that the putative 1,25-(OH)2 vitamin D3 response element(s) is located between 181 and 1103 bp upstream of the transcriptional initiation site.

Vitamin D3 responsive elements (VDRE) have been identified from many genes, including the human renal NaPi-IIa (NaPi-3) gene (50), the rat osteocalcin (OSC) gene, the mouse osteopontin (MOP) gene, the rat calbindin D-9k (CaBP) gene, and the human parathyroid hormone (PTH) gene (18, 46). We searched the human NaPi-IIb gene promoter region from −181 bp to −1103 bp for putative VDREs, but no classical VDR binding sequences were identified. This may therefore classify the human NaPi-IIb gene in a group of genes that are responsive to 1,25-(OH)2 vitamin D3 treatment but do not have classic VDRE sequences in their promoter regions (8, 46, 51). These data may also suggest that there is a novel VDRE present in this gene, or alternatively, the 1,25-(OH)2 vitamin D3 response could be mediated by a trans-acting factor that acts independently of the VDR.

In summary, we showed that the decrease in NaPi-I absorption during development correlates with decreased NaPi-Ib gene expression in the intestinal mucosa. We also demonstrated that 1,25-(OH)2 vitamin D3 treatment increases NaPi-IIb mRNA abundance in suckling rats and RIE cells and NaPi-IIb gene promoter activity in transfected RIE cells. Because actinomycin D treatment blocked 1,25-(OH)2 vitamin D3-induced increases in NaPi-IIb mRNA expression in RIE cells, we hypothesize that a transcriptional mechanism is likely involved. Further studies will focus on identification of the responsive region in the promoter and the trans-acting factors involved in regulation of the NaPi-IIb gene by 1,25-(OH)2 vitamin D3.

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