1,25 Dihydroxyvitamin D Amplifies Type A Natriuretic Peptide Receptor Expression and Activity in Target Cells

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1,25 dihydroxyvitamin D (VD) has been shown to exert a number of beneficial effects on cardiovascular function, including reduction in BP and inhibition of cardiac hypertrophy. In an effort to identify a possible mechanistic link between VD and these salutary effects, the role of VD in controlling the activity and expression of the type A natriuretic peptide receptor (NPR-A), a receptor that signals reductions in BP and suppression of cellular growth in the myocardium and vascular wall, was investigated. VD, as well as the nonhypercalcemic analogue RO-25-6760, increased NPR-A–dependent cyclic guanosine monophosphate production and NPR-A gene expression in cultured rat aortic smooth muscle cells. The increase in NPR-A expression was associated with an increase in NPR-A gene promoter activity that was critically dependent on the presence of a functional VD receptor response element located approximately 495 bp upstream from the transcription start site of the gene. This element was associated with the VD receptor/retinoid X receptor complex in vitro. Mutation of this element resulted in complete elimination of the VD-dependent induction of the NPR-A gene promoter but did not affect osmotic stimulation of the promoter. Treatment of rats with RO-25-6760 for 7 d increased the atrial natriuretic peptide–dependent excretion of sodium and cyclic guanosine monophosphate without affecting mean arterial BP or plasma calcium levels. This was associated with a twofold increase in NPR-A mRNA levels in the inner medulla. Amplification of NPR-A activity represents a plausible mechanism to account for at least some of the beneficial effects that VD exerts on cardiovascular function.


The natriuretic peptides constitute a family of closely related vasoactive hormones that play an important role in the regulation of cardiovascular, renal, and endocrine homeostasis (1). Atrial natriuretic peptide (ANP) and brain natriuretic peptide (BNP) are produced predominantly in the heart, whereas C-type natriuretic peptide (CNP) is produced in endothelial cells of the vasculature (2), reproductive tissues (3), growth plate of long bones (4), and the central nervous system (5). The natriuretic peptides exert their biologic effects through association with specific membrane-bound receptors. Natriuretic peptide receptor types A and B (NPR-A and NPR-B, respectively) each have a large extracellular ligand-binding domain connected to the intracellular signaling domain through a single membrane-spanning segment. The intracellular domain harbors a noncatalytic, kinase-like region linked to a particulate guanylyl cyclase at the carboxy terminus of the molecule (1). NPR-A serves as the cognate receptor for both ANP and BNP, whereas NPR-B binds selectively to CNP. Most of the important physiologic activities of these peptides seem to be mediated through activation of these guanylyl cyclase–linked receptors. A third receptor subtype, NPR-C, is thought to function largely, albeit not entirely (6), as a clearance receptor. Blockade of NPR-C has been shown to increase levels of endogenous natriuretic peptides in circulating plasma (7).

Vitamin D is a seco-steroid that bears a number of important similarities to hormonal ligands that act through nuclear mechanisms to control gene expression. Its most polar metabolite, 1,25 dihydroxyvitamin D (VD), binds specifically and with high affinity to a member of the nuclear receptor gene family termed the vitamin D receptor (VDR). The liganded VDR, when complexed with its heterodimeric partner, the retinoid X receptor (RXR), associates with specific regulatory elements on DNA called vitamin D response elements (VDRE). One such VDRE, DR3, is a direct repeat of the sequence AGGTCA separated by a three-nucleotide spacer (AGGTCAAGGTCA). DR3 displays selectivity in preferentially binding to the VDR-RXR heterodimer versus other nuclear receptor dimeric complexes (8).

A number of studies have identified VD as a potentially important regulator of BP and cardiovascular homeostasis. The mechanism underlying the inverse relationship between VD levels and BP is not completely understood; however, the recent studies of Li et al. (9) suggest that the renin-angiotensin system may be involved. Using genetically engineered VDR knockout mice, they showed that these animals were hypertensive relative to their VDR +/+ littermates and that this increase in BP was accompanied by apparent activation of the plasma renin-angiotensin system (RAS).

The data are equally supportive for an important regulatory role for VD in the heart. Rats made vitamin D–deficient display elevations not only in BP but also in ventricular hypertrophy (10). The former seems to be related, at least in part, to the
attendant hypocalcemia because calcium supplementation corrects the hypertension; however, the cardiac hypertrophy persists. Subsequent studies from the same group indicated that a major component of the hypertrophy seen in vitamin D–deficient animals reflects expansion of the interstitial compartment in the heart (predominantly cardiac fibroblasts) with increased production of extracellular matrix proteins (11). VD has been shown to reduce endothelin-stimulated ANP and BNP gene expression (markers of myocyte hypertrophy) and transcription in cultured neonatal rat atrial (12) and ventricular (13) myocytes and to suppress the hypertrophic response to endothelin, a well-known hypertrophic agonist in the cultured ventricular myocyte model (13). It is interesting that this suppressive activity seems to require structural features of the VDR that are more typically associated with the activation function of this receptor (14,15).

Like the liganded VDR, activated NPR-A has been shown to be both antihypertrophic and antihypertensive (16,17) in a number of different model systems. In the present study, we explored the possibility that VD might exert beneficial effects on the cardiovascular system through NPR-A. Our findings support this hypothesis in demonstrating that VD increases both the activity of the NPR-A protein and the expression of the NPR-A gene in cultured rat aortic smooth muscle (RASM) cells.

Materials and Methods

Materials

[3H]dCTP and the cyclic guanosine monophosphate (cGMP) RIA kit were purchased from Perkin-Elmer Life Sciences (Boston, MA). ANP was obtained from Phoenix Pharmaceuticals, Inc. (Mountain View, CA). RNeasy minikit was from Qiagen Inc. (Santa Clara, CA). Primer-it RMT kit, hybridization solution, and NucTrap push columns were purchased from Stratagene (La Jolla, CA). RO-25-6760 was provided by M. Uskokovic (Roche Pharmaceuticals). Other reagents were obtained through standard commercial suppliers.

Culture of Vascular Smooth Muscle Cells

Neonatal RASM cells, originally isolated by P. Lynch at the University of Southern California, were obtained at passage 20 from H. Ives at University of California San Francisco. Cells were cultured at 37°C in a 5% CO2-humidified incubator in DMEM supplemented with 10% FBS, 100 units/ml penicillin, and 100 mg/ml streptomycin, and 2% (vol/vol) broth, tryptose phosphate (18).

Measurement of ANP-Stimulated cGMP Levels

RASM cells were grown to approximately 80% confluence, then RASM cells were transfected with 5 μg of hVDR and hRXR or sham transfected using Lipofectin Reagent (Invitrogen) for 24 h. Cells were transfected using Prevec LUC with 0.2 μg of cytomegalovirus–β-galactosidase. After transfection, cells were plated on six-well plates in growth medium for 24 h. Medium was then changed to DMEM/serum substitute for the ensuing 24 h, at which point various concentrations of VD, or 10−8 M VD at different time intervals, were added. Luciferase activity was measured using the luciferase assay system (Promega). β-Galactosidase activity was assayed using the Galectolight Plus chemiluminescence assay (Tropix, Bedford, MA). Luciferase levels were normalized for β-galactosidase activity in the individual cultures.

Preparation of Nuclear Extracts

RASM cells were transfected with 5 μg of hVDR and hRXR or sham transfected using Lipofectin Reagent (Invitrogen) for 24 h. Cells were changed to serum substitute media and treated with vehicle or 10−8 M VD for 48 h. Nuclear extracts were prepared as described previously (24).
Electrophoretic Mobility Shift Assay

$^{35}$S-labeled hVDR and unlabeled hVDR and hRXRα were translated in vitro using a cell-free translation kit (TNT T7 Quick Master Mix kit; Promega) with $[^{35}]$methionine and unlabeled methionine, respectively. Double-stranded DR3 (5′-AGCTTCAGGAGGTAAGGCAAGGGAAG-3′; consensus VDRE half sites indicated in bold letters) and wild-type or mutant oligonucleotides derived from NRP-A promoter sequence spanning the region between −504 and −472 were generated for use in the electrophoretic mobility shift assay (EMSA). Two different protocols were used for EMSA.

Protocol 1
Two microliters each of unlabeled recombinant VDR and hRXRα or 3 μl of RASM cell nuclear extract (see above) were incubated in binding reaction buffer (10 mM HEPES [pH 7.9], 50 mM KCl, 0.2 mM EDTA, 2.5 mM DTT, 10% glycerol, and 0.05% NP-40) that contained 0.5 μg of poly(dl-dc) and $^{32}$P-end-labeled, double-stranded oligonucleotide, and 10 nM VD at room temperature for 30 min. For competition experiments, a 10- or 100-fold molar excess of unlabeled, double-stranded oligonucleotide was added to the binding reaction. All samples were resolved on 5% nondenaturing polyacrylamide gels. Gels were dried and exposed to x-ray film.

Protocol 2
Two microliters of $^{35}$S-labeled VDR (synthesized in vitro) was incubated in the presence of 2 μl of unlabeled hRXRα and 0.5 μg poly(dl-dc), with 20 ng of unlabeled, double-stranded DR3 or NRP-A oligonucleotide and 10 nM VD. Reactions were carried out in binding buffer (10 mM NaHPO$_4$ [pH 7.6], 0.25 mM EDTA, 0.5 mM MgCl$_2$, and 5% glycerol) for 20 min at 23°C. DNA–protein complexes were resolved by electrophoresis on 5% nondenaturing polyacrylamide gels run in TAE buffer (67 mM Tris-HCl [pH 7.5], 10 mM EDTA, and 33 mM dC), with 20 ng of unlabeled, double-stranded oligonucleotide, and exposed to x-ray film.

Site-Directed Mutagenesis
Mutations (M1, M2) were created in −1575 NRP-A-Luc by site-directed mutagenesis using a commercial kit (Stratagene). Briefly, mixtures that contained 50 ng of −1575 NRP-A-Luc, two mutagenic primers, dNTP, and Pfu DNA polymerase were added to the PCR buffer. PCR was carried out for 18 cycles using 30 s of denaturation at 95°C, 1 min of annealing at 55°C, and 2 min/kb extension at 68°C. After PCR, 1 μl of Dpn I was added to the reaction to cut parental DNA template, and 5 μl of this digest was used for transformation. Several candidate clones were identified and characterized by DNA sequencing.

Analysis of Renal ANP Activity In Vivo
Under anesthesia (intraperitoneal injection of Brevital sodium 50 mg/kg), a midscapular incision was made and Alzet osmotic minipump was implanted subcutaneously into adult male Sprague-Dawley rats. In the treatment group, the pumps were loaded with RO-25-6760 (0.8 μg/kg per d dissolved in 98% propylene glycol + 2% ethanol). In the control group, pumps were filled out with vehicle alone. The wound was closed with sutures, and the rats were returned to their cages after recovery from anesthesia. Over the ensuing 7 d, they were observed for any signs of calcium toxicity: Tremors, muscle spasms, seizures, anorexia, or weight loss. One week after pump implantation, each rat received an intraperitoneal injection of Inactin (100 mg/kg) to induce anesthesia. Animals were placed on a heated operating table. A tracheotomy was performed, and a segment of PE20 tubing was inserted into the trachea to facilitate clearance of secretions from the upper respiratory tract. The left jugular vein was exposed, and a catheter (PE50) was placed. A solution of normal saline that contained 1 mg/ml BSA was infused continuously at 25 μl/min through this catheter. The carotid artery was identified through a small paratracheal incision, and a tapered polyethylene catheter (PE50) was inserted for measurement of arterial pressure with a P23id Statham BP transducer attached to a direct writing recorder. A small catheter (PE10) was inserted into the right ureter near the hilum through a right flank incision for collection of urine from the right kidney. A curved 30-G needle attached to PE10 tubing was inserted into the left renal artery near its takeoff from the aorta through a left flank incision under the rib cage. Prompt reflux of arterial blood into the catheter was used to indicate successful needle placement. After completion of this preparative surgery, the rats received an infusion of normal saline that contained 5% BSA (total volume equal to 0.7% of total body weight) over 15 min to replace surgical fluid losses. BP was measured over the subsequent 30 min, and an average value of the electrical mean was taken to record mean arterial pressure. At that point, ANP (250 fmol/L per min) was infused into the left renal artery for 20 min. Urine was collected from both kidneys in the 20-min period before and during the 20-min period of intrarenal ANP infusion. At the end of study, the rats were killed, blood was collected and processed for measurement of plasma calcium, and the renal inner medulla from both kidneys was isolated for measurement of NRP-A mRNA. Urine volume was determined, and urinary sodium was measured by flame photometry (Model 943; Instrumentation Laboratories, Lexington, MA). Urine samples were then diluted 50-fold before measurement of cGMP (2-μl sample) using the method described above. All procedures were approved by the IAUCC at UCSF.

Real-Time PCR Analysis
Total RNA was prepared from frozen inner medullas of control and RO-25-6760–treated rats using the RNeasy kit. Two micrograms of total RNA was used to reverse transcribe cDNA using the Clontech reverse transcription kit. Rat NRP-A and glyceraldehyde phosphate dehydrogenase (GAPDH) (25) primer pairs and probes were synthesized by Applied Biosystems. Real-time PCR was performed using Taqman Master mix (A&B Applied Biosystems) with an ABI Prism 7700 (A&B Applied Biosystems). Negative controls without input cDNA were used to assess signal specificity. NRP-A transcript levels were quantified and normalized for GAPDH transcript levels in each sample.

Statistical Analyses
Data were analyzed by ANOVA using Bonferroni test to assess significance.

Results
To determine whether VD has a role in the regulation of NRP-A expression/activity, we examined the functional response of the receptor to ligand (ANP-dependent cGMP generation) in the presence or absence of VD. Forty-eight hours of VD treatment had no effect on basal cGMP levels in RASM cells (Chen et al., data not shown). ANP treatment of control cells led to an approximately fivefold increment in cGMP levels, relative to basal, over a 10-min incubation period. VD pretreatment nearly doubled this response with a net stimulation of cGMP levels of approximately 10-fold after ANP treatment (Figure 1A). A similar amplification of the response to ANP was seen
after pretreatment with the nonhypercalcemic VD analogue RO-25-6760. This increment in NPR-A activity resulted, at least in part, from an increase in NPR-A gene expression. As shown in Figure 1B, VD pretreatment led to an approximately five- to sixfold increase in steady-state NPR-A mRNA levels. Of note, the nonhypercalcemic analogue RO-25-6760 led to a similar increment (approximately fivefold) in NPR-A mRNA levels in cultured RASM cells (Figure 1C).

The increment in gene expression, in turn, seems to reflect an increase in NPR-A gene transcription. As shown in Figure 2, VD, as well as RO-25-6760, treatment of cultured RASM cells led to a significant increase in the activity of a transfected NPR-A promoter-luciferase reporter construct that was not seen with the transfected background vector (pGL3). This stimulation was ligand dependent (Figure 2A), VD receptor dependent (Figure 2B), and time-dependent (Figure 2B) and amplifiable with co-transfected RXR, the heterodimeric partner of VDR (Figure 2C).

A series of 5'-deletion mutants of the NPR-A promoter, extending from −1575 to −77 relative to the transcription start site, were generated and linked to the luciferase reporter. As shown in Figure 3, mutants that harbored as little as 706 bp of the 5' flanking sequence retained the ability to respond to VD; however, the −387 NPR-A-luciferase mutant displayed no induction with VD treatment. This suggests that the VD-sensitive regulatory element is positioned between −706 and −387 in the NPR-A gene promoter.

Examination of the DNA sequence spanning this region for the presence of conventional VD regulatory elements revealed five potential candidates, each displaying moderate to high homology to the DR3 motif (two AGGTCA motifs arrayed as direct repeats and separated by three spacer nucleotides [8]; Figure 4). Double-stranded oligonucleotides spanning each of these regions were incubated with radiolabeled VDR, unlabeled RXR, and the VD ligand (10⁻⁸ M) and fractionated on nondenaturing polyacrylamide gels to assess potential DNA-protein interactions (i.e., conventional EMSA assay). As shown in Figure 5A, NPR-A-1, which displayed the highest degree of homology to the consensus VDRE (homologous at 9 of 12 positions), failed to interact with the VDR-RXR complex. Similarly, NPR-A-2 and NPR-A-3 failed to associate with this receptor complex (Figure 5B); however, NPR-A-4 displayed a level of VDR-RXR binding that was readily discernible, albeit at a fraction (approximately 50%) of the intensity seen with the consensus DR3 motif. Binding to radiolabeled NPR-A-4 was almost completely inhibited by a 100-fold molar excess of unlabeled oligonucleotide (Figure 5C). Introduction of mutations into the downstream candidate site (NPR-A-4 M1) did not impair the ability of this oligonucleotide to inhibit VDR-RXR binding to NPR-A-4 (Figure 6A) (i.e., mutated sequence does not harbor the VDRE). Mutation of the upstream site (NPR-A-4 M2), however, resulted in near complete abrogation of the competition for NPR-A-4 binding to the heterodimeric receptor complex. The latter finding suggests that the liganded VDR-RXR complex binds to the upstream sequence that is targeted by the NPR-A-4 M2 mutation. This was confirmed in direct binding studies as shown in Figure 6B.

Figure 1. Vitamin D (VD) effects on natriuretic peptide receptor A (NPR-A) activity and expression. (A) Rat aortic smooth muscle (RASM) cells were cultured in serum substitute medium for 24 h and then treated with VD (10⁻¹⁰ to 10⁻⁷ M) or RO-25-6760 (10⁻⁸ M) for 48 h. Total RNA was isolated, and Northern blot analysis was performed as described in the Materials and Methods section. Pooled data from three independent experiments are shown. **P < 0.01 versus control in the presence of ANP. Control cGMP levels were 198 ± 21 pmol/mg soluble protein. (B) RASM cells were treated with various concentrations of VD for 48 h. Total RNA was isolated, and Northern blot analysis was performed as described in the materials and Methods section. Representative autoradiographs are shown. The bar graph displays findings (NPR-A mRNA/18S rRNA) from three separate experiments. **P < 0.01 versus control. (C) RASM cells were exposed to various concentrations of RO-25-6760 for 48 h. Total RNA was prepared, and Northern hybridization was carried out. NPR-A mRNA levels were normalized to 18S rRNA. VD and RO-25-6760 concentrations in all three panels presented as log [M]. Pooled data from three independent experiments are shown. **P < 0.01 versus control.
Figure 2. VD upregulates NPR-A promoter activity in RASM cells. (A) RASM cells were transfected with −1595 NPR-A LUC or pFOX LUC, and cytomegalovirus-β-galactosidase (CMV-β-gal) and cultured for 24 h. Medium was then changed to DMEM/serum substitute for the ensuing 24 h, at which point VD or RO-25-6760, at the concentrations indicated (all concentrations presented as log [M]), was added for the next 48 h. (B) −1595 NPR-A LUC and CMV-β-gal were co-transfected with increasing concentrations of VD receptor (VDR) expression vector into RASM cells. After transfection, cells were treated with 10⁻⁸ M VD for indicated time intervals. (C) A total of 0.5 μg of VDR and retinoid X receptor (RXR) was co-transfected with −1595 NPR-A LUC/CMV-β-gal into RASM cells. The transfected cells were incubated with vehicle or 10⁻⁸ M VD for 48 h. In all cases, lysates were generated and luciferase and β-gal activities were measured as described in the Materials and Methods section. Experiments were repeated three to six times. **P < 0.01, *P < 0.05 versus corresponding control.
Whereas wild-type NPR-A-4 and the M1 mutant associated with radiolabeled VDR in this assay, the M2 mutant was devoid of binding activity.

Binding of VDR to NPR-A-4 did not require ligand; binding of recombinant VDR/RXR to NPR-A-4, if anything, was reduced slightly in the presence of 1,25 dihydroxyvitamin D (Figure 7A). It is interesting that this differed from receptor expressed in a transfected cell. As shown in Figure 7B, extracts of cells that were transfected with hVDR plus hRXR showed a modest increase in binding to NPR-A-4 versus untransfected cells; however, the inclusion of ligand resulted in a dramatic increase in binding of the extract to the oligonucleotide, suggesting that other proteins in the extract may serve to stabilize the DNA-VDR/RXR complex.

Extending this analysis to the functional assay, mutation of the M1 site in the context of /H110021575 NPR-A luciferase failed to influence the VD-dependent stimulation of this promoter, relative to the wild-type sequence, whereas introduction of the M2 mutation into the same promoter context resulted in complete loss of the VD-dependent stimulation (Figure 8A). Noteworthy, the M2 mutation did not prevent induction of the NPR-A promoter by osmotic stimuli such as NaCl or sucrose (22,26) (Figure 8B), indicating that the targeted sequence selectively transduces the response to VD.

To assess the physiologic relevance of the in vitro findings, we attempted to extend these analyses into an in vivo model. We chose a model that we have used previously to examine the effects of ANP infused directly into the renal artery on urinary volume and sodium excretion (27). This model has the advantage of identifying ANP-induced changes in urinary sodium and water handling independent of changes in peripheral hemodynamics. As shown in Table 1, pretreatment of animals

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**Figure 3. Deletion analysis of VD induction of NPR-A promoter activity.** Different deletions of NPR-A-LUC were made as described in the Materials and Methods section and transfected into RASM cells. Twenty-four hours later, cells were incubated in presence or absence of 10⁻⁸ M VD for 48 h before generating lysates for luciferase and β-gal measurements. The experiments were repeated three to four times. **P < 0.01 versus corresponding control.

**Figure 4. Identification of candidate VD response elements (VDRE) in NPR-A promoter.** Consensus VDRE half sites in DR3 motif are identified at the top. Candidate VDRE sequences in NPR-A promoter are identified in bold type. Termini identify positions of the DNA fragments relative to the transcription start site; orientation is indicated by arrows positioned above. Mutated bases in VDRE of NPR-A4 (M1 and M2) are indicated in lowercase letters.

**Figure 5. Specific VDR/RXR binding activity resides in NPR-A4 sequence.** (A and B) ³⁵S-VDR and unlabeled RXR were incubated with unlabeled oligonucleotides encoding a consensus DR3 motif or one of four putative VDRE in the NPR-A promoter. All incubations were carried out in the presence of VD (10⁻⁸ M). DNA-protein complexes were fractionated by electrophoretic mobility shift assays (EMSA) as described in the Materials and Methods section. (C) Unlabeled VDR and RXR, along with VD, were incubated with ³²P-labeled NPR-A-4 oligonucleotide in the absence or presence of increasing concentrations of unlabeled oligonucleotide (10- or 100-fold molar excess). The reaction complexes were resolved on 5% non-denaturing polyacrylamide gels. The experiments were repeated two to three times. Representative autoradiographs are shown.

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with the nonhypercalcemic analogue RO-25-6760 for 7 d had no effect on basal mean arterial pressure relative to control. Serum calcium levels were measured in both the control and the analogue-infused animals. Calcium levels in the RO-25-6760–treated animals remained in the normal range and were not statistically different from the controls (Table 1). Infusion of ANP (250 fmol/kg per min) into the left renal artery had no effect on systemic arterial pressure (Table 1). It did, however, promote an increase in urinary sodium excretion that was not seen in the contralateral, noninfused kidney (Figure 9A). Pre-treatment with RO-25-6760 for 7 d resulted in a significant increase in ANP-stimulated urinary sodium excretion in the infused kidney, and this was accompanied by a significant increase in ANP-dependent urinary cGMP levels when compared with the control group (Figure 9B). In both instances, the magnitude of the increases (in UNa and UCMP) was larger in the RO-25-6760–treated versus vehicle-treated rats. A modest elevation in UNaV and UCMP was seen in urine that was collected from the contralateral kidney, presumably reflecting the effects of peptide that has either found its way into the systemic circulation or refluxed into the aorta from the infusion site. Collectively, these findings support our in vitro data in demonstrating that the VD analogue amplifies the activity of the natriuretic peptides in target cells.

To determine whether the increase in natriuretic peptide activity resulted from increased NPR-A expression, as documented in the in vitro system above, we measured NPR-A mRNA transcripts in inner medulla of control versus RO-25-6760–treated rats using real-time PCR. As shown in Figure 9C, treatment with RO-25-6760 for 7 d resulted in an approximately twofold increment in NPR-A mRNA levels in the inner medulla. Levels of the GAPDH transcript were essentially un-
changed. This suggests that the increment in activity of the liganded NPR-A noted above results from VD-dependent stimulation of NPR-A gene expression.

Discussion

The studies described above provide definitive evidence for transcriptional regulation of a cardiovascular gene by VD and identify an important mechanism through which this hormone may exert salutary effects in the cardiovascular system. Previous studies have suggested regulation of NPR-C expression by glucocorticoids (28); however, transcriptional regulation was not documented in those studies, and a specific glucocorticoid responsive element was not identified. The current study demonstrates VD1-dependent stimulation of NPR-A activity as well as NPR-A gene expression. The latter derives from an increase in NPR-A promoter activity that is driven by a solitary VDRE located approximately 495 bp upstream from the transcription start site.

Vitamin D activity is classically described in terms of its calcitropic properties in the intestine, kidney, and bone (29); however, more recently increased attention has been devoted to its ability to regulate physiologic events through pathways independent of those that it uses to control serum calcium and phosphorous concentrations. VD has immune-suppressant and anti-inflammatory properties (30), it reduces cell proliferation in a variety of in vitro models through mechanisms that are only partially understood (31,32), and it seems to play an important role in the growth and development of the epidermis and hair follicle (33,34). Data acquired through a number of different experimental and clinical studies indicate that VD also plays an important role in cardiovascular homeostasis. 25-Hydroxyvitamin D levels are inversely related to BP levels in selected forms of rodent (35) and human (36) hypertension, and administration of VD restores BP to normal or near-normal levels (37,38). Of note, the recent study of Teng et al. (39) demonstrated improved survival of patients who had ESRD and were on hemodialysis when they were treated with the nonhypercalcemic analogue of VD, paricalcitol, versus VD itself, suggesting the existence of important salutary effects of the seco-steroid that are independent of its calcitropic properties. Because the majority of patients with ESRD die of cardiovascular causes, the inference would be that the benefit accrued secondary to the beneficial cardiovascular effects of these drugs.

True vitamin D–deficiency in the rat is accompanied by acquired hypertension and associated ventricular hypertrophy (10), although in this model, the BP elevation abates with correction of the attendant hypocalcemia. The recent studies of Li et al. (9) suggest that hypertension results from defective VD action in general, rather than solely from hypocalcemia. In VDR null (−/−) mice, systolic BP was elevated approximately 20 mmHg versus controls, and this was associated with activation of the RAS. Because these animals were maintained on a high-calcium diet to prevent the development of hypocalcemia, it seems likely that the BP elevation is at least partially reflective of the defect in VDR signaling. Li et al. also presented data suggesting a direct effect of the liganded VDR on the renin gene promoter, although a discrete functional VDRE was not identified.

NPR-A plays a major role in controlling BP and extracellular fluid volume (1). In fact, the studies of DuBois et al. (40) suggest that most, if not all, of the renal effects of ANP are mediated through this receptor. That it mediates vasodilation, increased urinary excretion of sodium and water, and suppression of myocardial hypertrophy and fibrosis (17) makes it an attractive

![Figure 8. Mutation of the VDRE in the NPR-A gene promoter interferes with VD-induced NPR-A gene promoter activity. (A)](image-url)

A)

Wild-type −1595 NPR-A-LUC or one of two individual mutants of the NPR-A-4 sequence (M1 and M2) was co-transfected with CMV-β-gal into RASM cells. (B) −1595 NPR-A-LUC and M2 mutant along with CMV-β-gal were co-transfected into RASM cells. After 24 h, the cells were treated with 10⁻⁸ M VD for 48 h. Alternatively, 48 h after transfection, cells were treated with 150 mM sucrose or 75 mM NaCl for 24 h. Luciferase and β-gal activity was measured. Experiment was repeated three times. **P < 0.01 versus corresponding control.
candidate as a target of and mediator for VD action. Although this does not exclude a parallel role for VD in suppression of the RAS, it is noteworthy that the liganded NPR-A is also capable of suppressing the RAS at multiple points in the signaling cascade (41), including inhibition of renin gene expression. Therefore, it is conceivable that at least a portion of the reduction in renin expression could result from enhanced NPR-A expression and activity rather than through a direct liganded VDR-dependent suppression of the renin gene promoter. In fact, Li et al. (42) reported that NPR-A gene expression is reduced in VDR−/− mice. Additional studies will be required to assess whether the direct versus indirect model is operative here.

Finally, our previous demonstration that VD suppresses expression and secretion of ANP (12,13,43) seems, at first glance, to be physiologically inconsistent with the data presented here, because a reduction in ligand levels would be predicted to offset the increase in receptor activity. However, it is important to point out that the VD-dependent inhibition of ANP expression is largely confined to hypertrophy-dependent expression (e.g., that induced by endothelin treatment) and reflects a global antagonism of the hypertrophic process rather than isolated inhibition of the ANP gene. Activation of the ANP gene in hypertrophy represents an attempt, oftentimes futile (44), to reverse the hemodynamic stimuli that elicit the increase in myocardial mass and through primary (16,17) as well as secondary (1) effects control further progression of the hypertrophic process. On the basis of its ability to increase NPR-A activity, VD would be predicted to support ANP effects in this setting. Thus, the VD effects may be viewed as consistently aligned toward suppression of growth and hypertrophy in the cardiovascular system.

In summary, we have shown that the vasodilatory, antimitogenic receptor NPR-A is under the transcriptional regulatory control of 1,25 dihydroxyvitamin D. This effect may be responsible for a major component of the salutary effects that vitamin D exerts in the cardiovascular system.

Acknowledgments
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References

Table 1. Effect of RO-25-6760 on plasma Ca\(^{2+}\) and mean arterial BP before and after infusion of ANP in vivo\(^a\)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Infusion of Vehicle</th>
<th>Infusion of ANP</th>
<th>Plasma Ca(^{2+})</th>
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<td>Control</td>
<td>(n = 8)</td>
<td>105 ± 4.6 mmHg</td>
<td>106 ± 5.2 mmHg</td>
<td>9.1 ± 0.30 mg/dl</td>
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<tr>
<td>RO-25-6760</td>
<td>(n = 8)</td>
<td>102 ± 4.4 mmHg</td>
<td>101 ± 4.4 mmHg</td>
<td>9.7 ± 0.59 mg/dl</td>
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</tbody>
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\(\text{ANP, atrial natriuretic peptide.}\)

Figure 9. Effect of RO-25-6760 on ANP-stimulated \(U_{\text{NaV}}\), urinary cGMP, and NPR-A mRNA levels in vivo. (A) Intrarenal infusion of ANP (250 fmol/kg per min) for 20 min. Urine was collected from infused and contralateral kidney of control and RO-25-6760–treated rats before and after ANP infusion. Urine volume and sodium were measured. (B) Urinary cGMP levels were measured in aliquots of urine as described in the Materials and Methods section. Control cGMP levels were 533 ± 53 pmol/ml urine. (C) Real-time PCR analysis of NPR-A mRNA levels in rat inner medulla. Paired inner medullas from control and RO-25-6760–treated rats were isolated and snap-frozen in liquid nitrogen. Total RNA was prepared and reverse transcribed into cDNA. Real-time PCR was carried out as described in the Materials and Methods section. Pooled data (\(n = 8\)) are shown. **\(P < 0.01, *P < 0.05\) versus ANP-infused control; **\(P < 0.01\) versus ANP-infused control.


