Expression of the Vitamin D Receptor Is Increased in the Hypertrophic Heart

Songcang Chen, Denis J. Glenn, Wei Ni, Christopher L. Grigsby, Keith Olsen, Minobu Nishimoto, Christopher S. Law, David G. Gardner

Abstract—The liganded vitamin D receptor (VDR) is thought to play an important role in controlling cardiac function. Specifically, this system has been implicated as playing an antihypertrophic role in the heart. Despite this, studies of VDR in the heart have been limited in number and scope. In the present study, we used a combination of real-time polymerase chain reaction, Western blot analysis, immunofluorescence, and transient transfection analysis to document the presence of functional VDR in both the myocytes and fibroblasts of the heart, as well as in the intact ventricular myocardium. We also demonstrated the presence of 1-α-hydroxylase and 24-hydroxylase in the heart, 2 enzymes involved in the synthesis and metabolism of 1,25 dihydroxyvitamin D. VDR is shown to interact directly with the human B-type natriuretic peptide gene promoter, a surrogate marker of the transcriptional response to hypertrophy. Of note, induction of myocyte hypertrophy either in vitro or in vivo leads to an increase in VDR mRNA and protein levels. Collectively, these findings suggest that the key components required for a functional 1,25 dihydroxyvitamin D–dependent signaling system are present in the heart and that this putatively antihypertrophic system is amplified in the setting of cardiac hypertrophy. (Hypertension. 2008;52:1106-1112.)

Key Words: vitamin D ■ cardiac hypertrophy ■ nuclear receptors ■ BNP ■ cardiac myocyte

Vitamin D is a secosteroid that functions as a ligand for a nuclear receptor (vitamin D receptor, or VDR) to control the transcription of target genes in a positive or negative fashion. Vitamin D is either ingested in the diet or generated de novo through scission of cholesterol precursors in the skin by ultraviolet light. Vitamin D is activated through 2 sequential hydroxylation reactions. The first of these, a 25-hydroxylation, takes place largely in the liver to produce 25-hydroxyvitamin D. This molecule circulates bound to a plasma protein and is measured to assess the adequacy of vitamin D stores. The second hydroxylation, a 1-α-hydroxylation, takes place predominantly in the kidney to produce 1,25-dihydroxyvitamin D (VD3). It serves as the principal ligand for VDR in the nucleus (and extranuclear compartment) of target cells. Another enzyme, that is ubiquitously expressed 24-hydroxylase, is responsible for hydroxylating this ligand, leading to its inactivation and subsequent degradation.

Recent studies suggest that VD3, in addition to stimulating absorption of intestinal calcium and promoting mineralization of bone osteoid, may play an important role in controlling cardiac hypertrophy. We have shown that VD3, as well as a number of nonhypercalcemic analogues, act in both atrial and ventricular myocytes to inhibit the activation of phenotypic markers associated with hypertrophy in vitro. Endothelin (ET)-stimulated changes in fetal gene expression and promoter activity, cell size, and protein synthesis are partially reversed by VD3 or its nonhypercalcemic analogues. Similar findings have been reported by others using the cultured cardiac HL-1 myocytes. In animal studies, vitamin D deficiency in Sprague-Dawley rats leads to both hypertension and cardiac hypertrophy, whereas treatment of Dahl salt-sensitive rats with the vitamin D analogue paricalcitol reverses cardiac hypertrophy in that model. The VDR knockout mouse displays hypertension, cardiac hypertrophy with enlargement of individual myocytes, and elevations in atrial natriuretic peptide (ANP) expression. However, the elevated blood pressure precludes assigning the liganded VDR a primary antihypertrophic role at the level of the cardiac myocyte. Although VDR has been identified microscopically and functionally in the heart, our understanding of the role of the liganded VDR in the maintenance of cardiac function remains incomplete.

Although VDR is clearly present in the heart, we know little of the specific cell types in which it is expressed nor of the functional activities associated with the liganding of these receptors. In the present article, we demonstrate the presence of VDR- and VD3-dependent functional activity in both the...
myocytes and fibroblasts of the rat heart. We also demonstrate the presence of the 1-α-hydroxylase enzyme in both compartments. Noteworthy, application of a hypertrophic stimulus (ET in vitro and isoproterenol in vivo) leads to an increase in VDR gene expression with little or no effect on expression of the 1-α-hydroxylase. The results indicate that a functional vitamin D–dependent signaling system is present in both cardiac myocytes and fibroblasts and support a growing body of data defining an antihypertrophic role for this system in the heart.

Materials and Methods

Materials

ET was purchased from American Peptide (Sunnyvale, Calif). Paricalcitol and active hectorol were gifts from Joel Melnick (Abbott Laboratories, Abbott Park, Ill). VD3 and 25-hydroxyvitamin D3 were obtained from Calbiochem Inc (La Jolla, Calif). VDR antibody (sc-1008), procollagen I antibody (sc-8787), 24-hydroxylase antibody (a gift from Martin Hewison, Cedars-Sinai Hospital, Los Angeles, Calif), and GAPDH antibody (sc-32233) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 25-Hydroxvitamin D 1-α-hydroxylase antibody (PC290) was from The Binding Site (Birmingham, UK).

Isoproterenol-Induced Cardiac Hypertrophy

Wistar rats purchased from Charles River Laboratories (Wilmington, Mass) were anesthetized, and subcutaneous osmotic minipumps were implanted in the dorsum of the neck. Individual rats received vehicle alone or isoproterenol at a rate of 2.4 mg/kg per day for 7 days (n=8 each group). Animals were weighed and euthanized by CO2 narcosis followed by bilateral thoracotomy. Left ventricles were isolated and weighed. Left ventricular weight/body weight and left ventricular weight/tibial length were measured as indices of cardiac enlargement. All experiments were approved by the institutional animal care and use committee at University of California at San Francisco (UCSF).

Cell Culture

Ventricular myocytes and fibroblasts were prepared from 1- to 2-day-old neonatal Sprague-Dawley rats (Charles River Laboratories) as described previously. Both cell types were maintained in Dulbecco’s modified Eagle’s medium H-21 supplemented with 10% fetal bovine serum (Gemini Bioproducts; West Sacramento, Calif).

Immunoblotting

Myocytes and fibroblasts were changed from media containing 10% enriched calf serum to media containing 10% serum substitute for 2 days. Animals were weighed and euthanized by CO2 narcosis followed by bilateral thoracotomy. Left ventricles were isolated and weighed. Left ventricular weight/body weight and left ventricular weight/tibial length were measured as indices of cardiac enlargement. All experiments were approved by the institutional animal care and use committee at University of California at San Francisco (UCSF).

Materials

ET was purchased from American Peptide (Sunnyvale, Calif). Paricalcitol and active hectorol were gifts from Joel Melnick (Abbott Laboratories, Abbott Park, Ill). VD3 and 25-hydroxyvitamin D3 were obtained from Calbiochem Inc (La Jolla, Calif). VDR antibody (sc-1008), procollagen I antibody (sc-8787), 24-hydroxylase antibody (a gift from Martin Hewison, Cedars-Sinai Hospital, Los Angeles, Calif), and GAPDH antibody (sc-32233) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). 25-Hydroxvitamin D 1-α-hydroxylase antibody (PC290) was from The Binding Site (Birmingham, UK).

Immunoblotting

Myocytes and fibroblasts were changed from media containing 10% enriched calf serum to media containing 10% serum substitute for 24 hours. At that point, fresh media containing different concentrations of ET or VD3 were added. Cells were cultured for another 24 hours. At that point, the cells were collected and lysed. Luciferase activity was measured using the Dual-Luciferase kit (Promega; Madison, Wis). BNP luciferase activity was normalized for Renilla luciferase activity.
time PCR was performed on the ABI Prism 7900HT (Applied Biosystems).

**Statistical Analysis**

Data were analyzed by 1-way ANOVA using Student-Newman–Keuls post hoc test.

**Results**

VDR is expressed in the heart, both in the neonatal rat cardiac myocyte (Figure 1A) and fibroblasts (Figure 1B) that populate the myocardium. Immunoreactive protein of the appropriate size (~55 kDa) was identified in extracts of both cell populations as well as inner medullary collecting duct cells, a population that has been shown to respond to vitamin D in previous studies. This immunoreactivity was competed effectively by antigenic VDR peptide (Figure 1C). Interestingly, treatment of either the myocyte (Figure 1A) or fibroblast (Figure 1B) cultures with the prohypertrophic vasoactive peptide ET resulted in a significant increase in levels of the VDR protein. A similar stimulation of VDR mRNA transcripts by ET was seen in both myocytes and fibroblasts (Figure 1D). ET-stimulated VDR mRNA expression was seen as early as 4 hours, peaked at 14 hours, and gradually declined at 24 hours in both types of cells (data not shown).

To assess the effects of the liganded VDR on the nonmyocyte population in the heart, we treated cells with VD3 for 36 to 48 hours before assessing proliferative (ie, H-thymidine incorporation) as well as synthetic effects (ie, procollagen I levels) in these cultures. As shown in Figure 3, VD3 treatment resulted in a significant reduction in both H-thymidine incorporation (Figure 3A) as well as procollagen I levels (Figure 3B), demonstrating that VD3 has important biological effects in these cells and suggesting that it may have antifibrotic activity in the interstitial compartment of the myocardium.

To confirm the biological activity of VD3 in the myocyte cultures, we transfected an hBNP luciferase reporter into

![Figure 1](image1.png)

**Figure 1.** ET increases VDR protein and mRNA expression in cardiac myocytes and fibroblasts. ET stimulates VDR protein expression in a dose-dependent fashion in cardiac myocytes (n=3 to 5; A) and fibroblasts (n=4; B). C, VDR immunoreactivity was blocked by VDR-competing peptide (CP). The protein from cardiac myocytes (CM), fibroblasts (F), heart tissue (H), and inner medullary collecting duct (IMCD) cells were transferred onto membranes and incubated with VDR antibody alone or with the antibody-competing peptide mixture. D, ET (10⁻⁷ mol/L) increases VDR mRNA levels in cardiac myocytes and fibroblasts. Quiescent cells were treated with ET for 14 hours, and total RNA was collected (n=3). *P<0.05; **P<0.01 vs control. C indicates control.

![Figure 2](image2.png)

**Figure 2.** Immunocytochemistry of VDR in cardiac cells. VDR was visualized by immunofluorescence in cardiac myocytes (100x; oil immersion) and in cardiac fibroblasts (60x). A, Cardiac myocytes were double stained with a polyclonal anti-VDR antibody, visualized with Cy3 (red)-conjugated anti-rabbit antibody, and sarcomere-staining, with monoclonal anti-α-actinin (green), visualized by AG488-conjugated anti-mouse antibody. VDR colocalized to the nucleus, stained with 4’,6-diamidino-2-phenylindole (DAPI; blue). B, Cardiac fibroblasts were double stained with anti-VDR, visualized with Cy3-conjugated anti-rabbit antibody and anti-vimentin, visualized with AG488-conjugated anti-mouse antibody. VDR colocalized to the nucleus stained with DAPI (n=3).
these cells. BNP gene expression and BNP promoter activity serve as surrogate markers of hypertrophy.\textsuperscript{13,20,21} We then treated them with VD3 or 1 of 2 nonhypercalcemic analogues of VD3 (ie, paricalcitol or active hectorol), followed by the prohypertrophic peptide ET. As shown in Figure 4A, treatment of cells with 10\textsuperscript{-7} mol/L ET resulted in a 2-fold increase in promoter activity in these experiments. As shown in the same panel, pretreatment with VD3 or the nonhypercalcemic analogues of VD3 resulted in a reduction in basal hBNP promoter activity and a significant reduction in the ET-dependent stimulation of promoter activity. In each case, the effective dose range was similar among the 3 VDR ligands. We also tested the ability of the VD3 precursor 25-hydroxyvitamin D to suppress basal hBNP promoter activity. As shown in Figure 4B, 25-hydroxyvitamin D also led to a reduction in hBNP promoter activity, although, perhaps, with somewhat lower potency (compare effect of 10\textsuperscript{-10} mol/L VD3 in Figure 4A with effect of 10\textsuperscript{-10} mol/L 25-hydroxyvitamin D in Figure 4B). The ability of the precursor to mirror activity of VD3 implies that these cultures may have the requisite enzymatic machinery to convert 25-hydroxyvitamin D to VD3 (ie, 1\textsuperscript{-α}-hydroxylase). As shown in Figure 4C, this was confirmed by Western blot analysis and real-time PCR, which demonstrated immunoreactive 1\textsuperscript{-α}-hydroxylase protein and mRNA in the myocyte cultures. In this case, protein and mRNA levels were unaffected by ET treatment. Expression of 1\textsuperscript{-α}-hydroxylase mRNA was also seen in cultured fibroblasts and was not changed following ET treatment (VD3 1.14\textpm 0.17-fold induction versus control; mean\textpm SD; n=3).

To determine whether VDR was directly associated with the hBNP promoter, we performed DNA immunoprecipitation experiments.

---

**Figure 3.** VD3 inhibits fibroblast proliferation and procollagen I synthesis. A, VD3 reduces 3H-thymidine incorporation in cultured ventricular fibroblasts. Values are normalized to controls in each experiment (n=4). B, VD3 inhibits procollagen I synthesis in cultured ventricular fibroblasts. Cells were treated with VD3 for 48 hours in serum substitute (SS) media. Procollagen I protein was detected by Western blot (n=4). Values are normalized to control in each experiment. *P<0.05 versus control. C indicates control.

**Figure 4.** VD3, 25-hydroxyvitamin D, paracalcitol, and active hectorol inhibit -1595 BNP promoter activity in cardiomyocytes. A, -1595 BNP-Luc and Renilla-Luc were cotransfected with VDR/retinoid X receptor (RXR) expression vectors into the myocytes. Twenty-four hours after transfection, cells were treated with different doses of VD3, paricalcitol, active hectorol, or vehicle for 48 hours and ET for 24 hours before collecting cells (n=3). BNP luciferase activity was normalized for Renilla luciferase activity. B, Ventricular myocytes were transfected with -1595 BNP-Luc, Renilla-Luc, and VDR/RXR expression vectors for 24 hours and then treated with different doses of 25-hydroxyvitamin D for 48 hours (n=5). C, 1\textsuperscript{-α}-hydroxylase expressed in cardiomyocytes as assessed by Western blot and real-time PCR (n=3). *P<0.05; **P<0.01 vs control; +P<0.01 vs ET alone. C indicates control.
tion analysis of myocyte cultures, previously transfected with the -198 hBNP-luciferase reporter, and then treated with VD3 in the presence or absence of ET. As shown in Figure 5A, this construct responded to VD3 treatment in a qualitatively similar fashion to the longer promoter construct used above. Promoter activity was reduced by \( \approx 30\% \) by VD3 treatment alone and by >80% when the cells were cotransfected with VDR and retinoid X receptor (heterodimeric partner of VDR) before VD3 treatment. Figure 5B exhibits a direct association of VDR with the hBNP promoter. This association was undetectable at baseline but increased after treatment with the VDR ligand. Treatment with ET resulted in an increase in VDR association with the promoter, and this was further amplified by addition of the VDR ligand.

The ET-dependent induction of VDR was an unexpected finding and raised the possibility that the activation of VDR expression was further amplified by addition of the VDR ligand. This finding and raised the possibility that the activation of VDR expression was further amplified by addition of the VDR ligand. This association was undetectable at baseline but increased after treatment with the VDR ligand. Treatment with ET resulted in an increase in VDR association with the promoter, and this was further amplified by addition of the VDR ligand.

The ET-dependent induction of VDR was an unexpected finding and raised the possibility that the activation of VDR expression was further amplified by addition of the VDR ligand. This association was undetectable at baseline but increased after treatment with the VDR ligand. Treatment with ET resulted in an increase in VDR association with the promoter, and this was further amplified by addition of the VDR ligand.

hypertrophy by ET might be the driving force behind the increased expression of the putatively antihypertrophic VDR (ie, a closed-loop feedback system). To test this further, we investigated cardiac VDR expression in a rat model of myocardial hypertrophy after 7 days of continuous isoproterenol infusion. This infusion reliably generates significant increases in cardiac mass and hypertrophy-dependent gene expression (eg, ANP) over this time interval. As shown in Figure 6A, isoproterenol-treated rats experienced a significant increase in left ventricular size when normalized either to body weight or tibial length. This was accompanied by a significant increase in left ventricular size when normalized either to body weight or tibial length. This was accompanied by a significant increase in left ventricular size when normalized either to body weight or tibial length. This was accompanied by a significant increase in left ventricular size when normalized either to body weight or tibial length. This was accompanied by a significant increase in left ventricular size when normalized either to body weight or tibial length. This was accompanied by a significant increase in left ventricular size when normalized either to body weight or tibial length. This was accompanied by a significant increase in left ventricular size when normalized either to body weight or tibial length. This was accompanied by a significant increase in left ventricular size when normalized either to body weight or tibial length. This was accompanied by a significant increase in left ventricular size when normalized either to body weight or tibial length.

We also observed a significant increment in VDR mRNA and protein levels in the isoproterenol-treated animals (Figure 6C) but no increase in...
the expression of 1-α-hydroxylase, 24-hydroxylase or GAPDH protein (Figure 6D), or the 1-α-hydroxylase mRNA (1.04±0.25-fold induction relative to control; mean±SD; n=4).

Discussion
The key findings of the present report are: (1) the demonstration of VDR in both cardiac myocytes and fibroblasts, as well as ventricular myocardium, using a combination of biochemical, immunofluorescence, and functional assays, (2) the demonstration of 1-α-hydroxylase in the same sources and 24-hydroxylase protein in the intact heart, (3) the demonstration that VDR has the capacity to bind directly to the BNP gene promoter, and (4) the finding that activation of myocyte hypertrophy, either in vitro or in vivo, is associated with a significant increase in VDR expression.

Previous studies have suggested a role of the VD3/VDR system in the control of cardiac function. Our group has shown previously that VD3 as well as less hypercalcemic analogues of VD3 demonstrate antihypertrophic activity in cultured neonatal rat cardiac myocytes.5,14,16 The VDR gene knockout mouse displays significant cardiac hypertrophy and activation of hypertrophy-dependent gene expression, which can be linked directly to increased myocyte size.9 Moreover, Bodyak et al recently demonstrated that the VD3 analogue paricalcitol reduces cardiac hypertrophy, without affecting blood pressure, in the Dahl salt-sensitive rat.8 A variety of clinical studies lend additional support. Vitamin D deficiency in early childhood is associated with significant cardiomyopathy and congestive heart failure.24 Several independent studies in adult patients have linked congestive heart failure, and cultured cardiac HL-1 cells.6 To our knowledge, ours is the first report that demonstrates VDR expression in both the myocyte and cardiac fibroblast and the first to document hypertrophy-dependent stimulation of VDR expression. The presence of VDR in both myocytes and fibroblasts implies that the potential exists for widespread effects of the liganded receptor in the heart (eg, suppression of myocyte hypertrophy and interstitial fibrosis). It also engenders a need to be cautious in interpreting effects of vitamin D on the heart in the whole animal. Such effects could result from direct interactions with the cardiac myocyte, indirect hemodynamic effects (eg, reductions in blood pressure), or indirect paracrine effects resulting from ligand-dependent interactions with neighboring fibroblasts.

The presence of 1-α-hydroxylase in myocytes and fibroblasts implies that the heart has the capacity to synthesize the bioactive VD3 metabolite independent of its production in the kidney, using circulating plasma 25-hydroxyvitamin D as substrate.27 Similar 1-α-hydroxylase immunoreactivity has been demonstrated in inflammatory cells,28 breast,29 colon,30 and prostate cancer cells.31 This is an important consider-
Acknowledgment

We are grateful to Martin Hewison for providing the anti–24-hydroxylase antibody used in these studies.

Sources of Funding

This work was supported by National Institutes of Health grants HL45637 (D.G.G.) and F32 HL086158 (D.J.G.), American Heart Association grant 0825140F (W.N.), and a grant from Abbott Laboratories.

Disclosures

None.

References

Expression of the Vitamin D Receptor Is Increased in the Hypertrophic Heart

Songcang Chen, Denis J. Glenn, Wei Ni, Christopher L. Grigsby, Keith Olsen, Minobu Nishimoto, Christopher S. Law and David G. Gardner

Hypertension. 2008;52:1106-1112; originally published online October 20, 2008;
doi: 10.1161/HYPERTENSIONAHA.108.119602

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 2008 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://hyper.ahajournals.org/content/52/6/1106

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Hypertension can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Hypertension is online at:
http://hyper.ahajournals.org//subscriptions/