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# 1,25-Dihydroxyvitamin $D_3$ Regulation of Myocardial Growth and *c-myc* Levels in the Rat Heart*

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In this report we demonstrate that rats maintained on a calcium supplemented vitamin $D_3$-deficient diet for nine weeks showed an increase in heart to body weight ratio. Morphometric analysis indicated that vitamin $D_3$ deficiency produced cardiac myocytes that were smaller yet more numerous, indicating hyperplasia. Western blot analysis showed that vitamin $D_3$ deficiency also resulted in increased *c-myc* protein levels in the hearts of vitamin $D_3$-deficient rats. Furthermore, 1,25-dihydroxyvitamin $D_3$ reduced *c-myc* protein levels in primary cultures of ventricular myocytes from neonatal rat hearts. Our data suggest a possible relationship between myocyte hyperplasia and increased *c-myc* levels in the vitamin $D_3$-deficient rat heart. 

The identification of a high affinity, low capacity receptor for $1,25(OH)_2D_3$ in cultured cardiac myocytes and whole left ventricle (1, 2) was the first direct evidence for the heart as a target tissue for vitamin $D_3$. Our laboratory provided further evidence supporting the idea that the heart is a target for $1,25(OH)_2D_3$ and described the effects of vitamin $D_3$ depletion on cardiac morphology and function (3, 4, 5). We previously showed that vitamin $D_3$ deficiency increased myocardial contractility in rats (3, 4). Furthermore, the augmentation of ventricular contractility in response to vitamin $D_3$ deficiency was not caused by hypocalcemia, which normally accompanies vitamin $D_3$ deficiency. We also found vitamin $D_3$ deficiency induced cardiac hypertrophy, observed as an increase in the heart to body weight ratio (5). Again, this response was a direct effect of vitamin $D_3$ depletion, not hypocalcemia (5). Thus, there may be an

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association between the effects of vitamin $D_3$ on cardiac contractility and the structural and morphological changes in the heart caused by vitamin $D_3$ deficiency.

In the present study we evaluated the effects of vitamin $D_3$ deficiency on myocardial growth. Our data suggest that cardiac hypertrophy produced by vitamin $D_3$ deficiency (5) is accompanied by myocyte hyperplasia. Further, we found that vitamin $D_3$ deficiency increased *c-myc* protein levels in the hearts of vitamin $D_3$-deficient rats. Finally, we observed that $1,25(OH)_2D_3$ directly decreased *c-myc* protein levels in primary cultures of ventricular myocytes.

## Methods

*Animals*

Sprague-Dawley 21-day-old male weanling rats (Holtzman Rats Co., Madison, WI) from mothers fed low vitamin $D_3$ diets were raised for nine weeks on 15 g/day of a vitamin $D_3$-sufficient (2 IU/g of food/day) or -deficient diet, as previously described (3, 4). The diets contained 2.5% calcium and 1.5% phosphate (TEKLAD diet #TD 85029), which maintained normal serum concentrations of these ions, preventing secondary effects of vitamin $D_3$ deficiency due to altered serum calcium and phosphate levels (3, 6). Rats were killed by rapid decapitation, and blood samples were collected from the trunk of the animal. All procedures for animal care and experimentation were stringently reviewed and approved by the University of Michigan Animal Research Committee.

*Culture of Cardiac Myocytes*

Myocytes were obtained from hearts of day old rats by the serial trypsinization technique as previously described (7). Briefly, animals were killed and the hearts removed, minced and placed in a trypsin solution at room temperature. Cells were dissociated by gentle pipetting. Dissociated cells were removed, washed and placed in Dulbecco's modified eagle medium (DMEM) containing 10% fetal calf serum (FCS) and 10,000 U/ml penicillin - 10 mg/ml streptomycin (PS), and the cycle repeated until the cells were completely dissociated. Contaminating nonmuscle cells were removed by preplating for 3 h. The unattached myocytes were plated in DMEM with 10% FCS and PS, 5 mM taurine, 5 mM creatine, 2 mM L-carnitine and 10 $\mu$M ara-C for approximately 36 h. The medium was then changed and myocytes were grown in DMEM with 10% FCS, PS, 5 mM taurine, 5 mM creatine, and 2 mM L-carnitine and treated with vehicle (0.1% EtOH) or increasing concentrations of $1,25(OH)_2D_3$ (0.1 nM, 1 nM, 10 nM or 100 nM) for 72 h.

*Measurements of Serum Calcium and $1,25(OH)_2D_3$*

Blood samples were allowed to clot and the serum was decanted after centrifugation. Serum calcium was determined by colorimetric analysis by the method of Kessler and Wolfman (8). Serum $1,25(OH)_2D_3$ was measured according to the methods of Reinhardt *et al.* (9). Vitamin $D_3$ metabolites were extracted using acetonitrile and then partially purified using a $C_{18}$ cartridge. A $1,25(OH)_2D_3$-receptor-protein competitive binding assay (Immuno Nuclear, Stillwater, MN) was used to quantitate $1,25(OH)_2D_3$ levels. The limit of detection was 10.0 pg/ml $1,25(OH)_2D_3$.

*Quantitative assessment of morphological changes in myocardial tissue*

Changes in myocardial morphology were measured using methods described by Rubin and Roberts (10). Preparation of tissue sections was previously described (5). Sections of ventricular muscle were examined under a Carl Zeiss microscope (model III RS) with a high-resolution camera integrated

with a Reichert image analysis system. The area of specific regions within a section could be determined with a digitizer tablet calibrated at an overall magnification of X2,350. Three or four sections per specimen were evaluated, and for each section two regions were examined (6-8 measurements per specimen). The following parameters were measured: total myofibril area, average area and maximum diameter of each myofibril, number of myofibrils per region and total nonmyofibrillar area.

*Western Blot Analysis of c-myc*

For analysis of *c-myc* protein rats were killed, and the heart was rapidly excised and perfused free of blood. The heart was weighed (wet weight) and sectioned. Left ventricular tissue was placed in PBSTDS (100 mM $Na_2PO_4$, 150 mM NaCl, 1% (v/v) Triton X-100, 0.5% sodium deoxycholate, 0.1% lauryl sulfate (SDS), 0.2% $NaN_3$ and 0.004% NaF, pH 7.4). Protein concentration was determined by the method of Bradford (11). 1 μg/ml of *c-myc* antibody (*c-myc* AB-3, Oncogene Science, Manhasset, NY) was added to 4 mg of cytosolic protein and the samples rotated overnight at 4°C. For analysis of *c-myc* protein levels in primary cultures of ventricular myocytes, cells were washed two times with PBS and removed from the dish using a rubber policeman. The cells were then counted and washed again in PBS. *c-myc* protein was immunoprecipitated from an equal number of cells in each condition by first lysing the cells in PBSTDS. 1 μg/ml of *c-myc* antibody (*c-myc* AB-3) was added to the lysed cells and the samples rotated overnight at 4°C. After overnight incubation with the antibody, the procedure was the same. Protein A-Sepharose was added to the samples and incubated for 2 h at 4°C. The Protein A-Sepharose was pelleted and then washed 3X with PBSTDS. Samples were run on a 10% polyacrylimide gel and the proteins transferred to Immobilon paper (Millipore, Bedford, MA). The blot was blocked with buffer containing 1% bovine serum albumin (10 mM tris, 0.1% tween-20, and 1% BSA, pH 7.4). The blot was probed for 1.5 h with the primary antibody (*c-myc* AB-3), then washed 3X with blocking buffer and incubated for 1 h with a secondary antibody conjugated with horse radish peroxidase. The blot was then washed 5X with tween-TBS (10 mM tris and 0.2% tween-20, pH 7.4). Finally, the blot was developed using enhanced chemiluminessence (Amersham, Clearbrook, IL) and exposed to X-ray film.

## Results

*Effects of vitamin $D_3$ depletion on heart size and morphology*

As shown in Table 1, a significant increase in the heart to body weight ratio was observed in vitamin $D_3$-deficient rats raised on a high calcium (2.5%), vitamin $D_3$-deficient diet (body weight was not significantly altered by vitamin $D_3$ deficiency, data not shown). By nine weeks, the rats were depleted of $1,25(OH)_2D_3$, (Table 1) and since both the vitamin $D_3$-sufficient and -deficient rats were normocalcemic (Table 1), cardiac hypertrophy appears to be a direct result of vitamin $D_3$ deficiency. This agrees with previous reports in which we found that vitamin $D_3$ deficiency induced cardiac hypertrophy (5). In addition, we examined liver, kidney, spleen, and stomach organ to body weight ratios in the same rats. However, we found no significant changes in these ratios as a result of vitamin $D_3$ depletion (data not shown).

Morphometric analysis was performed on thin sections of hearts from both vitamin $D_3$-sufficient and -deficient rats. Thin sections prepared from

**Table 1.** Serum $1,25(OH)_2D_3$ and calcium levels and heart/body weight ratios for rats maintained on a vitamin $D_3$-deficient or -sufficient diet

| Diet Type | Weeks on Diet | $1,25(OH)_2D_3$ (pg/ml) | Calcium (mg/dl) | Heart/Body Wt. Ratio[a] |
|---|---|---|---|---|
| Sufficient | 3 | 70.3 ± 15.2 | 10.37 ± 1.06 | N.D.[c] |
| Deficient | 3 | 84.2 ± 15.8 | 9.01 ± 0.42 | N.D. |
| Sufficient | 9 | 86.8 ± 5.2 | 8.00 ± 0.60 | 3.07 ± 0.07 |
| Deficient | 9 | 11.1 ± 5.0[b] | 9.13 ± 0.22 | 3.50 ± 0.10[d] |

Data represent the mean ± S.E.M. for at least three rats in each condition. [a]Value is x $10^{-3}$; [b]$p = 0.0001$; [c]N.D. = not determined; [d]$p = 0.024$.

Male weanling rats were fed either a vitamin $D_3$-sufficient or -deficient diet (see methods). Serum $1,25(OH)_2D_3$ and calcium levels were measured as outlined in the methods section. Heart to body weight ratio was determined by weighing rats, then killing the rat and removing the heart, washing with phosphate buffered saline, drying and weighing the heart. Statistical analysis was performed using the two-tailed Students t Test and significant differences are noted.

vitamin $D_3$-deficient rats had a decrease in average myofibrillar area of 64.4 $\mu m^2$ ($p < 0.05$) (Table 2) as well as a reduction in average maximum fibril diameter of 4.4 $\mu m$ ($p < 0.05$) (Table 2). However, vitamin $D_3$-deficient rats showed an increase in average number of myofibrils per region analyzed (Table 2). This suggests that vitamin $D_3$ deficiency induced hyperplasia in the developing rat heart.

**Table 2.** Morphometric analysis of hearts from rats maintained on a vitamin $D_3$-deficient or -sufficient diet

| Measurement | Vitmain $D_3$-Sufficient Rats | Vitamin $D_3$-Deficient Rats |
|---|---|---|
| Average Area ($\mu m^2$) | 169.5 ± 24.1 | 105.1 ± 9.3[a] |
| | (6) | (5) |
| Average Max Diam ($\mu m$) | 17.7 ± 1.5 | 13.3 ± 0.6[a] |
| | (6) | (5) |
| Number/Region | 58 ± 7 | 75 ± 6[a] |
| | (6) | (5) |

Data represent the mean ± S.E.M. for the number of measurements shown in parentheses. [a]$p < 0.05$.

Male weanling rats were fed either a vitamin $D_3$-sufficient or -deficient diet (see methods). Determinations of average myofibril area, average maximum myofibril diameter and number of myofibrils in the region analyzed were made. Statistical analysis was performed using the two-tailed Students t Test and significant differences are noted.

*c-myc protein levels in hearts from vitamin $D_3$-sufficient and -deficient rats and in primary cultures of ventricular myocytes treated with 1,25(OH)$_2$D$_3$*

To characterize the effect of vitamin $D_3$ deficiency on *c-myc* protein levels in the heart, rats were maintained on either a high calcium vitamin $D_3$-sufficient or -deficient diet. Western blot analysis was performed to detect differences in myocardial *c-myc* protein levels. Figure 1 shows a clear increase in the myocardial *c-myc* protein levels in vitamin $D_3$-deficient rats.

We also analyzed *c-myc* protein levels in primary cultures of ventricular myocytes treated with 1,25(OH)$_2$D$_3$. Our cultures were at least 90% myocytes as determined by their morphology and number of beating cells (personal observation). Myocytes were maintained in an immature and proliferating state by growing the cells in medium supplemented with 10% FCS (12). The levels of the *c-myc* protein were analyzed by Western blot analysis as shown in Figure 2. Figure 2 demonstrates that 1,25(OH)$_2$D$_3$ decreased the levels of the *c-myc* protein in a concentration-dependent manner, suggesting that 1,25(OH)$_2$D$_3$ directly regulates *c-myc* protein levels in cardiac myocytes.

## Discussion

The experiments described herein characterized the effects of vitamin $D_3$ deficiency on cardiac muscle structure and *c-myc* levels. Our results demonstrate that vitamin $D_3$ deficiency increased heart to body weight ratios in the developing rat, in agreement with our previous report (5). This finding was not secondary to vitamin $D_3$ induced hypocalcemia, since serum calcium was maintained at normal levels throughout the study. Additionally, we found that the increase in organ to body weight ratio was specific to the heart in that liver, spleen, kidney and stomach organ to body weight ratios were not changed by vitamin $D_3$ deficiency. This study also demonstrated that vitamin $D_3$ deficiency induced myocyte hyperplasia. We previously reported an increase in myocardial collagen content in response to vitamin $D_3$ deficiency (5). Therefore,

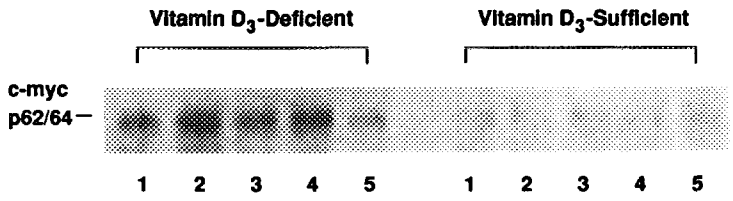


**Figure 1.** Effect of vitamin $D_3$ depletion on *c-myc* protein levels in rat heart. Rats were maintained for nine weeks on either a high calcium (2.5%) vitamin $D_3$-sufficient or -deficient diet. *c-myc* protein levels were determined by Western blot after immunoprecipitation of *c-myc* from whole heart tissue. The figure shows a representative Western blot depicting the *c-myc* 62/64 kD doublet. Each lane represents protein samples from 1 rat.

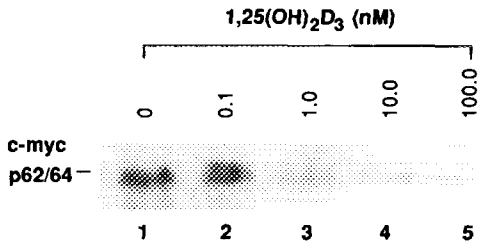


**Figure 2**. Effect of $1,25(OH)_2D_3$ on *c-myc* protein levels in primary cultures of ventricular myocytes. *c-myc* protein levels were determined by Western blot after immunoprecipitation from primary cultures of rat ventricular myocytes treated with: Lane 1) 0.1% ethanol (all groups contained 0.1% ethanol), Lane 2) 0.1 nM $1,25(OH)_2D_3$, Lane 3) 1 nM $1,25(OH)_2D_3$, Lane 4) 10 nM $1,25(OH)_2D_3$ or Lane 5) 100 nM $1,25(OH)_2D_3$ for 72 h. The figure shows a representative Western blot depicting the *c-myc* 62/64 kD doublet.

it appears that cardiac hypertrophy due to vitamin $D_3$ deficiency may be at least partly caused by a combination of myocyte hyperplasia and increased collagen deposition in the extracellular matrix.

This study also shows that in the rat heart, *c-myc* protein levels are regulated by vitamin $D_3$ deficiency. The increase in myocardial *c-myc* protein levels is correlated with the appearance of cardiac hypertrophy in vitamin $D_3$-deficient rats. This is consistent with data that demonstrate that *c-myc* protein levels are reduced by $1,25(OH)_2D_3$ in primary cultures of ventricular myocytes. These observations, taken together, strongly suggest that $1,25(OH)_2D_3$ acts directly on cardiac myocytes to downregulate *c-myc* protein levels in the heart.

Several studies suggest a correlation between *c-myc* expression and the induction of cardiac hypertrophy. Bauters *et al.* showed that *c-myc* expression rose proportionally with increased coronary flow in isolated, perfused, beating hearts, but was not expressed in ischemic or KCl-arrested hearts (13). Pressure overload was shown to rapidly increase *c-myc* expression and to induce cardiac hypertrophy (14). Furthermore, Starksen *et al.* demonstrated that cardiac hypertrophy resulting from catecholamine administration was associated with elevated *c-myc* mRNA levels in primary cultures of cardiac myocytes (15). Thus, it is possible that depletion of vitamin $D_3$ might increase *c-myc* expression in the mature heart that, in turn, would result in cardiac hypertrophy. However, the down regulation of *c-myc*, while not a causative factor leading to myocyte maturation (16, 17), may play a role in regulating myocyte proliferation (18, 19). Specifically, Schneider *et al.*, who studied the expression of *c-myc* during heart development, reported that *c-myc* was expressed at high levels during early embryogenesis, when cardiac myocytes are actively replicating (18). As fetal development continued, *c-myc* expression steadily declined until it was virtually undetectable eight to 16 weeks

postpartum. Additionally, it was demonstrated in transgenic mice that maintaining high levels of *c-myc* expression throughout myocardial development leads to myocyte hyperplasia (19). This finding is in direct agreement with our finding that increased *c-myc* protein levels led to myocyte hyperplasia. Therefore, from these studies it appears that depletion of vitamin $D_3$ leads to a chronic elevation of *c-myc* protein levels that may lead to myocyte hyperplasia and myocardial hypertrophy in the vitamin $D_3$-deficient heart.

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