Published in final edited form as:
J Steroid Biochem Mol Biol. 2021 February ; 206: 105804. doi:10.1016/j.jsbmb.2020.105804.

Early-life persistent vitamin D deficiency-induced cardiovascular dysfunction in mice is mediated by transient receptor potential C channels

Kimberly Stratford[1], **Najwa Haykal-Coates**[2], **Leslie Thompson**[3], **Aimen Farraj**[3], **Mehdi Hazari**[3,*]

[1]Curriculum in Toxicology and Environmental Medicine, University of North Carolina – Chapel Hill, Chapel Hill, NC, 27599

[2]Inhalation Toxicology Facilities Branch, Public Health and Integrated Toxicology Division, Center for Public Health and Environmental Assessment, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711

[3]Cardiopulmonary and Immunotoxicology Branch, Public Health and Integrated Toxicology Division, Center for Public Health and Environmental Assessment, U.S. Environmental Protection Agency, Research Triangle Park, NC 27711

Abstract

Background -—Studies indicate that chronic vitamin D deficiency (VDD) may predispose to hypertension, yet, there is very little data characterizing its direct cardiac effects. Vitamin D modulates the function of transient receptor potential C cation channels (TRPC), which is a mechanosensitive cation channel that plays a role in cardiac slow-force responses to hemodynamic changes. The purpose of this study was to determine the cardiac effects of VDD and the potential role of TRPC.

Methods -—Three-week old mice were placed on a VDD or normal diet (ND) for 19 weeks. Mice were then implanted with radiotelemeters for the measurement of heart rate (HR) and heart rate variability (HRV), while a separate group was anesthetized to measure blood pressure (BP) and left ventricular function using an intraventricular probe. Animals were treated with a TRPC antagonist or vehicle after which they were challenged with dobutamine to measure cardiac responses.

Results -—VDD mice had significantly increased BP (72 ± 3 mmHg vs. 62 ± 2 mmHg) and left ventricular pressure (LVP) (84.6 ± 0.8 mmHg vs. 78.2 ± 2.0 mmHg), and decreased cardiac contractility (− 3% vs. + 11%) and HR response (+ 8% vs. + 13%) to dobutamine when compared

[*]Corresponding author: Mehdi S. Hazari, Public Health and Integrated Toxicology Division, Center for Public Health and Environmental Assessment, USEPA, 109 Alexander Drive, B105; Research Triangle Park, NC 27711; (Phone: 919-541-4588; Fax: 919-541-0034; hazari.mehdi@epa.gov).

The Author(s) declare(s) that there is no conflict of interest.

to ND. These responses were blocked by the TRPC antagonist. HRV decreased with increasing dobutamine doses in ND but not VDD mice, however, the antagonist had no effect.

Conclusion -—VDD increases BP and alters cardiac mechanical function in mice, the latter appears to be mediated by TRPC, in particular TRPC6. Although the cardiac effects might be due to increased BP, it is likely that VDD also affects the function of the heart directly. This is the first study to demonstrate the potentially deleterious effects of VDD on cardiac function and the role of TRPC6 in this response.

Keywords

Vitamin D deficiency; cardiovascular; hypertension; TRPC

Introduction

Vitamin D is one of the only micronutrient molecules that plays a critical homeostatic role in all organ systems and is endogenously produced in the body[1,2]. It is a fat-soluble vitamin synthesized in the skin after exposure to ultraviolent B radiation, or acquired through the diet from a variety of foods like milk, fish, and cheese[3]. Vitamin D receptors (VDR) are present on numerous tissues and cells throughout the body, including cardiomyocytes. VDR exert their effects by forming a heterodimer with the retinoid-x receptor, which then binds to vitamin D response elements (VDRE) on DNA[1,4], and as a result influences expression of a wide variety of genes (e.g. calcium homeostasis, antioxidant production, protein synthesis), including those involved in cardiovascular regulation.[4–7]

Reduced sun exposure, insufficient nutrition, as well as a myriad of other factors result in vitamin D deficiency (VDD)[1,8], which has become a global public health concern affecting 8% of the pediatric population in the United States[9]. In addition to commonly described bone-related maladies, a few animal and epidemiological studies have demonstrated that VDD might be linked to the development of cardiovascular disease and can increase the risk of hypertension[2,10–15]. Vitamin D has been shown to be a negative regulator of the renin-angiotensin-aldosterone system (RAAS)[16], which is activated in hypertensive and atherosclerotic mouse models fed a VDD diet[15]. In addition, early-life or childhood VDD can lead to vascular dysfunction, hypertension and other cardiac abnormalities[17,18]. However, despite this recent data[19,20], the association with disease development and an underlying mechanism are still not firmly established, nor is its precise long-term effect on autonomic balance and cardiac mechanical function.

More and more studies have documented the role of transient receptor potential cation channels in cardiovascular function, particularly transient receptor potential canonical 6 (TRPC6) channels, which respond to mechanical stress and are expressed in cardiomyocytes and vascular smooth muscle cells[21–23]. Increased expression of TRPC6 has been linked to cardiovascular disease, hypertrophy and hypertension[21,24–25]. Moreover, mice treated with isoproterenol to induce cardiac stress and hypertrophy, similar to what is observed in hypertension, have increased TRPC6 mRNA expression in the heart. Previous studies also suggest that inhibition or deletion of TRPC6 in such states is cardioprotective and preserves normal function[26]. More importantly, vitamin D appears to decrease TRPC6 expression[27].

Together, these data suggest that limited expression of TRPC6 in the heart is normal under healthy conditions, however, it's increase and altered function due to persistent cardiac stress (e.g. high aortic blood pressure, ischemia-reperfusion), or possibly due to VDD, may be potentially detrimental.

The purpose of this study was to determine the role of TRPC6 in VDD-induced cardiovascular mechanical changes and responses to dobutamine challenge stress in adult mice. We hypothesized that mice with persistent VDD would have increased blood pressure when compared to normal mice, and that VDD would cause baseline cardiac mechanical changes that are mediated by TRPC6.

Materials and Methods

Animals -

Female C57BL/6 mice (Jackson Laboratory, Raleigh, NC) were housed five per cage and maintained on a 12-hr light/dark cycle at approximately 22°C and 50% relative humidity in an AAA-LAC-approved facility. Food (Prolab RMH 3000; PMI Nutrition International, St. Louis, MO) and water were provided ad libitum during the quarantine period (3 days) after arrival. All protocols were approved by the Institutional Animal Care and Use Committee of the U.S. Environmental Protection Agency and are in accordance with the National Institutes of Health Guides for the Care and Use of Laboratory Animals. The animals were treated humanely and with regard for alleviation of suffering.

Diet -

Mice were maintained ad libitum on either a vitamin D deficient (VDD) (D10073001-Research Diets Inc.) or normal diet (ND) (D10012G-Research Diets Inc.) for 19 weeks. The VDD diet had no added vitamin D but had 40% kcal vitamin mix V10037. The ND had 1000 IU per 10 grams of vitamin D. We previously showed that this model causes circulating vitamin D levels to be low in VDD mice when compared to ND mice[28]. The diets had equal levels of all other vitamins and minerals including calcium, which was at the concentration specified by the American Institute of Nutrition[29]. Water was provided ad libitum throughout the diet regimen.

Experimental Design and Groups -

At the beginning of the study, three-week old mice were randomly assigned into a ND (n = 20–25) or VDD (n = 20–25) group and maintained on those diets for the extent of the study. Of those animals, 9 of each diet were randomly chosen and implanted with radiotelemeters at 22 weeks of age (Group A). The remaining mice from each diet group were surgically implanted with a Millar probe (SPR-671, ADInstruments, Colorado Springs, CO) and used for intraventricular assessments (Group B). The study design is shown in Fig. 1. See Supplemental Material for echocardiographical assessments of ND and VDD mice.

Drugs -

TRPC6 antagonist GsMTx4 (2mg/kg, Tocris, Minneapolis, MN) was dissolved in saline. Two doses of freshly diluted dobutamine hydrochloride (Hospira, Lake Forest, IL) were

prepared and administered intraperitoneally at 0.5 (low dose) and 1.5 mg/kg (high dose). Previous studies describe increased HR after acute intraperitoneal administration of dobutamine[30–31]. Dobutamine stress test was performed on Group A mice at 0.5 mg/kg (low) and 1.5 mg/kg (high). Twenty-four hours later Group A mice were pre-treated with the TRPC6 antagonist (2 mg/kg, i.p.) for 20 minutes prior to repeating the dobutamine stress test. For Group B, after insertion of the Millar probe, mice were pre-treated with either vehicle or the TRPC6 antagonist for 20 minutes prior to the dobutamine stress test.

Surgical Implantation of Radiotelemeters -

Mice were implanted with radiotelemeters as previously described[32]. Briefly, animals were anesthetized using inhaled isoflurane (Isothesia, Butler Animal Health Supply, Dublin, OH). Anesthesia was induced by spontaneous breathing of 2.5% isoflurane in pure oxygen at a flow rate of 1 L/min and then maintained by 1.5% isoflurane in pure oxygen at a flow rate of 0.5 L/min; all animals received the analgesic buprenorphrine (0.03 mg/kg, i.p. manufacturer). Using aseptic technique, each animal was implanted subcutaneously with a radiotelemeter (ETA-F10, Data Sciences International, St Paul, MN); the transmitter was placed under the skin to the right of the midline on the dorsal side. The two electrode leads were then tunneled subcutaneously across the lateral dorsal sides; the distal portions were fixed in positions that approximated those of the lead II of a standard electrocardiogram (ECG). Body heat was maintained both during and immediately after the surgery. Animals were given food and water post-surgery and were housed individually. All animals were allowed 7–10 days to recover from the surgery and reestablish circadian rhythms.

Radiotelemetery Data Acquistion -

Radiotelemetry methodology (Data Sciences International, Inc., St. Paul, MN) was used to track changes in cardiovascular function by monitoring heart rate (HR) and ECG waveforms immediately following telemeter implantation and through exposure until the end of exposure. This methodology provided continuous monitoring and collection of physiologic data from individual mice to a remote receiver. Sixty-second ECG segments were recorded every 15 minutes during the 24 hours pre-experiment and immediately (imm.) before dobutamine stress test experiment periods and continuously during the experiment; HR was automatically obtained from the waveforms (Dataquest ART Software, version 4.0, Data Sciences International, St. Paul, MN). Baseline was defined as the 4 hours before the start of dobutamine stress test.

Measurement of Intraventricular Pressure -

Mice were anesthetized with urethane (1.5mg/kg intraperitoneally, Sigma) and then prepared for intraventricular measurements. While in a supine position and after achieving the appropriate anesthetic plane, the cervical region of the mouse was dissected exposing the right carotid artery. After isolation, the artery was catheterized with a 1.4 French Millar transducer (SPR-671, ADInstruments, Colorado Springs, CO). The probe was connected via a Pressure Control Unit (PCU- 2000) to a receiver (Powerlab 8/30) and a computer acquiring data at 1000 Hz (LabChart Pro. 7.3.8). The probe was advanced while continuously monitoring the pressure trace until entry into the left ventricle was confirmed. Once in the left ventricle, a 4-min baseline was measured followed by dobutamine administration.

Left ventricular pressure (LVP), dP/dTmax or the rate of left ventricular pressure rise and dP/dTmin or the rate of left ventricular pressure decrease were measured continuously during the baseline period, dobutamine stress test and recovery. Adequate time was allowed for cardiac parameters to return to baseline between successive dobutamine doses.

Heart Rate Variability Analysis -

Heart rate variability (HRV) was calculated as the mean of the differences between sequential RR intervals for the complete set of ECG waveforms using ECGAuto. For each 1-min stream of ECG waveforms, mean time between successive QRS complex peaks (RR interval), mean HR, and mean HRV-analysis–generated time-domain measures were acquired. The time-domain measures included standard deviation of the time between normal-to-normal beats (SDNN), and root mean squared of successive differences (RMSSD). HRV analysis was also conducted in the frequency domain using a Fast-Fourier transform. The spectral power obtained from this transformation represents the total harmonic variability for the frequency range being analyzed. In this study, the spectrum was divided into low-frequency (LF) and high-frequency (HF) regions. The ratio of these two frequency domains (LF/HF) provides an estimate of the relative balance between sympathetic (LF) and vagal (HF) activity.

Tissue Collection and Analysis -

Radiotelemeter implanted mice were euthanized 24 hours after dobutamine stress test and blood collected, processed and analyzed. Vitamin D concentrations were confirmed in the serum spectro-photometrically using a Vitamin D EIA Kit (Cayman Chemical, Ann Arbor, Michigan) and were similar to levels previously observed and published (Stratford, 2018).

Statistics -

All data were analyzed using SAS 9.4 (SAS Institute Inc., Cary, NC) software. Mixed-model ANOVAs followed by Tukey’s procedure for the post hoc comparisons were used to examine the statistical differences between TRPC6 antagonist and diet, and repeated measures analysis was used as needed. The statistical significance was set at $p < 0.05$.

Results

Heart rate –

Before the start of the dobutamine stress test, the baseline HR of ND and VDD mice was similar. Dobutamine caused an increase in the HR of ND mice, albeit not in a dose-dependent manner, and the response was significantly decreased in VDD mice. Treatment with the TRPC6 antagonist had no effect on the baseline HR of either diet group, however, it blocked the decreased response to dobutamine in VDD mice making it similar to ND. There was no effect of the TRPC6 antagonist on the dobutamine response of ND mice (Figure 2).

Heart rate variability –

During the baseline, SDNN, which indicates overall autonomic function, RMSSD, which indicates parasympathetic influence on the heart, and LF and HF, which represent the

balance between both autonomic branches and other inputs into the heart, were not significantly different between ND and VDD mice. Dobutamine caused a decrease in SDNN and LF only at the high dose in ND mice, but there were no effects on RMSSD or HF. In contrast, both low and high dobutamine caused a significant decrease in SDNN, RMSSD, LF and HF in VDD mice. Pretreatment of ND mice with the TRPC6 antagonist caused SDNN and LF to become further decreased during dobutamine challenge and caused RMSSD and HF to decrease with the high dose. On the other hand, the TRPC6 antagonist had no effect on SDNN in VDD mice but blocked the decreases in LF and HF (Figure 3).

Blood pressure and baseline left ventricular function –

There was no difference in the diastolic pressure between the diets (Figure 4A), however, VDD mice had increased systolic pressure (Figure 4B) and mean arterial pressure (Figure 4C) when compared to ND. Similarly, baseline LVP was increased in VDD mice, this response was blocked by the TRPC6 antagonist (Figure 4D). The effect of the TRPC6 antagonist on blood pressure was not measured because the drug was administered after the probe was advanced into the left ventricle.

Left ventricular contractility and relaxation –

Figure 5 shows the percent change in dP/dTmax and dP/dTmin from baseline during dobutamine challenge in ND and VDD mice. Low dobutamine increased dP/dTmax or contractility in ND mice, this was not significantly different for VDD mice nor did the TRPC6 antagonist have an effect on either diet (Figure 5A). High dobutamine also caused contractility to increase in ND mice but not as much as the low dose. In contrast, VDD mice had a significantly decreased response to the high dose when compared to ND and this effect was blocked by the TRPC6 antagonist (Figure 5B). Although not statistically significant, VDD mice had a trend of decreased dP/dTmin or ventricular relaxation during the dobutamine challenge which appeared to be reversed by TRPC6 blockade (Figure 5C and D). Raw data for intraventricular function are in Table S4 in the Supplemental Material.

Echocardiography was performed on mice after 13 weeks on either the normal or vitamin D deficient diets. The differences were minor and can be found in the Supplemental Material. Echocardiography was not performed on animals treated with the TRPC6 antagonist, future studies will examine these effects.

Discussion

This study demonstrates that persistent VDD in mice causes cardiac dysfunction and altered responses to stress. Although chronic VDD was previously shown to increase the risk of hypertension, it was unknown whether there were any cardiac effects, either due to direct impacts on the heart or as a result of the increased blood pressure. Our results confirm that VDD causes increased blood pressure, and further show that cardiac contractile mechanics are altered. Moreover, this is the first study to demonstrate that VDD-induced mechanical changes in the heart are mediated by TRPC6, which is known to be involved in cardiac hypertrophic dysfunction.

In this study, dobutamine was used to increase heart rate and contractility, and thus, unmask latent cardiac effects that otherwise may not have been evident. Heart rate is an intrinsic and dynamic characteristic of the heart and therefore the degree to which it fluctuates during sudden changes can be a profound indicator of cardiac dysfunction. HR was not significantly different between normal and VDD mice at rest, however, the latter had decreased HR responses to both the low and high dose of dobutamine. In a study of middle-aged men, blunted heart rate response to exercise was associated with increase cardiovascular mortality[33]. Furthermore, other pharmacological cardiac stress tests, for instance with dipyridamole, have shown the same heightened risk of cardiovascular complications when people had a blunted heart rate response[34], which could be due to altered autonomic regulation[35] or change in the expression and signal transduction of β1-adrenergic receptors, which rely on the presence of vitamin D both locally as well as centrally[36,37].

The blunted heart rate response to dobutamine of VDD mice was blocked by the TRPC6 antagonist. Interestingly, the drug had no effect on resting heart rate in either normal or VDD mice. This suggests that the role of TRPC6 is related to neurohormonal or mechanical stress-induced cardiac changes[25] that do not necessarily occur under normal physiological conditions but rather during pressure overload and/or oxidative stress. The fact that we did not see a dose-response to dobutamine or a more robust response, both with and without the drug, indicates that mouse sensitivity to this challenge is quite low[38]. The TRPC6 antagonist likely prevented calcium overload and raised the mechanical threshold for activation, which appropriately explains the restoration of responsiveness to dobutamine[39,40].

The chief physiological role of TRPC6 in the heart is calcium[41], which contributes to pacemaker function and stretching of myocardium for the ejection of blood against afterload. An increase in TRPC6 expression even in the sinoatrial node and right atria contributes to its pathological role and is associated with cardiovascular disease[21,22]. Interestingly, increased calcium handling and TRPC6 expression also increases calcium sensing receptor expression, which has consequently been shown to be reversed by vitamin D supplementation[42]. As far as our model is concerned, it is unclear whether vitamin D supplementation would reverse the effect of TRPC6 on the function of the heart, whether through the pathway described above or otherwise. Calcium is likely the key mediator in VDD-induced cardiovascular dysfunction and increased TRPC6 expression. TRPC6 expression in the heart showed an increasing trend in VDD mice in this study (data not shown).

Persistent VDD can result in autonomic imbalance and hypertension, even in otherwise healthy adults[23,43]. This is not surprising given HRV is not only influenced by HR but also blood pressure and respiratory patterns[44]. SDNN and LF, both of which indicate sympathetic modulation of the heart, appeared to be more decreased during dobutamine challenge in VDD mice than normal. A similar trend was observed with RMSSD and HF, which reflect parasympathetic modulation. VDD-induced LF and HF changes during dobutamine, which partly reflect blood pressure regulation, were blocked by the TRPC6 antagonist. Similar to the HR response, blockade of TRPC6 had no effect on any HRV parameter at rest, which supports the contention that physiological changes (i.e. blood pressure) during a stress

challenge could have mediated the decreases in LF and HF. Another possible explanation for the efficacy of the TRPC6 antagonist in blocking the LF and HF in VDD mice is the role TRPC6 plays in maintaining mechanical stress-induced vascular tone[45], which has a direct impact on blood pressure and therefore the variability in heart rate.

Although blood pressure assessments were performed under anesthesia, there was a significant difference between ND and VDD. Low vitamin D levels impact systolic blood pressure in particular because vitamin D increases the activity of endothelial nitric oxide synthase, which leads to the synthesis of nitric oxide (NO) and subsequent vasodilation[46] and inhibits the release of vasoconstrictor mediators[47]. Yet, with respect to TRPC6, although the antagonist appeared to block the increased blood pressure in VDD mice (not shown), such a response is not conclusive because we had to make the measurement after dobutamine challenge and withdrawal of the probe from the left ventricle. It is conceivable that TRPC6 plays a role in the VDD-induced hypertension because its expression in vascular smooth muscle in humans and rodents is associated with higher blood pressure[48].

To our knowledge, there is no study describing the effects of VDD on cardiac mechanical function. Indeed, VDD mice had increased baseline LVP, these changes may be due to altered vascular tone because the heart needs to consistently develop a higher pressure in order to “push” blood out into the vasculature. Elevated baseline LVP can predispose the heart to arrhythmia and even failure in the long-term due to hypertrophy[49]. It also alters the degree to which the heart can increase its contractility during periods of stress like the conditions created by dobutamine. Blockade of TRPC6 attenuated the increased LVP in VDD mice and pointed to stress-induced modification of pressure dynamics, which have been shown to be mediated by TRPC6[26], which facilitates greater influx of calcium into cardiac myocytes during prolonged mechanical stress[50]. As a result, cardiac contractility was significantly decreased in VDD mice when challenged with high dobutamine and was restored by the TRPC6 antagonist, possibly due to the restoration of a normal LVP. In contrast, lusitropy was not significantly decreased in VDD mice during the challenge although there was a trend towards this and recovery with blockade of TRPC6.

Conclusions

Taking into account increased left ventricular, systolic and mean arterial pressure it is certainly likely that VDD mice exhibit signs of essential hypertension, which is a risk factor for heart failure because it can cause pressure overload and when sustained can lead to ventricular remodeling and hypertrophy. In any case, the HR, pressure and contractility changes due to VDD are independently significant indicators of increased cardiac risk. Yet, it is not known whether treatment of VDD mice with vitamin D would reverse these effects, particularly given the significant amount of data showing a lack of efficacy in large epidemiological and human studies. When comparing the human findings with what is presented here, it bears mentioning that VDD-induced responses may vary due to baseline differences in cardiovascular physiology, especially as it relates to ion flow and autonomic regulation[51]. In addition, the functional changes demonstrated here were acquired using highly-invasive, but sensitive, techniques, which are not possible in humans, and although more work is still needed to determine the complete mechanism behind

VDD-induced cardiovascular dysfunction, this study points to the role TRPC channels as potential mediators of VDD-induced cardiovascular mechanical dysfunction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements:

We are very grateful to Drs. Ian Gilmour and Molly Harmon for their reviews of this manuscript.

Funding:

All funding for this study is from U.S. Environmental Protection Agency

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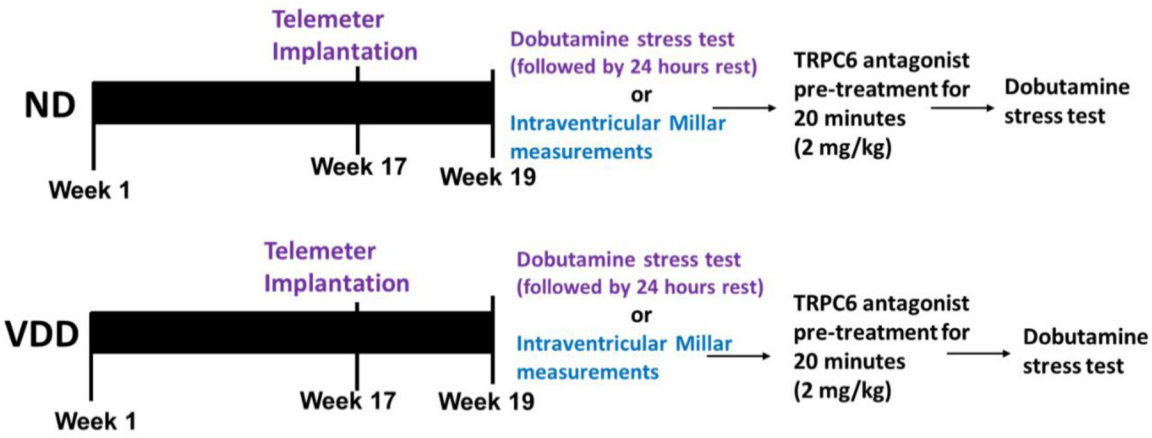

Figure 1. Experimental design and timeline.

ND and VDD mice were put on their respective diets for 19 weeks. Cardiovascular function was measured in separate groups of mice (n = 9–11) using radiotelemetry or a Millar pressure probe at baseline and during dobutamine challenge.

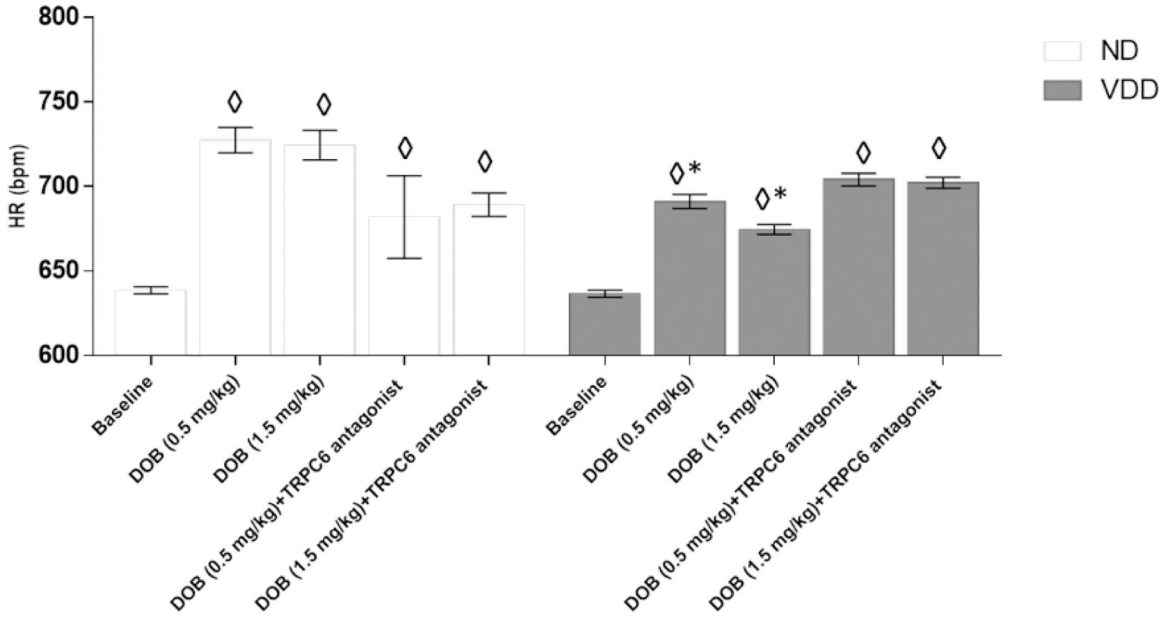

Figure 2. Heart rate response to dobutamine challenge in ND and VDD mice.

Dobutamine increased HR in ND mice, this response was significantly decreased in VDD mice. Pre-treatment with the TRPC6 antagonist blocked the decreased response of VDD mice to dobutamine but had no effect in ND mice. *significantly different from ND ($p < 0.05$). $^{\lozenge}$significantly different from baseline ($p < 0.05$), values represent means ± SEM.

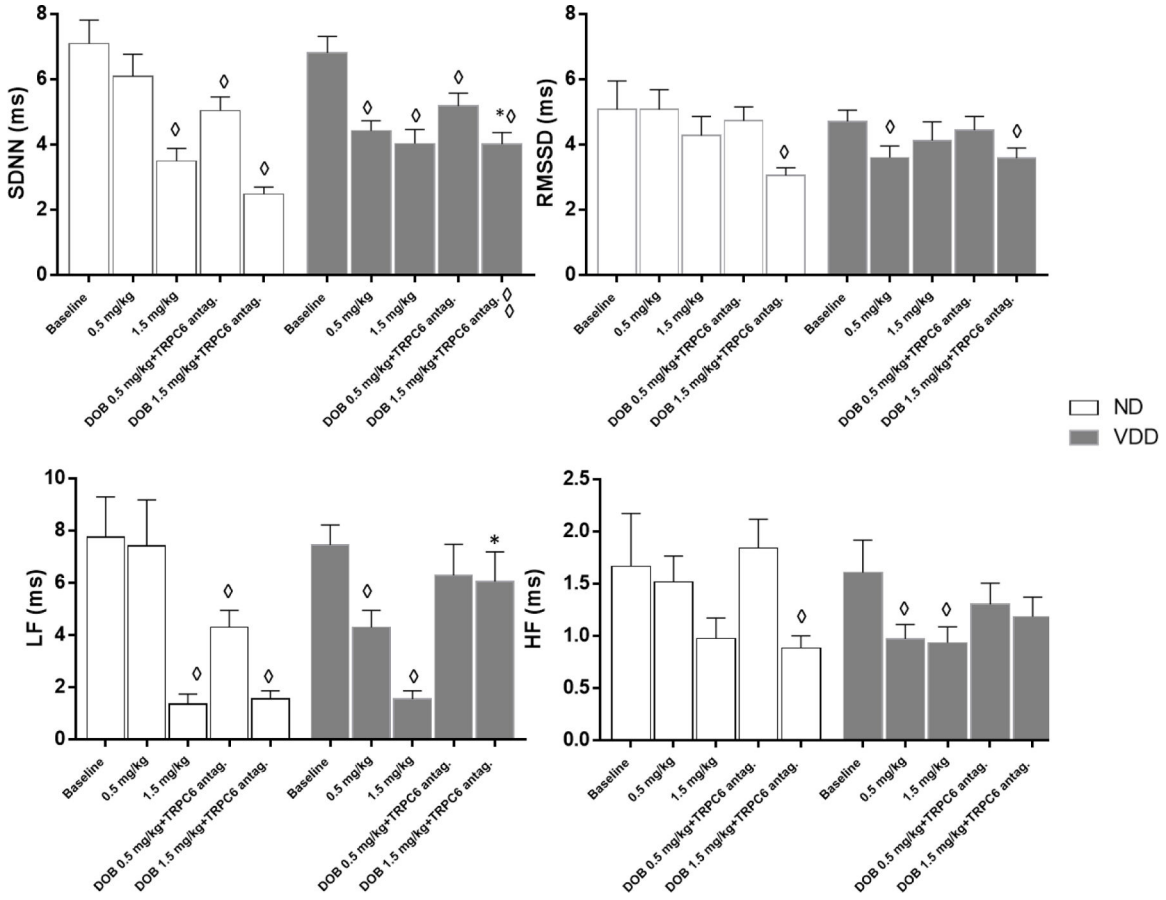

Figure 3. Autonomic responses to dobutamine challenge in ND and VDD mice.

There were no differences in SDNN, RMSSD, LF or HF between ND and VDD mice at baseline. Dobutamine challenge caused a significant decrease in SDNN and LF in ND and SDNN, RMSSD, LF and HF in VDD mice. Pretreatment of ND mice with TRPC6 caused further decreases in all HRV parameters, whereas it blocked decreases in LF and HF in VDD mice. *significant change from ND ($p < 0.05$). ◊significantly different from baseline ($p < 0.05$) Values represent means ± SEM.

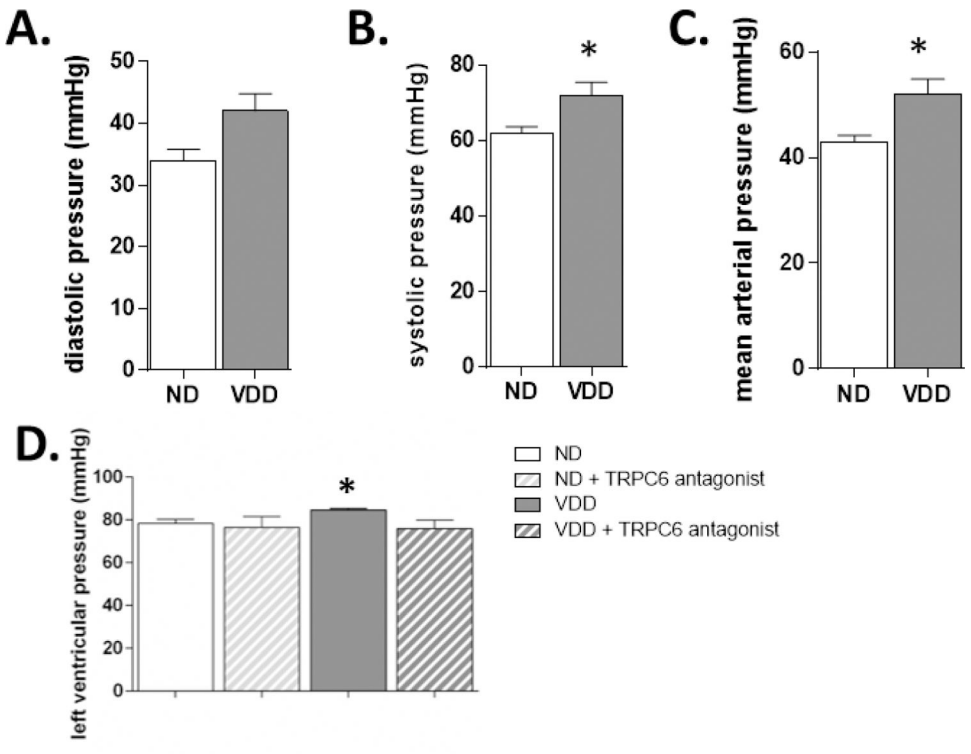

Figure 4. Hemodynamic and cardiac mechanical effects of vitamin D deficiency.

A.) There was no difference in the diastolic pressures of ND and VDD mice. However, systolic and mean arterial pressure were significantly increased in VDD when compared to controls **(B.** and **C.) D.)** Baseline LVP was significantly increased in VDD but blocked by TRPC6 antagonist. *significantly different from ND ($p < 0.05$). Values represent means ± SEM.

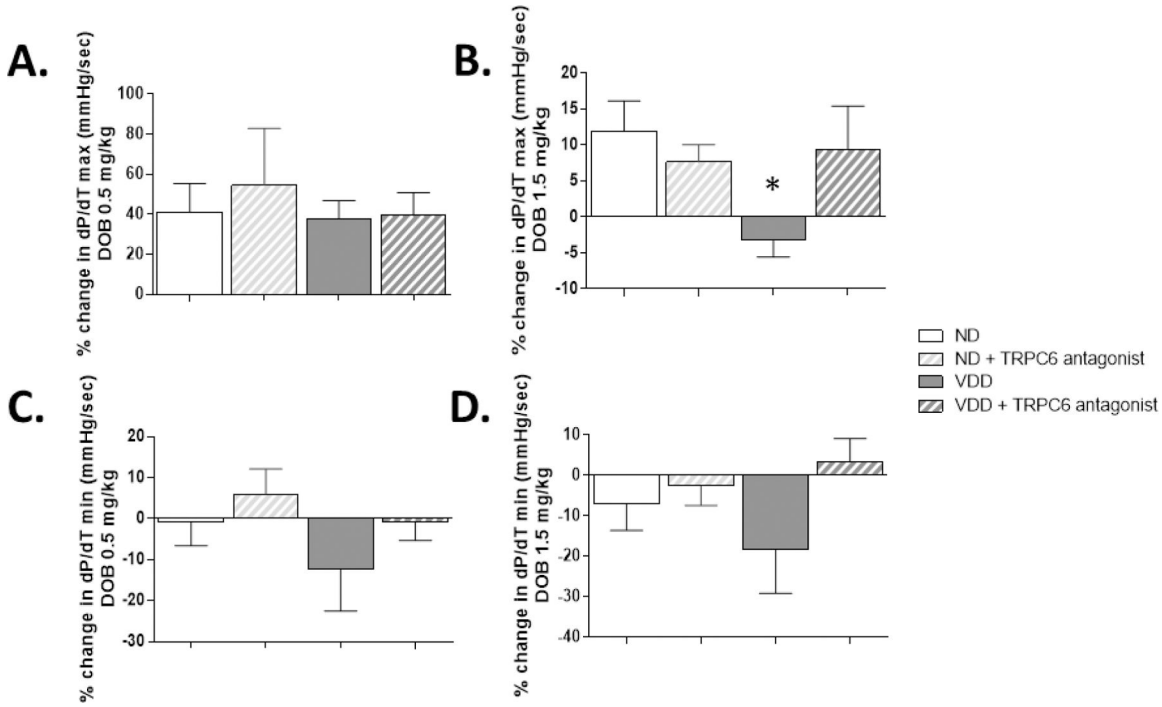

Figure 5. Ventricular contractile effects of vitamin D deficiency and role of TRPC6.

A.) Low dobutamine caused increase in dP/dTmax in ND but there was no difference when compared to VDD, nor with TRPC6 pretreatment. **B.)** In contrast, VDD mice had significantly decreased responses to high dobutamine when compared to ND and the response was blocked by the TRPC6 antagonist. **C-D.)** VDD mice had a trend of decreased dP/dTmin during dobutamine challenge, this was not apparent after TRPC6 blockade. *significantly different from ND ($p < 0.05$). Values represent means ± SEM.