Alfacalcidol suppresses α-receptor–mediated vasoconstriction via an endothelium dependent mechanism

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Aim: Vitamin D level could be related to blood pressure and cardiovascular health. However, the direct suppressor effect of vitamin D on vascular contraction has not yet been explored. Therefore, the effects of alfacalcidol (1α-hydroxyvitamin D₃) and calcitriol (1α,25-dihydroxyvitamin D₃) on contractions induced by phenylephrine (10⁻⁸ to 10⁻⁵ M) in mice aorta were investigated.

Materials and methods: Arterial rings were suspended in isolated organ chambers filled with Krebs bicarbonate solution gassed with 95% O₂ and 5% CO₂ maintained at 37 °C. Tissue responses were recorded isometrically with a force transducer and displayed on an acquisition system. Alfacalcidol and calcitriol were applied on phenylephrine-induced active tone. Furthermore, aortic rings were preincubated with alfacalcidol and calcitriol and thereafter contracted with phenylephrine. Incubation studies were conducted by endothelium removal and in the presence of L-nitro-arginine methyl ester (L-NAME).

Results: Phenylephrine-induced active tones were significantly suppressed by alfacalcidol (P < 0.05 and P < 0.01) and calcitriol (both 10⁻⁵ and 10⁻⁶ M). The pEC₅₀ values of alfacalcidol and calcitriol were 7.06 ± 0.91 and 7.13 ± 0.60, respectively. Furthermore, preincubation of aortic rings with alfacalcidol (10⁻⁵ M) but not calcitriol (10⁻⁵ M) significantly inhibited phenylephrine-induced contractions (10⁻⁸ to 10⁻⁵ M, P < 0.05 and P < 0.001). The suppressive effect of alfacalcidol incubation disappeared with endothelium removal. Furthermore, alfacalcidol was unable to inhibit phenylephrine-induced contractions in the presence of L-NAME, an inhibitor of nitric oxide biosynthesis.

Conclusion: In this study we report for the first time that alfacalcidol may prevent phenylephrine-induced vasoconstriction in mice aorta with an endothelium-dependent nitric oxide-mediated mechanism.

Key words: Alfacalcidol, calcitriol, mice, aorta, contraction

1. Introduction
A growing body of evidence suggests a possible association between vitamin D deficiency and many cardiovascular disorders, including hypertension, peripheral vascular disease, diabetes mellitus, metabolic syndrome, coronary artery disease, and heart failure (1,2). It has been reported that alfacalcidol treatment significantly and moderately lowers blood pressure in hypertensive and normotensive patients, respectively (3). However, the underlying mechanisms still remain unclear. Recently, some rapid nongenomic effects of hormones such as estrogen, progesterone, testosterone, and vitamin D have been explored beyond the classical genomic effects that usually occur over a long period of time (4). These rapid, nongenomic steroid actions are likely to be transmitted via specific membrane receptors (4).

Calcitriol is a secosteroid whose genomic mechanism of action is similar to that of other steroid hormones (5). Nongenomic steroid action may find applications in various clinical areas such as cardiovascular and central nervous disorders, electrolyte homeostasis, and infertility (4). Accordingly, we have previously demonstrated that testosterone could relax the human internal mammary artery in vitro, and some cardiovascular risk factors modulate the degree of this relaxation (6,7).

Vitamin D derivatives acutely reduce endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat (8). The vitamin D receptor, a member of the steroid/thyroid hormone nuclear receptor superfamily, is responsible for most of the biological activities of vitamin D in the body (9). In addition, the presence of the vitamin D receptor in endothelial cells has been reported (10). Furthermore, annexin II, the expression of which has been demonstrated in endothelial cells (11,12), has been suggested as a membrane receptor for rapid actions of calcitriol (13). These rapid actions include a variety of...
signal transduction systems including Ca²⁺ influx; release of Ca²⁺ from intracellular stores; modulation of adenylate cyclase, phospholipase C, and protein kinase activities; and changes in the phosphorylation status of cellular proteins (14). However, knowledge about the nongenomic action of another vitamin D form, alfacalcidol, is limited.

Renovascular diseases and chronic kidney disease (CKD) are secondary causes of hypertension. The issue of sympathetic overactivity in CKD has been the topic of well-documented reviews (15,16). It has been shown that oral administration of alfacalcidol for predialysis CKD patients is associated with reduced risk for cardiovascular diseases (17). Several mechanisms of action of vitamin D in hypertensive vascular disease have been proposed, such as an increase in intracellular Ca²⁺ leading to decreased renin activity, calcitriol suppression of the renin promoter gene, and alteration of the sensitivity of vascular smooth muscle cells (1). However, direct actions of vitamin D on vascular tissues have not been well documented. Therefore, in the present study we aimed to explore whether alfacalcidol and calcitriol have any direct effects on precontracted mice aorta. For that reason, we tested the effects of 2 vitamin D forms, alfacalcidol and calcitriol, on mouse aortic ring contractions due to α₁ receptor agonist phenylephrine. Any possible contribution of the endothelial integrity to the effects of vitamin D was also tested.

2. Materials and methods
2.1. Animals
Male BALB/c mice weighing 35–40 g were obtained from the Experimental Medicine Unit of the Mersin University Medical Faculty. The mice were caged separately under a 12-h light/12-h dark photoperiod and a constant temperature (23 ± 1 °C) and they received standard mice chow ad libitum. The study was performed in accordance with the Guide for the Care and Use of Laboratory Animals of the Experimental Medicine Unit of the Medical Faculty of Mersin University.

2.2. Drugs and chemicals
Phenylephrine, potassium chloride, carbachol, Nω-nitro-L-arginine methyl ester (L-NAME), alfacalcidol, and calcitriol were purchased from Sigma Chemical Co. (St. Louis, MO, USA). The alfacalcidol and calcitriol were dissolved in ethanol and the other chemicals were in distilled water.

2.3. Tissue preparation
The mice were killed by a blow to the head and exsanguinated. The aorta was immediately isolated and adherent tissues were carefully removed in a petri dish containing Krebs solution (composition in mM: NaCl 118, KCl 4.8, CaCl₂ 2.5, MgSO 1.2, NaHCO₃ 25, KH₂PO₄ 1.2, glucose 11, and Na₂EDTA 0.01). Arterial ring segments (approximately 3 mm wide) were prepared fine scissors, keeping the endothelium intact. In some experiments, the endothelium was gently damaged in order to evaluate endothelial contribution to vitamin D action.

2.4. Experimental procedure
The aortal rings were suspended in organ baths filled with Krebs solution (37 °C) and gassed with 95% O₂ and 5% CO₂ under 0.5 g of initial tension. Tissue responses were recorded isometrically with a force transducer (COMMAT, Ankara, Turkey) and displayed on a Biopac acquisition system (Biopac Systems, Goleta, CA, USA). The vessels were allowed to equilibrate at optimum resting tensions for 60 min before experiments were carried out, during which time the bath was replaced with fresh Krebs solution every 20 min. Following equilibration, the arterial rings were contracted using 80 mM KCl. After a steady state of contraction was obtained, the rings were washed and allowed to rest for 45 min more. Thereafter, the rings were contracted using phenylephrine (10⁻⁵ M) and relaxed with carbachol (10⁻⁴ M) to test the endothelial integrity. In all of the performed experiments, the final concentration of ethanol in the organ baths did not exceed 0.02%, which had no effect by itself (data not shown).

2.5. Observation of alfacalcidol administration on phenylephrine-induced active tone
After the above-mentioned procedure was conducted, a vehicle (ethanol), alfacalcidol, or calcitriol (each at 10⁻⁸, 10⁻⁷, 10⁻⁶, and 10⁻⁵ M) was added on aortic rings that had been precontracted with phenylephrine (10⁻⁵ M). Arterial tones were monitored for 1 h, and at the end of each 15 min period, the vascular tones were obtained and expressed as percentages of phenylephrine-induced contractions (0-min value). Each arterial segment was used to obtain a response from only one concentration of vehicle, alfacalcidol, or calcitriol.

2.6. Alfacalcidol and calcitriol incubation studies
Intact endothelium or mechanically damaged arterial rings were incubated with alfacalcidol (10⁻⁵ M), calcitriol (10⁻⁵ M), or their vehicle ethanol (0.02%) for 1 h. Following that, the tissues were contracted with phenylephrine (10⁻⁸ to 10⁻⁵ M). Contractile responses to the phenylephrine were shown as a percentage of 80 mM KCl-induced contractions. Endothelial integrity was tested with relaxation to carbachol (10⁻⁴ M).

2.7. Studies with L-NAME
While the control group was incubated with L-NAME (2 × 10⁻⁴ M) plus ethanol (incubation period of 1 h), the experiment group was incubated with L-NAME (2 × 10⁻⁴ M) plus alfacalcidol (10⁻⁵ M).

2.8. Statistical analyses
Arterial tones obtained after 15, 30, 45, and 60 min of
vehicle, alfacalcidol, and calcitriol administration were expressed as percent of phenylephrine-induced tone (0-min value regarded as 100%). For the incubation studies, the phenylephrine-induced contractions were expressed as a percent of 80 mM KCl-induced tone and shown as mean ± standard error of the mean (SEM). For statistical analysis, one-way analysis of variance (ANOVA) followed by the Bonferroni post hoc test or Student’s t-test, if appropriate, was used. P-values of less than 0.05 were considered significant.

3. Results

3.1. Effect of alfacalcidol and calcitriol on phenylephrine-induced active tone

Figures 1A–1F show original tracings of phenylephrine precontracted and phenylephrine-induced contractions of mice aorta. In the control group there was an increase in phenylephrine-induced active tone at the end of 1 h of observation (Figure 1A). Alfacalcidol (10⁻⁵ to 10⁻⁶ M) and calcitriol (10⁻⁵ to 10⁻⁶ M) significantly decreased or relaxed phenylephrine-induced (10⁻⁵ M) active tone in mice aorta (Figures 1B, 1C, and 2A, P < 0.05). The relaxations occurred slowly (Figures 1B and 1C). The most appropriate point to calculate alfacalcidol and calcitriol pEC₅₀ values was at 30 min after application, due to statistical significance (Figures 2A and 2B). The pEC₅₀ values for the alfacalcidol and calcitriol groups at 30 min after application were 7.06 ± 0.91 and 7.13 ± 0.60, respectively.

3.2. Effect of alfacalcidol and calcitriol incubation on phenylephrine-induced contractions

Preincubation of aortal rings with alfacalcidol (10⁻⁵ M) significantly inhibited the phenylephrine-induced contractions (10⁻⁸ to 10⁻⁵ M, P < 0.05 and P < 0.001) (Figures 1D, 1E, and 3A). The E_max values obtained with 10⁻⁵ M phenylephrine as a percentage of 80 mM KCl-induced tone were 130.83 ± 3.77 (n = 6) and 77.78 ± 9.25 (n = 5) for the control and alfacalcidol groups, respectively.

To evaluate whether alfacalcidol could be converted...
into its active form, calcitriol, and thereafter produce its inhibitory effect, we also preincubated the rings with calcitriol. However, calcitriol incubation (10–5 M) did not suppress phenylephrine-induced contractions (10–8 to 10–5 M) (Figures 1F and 3B). The E_{max} values obtained with 10–5 M phenylephrine as a percentage of 80 mM KCl-induced tone were 131.32 ± 26.42 (n = 10) and 122.59 ± 28.01 (n = 8) for the control and calcitriol groups, respectively.

3.3. Effect of endothelium removal on suppression of phenylephrine-induced contraction with alfacalcidol preincubation

In arteries with mechanically destroyed endothelium, alfacalcidol (10–5 M) was unable to inhibit the phenylephrine-induced contractions (10–8 to 10–5 M), indicating that the endothelium has a role in the inhibitory effect of alfacalcidol (Figure 4). In the alfacalcidol

Figure 2. The effect of alfacalcidol and calcitriol on phenylephrine-induced contractions during the 1-h period after vitamin D application. Alfacalcidol (A) and calcitriol (B) significantly reduced the phenylephrine-induced tone of the arterial rings. After the rings were contracted with phenylephrine (10–5 M), alfacalcidol (10–5 to 10–8 M), calcitriol (10–5 to 10–8 M), or the vehicle (ethanol) were added to the organ bath. Thereafter, at the end of 15, 30, 45, and 60 min, the phenylephrine-induced tones were obtained and expressed as a percentage of phenylephrine-induced contractions (0-min value). For statistical analysis, Student's t-test was used and data are shown as the mean ± SEM of 4-8 observations. * P < 0.05, ** P < 0.01.
preincubated group, there was a slight but not significant increase in the phenylephrine-induced contractions. The $E_{\text{max}}$ values obtained with $10^{-5}$ M phenylephrine as a percentage of 80 mM KCl-induced tone were 139.11 ± 18.52 (n = 5) and 158.89 ± 22.09 (n = 8) for the control and alfacalcidol groups, respectively.

### 3.4. Effect of L-NAME incubation on alfacalcidol-mediated inhibition of phenylephrine-induced contractions

The application of L-NAME ($2 \times 10^{-4}$ M) to the organ bath gradually increased the resting tension of the arteries, which was not significantly different between the control and alfacalcidol groups. With the presence of L-NAME ($2 \times 10^{-4}$ M), alfacalcidol ($10^{-5}$ M) was unable to inhibit the phenylephrine-induced contractions ($10^{-9}$ to $10^{-5}$ M), indicating that nitric oxide (NO) has a role in the inhibitory effect of alfacalcidol (Figure 5). The $E_{\text{max}}$ values obtained with $10^{-5}$ M phenylephrine as a percentage of 80 mM KCl-induced tone were 102.21 ± 19.02 (n = 5) and 102.69 ± 8.32 (n = 5) for the control and alfacalcidol groups, respectively.
4. Discussion

The present study is the first to report that alfacalcidol suppresses phenylephrine-induced vascular contraction. Calcitriol suppressed or relaxed when applied to arteries precontracted by phenylephrine; however, its preincubation did not suppress contractions induced by the α₁ agonist. Interestingly, endothelium removal prevented the suppressor effect of alfacalcidol, indicating the role of this vessel layer. Furthermore, the nitric oxide synthase (NOS) inhibitor L-NAME also abolished the suppressor effect of alfacalcidol, suggesting that this vasodilatation could be NO-mediated.

Increased sympathetic nerve activity is a major actor in the development of essential hypertension (18,19). Phenylephrine is a sympathomimetic agent and increases blood pressure via selective α₁-adrenergic receptor stimulation. The relaxant effects of hormones, which are generally observed in high doses that exceed the physiological concentrations, are slow to develop when compared to other potent relaxants but still rapid in respect to the genomic effects (6,20). The observed suppressive or relaxant effect of alfacalcidol on phenylephrine-induced active tone was a slowly developing effect. However, the suppression of phenylephrine-induced contractions was more pronounced when the aortic rings were preincubated with alfacalcidol.

In healthy individuals, vitamin D derived from nutritional sources or produced in the skin is converted to 25-hydroxyvitamin D in the liver and then to 1,25-dihydroxyvitamin D in the kidney by 25-α-hydroxylase and 1-α-hydroxylase, respectively. Vitamin D receptors and 1-α-hydroxylase, which convert vitamin D into the hormonal 1,25-dihydroxyvitamin D form, are present in many tissues, including endothelial cells (10). However, the presence of 25-α-hydroxylase in endothelial cells has not yet been reported. Vascular endothelial cells are known to harbor the CYP27A product (21), whose enzyme is thought to be responsible for 25-hydroxylation of vitamin D₃ (22). CYP27A1, however, has a low affinity for vitamin D₃, does not 25-hydroxylate vitamin D₃, and, when mutated, results in cerebrotendinous xanthomatosis, not rickets (23). In vitro, many nonrenal tissues, including bone, placenta, prostate, keratinocytes, macrophages, T-lymphocytes, dendritic cells, and several cancer cells, can enzymatically convert 25-hydroxyvitamin D to 1α,25-dihydroxyvitamin D (23). Based on our findings, alfacalcidol exerted its suppressive effect in the mouse aorta without converting to calcitriol because the incubation with calcitriol did not decrease phenylephrine-induced contractions.

Although the beneficial effect of vitamin D supplementation on hypertension has been reported (24), another study also reported that it might protect against cardiovascular disease but involve some mechanisms other than blood pressure (25). The ineffectiveness of calcitriol preincubation to suppress phenylephrine-induced contraction is in accordance with the findings of Wong et al. (8). Further investigation is needed to explain the discrepancy observed with the application procedure of calcitriol, i.e. with preincubation and after the active tone.

Low concentrations of vitamin D have been linked to the pathogenesis of several chronic diseases with cardiovascular risk factors, such as hypertension, heart failure, atherosclerosis, and peripheral arterial disease (1,2,26). Moreover, it has been reported that vitamin D analogs and supplements may potentially be agents for controlling renin production and blood pressure (27). However, little is known about the mechanism of action of vitamin D regarding cardiovascular effect when compared to other steroids such as testosterone, estrogen, and progesterone.

The nongenomic vascular effects of sex hormones include both endothelium-dependent and endothelium-independent mechanisms involving direct effects on vascular smooth muscle (20). Sex steroid hormones (estrogen and testosterone) increase the production and release of endothelium-derived nitric oxide in both men and women (28). Since vitamin D replacement in deficient subjects significantly improved flow-mediated dilatation of the brachial artery, Tarcan et al. (29) suggested that vitamin D plays a role in endothelial function. Here we report that alfacalcidol suppresses contractions via an endothelium-dependent mechanism. As vitamin D has long been identified to regulate Ca²⁺ homeostasis (30), it may modulate Ca²⁺ transport in endothelial cells (8). This suppression of contractions may occur in part via nitric oxide. According to these findings, the idea that alfacalcidol may increase NOS activity is in accordance with the literature (17,30). However, endothelial NOS activity and the calcium levels in the endothelial cells of aortic rings after vitamin D exposure still have to be investigated.

In summary, the aim of this study was to determine the direct acute effect of vitamin D on mice aorta. Alfacalcidol and calcitriol inhibited or relaxed phenylephrine-induced contractions. In addition, incubation of arteries with alfacalcidol caused a significant decrease in the α₁-sympathomimetic-induced contractions achieved by phenylephrine. Alfacalcidol-induced suppression of contractions is likely to be mediated without its conversion to calcitriol because calcitriol preincubation itself was ineffective. This suppressive effect might be mediated at least in part via an endothelium-dependent NO-mediated mechanism because the removal of the endothelium and the NOS inhibitor, L-NAME, prevented this effect. However, whether the mechanism of calcitriol inhibition
of phenylephrine-induced active tone is different from that of alfacalcidol still needs to be investigated.

In conclusion, vitamin D may be able to suppress vasoconstriction due to phenylephrine in the mouse aorta.

References


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