Vitamin D derivatives acutely reduce endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat


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Vitamin D derivatives acutely reduce endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat. Am J Physiol Heart Circ Physiol 295: H289–H296, 2008. First published May 16, 2008; doi:10.1152/ajpheart.00116.2008.—The available evidence suggests that vitamin D has cardiovascular effects besides regulating calcium homeostasis. To examine the effect of 1,25-dihydroxyvitamin D3, the major metabolite of vitamin D, on endothelium-dependent contractions, aortic rings of spontaneously hypertensive rats (SHRs) were suspended in organ chambers for isometric force measurements. Rings were incubated with Nω-nitro-L-arginine methyl ester (L-NAME) and then exposed to increasing concentrations of acetylcholine, ATP, or the calcium ionophore to trigger contractions. This was done in the absence or presence of 1,25-dihydroxyvitamin D3. The release of prostacyclin after acetylcholine or A-23187 stimulation was also measured. The cytosolic-free calcium concentration was measured by confocal microscopy after incubation with the fluorescent dyes fluo-4 and fura red. The presence of vitamin D receptors was confirmed using immunohistochemistry. Acetylcholine- and ATP-induced endothelium-dependent contractions were significantly reduced compared with those obtained in the absence of the drug. This effect was not present if A-23187 was used as an agonist. The acetylcholine- but not the A-23187-induced release of prostacyclin was reduced by the acute administration of 1,25-dihydroxyvitamin D$_3$. Exposure to 1,25-dihydroxyvitamin D$_3$ reduced the increase in cytosolic-free calcium concentration caused by acetylcholine but not by A-23187 in cells. Vitamin D receptors were densely distributed in the endothelium. Inecalcitol (19-nor-14-epi-23-yne-1,25-dihydroxyvitamin D$_3$), a synthetic analog of vitamin D, caused a comparable depression of endothelium-dependent contractions as 1,25-dihydroxyvitamin D$_3$. The direct effects of 1,25-dihydroxyvitamin D$_3$ inhibit renin synthesis in vivo (25).

The endothelium modulates vascular tone by releasing substrates including nitric oxide and prostacyclin, which relax the underlying vascular smooth muscle. They are referred to as endothelium-derived relaxing factors (EDRFs) (11, 24). Under certain circumstances, the endothelium can also elicit endothelium-dependent contractions (7, 11, 41). In the rat aorta, endothelium-dependent contractions are caused by the production of metabolites of arachidonic acids, mainly endoperoxide and prostacyclin, which then activate thromboxane-prostanoid (TP) receptors of the vascular smooth muscle cells (12, 21, 35, 39). The healthy endothelium has a balanced production of EDRF and endothelium-derived contracting factor (EDCF). Endothelial dysfunction disturbs the balance and favors the production of EDCF. Such unbalance has been observed in blood vessels of humans with atherosclerosis, myocardial infarction, and hypertension (3, 30, 38) and in animals, including the adult spontaneously hypertensive rats (SHRs) (20), aging normotensive animals (17), and diabetic rats (28). The production of EDCF is a calcium-dependent process in which the calcium influx activates phospholipase A$_2$, which converts the membrane phospholipids to arachidonic acid. The presumably membrane-bound cyclooxygenase-1 (COX-1) enzyme then catalyzes arachidonic acid into endoperoxides, the precursor of EDCF (33, 34). Therefore, the endothelial calcium concentration is an important factor in EDCF-mediated responses.

The aim of the present study was to test the hypothesis that vitamin D$_3$ directly modulates endothelium-dependent responses in a calcium-dependent manner.

METHODS

Animals and tissue preparation. Adult SHR (410 g) and Wistar-Kyoto (WKY; 470 g) rats of 37–39 wk old were used. All animals were housed in a room with standardized temperature (21 ± 1°C) and exposed to a 12-h:12-h dark-light cycle. Animals had free access to a standardized diet (LabDiet 5053) and tap water. The rats were anesthetized with pentobarbital sodium (30 mg·ml$^{-1}$·kg$^{-1}$·ip). The mean arterial blood pressure of the rats was measured by a polyethylene cannula inserted into the left carotid artery and connected to a pressure transducer (Gould P23 ID, Statham). The mean blood pressures of SHRs and WKY rats were 201.4 ± 5.4 and 122.8 ± 3.8 mmHg.

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respectively. After the blood pressure measurement was taken, the rats were euthanized with pentobarbital sodium (70 mg·ml⁻¹·kg⁻¹) and their thoracic aortae were isolated and placed immediately into cold Krebs-Ringer buffer with the following composition: (in mM) 118 NaCl, 4.7 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 1.2 KH₂PO₄, 25 NaHCO₃, and 11.1 glucose (control solution). The adhering fat and connective tissue were cleaned. The aortae were cut into rings (length, ~3 mm). In some rings, the endothelium was removed mechanically by gently rubbing the intimal surface of the rings with a syringe needle. Some of the rings were used to measure isometric force, whereas the others were used to measure the release of prostacyclin or cytosolic-free calcium in the endothelial cells. This investigation was approved by the Committee on the Use of Laboratory Animals for Teaching and Research of the University of Hong Kong.

Isometric force. The rings were suspended in organ chambers that contained 5 ml of control solution (37°C aerated with 95% O₂-5% CO₂ gas mixture (pH 7.4). Each ring was connected to a force transducer (model MLT0201/D, AD Instruments) for isometric force recording. The rings were stretched to an optimal tension of 2.5 g (determined in preliminary experiments; data not shown) and allowed to equilibrate for 90 min. They were then exposed to 60 mM KCl to obtain a submaximal contraction. All further contractions were expressed in percentage of this reference response. Some rings with endothelium were first contracted with phenylephrine and then exposed to an increasing concentration of acetylcholine (10⁻¹⁰–10⁻³ M). Other rings with endothelium were incubated with Nω-nitro-l-arginine methyl ester (l-NAME; 10⁻⁴ M) for 30 min to optimize endothelium-dependent contractions (1, 31). They were then exposed to cumulative concentrations of acetylcholine (10⁻⁸–10⁻⁵ M), adenosine triphosphate (ATP, 10⁻⁸–10⁻⁵ M), or the calcium ionophore A-23187 (10⁻⁸–3 × 10⁻⁶ M). Rings without endothelium were exposed to increasing doses of 15(S)-hydroxy-11,9-(epoxymethano) prostadienoic acid (U-46619, thromboxane mimetic, 10⁻¹⁰–10⁻⁶ M) or phenylephrine (α₁-adrenergic agonist; 10⁻⁷–10⁻⁵ M) to obtain endothelium-independent contractions. The responses to the different agonists were examined in parallel rings incubated for 30 min in the absence or presence of 1,25-dihydroxyvitamin D₃ (10⁻⁷ M) or inecalcitol (19-nor-14-epi-23-yno-1,25-dihydroxyvitamin D₃; 10⁻⁷ M), a synthetic analog of vitamin D (10, 36, 37). This concentration was selected because it was the lowest concentration that gave a significant effect in organ chamber experiments (preliminary data not shown). Both 1,25-dihydroxyvitamin D₃ and inecalcitol were dissolved in ethanol (0.1% vol/vol), which had no effect by itself (preliminary data not shown). For certain experiments, followed by incubation, 1,25-dihydroxyvitamin D₃ was washed away before triggering endothelium-dependent contractions.

Release of prostacyclin. To measure the release of prostacyclin, rings were placed in small chambers containing 1 ml of Krebs solution at 37°C aerated with 95% O₂-5% CO₂ gas mixture. They were allowed to equilibrate for 90 min. Drugs, including l-NAME and 1,25-dihydroxyvitamin D₃, were given 30 min before the rings were exposed to a single concentration of either acetylcholine (10⁻⁵ M) or the calcium ionophore A-23187 (10⁻⁶ M). The rings were then removed, and the solution was diluted 500 times before measuring the 6-keto-prostaglandin F₁α, concentration by an EIA kit. The assays were performed according to the manufacturer’s instructions. The release of prostacyclin was expressed in picograms per millimeter rings per milliliters (13).

Cytosolic-free calcium. Cytosolic-free calcium changes were monitored and measured by confocal microscopy after incubation with two fluorescent dyes: fluo-4 AM (2 × 10⁻⁵ M) and fura red AM (4 × 10⁻⁵ M) to minimize the effects of artifacts. The protocol was modified from previous studies (4, 22, 33). The dyes were dissolved in 0.1% (vol/vol) dimethyl sulfoxide (DMSO), first followed by the dilution with a control solution containing 0.02% (vol/vol) pluronic acid F-127. Each aortic ring was incubated in 0.5 ml of the dye mixture for 1 h to allow the penetration into the tissue and cleavage of acetyloxymethyl ester. After incubation, the rings were cut longitudinally.

Fig. 1. A: contractions to cumulative concentrations of acetylcholine in different concentrations of 1,25-dihydroxyvitamin D₃ in rings of spontaneously hypertensive rat (SHR) aorta with endothelium. B: corresponding data in area under the curve (in arbitrary units). C: rings in the absence (control) and presence of 1,25-dihydroxyvitamin D₃ (10⁻⁷ M 1,25-dihydroxyvitamin D₃) and in rings previously incubated with 1,25-dihydroxyvitamin D₃, followed by washing away of the drug (washout). Data are expressed as percentages of the change of the reference contraction to 60 mM KCl and shown as means ± SE (n = 6 rats). *P < 0.05, statistically significant difference from control.
nally into strips and fixed into a transparent chamber with the intimal side faced downward, leaving a 1-mm gap between the endothelium and the coverslip. The whole system was connected to a peristaltic pump for a continuous perfusion with oxygenated control solution (95% O₂-5% CO₂) loaded with different drugs. The incoming solution was maintained at 37°C by a temperature bath, and the perfusion rate was 2 ml/min. The tissues were first perfused with control solution for 10 min to wash away excess dye. A single dose, which caused maximal contractions in organ chamber experiments, of either acetylcholine (10⁻⁵ M) or A-23187 (10⁻⁶ M) was added. Real-time cytosolic-free calcium concentrations in the endothelium cells were monitored using a laser confocal microscope at 3-s intervals for 400 s and was expressed as the ratio of fluo-4 to fura red. The maximal change in fluorescence ratio was calculated (expressed as percent increase over the basal fluorescence ratio) and compared between the control and treated (1,25-dihydroxyvitamin D₃) groups. To mimic the organ chamber experiments, all strips were incubated with L-NAME for 30 min before the addition of either acetylcholine or A-23187. Terutroban (S18886 or Triplion, TP receptor antagonist; 10⁻⁷ M) was added together with L-NAME to prevent the contraction of the smooth muscle and thus to avoid movements that can alter the microscopic image. At this concentration, terutroban does not affect the movement of calcium in endothelial cells (33).

Fig. 2. Contractions to cumulative concentrations of acetylcholine (A), ATP (B), or the calcium ionophore A-23187 (C) in rings of SHR (left) or Wistar-Kyoto (WKY; right) aorta with endothelium in the absence (control) or presence of 1,25-dihydroxyvitamin D₃ (10⁻⁷ M). Data are expressed as percentages of the change of the reference contraction to 60 mM KCl and shown as means ± SE (n = 6 rats). *P < 0.05, statistically significant difference from control.
The microscope used in this study was an inverted Nikon Eclipse TE300 with a ×10 objective. It was attached to a confocal argon ion Radiance-2100 laser scanning unit (Bio-Rad, Hertfordshire, England). An argon ion laser line (488 nm) was directed to the sample to excite the fluorescent probes. The emitted fluorescence was split by a dichroic beam splitter to reach two photomultiplier tubes concomitantly. Fluo-4 emission was detected via a band-pass filter (510–525 nm), whereas fura red emission was detected via a long-pass filter (>590 nm). An image field of 256 × 256 pixels was selected randomly on the aortic segment. The resulting fluorescence was a mean of all pixels in this chosen field. The LaserSharp 2000 program (Bio-Rad) was used to record the real-time changes in mean intensity of the images. The parameters of the confocal laser scanning were kept constant for all experiments, as follows: 1) zoom factor = 4, 2) iris = 1.2, 3) gain = 98, and 4) intensity = 36.4.

Immunohistochemistry. Some aortic segments were used to make sections of 3 mm. The sections were then dehydrated with incremental concentration of alcohol, followed by xylene and paraffin, and 5-μm slices were prepared. Slices were first treated with hydrogen peroxide and methanol to block endogenous peroxides, followed by a serial exposure to serum-free protein block (10 min, DakoCytomation, Zug, Switzerland), primary rabbit anti-vitamin D receptor antibody (2 h, VDR Ab-2, NeoMarkers), secondary biotinylated anti-rabbit antibody (30 min, Vector, Burlingame, CA), Vectastain ABC reagent (30 min, Vector). They finally were treated with peroxidase substrate solution (Vector) until the desired stain intensity had developed.

Data analysis. Results were presented as means ± SE with n referring to the number of rats used. Statistical analysis was performed by Student’s t-test for comparison of two groups or two-way ANOVA followed by the Bonferroni posttest for multiple comparisons. All statistical analysis and the area under the curve calculation were performed using Prism version 3a (GraphPad Software, San Diego, CA). Differences were considered to be statistically significant when P < 0.05.

Chemicals. Acetylcholine, ATP, A-23187, l-NAME, pluronic acid F-127, phenylephrine, and 1,25-dihydroxyvitamin D₃ were purchased from Sigma Chemical (St. Louis, MO). Inecalcitol was a kind gift of Hybrigenics (Paris, France). U-46619 was purchased from Biomol (St. Louis, MO). DMSO was purchased from Merck (Darmstadt, Germany). EIA kits were purchased from Cayman Chemical (Ann Arbor, MI). Fluo-4 AM and fura red AM were purchased from Molecular Probes (Eugene, OR). Terutroban (S18886, Triplion), a selective TP-receptor antagonist at the concentration used (29, 32, 40), was a kind gift from the Institut de Recherches Servier (Suresnes, France). All drugs, except 1,25-dihydroxyvitamin D₃, U-46619, and the calcium ionophore A-23187, were prepared daily (diluted from stock solutions with control solution). 1,25-Dihydroxyvitamin D₃ was finally dissolved in absolute DMSO (0.1% in the organ bath) before dilution with control solution. U-46619 and the calcium ionophore A-23187 were first dissolved in absolute DMSO (0.1% in the organ bath) and further diluted with control solution. Concentrations were expressed as final molar concentrations.

RESULTS

Endothelium-dependent contractions. In SHR rings with endothelium, acetylcholine (10⁻⁸–10⁻⁵ M) caused endothelium-dependent contraction in a concentration-dependent manner (Fig. 1). Acute administration of 1,25-dihydroxyvitamin D₃ showed a concentration-dependent inhibitory effect of the endothelium-dependent contractions to acetylcholine (Fig. 1, A and B). The effect was significant with 0.1 μM of 1,25-dihydroxyvitamin D₃, and it disappeared if the drug was washed out (Fig. 1C). ATP (10⁻⁸–10⁻³ M) and the calcium ionophore A-23187 (10⁻⁸–3 × 10⁻⁶ M) also caused endothelium-dependent contractions. The maximal contractions in

Fig. 3. A: contractions to cumulative concentrations of acetylcholine in different concentrations of inecalcitol in rings of SHR aorta with endothelium. B: corresponding data in area under the curve (in arbitrary units). C: contractions to cumulative concentrations of ATP in rings of SHR aorta with endothelium in the absence (control) or presence of inecalcitol (10⁻⁷ M). Data are expressed as percentages of the change of the reference contraction to 60 mM KCl and shown as means ± SE (n = 6 rats). *P < 0.05, statistically significant difference from control.
response to ATP were also reduced significantly by 0.1 μM of 1,25-dihydroxyvitamin D₃. This was not the case for contractions to A-23187 (Fig. 2). A similar pattern was obtained in rings from age-matched WKY rats, but the endothelium-dependent contractions were significantly smaller than those obtained in SHR rings. The same concentration of 1,25-dihydroxyvitamin D₃ (0.1 μM) also reduced endothelium-dependent contractions caused by acetylcholine and ATP but not that caused by A-23187 (Fig. 2). Inecalcitol at a concentration of 0.1 μM had a comparable effect as 1,25-dihydroxyvitamin D₃ on endothelium-dependent contractions (Fig. 3).

**Endothelium-independent contractions.** In rings without endothelium, U-46619 (10⁻¹⁰-10⁻⁶ M) and phenylephrine (10⁻⁹-10⁻⁵ M) produced endothelium-independent contractions in a concentration-dependent manner. There was no significant difference in these responses in the absence or presence of 1,25-dihydroxyvitamin D₃. The responses were comparable in SHR and WKY rings (Fig. 4).

**Release of prostacyclin.** Both acetylcholine and A-23187 evoked the release of 6-keto-prostaglandin F₁α. In the presence of 1,25-dihydroxyvitamin D₃, the production of prostacyclin induced by acetylcholine was reduced but not that caused by A-23187 (Fig. 5).

**Cytosolic-free calcium.** Acetylcholine (10⁻⁵ M) caused an increase in cytosolic-free calcium concentration. The increase in the acetylcholine-induced calcium influx after 1,25-dihydroxyvitamin D₃ incubation was reduced significantly compared with control. However, 1,25-dihydroxyvitamin D₃ did not significantly affect the increase in cytosolic-free calcium concentration evoked by A-23187 (Fig. 6).

**Immunohistochemistry.** Vitamin D receptor immunoreactivity appeared as a brown color in the studied sections. Vitamin D receptors were found in both endothelial cells and vascular smooth muscle cells. They were distributed densely in the endothelium and were more closely packed than those in the smooth muscle cells (Fig. 7). A similar distribution was observed in aortas of SHR and WKY rats.
DISCUSSION

The present experiments demonstrate a novel vascular effect of the main active metabolite of vitamin D3. The major finding of the study was that 1,25-dihydroxyvitamin D3 and its synthetic analog inecalcitol modulate vascular tone by reducing endothelium-dependent contractions. This inhibitory effect was observed in aortae of both SHR and WKY rats. The acute effect of 1,25-dihydroxyvitamin D3 was concentration dependent. At 0.1 μM, 1,25-dihydroxyvitamin D3, a significant reduction of the endothelium-dependent contractions to acetylcholine, was observed. When 1,25-dihydroxyvitamin D3 was washed out after an incubation for 30 min, the endothelium-dependent contractions were restored to the control level, indicating that the acute effect of 1,25-dihydroxyvitamin D3 is reversible.

1,25-Dihydroxyvitamin D3 reduces endothelium-dependent contractions by acting on the endothelium rather than the underlying vascular smooth muscles. This conclusion is based on the observations that the acetylcholine-induced endothelium-dependent contractions were reduced by a previous incubation of the rings with 1,25-dihydroxyvitamin D3, but those of rings without endothelium to the thromboxane mimetic, U-46619 or the α1-adrenergic agonist phenylephrine, were not affected.

The effect of 1,25-dihydroxyvitamin D3 is not specific for the activation of endothelial muscarinic receptors since the endothelium-dependent contractions to ATP, which binds to purinergic P2Y receptor (16), were also reduced. In contrast, endothelium-dependent contractions to the calcium ionophore A-23187 were unaffected. Endothelium-dependent contractions elicited by acetylcholine, ATP, and A-23187 share many common characteristics in that they all rely on an increase of cytosolic-free calcium (33) and the major endothelium-dependent contraction factor produced by...
these agonists is prostacyclin in SHR aorta (13). However, the calcium-triggering effect of acetylcholine and ATP is receptor mediated, whereas the calcium entry by A-23187 is receptor independent. A-23187 causes the cells to release potassium rapidly in exchange for an uptake of calcium (26). Thus the observations that the SHR aortae previously incubated with 1,25-dihydroxyvitamin D₃ exhibit a reduced endothelium-dependent response in contraction to acetylcholine and ATP, but not to the calcium ionophore, suggest that 1,25-dihydroxyvitamin D₃ acts upstream of the increase in the calcium concentration inside the endothelial cells. The same conclusion can be reached when comparing the release of prostacyclin, which is an endothelium-derived vasoconstrictor in the SHR aorta (13), during acetylcholine or A-23187 stimulation.

Inecalcitol, a synthetic analog of vitamin D₃, considered to be a superagonist at the vitamin D receptor (10, 36, 37), mimicked the actions of 1,25-dihydroxyvitamin D₃ in the organ chamber experiments, suggesting that the binding to the vitamin D receptor is crucial in the inhibitory effect of 1,25-dihydroxyvitamin D₃ or its analog on endothelium-dependent contractions. Therefore, the present study implies that functional vitamin D receptors are present in endothelial cells of both SHR and WKY aortae. This conclusion is supported by the immunohistochemical demonstration of the presence of vitamin D receptors in the endothelial cells of both SHR and WKY aortae. Indeed, the vitamin D receptor, one of the steroid/thyroid hormone nuclear receptor superfamilies, is responsible for most of the biological activities of vitamin D in body (15).

The cytosolic-free calcium concentration plays a key role in endothelium-dependent contractions (33). The present study confirms that acetylcholine and A-23187 at concentrations causing maximal EDCF-mediated contractions cause an increase in intracellular-free calcium (33). The current findings demonstrated that 1,25-dihydroxyvitamin D₃ modulates endothelium-dependent contractions through a calcium-dependent mechanism since it inhibited the acetylcholine-induced increase in calcium influx. This partial inhibition of the endothelial calcium influx then explains the reduced production of EDCFs and hence the decreased endothelium-dependent contractions. The absence of an effect on the A-23187-induced increase in calcium concentration supports the conclusion that 1,25-dihydroxyvitamin D₃ acts upstream of the increase in calcium in the endothelial cells. Indeed, vitamin D has long been identified to regulate calcium homeostasis (6), so it is not surprising that it may modulate the calcium transport in endothelial cells. Although 1,25-dihydroxyvitamin D₃ and inecalcitol have divergent effects on calcemia, with the latter causing a lesser hypercalcemic response than the former (37), the present experiments predict that the two analogs of vitamin D will share the same beneficial cardiovascular effects (19, 41).

In summary, the present study demonstrates that the major active metabolite of vitamin D, 1,25-dihydroxyvitamin D₃, reduces endothelial dysfunction by decreasing endothelium-dependent contractions. It does so by reducing the increase in cytosolic-free calcium concentration in the endothelial cells. If this were to occur in vivo at the level of the resistance vessels, it may help to explain the link between lower levels of vitamin D and hypertension (27, 30).

GRANTS

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