Chronic treatment with vitamin D lowers arterial blood pressure and reduces endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat

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Wong MS, Delansorne R, Man RYK, Svenningsen P, Vanhoutte PM. Chronic treatment with vitamin D lowers arterial blood pressure and reduces endothelium-dependent contractions in the aorta of the spontaneously hypertensive rat. *Am J Physiol Heart Circ Physiol* 299: H1226–H1234, 2010. First published August 6, 2010; doi:10.1152/ajpheart.00288.2010.—Vitamin D has cardiovascular protective effects besides regulating calcium homeostasis. To examine the chronic in vivo effect of a physiological dose of 1,25-dihydroxyvitamin D_3 on the occurrence of endothelium-dependent contractions, spontaneously hypertensive rats (SHR) and Wistar-Kyoto rats (WKY) were treated with the vitamin D derivative for 6 wk. The serum 1,25-dihydroxyvitamin D_3 level of both treated WKY and SHR was significantly higher than in untreated rats while the mean arterial blood pressure of the treated SHR was significantly lower than that of control SHR. Aortic rings with or without endothelium were studied in conventional organ chambers for isometric force measurement. Confocal microscopy was used to measure the cytosolic free calcium concentration (with the fluorescent dye fluo 4) and reactive oxygen species (ROS; with dichlorodihydrofluorescein diacetate). Reverse transcription PCR and Western blotting were used to determine the mRNA and protein expression level of cyclooxygenase-1 (COX-1), prostacyclin synthase, and thromboxane synthase. The endothelium-dependent concentration-contraction curves to both acetylcholine- and A-23187-induced contractions were shifted to the right in aortas from treated SHR but not from treated WKY. The chronic treatment normalized the relaxations of contracted preparations to acetylcholine. There were no significant differences in the increases in cytosolic free calcium concentration evoked by acetylcholine and A-23187 between control and treated groups. The endothelial ROS level was higher in SHR than WKY aortas and reduced by the chronic treatment. The gene and protein expression studies indicated that the overexpression of COX-1 observed in SHR aorta was reduced by the chronic treatment. These results demonstrate that chronic treatment with 1,25-dihydroxyvitamin D_3 modulates vascular tone and this modulation is accompanied by a lowered blood pressure, reduced expression of COX-1 mRNA and protein, and reduced ROS level in SHR. The reduction in endothelium-dependent contractions does not involve the surge in endothelial cytosolic calcium concentration that initiates the contractions.

1,25-dihydroxyvitamin D_3; endothelium-derived contracting factors; cyclooxygenase-1; reactive oxygen species

VITAMIN D DEFICIENCY OCCURS in 30–50% of the population (24), and it can lead to various cardiovascular disorders (12, 17, 32).

The serum concentration of 1,25-dihydroxyvitamin D_3, the most active metabolite of vitamin D, is inversely related to arterial blood pressure (22), and people with suboptimal production and/or intake of vitamin D have a higher risk of hypertension (7, 18, 38). In rats, vitamin D deficiency has been linked to cardiac fibrosis and hypertrophy (56, 57).

The endothelium modulates vascular tone by releasing vasodilator substances to control the underlying smooth muscle cells (53). Among these substances, nitric oxide (NO), produced by endothelial NO synthase (eNOS), plays a major role as endothelium-derived relaxing factor (EDRF) (8, 30, 53). When the production of NO by eNOS is reduced, with ageing or in the course of diseases such as diabetes, endothelial dysfunction ensues (53). In that regard, 1,25-dihydroxyvitamin D_3 has favorable effects on endothelial cells by protecting them against the deleterious effects of glycation end products and increasing the activity of eNOS (44). In addition to the release of NO, under certain circumstances, in particular when the NO production is reduced, the endothelial cells release vasoconstrictor prostanoids that elicit endothelium-dependent contractions (8, 51, 53, 54). In particular, in the aorta of the spontaneously hypertensive rat (SHR), such endothelium-dependent contractions are caused mainly by endothelium-derived endoperoxides and prostacyclin, which activate thromboxane-prostanoid (TP) receptors of the vascular smooth muscle cells (10, 13, 27, 46, 51, 52, 61). The unbalanced augmented production of endothelium-derived contraction factor (EDCF) is a characteristic of endothelial dysfunction (53–55), and such imbalance has been observed in blood vessels of humans [with atherosclerosis, myocardial infarction, and hypertension (3, 43, 53, 55)] and in animals [including the adult SHR (26), aging normotensive animals (21, 59), and diabetic rats (39)]. Previous in vitro studies of the laboratory showed that supraphysiological concentrations of 1,25-dihydroxyvitamin D_3 acutely reduce endothelium-dependent contractions in the SHR aorta (58). This pharmacological observation prompted the present experiments, which were designed to determine whether or not chronic in vivo treatment with a physiological dose of 1,25-dihydroxyvitamin D_3 affects endothelium-dependent contractions in the rat aorta.

METHODS

Animals and tissue preparation. Adult SHR and Wistar-Kyoto (WKY) rats (36 wk old) were anesthetized (pentobarbital sodium, 30 $mg \cdot ml^{-1} \cdot kg^{-1}$, ip injection), and mini-osmotic pumps (model 2006; Alzet, Cupertino, CA) were implanted subcutaneously. In the treatment group, the pumps were loaded with 1,25-dihydroxyvitamin D_3,

Table 1. *Sequences of PCR primer pairs and anticipated size of the amplified products for the genes of COX-1, prostacyclin synthase, thromboxane synthase, and β-actin*

Gene Name	Sense	Antisense	Size, bp
COX-1	5′-GGGAAACTTAAGTACCAGGTG	5′-CATCTGCTTTTCGGGCGGGAC	108
Prostacyclin synthase	5′-TCTGCAGCTTCCAAATTTCAA	5′-CTGCCTCCTTCTGTGGAGTCA	144
Thromboxane synthase	5′-GGGGCTTCTCAAGTTCGAAGT	5′-CCCAACTTCCTCAGTCTTGAG	117
β-Actin	5′-CGAGTCCGCGTCCACCCGCGA	5′-GACGACGAGCGCAGCGATATC	106

COX-1, cyclooxygenase-1.

dissolved in propylene glycol, which diffused out at a rate of 10 ng·100 g body wt^{-1}·day^{-1} (60). In the control group, they were filled with propylene glycol alone. The rats were returned to their cages after recovery from anesthesia and were housed in a room with standardized temperature (21 ± 1°C) and exposed to a 12:12-h dark-light cycle. They had free access to standardized diet (LabDiet 5053, Philadelphia, PA) and tap water. After 6 wk, the rats were anesthetized again, and their arterial blood pressure was measured by means of a polyethylene cannula inserted in the left carotid artery and connected to a pressure transducer (P23 1D; Gould Statham, Oxnard, CA). Next, the animals were killed, and their blood was collected for the determination of the serum level of 1,25-dihydroxyvitamin D$_3$. The thoracic aorta was isolated and placed immediately in cold Krebs-Ringer buffer with the following composition (in mM): 118 NaCl, 4.7 KCl, 2.5 CaCl$_2$, 1.2 MgSO$_4$, 1.2 KH$_2$PO$_4$, 25 NaHCO$_3$, and 11.1 glucose (control solution). The adhering fat and connective tissue were removed. The aortas 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. The endothelial layer of some rings was collected for reverse transcription PCR analysis and Western blotting. The other rings were used to measure isometric force or the changes in either cytosolic free calcium or reactive oxygen species (ROS) 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.

1,25-Dihydroxyvitamin D$_3$ radioimmunoassay. 1,25-Dihydroxyvitamin D$_3$ was purified from serum by monoclonal immunoextraction and quantified using a ^{125}I radioimmunoassay according to the manufacturer's instructions (Immunodiagnostic Systems, Fountain Hills, AZ).

Isometric force. The rings were suspended in organ chambers that contained 5 ml of control solution (37°C) aerated with a 95% O$_2$-5% CO$_2$ gas mixture (pH 7.4). Each ring was connected to a force transducer (model MLT0201/D; AD Instruments, Colorado Spring, CO) 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 incubated with N^{ω}-nitro-L-arginine methyl ester (L-NAME; 10^{-4} M) for 30 min to optimize endothelium-dependent contractions (1). They were then exposed to cumulative concentrations of acetylcholine (10^{-8} to 10^{-5} M) or of the calcium ionophore A-23187 (10^{-8} to 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} to 10^{-6} M) to obtain endothelium-independent contractions. To obtain endothelium-dependent relaxations, some rings with endothelium were first contracted with phenylephrine (10^{-6} M) and then exposed to increasing concentration of acetylcholine (10^{-10} to 10^{-3} M). Relaxations were expressed in percentage of phenylephrine-induced contractions.

Cytosolic free calcium and ROS levels. Cytosolic free calcium change was monitored and measured by confocal microscopy after incubation with fluo 4-AM [2 × 10^{-5} M (29, 48, 58)]. ROS were labeled with dichlorodihydrofluorescein diacetate (DCF; 4 × 10^{-5} M). The dyes were dissolved in 0.1% (vol/vol) dimethyl sulfoxide (DMSO) first followed by dilution with control solution containing 0.02% (vol/vol) pluronic acid F-127. Aortic rings for calcium measurement were incubated in the dye mixture for 1 h, whereas those for ROS measurement were incubated for 10 min to allow penetration of the dyes in the tissue and cleavage of acetyloxymethyl ester. After incubation, the rings were cut longitudinally into strips and fixed into a transparent chamber with the intimal side faced downward, leaving a 1-mm gap between the endothelium and the cover slip. The whole system was connected to a peristaltic pump for continuous perfusion with oxygenated control solution (95% O$_2$-5% CO$_2$) loaded with different drugs. The incoming solution was maintained at 37°C, and the perfusion rate was 2 ml/min. The tissues were first superfused with control solution for 10 min to wash away excess dye. For calcium measurements, three different concentrations (selected from the concentration-contraction curves obtained in the organ chambers) of either acetylcholine (10^{-7}, 10^{-6}, and 10^{-5} M) or A-23187 (10^{-7}, 3 × 10^{-7}, and 10^{-6} M) were added. Real-time changes in cytosolic free calcium concentration in the endothelial cells were monitored using a laser confocal microscope at 1-s intervals for 300 s. For ROS measurements, single still images were taken to quantify the basal ROS level since time series scanning by the laser confocal may lead to intense background fluorescence. The maximal change in fluorescence intensity was calculated (expressed as percent increase over the basal fluorescence intensity) and compared between the control and treated 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.

The microscope used in this study was an inverted Nikon Eclipse TE300 with ×10 objective. It was attached to a confocal argon ion Radiance-2100 laser scanning unit (Bio-Rad, Hertfordshire, UK). An

Table 2. *Mean arterial blood pressure and serum 1,25-dihydroxyvitamin D$_3$ levels of treated [1,25-dihydroxyvitamin D$_3$ (10 ng·100 g body wt^{-1}·day^{-1})] and untreated (control) SHR and WKY*

Strain	Arterial Mean Blood Pressure, mmHg		Serum 1,25-Dihydroxyvitamin D$_3$, pg/ml	
	Control	Treated	Control	Treated
WKY	130.5 ± 7.2	128.8 ± 6.8	23.8 ± 2.3	48.4 ± 9.8*
SHR	198.3 ± 5.4	165.6 ± 12.8*	20.9 ± 1.3	54.9 ± 11.6*

Data are shown as means ± SE; n = 6 rats in each group. WKY, Wistar-Kyoto rats; SHR, spontaneously hypertensive rats. *Statistically significant differences with the controls (P < 0.05).

argon ion laser line (488 nm) was directed to the sample to excite the fluorescent probes. The emitted fluorescence was detected via a band pass filter (510–525 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, Hercules, CA) 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.

RNA extraction. The aorta was cut open with the endothelial cell layer facing upward. The endothelial cells were collected by scraping with a spatula (Cell Lifter; Corning Costar, New York, NY) and were pooled in 1 ml of Tri Reagent (Molecular Research Center, Cincinnati, OH). To improve the efficiency of RNA isolation, cells were triturated by thrusting the Tri Reagent cell mixture up and down a syringe needle to facilitate cell rupture. Total RNA was isolated with Tri Reagent according to the manufacturer's instructions.

Real-time PCR. Total RNA (500 ng) was used for reverse transcription. The total reverse transcription product (1 μl) was added to the PCR reaction mixture (20 μl) containing 10 μl 2× SYBR Green PCR master mix (Applied Biosystems) and the primers (sense and antisense; 1 μM) for PCR. The real-time PCR reactions were monitored using the 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The primer sequences are listed in Table 1. Standard reference curves were obtained concomitantly with the unknown samples for the calculation of gene copy numbers in each real-time PCR experiment. Gene expressions were normalized to that of β-actin.

Protein extraction and immunoblotting. The aorta was cut open, and the endothelial cells were collected by scraping with a spatula (Cell Lifter; Corning Costar) and pooled into lysis buffer (20 mmol/l Tris·HCl, 1% Triton X-100, 150 mmol/l NaCl, 1 mmol/l EDTA, 1 mmol/l EGTA, 2.5 mmol/l sodium pyrophosphate, 1 mmol/l β-glycerophosphate, and 1 mmol/l sodium orthovanadate) supplemented with a cocktail of protease inhibitors (100 mmol/l phenylmethylsulfonyl fluoride, 10 μg/ml trypsin inhibitor, 1 mg/ml leupeptin, and 2 μg/ml pepstatin A). The mixture was centrifuged at 5,000 rpm at 4°C for 3 min, and the supernatant was kept at −80°C until use. For gel electrophoresis, 20 μg of tissue homogenate protein were used. The samples were mixed with 1× sample buffer (NuPAGE LDS Sample Buffer 4×; Invitrogen, Carlsbad, CA) and 1× reducing agent (10× Reducing Agent; Invitrogen) and diluted with ultrapure water to obtain 40 μl. The samples were boiled for 10 min at 95°C and subsequently separated by SDS-PAGE (10%) at 200 V, 500 mA for 1 h. The proteins were transferred electrophoretically onto nitrocellulose membranes. The blotting was performed at 1,000 V, 300 mA for 2 h. Subsequently, the membranes were blocked in Tris-buffered saline with 5% dry milk at room temperature for 2 h, washed in Tris-buffered saline Tween 20 (TBST), and then incubated with primary antibodies (1:200) overnight at 4°C. Next, the membranes were incubated with either horseradish peroxidase-conjugated anti-rabbit antibody for thromboxane synthase or anti-mouse antibody (1:3,000 in milk, room temperature, for 2 h; Amersham Biosciences, Piscataway, NJ) for cyclooxygenase (COX)-1 and prostacyclin synthase. Bound secondary antibody was detected by chemiluminescence (Amersham Biosciences) and exposed to X-ray film. To reprobe β-actin, membranes were washed with Tris-Tween buffered saline and incubated with the monoclonal β-actin antibody (Sigma, St. Louis, MO). The optical densities of the protein bands were determined with a computerized program (MultiAnalysis; Bio-Rad Laboratories, Irvine, CA). Densitometric analysis was normalized to the immunoreactive β-actin band.

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

Chemicals. Acetylcholine, A-23187, DCF, L-NAME, pluronic acid F-127, and 1,25-dihydroxyvitamin D$_3$ were purchased from Sigma Chemical. U-46619 was purchased from Biomol (St. Louis, MO). Dimethyl sulfoxide (DMSO) was purchased from Merck (Darmstadt, Germany). EIA kits were purchased from Cayman Chemical (Ann Arbor, MI). Fluo 4-AM was purchased from Molecular Probes (Eugene, OR). All drugs, except U-46619 and the calcium ionophore A-23187, were prepared daily by dissolving in absolute DMSO (0.1% in the organ bath) and further diluted with control solution.
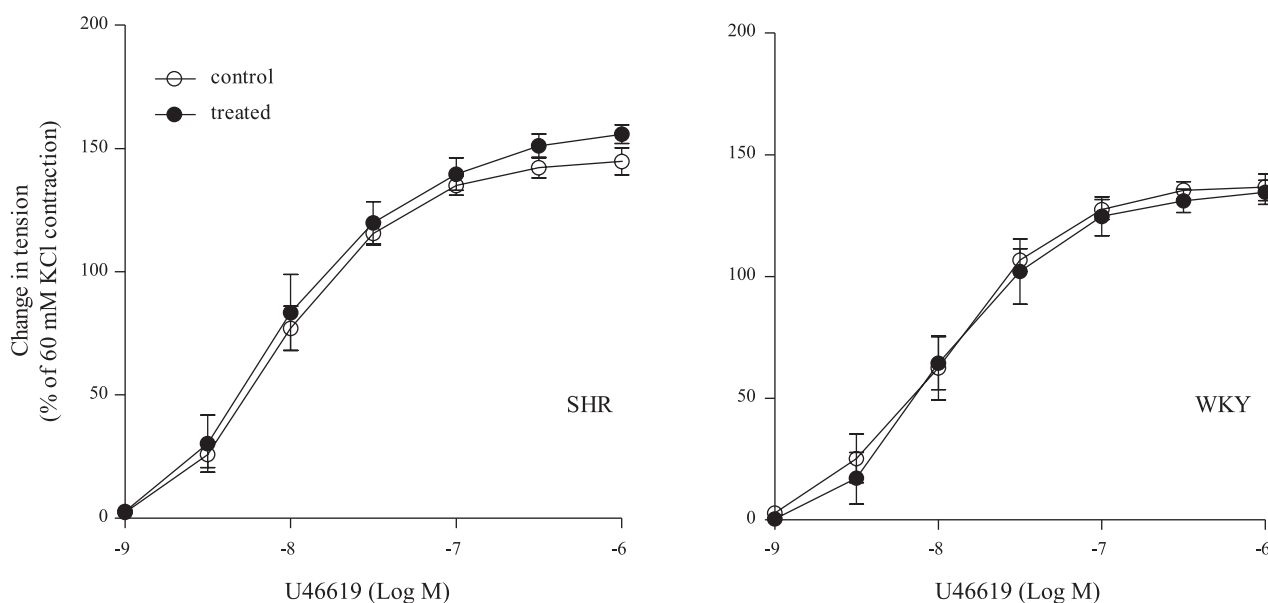

Fig. 1. Contractions to cumulative concentrations of U-46619 in rings of spontaneously hypertensive rats (SHR, *left*) and Wistar-Kyoto rats (WKY, *right*) aortas without endothelium taken from control rats or rats chronically treated with 1,25-dihydroxyvitamin D$_3$ (10 ng 100 g body wt^{-1} day^{-1}). Data are expressed as a percentage of the reference contraction to 60 mM KCl and shown as means ± SE ($n = 6$).

RESULTS

Blood pressure. The mean arterial blood pressure of untreated SHR was significantly higher than that of WKY (Table 2). The mean arterial blood pressure was significantly lower in treated SHR compared with untreated rats of the same strain, whereas no significant changes were observed in WKY (Table 2).

Serum 1,25-dihydroxyvitamin D_3. The serum level of 1,25-dihydroxyvitamin D_3 was significantly higher in the treated rats compared with their respective controls. There were no significant differences between SHR and WKY (Table 2).

Isometric force measurements. In rings without endothelium, U-46619 evoked comparable concentration-dependent contractions in aortas of both SHR and WKY (Fig. 1). No significant difference was found between control and treated rats of either strain.

In aortic rings with endothelium, acetylcholine and A-23187 evoked concentration-dependent, endothelium-dependent contractions. The responses were significantly greater in preparations from SHR than in those from WKY. Both acetylcholine- and the A-23187-induced contractions were inhibited significantly in aortas from the treated SHR group compared with the SHR controls. There was no significant difference in contractions to either acetylcholine or A-23187 between aortas from control and treated WKY (Fig. 2).

In phenylephrine (10^{-6} M)-contracted SHR rings with endothelium, acetylcholine (10^{-10} to 10^{-3} M) evoked concentration-dependent, triphasic responses (relaxation-contraction-relaxation). The secondary contraction starting at 10^{-6} M was absent in aortas of the treated SHR group (Fig. 3).

Cytosolic free calcium. Both acetylcholine (10^{-7}, 10^{-6}, and 10^{-5} M) and A-23187 (10^{-7}, 3×10^{-7}, and 10^{-6} M) caused
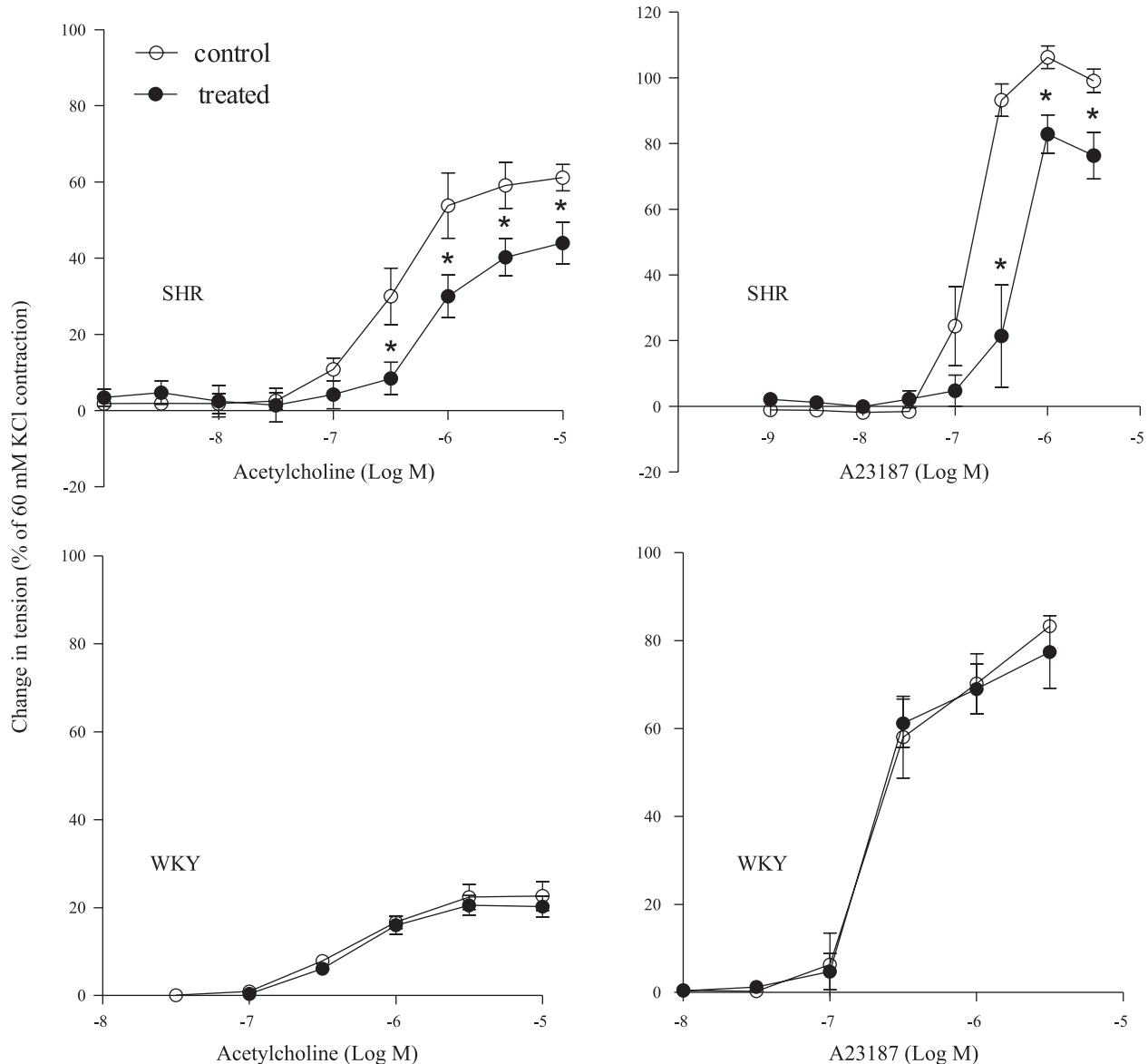

Fig. 2. Contractions to cumulative concentrations of acetylcholine (*left*) or A-23187 (*right*) in rings of SHR (*top*) or WKY (*bottom*) aortas with endothelium taken from control rats or rats chronically treated with 1,25-dihydroxyvitamin D_3 (10 ng·100 g body wt^{-1}·day^{-1}). Data are expressed as a percentage of the reference contraction to 60 mM KCl and shown as means ± SE ($n = 6$). *Statistically significant differences with controls ($P < 0.05$).
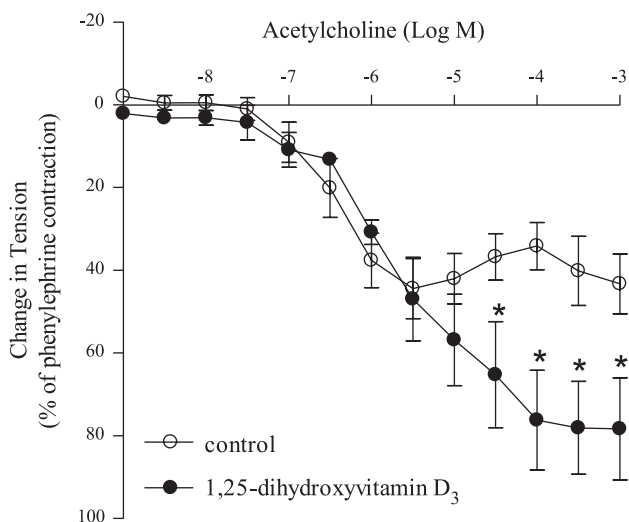
Fig. 3. Relaxation to cumulative concentrations of acetylcholine in phenylephrine-contracted rings of SHR aorta with endothelium taken from control rats or rats chronically treated with 1,25-dihydroxyvitamin D_3 (10 ng·100 g body wt^{-1}·day^{-1}). Data are expressed as a percentage of the contraction to phenylephrine (10^{-6} M) and shown as means ± SE ($n = 5$). *Statistically significant differences with controls ($P < 0.05$).

concentration-dependent increases in cytosolic free calcium concentration in aortas of both SHR and WKY. The increases in calcium concentration caused by 10^{-6} and 10^{-5} M acetylcholine were significantly higher in SHR compared with WKY aortas. Chronic treatment with 1,25-dihydroxyvitamin D_3 did

not significantly affect these increases in calcium concentration in either strain (Fig. 4).

ROS level. The ROS level was significantly higher in the endothelial cells of SHR than in those of WKY. Chronic treatment of 1,25-dihydroxyvitamin D_3 significantly reduced the endothelial ROS level in the SHR but not in WKY aortas (Fig. 5).

Gene and protein expressions. Real-time PCR (Fig. 6) and Western blotting (Fig. 7) revealed a significant decrease in the mRNA and protein expression of COX-1 in endothelial cells of SHR treated with 1,25-dihydroxyvitamin D_3 compared with control SHR, whereas it was comparable in treated and control WKY. No differences in gene and protein expression were observed for prostacyclin and thromboxane synthases after chronic vitamin D treatment in either SHR or WKY.

DISCUSSION

The present experiments were designed to determine whether or not chronic in vivo treatment with a physiological dose of 1,25-dihydroxyvitamin D_3 affects endothelium-dependent contractions.

The levels of mRNA and protein of COX-1 are comparable in the aorta of 5- and 10-wk-old WKY and SHR (33), but these levels are significantly higher in preparations of 36 (10)- or 40 (33)-wk-old (33) SHR than in those of age-matched WKY. Because overexpression of COX-1 is the major factor that contributes to the overproduction of EDCF in the SHR (47), the present experiments were performed on aortas of 36-wk-old rats. The rat aorta is a standard preparation to measure
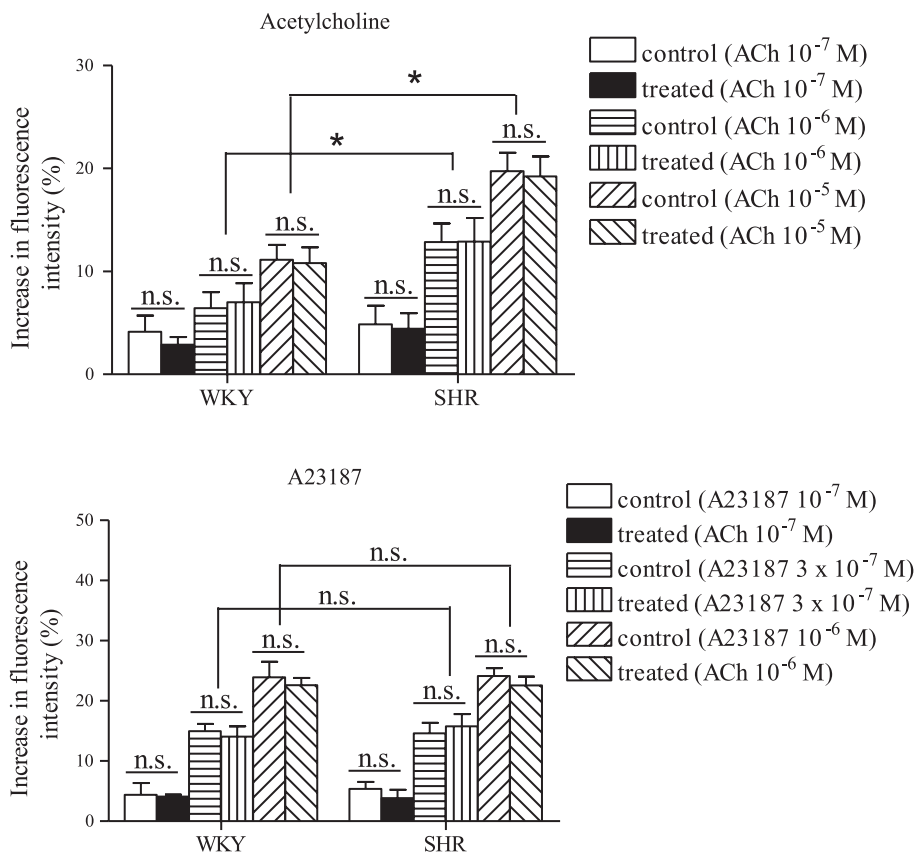
Fig. 4. Bar graphs showing increases in fluorescence intensity (representing the cytosolic free calcium concentration) in response to three concentrations of either acetylcholine (ACh; *top*) or A-23187 (*bottom*) as a percentage of the basal calcium level in aortic endothelial cells from control and 1,25-dihydroxyvitamin D_3-treated (10 ng·100 g body wt^{-1}·day^{-1}) WKY and SHR. Data are expressed as means ± SE ($n = 5$). *Statistically significant differences between WKY and SHR. NS, not significantly different.
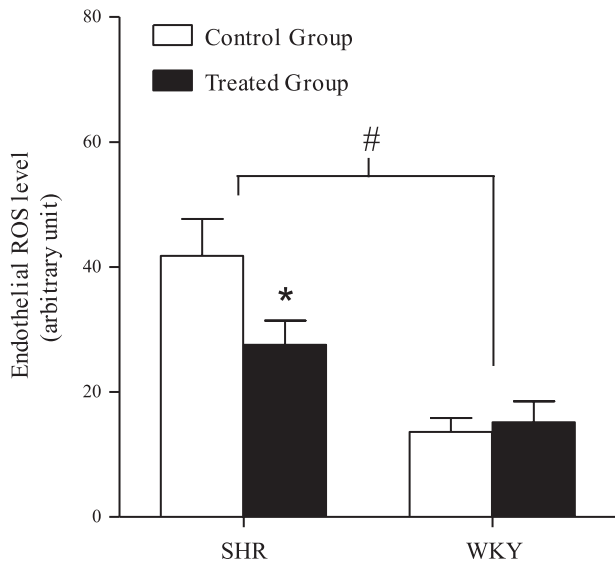
Fig. 5. Levels of reactive oxygen species (ROS) in aortic endothelial cells of control and 1,25-dihydroxyvitamin D$_3$ (10 ng·100 g body wt^{-1}·day^{-1})-treated SHR and WKY. Data are expressed as means ± SE ($n = 5$). *Statistically significant difference with controls ($P < 0.05$). #Statistically significant differences between SHR and WKY aortas ($P < 0.05$).

EDCF-mediated responses (1, 14, 26, 47) and allows the measure of the change in cytosolic free calcium and ROS levels (48, 58).

The in vivo treatment with 1,25-dihydroxyvitamin D$_3$ did not affect ex vivo contractions evoked by the TP receptor agonist U-46619 in SHR preparations without endothelium but reduced the contractions to both acetylcholine and the calcium ionophore A-23187 in those with endothelium. These observations demonstrate that the chronic intake of vitamin D
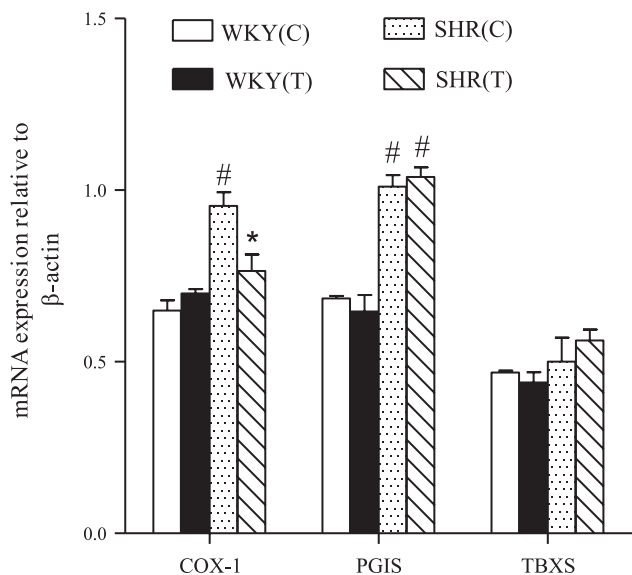

Fig. 6. Real-time PCR results showing the gene expression levels of cyclooxygenase-1 (COX-1), prostacyclin synthase (PGIS), and thromboxane synthase (TBXS) in aortic endothelial cells in control (C) or 1,25-dihydroxyvitamin D$_3$ (10 ng·100 g body wt^{-1}·day^{-1})-treated (T) SHR and WKY. Data are expressed as means ± SE ($n = 6$). *Statistically significant difference with controls ($P < 0.05$). #Statistically significant differences between SHR and WKY aortas ($P < 0.05$).
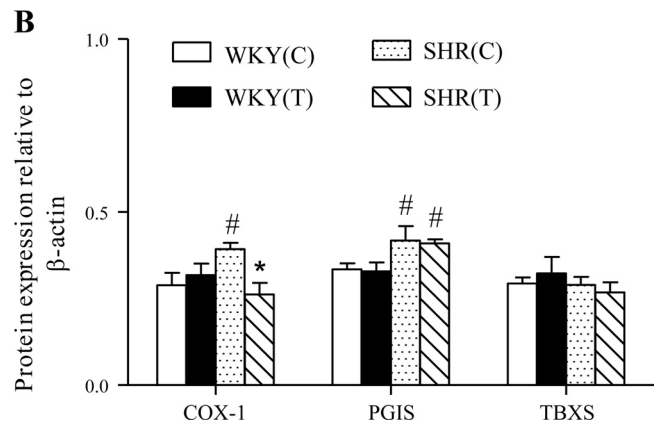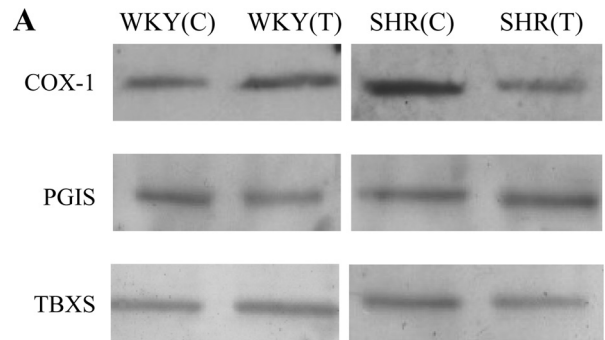

Fig. 7. A: Western blot showing the protein expression of COX-1, PGIS, and TBXS in endothelial cells. B: the protein expressions of these enzymes relative to β-actin are expressed as a bar graph. Data are expressed as means ± SE ($n = 6$). *Statistically significant difference with controls ($P < 0.05$). #Statistically significant differences between SHR and WKY aortas ($P < 0.05$).

reduces endothelium-dependent contractions without affecting the responsiveness of the vascular smooth muscle of the SHR aorta.

An increase in cytosolic calcium concentration in the endothelial cells is the first step in the process leading to EDCF-mediated responses (48, 58). The calcium-triggering effect of acetylcholine is receptor-mediated, whereas the calcium ionophore A-23187 causes the cells to release potassium rapidly in exchange for an uptake of calcium (36). Thus the observations that the aortas of treated SHR exhibit a reduced endothelium-dependent response to both the muscarinic agonist and the calcium ionophore suggest that 1,25-dihydroxyvitamin D$_3$ acts downstream of the surge in calcium concentration. This interpretation was confirmed by measuring changes in calcium fluorescence in the endothelial cells exposed to acetylcholine and A-23187. Indeed, both agonists evoked concentration-dependent increases in cytosolic calcium concentration, and these increments were not affected by the chronic treatment with vitamin D. This contrasts with the previous findings that acute exposure to a supraphysiological concentration of 1,25-dihydroxyvitamin D$_3$ reduces the increase of calcium in the endothelial cells in response to acetylcholine but not to A-23187. The present findings confirm that acetylcholine, but not A-23187, causes larger increases in endothelial calcium concentration in the SHR than in the WKY aorta (48). They demonstrate that the increase in calcium concentration evoked by both agonists is not affected by the chronic treatment with

vitamin D and thus that the latter does not interfere with the surge in calcium required to initiate endothelium-dependent contractions.

The triphasic (relaxation-contraction-relaxation) response to acetylcholine demonstrated in the present study is in line with previous observations in the same preparation (26, 45). The secondary contraction phase illustrates the EDCF-mediated component since it can be prevented by the cyclooxygenase inhibitor indomethacin (26, 42). The present study demonstrates that the secondary contraction was absent in the aortas of treated SHR, which further confirms that chronic treatment with 1,25-dihydroxyvitamin D_3 prevents the occurrence of endothelium-dependent contractions, and thus the endothelial dysfunction of this animal model of essential hypertension. The lack of effect of the chronic treatment on the first relaxing phase shows that it has minimal effects on the NO-dependent pathway. Earlier bioassay studies have demonstrated that the release of EDRF/NO is normal in aortas of SHR treated with indomethacin (25).

Acetylcholine- and A-23187-induced endothelium-dependent contractions were reduced by the chronic treatment with vitamin D in the SHR but not in their normotensive counterpart WKY. These results indicate that the chronic action of vitamin D is specifically targeting the disease model. When considering the differences between SHR and WKY, the major enzymes downstream of the initial calcium surge, which are involved in the generation of EDCF, were studied (49, 51, 53). They included COX-1, prostacyclin synthase, and thromboxane synthase. The expression levels of COX-1 and prostacyclin synthase were augmented in the aortas of untreated SHR compared with those of WKY, which confirms results from previous studies and explains the overproduction of EDCF in the SHR aorta (10, 11, 33, 49). Both the mRNA expression and protein presence of COX-1 was reduced in the treated SHR, but no such reduction was observed in preparations from WKY despite similar increases in the plasma level of vitamin D_3, demonstrating that 1,25-dihydroxyvitamin D_3 chronically prevents the overexpression of COX-1, hence reducing the overproduction of EDCF in the disease model without altering the constitutive expression of the enzyme in the normotensive animals. 1,25-Dihydroxyvitamin D_3 downregulates the mRNA expression of COX-2 in human neonatal monocytes (34) and prostate cancer cells (31). COX-1 and COX-2 are the two major isoforms of cyclooxygenase, and both are heme proteins with comparable ability in transforming arachidonic acid into endoperoxides (9, 50), the precursor of vasoconstrictor prostanoids that, per se, can act as EDCF (10). Selective COX-1 inhibitors abolish endothelium-dependent contractions of the SHR aorta, whereas preferential COX-2 inhibitors only modestly reduce the response (10, 62). In addition, endothelium-dependent contractions can be induced in aortas of COX-2 knockout but not of COX-1 knockout mice (47). Taken in conjunction, those previous findings demonstrate that COX-1 is responsible for the production of EDCF in rodents (51, 54). The present data suggest that the ex vivo inhibition of endothelium-dependent contractions afforded by the chronic in vivo treatment with 1,25-dihydroxyvitamin D_3 may well be because of a direct effect of the vitamin preventing the overexpression of the COX-1 gene and the resulting increased protein presence of the enzyme, which is the major source of EDCF.

The present study demonstrates a reduction of the basal ROS level in the endothelial cells of SHR treated chronically with 1,25-dihydroxyvitamin D_3, which may explain the normalization of the COX-1 expression in the hypertensive animals. Oxidative stress contributes to the upregulation of cyclooxygenases (4, 19). In arteries of rats with streptozotocin-induced diabetes, both COX-1 and COX-2 are upregulated, and this can be prevented by antioxidant treatment (40). Vitamin D possesses antioxidant activities and protects cells from oxidative stress (5, 16, 41). Besides vitamin D, several antioxidants reduce arterial blood pressure in various hypertensive models (2, 6, 20, 63).

In male Sprague-Dawley rats, the normal serum vitamin D concentration decreases with aging and is around 58 pg/ml in 12-mo-old rats (60). The serum vitamin D level of normal WKY in the present study is ∼24 pg/ml, which appears to be relatively low. This may be because of the different strain of the rat used, different composition of the diet, or different housing conditions. The vitamin D level in both strains increased after treatment but remained within the physiological range (15, 60). Although the serum level of 1,25-dihydroxyvitamin D_3 was comparable in the treated groups of both the normotensive and hypertensive rats, only the treated SHR showed a substantial decrease in arterial blood pressure, suggesting that the chronic administration of vitamin D interferes with the disease process without affecting the cardiovascular system under normal conditions. Vitamin D lowers blood pressure in hypertensive patients (35). The underlying mechanisms include suppression of the renin-angiotensin-aldosterone system (23), parathyroid hormone secretion (37), and improvement of endothelial function in vascular cells (28, 64). Earlier work suggests that the overexpression of COX-1 in the aorta of the SHR follows rather than precedes the increase in arterial blood pressure (11, 33). Therefore, it is possible that the reduction in COX-1 expression and/or the endothelium-dependent contractions caused by the chronic treatment of vitamin D observed in the present study may be secondary to the reduced arterial blood pressure in the treated SHR.

In conclusion, chronic treatment of 1,25-dihydroxyvitamin D_3 reduces endothelium-dependent contractions in the SHR aorta. The inhibitory effect is accompanied by a lowered blood pressure, reduction in the basal endothelial ROS level, and downregulation of COX-1 expression. These changes are not seen in normotensive animals.

DISCLOSURES

No conflicts of interest are declared by the authors.

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