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$1,25(OH)_2D_3$ Induces Placental Vascular Smooth Muscle Cell Relaxation by Phosphorylation of Myosin Phosphatase Target Subunit 1^{Ser507}: Potential Beneficial Effects of Vitamin D on Placental Vasculature in Humans[1]

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ABSTRACT

Placental vascular dysfunction has been linked to insufficiency/deficiency of maternal vitamin D levels during pregnancy. In contrast, sufficient maternal vitamin D levels have shown beneficial effects on pregnancy outcomes. To study the role of vitamin D in pregnancy, we tested our hypothesis that vitamin D exerts beneficial effects on placental vasculature. We examined expression of CYP2R1, CYP27B1, vitamin D receptor (VDR), and CYP24A1 in placental vascular smooth muscle cells (VSMCs) in response to $1,25(OH)_2D_3$. We found that VDR expression was inducible, CYP27B1 expression was dose-dependently down-regulated, and CYP24A1 expression was dose-dependently up-regulated in cells treated with $1,25(OH)_2D_3$. These data suggest a feedback autoregulatory system of vitamin D existing in placental VSMCs. Using a VSMC/collagen-gel contraction assay, we evaluated the effect of $1,25(OH)_2D_3$ on placental VSMC contractility. We found that, similar to losartan, $1,25(OH)_2D_3$ could diminish angiotensin II-induced cell contractility. The mechanism of $1,25(OH)_2D_3$-mediated VSMC relaxation was further explored by examination of Rho-associated protein kinase 1 (ROCK1)/phosphorylation of myosin phosphatase target subunit 1 (MYPT1) pathway molecules. Our results showed that p-MYPT1^{Thr853} and p-MYPT1^{Thr696} were undetectable. However, p-MYPT1^{Ser507}, but not p-MYPT1^{Ser668}, was significantly up-regulated in cells treated with losartan plus angiotensin II. Similar effects were also seen in cells treated with $1,25(OH)_2D_3$ plus angiotensin II or $1,25(OH)_2D_3$ plus losartan plus angiotensin II. Because MYPT1 serine phosphorylation could activate myosin light chain phosphatase (MLCP), and MLCP activation is an important regulatory machinery of smooth muscle cell relaxation, up-regulation of MYPT1^{Ser507} phosphorylation could be a mechanism of vitamin D and/or losartan mediated placental VSMC relaxation.

MYPT1, placenta, pregnancy, vitamin D, VSMC contractility

INTRODUCTION

In recent years, vitamin D has increasingly been recognized as having significant effects on organ development and cardiovascular health. Epidemiologic studies revealed that there is an inverse correlation between blood levels of vitamin D and many chronic or acute cardiovascular-related diseases, including hypertension, coronary artery and peripheral vascular diseases, myocardial infarction, heart failure, stroke, hyperlipidemia, diabetes, and others [1–4], suggesting that vitamin D deficiency is an independent risk factor for cardiovascular events [2]. Vitamin D insufficiency/deficiency during pregnancy has also been found to be associated with increased risks of maternal and fetal morbidities such as pre-eclampsia, intrauterine growth restriction, gestational diabetes, and preterm birth [5–8]. Conversely, increasing vitamin D intake during pregnancy appears to afford protection to both the mother and the developing fetus, with lower risk of pregnancy complications [9–11].

Placental vascular development and proper placental blood perfusion are fundamental for a successful pregnancy. It is well known that placental vascular dysfunction negatively affects fetal growth [12], and importantly, placental vascular dysfunction was found to be linked to maternal vitamin D insufficiency/deficiency during pregnancy [13]. We previously reported that vitamin D metabolic components, including vitamin D binding protein (VDBP), vitamin D 25-hydroxylase (CYP2R1), 25-hydroxyvitamin D_3 1-alpha-hydroxylase (CYP27B1), vitamin D receptor (VDR), and 1,25-dihydroxyvitamin D_3 24-hydroxylase (CYP24A1), were all present in placental trophoblasts [14]. CYP2R1 and CYP27B1 are 2 vitamin D-converting enzymes. The former enzyme converts cholecalciferol into 25-hydroxyvitamin D and the latter converts 25-hydroxyvitamin D into 1,25-hydroxyvitamin D ($1,25[OH]_2D_3$). The function of CYP24A1 is to degrade $1,25(OH)_2D_3$. Altered trophoblast expression of VDBP, CYP2R1, and VDR was found to be associated with increased oxidative stress in pre-eclampsia [14], a pregnancy disorder relating to placental vascular dysfunction. Our recent study also revealed that vitamin D could promote angiogenic activity of

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placental vessel endothelial cells [15]. However, little information is available about effects of vitamin D on vasoactivity of placental vasculature. To explore the potential role of vitamin D in placental vascular function, in the present study we isolated vascular smooth muscle cells (VSMCs) from normal-term placentas and specifically examined vitamin D metabolic component expression, including CYP2R1, CYP27B1, VDR, and CYP24A1 in placental VSMCs. Using a collagen-gel contraction assay, we determined effects of bioactive vitamin D $1,25(OH)_2D_3$ on contractility of placental VSMC stimulated with angiotensin II. Our results showed that $1,25(OH)_2D_3$ not only reduced placental VSMC contractility induced by angiotensin II but also potentiated losartan to promote placental VSMC relaxation. The mechanism of $1,25(OH)_2D_3$-mediated placental VSMC relaxation was further assessed by determination of Rho-associated protein kinase 1 (ROCK1)/myosin phosphatase regulatory target unit 1 (MYPT1) pathway molecule expression. Strikingly, we found that $1,25(OH)_2D_3$ in the presence of angiotensin II down-regulated ROCK1 expression and up-regulated MYPT1 phosphorylation at serine 507 (p-MYPT1^{Ser507}) expression, similar to the effect found with losartan when it is combined with angiotensin II. Because MYPT1 is the major subunit involved in myosin light chain phosphatase (MLCP) activation, which is essential for vessel vasodilation [16], phosphorylation of MYPT1^{Ser507} induced by $1,25(OH)_2D_3$ could be a cellular and molecular mechanism of $1,25(OH)_2D_3$-mediated relaxation of placental VSMCs.

MATERIALS AND METHODS

Chemicals and Reagents

Dulbecco modified Eagles medium (DMEM), $1,25(OH)_2D_3$, losartan, and angiotensin II were purchased from Sigma Chemicals (St. Louis, MO). Antibodies for CYP27B1 (product H-90; code sc-67261), VDR (product D-6; code sc-13133), CYP24A1 (product H-87; code sc-66851), AT-1 (product N-10; code sc1173), AT-2 (product H-143; code sc-9040), CD31 (product M-20; code sc-1506), vimentin (product V-9; code sc-6260), and ROCK1 (product G-6; code sc-17794) were purchased from Santa Cruz Biotechnology (Dallas, TX). Antibody for CYP2R1 (product S1968) was from Epitomics (Burlingame, CA), and anti-human smooth muscle actin (product M0851) was from Dako Denmark (Carpinteria, CA). Antibodies for RhoA (products 67B9 and 2117P), MYPT1 (products D6C1 and 8574S), p-MYPT1^{Thr853} (at threonine 853 [product 4563P]), p-MYPT1^{Thr696} (product 5163P), p-MYPT1^{Ser668} (product 5143P), and p-MYPT1^{Ser507} (product 3040P) were obtained from Cell Signaling Technology (Danvers, MA). β-actin antibody was from Sigma Chemicals. All other chemicals and reagents were from Sigma Chemicals unless otherwise noted.

Placental VSMC Isolation and Rat Tail Type I Collagen Extraction

Placental VSMCs were isolated from tertiary chorionic plate arteries from normal-term placentas as previously described [17], and VSMCs at passages 3–5 were used in the present study. Placental VSMCs were cultured with DMEM supplemented with 10% fetal bovine serum and antibiotics in a humidified cell culture incubator at 37°C with 5% CO_2. Isolated placental VSMCs exhibited positive staining for human smooth muscle actin and vimentin and negative staining for CD31. Collection of placentas was approved by the Institutional Review Board for Human Research at Louisiana State University Health Science Center-Shreveport.

Rat tail type I collagen was prepared as previously described [17, 18]. Briefly, rat tail type I collagen was extracted by digestion of rat tail tendons in sterile 4 mM acetic acid for 48 h at 4°C. Undigested tendon pieces were removed by filtration through 250-μm nylon filter (Spectrum Labs, Rancho Dominguez, CA). After centrifugation, collagen solution was divided into aliquots which were lyophilized by using a bench-top manifold freeze-dryer unit (Millrock Technology, Kingston, NY) and stored at −20°C for future use.

Preparation of Placental VSMC/Collagen Gel

Lyophilized collagen was solubilized with cold 0.012 M hydrochloric acid under constant agitation overnight at 4°C, until all collagen resolved. On the day of assay, the pH of collagen solution was quickly titrated at 7.3–7.4 by 1 N NaOH on ice. Placental VSMCs pretreated with $1,25(OH)_2D_3$ (100 nM for 24 h) were harvested by trypsin-EDTA, suspended in serum-free DMEM, and then quickly added to collagen solution on ice. The final concentration of placental VSMC/collagen mixture was 10^5 cells/2.5 mg of collagen/ml. An aliquot of 500 μl placental VSMC/collagen mixture was then loaded per well into 24-well plates. The placental VSMC/collagen mixture was allowed to polymerize at 37°C for 1 h, and then 1.0 ml of serum-free DMEM supplemented with different agents (see below) was added to each well. Gels were detached from the edge of the wells with an L-shaped blunted glass pipette tip and allowed to float freely [17, 18].

Placental VSMC/Collagen Gel Contraction Assay

To study effects of vitamin D on VSMC contractile activity, placental VSMC/collagen gel contraction assays were performed. Because renin-angiotensin system is present in the placenta, angiotensin II (10^{-7} M) was used to stimulate VSMC contraction, and losartan (10^{-4} M) was used to block angiotensin II receptor activity. $1,25(OH)_2D_3$ (100 nM) was used as a bioactive vitamin D. VSMC/collagen gel was incubated with 1 ml serum-free DMEM with different treatments in 6 groups, as follows: 1) untreated control, 2) angiotensin II alone, 3) losartan plus angiotensin II, 4) $1,25(OH)_2D_3$ alone, 5) $1,25(OH)_2D_3$ plus angiotensin II, and 6) $1,25(OH)_2D_3$ plus losartan plus angiotensin II. Gels were incubated at 37°C in 5% CO_2 for 7 days. Angiotensin II, losartan, or $1,25(OH)_2D_3$ was added to culture wells every 24 h. Plates were scanned every 24 h using an Epson flatbed scanner (Long Beach, CA) over 7 days to monitor changes of gel area. For data analysis, the area of the gel in each well was measured by ImageJ imaging analysis software (National Institutes of Health, Bethesda, MD). Gel contractility (X) was defined as the change in gel surface area on Day 7 (Y) as a fraction of its area measured on Day 0 (Z) and then normalized to the internal control in each assay ($X = [1 - Y/Z] \times 100\%$) [18]. Gel contraction of control wells on Day 7 was set as 100% contraction. Final data were expressed as mean ± SEM from 5 independent experiments with triplicate wells in each group.

Protein Expression

To study vitamin D metabolic system in placental VSMCs, confluent cells were treated with $1,25(OH)_2D_3$ at concentrations of 0, 5, 10, 20, 50, or 100 nM for 24 h. Cells were then lysed with lysis buffer containing 50 mmol/L Tris, 0.5% NP40, 0.5% Triton X-100 with protease, and phosphatase inhibitors, and total cellular protein was collected. Protein expression levels for CYP2R1, CYP27B1, VDR, and CYP24A1 were determined by Western blotting. To explore the mechanism of vitamin D-induced relaxation of placental VSMCs, RhoA and ROCK1 expression and phosphorylation of MYPT1 signaling pathway molecule expression for MYPT1, p-MYPT1^{Thr853}, p-MYPT1^{Thr696}, p-MYPT1^{Ser668}, and p-MYPT1^{Ser507} were determined by Western blot analysis. In addition, protein expression for VDR, AT-1, and AT-2 were also determined. For Western blotting, 10 μg of cellular protein per sample was subject to electrophoresis (Bio-Rad Laboratories, Hercules, CA) and then transferred to nitrocellulose membranes. After being blocked, membranes were probed with a specific antibody, followed by a matched secondary antibody. The bound antibody was visualized with an enhanced chemiluminescent detection kit (Amersham Corp., Arlington Heights, IL) and exposed by sandwiching with X-ray film. Membranes were then stripped, blocked, and reprobed with different antibodies. The density was scanned and analyzed using ImageJ analysis software. β-actin or MYPT1 expression was used to normalize the target protein expression for each sample. Data are mean ± SEM from 3 to 5 independent experiments.

Statistical Analysis

Data are mean ± SEM and were analyzed by using analysis of variance (ANOVA) with Student-Newman Keuls test as post hoc test or by paired Student *t*-test with Prism 5 software (GraphPad, Inc., La Jolla, CA). A probability level less than 0.05 was considered statistically significant.

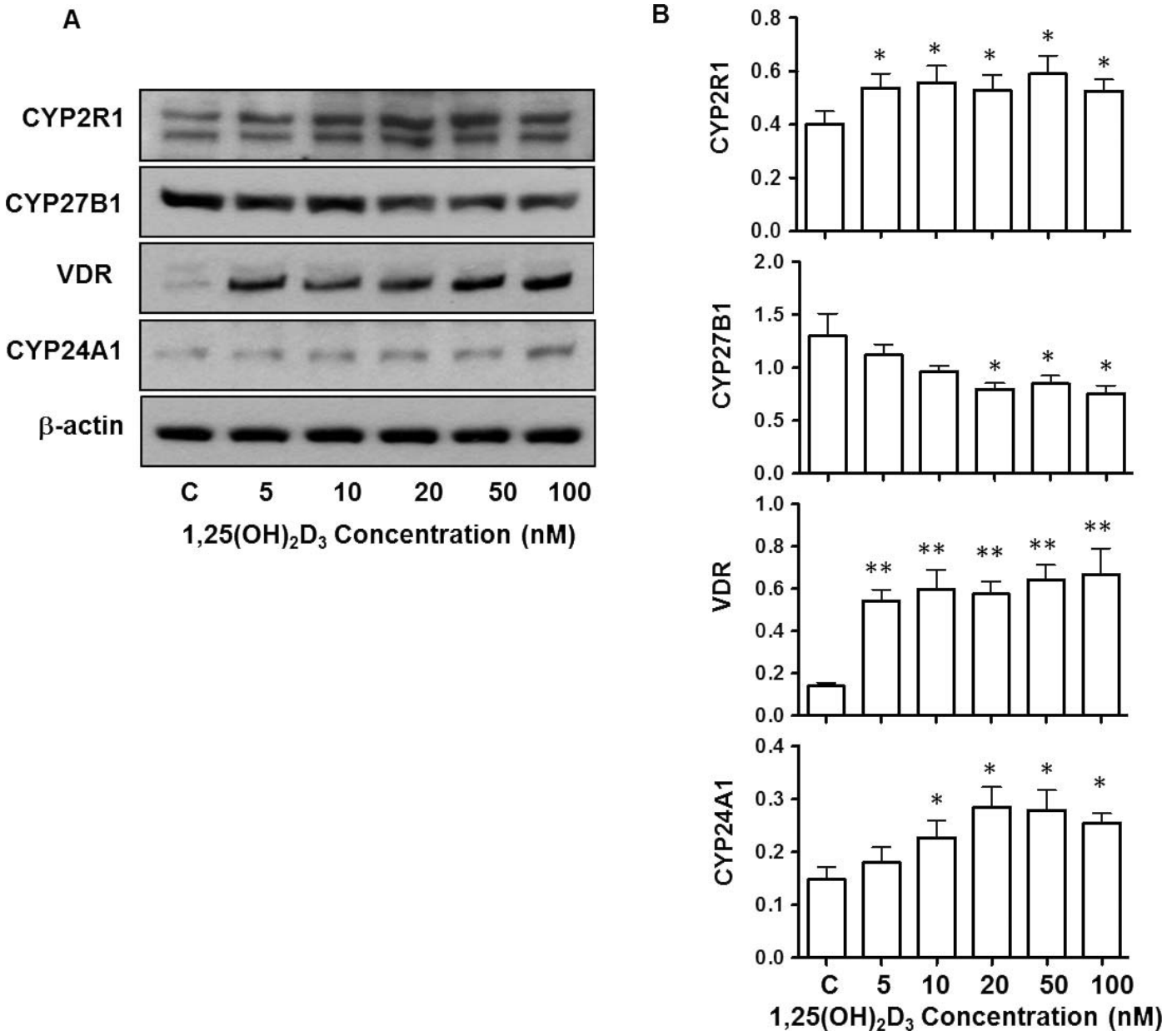

FIG. 1. Protein expression of vitamin D metabolic system is shown in placental VSMCs. **A**) Representative Western blots are shown for levels of CYP2R1, CYP27B1, VDR, and CYP24A1 expression in placental VSMCs treated with different concentrations of $1,25(OH)_2D_3$. **B**) Relative density of protein expression levels for CYP2R1, CYP27B1, VDR, and CYP24A1 are shown after normalization with β-actin expression for each sample. Data are means from 4–5 independent experiments. CYP27B1 expression was dose-dependently down-regulated, and CYP24A1 expression was dose-dependently up-regulated in cells treated with $1,25(OH)_2D_3$ ($P < 0.05$). VDR expression was inducible in cells treated with $1,25(OH)_2D_3$. *$P < 0.05$; **$P < 0.01$ for $1,25(OH)_2D_3$-treated cells versus control.

RESULTS

Effects of $1,25(OH)_2D_3$ on Vitamin D Metabolic System in Placental VSMCs

We first examined expression of the vitamin D metabolic system including CYP2R1, CYP27B1, VDR, and CYP24A1 in placental VSMCs treated with or without $1,25(OH)_2D_3$. Figure 1A shows representative Western blots for CYP2R1, CYP27B1, VDR, and CYP24A1 in control cells and in cells treated with different concentrations of $1,25(OH)_2D_3$. Figure 1B shows the relative density of expression levels for proteins CYP2R1, CYP27B1, VDR, and CYP24A1 after normalization with β-actin expression for each sample. Data are means from 4 to 5 independent experiments. Our results clearly showed that placental VSMCs expressed comparative amounts of CYP2R1, CYP27B1, and CYP24A1 in control cells. CYP2R1 expression was up-regulated in cells treated with $1,25(OH)_2D_3$ ($P < 0.05$). Conversely, CYP27B1 expression was dose-dependently down-regulated in cells treated with $1,25(OH)_2D_3$ ($P < 0.05$). Although VDR was weakly expressed in control cells, VDR expression was dramatically up-regulated in cells treated with $1,25(OH)_2D_3$ ($P < 0.01$). The pattern for CYP24A1 expression was also dose dependently up-regulated in cells treated with $1,25(OH)_2D_3$ ($P < 0.05$). These results indicate that vitamin D metabolic components exist in placental VSMCs and that the local level of $1,25(OH)_2D_3$ concentration could not only regulate

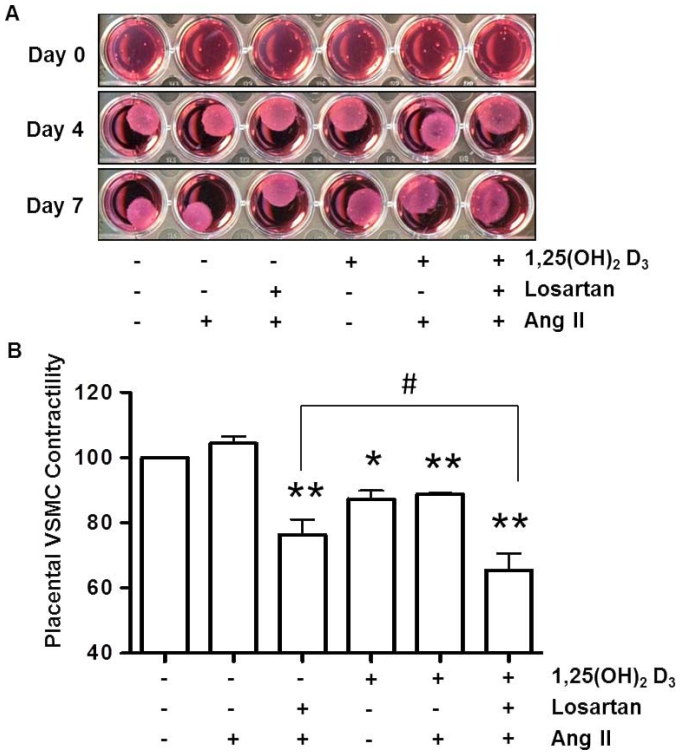

FIG. 2. Effect is shown of $1,25(OH)_2D_3$ on placental VSMC contractility under angiotensin II stimulation. Placental VSMC contractility was evaluated by collagen gel contraction assay. **A**) Representative collagen gel was recorded on Days 0, 4, and 7 with or without angiotensin II (10^{-7} M) treatment in the presence of $1,25(OH)_2D_3$ (100 nM) or losartan (10^{-4} M) separately or in combination. The surface area of PVSMC/collagen gel represents tonic contraction of smooth muscle cells. Gels maintained established levels of tonic contractility and did not exhibit relaxation or "fatigue," once contracted. **B**) Graph shows mean VSMC contractility of collagen gel on Day 7 (percentage of gel surface area changes). Data are means from 5 independent assays, each done in triplicate. $^*P < 0.05$ and $^{**}P < 0.01$ for losartan plus angiotensin II, $1,25(OH)_2D_3$, $1,25(OH)_2D_3$ plus angiotensin II, and $1,25(OH)_2D_3$ plus losartan plus angiotensin II versus untreated control cells or cells treated with angiotensin II; $\#P < 0.05$ for $1,25(OH)_2D_3$ plus losartan plus angiotensin II versus losartan plus angiotensin II, respectively.

VDR expression it could also affect expression of key enzymes for vitamin D synthesis or degradation.

$1,25(OH)_2D_3$ Attenuated Angiotensin II-Induced Placental VSMC Contractility

Because vitamin D has beneficial effects on cardiac vasculature [19] and because vitamin D metabolic components are present in placental VSMCs, we then determined whether vitamin D exerted beneficial effects on placental VSMCs. More specifically, we determined the role of vitamin D in placental VSMC contractility. This was evaluated by VSMC/collagen gel contraction assay. Angiotensin II was used as the stimulator to induce cell contraction. As a comparison, the angiotensin II receptor-1 (AT-1) blocker losartan was used to inhibit angiotensin II-induced placental VSMC contractility. Figure 2A shows representative collagen gel recordings on Days 0, 4, and 7 with or without angiotensin II treatment in the presence of $1,25(OH)_2D_3$ or losartan separately and in combination. Figure 2B graph shows quantitative results of VSMC contractility of collagen gel on Day 7. Our results showed that placental VSMC contractility increased 5% in cells treated with angiotensin II alone (105%) compared to untreated control cells. Contractility induced by angiotensin II was suppressed by losartan (76%). Interestingly, the gel area of $1,25(OH)_2D_3$-treated cells was significantly larger than that of untreated controls (100%), indicating that less contractility of the cells when exposed to $1,25(OH)_2D_3$ (87%; $P < 0.05$). VSMC contractility was also significantly reduced when cells were treated with $1,25(OH)_2D_3$ plus angiotensin II (89%) versus when cells were treated with angiotensin II alone ($P < 0.01$). Moreover, cell contractility was further reduced in cells treated with angiotensin II in the presence of both $1,25(OH)_2D_3$ and losartan (66%) versus in angiotensin II alone ($P < 0.01$) or versus angiotensin II plus losartan ($P < 0.05$). These results suggest that vitamin D not only modulates tone of placental VSMCs but also potentiates the pharmacological action of losartan to counteract angiotensin II induced VSMC contraction.

Effects of $1,25(OH)_2D_3$ on Angiotensin II Receptor Expression in Placental VSMCs

To determine whether $1,25(OH)_2D_3$ had any effect on angiotensin II receptor AT-1 and AT-2 expression, placental VSMCs were cultured in 6-well plates and treated with $1,25(OH)_2D_3$ in the presence or absence of angiotensin II with or without losartan in culture. Untreated cells or cells treated with angiotensin II alone or losartan plus angiotensin II were used as comparisons. These experiments were parallel to the placental VSMC/collagen gel contraction assay. Results are shown in Figure 3. VDR expression was significantly up-regulated in cells treated with $1,25(OH)_2D_3$, even in the presence of angiotensin II and/or losartan in culture, $P < 0.01$. However, $1,25(OH)_2D_3$ had no effect on AT-1 and AT-2 expression, indicating that the amount of AT-1 or AT-2 protein was not influenced by $1,25(OH)_2D_3$ exposure. This was similar to what was found for AT-1 and AT-2 protein expression in cells treated with angiotensin II alone or treated with losartan plus angiotensin II in placental VSMCs.

Role of $1,25(OH)_2D_3$ in ROCK1-MYPT1 Pathway Molecule Expression

MYPT1 is a major subunit in MLCP. To further test whether MYPT1 pathway molecule phosphorylation induced by $1,25(OH)_2D_3$ mediated placental VSMC relaxation, we determined expression levels of RhoA, ROCK1, MYPT1, p-MYPT1^{Thr853}, p-MYPT1^{Thr696}, p-MYPT1^{Ser668}, and p-MYPT1^{Ser507} in cells treated with $1,25(OH)_2D_3$ with or without angiotensin II stimulation. Untreated cells and cells treated with losartan plus angiotensin II served as comparisons. Results are shown in Figure 4. Figure 4A shows representative Western blots for RhoA, ROCK1, MYPT1, p-MYPT1^{Ser668}, and p-MYPT1^{Ser507} expression levels in cells treated with $1,25(OH)_2D_3$ and/or losartan plus angiotensin II stimulation. Figure 4B shows relative protein expression for RhoA, ROCK1, p-MYPT1^{Ser668}, and p-MYPT1^{Ser507} expression. Our results showed that protein expression levels for RhoA and total MYPT1 were not different in cells treated with $1,25(OH)_2D_3$ or losartan in the presence or absence of angiotensin II in culture. Expression for ROCK1 was significantly up-regulated in cells treated with angiotensin II, $P < 0.05$. The increased ROCK1 expression induced by angiotensin II could be attenuated by losartan or/and $1,25(OH)_2D_3$, $P < 0.05$. p-MYPT1^{Thr853} and p-MYPT1^{Thr696} were undetectable (data not shown). Interestingly, expression of p-MYPT1^{Ser507}, but not p-MYPT1^{Ser668}, was significantly up-regulated in cells treated with losartan in the presence of angiotensin II, $P < 0.01$. Similarly, p-MYPT1^{Ser507} was also

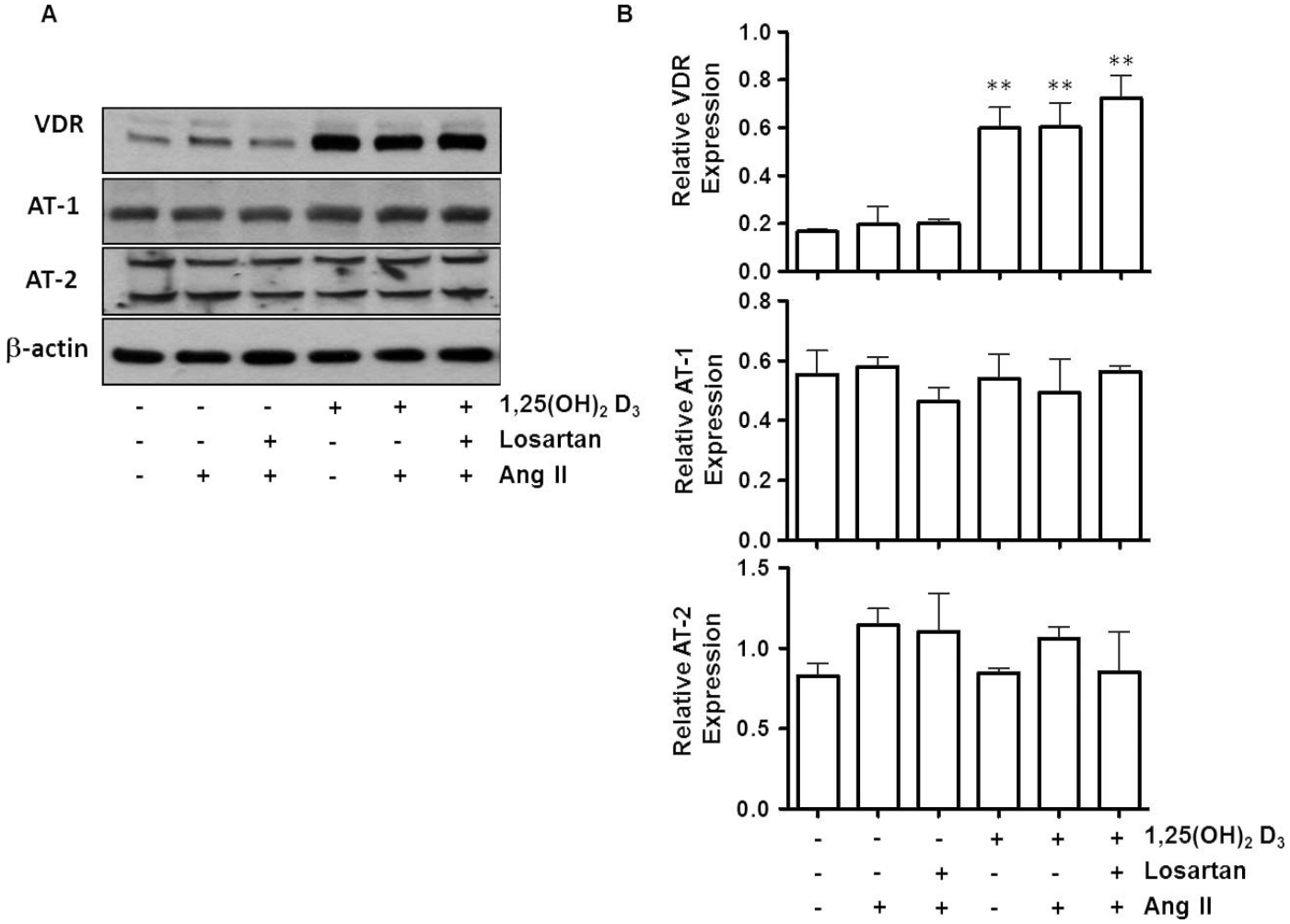

FIG. 3. Effect of $1,25(OH)_2D_3$ on AT-1, AT-2, and VDR expression levels in placental VSMCs. **A**) Representative Western blots are shown for AT-1, AT-2, and VDR expression in VSMCs treated with or without angiotensin II in the presence of $1,25(OH)_2D_3$ or losartan separately or in combination. **B**) Relative density levels of expression for proteins AT-1, AT-2, and VDR are shown after normalization with β-actin expression for each sample. Data are means from 3 independent experiments. VDR expression was significantly up-regulated in cells treated with $1,25(OH)_2D_3$, even in the presence of angiotensin II and/or losartan in culture. $^{**}P < 0.01$ for cells treated with $1,25(OH)_2D_3$ versus cells not treated with $1,25(OH)_2D_3$. However, $1,25(OH)_2D_3$ had no effect on expression of AT-1 and AT-2.

significantly up-regulated in cells treated with $1,25(OH)_2D_3$ plus angiotensin II, $P < 0.05$, and $1,25(OH)_2D_3$ plus losartan plus angiotensin II, $P < 0.01$, respectively.

DISCUSSION

Vitamin D Biosynthesis System Is Present in Placental VSMCs

In this study, we first determined whether the vitamin D metabolic system was present in placental VSMCs. We examined CYP2R1, CYP27B1, VDR, and CYP24A1 expression in cells treated with $1,25(OH)_2D_3$. We found that CYP2R1 and CYP27B1 were strongly expressed and that CYP24A1 was weakly expressed in untreated control cells. Furthermore, we noticed that expression of CYP27B1 and CYP24A1 were inversely related when cells were treated with $1,25(OH)_2D_3$. CYP27B1 expression was dose-dependently down-regulated, and CYP24A1 expression was dose-dependently up-regulated in cells treated with $1,25(OH)_2D_3$. It is known that CYP27B1 and CYP24A1 are important regulators for $1,25(OH)_2D_3$ homeostasis. The former enzyme converts $25(OH)_2D_3$ to $1,25(OH)_2D_3$, and the latter degrades $1,25(OH)_2D_3$ to form calcitroic acid. The phenomena of $1,25(OH)_2D_3$-induced down-regulation of CYP27B1 and up-regulation of CYP24A1 suggest that extracellular vitamin D levels are able to influence cellular vitamin D biosynthesis and metabolism in placental VSMC. We also noticed that VDR was weakly expressed in control cells but that VDR expression was significantly up-regulated in cells treated with $1,25(OH)_2D_3$. This observation is very important because up-regulation of VDR expression is critical for producing downstream biological actions induced by vitamin D, even though CYP27B1 expression was down-regulated by $1,25(OH)_2D_3$. The phenomenon of inducible VDR by $1,25(OH)_2D_3$ was also reported in pre-adipocytes [20] and in endothelial cells [15]. In the present study, we did not examine biosynthesis of vitamin D in placental VSMCs. However, the presence of CYP2R1, CYP27B1, and CYP24A1 in PVSMC, together with our finding of inducible VDR expression by$1,25(OH)_2D_3$, suggests that a vitamin D biosynthesis and feedback autoregulatory system is present in placental VSMCs.

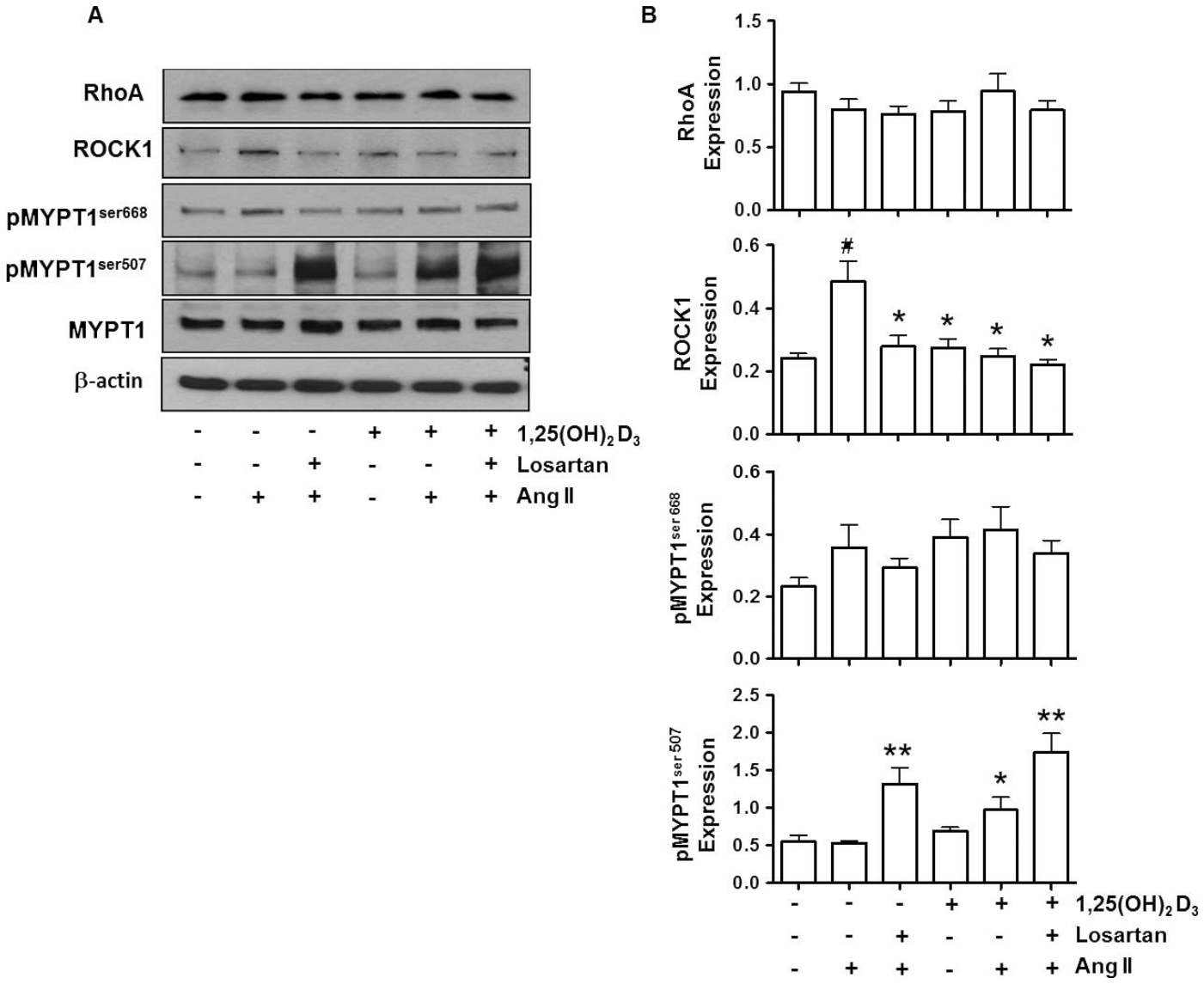

FIG. 4. Effect of $1,25(OH)_2D_3$ in RhoA/ROCK1-MYPT1 pathway molecule expression in placental VSMCs is shown. **A**) Representative Western blots for RhoA, ROCK1, MYPT1, p-MYPT1^{Ser668}, and p-MYPT1^{Ser507} expression in cells treated with $1,25(OH)_2D_3$ and/or losartan plus angiotensin II stimulation are shown. **B**) Relative protein expression levels for RhoA, ROCK1, p-MYPT1^{Ser668}, and p-MYPT1^{Ser507} are shown after normalization. Data are means from 3–5 independent experiments. Expression levels for RhoA and ROCK1 were normalized with β-actin expression. Expression levels for p-MYPT1^{Ser668} and p-MYPT1^{Ser507} were normalized with MYPT1 expression. ROCK1 expression was significantly increased in cells treated with angiotensin II compared to that in control cells (#$P < 0.05$), but no change was detected in $1,25(OH)_2D_3$ and/or losartan plus angiotensin II-treated cells. There were no differences for RhoA and MYPT1 expression levels in cells treated with $1,25(OH)_2D_3$ or losartan in the presence or absence of angiotensin II, whereas expression of p-MYPT1^{Ser507}, but not p-MYPT1^{Ser668}, was significantly up-regulated in cells treated with $1,25(OH)_2D_3$ and/or losartan in the presence of angiotensin II; *$P < 0.05$ and **$P < 0.01$ for losartan and/or $1,25(OH)_2D_3$ plus angiotensin II vs. cells treated with angiotensin II alone.

Vitamin D Reduces Placental VSMC Contractility

Both human and animal studies have shown that vitamin D levels are inversely associated with blood pressure/hypertension. A cohort study conducted by Griffin et al. [21] showed that vitamin D insufficiency was associated with increased risk of systolic hypertension in women later in life. An animal study by Li et al. [22] found a mechanism of vitamin D as a negative regulator for renin-angiotensin system. Their study revealed that, in wild-type mice, inhibition of $1,25(OH)_2D_3$ synthesis led to an increase in renin expression, whereas $1,25(OH)_2D_3$ administration led to renin suppression. In a cell culture system, these investigators also noticed a marked suppression of renin transcription by $1,25(OH)_2D_3$ through a VDR-mediated mechanism [22]. Wong et al. [23] also reported that vitamin D could modulated the vascular tone by reducing endothelium-derived contracting factor production. These findings suggest that suppression of renin-angiotensin system or reduction of endothelium-derived contracting factor production could be part of the cellular response to modulate vascular tone by vitamin D.

Pre-eclampsia is a placental disorder with increased vascular reactivity and reduced uterine blood perfusion. Altered renin-angiotensin system and presence of AT-1 autoantibody have been considered to contribute to increased placental vascular resistance in pre-eclampsia [24–26]. To investigate whether vitamin D may exert beneficial effects on placental vasculature, we specifically tested effects of $1,25(OH)_2D_3$ on placental VSMC contractility by using collagen gel contraction assay as the testing model. The collagen gel contraction assay has been

widely used to study contractile function in multiple cell types, including vascular smooth muscle cells [17, 18, 27, 28]. Although this model does not completely mimic isolated intact vessel contraction, it allows us to study contractile activity of a homogenous population of placental VSMC in response to angiotensin II, losartan, and/or $1,25(OH)_2D_3$ separately or in combination. Moreover, this model offers an excellent condition with which to study VSMC function without the influence of external mechanical load such as sheer stress and/or effects of endothelial- or matrix-derived mediators on smooth muscle cells, as those occur in the intact vessel. Interestingly, using AT-1 receptor blocker losartan as the comparator, we found that, similar to losartan, $1,25(OH)_2D_3$ could diminish angiotensin II-induced placental VSMC contractility, and most significantly, the inhibitory effect of losartan on angiotensin II-induced placental VSMC contraction could be potentiated when $1,25(OH)_2D_3$ was present in culture. According to Poiseuille's law, the increase in flow is proportional to the fourth power of the diameter of a vessel, hence a relative change in vessel diameter will cause an extreme change in blood flow. Therefore, in an in vivo situation, sufficient vitamin D and/or proper vitamin D metabolism in placental vasculature may exert beneficial effects by reducing placental vascular tone and resistance and subsequently result in an increase in placental blood compliance and perfusion. These findings may also have an impact on systemic vasculature, which may provide an explanation of inverted relationship of vitamin D deficiency and hypertension found in epidemiologic studies [1, 29].

Potential Mechanism of Vitamin D Induced Placental VSMC Relaxation

Vascular resistance depends on the balance between contraction and relaxation of VSMC to establish vascular tone. Smooth muscle contraction results from interaction of myosin and actin filament, essential to this interaction is the phosphorylation of MLC. MYPT1 is the main regulatory subunit of MLCP, which will dephosphorylate MLC and result in relaxation. An animal study conducted by Qiao et al. [30] revealed that deletion of MYPT1 in VSMCs enhanced myosin phosphorylation, thereby induced VSMC contractility and increased blood pressure in study animals, which indicates the important role of MYPT1 in maintaining VSMC relaxation. The activity of MYPT1 is found largely regulated by its own phosphorylation; threonine (Thr-696 and Thr-853) phosphorylation can inhibit its activity whereas serine (Ser-507 and Ser-668) phosphorylation can promote its effect in MLCP. ROCK1 activation by RhoA promotes phosphorylation of regulatory MLC and increase in cell contractility [31, 32]. It has been demonstrated that activation of AT-1 by angiotensin II could increase free intracellular Ca^{2+} concentration, which up-regulates ROCK1 expression and leads to the phosphorylation of MYPT1 at Thr-696 or Thr-853 and subsequently smooth muscle contraction [30, 33–35]. Recently, studies found that vitamin D or vitamin D receptor agonist could inhibit RhoA/ROCK-MYPT1 pathway [36]. To determine whether $1,25(OH)_2D_3$ had any effects on RhoA/ROCK1-MYPT1 signaling pathway molecules, we examined protein expression for RhoA, ROCK1, and MYPT1, as well as p-MYPT1^{Thr853}, p-MYPT1^{Thr696}, p-MYPT1^{Ser507}, and p-MYPT1^{Ser668}. Strikingly, we found that there was no change for RhoA and total MYPT1 expression in cells with or without angiotensin II and losartan treatment, nor for cells treated with $1,25(OH)_2D_3$ in the presence or absence of angiotensin II. ROCK1 expression was significantly up-regulated in cells treated with angiotensin II. The increased ROCK1 expression induced by angiotensin II could be attenuated by either losartan or $1,25(OH)_2D_3$ combined with angiotensin II. MYPT1 phosphorylation at Thr-853 and Thr-696 was undetectable. However, p-MYPT1^{Ser507}, but not p-MYPT1^{Ser668}, was significantly up-regulated in cells treated with losartan in the presence of angiotensin II. A similar effect was also seen in cells treated $1,25(OH)_2D_3$ or combined with losartan in the presence of angiotensin II. It is very likely that inhibition of ROCK1 expression and phosphorylation of MYPT1 at Ser-507 by losartan or $1,25(OH)_2D_3$ in the presence of angiotensin II leads to activation of MLCP, and subsequently results in VSMC relaxation.

In conclusion, we have made several important findings in this study. First, we found that vitamin D biosynthesis components are present in placental VSMCs. The observations of inverse expression of CYP27B1 and CYP24A1 together with inducible VDR expression by $1,25(OH)_2D_3$ provide evidence of a feedback autoregulatory system of vitamin D existing in placental VSMCs. Second, using angiotensin II as a vasoconstrictive stimulator and AT-1 blocker losartan as a contrast, we found that $1,25(OH)_2D_3$ could not only reduce contractility of placental VSMCs stimulated by angiotensin II, but also potentiate the inhibitory effect mediated by losartan on angiotensin II-induced placental VSMC contraction. Third, we found for the first time that $1,25(OH)_2D_3$ exerts an effect similar to that of losartan on RhoA/ROCK1-MYPT1, that is, to inhibit angiotensin II-induced ROCK1 expression and to induce MYPT1 phosphorylation at Ser-507. ROCK1 can promote contractile force generation by inhibiting MLCP activity. Contrarily, MYPT1 serine phosphorylation could activate MLCP, and MLCP activation is an important regulatory mechanism of smooth muscle cell relaxation. Therefore, ROCK1 inhibition and MYPT1 phosphorylation at Ser-507 may be a plausible mechanism of vitamin D and losartan induced VSMC relaxation that counteracts angiotensin II/AT-1 induced increased VSMC contraction. Whether vitamin D exerts similar effects on the systemic vasculature/VSMCs warrant further investigation.

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