Vitamin D Depletion Aggravates Hypertension and Target-Organ Damage

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Background—We tested the controversial hypothesis that vitamin D depletion aggravates hypertension and target-organ damage by influencing renin.

Methods and Results—Four-week-old double-transgenic rats (dTGR) with excess angiotensin (Ang) II production due to overexpression of the human renin (hREN) and angiotensinogen (hAGT) genes received vitamin D-depleted (n=18) or standard chow (n=15) for 3 weeks. The depleted group had very low serum 25-hydroxyvitamin D levels (mean±SEM; 3.8±0.29 versus 40.6±1.19 nmol/L) and had higher mean systolic BP at week 5 (158±3.5 versus 134.6±3.7 mm Hg, P<0.001), week 6 (176.6±3.3 versus 162.3±3.8 mm Hg, P<0.01), and week 7 (171.6±5.1 versus 155.9±4.3 mm Hg, P<0.05). Vitamin D depletion led to increased relative heart weights and increased serum creatinine concentrations. Furthermore, the mRNAs of natriuretic peptides, including ANP and the counter-regulatory breakdown product Ang 1 to 7, were significantly up-regulated in the vitamin D-depleted groups, while ACE-1 and ACE-2 activities were not affected.

Conclusions—Short-term severe vitamin D depletion aggravated hypertension and target-organ damage in dTGR. Our data suggest that even short-term severe vitamin D deficiency may directly promote hypertension and impacts on renin-angiotensin system components that could contribute to target-organ damage. The findings add to the evidence that vitamin D deficiency could also affect human hypertension. (J Am Heart Assoc. 2015;4:e001417 doi: 10.1161/JAHA.114.001417)

Key Words: hypertension • renin • vitamin D

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promoter. Finally, vitamin D receptor knockout mice had significantly elevated renin and angiotensin (Ang) II concentrations. The mice also developed hypertension and target-organ-damage. In human cross-sectional studies, lower vitamin D levels were associated with higher plasma renin activity (PRA), higher Ang II concentrations, altered responses to Ang II, and higher RAS activity in vascular tissue. Little is known about the effect of vitamin D on other RAS components, particularly metabolites of angiotensinogen. Ang 1 to 7 appears to counteract the actions of Ang II. We performed studies in double-transgenic rats expression human renin (hREN) and angiotensinogen (hAGT) to investigate an interaction between vitamin D deficiency and the human RAS in a rat model.

**Methods**

We purchased dTGR rats (n=33) and non-transgenic control Sprague-Dawley rats (n=12) from Harlan A/S. Our local review board (State Office of Health and Social Affairs [LAGeSo], permit number G160/09) approved the studies that complied with guidelines from the American Physiology Society. Age-matched male dTGR and non-transgenic Sprague-Dawley rats at weaning (age 4 weeks) were given free access to drinking water and either a vitamin D-deficient diet with normal calcium and phosphorus, or a diet with normal vitamin D amounts (AIN93G; SSNFF, Germany). Systolic BP was measured under light anesthesia weekly by tail-cuff. The “Tail cuff” apparatus was built by the workshop of our institute. Light anesthesia was employed to ensure correct and safe handling of the rats during the procedure. The animals were placed in metabolic cages once weekly, and 24-hour urine albumin excretion (ELISA; CellTrend) was measured. Rats were killed by guillotine at age 7 weeks, after light ether anesthesia to ensure safe and correct handling. Blood for hormone analysis was drawn by aortic puncture into pre-chilled tubes containing EDTA (6.25 mmol/L) and phenanthroline (26 mmol/L) as anticoagulant and inhibitor of Ang II breakdown in vitro, respectively. Serum was collected for further analysis. The heart, liver, and kidneys were washed with ice-cold saline, blotted dry, and weighed. For Western blot, and RT-PCR analysis, the tissues were snap-frozen in liquid nitrogen, for immunohistochemistry in isopentane (−35°C), and stored at −80°C.

Total cardiac and kidney RNA was isolated with TRIZOL followed by the Qiagen protocol, and TaqMan reverse-transcription polymerase chain reaction was performed as recommended by the manufacturer. The sequences of the primers are described earlier. Quantitative analysis of target mRNA expression was performed with real-time PCR using the relative standard curve method. GAPDH or 18S were chosen as the endogenous control (“housekeeping gene”). The expression level of the target genes ANP and BNP in the heart was normalized by GAPDH. The expression level of the target genes neutrophil gelatinase-associated lipocalin (NGAL), hREN, rRen, hAGT, and rAgt in the kidney was normalized by 18S.

Techniques for measuring plasma renin activity (PRA), plasma renin concentrations (PRC), and Ang I generation are outlined in detail elsewhere. We used mass spectrometry to investigate the effect of vitamin D depletion on Ang metabolites. This technique allows for measurement of 10 metabolites, including Ang 1 to 10 (Ang I), Ang 1 to 8 (Ang II), Ang 1 to 7 (MAS receptor agonist), Ang 2 to 8 (Ang III) and Ang 3 to 8 (Ang IV). Plasma samples were spiked with 100 pg/mL stable-isotope-labeled internal standards and subjected to solid-phase extraction using Sep-Pak cartridges (Waters) according to manufacturer’s protocol. Following elution and solvent evaporation, samples were reconstituted in 50 µL 50% acetonitrile/0.1% formic acid and subjected to LC-MS/MS analysis using a reversed-phase analytical column (Luna C18, Phenomenex) using a gradient ranging from 10% acetonitrile/0.1% formic acid to 70% acetonitrile/0.1% formic acid in 9 minutes. The eluate was analyzed in line with a QTRAP-4000 mass spectrometer (AB Sciex) operated in the MRM mode using dwell times of 25 msec at a cone voltage of 4000 volts and a source temperature of 300°C. For each peptide and corresponding internal standards, 2 different mass transitions were measured. Ang peptide concentrations were calculated by relating endogenous peptide signals to internal standard signals provided that integrated signals achieved a signal-to-noise ratio above 10. We calculated ACE1 and ACE2 activity according to the established ratio; ACE-1 activity was estimated as Ang II/Ang 1 to 10 and ACE-2 activity was estimated as Ang 1 to 7/Ang 1 to 8. For vitamin D determinations, serum was stored at −20°C until analysis, which was performed as previously reported for human serum. Briefly, vitamin D2 and D3 were measured as individual components in one single analysis on a TurboFlow LC-MS/MS system (TLX2 HPLC with a Vantage TSQ MS, all from Thermo Scientific). For internal standard, d3-25-OH-vitamin D3 (Sigma-Aldrich) was used. Calibration curves were verified with NIST 972 and 2972.

Spleens and heparinized blood for analysis of regulatory T cells were collected from dTGR in the 2 groups (depletion and control) at the time of death. Tissues were minced through 70 µm sieves and washed with cold PBS containing 0.1% FCS. Flow-through and blood were subjected to osmotic lysis of red blood cells at 37°C. Next, cell suspension was filtered through 40 µm sieves, diluted and centrifuged at 300g for 10 minutes, at 4°C. Cells were then washed twice using cold PBS/ FCS buffer. Cells in single-cell suspension were stained with anti-CD4, anti-CD25, and permeabilized and stained with anti-FoxP3 (FoxP3 kit from BioLegend, San Diego, CA, CD4-FITC.
Results

After 3 weeks, the group receiving vitamin D-depleted chow (VDd-dTGR) had very low serum 25(OH)D$_3$ levels of 8.0±0.7 nmol/L, whereas the controls (ctl-dTGR) had levels of 40.6±2.9 nmol/L (Figure 1A). None of the groups had detectable levels of 25(OH)D$_2$. Systolic BP was higher in the VDd-dTGR group relative to ctl-dTGR throughout the study. Already at week 5, VDd-dTGR had higher systolic BP at 158±3.5 versus 134.6±3.7 mm Hg, P<0.001, week 6 176.6±3.3 versus 162.3±3.8 mm Hg, P<0.01, and week 7 171.6±5.1 versus 155.9±4.3 mm Hg, P<0.05 (Figure 1B).

Both depleted and control dTGR displayed substantial cardiac hypertrophy. Log-transformed relative heart weight was significantly higher for VDd-dTGR (0.75±0.01 versus 0.71±0.01 mg/g, P<0.01) (Figure 2A). Relative expression of markers of heart failure were significantly up-regulated in heart tissue from the depletion group, atrial natriuretic peptide (ANP) at 0.80±0.06 versus 0.60±0.05 arbitrary units (AU), P<0.05 and brain natriuretic peptide (BNP) at 0.83±0.06 versus 0.57±0.07 AU, P<0.01 (Figure 2B). Albuminuria was present in both groups, but did not differ significantly at any time point (Figure 3A). The VDd-dTGR group exhibited substantially higher relative mRNA expression of neutrophil gelatinase-associated lipocalin (N-GAL) in kidney tissue than the ctl-dTGR, at 2.10±0.3 versus 1.30±0.15 AU, P<0.05 (Figure 3B), and higher plasma creatinine levels at 22.43±0.48 versus 18.83±0.87 μmol/mL, P<0.01 as well as a tendency towards higher plasma cystatin C levels at 2260±146 versus 1929±110 ng/mL, P=0.1. Tissue analyses for inflammation and fibrosis by immunohistochemistry showed no difference between depleted and control dTGR in heart and kidney tissue (Figures 4 and 5). Vitamin D depletion in non-transgenic Sprague-Dawley rats had no effect on blood pressure, proteinuria, GFR, and relative heart weight, renal N-GAL, and cardiac BNP expression, compared with control rats with normal diet (data not shown). We did not observe an effect on renal rRenin and liver rAogen expression by vitamin D depletion (data not shown).

To investigate the vitamin D-mediated molecular mechanism, we investigated whether the RAS was induced by vitamin D depletion. Analysis of mRNA showed that both human and rat renin were up-regulated in the VDd-dTGR compared to ctl-dTGR, human renin at 1.81±0.33 versus 0.89±0.13 AU, P<0.05 and rat renin at 1.16±0.14 versus 0.77±0.08 AU, P<0.05 (Figure 6A). Liver tissue was examined for relative mRNA expression of human and rat angiotensinogen, however there were no significant differences in expression between VDd-dTGR and ctl-dTGR (Figure 6B).

PRC and PRA were increased in VDd-dTGR (Table 1). Similarly, Ang I generation was significantly higher in the circulation of VDd-dTGR compared with controls. None of 10 angiotensin metabolites (Table 2) were significantly up-regulated in the VDd-dTGR group, including Ang II and Ang 1 to 7.

Figure 1. Serum 25(OH)D$_3$ concentration and systolic blood pressure. A, Vitamin D-depleted rats had a significant decrease in serum 25-hydroxyvitamin D3 after 3 weeks on vitamin D-depleted chow. Results are expressed as mean±SEM of at least 6 animals per group. B, Systolic BP increased progressively in vehicle-treated dTGR from week 5 to week 7. Vitamin D depletion increased BP from week 5 to 7. BP indicates blood pressure; ctl-dTGR, controls double-transgenic rats; VDd, vitamin D-depleted.
Moreover, ACE-1 activity (estimated as Ang II/Ang 1 to 10) and ACE-2 activity (estimated as Ang 1 to 7/Ang 1 to 8) were not different between the 2 groups. Because vitamin D is known to induce regulatory T cells, we investigated whether or not vitamin D deficiency would affect regulatory T cells (CD4+CD25+FoxP3). No difference in the spleen or in peripheral blood was found (Figure 7).

**Discussion**

We found that short-term dietary depletion of vitamin D aggravated both hypertension and cardiovascular target-organ damage in dTGR rats. We attribute these effects to up-regulation of both the hREN transgene and the endogenous rRen in the VDd-dTGR group, leading to increased generation of various angiotensin peptides, after only 3 weeks of vitamin D depletion. However, neither hATG or rAgt expression nor ACE-1 nor ACE-2 activity were altered by vitamin D depletion. Moreover, vitamin D depletion did not alter the relative percentages of regulatory T cells, suggesting that regulatory T-cell deficiency was not responsible for the higher BP and target-organ damage.

Several rodent studies have been conducted to investigate the effect of vitamin D supplementation on hypertension and target-organ damage, using the Dahl salt-sensitive rats26–28 and models of renal failure and nephropathy.29–31
Figure 4. A and B, Masson Goldner staining for fibrosis in formalin fixed paraffin-embedded kidney sections (top: lower magnification, middle: higher magnification). C, Macrophage immunofluorescent staining (ED1) in formalin-fixed paraffin-embedded Kidney Sections and quantification of ED1-positive Cells. All measurements were performed in double transgenic rats with normal diet (VDs-dTGR) and after vitamin D depletion (VDd-dTGR). dTGR indicates double-transgenic rats; VDd, vitamin D-depleted.
Figure 5. A and B, Picro Sirius Red staining for fibrosis in formalin fixed paraffin-embedded heart sections (top: lower magnification, middle: higher magnification). Quantification of Fibrosis in Picro Sirius Red staining (C) Immunofluorescence staining of macrophages (ED1) in formalin-fixed paraffin-embedded heart sections and quantification (% Area Measured as Area related to whole LV). All measurements were performed in double transgenic rats with normal diet (VDs-dTGR) and after vitamin D depletion (VDd-dTGR). dTGR indicates double-transgenic rats; LV, left ventricle; VDd, vitamin D-depleted.
Most of these studies found an ameliorating effect. Li et al showed that vitamin D-receptor deletion in mice leads to up-regulation of both rodent renin mRNA and protein expression in the kidney. These mice had elevated renin and Ang II concentration, as well as hypertension and target-organ damage. Furthermore, 1,25–dihydroxyvitamin D3 (1,25(OH)2D3), is involved in transcriptional repression of renin expression, through a cyclic AMP response element in the renin gene promoter. A mouse model of 1α-hydroxylase-gene deletion showed that the enzyme protects the cardiovascular system by suppressing the RAS. Six-week vitamin D depletion in LDL-receptor and apolipoprotein E-null mice resulted in reversible increases in systolic and diastolic BP with elevated plasma renin. The null mice develop substantial renal and cardiovascular target-organ damage compared to controls. The induction of renin is independent of calcium and parathyroid hormone and is a direct effect of altered 1,25(OH)2D3 levels. In juxtaglomerular cells 1,25(OH)2D3 directly suppressed renin gene transcription, which was dependent on the vitamin D receptor. Treatment of juxtaglomerular cells with 1,25(OH)2D3 decreased promoter activity of a transfected renin promoter.

### Figure 6. Renin and angiotensinogen expression

A, Vitamin D depletion resulted in a significant increase in mRNA expression of human renin (left) and rat renin (right) in the kidney. B, mRNA expression of human angiotensinogen (left) and rat angiotensinogen (right) were similar between VDd- and ctl-dTGR. mRNA levels of the target genes were normalized for the housekeeping gene 18S. Results are expressed as arbitrary units (AU) with mean±SEM of at least 7 animals per group. ctl-dTGR indicates controls double-transgenic rats; hAGT, human angiotensinogen; VDd, vitamin D-depleted.

### Table 1. Plasma Renin Concentration and Activity (Mean±SEM)

<table>
<thead>
<tr>
<th></th>
<th>ctl-dTGR</th>
<th>VDd-dTGR</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PRC (sheep substrate) ng Ang I/(mL per h)</td>
<td>16.45±7.3</td>
<td>26.58±12.5</td>
<td>0.05</td>
</tr>
<tr>
<td>PRC (rat substrate) ng Ang I/(mL per h)</td>
<td>11.47±3.4</td>
<td>23.29±9.1</td>
<td>0.01</td>
</tr>
<tr>
<td>PRA ng Ang I/(mL per h)</td>
<td>35.79±35.81</td>
<td>54.22±38.8</td>
<td>0.34</td>
</tr>
<tr>
<td>Ang I ng/mL</td>
<td>4.98±1.6</td>
<td>8.58±2.5</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Ang I indicates angiotensin I; ctl-dTGR, controls double-transgenic rats; PRA, plasma renin activity; PRC, plasma renin concentration; VDd, vitamin D-depleted.
Table 2. Angiotensin Metabolites in the Circulation, Measured By Mass Spectroscopy (Mean±SEM)

<table>
<thead>
<tr>
<th>Parameter (pg/mL)</th>
<th>ctl-dTGR (Mean±SEM)</th>
<th>VDd-dTGR (Mean±SEM)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ang 1 to 5</td>
<td>5.0±0.0</td>
<td>6.1±0.3</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ang 1 to 7</td>
<td>25.2±4.6</td>
<td>48.3±5.9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ang 1 to 8</td>
<td>748.3±35.0</td>
<td>1512.0±180.3</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ang 1 to 9</td>
<td>6.7±0.3</td>
<td>8.4±0.2</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Ang 1 to 10</td>
<td>918.6±77.9</td>
<td>1764.0±223.0</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ang 2 to 7</td>
<td>2.0±0.0</td>
<td>2.9±0.1</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ang 2 to 8</td>
<td>70.9±4.7</td>
<td>125.5±12.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Ang 2 to 10</td>
<td>21.9±4.1</td>
<td>41.1±8.9</td>
<td>0.074</td>
</tr>
<tr>
<td>Ang 3 to 7</td>
<td>2.1±0.1</td>
<td>3.0±0.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Ang 3 to 8</td>
<td>42.3±2.5</td>
<td>88.4±10.5</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>ACE2 Activity (Ang 1 to 7/Ang 1 to 8)</td>
<td>0.035±0.007</td>
<td>0.032±0.001</td>
<td>0.72</td>
</tr>
<tr>
<td>ACE1 Activity (Ang 1 to 8/Ang 1 to 10)</td>
<td>0.851±0.079</td>
<td>0.865±0.037</td>
<td>0.87</td>
</tr>
</tbody>
</table>

ACE indicates angiotensin converting enzyme; ANG, angiotensin; ctl-dTGR, controls double-transgenic rats; VDd, vitamin D-depleted.

In infants, reversible cardiomyopathy has been reported in infants with nutritional rickets, and simultaneously low vitamin D and calcium levels.37,38 Furthermore, a recent study of taketsubo-cardiomyopathy in adult women also suggested an association to low vitamin D levels.39 Randomized clinical trials of vitamin D supplementation for reduction of BP in humans are conflicting. A recent trial meta-analysis of the effect of oral vitamin D supplementation on BP showed a trend towards a reduction in diastolic BP and a small, but significant reduction in diastolic BP, in patients with preexisting cardiovascular risk.9 In a trial with hypertensive patients, the group receiving vitamin D supplementation showed a 4 mm Hg decrease in central systolic BP. In patients with baseline 25OHD <80 nmol/L, a decrease in both systolic and diastolic BP was observed.40 Others found a significant decrease in systolic BP with calcitriol intervention in hypertensive patients41 or paricalcitol intervention in diabetic patients.42 However, The Women’s Health Initiative trial with vitamin D and calcium intervention, failed to show an effect of vitamin D on BP or incident hypertension.43 Another trial of chronic renal disease patients found no effect of paricalcitol therapy on BP.44,45 Our study supports the idea that profoundly low 25(OH)D levels may aggravate hypertension and adds mechanistic information. To our knowledge, no studies have previously focused on the effect of short-term vitamin D depletion in humans.

In earlier studies, we showed that both human and rat renin mRNA were expressed in the kidneys of dTGR.46 However, the 2 renin mRNAs were decreased, indicating a physiological down-regulation probably by high BP. Our current experiments show that the up-regulation of rat renin by vitamin D depletion is important and pronounced, since it overcomes the expected down-regulation by high BP.46 We observed an effect on BP after only 3 weeks of depletion, which points towards a very rapid effect of vitamin D depletion on hypertension. This finding is clinically interesting as it suggests that the human renin-angiotensin axis may be quite sensitive to changes in serum levels of 25(OH)D.

Vitamin D-binding protein and serum albumin both bind 25(OH)D, and a measurement of the 2 binding proteins could have enabled us to calculate the amounts of bioavailable 25(OH)D in the rat circulations.47 However, in this study, depletion of dTGR rats resulted in very low levels of vitamin D (8±0.7 nmol/L versus 40.6±2.9 nmol/L) and it is reasonable to assume that the proportion of bioavailable vitamin D would be correspondingly very low, if not negligible. Calculation of the bioavailable fraction might be of more value in an intervention study with supplementation, where it is of interest to find the increase in bioactive vitamin D. A further limitation to our study is not using radiotelemetry for measuring blood pressure. Yet, the tail cuff method has been used by the group in research with this transgenic rodent model for many years, and is reliable and reproducible.
particularly seeing as the animals develop very high blood pressures. However, radiotelemetry could have been more accurate in this experimental setting.

Our group previously demonstrated that adoptive regulatory T-cell transfer could ameliorate angiotensin-II induced target-organ damage. In addition, vitamin D has been shown to regulate relative numbers and activity of regulatory T cells in experimental and clinical studies. These observations caused us to investigate regulatory T cells in the current study. However, we did not find any evidence that splenic or peripheral numbers of regulatory T cells differed between groups.

We also observed that the Ang ratios, indicating ACE-1 and ACE-2, activity were not influenced by vitamin D depletion. We determined the expression of rat and human renin and angiotensinogen in liver and kidney and measured the concentrations of Ang metabolites in the serum by mass spectroscopy. Our findings lead us to conclude that the differences induced by vitamin D depletion were based solely on the up-regulation of renin expression and activity. The induction of renin subsequently leads to a higher serum concentration of Ang metabolites, while other enzymes of the RAS were not induced by vitamin D depletion. Instead, we observed a direct effect on the hREN transgene. Interestingly, the hREN transgene harbors the human renin gene promoter including all its flanking regions, the proposed site of 1,25(OH)2D3 action.

**Conclusion**

Blockade of the renin-angiotensin system with ACE inhibitors, AT1 receptor blockers, or direct renin inhibitors, all increase circulating renin. Conceivably, vitamin D supplementation or substitution in severe vitamin D deficiency could blunt this
increase. This hypothesis could be tested in subsequent translational studies. Furthermore, by adding anti-hypertensive drugs not targeting the RAS system to studies with dGFR rats, the degree of non-hypertensive Ang II induced organ damage could be established.

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Disclosures

None.

References


Vitamin D Depletion, Hypertensive Damage  Andersen et al


Vitamin D Depletion Aggravates Hypertension and Target–Organ Damage
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