25-Hydroxyvitamin D Deficiency Is Associated With Inflammation-Linked Vascular Endothelial Dysfunction in Middle-Aged and Older Adults

Kristen L. Jablonski, Michel Chonchol, Gary L. Pierce, Ashley E. Walker, Douglas R. Seals

Abstract—We tested the hypothesis that vascular endothelial function, assessed by endothelium-dependent dilation, is related to serum vitamin D status among middle-aged and older adults without clinical disease, and that this is linked to inflammation. Brachial artery flow-mediated dilation, a measure of endothelium-dependent dilation, was lower (P<0.01) in vitamin D–insufficient (3.7±0.2%; serum 25-hydroxyvitamin D [25(OH)D]: 20 to 29 ng/mL; 62±1 years of age; n=31; mean± SE) and vitamin D–deficient (3.2±0.3%; 25(OH)D: <20 ng/mL; 63±2 years of age; n=22) versus vitamin D–sufficient (4.6±0.4%; 25(OH)D: >29 ng/mL; 61±1 years of age; n=22) subjects, whereas endothelium-independent dilation (brachial dilation to sublingual nitroglycerine) did not differ (P=0.45). Among all subjects, brachial flow-mediated dilation was positively related to serum 25(OH)D (%Δ: r=0.35; P<0.01) but not 1,25-dihydroxyvitamin D (r=−0.06; P=0.61), the active form of vitamin D. Vascular endothelial cell expression of the proinflammatory transcription factor nuclear factor κB was greater in deficient versus sufficient subjects (0.59±0.07 versus 0.44±0.05; P<0.05), and inhibition of nuclear factor κB (4 days oral salsalate) improved flow-mediated dilation to a greater extent in subjects with lower versus higher 25(OH)D (+3.7±0.6 versus +2.0±0.2%; P<0.05). Endothelial cell expression of the downstream proinflammatory cytokine interleukin-6 also was higher in deficient versus sufficient subjects (0.67±0.08 versus 0.47±0.05; P<0.01) and inversely related to serum 25(OH)D level (r=−0.62; P<0.01), whereas vitamin D receptor and 1-α hydroxylase, the 25(OH)D to 1,25-dihydroxyvitamin D converting enzyme, were lower (P<0.05). Inadequate serum 25(OH)D is associated with vascular endothelial dysfunction among healthy middle-aged/older adults, and this is mediated in part by nuclear factor κB–related inflammation. Reduced vitamin D receptor and 1-α hydroxylase may be molecular mechanisms linking vitamin D insufficiency to endothelial dysfunction. (Hypertension. 2011;57:63-69.) ● Online Data Supplement

Key Words: aging ■ endothelium-dependent dilation ■ NFκB ■ interleukin-6 ■ 1-α hydroxylase ■ VDR

Cardiovascular diseases (CVDs) remain the leading cause of death in modern societies, and advancing age is the major risk factor for CVD.1–3 The increase in CVD risk with aging is attributable in large part to the development of vascular endothelial dysfunction, most commonly assessed as impaired endothelium-dependent dilation.2,4–5 Consistent with this, middle-aged and older adults demonstrate reduced brachial artery FMD, a measure of endothelium-dependent dilation (FMD), among healthy middle-aged/older adults, and this is mediated in part by nuclear factor κB–related inflammation. Reduced vitamin D receptor and 1-α hydroxylase may be molecular mechanisms linking vitamin D insufficiency to endothelial dysfunction.

In the present study, we tested the hypothesis that brachial artery FMD is inversely related to vitamin D status (as reflected by serum concentrations of 25-hydroxyvitamin D...
[25(OH)D] among middle-aged/older adults in the absence of clinical diseases. We also tested the hypothesis that vitamin D deficiency and impaired brachial FMD are associated with vascular inflammation in this group. To do so, we determined brachial artery FMD in healthy middle-aged/older men and women differing in serum 25(OH)D levels. We then assessed the expression of NFκB and IL-6 in vascular endothelial cells obtained from subgroups of these subjects, as well as NFκB signaling-associated suppression of brachial artery FMD using salsalate, an inhibitor of NFκB activation. To provide additional insight into the molecular mechanisms linking circulating 25(OH)D to endothelial function, we measured 1α-hydroxylase, the enzyme that converts 25(OH)D to 25,15-dihydroxyvitamin D [1,25(OH)2D], the biologically active form of vitamin D] and vitamin D receptor in endothelial cells obtained from subgroups of vitamin D–sufficient and vitamin D–deficient subjects.

Methods

Subjects

Subjects were 75 middle-aged/older (50 to 79 years) men (n=47) and postmenopausal women (n=28; 92% white, 5% Asian, and 3% Hispanic). Subjects had a systolic blood pressure <159 mm Hg, diastolic blood pressure <99 mm Hg, and were otherwise free of CVD, diabetes, kidney disease, and other chronic clinical disorders as assessed by medical history, physical examination, blood chemistries, and resting and exercise ECG. All subjects were nonsmokers not taking medications (prescription or over the counter), hormone replacement therapy, or dietary supplements (including those with antioxidants) and were not performing regular aerobic exercise (ie, <30 minutes per day, <2 days per week for ≥2 years before study participation). All procedures were approved by the Human Research Committee of the University of Colorado at Boulder. The nature, benefits, and risks of the study were explained to the volunteers, and their written informed consent was obtained before participation.

Study Procedures

All measurements were performed at the University of Colorado at Boulder Clinical and Translational Research Center after an overnight fast (water only) and a 24-hour abstinence from alcohol and vigorous physical activity.

Subject Characteristics, Blood Assays, and Dietary Analysis

Subjects were classified by 25(OH)D concentration as deficient (<20 ng/mL), insufficient (20 to 29 ng/mL), or sufficient (≥29 ng/mL) in accordance with recent recommended guidelines.18 (For details of subject characteristics, blood analyses, and dietary analysis, please see the online supplement, available at http://hyper.ahajournals.org.)

Brachial Artery FMD and Endothelium-Independent Dilation

Brachial artery FMD (upper forearm cuff position), peak shear rate during FMD, and endothelium-independent dilation (brachial artery dilation in response to sublingual nitroglycerin) were determined using duplex ultrasonography (Power Vision 6000; Toshiba) with a linear array transducer as described previously.19,20 Responses are expressed as millimeters and percent change from baseline diameter per recent recommended guidelines.21

Vascular Endothelial Cell Protein Expression

Vascular endothelial cells were obtained from antecubital venous sampling and analyzed for expression of proteins (IL-6, NFκB p65, tumor necrosis factor-α [TNF-α], vitamin D receptor, or 1α-hydroxylase) by quantitative immunofluorescence as described previously.20,23–24 Interindividual and group differences in protein expression using this procedure reflect expression in endothelial cells obtained from brachial artery sampling in the same subject.25 (For details of the methodology, see the online supplement.)

Salsalate Administration

To determine the contribution of NFκB-associated proinflammatory signaling to 25(OH)D-related differences in endothelium-dependent dilation, a subgroup of 21 middle-aged/older (50 to 68 years) men (n=17) and postmenopausal women (n=4) were randomly assigned to receive oral doses of salsalate, an NFκB inhibitor, or placebo using a 4-day cross-over design as described previously.20 (For details, see the online supplement.)

Statistics

All of the data are presented as means±SEM. (For details on statistical analysis, please see the online supplement.)

Results

Subject Characteristics

General subject characteristics, blood pressure, metabolic factors, and kidney function are presented in Table 1. Mean serum 25(OH)D in the overall sample was 25.3±1.0 ng/mL.
Humoral Factors

<table>
<thead>
<tr>
<th>Humoral Factors</th>
<th>Deficient (&lt;20 ng/mL)</th>
<th>Insufficient (20–29 ng/mL)</th>
<th>Sufficient (&gt;29 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP (mg · L⁻¹)</td>
<td>1.3±0.3</td>
<td>1.7±0.3</td>
<td>1.0±0.3</td>
</tr>
<tr>
<td>IL-6 (pg · mL⁻¹)</td>
<td>1.8±0.2</td>
<td>1.4±0.2</td>
<td>1.1±0.1*</td>
</tr>
<tr>
<td>TNF-α (pg · mL⁻¹)</td>
<td>1.5±0.1</td>
<td>1.3±0.1</td>
<td>1.7±0.3</td>
</tr>
<tr>
<td>Oxidized LDL (U · L⁻¹)</td>
<td>53.3±3.6</td>
<td>62.6±2.6</td>
<td>58.1±2.7</td>
</tr>
<tr>
<td>Total antioxidant status (mmol · L⁻¹)</td>
<td>1.19±0.04</td>
<td>1.22±0.03</td>
<td>1.24±0.06</td>
</tr>
<tr>
<td>ET-1 (pg · mL⁻¹)</td>
<td>6.5±0.5</td>
<td>6.3±0.2</td>
<td>7.0±0.9</td>
</tr>
<tr>
<td>Norepinephrine (mg · dL⁻¹)</td>
<td>311±28</td>
<td>311±31</td>
<td>275±28</td>
</tr>
</tbody>
</table>

Data are mean±SE. *P<0.01 vs deficient. CRP indicates C-reactive protein; ET-1, endothelin-1.

(range 7 to 47): 29% of subjects were 25(OH)D deficient, and this was similar in men versus women (30% and 29%). The 25(OH)D-deficient, 25(OH)D-insufficient and 25(OH)D-sufficient groups did not differ significantly in age, percent body fat, hip circumference, waist-to-hip ratio, physical activity, total bone mineral density, systolic and diastolic blood pressure, plasma lipids and lipoproteins, plasma glucose and insulin, homeostasis model assessment, estimated glomerular filtration rate, or 1,25(OH)₂D, whereas there were selective group differences in body mass index and waist circumference (P<0.05). The season in which blood samples were collected (data not shown) was only weakly related to serum 25(OH)D (eta coefficient 0.29) and 1,25(OH)₂D (eta coefficient 0.16) in the overall sample.

Humoral Factors

Humoral factors are shown in Table 2. C-Reactive protein, TNF-α, oxidized LDL, total antioxidant status, endothelin-1, and norepinephrine did not differ among the groups. Plasma IL-6 was higher in the deficient group (P<0.01 versus sufficient) and was inversely related to 25(OH)D in the overall sample (r=−0.40; P<0.01).

Diet Composition

Dietary analysis is shown in supplemental Table I. Total energy intake, percent macronutrient intake, and cholesterol, vitamin C, calcium, potassium, and sodium intake were similar in the 3 groups. Mean values for dietary vitamin D tended to be progressively greater in the 25(OH)D-deficient, 25(OH)D-insufficient, and 25(OH)D-sufficient groups (P=0.20) and tended to be related to serum 25(OH)D in the overall sample (r=0.31; P=0.06).

Brachial Artery FMD and Endothelium-Independent Dilation

Baseline brachial artery diameter and peak shear rate during FMD did not differ among groups (see online supplement) or relate to serum 25(OH)D level (P≥0.30). Brachial artery FMD was lower in the 25(OH)D-deficient and 25(OH)D-insufficient versus 25(OH)D-sufficient groups (−30% [%Δ] and −29% [mmΔ] deficient versus sufficient; P<0.05; Figure 1). Endothelium-independent dilation did not differ among groups (P=0.40; see online supplement). In all subjects, brachial artery FMD was positively related to serum 25(OH)D level (%Δ: r=0.35, P<0.01; mmΔ: r=0.29, P=0.01; Figure 1). This relationship remained significant after correcting for subject characteristics in Table 1 (%Δ: R²=0.18, P<0.01; β=0.43, SE=0.03, P<0.01; mmΔ: R²=0.25, P<0.01; β=0.40, SE=0.002, P<0.01). Season of measurement only weakly related to FMD (eta coefficient 0.19) and the relationship between FMD and serum 25(OH)D was unaffected after adjusting for season of measurement (partial correlation coefficient [%Δ r=0.36, P<0.01; mmΔ r=0.30, P<0.01]).

In contrast to 25(OH)D, brachial artery FMD did not differ across tertiles of serum 1,25(OH)₂D and was not related to serum 1,25(OH)₂D in the overall sample (%Δ r=−0.06, P=0.61; mmΔ r=−0.06, P=0.61; see online supplement). Endothelium-independent dilation also did not differ across tertiles of serum 1,25(OH)₂D levels (P≥0.60).
Vascular Endothelial Cell NFκB and Brachial Artery FMD
Expression of NFκB was greater in vascular endothelial cells of the 25(OH)D-deficient compared with the 25(OH)D-sufficient group (P<0.05; Figure 2, left panel).

When subjects were divided by the median value of 25(OH)D, brachial FMD was 42% lower in the group with lower compared with higher circulating vitamin D (P<0.05; Figure 2, middle panel). NFκB inhibition with salsalate improved FMD in both groups (P<0.05 versus placebo), but the increase was greater (P<0.05) in subjects with lower (+168%) compared with higher (+50%) serum 25(OH)D. As a result, FMD was similar in the 2 groups after treatment with salsalate. There were no differences in baseline diameter or shear rate between groups or conditions (salsalate versus placebo). Groups did not differ in any subject characteristics, and serum 25(OH)D concentrations did not differ between placebo and salsalate conditions. The results were similar when mmΔ was used instead of %Δ.

Salsalate treatment reduced the expression of NFκB in vascular endothelial cells obtained from subjects with lower (P=0.06) but not from those with higher serum 25(OH)D (Figure 2, right panel). There were no differences in endothelial cell expression of NFκB in the 2 groups after treatment with salsalate.

Vascular Endothelial Cell IL-6 and TNF-α Expression
25(OH)D-deficient subjects had 81% greater endothelial cell protein expression of IL-6 compared with sufficient subjects (P<0.01; Figure 3). IL-6 was inversely related to 25(OH)D among all subjects (r=−0.62; P<0.01). In contrast, endothelial cell expression of TNF-α did not differ among groups (P=0.60; Figure 3) or correlate with 25(OH)D (r=−0.03; P=0.86).

Vascular Endothelial Cell Vitamin D Receptor and 1-α Hydroxylase Expression
Serum 25(OH)D-deficient subjects had lower endothelial cell protein expression of vitamin D receptor compared with sufficient subjects (P<0.05; Figure 4). Endothelial cell protein expression of 1-α hydroxylase also was lower in serum 25(OH)D-deficient versus 25(OH)D-sufficient subjects (P<0.05) and was positively related to FMD (%Δ r=0.82, P<0.01; mmΔ r=0.92, P<0.01; Figure 4).
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Vitamin D and Vascular Inflammation

The findings of the present study are the first to show that vascular endothelial function is related to vitamin D status (as reflected by serum 25(OH)D) among middle-aged/older adults free of clinical disease. Our results also provide the first evidence in humans that lower serum 25(OH)D is associated with vascular endothelial inflammation, increased NFκB signaling–related suppression of vascular endothelial function, and reduced vascular endothelial vitamin D receptor and 1-α hydroxylase expression. In contrast to 25(OH)D, our data indicate that brachial artery FMD is not related to serum 1,25(OH)2D in this group.

Vitamin D and Vascular Endothelial Dysfunction With Aging

The results of the present study demonstrate that brachial artery FMD, a measure of endothelium-dependent dilation and vascular endothelial function, is inversely related to serum vitamin D status among middle-aged/older adults without clinical disease. This is consistent with recent work in patients with diabetes and chronic kidney disease and in young adults with severe vitamin D deficiency. Our findings extend these observations by showing an inverse association between circulating vitamin D and vascular endothelial function that is evident in healthy adults within a typical range of serum 25(OH)D.

Our data indicate a specific relationship between brachial artery FMD and serum 25(OH)D, the metabolite used to determine vitamin D status, but not 1,25(OH)2D, the biologically active form of vitamin D. 1,25(OH)2D is formed via conversion of 25(OH)D by the enzyme 1-α hydroxylase, a process historically viewed as occurring in the kidney with the presumed aim of maintaining muscle and bone health. However, it has been recognized recently that this conversion also can occur locally in several different cells and tissues. This implies that 1,25(OH)2D can exert autocrine/paracrine cell-specific functions when produced outside of the kidney.

In the context of the present work, vascular endothelial cells in culture express vitamin D receptors and 1-α hydroxylase. Here, we demonstrate for the first time expression of these proteins in vascular endothelial cells collected from human subjects. Because circulating 1,25(OH)2D did not differ between groups and was not related to brachial FMD, our results suggest that the association between serum 25(OH)D level and FMD could be mediated in part by vascular endothelial cell conversion of 25(OH)D to 1,25(OH)2D. That endothelial cells from vitamin D–deficient subjects had reduced expression of vitamin D receptors and 1-α hydroxylase compared with vitamin D–sufficient subjects and that 1-α hydroxylase was strongly related to FMD are consistent with this possibility. Thus, reduced expression of vitamin D receptors and 1-α hydroxylase in vitamin D–deficient subjects may have limited the conversion of 25(OH)D to 1,25(OH)2D and attenuated vitamin D signaling in the vascular endothelium, thus contributing to reductions in FMD. If so, this represents a novel mechanism by which circulating vitamin D deficiency may be linked to vascular endothelial dysfunction.

It should be noted that previous investigations in patients with chronic kidney disease on hemodialysis and healthy young adults with severe vitamin D deficiency found that brachial FMD was related to both serum 25(OH)D and 1,25(OH)2D levels. The differences between our results and those of previous investigations on this point may be explained by differing kidney function (ie, kidney disease versus healthy) or the degree of vitamin D deficiency. Differences in oxidative stress between vitamin D–sufficient and vitamin D–deficient groups also may have been greater in these previous studies, as reflected by increased circulating markers of oxidant damage. In contrast, plasma-oxidized LDL, a marker of oxidant modification of lipids, did not differ among the groups in the present study.

Vitamin D and Vascular Inflammation

Our results are the first to show a relation between serum vitamin D status and vascular endothelial inflammation in humans. Specifically, we found that vascular endothelial cell expression of the p65 subunit of NFκB, a major proinflammatory nuclear transcription factor, and IL-6, a proinflamma-

Figure 4. Protein expression of vitamin D receptor (VDR; left) and 1-α hydroxy (1-OHase; middle) in vascular endothelial cells obtained from 25(OH)D-deficient and 25(OH)D-sufficient subjects; representative images shown below. Relationship between endothelial cell protein expression of 1-OHase and FMD (mm); right). For all proteins, values are venous endothelial cell expression relative to human umbilical vein endothelial cell control. *P < 0.05 vs deficient.
tory cytokine and downstream target of NFκB, were greater in vitamin D–deficient compared with vitamin D–sufficient middle-aged/older adults. Indeed, IL-6 expression in endothelial cells was strongly inversely related to serum 25(OH)D. In contrast, the expression of TNF-α, a proinflammatory cytokine and (upstream) activator of NFκB, was not associated with 25(OH)D, suggesting no obvious involvement in vitamin D–related effects on endothelial cell inflammation.

Previous investigations have reported a positive correlation between circulating proinflammatory cytokines and serum 25(OH)D levels, and that vitamin D inhibits experimental activation of NFκB, as well as IL-6 release, in endothelial cell culture. Our findings extend this previous work to show an inverse association between serum 25(OH)D level and proinflammatory NFκB–IL-6 activation in vascular endothelial cells obtained from human subjects.

To determine whether differences in NFκB-related signaling in the vascular endothelium between subjects with lower compared with higher 25(OH)D may have contributed to differences in vascular endothelial function, we inhibited NFκB activation using short-term treatment with salsalate. Salsalate reduces endothelial cell NFκB in adults with elevated baseline expression. Consistent with this, in the present study, we observed a selective reduction in NFκB in endothelial cells obtained in subjects with lower serum 25(OH)D concentrations, a group with elevated baseline expression of NFκB. Most important, we found that the tonic suppression of endothelium-dependent dilation by NFκB signaling, as assessed by the improvement in brachial artery FMD from placebo control in response to short-term treatment with salsalate, was greater in the subgroup with lower circulating 25(OH)D, and that salsalate treatment abolished group differences in FMD. This provides direct evidence of NFκB activation–related vascular endothelial dysfunction in middle-aged/older adults with lower serum 25(OH)D.

Limitations

In the present study, selective subject characteristics were or tended to be different between groups. However, these trends did not explain group differences in brachial FMD because correcting for these factors did not weaken the relationship between FMD and serum 25(OH)D concentrations. Parenthetically, the tendency for differences in such characteristics in the present study among healthy adults is consistent with evidence in human studies and animal models (eg, vitamin D receptor knockout mice) that vitamin D deficiency is associated with cardiovascular risk factors such as increased blood pressure and insulin resistance.

Expression of proteins, including NFκB, in endothelial cells obtained from the antecubital vein generally reflects expression in cells obtained from the brachial artery of the same subject. However, in the present study, we assessed only cells from venous sampling and, thus, cannot confirm the presence of differences in arterial endothelial cells among groups differing in serum vitamin D status.

Finally, we did not measure for proteinuria, and kidney function was estimated by the Modification of Diet in Renal Disease equation, which is less accurate in persons with normal or mildly impaired kidney function compared with patients with more severe dysfunction. Therefore, we cannot rule out differences in kidney function among the 3 serum 25(OH)D groups.

Conclusions

In conclusion, the results of the present study support the hypothesis that serum 25(OH)D status is associated with vascular endothelial function among middle-aged/older adults in the absence of clinical disease. Our findings also demonstrate that lower 25(OH)D status is associated with increased vascular endothelial cell expression of NFκB and IL-6 and increased NFκB-related suppression of vascular endothelial function. Our data indicate that, in contrast to 25(OH)D, FMD is not associated with serum 1,25(OH)2D in this group. Finally, our results show for the first time that vascular endothelial cell expression of vitamin D receptor and 1-α-hydroxylase are decreased with 25(OH)D deficiency and are related to vascular endothelial function.

Perspectives

These observations provide an experimental basis for intervention trials aimed at determining the efficacy of vitamin D treatment for reversing vascular endothelial dysfunction in 25(OH)D-deficient and 25(OH)D-insufficient middle-aged/older adults to prevent CVDs and the role of inhibition of inflammatory signaling in any such benefits.

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Disclosures

None.

References


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25-Hydroxyvitamin D deficiency is associated with inflammation-linked vascular endothelial dysfunction in middle-aged and older adults

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Supplemental Methods.

**Subject Characteristics, Blood Assays and Dietary Analysis.** Body mass index (BMI) was calculated from height and weight to the nearest 0.1 kg, and waist and hip circumferences were measured by anthropometry. Total body fat and bone mineral density were determined using dual energy x-ray absorptiometry (DPX-IQ, GE/Lunar, Inc.). Arterial blood pressure was measured over the brachial artery during seated rest using a semi-automated device (Dynamap XL, Johnson and Johnson). Habitual physical activity was assessed from estimates of daily energy expenditure using the Stanford Physical Activity Questionnaire as previously described. Diet composition and caloric intake were estimated from 3-day food intake records (The Food Processor 8.2, ESHA Research) as described previously.

Serum samples were analyzed for 25(OH)D and 1,25(OH)D₂ by the Associated Regional and University Pathologists (ARUP) laboratories (Salt Lake City, UT) using liquid chromatography/tandem mass spectrometry and radioimmunoassay, respectively. The inter- and intra-batch coefficients of variation averaged 7.0% and 4.5% for 25(OH)D. Because serum 25(OH)D levels fluctuate seasonally, the sampling date for each subject was classified according to season (winter (December-February), spring (March-May), summer (June-August), autumn (September-November)).

All other assays were performed by the University of Colorado CTRC core laboratory. Plasma total cholesterol, low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglycerides, glucose and insulin were determined using standard assays. The homeostasis model of insulin resistance (HOMA-IR) was calculated by the formula: [fasting glucose (mg/dl) x fasting insulin μU/L]/405. Estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease (MDRD) formula: 186.3 x (serum creatinine)⁻¹.₁⁵₄ x age⁻₀.₂₀₃ x (0.742 for women) x (1.21 if African American), with serum creatinine measured using an enzymatic assay (Beckman Coulter Inc.).

Serum concentrations of C-reactive protein were measured using a high-sensitivity Chemistry Immuno Analyzer (AU400e, Olympus America, Inc.). Serum interleukin-6 (IL-6; R&D Systems, Inc), tumor-necrosis factor-α (TNF-α, R&D Systems, Inc), oxidized LDL (Alpco, Inc), and endothelin-1 (Peninsula Laboratories, Inc), were measured by ELISA. Total antioxidant status was measured using an enzymatic kit (Randox Laboratories, Inc.), and plasma norepinephrine concentrations were measured by high performance liquid chromatography (BioRad Laboratories).

**Vascular Endothelial Cell Protein Expression.** Briefly, J-wires were advanced into the antecubital vein ~4 cm beyond the tip of an 18-guage catheter and withdrawn. Cells were recovered by washing and centrifugation, fixed with 3.7% formaldehyde and plated on slides. Non-specific binding sites were blocked with 5% donkey serum (Jackson Immunoresearch) and cells were incubated with monoclonal antibodies for IL-6 (Santa Cruz) (n=24), NFκB p65(Novus) (n=64), TNFα (Abcam) (n=37), vitamin D receptor (Santa Cruz) (n=12) or 1-α hydroxylase (The Binding Site) (n=10). Slides were systematically viewed with a fluorescence microscope (Eclipse 600, Nikon, Melville, NY).
to identify endothelial cells (positive staining for von Willebrand factor) and nuclear integrity was confirmed with DAPI (4′,6′-diamidino-2-phenylindole hydrochloride) staining. Images were then captured and analyzed with Metamorph Software (Universal Imaging Corp, Downington, PA) to quantify the intensity of CY3 staining (i.e., average pixel intensity). The values for each protein are reported as a ratio of endothelial cell to human umbilical vein endothelial cell (HUVEC) average pixel intensity, which minimizes the possible confound of differences in intensity of staining among different staining sessions. A single investigator analyzed each batch of cells and was blinded to subject identification.

**Salsalate Administration.** Salsalate was administered in a total daily dose (2500-4500 mg) that has been used clinically to inhibit NFκB by producing steady state plasma salicylate concentrations in the therapeutic range of 10 to 30 mg/100 mL⁷,⁸, while minimizing gastrointestinal and other side effects⁹,¹⁰. Subjects received a standardized diet prepared by the CTRC bionutritionist for 3 days prior to the vascular testing sessions.

**Statistics.** Statistical analyses were performed with PASW Statistics (version 18.0). For the main study, differences across the three groups of 25(OH)D and tertiles of 1,25(OH)ΔD were determined by one-way ANOVA and between two groups of 25(OH)D using an unpaired t-test. Least squares difference post hoc tests were used where appropriate. Differences in the categorical variable (season) across groups were determined using the chi-squared test of independence. Potential bivariate relations of interest between variables, including relations between subject characteristics (potential confounders) and the independent (25(OH)D) and dependent (FMD) variables, were assessed using Pearson product-moment correlation analyses. The relation between non-dichotomous nominal and interval variables was assessed by the eta correlation. Stepwise multiple regression analyses were then conducted, with variables chosen based on subject characteristics that tended to differ in Table 1 and those variables known or thought to be associated with vascular endothelial function, based on published observations (characteristics included in the model were age, mass, BMI, body fat, waist circumference, waist-to-hip ratio, physical activity, systolic blood pressure, diastolic blood pressure, total cholesterol, LDL cholesterol, triglycerides, glucose, insulin and HOMA). Partial correlation analysis was used to statistically remove the potential influence of a variable on the relation between 25(OH)D and FMD. For the salsalate sub-protocol, subjects were divided into higher and lower vitamin D groups based on the median value for 25(OH)D and a t-test was used for independent sample comparisons. Statistical significance for all analyses was set at P < 0.05.
Supplemental References.


Supplemental Results.

Figure Legends

**Figure S1.** FMD (percent change (%Δ), top; absolute change (mmΔ); bottom) across tertiles of serum 1,25(OH)₂D (1: <40 pg/mL; 2: 41-53 pg/mL; 3: ≥54 pg/mL) groups (left); relation between serum 1,25(OH)₂D levels and FMD in the overall sample (%Δ, top; mmΔ; bottom) (right).
Table S1.

<table>
<thead>
<tr>
<th>Diet Composition</th>
<th>Deficient (&lt;20 ng/mL)</th>
<th>Insufficient (20-29 ng/mL)</th>
<th>Sufficient (&gt;29 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total kilocalories/day</td>
<td>2143±140</td>
<td>2147±133</td>
<td>1990±211</td>
</tr>
<tr>
<td>Fat (% of total kcals/day)</td>
<td>34±1</td>
<td>33±1</td>
<td>32±2</td>
</tr>
<tr>
<td>Protein (% of total kcals/day)</td>
<td>17±1</td>
<td>16±1</td>
<td>16±1</td>
</tr>
<tr>
<td>Carbohydrates (% of total kcals/day)</td>
<td>48±3</td>
<td>51±3</td>
<td>51±4</td>
</tr>
<tr>
<td>Cholesterol (mg/day)</td>
<td>286±46</td>
<td>288±36</td>
<td>281±47</td>
</tr>
<tr>
<td>Vitamin C (mg/day)</td>
<td>116±10</td>
<td>158±23</td>
<td>101±16</td>
</tr>
<tr>
<td>Vitamin D (IU/day)</td>
<td>77±25</td>
<td>145±51</td>
<td>191±55</td>
</tr>
<tr>
<td>Calcium (mg/day)</td>
<td>814±82</td>
<td>987±82</td>
<td>951±119</td>
</tr>
<tr>
<td>Potassium (mg/day)</td>
<td>2754±213</td>
<td>3101±361</td>
<td>2556±468</td>
</tr>
<tr>
<td>Sodium (mg/day)</td>
<td>3039±271</td>
<td>3218±417</td>
<td>3129±440</td>
</tr>
</tbody>
</table>

Data are mean ± S.E. kcal, kilocalories.

Table S2.

<table>
<thead>
<tr>
<th>Brachial Artery Parameters</th>
<th>Deficient (&lt;20 ng/mL)</th>
<th>Insufficient (20-29 ng/mL)</th>
<th>Sufficient (&gt;29 ng/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline Diameter (mm)</td>
<td>3.9±0.2</td>
<td>3.8±0.1</td>
<td>3.8±0.2</td>
</tr>
<tr>
<td>Peak Shear Rate (sec⁻¹)</td>
<td>485±46</td>
<td>453±24</td>
<td>470±39</td>
</tr>
<tr>
<td>Dilation to NTG (%)</td>
<td>22.2±1.6</td>
<td>23.9±1.3</td>
<td>24.9±1.6</td>
</tr>
<tr>
<td>Dilation to NTG (mmΔ)</td>
<td>0.83±0.04</td>
<td>0.92±0.05</td>
<td>0.90±0.06</td>
</tr>
</tbody>
</table>

Data are mean ± S.E. NTG, nitroglycerin.
Figure S1.

The figure shows the relationship between brachial artery FMD and serum 1,25(OH)2D levels. The left panels depict the mean FMD values across tertiles of serum 1,25(OH)2D, with error bars indicating the standard error of the mean. The right panels show scatter plots correlating serum 1,25(OH)2D levels with brachial artery FMD values, with regression lines and correlation coefficients (r) and p-values provided.