Vitamin D and Parathyroid Hormone Relationships with Urinary Nitric Oxide Metabolites and Plasma Isoprostanes in African-Americans

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Key Words
Vitamin D • Parathyroid hormone • Nitric oxide • Endothelial dysfunction • Isoprostanes

Abstract

Background: Vitamin D deficiency and secondary rises in parathyroid hormone (PTH) are highly prevalent in obese African-Americans. Endothelial dysfunction related to oxidative stress is more common in African-Americans compared to whites. Currently, the association of vitamin D (25-hydroxyvitamin D, 25-OH D) and PTH to nitric oxide metabolites (NOx) – nitrate and nitrite – and oxidative stress in African-Americans is unknown. Objective: A cross-sectional design was utilized to determine the association of 25-OH D and PTH with urinary NOx (UNOx) (n = 101) and plasma isoprostanes (n = 125), an oxidative stress marker, in overweight (body mass index of 25–39.9), normotensive African-Americans aged ≥ 35 years. Measurements: Multivariable linear regression analysis adjusted for age, sex, body mass index, and season was used to determine the relationship of 25-OH D and PTH to UNOx and isoprostanes. General linear models, adjusted for the same covariates, contrasted UNOx across three mutually exclusive vitamin D/PTH groups: (1) normal 25-OH D (51–249 nmol/l) and normal PTH (< 65 pg/ml); (2) low 25-OH D and normal PTH, and (3) low 25-OH D and high PTH. Results: 25-OH D was directly associated with UNOx before (p = 0.02) and after (p = 0.03) adjustment for PTH levels. A borderline significant association was observed between PTH and isoprostanes (p = 0.08). UNOx was 424, 290, and 270 μmol/8 h, respectively, across vitamin D/PTH groups 1–3 (p = 0.08). Conclusion: 25-OH D was directly associated with NO availability and PTH was positively, though borderline, associated with isoprostanes in overweight, normotensive adult African-Americans.

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Introduction

Hypertension disproportionately affects African-Americans compared to whites [1], an observation that is plausible, at least in part, because of endothelial dysfunction that is attributable to low bioavailability of nitric oxide (NO). African-Americans, including those with normal blood pressure (BP) levels [2], have greater impairment of endothelium-derived NO function than whites [3]. In addition, African-Americans have higher levels of oxidative stress that likely contribute to the destruction of NO, thereby reducing NO bioavailability [4, 5]. Both hypovitaminosis D and secondary hyperparathyroidism (SHPT) are excessively prevalent in obese African-Americans [6, 7]. Obesity disproportionately affects African-Americans, particularly women [8], and has been linked to endothelial dysfunction [9], depressed vitamin D levels, SHPT, and hypertension [1, 6, 7, 10]. Accordingly, weight loss improves endothelial dysfunction in severely obese individuals [11]. It has also been suggested that elevated parathyroid hormone (PTH) induces oxidative stress [12] by causing intracellular calcium overload [13]. Moreover, a recent study supported a link between vitamin D deficiency and attenuated brachial artery flow-mediated dilatation, an indication of endothelial dysfunction, and augmented levels of lipid peroxidation [14]. Furthermore, vitamin D3 treatment improved endothelial function and lowered oxidative stress in these same vitamin D deficient participants.

The data regarding vitamin D supplementation and endothelial dysfunction have not been entirely consistent as one study in rabbits reported that vitamin D2 supplementation actually caused endothelial dysfunction and increased levels of oxidative stress [15]. The relationships of 25-hydroxyvitamin D (25-OH D) and PTH to levels of NO metabolites (NOx) and oxidative stress in African-Americans have not been established. There are also, to our knowledge, no available data regarding the impact of augmentation of vitamin D levels on NOx.

Thus, the major goal of this study was to determine the associations of circulating 25-OH D and PTH with NOx and isoprostanes, a marker of oxidative stress, in overweight, normotensive African-Americans. This healthy cohort of African-Americans afforded the opportunity to investigate these relationships prior to the onset of clinically detectable cardiovascular disease.

Methods

A cross-sectional study design was utilized to determine the relationship of 25-OH D and PTH to NOx and isoprostanes in overweight [body mass index (BMI) 25–39.9], normotensive (BP <140/90 mm Hg) African-Americans recruited in Detroit, Michigan, for participation in a National Institute of Environmental Health Sciences salt sensitivity/weight loss study. The cohort was otherwise healthy with no cardiac disease or kidney disease with estimated glomerular filtration rate (GFR) >60 ml/min/1.73 m². Participants were also not taking any BP or cardiovascular medications including lipid-lowering drugs, oral steroids, nitrates or non-steroidal anti-inflammatory drugs >4 days/week. Further details of the study population have been described previously [6]. Personal identifiers were removed and data were imported into a research database for analysis. This study was reviewed and approved by the Wayne State University Institutional Review Board. Signed informed consent was obtained from all participants prior to their participation.

Study Measures

Venous blood sampling was performed to determine 25-OH D, PTH, and isoprostanes. Urine was collected during nighttime for a period of 8 h. Specimens were stored at –80°C. Biological specimens were processed in batches to minimize technical and overall variability.
25-OH Vitamin D
Serum 25-OH D was determined using liquid chromatography-tandem spectrometry at Mayo Medical Laboratories, Rochester, Minn., USA. Functional sensitivity of the assay was <10 nmol/l and the mean interassay coefficient of variation (CV) was 3.8%.

Intact PTH
Serum intact PTH was determined using automated chemiluminescent immunometric assay and electrochemiluminescence method using Roche Cobas assay because of methodology change at Mayo Medical Laboratories, Rochester, Minn., USA. Functional sensitivities of the assays were <5 and <6 pg/ml, respectively, and mean interassay CVs were 8 and 6–7%, respectively. Detailed PTH processing has been described previously [6].

Total NOx
Participants were guided by dieticians to adhere to a low nitrate diet for 4 days prior to urine collection. Urinary nitrate and nitrite (UNOx) was determined using Cayman’s Nitrate/Nitrite colorimetric assay kit (Cayman Chemical Company, Ann Arbor, Mich., USA). The detection limit for nitrate/nitrite assay (80 µl) and nitrite assay (100 µl) was 2.5 and 2.0 µmol/l, respectively, and the mean interassay CV was 3.4%. Participants with urinary nitrite levels >1% (likely due to contamination) or with no UNOx data were excluded leaving 101 participants for analyses. UNOx was expressed as an excretion rate in units of µmol/8 h.

Isoprostanes
Plasma isoprostanes were measured using a competitive enzyme-linked immunoassay (ELISA) for 15-isoprostanes F3, also known as 8-isoprostanes (Oxford Biomedical Research, Oxford, Mich., USA). A colorimetric plate reader was used to measure absorbance at 450 nm.

Dietary Data
The Block 98 Food Frequency Questionnaire was used to estimate daily dietary intake of vitamin D and calcium over the last 12 months. Food Frequency Questionnaire records were analyzed with the Nutrition Data System, research version of the software (University of Minnesota, Nutrition Coordinating Center).

Body Mass Index
BMI was calculated as body weight (using a standard balance beam scale to the nearest 0.1 kg) divided by the square of height (using a stadiometer to the nearest 0.001 m).

Waist and Hip Circumference
Waist circumference was measured to the nearest 0.1 cm by applying the measuring tape horizontally midway between the lowest rib and the iliac crest after a normal expiration. Hip circumference was measured at the point yielding the maximum circumference over the greater trochanter.

Body Composition
Fat mass was determined using a dual-energy X-ray absorptiometry (DEXA, QDR 4500 Acclaim Series Elite, Hologic Inc., Bedford, Mass., USA).

Blood Pressure
Three seated BP readings were recorded from the right arm, using a mercury sphygmomanometer and the bell of the stethoscope, and averaged.

Statistical Analysis
Continuous data were summarized as means, medians, standard deviations, standard errors, and 95% confidence intervals (CI), while categorical variables were summarized as frequencies and counts. The distributions of all continuous variables were examined for skewness/normality using Shapiro-Wilk statistic. Continuous data that deviated significantly from normality were log transformed prior to analysis. Least square means and corresponding 95% CI were reported. Geometric means were calculated by exponentiating log-transformed means. Spearman rank-order correlations were utilized to assess uni-
Valiña-Tóth et al.: Vitamin D and Parathyroid Hormone Relationships with Urinary Nitric Oxide Metabolites and Plasma Isoprostanes in African-Americans

variate relationships of 25-OH D and PTH with UNOx and isoprostanes. Primary analyses utilized multivariable linear regression models adjusted for covariates to characterize the relation of 25-OH D and PTH to UNOx and isoprostanes.

Secondary analyses were performed utilizing general linear models, adjusted for selected covariates, contrasting UNOx and isoprostanes across three mutually exclusive vitamin D/PTH groups: (1) normal: 25-OH D = 51–249 nmol/l and PTH ≥ 65 pg/ml (1 nmol/l = 0.4 ng/ml; 1 pg/ml = 1 ng/l); (2) LN: low 25-OH D and normal PTH, and (3) SHPT: low 25-OH D and high PTH.

Bonferroni’s correction was applied to maintain the overall α level at 0.05 across the multiple contrasts. General linear models was also utilized to compare between groups with normal or high vitamin D and low vitamin D. Statistical analyses were performed using SAS statistical software (SAS, version 9.2, SAS Institute Inc., Cary, N.C., USA). Statistical significance was set at p < 0.05.

Results

Participant Characteristics (table 1)

The majority of participants were women (82%). Participants on average were obese (BMI ≥ 30) with normotension (BP < 140/90 mm Hg). In addition, the study group on average consumed amounts of calcium and dietary vitamin D that were below the daily recommended intake. Moreover, the average circulating serum 25-OH D level was in the range of vitamin D deficiency (25-OH D ≤ 50 nmol/l) [16]. The mean circulating PTH level was within high normal range. There were no mean differences in 25-OH D and PTH levels between men and women.

Relationships of 25-OH D and PTH to NOx and Isoprostanes (table 2)

PTH and 25-OH D were regressed on UNOx and isoprostanes as dependent variables using multivariable linear regression analyses adjusted for age, sex, BMI, and season. No significant interactions between sex and PTH or 25-OH D were detected. Similar findings were observed performing the above analysis with female participants only.

Univariate analysis using Spearman correlations did not support an association of either 25-OH D or PTH with UNOx and isoprostanes. However, multivariable regression analysis predicting UNOx showed a statistically significant direct relationship between 25-OH D and UNOx (p = 0.02, model 1). The inverse association between PTH and UNOx was not statistically significant (model 2). After adjustment for PTH levels, 25-OH D remained positively
related to UNOx (p = 0.03, model 3). However, adjustment for 25-OH D levels did not change the lack of relationship of PTH to UNOx (model 3).

A borderline significant association was observed between PTH and isoprostanes (p = 0.08, model 2). However, after adjustment for 25-OH D levels, the direct association between PTH and isoprostanes did not attain statistical significance (p = 0.12, model 3). 25-OH D was unrelated to isoprostanes even after adjustment for PTH levels.

**Table 2. Multivariable linear regression analysis of UNOx and isoprostanes on 25-OH D and PTH**

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Model</th>
<th>Independent variable</th>
<th>Regression coefficient</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>log [UNOx, μmol/8 h], (n = 101)</td>
<td>1</td>
<td>25-OH D</td>
<td>0.01</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>PTH</td>
<td>–0.002</td>
<td>0.41</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25-OH D</td>
<td>0.01</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTH</td>
<td>–0.001</td>
<td>0.72</td>
</tr>
<tr>
<td>log [isoprostanes, ng/ml], (n = 125)</td>
<td>1</td>
<td>25-OH D</td>
<td>–0.003</td>
<td>0.38</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>PTH</td>
<td>0.004</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>25-OH D</td>
<td>–0.001</td>
<td>0.65</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PTH</td>
<td>0.003</td>
<td>0.12</td>
</tr>
</tbody>
</table>

Multivariable linear regression models adjusted for age, sex, BMI, and season. Models 1, 2 and 3 displayed the association of 25-OH D, PTH, and both to either NOx and isoprostanes, respectively.

**Table 3. Contrast of study variables between low vitamin D group (LN + SHPT groups) and high vitamin D group (normal group)**

<table>
<thead>
<tr>
<th>Dependent variable</th>
<th>Low vitamin D</th>
<th>High vitamin D</th>
<th>p value</th>
</tr>
</thead>
<tbody>
<tr>
<td>25-OH D, nmol/l†</td>
<td>31.7 (29.1–34.2)</td>
<td>67.4 (62.9–71.9)</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>PTH, pg/ml‡</td>
<td>44.6 (38.9–50.2)</td>
<td>33.0 (23.2–42.8)</td>
<td>0.04</td>
</tr>
<tr>
<td>UNOx, μmol/8 h‡, §</td>
<td>299 (245–330)</td>
<td>446 (317–570)</td>
<td>0.02</td>
</tr>
<tr>
<td>Isoprostanes, ng/ml†, §</td>
<td>2.71 (2.44–3.03)</td>
<td>2.49 (2.00–3.56)</td>
<td>0.49</td>
</tr>
</tbody>
</table>

Values are expressed as least square means with corresponding 95% CI in parentheses. General linear models: † adjusted for age, sex, BMI, and season; ‡ adjusted for age and sex. § Geometric means were calculated by taking the exponential of log-transformed means.
Discussion

The main new finding of our cross-sectional study in overweight, normotensive African-Americans was that 25-OH D was directly related to UNOx, while PTH was borderline positively related to isoprostanes; observations that extend the previously reported relationships by Tarcin et al. [14] between depressed vitamin D and impaired brachial artery flow-mediated dilation. Furthermore, our data documented that the combination of depressed 25-OH D and raised PTH was associated with lower UNOx. However, we were unable to show an inverse relationship of PTH, a known potentiator of oxidative stress, with UNOx. To date, studies on endothelial dysfunction in African-Americans have not associated 25-OH D and/or PTH levels to biological measures of NOx and oxidative stress.

African-Americans compared to whites have higher rates of hypertension, microvascular and macrovascular structural and functional abnormalities, including endothelium (NO)-dependent vascular dysfunction, oxidative stress, inflammation, obesity, and obesity-related diseases (e.g. hypertension, diabetes, and heart failure) [1, 8]. Even normotensive African-Americans have more impaired endothelium-dependent and endothelium-independent NO-mediated vasodilation in response to vasodilatory agonists or stimuli than whites [2, 3, 9, 17]. Furthermore, African-Americans manifest greater intima media thickening of large resistance vessels, greater large artery stiffening, and lower arterial compliance in addition to blunted vasodilatory response to vasodilatory stimuli [2, 18, 19].

Reduced NO bioavailability in African-Americans compared to whites does not appear to be a consequence of reduced NO production [4]. Kalinowski et al. [4] documented upregulation of endothelial NO synthase (eNOS), greater NO production, but also more release of superoxide (O$_2^-$) and peroxynitrate (ONOO$^-$) from single human umbilical vein endothelial cells in African-Americans compared to whites. Thus, reduced NO bioavailability was plausibly a consequence of increased NO destruction by superoxide anion and also by peroxynitrate-induced uncoupling of eNOS.

There are multiple possible pathways through which vitamin D/PTH potentially influence NO bioavailability as depicted in a conceptual model in figure 1 [20]. In the setting of hypovitaminosis D, raised PTH activates renal 1α-hydroxylation of 25-OH D to produce 1,25-(OH)$_2$ D in the kidneys, augmenting intracellular calcium which, in turn, promotes reactive oxygen species [12, 13, 21]. Angiotensin II induces superoxide formation via angiotensin type [1] receptor activation of NADPH oxidase [22]. Consequently, enhanced superoxide production augments inflammation and peroxynitrate production when eNOS is stimulated and, in turn, peroxynitrate increases eNOS uncoupling, which also increases superoxide anion synthesis [23]. Angiotensin II has also been shown to attenuate NO production and endothelium-dependent vasodilation by increasing tyrosine phosphorylation of eNOS [24]. Vitamin D, however, suppresses renin-angiotensin system (RAS) activity by suppression of renin gene transcription [25] and also potently suppresses PTH, which should lead to reduced angiotensin II and also theoretically to greater NO production, less NO destruction, and increased NO bioavailability.

Both hypovitaminosis D and SHPT are highly prevalent in obese African-Americans [6] in comparison to their white counterparts [7]. Obesity, which disproportionately affects African-Americans, has been linked to endothelial dysfunction, oxidative stress, and inflammatory stress [9, 26]. Adipose tissue is a potential source of NO production given the findings of endothelial and inducible NOS in adipose tissue [27]. Although obesity has been shown to increase NO production [28], the NO bioavailability may be depressed secondary to the presence of low vitamin D levels. Elevated PTH as a consequence of depressed vitamin D may contribute to endothelial dysfunction by augmenting oxidative stress.
Fig. 1. Proposed conceptual model linking low 25-OH D and raised PTH to greater oxidative stress and lower NO bioavailability [20].

a Dark skin and adipocytes decrease vitamin D_3_. Low 25-OH D is initially associated with low 1,25-(OH)_2 D decreasing serum ionized calcium (Ca^{2+}), which consequently increases PTH release.

b Raised PTH activates renal 1α-hydroxylation of 25-OH D to produce 1,25-(OH)_2 D in the kidneys, RAS, and intracellular calcium overload [12, 13, 21, 25]. The paradoxically increased circulating 1,25-(OH)_2 D augments intracellular calcium in adipocytes by activating the putative membrane vitamin D receptor (MARRS) [21]. Angiotensin II and calcium influx generate reactive oxygen species (ROS) by activating nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, myeloperoxidase, and protein kinase C that stabilizes NADPH oxidase [22]. 1,25-(OH)_2 D binds to nuclear VDR downregulating uncoupling protein 2 (UCP2) activity, which decreases ROS clearance [21].

c Raised superoxide radicals (O_2^–) consume NO producing peroxynitrite (ONOO^–), which in turn causes eNOS uncoupling [24]. O_2^– also promotes inflammation via overwhelming superoxide dismutase to release hydrogen peroxide (H_2O_2) that activates nuclear factor kappa beta (NFκB) enhancing ONOO^– [23]. Overall, inflammatory stress and oxidative stress generate lipid peroxidation, such as isoprostanes, leading to NO destruction and, as a consequence, decreased NO bioavailability.
**Limitations**

Our study was cross sectional and therefore the direction of causality could not be established. We enrolled a relatively modest sample size and therefore may have inadequate power to detect important associations. The majority, though not all, of our participants were women. We did not determine the menopausal state of women, which may have an effect on circulating 25-OH D levels. Despite controlling diet, the UNOx is a crude measurement of NO from various sources including endothelial NO production. Also, we did not index UNOx to urinary creatinine. The lack of indexing UNOx to creatinine may diminish our ability to correct for errors in the absolute time of collection. However, such timing errors would unlikely occur differentially across vitamin D/PTH groups and when they did occur would likely bias contrasts across these groups to the null.

**Conclusion**

Our cross-sectional study supports a direct association between 25-OH D and UNOx and suggests a positive association between PTH and isoprostanes in normotensive, overweight African-Americans. Our findings also suggest that the combined low 25-OH D and high PTH is linked to lower UNOx. Future studies are warranted to assess whether raising 25-OH D levels via supplementation, and thus suppressing PTH levels, will attenuate oxidative stress augmenting NO production in African-Americans. These data may be of particular relevance in African-Americans who manifest a high prevalence of vitamin D deficiency and depressed NO vascular effectiveness that plausibly contribute to elevated BP.

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**Disclosure Statement**

None of the authors had a conflict of interest.

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