NEWS & VIEWS

In African American Type 2 Diabetic Patients, Is Vitamin D Deficiency Associated with Lower Blood Levels of Hydrogen Sulfide and Cyclic Adenosine Monophosphate, and Elevated Oxidative Stress?

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Abstract

African Americans (AA) have a higher incidence of cardiovascular disease and vitamin D (VD) deficiency compared with Caucasians. Hydrogen sulfide (H_2S) is an important signaling molecule. This study examined the hypothesis that blood levels of H_2S are lower in AA type 2 diabetic patients (T2D). Fasting blood was obtained from T2D and healthy controls. Results showed a significant decrease in plasma levels of cyclic adenosine monophosphate (cAMP) and H_2S in AA T2D but not in Caucasian T2D when compared with those of respective age- and race-matched healthy controls. Plasma VD levels were significantly lower in AA T2D compared with Caucasian T2D. Cell culture studies demonstrate that $1,25(\text{OH})_2\text{-VD}$ supplementation significantly increased expression of cystathionine- γ -lyase (CSE), H_2S formation, and cAMP secretion, but decreased reactive oxygen species in high glucose-treated U937 monocytes. This suggests that VD supplementation upregulates CSE and H_2S formation and decreases oxidative stress, and that VD deficiency may contribute to the malfunctioning of H_2S signaling and thus a higher incidence of vascular inflammation in AA. These results lead to the hypothesis that VD supplementation can replenish blood concentrations of H_2S and cAMP and lower oxidative stress and cardiovascular disease in AA T2D. *Antioxid. Redox Signal.* 18, 1154–1158.

Introduction

HYPERTENSION, DIABETES, AND cardiovascular morbidity and mortality are more prevalent in African Americans (AA) than in Americans of European descent (Caucasians) (2). AA also experience much higher rates of vitamin D (VD) deficiency (2). Epidemiological studies have provided evidence of a relationship between VD deficiency and an increased risk of chronic metabolic and cardiovascular diseases in humans (1, 6). However, a scientific explanation for the beneficial role of VD supplementation in the prevention and treatment of chronic non-skeletal diseases is lacking (6).

Hydrogen sulfide (H_2S) is an important signaling molecule (3, 4, 7–9). H_2S is produced *in vivo* from L-cysteine by the action of two main enzymes, cystathionine β -synthase (CBS) and cystathionine- γ -lyase (CSE) (4, 7, 8). CSE is mainly expressed in the thoracic aorta, portal vein, ileum, heart, liver, kidney, and vascular smooth muscle, whereas CBS is highly expressed in the central and peripheral nervous systems (4). CSE is a major H_2S -producing enzyme in the cardiovascular

system (8). Genetic deletion of the CSE enzyme in mice markedly reduces H_2S levels in the serum and other tissues, and mice lacking CSE display pronounced hypertension and diminished endothelium-dependent vasodilation (8). Further, exogenous H_2S supplementation reduces blood pressure and prevents progression of diabetic nephropathy in spontaneous hypertensive rats and reduces atherosclerotic plaque size in apolipoprotein E knockout mice compared with those of controls (7, 8). Several recent reviews discuss the potential benefits of H_2S in biological systems and its potential role in certain disease processes, such as the physiological effects mediated by H_2S in the protection of mitochondrial function and cardiovascular pathophysiology (4, 5). H_2S inhibits oxidative stress, decreases pro-inflammatory cytokines, and slows the progression of atherosclerosis (4, 7–9).

In this study, we report that blood levels of H_2S and cyclic adenosine monophosphate (cAMP) are lower, and those of oxidative stress higher, in AA type 2 diabetic patients (T2D) but not in Caucasians T2D in comparison to those of respective age- and race-matched healthy subjects. Plasma VD levels

Innovation

Vitamin D (VD) deficiency and cardiovascular disease are more prevalent in African Americans (AA) than in Caucasians. This study reports for the first time that blood levels of the anti-inflammatory biomarkers hydrogen sulfide (H_2S) and cyclic adenosine monophosphate (cAMP) are significantly lower and oxidative stress higher in AA diabetic patients, but not in Caucasian diabetics, compared with age- and race-matched healthy subjects. VD supplementation causes cystathionine- γ -lyase upregulation, H_2S formation, and cAMP secretion, and it decreases reactive oxygen species in high glucose-treated monocytes. This study provides a scientific explanation for the health benefits of VD and a novel link between impaired H_2S signaling and the VD deficiency in AA.

were significantly lower in AA T2D in comparison to Caucasian T2D. Cell culture studies demonstrated that VD supplementation increased expression of CSE, H_2S formation, and cAMP secretion, but it decreased reactive oxygen species (ROS) formation in U937 monocytes. These results suggest that VD upregulated CSE and H_2S formation, and that its deficiency may contribute to the lower blood levels of cAMP and H_2S , and higher risk of vascular inflammation, associated with AA compared with Caucasians.

Results, Discussion, and Future Directions

The ages (mean $\pm$ standard error [SE]) among AA T2D (46 ± 1.3 years, $n=52$) and healthy subjects (42 ± 3 years, $n=18$) and Caucasian T2D (49.9 ± 2 years, $n=24$) and healthy subjects (43.4 ± 3 years, $n=18$) were similar. The body mass index (mean $\pm$ SE, kg/m^2) was significantly higher ($p < 0.03$) in T2D subjects than in respective healthy subjects in both AA (37 ± 1.2 vs. 31 ± 2) and Caucasians (36.5 ± 2.7 vs. 29 ± 1.4). Fasting blood glucose levels (mean $\pm$ SE, mg/dl) were higher ($p < 0.02$) in T2D subjects than in respective healthy subjects in both AA (140 ± 9 vs. 101 ± 6) and Caucasians (128 ± 9 vs. 86 ± 4). Glycated hemoglobin A_{1C} (HbA $_{1C}$) (%) (mean $\pm$ SE) were higher ($p < 0.01$) in T2D subjects than in respective healthy subjects in both AA (8.13 ± 0.3 vs. 5.62 ± 0.33) and Caucasians (7.12 ± 0.2 vs. 4.8 ± 0.2). Blood levels of VD (mean $\pm$ SE, nM) were significantly lower ($p < 0.04$) in AA T2D (13.5 ± 0.9) compared with those of Caucasian T2D (17.8 ± 2.2).

Figure 1 illustrates blood levels of H_2S (Fig. 1A), protein oxidation (Fig. 1B), and cAMP (Fig. 1C) in AA and Caucasian T2D. Blood levels of H_2S and cAMP are significantly lower in AA T2D but not in Caucasian T2D patients compared with those of respective age- and race-matched healthy controls. The level of oxidative stress (protein oxidation) was significantly elevated in AA T2D but not in Caucasian T2D (Fig. 1). This is the first report to demonstrate that AA with T2D have lower levels of circulating H_2S . This leads to the question whether VD deficiency is linked to lower H_2S levels in AA T2D, and ultimately to the question whether VD supplementation increases H_2S formation and lowers oxidative stress.

Figure 2 illustrates that 1,25(OH) $_2$ VD supplementation upregulates CSE expression (Fig. 2A), CSE/actin ratio (Fig. 2B), and H_2S formation (Fig. 2C) in monocytes exposed to high glucose (HG) concentrations. This demonstrates for the

FIG. 1. Lower H_2S and cAMP, and elevated protein oxidation levels in blood of African American type 2 diabetic patients. Blood levels of H_2S (A), protein oxidation (B), and cAMP (C) in AA T2D ($n=52$) and Caucasian American T2D ($n=24$) and respective age-matched healthy AA ($n=18$) and Caucasian ($n=18$) subjects. Values are mean $\pm$ SE. AA, African Americans; cAMP, cyclic adenosine monophosphate; H_2S , hydrogen sulfide; NS, not significant; SE, standard error; T2D, type 2 diabetic patients.

first time that VD supplementation increases H_2S formation presumably by upregulating CSE. Figure 3 shows that HG increased ROS formation (Fig. 3A) and decreased cAMP (Fig. 3B). Supplementation with VD caused a significant reduction in ROS and increase in cAMP secretion in HG-treated monocytes. Mannitol used as an osmolarity control showed no effect on CSE expression, H_2S formation, cAMP secretion, or ROS in monocytes (data not shown here). We focused our initial studies on CSE because this is a major source of H_2S in the vasculature. VD treatment had no effect on cell viability.

FIG. 2. Vitamin D upregulates CSE and H₂S formation. Effect of 1,25(OH)₂ VD supplementation on CSE expression Western blot (A), the CSE/actin ratio (B), and H₂S formation (C) in U937 monocytes treated with and without HG. Cells were pretreated with 1,25(OH)₂ VD for 24 h and subsequently with HG for another 24 h. Values are mean ± SE (*n* = 3). CSE, cystathionine-γ-lyase; HG, high glucose; VD, vitamin D.

Many studies report that VD has important health benefits through paracrine and autocrine mechanisms and that higher blood 25-OH-VD levels are associated with better health outcomes (6). Darker skin pigmentation is a factor known to impair the body's ability to synthesize adequate amounts of VD, which may explain the higher incidence of VD deficiency in AA (6). Circulating 25(OH) VD has a half-life of 2–3 weeks and is considered a reliable and stable indicator of the VD status in the body (6). 1,25(OH)₂ is the biologically active form of VD (5). The well established systemic effects of VD are the maintenance of serum calcium and phosphate homeostasis *via* the control of intestinal absorption of calcium, renal resorption of phosphate, and the release of calcium from the skeleton (6). However, a scientific explanation for the beneficial role of VD supplementation in the prevention and treatment of chronic non-skeletal diseases, such as hypertension and diabetes, remains to be determined.

This study demonstrates that the blood levels of H₂S and cAMP were significantly lower and those of protein oxidation significantly higher in AA T2D but not in Caucasian T2D compared with those of age- and race-matched healthy controls. This suggests that AA T2D are at greater risk of lower

FIG. 3. Vitamin D decreases oxidative stress and increases cAMP. Effect of 1,25(OH)₂ VD supplementation on ROS formation (A) and cAMP secretion (B) in U937 monocytes treated with and without HG. Cells were pretreated with 1,25(OH)₂ VD for 24 h and subsequently with HG for another 24 h. Values are mean ± SE (*n* = 3). ROS, reactive oxygen species.

circulating level of H₂S. This led us to question whether VD deficiency is linked to lower H₂S levels in AA T2D. Interestingly, supplementation with 1,25(OH)₂ VD caused the upregulation of CSE and H₂S formation in monocytes exposed to HG. This suggests that VD deficiency, which is more prevalent in AA, plays a role in the lower H₂S formation in AA.

H₂S is an important signaling molecule whose blood levels have been shown to be lower in various diseases associated with a higher incidence of vascular inflammation, such as those in hypertensive animals and patients, diabetic animals and patients, and asthmatic patients (3, 7). Several studies demonstrate that H₂S is an antioxidant that can regenerate glutathione (GSH) and lower oxidative stress (4). Previous studies demonstrate that supplementation with an exogenous H₂S donor decreases secretion of the pro-inflammatory biomarkers IL-8, MCP-1, TNF-α, and ROS (3). In addition, it increases anti-inflammatory biomarkers GSH and cAMP formation in cell culture studies, and it reduces the progression of endothelial dysfunction and atherosclerosis in animal studies (3, 4). Increasing evidence indicates that abnormal H₂S homeostasis can contribute to the pathogenesis of vascular inflammation and atherosclerosis.

Hyperglycemia in diabetes increases oxidative stress. VD deficiency further adds to the oxidative insult and increases susceptibility to oxidative stress by lowering cAMP and H₂S levels in the AA population. Oxidative stress associated with diabetes increases nuclear factor-κB (NF-κB), which then can activate pro-inflammatory cytokines. Some of the effects of VD may be mediated through formation of H₂S and inhibition of oxidative stress and others may be independent of H₂S formation. Oxidative stress is known to decrease cAMP levels,

whereas H₂S can increase cellular cAMP (4, 8). An increase in cAMP can upregulate AMP kinase and thereby increase glucose metabolism (5). This study provides evidence for a novel molecular mechanism by which VD upregulates CSE and H₂S formation, while its deficiency contributes to lower blood levels of cAMP and H₂S, and a higher risk of vascular inflammation in AA compared with Caucasians.

The lower levels of circulating H₂S in AA diabetic patients could represent a novel mechanism responsible for the higher incidence of diabetes-associated vascular complications seen in this population. This study reports a unique link between VD, CSE activation, and H₂S formation. Cell culture studies demonstrate that VD activates enzymes that catalyze H₂S formation. Future clinical trials are needed to investigate whether supplementation with VD replenishes blood concentrations of H₂S and cAMP and lowers oxidative stress and vascular inflammation in the AA T2D population.

Notes

Patient enrollment and blood collection

Informed written consent was obtained from all patients according to the protocol approved by the Louisiana State University Health Sciences Center (LSUHSC) Institutional Review Board. All patients included in this study were adult T2D attending clinics at LSUHSC. Patients were excluded if they had any history of cardiovascular disease, sickle cell disease, treatment with insulin, or metabolic disorders, including uncontrolled hypertension, hypothyroidism, or hyperthyroidism. Patients were excluded if they showed signs of significant hepatic dysfunction, defined as any underlying chronic liver disease or liver function tests greater than 1.5 times the upper limit of normal or renal dysfunction, defined as a serum creatinine greater than 1.5 mg/dl. Women with a positive pregnancy test or those nursing infants were also excluded. Subjects who were taking any supplemental vitamins or herbal products were not included in this study. All patients who gave written informed consent were invited to return to have blood drawn after an overnight fast (8 h). Following blood collection, serum tubes for chemistry profile, ethylenediaminetetraacetic acid (EDTA) tubes for HbA_{1c}, and complete blood counts were promptly delivered to the LSUHSC clinical laboratories. Additional tubes of EDTA-blood were brought to the research laboratory. Clear plasma was separated *via* centrifugation of blood at 3000 rpm (1500 g) for 15 min. Plasma samples were analyzed in duplicate for the biochemical parameters.

Human pro-monocytic cell line

The U937 monocyte cell line was obtained from American Type Culture Collection. These cells were maintained at 37°C in RPMI 1640 medium containing 7 mM glucose, 10% (v/v) heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 12 mM sodium carbonate, 12 mM HEPES, and 2 mM glutamine in a humidified atmosphere containing 5% (v/v) CO₂. For treatments, cells were washed once in plain RPMI 1640 before being suspended in fresh medium (complete) containing serum and other supplements (3).

Treatment with HG and VD

Cells were treated with normal glucose (7 mM) and HG (25 mM) with and without 1,25(OH) VD. In this study control

cells were exposed to media containing 7 mM glucose. In humans, glucose is continuously degraded and formed to maintain a 5 mM blood glucose level. However, in cell culture studies, we observed that incubating cells with media containing a 5 mM glucose concentration for 24 h caused a decrease in glucose concentration levels lower than 2 mM. In cell culture studies, glucose is metabolized but not replaced. Our experience has shown that a 7 mM glucose concentration does not lead to glucose deficiency at 24 h incubation. In HG studies, cells were exposed to a HG concentration of 25 mM. Many previous studies have reported that glucose concentrations as high as 50 mM have been found in the blood of patients with uncontrolled diabetes (3). It is true that blood glucose levels in patients are not likely to stay as high as 25 mM for 24 h. However, tissue damage in diabetic patients occurs over many years of countless hyperglycemic episodes. Thus, the glucose concentration of 25 mM used in this cell culture study does not seem unreasonable. Cells (one million/ml) were pretreated with different concentrations of VD for 24 h followed by HG exposure for the next 24 h. Mannitol was used as an osmolarity control. In the mannitol-treated group, cells were exposed to 18 mM mannitol since the media contains 7 mM glucose. Cell viability of treated monocytes was also determined using the Alamar Blue reduction bioassay (Alamar Biosciences). This method is based upon Alamar Blue dye reduction by live cells.

Detection of intracellular ROS level

Intracellular ROS levels were measured using the fluorescent dye H₂DCFDA (2', 7' dichlorofluorescein diacetate [DCFH-DA]). Briefly, after treatment, cells were washed once with phosphate-buffered saline (PBS) and then loaded with 5 µM H₂DCFDA in PBS with 4% FBS. The cells were incubated at 37°C for 30 min in the dark and subsequently washed with PBS, harvested in PBS with 0.5% Triton X-100, centrifuged at 12,000 g for 10 min at 37°C, and the supernatant collected. The intensity of DCF fluorescence in the supernatant was read at excitation and emission wavelengths of 488 and 530 nm, respectively, using a multidetection microplate reader (Synergy HT; Biotek). The change in intracellular ROS level was plotted as mean fluorescence intensity. The oxidative stress sensitive dye DCFH-DA passively diffuses through the cellular membrane. Intracellular esterase activity causes the formation of DCFH, a nonfluorescent compound, which emits fluorescence when it is oxidized to DCF.

VD, cAMP, protein oxidation, and H₂S assays

cAMP and 25 hydroxy VD levels were determined by the sandwich enzyme linked immunosorbent assay method using commercially available kits from Fisher Thermo Scientific Co. Protein oxidation was determined by assessing levels of carbonyl protein using an ELISA kit (ENZO Life Sciences, Inc.). All appropriate controls and standards as specified by the manufacturer's kit were used. Control samples were analyzed each time to check the variation from plate to plate on different days of analysis. H₂S concentrations in the fresh plasma or H₂S formation in cultured monocyte supernatant was determined following the method described earlier (5), which is based upon the formation of methylene blue. Briefly, 200 µl culture medium was mixed with 600 µl 1% (w/v) zinc acetate to trap H₂S during formation of zinc sulfide, followed by the addition of 400 µl N-dimethyl-*p*-phenylenediamine sulfate (20 mM in 7.2 M HCl) and

400 μ l FeCl₃ in (30 mM in 1.2 M HCl) to the test tube. After the reaction mixture was incubated in the dark at room temperature for 20 min, 250 μ l 10% (w/v) trichloroacetic acid was added to precipitate any protein that might be present in the culture media. Subsequently the reaction mixture was centrifuged at 10,000 g for 10 min. The absorbance of the resulting solution was determined at 670 nm with a microplate reader. All samples were assayed in duplicate, and the H₂S concentrations were calculated using standard Na₂S (0–62.5 μ M).

Immunoblotting

For Western blotting, treated cells were lysed in radio-immunoprecipitation assay buffer (50 mM Tris pH 8, 150 mM NaCl, 1% NP-40, 0.5% deoxycholic acid, and 0.1% sodium dodecylsulfate [SDS]) supplemented with protease and phosphatase inhibitors (1 mM phenylmethylsulfonyl fluoride, 5 μ g/ml leupeptin, 2 μ g/ml aprotinin, 1 mM EDTA, 10 mM NaF, and 1 mM NaVO₄). Lysates were cleared by centrifugation and total protein concentrations were determined by bicinchoninic acid assay (Pierce/Thermo Scientific). All samples, which contained approximately the same amount of protein (~20–40 μ g) were run on 8%–10% SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Membranes were blocked at room temperature for 2 h in blocking buffer containing 1% bovine serum albumin to prevent nonspecific binding and then incubated with anti-CSE (1:1000) primary antibodies at 4°C overnight. The membranes were washed in Tris-buffered saline and Tween-20 (50 mM Tris-HCl, pH 7.6, 150 mM NaCl, and 0.1% Tween 20) for 30 min and incubated with the appropriate horseradish peroxidase conjugated secondary antibody (1:5000 dilution) for 2 h at room temperature and developed using the ultrasensitive enhanced chemiluminescence substrate. The intensity of each immunoblotting band was measured using the histogram tool of Adobe Photoshop CS5.

All chemicals were purchased from Sigma Chemical Co. unless otherwise mentioned. CSE antibody was purchased from Abcam, Inc. Data from cell culture studies were statistically analyzed using one way analysis of variance with Sigma Stat statistical software (Jandel Scientific). When data passed a normality test, all groups were compared using the Student–Newman–Keuls method. A difference was considered significant at the $p < 0.05$ level.

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References

- Devaraj S, Yun JM, Duncan-Stanley CR, and Jialal I. Low vitamin D levels correlate with the proinflammatory state in type 1 diabetic subjects with and without microvascular complications. *Am J Clin Pathol* 135: 429–433, 2011.
- Garrett-Mayer E, Wagner CL, Hollis BW, Kindy MS, and Gattioni-Celli S. Vitamin D3 supplementation (4000 IU/d for 1 y) eliminates differences in circulating 25-hydroxyvitamin D between African American and white men. *Am J Clin Nutr* 96: 332–336, 2012.
- Jain SK, Bull R, Rains JL, Bass PF, Levine SN, Reddy S, McVie R, and Bocchini JA. Low levels of hydrogen sulfide in the blood of diabetic patients and streptozotocin-treated rats cause vascular inflammation. *Antioxid Redox Signal* 12: 1333–1337, 2010.
- Kimura H, Shibuya N, and Kimura Y. Hydrogen sulfide is a signaling molecule and a cytoprotectant. *Antioxid Redox Signal* 17: 45–57, 2012.
- Manna P and Jain SK. Hydrogen sulfide and L-cysteine increase phosphatidylinositol 3,4,5-trisphosphate (PIP3) and glucose utilization by inhibiting phosphatase and tensin homolog (PTEN) protein and activating phosphoinositide 3-kinase (PI3K)/serine/threonine protein kinase (AKT)/protein kinase C ζ /λ (PKC ζ /λ) in 3T3L1 adipocytes. *J Biol Chem* 286: 39848–39859, 2011.
- Rosen, CJ, Adams JS, Bikle DD, Black DM, Demay MB, Manson JE, Murad MH, and Kovacs CS. The nonskeletal effects of vitamin D: an endocrine society scientific statement. *Endocr Rev* 33: 456–492, 2012.
- Suzuki K, Olah G, Modis K, et al. Hydrogen sulfide replacement therapy protects the vascular endothelium in hyperglycemia by preserving mitochondrial function. *Proc Natl Acad Sci U S A* 108: 13829–13834, 2011.
- Wang R. Hydrogen sulfide: the third gasotransmitter in biology and medicine. *Antioxid Redox Signal* 12: 1061–1064, 2010.
- Whiteman M, Gooding KM, Whatmore JL, Ball CL, Mawson D, Skinner K, Tooke JE, and Shore AC. Adiposity is a major determinant of plasma levels of the novel vasodilator hydrogen sulfide. *Diabetologia* 53: 1722–1726, 2010.

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Abbreviations Used

AA	= African Americans
cAMP	= cyclic adenosine monophosphate
CBS	= cystathionine- β -synthase
CSE	= cystathionine- γ -lyase
DCFH-DA	= 2', 7' dichlorofluorescein diacetate
EDTA	= ethylenediaminetetraacetic acid
FBS	= fetal bovine serum
GSH	= glutathione
HbA _{1C}	= glycated hemoglobin A _{1C}
HG	= high glucose
H ₂ S	= hydrogen sulfide
NS	= not significant
PBS	= phosphate-buffered saline
ROS	= reactive oxygen species
SDS	= sodium dodecylsulfate
SE	= standard error
T2D	= type 2 diabetic patients
VD	= vitamin D