Tetrahydrobiopterin lowers muscle sympathetic nerve activity and improves augmentation index in patients with chronic kidney disease

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Park J, Liao P, Sher S, Lyles RH, Deveaux DD, Quyyumi AA. Tetrahydrobiopterin lowers muscle sympathetic nerve activity and improves augmentation index in patients with chronic kidney disease. Am J Physiol Regul Integr Comp Physiol 308: R208–R218, 2015; First published December 4, 2014; doi:10.1152/ajpregu.00409.2014.—Chronic kidney disease (CKD) is characterized by overactivation of the sympathetic nervous system (SNS) that contributes to cardiovascular risk. Decreased nitric oxide (NO) bioavailability is a major factor contributing to SNS overactivity in CKD, since reduced neuronal NO leads to increased central SNS activity. Tetrahydrobiopterin (BH4) is an essential cofactor for nitric oxide synthase that increases NO bioavailability in experimental models of CKD. We conducted a randomized, double-blinded, placebo-controlled trial testing the benefits of oral sapropterin dihydrochloride (6R-BH4, a synthetic form of BH4) in CKD. 36 patients with CKD and hypertension were randomized to 12 wk of 1) 200 mg 6R-BH4 twice daily + 1 mg folic acid once daily; vs. 2) placebo + folic acid. The primary endpoint was a change in resting muscle sympathetic nerve activity (MSNA). Secondary endpoints included arterial stiffness using pulse wave velocity (PWV) and augmentation index (AIx), endothelial function using brachial artery flow-mediated dilation and endothelial progenitor cells, endothelium-independent vasodilatation (EID), microalbuminuria, and blood pressure. We observed a significant reduction in MSNA after 12 wk of 6R-BH4 (−7.5 ± 2.1 bursts/min vs. +3.2 ± 1.3 bursts/min; P = 0.003). We also observed a significant improvement in AIx by −5.8 ± 2.0% vs. +1.8 ± 1.7% in the placebo group, P = 0.007. EID increased significantly (by +2.0 ± 0.59%; P = 0.004) in the 6R-BH4 group, but there was no change in endothelial function. There was a trend toward a reduction in diastolic blood pressure by −4 ± 3 mmHg at 12 wk with 6R-BH4 (P = 0.055). 6R-BH4 treatment may have beneficial effects on SNS activity and central pulse wave reflections in hypertensive patients with CKD.

sympathetic activity; autonomic function; blood pressure; nitric oxide; vascular stiffness

CHRONIC KIDNEY DISEASE (CKD) is characterized by chronic sympathetic nervous system (SNS) overactivation (14, 21, 45), which is a major contributing factor to increased cardiovascular (CV) risk in this population. SNS overactivity increases CV risk not only by increasing blood pressure (BP), but also by factors independent of increased BP, including vascular inflammation and hypertrophy (9, 55), arterial stiffness (19), arrhythmogenesis (60), insulin resistance (35), and progression of myocardial and renal sclerosis (4, 46).

Current therapeutic options to combat SNS overactivation include central (clonidine) and peripheral (β-blockers and α-blockers) sympatholytic medications. However, treatment with these agents in CKD patients is often complicated by adverse side effects, including hypotension, orthostasis, fatigue, and erectile dysfunction. In addition, peripheral sympatholytics cause a reflex increase in central sympathetic nerve activation (12, 22, 57), as evidenced by increased muscle sympathetic nerve activity (MSNA), which reflects central SNS activity. Therefore, these medications may have adverse or equivocal effects on long-term CV risk. Indeed, there is no evidence that β-blocker therapy improves mortality risk in CKD patients without heart disease (5, 7), and long-term β-blocker use is associated with development of insulin resistance and hyperlipidemia (6, 12). Therefore, there is a need to investigate adjunctive or alternative therapies that safely ameliorate SNS overactivation in CKD.

One potential alternative strategy for reducing SNS activity is by increasing nitric oxide (NO) bioavailability. Neuronal NO has a tonic inhibitory effect on central sympathetic activation (8, 51). Therefore, a reduction in NO bioavailability leads to overactivation of the SNS when the sympathoinhibitory effect of NO is reduced; conversely, increasing NO bioavailability results in greater restraint of central SNS activity. CKD patients have significantly decreased NO bioavailability (68), and NO deficiency is a major contributing factor in the pathogenesis of chronic SNS overactivity in CKD (65). In addition, decreased NO bioavailability contributes to other CV risk factors in CKD, including endothelial dysfunction, arterial stiffness, and hypertension (11, 51). Thus, strategies to improve NO bioavailability in CKD have the potential to impact CV risk by reducing central SNS activation, as well as improving arterial compliance, endothelial function, and BP.

A novel therapeutic approach for improving NO bioavailability is via treatment with sapropterin dihydrochloride (6R-BH4, BioMarin). 6R-BH4 is the synthetic form of the naturally occurring enzyme tetrahydrobiopterin (BH4), an essential cofactor for nitric oxide synthase (NOS) in the catalytic reaction that forms NO (44, 52, 54). BH4 supplementation has been shown to increase NO bioavailability and improve BP and endothelial function in animal models of chronic renal failure (48, 49, 64); however, 6R-BH4 has never previously been tested in humans with kidney disease. In addition, the effects of 6R-BH4 supplementation on SNS activity are unknown. Therefore, we conducted a 12-wk randomized, placebo-controlled double-blinded clinical trial (NCT 1356966), testing the hypothesis that 6R-BH4 supplementation lowers SNS activity,
as well as improves arterial stiffness, endothelial function, and BP in patients with CKD.

Methods

Study Population

Forty-nine male veteran participants with hypertension and CKD Stage 2 or 3 were recruited and enrolled from outpatient clinics at the Atlanta Veterans Affairs (VA) Medical Center. Stage 3 CKD was defined as an estimated glomerular filtration rate (eGFR) (34) between 60 and 89 ml·min\(^{-1}\)-1·m\(^{-2}\), and Stage 2 CKD was defined as an eGFR between 80 and 69 ml·min\(^{-1}\)-1·m\(^{-2}\) with concomitant urinary microalbumin:creatinine ratio greater than 30 mg/g. Inclusion criteria included stable renal function defined as no greater than a 10% fluctuation in eGFR within the prior 3 mo and stable antihypertensive medication regimen for 3 mo prior to enrollment. Exclusion criteria included severe CKD (eGFR <30 cm\(^{-1}\)-1·min\(^{-1}\)), diabetes; HIV infection; clinical evidence of congestive heart failure or ejection fraction below 35%; any history of past coronary artery; cerebrovascular, aortic, or peripheral vascular disease; symptomatic heart disease determined by electrocardiogram, stress test, and/or history; hepatic enzyme concentrations greater than 2 times the upper limit of normal; severe anemia with hemoglobin level <10 g/dl; history of nephrolithiasis; any serious systemic disease that might influence survival; current treatment with clonidine, levodopa, or methotrexate; BP greater than 160/90 mmHg; BP less than 110/60; change in medications or surgery within the past 3 mo; drug or alcohol abuse; previous treatment or hypersensitivity to 6R-BH4.

Study Design

This was a prospective, randomized, placebo-controlled, double-blind clinical trial (clinicaltrials.gov identifier NCT1356966). After obtaining written informed consent, baseline MSNA, office and ambulatory BP, basic metabolic panel, and urinary microalbumin and creatinine were obtained. On a separate day, baseline brachial artery flow-mediated dilatation (FMD), endothelium-independent vasodilation (EID), pulse wave analysis, pulse wave velocity, and blood samples for progenitor cell (PC) were obtained. All measurements were obtained in a quiet, temperate (−21°C) environment, after abstaining from food, caffeine, smoking, and alcohol for at least 12 h, and exercise for at least 24 h. Participants in whom an adequate baseline MSNA neurogram was unable to be obtained, did not proceed to the clinical trial. After baseline assessments, participants were randomly assigned to receive 6R-BH4 200 mg twice daily with folic acid 1 mg daily, or an identical placebo twice daily with folic acid 1 mg daily. All participants were given folic acid because folic acid enhances the binding affinity of BH4 to NOS and also enhances the regeneration of BH4 from inactive BH2 (41). Participants were instructed to dissolve study pills in water or apple juice and to take with food. Interim visits were performed at weeks 1, 3, 6, 9, and 12, to assess compliance via pill count, assess for adverse effects, and measure BP and laboratory measures of serum electrolytes and kidney function. At the end of the trial, between weeks 9 and 12, baseline measurements were repeated under identical conditions. Participants were instructed not to change medication regimen, dietary, or exercise habits for the duration of the study. This study was approved by the Emory University Institutional Review Board, and the Atlanta VA Medical Center Research and Development Committee.

Measurements and Procedures

Blood pressure. Office BP was measured at baseline and at each follow-up visit after 5 min of rest in a seated position with the arm supported at heart level using an appropriately sized cuff. BP was measured with an automated device (Dinamap) using the oscillometric method. Each data point of BP was obtained as an average of three consecutive BP measurements separated by 5 min.

Muscle sympathetic nerve activity. Multiunit postganglionic MSNA was recorded directly from the peroneal nerve by microneurography, as previously described (58, 61). Participants were placed in a supine position, and the leg was positioned for microneurography. A tungsten microelectrode (tip diameter 5–15 μm) (Bioengineering, University of Iowa) was inserted into the nerve, and a reference microelectrode was inserted subcutaneously 1–2 cm from the recording electrode. The signals were amplified (total gain: 50,000–100,000), filtered (700-2,000 Hz), rectified, and integrated (time constant 0.1 s) to obtain a mean voltage display of sympathetic nerve activity (Nerve Traffic Analyzer, model 662C-4, University of Iowa, Bioengineering) that was recorded by the LabChart 7 Program (PowerLab 16sp, ADInstruments). Continuous ECG was recorded simultaneously with the neurogram using a bioamp system. Beat-to-beat arterial BP was measured concomitantly using a noninvasive monitoring device that detects digital blood flow via finger cuffs and transduces blood flow oscillations into continuous pulse waveforms and beat-to-beat values of BP (CNAP, CNSystems) (29, 30). Absolute values of BP were internally calibrated using a concomitant upper arm BP reading and were calibrated at the start and every 15 min throughout the study. The tungsten microelectrode was manipulated to obtain a satisfactory nerve recording that met previously established criteria (16, 17, 38). After 10 min of rest, baseline BP, heart rate, respiratory rate, and MSNA were recorded continuously for 10 min.

Arterial stiffness. Pulse wave analysis and PWV were assessed using ultrasound measurements of the radial, carotid, and femoral arteries, using the SphygmoCor Pulse Wave Velocity system (PWV Medical). In brief, peripheral pressure waveforms were recorded from the radial artery at the wrist, using applanation tonometry with a high-fidelity micromanometer. After 20 sequential waveforms were acquired, a validated generalized transfer function was used to generate the corresponding central aortic pressure waveform, and the degree of pressure augmentation due to the reflected wave form from the periphery was measured. Central AIx was calculated as the augmented pressure divided by the central pulse pressure from the central pressure waveform. Central AIx was also corrected for heart rate using a standardized value of 75 beats per min (bpm). Carotid-femoral artery PWV was determined by measuring transcutaneous Doppler flow velocity recordings carried out over the common carotid artery and the femoral artery. The distance between the recording sites was measured manually and divided by the time interval between the EKG R-waves from the recorded waveforms at each site, to arrive at a measurement of velocity (m/s).

Brachial artery flow-mediated dilatation. Participants were placed in a supine position, and a forearm occlusion cuff was placed around the forearm. For each measurement, baseline images of the brachial artery were obtained using an 13-MHz high-resolution ultrasound transducer (Acuson) longitudinally 2–10 cm above the antecubital fossa. Electrocardiogram gating was used to capture end-diastolic arterial diameters, and the average diameter and blood velocity for three cardiac cycles was recorded and analyzed for baseline values. The cuff was then rapidly inflated to suprasystolic levels (50 mmHg above systolic pressure) for 5 min (E-20 rapid cuff inflator; D. E. Hokanson) to create a flow stimulus in the brachial artery. Doppler ultrasound was used to measure peak hyperemic blood velocity during the first 10 s after cuff release, and then diameter measurements were captured at 60 and 90 s after cuff release. Calculations of FMD were based on the 60- and 90-s measurements, and at peak hyperemic diameters. Images were digitized, and arterial diameters were measured with customized software (Medical Imaging Applications). All measurements were obtained and analyzed by a single investigator (SS) blinded to the clinical status of the participant. FMD was expressed as the % change in artery diameter from baseline: (peak hyperemic diameter − baseline diameter)/baseline diameter. Shear rate was calculated as 4× peak blood velocity/arterial diameter, both at baseline and at peak hyperemia. FMD values were also normalized...
for peak hyperemic shear rate. After 20 min of rest to reestablish baseline conditions, EID was assessed by obtaining ultrasound images of the brachial artery just before, and 3–5 min after the administration of 0.4 mg of sublingual nitroglycerin. EIC is expressed as the % change in artery diameter from baseline.

**Progenitor cells.** Circulating progenitor-enriched populations were estimated by measuring the expression of surface antigens using direct flow cytometry analysis (BD FACSCanto II flow cytometer), as previously described (3). Approximately 300 ml of venous blood (anticoagulant: EDTA) was incubated with fluorochrome-labeled monoclonal mouse anti-human antibodies, namely, FITC-CD34 (BD Biosciences), PerCP-CD133 (BD Biosciences), PE-VEGF2R (R&D system also known as “Kinase insert domain receptor-KDR”), and APC-CD133 (Miltenyi) for 15 min. Red blood cells were removed by lysis in 1.5 ml of ammonium chloride lysis buffer, which was added to the sample and incubated for an additional 10 min. The lysis process was stopped by adding 1.5 ml of staining medium (PBS with 3% heat-inactivated serum and 0.1% sodium azide). Five million events were acquired from the cytometer with FlowJo software (TreeStar) used for subsequent analysis of accumulated data. Absolute cell counts for target cell subsets were determined using an indirect cell estimation method. The total absolute white cell count and absolute counts for lymphocytes, monocytes, and granulocytes were determined using a Coulter ACT/Diff cell counter (Beckman Coulter). The absolute mononuclear cell count was then estimated as the sum of lymphocytes and monocytes. The frequency of our target cell populations was reported as a subset of the mononuclear cell compartment. The frequency of our target cell population represents hematopoietic progenitors from which subsequent lineages, including endothelial progenitors, are derived. The CD34<sup>+</sup> population represents hematopoietic progenitors from which subsequent lineages, including endothelial progenitors, are derived. The CD34<sup>+</sup> subset of CD34<sup>+</sup> cells are believed to include immature or early progenitors as the marker subsequently disappears as they mature, whereas CD34 positivity with VEGF2R markers is widely considered to be a population enriched for endothelial progenitors. Thus, we examined the frequency in the peripheral circulation of CD34<sup>+</sup>, dual-positive CD34<sup>+</sup>/CD133<sup>+</sup>, dual-positive CD34<sup>+</sup>/VEGF2R<sup>+</sup>, and triple-positive CD34<sup>+</sup>/CD133<sup>+</sup>/VEGF2R<sup>+</sup> cell populations among the CD45<sup>+</sup> population. Samples were repeatedly analyzed on two occasions by two technicians.

**Study Endpoints**

The primary endpoint was the change in MSNA from baseline to end-of-study. Secondary outcomes included central AIx, PWV, brachial artery FMD, EID, endothelial PC counts, BP measurements at interval visits, and microalbuminuria.

**Data Analysis**

*Muscle sympathetic nerve activity.** MSNA and ECG data were exported from the LabChart data acquisition system to WinCPRS (Absolute Aliens, Turku, Finland) for analysis. R-waves were detected and marked from the continuous ECG recording. MSNA bursts were automatically detected by the program using the following criteria: 3:1 burst-to-noise ratio within a 0.5-s search window, with an average latency in burst occurrence of 1.2–1.4 s from the previous R-wave. After automatic detection, the ECG and MSNA neurograms were visually inspected for accuracy of detection by a single investigator (J. Park). MSNA was expressed as burst frequency (bursts/min), and burst incidence (bursts/100 heartbeats).

**Statistical Analysis**

Statistical analysis was performed using the R Language 3.0.1. Unless otherwise noted, within-group changes in variables of interest are reported in terms of means ± SE. Within-group changes were assessed via nonparametric Wilcoxon signed rank tests, while two-group Wilcoxon rank sum tests were conducted to assess whether median within-group changes differed between the 6R-BH4 and placebo groups. For the primary outcome variable (change in MSNA from baseline to 12 wk), multiple linear regression was used to determine whether a significant unadjusted two-group comparison was maintained after controlling for age and body mass index (BMI) as covariates. For outcome variables that displayed no difference in within-group changes from baseline to 12 wk, mixed linear models were also applied to estimate trends over time within groups and to compare these trends across groups.

**RESULTS**

**Study Enrollment and Baseline Characteristics**

Among the 49 patients who met the eligibility criteria and were enrolled, 32 were ultimately randomized to receive 6R-BH4 (n = 18) vs. placebo (n = 14) (Fig. 1). All participants also received folic acid 1 mg daily. Table 1 depicts baseline and clinical characteristics of the study population. All participants were male and African-American, except for one Caucasian in the 6R-BH4 group, and one Caucasian in the placebo group. Mean serum creatinine and estimated glomerular filtration rate (eGFR) were similar between groups, and the cause of CKD was hypertension in the majority of both groups. The mean number of antihypertensive medications was 2.2 among the 6R-BH4 group, and 2.4 among the placebo group, and the majority of both groups were treated with calcium channel blockers and angiotensin-converting enzyme inhibitors or angiotensin receptor blockers (ACE/ARB). Baseline systolic BP was significantly lower in the 6R-BH4 group by ~10 mmHg compared with the placebo group, and the baseline serum potassium was slightly lower in the placebo group. All other baseline hemodynamics, MSNA, measures of endothelial function, arterial stiffness, and oxidative stress, were similar between groups.

**Primary End Point**

The primary endpoint was the change in resting levels of MSNA assessed via direct peroneal nerve recordings of efferent sympathetic nerve discharge directed to the muscle using the microneurography technique. After 12 wk of treatment, there was a significant difference in change in resting MSNA between groups (P = 0.003; Fig. 2). Whereas MSNA decreased significantly by −7.5 ± 2.1 bursts/min; P = 0.007) in the 6R-BH4 group, there was no significant change in MSNA in the placebo group (+3.2 ± 1.3 bursts/min P = 0.175). Results were similar when analyzed as burst incidence (bursts/100 heartbeats). After adjustment for age and BMI, the difference remained significant (P = 0.032). Results were also similar in subgroup analyses among patients treated and not treated with ACE/ARB. 6R-BH4 treatment was associated with a significant reduction in MSNA in both patients treated with ACE/ARB and those not treated with ACE/ARB (data not shown).
Secondary End Points

Secondary end points included arterial stiffness measured by central AIX and PWV; endothelial function assessed by brachial artery flow-mediated dilatation (FMD) and endothelial PC; EID assessed via brachial artery vasodilatory response to nitroglycerin (NTG); microalbuminuria; and BP throughout the study period. There was a significant difference in change in arterial stiffness measured as central AIX, and central AIX corrected for heart rate (AIX at 75) between the groups (Fig. 3). Central AIX decreased in the 6R-BH4 group (by −5.8 ± 2.0% vs. +1.8 ± 1.7% in placebo group; P = 0.007), and similarly, central AIX at 75 decreased in the 6R-BH4 group (by −3.2 ± 2.1% vs. +1.1 ± 1.2%; P = 0.046). There was no significant difference in the change in PWV between the groups. (P = 0.53; Fig. 3A). Brachial artery diameters, peak blood velocities, and shear rates at baseline and peak hyperemia are given in Table 2. There was no significant difference in change in brachial artery FMD (P = 0.878) and FMD normalized for hyperemic shear rate (P = 0.439) between groups. In contrast, there was a significant increase in EID (by +2.0 ± 0.59%; P = 0.004) in the 6R-BH4 group, whereas no significant change was observed within the control group (Fig. 3B). Table 2 also gives central aortic pressures before and after treatment. There were no significant differences in change in central systolic, diastolic (CDP), and central mean pressure between the groups. There was a trend toward a reduction in CDP among the 6R-BH4 group compared with the placebo group, but this did not reach statistical significance (P = 0.055; Fig. 4B).

There were no significant differences in change in kidney function, microalbuminuria, or PC counts between groups (Table 3), although there was a small but significant decrease in serum potassium within the 6R-BH4 group. For variables that showed no significant within-group change, analyses based on mixed linear models utilizing outcome variable assessments at intervening time points yielded qualitatively similar conclusions to the simpler baseline vs. 12 wk comparisons (results not shown).

Adverse Events

Overall, 6R-BH4 was well tolerated with few adverse events. Among patients assigned to 6R-BH4, one patient withdrew from the study due to nausea and gastroesophageal reflux, which resolved after cessation of the study drug. Another patient was withdrawn by the PI for acute kidney injury (AKI) after week 3 of the study. The patient was noted to have an increase in serum creatinine from a baseline of 2.23 mg/dl to 2.75 mg/dl at the week 6 interim visit, which was subsequently repeated and confirmed. Concomitantly, he had a reduction in SBP from 141 mmHg at baseline to 113 mmHg, and DBP from 86 mmHg to 64 mmHg. The cause of AKI was deemed secondary to decreased renal perfusion from low BP. After cessation of the study drug, patient’s BP and renal function returned to baseline. Among patients assigned to placebo, one patient withdrew due to dysgeusia, and a second patient withdrew due to trauma unrelated to the study. An additional two patients from the treatment group and one patient from the placebo group were not included in the primary analysis because an adequate MSNA neurogram was unable to be obtained at the end of study.
DISCUSSION

This is the first investigation testing the effects of 6R-BH4 therapy on SNS activity in humans. In this randomized, double-blinded, placebo-controlled trial, we demonstrate that 12 wk of 6R-BH4 treatment significantly lowers resting MSNA in CKD patients. We also demonstrate that 6R-BH4 concomitantly and significantly improves arterial wave reflection and bioavailability, as characteristic of chronic renal failure, can lead to overactivation of sympathetic nerve activity. BH4 is a cofactor for NO function, essential for the pathway leading to hydroxylation of L-arginine, resulting in formation of L-citrulline and NO (44). When there is a relative deficiency of BH4, a state of “NOS uncoupling” from L-arginine occurs, in which NO production becomes inefficient, leading to a shift in balance, resulting in depletion of NO and a state of mitochondrial dysfunction (15).

Our findings demonstrate that 6R-BH4 may be one novel strategy for lowering SNS activity in humans with CKD. CKD is characterized by chronic SNS overactivation that contributes to and correlates with mortality risk in this population (69). Since SNS overactivity increases CV risk via multiple mechanisms both dependent and independent of BP (35, 55, 60), novel strategies for lowering sympathetic nerve activity could have clinical implications on long-term risk in these patients. The mechanisms by which 6R-BH4 lowers SNS activity in CKD patients are unclear, but increased neuronal NO likely plays a role. Neuronal NO has a tonic inhibitory effect on central SNS activation (8, 51), and therefore, decreased NO bioavailability, as characteristic of chronic renal failure, can lead to overactivation of sympathetic nerve activity. BH4 is a cofactor for NO function, essential for the pathway leading to hydroxylation of L-arginine, resulting in formation of L-citrulline and NO (44). When there is a relative deficiency of BH4, a state of “NOS uncoupling” from L-arginine occurs, in which superoxide is generated from the oxygenase domain of NOS rather than NO. Thus, in BH4-deficient states, NOS uncoupling leads to a shift in balance, resulting in depletion of NO and...
generation of oxidative stress. BH4 deficiency contributes to reduced NO bioavailability in a variety of diseases, including hypertension, diabetes, smoking, hyperlipidemia, atherosclerosis, aging, and chronic renal failure (44, 52, 54). Endogenous BH4 levels were significantly lower in humans with kidney disease (66), and BH4 treatment increased NO bioavailability in animal models of chronic renal failure. Together, these findings suggest that CKD patients have a relative deficiency in BH4 that leads to NOS uncoupling, decreased NO bioavailability, and subsequent overactivation of central SNS activity, which can be ameliorated with long-term supplementation of 6R-BH4. Further, our finding that 6R-BH4 reduces SNS activity but has no effect on endothelial function suggests that BH4 may have a relatively greater effect on neuronal (nNOS) than endothelial NOS (eNOS).

Concomitant with a reduction in SNS activity, we observed that 6R-BH4 treatment significantly improves central AIx, a measure of the augmentation of central aortic pressure by the reflected pulse wave. Although PWV, the gold-standard measurement of arterial stiffness, was not significantly altered by 12 wk of 6R-BH4 treatment, we observed a significant improvement in AIx, which is another validated measure of arterial stiffness (42). Increased arterial stiffness leads to an increase in the amplitude of the reflected pressure wave to the left ventricle (i.e., central AIx), leading to increased wall stress, elevated left ventricular afterload, and potentiation of atherosclerosis, thereby increasing CV risk (42). Indeed, AIx is an independent risk factor for early coronary atherosclerotic disease, CV events, and all-cause mortality (59, 63). In CKD, increased AIx independently predicts CV and all-cause mortality (36) and is associated with a more rapid progression of renal failure (62). Thus, our finding that 6R-BH4 treatment improves central AIx in CKD patients may have clinical implications that could impact long-term CV and renal outcomes.

Although PWV and AIx are both measures of arterial stiffness, we observed a reduction in central AIx that was independent of a change in PWV after treatment with 6R-BH4. PWV is largely reflective of stiffness of large arteries, whereas AIx may also be influenced by vascular tone of the small muscular arteries and arterioles (32). Prior studies have shown that NTG and ANG II (vasoactive substances that act predominantly to alter the tone of the small muscular arteries and arterioles, with little effect on the aorta) are capable of producing large changes in AIx without any effect on PWV. Indeed, sympathetic nerve activation may have greater effect on vascular tone of small arteries and arterioles, which may explain why 6R-BH4 treatment was associated with a significant reduction in sympathetic nerve activity with a concomitant reduction in AIx, but without a significant change in PWV.

One mechanism by which 6R-BH4 treatment may improve AIx is via reduction in sympathetic nerve activity, as demonstrated in this study. Prior studies have demonstrated a significant positive relationship between MSNA and AIx in normo-
The role of NO in modulation of arterial stiffness is unresolved. The reasons underlying the discrepancy in our findings, which demonstrate a lack of improvement in endothelial function with 6R-BH4 treatment compared with prior reports in other patient populations, are unclear, but we speculate on several possibilities. First, our study is the first to examine the effects of 6R-BH4 on endothelial function in CKD patients, who have vascular abnormalities that are distinct from other patient groups. In addition to intimal lesions, patients with reduced renal function are particularly prone to medial vascular calcification due to abnormalities in calcium and phosphorus metabolism, treatment with activated vitamin D, and deficiency of calcification inhibitors (18). In such a setting, 6R-BH4 may have less capacity to induce changes in vasodilatory response to endothelial NO. Second, we conducted a randomized double-blind placebo-controlled trial testing chronic effects after long-term (12 wk) oral supplementation, whereas most previous reports did not include a placebo group, were not blinded, or tested only acute effects after a single intravenous infusion of BH4. One prior study in hyperlipidemic patients (15) did utilize a randomized controlled design in a smaller number of patients with hypercholesterolemia (15). Acute intravenous infusions of 6R-BH4 improved endothelial function via increased NO bioavailability in patients with type 2 diabetes (26), coronary artery disease (37), and smokers (25).

The reasons underlying the discrepancy in our findings, which demonstrate a lack of improvement in endothelial function with 6R-BH4 treatment compared with prior reports in other patient populations, are unclear, but we speculate on several possibilities. First, our study is the first to examine the effects of 6R-BH4 on endothelial function in CKD patients, who have vascular abnormalities that are distinct from other patient groups. In addition to intimal lesions, patients with reduced renal function are particularly prone to medial vascular calcification due to abnormalities in calcium and phosphorus metabolism, treatment with activated vitamin D, and deficiency of calcification inhibitors (18). In such a setting, 6R-BH4 may have less capacity to induce changes in vasodilatory response to endothelial NO. Second, we conducted a randomized double-blind placebo-controlled trial testing chronic effects after long-term (12 wk) oral supplementation, whereas most previous reports did not include a placebo group, were not blinded, or tested only acute effects after a single intravenous infusion of BH4. One prior study in hyperlipidemic patients (15) did utilize a randomized controlled design in a smaller number of
Whether 6R-BH4 improves arterial baroreflex sensitivity remains unknown.

Although we observed no changes in NO-mediated vasodilation, we observed a small but significant improvement in EID with 6R-BH4. EID, or the vasodilatory response to NTG, is dependent on structural and functional properties of vascular smooth muscle cells, rather than on endothelium-derived NO (1). Increased SNS activation increases vascular smooth muscle cell contraction and restrains vasodilatory responses (24, 28, 40). As such, the decrease in sympathetic nerve activity concomitant with an improvement in EID observed with 6R-BH4 suggests that decreased sympathetic innervation may lead to a greater capacity for vasodilation in response to NTG when neurogenic constraint is reduced.

Despite a significant reduction in sympathetic activity, there were no significant differences in change in SBP or MAP with treatment between the groups; however, we did observe a trend toward a reduction in DBP ($P = 0.055$). A larger sample size may have detected significant reductions in DBP among the treatment group. In addition, the baseline BP was well controlled in the study population, making it less likely to observe significant changes in interval BP measurements, in contrast to prior studies that were conducted in patients with poorly controlled BP at baseline (50). Alternatively, the degree of inhibition of sympathetic nerve activity with this dose of 6R-BH4 may not have been sufficient to significantly affect the variability of BP under normal conditions. Adaptations such as altered vascular alpha adrenergic sensitivity or activation of the renin-angiotensin-aldosterone system may have also prevented a significant change in resting BP. Interestingly, one CKD patient for a shorter duration, but a larger dosage (400 mg twice daily) of BH4. Whether larger doses of 6R-BH4 might modulate endothelial function in CKD is unclear. Our finding that 6R-BH4 significantly lowers sympathetic nerve activity, but has no effect on endothelial function or microalbuminuria, suggests that 6R-BH4 treatment may have a relatively greater effect on neuronal NOS, compared with eNOS or renal NOS. Alternatively, rather than exerting direct central effects, 6R-BH4 may act peripherally to improve arterial baroreflex function, thereby reducing resting sympathetic nerve activity.

<table>
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<th>Table 3. Laboratory data</th>
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</tr>
<tr>
<td>Placebo group</td>
<td>$3.6 \pm 0.2$</td>
<td>$3.7 \pm 0.1$</td>
<td>0.682</td>
<td></td>
</tr>
<tr>
<td>Urinary microalbumin:creatinine, mg/g</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6R-BH4 group</td>
<td>$148 \pm 54$</td>
<td>$157 \pm 40$</td>
<td>0.348</td>
<td></td>
</tr>
<tr>
<td>Placebo group</td>
<td>$163 \pm 58$</td>
<td>$137 \pm 61$</td>
<td>0.278</td>
<td></td>
</tr>
<tr>
<td><strong>Progenitor Cells</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CD34$^+$, cells/μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6R-BH4 group</td>
<td>$4.17 \pm 0.94$</td>
<td>$3.94 \pm 0.71$</td>
<td>0.898</td>
<td></td>
</tr>
<tr>
<td>Placebo group</td>
<td>$3.07 \pm 0.71$</td>
<td>$3.09 \pm 0.63$</td>
<td>0.898</td>
<td></td>
</tr>
<tr>
<td>CD34$^+$/CD133$^+$, cells/μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6R-BH4 group</td>
<td>$2.00 \pm 0.40$</td>
<td>$1.97 \pm 0.39$</td>
<td>0.966</td>
<td></td>
</tr>
<tr>
<td>Placebo group</td>
<td>$1.74 \pm 0.44$</td>
<td>$1.64 \pm 0.38$</td>
<td>0.831</td>
<td></td>
</tr>
<tr>
<td>CD34$^+$/VEGF2R$^+$, cells/μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6R-BH4 group</td>
<td>$0.05 \pm 0.01$</td>
<td>$0.01 \pm 0.006$</td>
<td>0.813</td>
<td></td>
</tr>
<tr>
<td>Placebo group</td>
<td>$0.04 \pm 0.009$</td>
<td>$0.03 \pm 0.006$</td>
<td>0.759</td>
<td></td>
</tr>
<tr>
<td>CD34$^+$/CD133$^+$/VEGF2R$^+$, cells/μl</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6R-BH4 group</td>
<td>$0.01 \pm 0.006$</td>
<td>$0.01 \pm 0.004$</td>
<td>0.389</td>
<td></td>
</tr>
<tr>
<td>Placebo group</td>
<td>$0.01 \pm 0.004$</td>
<td>$0.02 \pm 0.004$</td>
<td>0.344</td>
<td></td>
</tr>
</tbody>
</table>

Values are expressed as the means ± SE. VEGF2R, vascular endothelial growth factor 2 receptor. A significant $P$ value is shown in bold.
patient randomized to 6R-BH4 was withdrawn from the study after experiencing hemodynamically significant reduction in BP, which resolved after cessation of the study drug, suggesting that 6R-BH4 may have a BP-lowering effect in some CKD patients.

We acknowledge several limitations. First, the study population was composed of only men, without diabetes, and primarily African-American, which may limit generalizability. Whether 6R-BH4 improves SNS overactivity in a more typical population with CKD, including women, diabetics, and other racial groups, is unknown. Secondly, the study population had well-controlled BP and low levels of microalbuminuria at baseline. Whether 6R-BH4 treatment might have greater effects on BP and proteinuria in CKD patients with higher levels of these parameters at baseline is unknown, but likely. Similarly, patients were on multiple antihypertensive medications that were not discontinued for participation in the study. There were no significant differences in antihypertensive regimen between the treatment and control groups, and participants were carefully followed and monitored to ensure that no adjustments of BP medications occurred during the study period. However, the majority of patients in both groups were treated with inhibitors of the renin-angiotensin system, which have been shown to improve endothelial function and reduce SNS activity (33). Therefore, 6R-BH4 treatment may have less of an effect on these parameters in patients already treated with these agents. In addition, only one dose (200 mg twice per day) of 6R-BH4 was tested. This dosage was chosen on the basis of a prior study in hypertensive patients that reported that a dosage of 200 mg twice daily of 6R-BH4 led to significant improvements in BP and endothelial function (50). In the same study, a smaller total daily dosage of 200 mg did not result in improved BP or endothelial function. However, even higher doses (400 mg twice per day) were used in a different study in hyperlipidemic patients that demonstrated improvements in endothelial function and inflammatory biomarkers (15) and whether doubling the dose might have greater effects on endothelial function, BP, and oxidative stress in CKD patients remains unknown. Lastly, FMD was determined by measuring brachial artery diameter intermittently, rather than continuously, after cuff release; therefore, peak hyperemia may have occurred at a time point that was not captured in this technique. Although FMD measurements were performed uniformly in all subjects across time by a single investigator, continuous measurements of arterial diameter may have improved the detection of peak hyperemia.

In conclusion, this study demonstrates that 6R-BH4 significantly lowers sympathetic activity and improves AIx and EID in patients with CKD Stage II and III. We also observed a trend toward a reduction in DBP with 6R-BH4 treatment. The treatment was safe and well tolerated, with few adverse effects. These findings warrant larger studies testing the potential benefits of 6R-BH4 treatment on long-term CV and renal outcomes in patients with CKD.

ACKNOWLEDGMENTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS


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