Vascular Dysfunction, Oxidative Stress, and Inflammation in Chronic Kidney Disease

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Abstract

**Background:** Increased arterial stiffness and vascular endothelial dysfunction are important non-traditional cardiovascular risk factors evident in patients with chronic kidney disease (CKD). Vascular oxidative stress and inflammation may contribute to vascular dysfunction in CKD, but direct evidence is lacking.

**Methods:** We assessed carotid-femoral pulse-wave velocity (arterial stiffness) and brachial artery flow-mediated dilation (vascular endothelial function) in participants with moderate-to-severe CKD (estimated glomerular filtration rate 15-59 ml/min/1.73 m²) and in healthy controls. Change in brachial artery flow-mediated dilation following an acute infusion of ascorbic acid to inhibit vascular oxidative stress (vs. saline) was also measured. Protein expression of vascular endothelial cells collected from a peripheral vein and enzyme-linked immunosorbent assays to assess circulating markers were also performed.

**Results:** Sixty-four CKD participants (65±8 years [mean±S.D.]) and 17 healthy controls (60±5 years) were included. Carotid-femoral pulse-wave velocity was greater in CKD participants compared to healthy controls (1071±336 vs. 732±128 cm/sec; p<0.001). Brachial artery flow-mediated dilation was lower in CKD participants compared to healthy controls (3.5±2.8% vs. 5.5±3.2%; p=0.02). Circulating inflammation markers (C-reactive protein and interleukin-6) were elevated in the CKD group (p≤0.02).

Endothelial cell protein expression of NADPH (1.48±0.28 vs. 1.25±0.31 [intensity vs. human umbilical vein endothelial cell control]; p=0.05) was greater in CKD participants. However, ascorbic acid significantly improved brachial artery flow-mediated dilation in
control participants (saline: 5.5±3.2; ascorbic acid: 6.8±3.6); as compared to CKD participants (saline: 3.5±2.8; ascorbic acid: 3.6±3.2) (p-interaction [group*condition] = 0.04), suggesting vascular oxidative stress could not be overcome with ascorbic acid in CKD participants.

**Conclusions:** Vascular oxidative stress is present in CKD, which cannot be overcome with acute infusion of ascorbic acid.
Introduction

Patients with chronic kidney disease (CKD) are more likely to die of cardiovascular disease (CVD) than to progress to end-stage kidney disease,(1) and the risk of cardiovascular mortality or a cardiovascular event is significantly increased compared to the general population.(2) However, while patients with CKD exhibit a high presence of traditional CVD risk factors, they only partially explain the increased incidence of CVD in this population.(3, 4)

Patients with CKD exhibit vascular endothelial dysfunction (impaired endothelium-dependent dilation, commonly assessed as brachial artery flow-mediated dilation [FMDBA])(5-7) and increased arterial stiffness (commonly assessed as carotid-femoral pulse-wave velocity [PWV]),(8-10) as well as chronic oxidative stress and inflammation.(11, 12) Oxidative stress and inflammation are important non-traditional risk factors for CVD(4) and may contribute to the development of vascular dysfunction; however, the mechanisms contributing to vascular dysfunction in CKD are incompletely understood.

Circulating markers of oxidative stress are associated with endothelial dysfunction in patients with CKD,(13) and evidence suggests that oxidative stress may contribute to cutaneous microvascular dysfunction in patients with stage 3-4 CKD.(14) However, the role of vascular oxidative stress in large conduit arteries is currently unclear.(5, 15, 16) It is plausible but currently unknown if local vascular endothelial oxidative stress and inflammation are increased in CKD.
We sought to compare vascular function and measures of vascular oxidative stress and inflammation in a group of participants with moderate-to-severe CKD and a group of age-matched healthy controls. We employed novel methods to assess FMD_{BA} during normal versus inhibited oxidative stress (via an acute supraphysiological infusion of ascorbic acid) and by measuring expression of proteins involved in oxidative stress and inflammation in endothelial cells collected from participants. We hypothesized that CKD participants would exhibit increased vascular oxidative stress and inflammation in conjunction with vascular dysfunction.

**Materials and Methods**

**Study Design and Participants**

This was a cross-sectional study assessing mechanisms of vascular dysfunction in adults with moderate-to-severe CKD as compared to age-matched healthy controls. CKD patients had participated in one of two randomized, placebo controlled trials: administration of an interleukin-1 inhibitor (rilonacept; n=10)(17) (trial 1) or lanthanum carbonate (NCT02209636; n=54; trial 2). Included data were collected at baseline. Trial 1 enrolled between September 2012 and September 2014 and trial 2 enrolled between September 2014 and December 2018. Healthy controls were prospectively recruited through advertisements at the University and in the community, with enrollment between December 2015 and November 2018. The study was conducted at the University of Colorado Anschutz Medical Campus Division of Renal Diseases and Hypertension Clinical Vascular Physiology.
Laboratory. Analysts were blinded to status (CKD or healthy control) when assessing outcome measures (vascular function and circulating/cellular markers).

All CKD participants in either clinical trial who had successful baseline mechanistic vascular measurements (i.e., change in FMD$_{BA}$ with acute infusion of ascorbic acid and/or endothelial cell protein expression [see details below]) were included in this analysis, in order to focus the analysis on these novel parameters. Inclusion criteria for trial 1 were: 18-80 years of age, estimated glomerular filtration rate (eGFR; Modified Diet Renal Disease [MDRD] equation) 15-59 ml/min/1.73 m$^2$, and evidence of chronic inflammation (high-sensitivity C-reactive protein $>$2.0 mg/L on at least two consecutive weekly determinations). All women from this trial included in present analysis were post-menopausal for better matching to trial 2 and healthy controls. Inclusion criteria for trial 2 were: 40-79 years of age (postmenopausal for women), MDRD eGFR 15-45 ml/min/1.73 m$^2$, and baseline serum phosphorous 2.8-5.5 mg/dL (stable in the past month and not taking phosphate binders). All CKD participants were on optimal, stable, antihypertensive, diabetic, and lipid-lowering regimens as appropriate for at least 1 month before inclusion. To eliminate the influence of smoking, all participants included in this analysis were non-smokers. Individuals who participated in both trials (n=2) were only included in the analysis, with their most recent trial participation (CKD trial 2), as this time point was the most likely to have sufficient remaining samples (e.g., endothelial cells, blood).
Healthy control participants were 50-72 years of age (recruited to best match the age of CKD participants following partial completion of CKD enrollment; women were postmenopausal). Inclusion criteria were: healthy (i.e., free from kidney disease, CVD, diabetes, and other chronic disease [assessed via self-report, physical exam including a resting 12-lead electrocardiogram, and screening labs], free from hypertension based on guidelines at the time (blood pressure [BP] <140/90 mmHg and no antihypertensive agents), an eGFR ≥60 ml/min/1.73 m² by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation(18), and non-smoking.

**Procedures**

**Vascular Measurements.** The number of participants in each group with each outcome measurement are shown in **Supplemental Table 1**. All measurements were made under supine, overnight fasted (water only) conditions, following standard recommendations including 24-hr abstention from physical activity, and in a climate-controlled room.(19) Participants refrained from non-prescription medications for 48-hours prior to testing, but prescription medications were not withheld, in order to maintain blood pressure control. FMD<sub>BA</sub> was determined using duplex ultrasonography (Xario 200, Toshiba, Tustin, CA) with ECG-gated end-diastolic ultrasound images analyzed by a single blinded analyst using a commercially available software package (Vascular Analysis Tools 5.8.1, Medical Imaging Applications, Coralville, IA), as described in detail previously.(17, 20, 21) Doppler flow of the brachial artery was also measured and peak shear rate was calculated as a potential covariate.(17, 20, 21) Endothelium-independent dilation
(brachial artery dilation to 0.4 mg of sublingual nitroglycerin) was assessed as a standard index of smooth muscle cell sensitivity to exogenous nitric oxide.(17, 20, 21) 14 control and 48 CKD participants were administered nitroglycerin (missing due to low HR and/or low systolic BP [n=2 control; n=12 CKD]; contraindication [n=2 CKD]; arrhythmia precluding analysis [n=1 CKD]; failed i.v. [n=1 control]).

The assessment of carotid-femoral PWV has been described in detail previously.(17, 20, 21) Briefly, carotid-femoral PWV and carotid-radial PWV (an index of peripheral stiffness) were non-invasively measured by positioning a transcutaneous custom tonometer (Noninvasive Hemodynamics Workstation [NIHem], Cardiovascular Engineering Inc., Norwood, MA) at the carotid, brachial, radial and femoral arteries. Distances between sites were measured using a custom raised ruler (NIHem, Cardiovascular Engineering Inc., Norwood, MA [suprasternal notch and femoral artery]) or tape measure (all other distances). The distance from the suprasternal notch to the carotid was subtracted from the distance between the two recording sites, and carotid-femoral PWV was calculated as the distance divided by time between the foot of waveforms recorded at each site, as described previously.(22) N=61 CKD participants and n=16 control participants had successful carotid-femoral PWV (n=62 and n=17 for carotid-radial PWV) that met quality assurance.

Ultrasound imaging of the carotid artery was obtained in conjunction with the tonometry to provide blinded assessment of carotid artery compliance and carotid artery \( \beta \)-stiffness index (secondary indices of arterial stiffness), as described previously (n=60
CKD participants and n=17 control participants).(17, 20) Carotid systolic BP and carotid intimal medial thickness (cIMT) were also assessed (n=62 CKD and n=17 control).(17, 20)

An acute supraphysiological dose of ascorbic acid or isovolumic saline was infused to determine the influence of oxidative stress on FMD\textsubscript{BA}. FMD\textsubscript{BA} was measured during the “drip infusion” when peak plasma concentrations occur, as described previously.(17, 20, 24) The plasma concentrations with this dose have been shown to inhibit superoxide production \textit{in vitro}.(23) A priming bolus of 0.075 g of ascorbic acid/kg of fat free mass was dissolved in 150 mL of saline and infused intravenously at 5 mL/min for 20 min (maximal dosage was set at 5.0 g). This was immediately followed by a “drip-infusion” of 0.5 mL/min and FMD\textsubscript{BA} was again measured. All 17 controls and 60 CKD participants received infusions. Before and after the ascorbic acid infusion, plasma ascorbic acid levels were measured (by ARUP laboratories; quantitative high performance liquid chromatography) to demonstrate effective elevation of circulating levels in a small sub-group of CKD (n=4) and control (n=5) participants.

\textbf{Cellular Markers of Oxidative Stress and Inflammation.} We have described the details and rigor of the technique to measure endothelial cell protein expression previously.(17, 21, 25-27) Vascular endothelial cells from the intima of an antecubital vein were obtained immediately prior to vascular measurements (n=8-11 control participants and n=24-38 CKD participants per protein analyzed; not available for all participants and all proteins due to i.v. failure or low cell yield; additionally, only limited
endothelial cells were available from CKD trial 1, as most slides were previously analyzed using a different microscope). Cells were recovered and fixed. Slides were prepared then frozen for subsequent staining. VE cadherin primary antibody (1:500, Abcam, Cambridge, MA) was used to identify endothelial cells. Primary antibodies used for the assessment of markers included NAD(P)H oxidase (p47phox; 1:1000, Millipore, Billerica, MA), interleukin-6 (IL-6; 1:50, Santa Cruz, Dallas, TX); nuclear factor κ B (NF κ B; 1:300, Santa Cruz, Dallas, TX), and phosphorylated endothelial nitric oxide synthase (PeNOS; 1:100, Cell Signaling, Danvers, MA). Expression of these proteins was determined by a blinded analyst using immunofluorescence (Nikon Eclipse Ti, Melville, NY), as described previously.(17, 20, 21, 25) These markers were selected as indicators of oxidative stress, inflammation, and vascular endothelial nitric oxide production.

**Circulating Markers of Oxidative Stress and Inflammation.** ELISA (MSD, Rockville, MD) was used to measure serum C-reactive protein (CRP) and IL-6 concentrations as markers of inflammation. Oxidized LDL was also measured by ELISA (Mercodia, Upsala, Sweden) as an index of oxidative stress. Stored samples were not available from n=1 from CKD trial 1 and n=5 from CKD trial 2, thus n=57-58 CKD and n=17 (all) controls were included in the assessment of these circulating markers.

**Statistical Analyses**

The Shapiro-Wilk test was used to test for normality. Independent sample t-tests, Chi-square tests, or Fisher's exact tests were used to evaluate differences
between groups in baseline variables. An independent samples t-test was used to determine differences between groups in vascular parameters and circulating markers. A 2x2 ANOVA was used to assess group differences in change in FMD\textsubscript{BA} following ascorbic acid infusion. ANCOVA was used to evaluate the influences of mean arterial pressure on carotid-femoral PWV\textsuperscript{(28)} and shear rate and baseline diameter on FMD\textsubscript{BA}. Log-transformation was performed on non-normally distributed variables prior to analysis. All data are reported as means±S.D. or medians (interquartile range) unless otherwise noted, with figures presented as means±S.E. Analysis was completed only on individuals with complete data for the outcome of interest (missing data for any variables is described above). Analyses were performed using SPSS 25 and statistical significance was set at p<0.05. Adjustment was not made for multiple comparisons, as the study was mechanistic and hypothesis-generating.

A sample size of 17 control subjects was calculated based on ~90% power at an alpha level of 0.05 (two-sided) in order to detect a group difference of 1.9 for the outcome of change in FMD\textsubscript{BA} following ascorbic acid infusion. This calculation was based on previously published data assessing change in FMD\textsubscript{BA} following ascorbic acid infusion in healthy older adults compared to young healthy controls (mean±S.D. change in percent FMD\textsubscript{BA}: young healthy controls: 0.2±2.0; older adults: 2.1±0.9);\textsuperscript{(24)} we assumed a similar effect size in CKD. While only 17 CKD participants were required to provide ~90% power, we included all participants from the two clinical trials in our CKD group. Based on previous publications in CKD, these sample sizes (n=17 controls and n=62 individuals with CKD) also provided 99% power to detect a group difference of
2.3±0.5 in percent FMD_{BA}(7) and 99% power to detect a group difference of 390±275 cm/sec in carotid-femoral PWV.(8)

**Study Approval**

All procedures were approved by the Colorado Multiple Institutional Review Board and adhere to the *Declaration of Helsinki*. The nature, benefits and risks of the study were explained to and volunteers provided written informed consent prior to study participation.

**Results**

**Demographic and Clinical Characteristics**

Sixty-four individuals with CKD from two previous clinical trials were included in this analysis. Twenty-two control participants were assessed for eligibility for the current study. Five were excluded from enrollment due to not meeting inclusion/exclusion criteria, for a total cohort of 17. Individuals in the CKD group were slightly older, more likely to be male (trial 2 was a mostly Veteran population), more likely to be a former smoker, and had higher blood pressure, higher cholesterol, higher BMI, and lower eGFR than healthy controls (Table 1). The majority of CKD participants had a history of hypertension and blood pressure was controlled. Due to inclusion criteria for enrollment, no control participants were hypertensive. CKD participants were more likely to use antihypertensive agents and statins. Use of other medications did not differ between groups, nor did race/ethnicity. Etiology of CKD
was attributed to diabetes (45%), hypertension (27%), nephrolithiasis (3%), ADPKD (3%), drugs or toxins (3%), acute kidney injury (3%), and/or other or unknown (34%).

**Vascular Parameters**

CKD participants had a 36% lower FMD_{BA}, indicating impaired endothelium dependent dilation, and 46% higher carotid-femoral PWV, indicating greater aortic stiffness compared to healthy controls (Table 1). The time to peak FMD_{BA} was also longer in CKD participants compared to healthy controls (p=0.03). Peak hyperemic (p=0.01) but not resting (p=0.39) shear rate differed between CKD participants and healthy controls; the fold-increase in shear rate during reactive hyperemia was thus greater in healthy controls (6.9±1.3) than in the CKD group (5.0±1.5; p<0.001). The difference in FMD_{BA} was no longer significant between groups after adjustment for peak shear rate (p=0.47). Endothelium-independent dilation to sublingual nitroglycerin was reduced in CKD participants compared to healthy controls (p=0.01). CKD participants also had greater carotid systolic BP, cIMT, and carotid β-stiffness index compared to controls, with no difference in carotid-radial PWV (an index of peripheral stiffness) or supine brachial artery mean arterial pressure. Consistent with the lack of difference in mean arterial pressure between groups, carotid-femoral PWV remained significantly different between groups after statistically adjusting for mean arterial pressure (p<0.001).

**Acute Inhibition of Vascular Oxidative Stress**

Following an acute infusion of ascorbic acid previously shown inhibit superoxide production *in vitro*, plasma ascorbic acid levels were significantly elevated
in both the control (pre: 73±7 µmol/L; post: 1236±81 µmol/L; 17-fold increase; 
p<0.001) and CKD group (pre: 36±7 µmol/L; post: 1664±429 µmol/L; 48-fold 
increased; p<0.01). However, the infusion (compared to isovolumetric saline) 
differentially improved FMD_{BA} in healthy controls as compared CKD participants 
(absolute change in percent FMD_{BA}: healthy control = 1.3±0.6; CKD = 0.12±0.2 
[mean±S.E.]; group*condition interaction p=0.04) (Figure 1).

**Cellular and Circulating Markers of Oxidative Stress and Inflammation**

**Cellular Markers**

Figure 2 displays vascular endothelial cell protein expression of NADPH 
oxidase (Panel A), IL-6 (Panel B), NFκB (Panel C), and PeNOS (Panel D). 
Expression of the oxidant enzyme NADPH oxidase was greater in the CKD compared 
to control group (1.48±0.05 vs. 1.25±0.11 [intensity vs. HUVEC control; mean±S.E.]; 
p=0.05). The pro-inflammatory transcription factor NFκB (0.78±0.02 vs. 0.67±0.08; 
p=0.19), pro-inflammatory cytokine IL-6 (0.94±0.02 vs. 0.98±0.05; p=0.43), and 
PeNOS (1.34±0.04 vs. 1.23±0.10; p=0.34) did not differ in the CKD group compared 
to controls.

**Circulating Markers**

Circulating pro-inflammatory markers CRP and IL-6 were elevated in the CKD 
compared to control group, with no difference in the marker of oxidative damage, 
oxidized LDL (Table 2).

**Discussion**
In this translational study comparing adults with moderate-to-severe CKD and middle-aged and older healthy controls, we confirmed the presence of vascular dysfunction (impaired FMD_{BA} and increased carotid-femoral PWV). Additionally, while hypothesis-generating in nature, we have provided the first direct evidence in humans with CKD suggesting vascular oxidative stress. Endothelial cell protein expression of the oxidant enzyme NADPH oxidase was increased with CKD, providing the first cellular evidence that vascular oxidative stress may be increased in adults with moderate-to-severe CKD.

Additionally, we administered an acute supraphysiological infusion of ascorbic acid that produces plasma concentrations known to inhibit superoxide production in vitro.(23) This infusion failed to improve FMD_{BA} in the CKD participants, despite improvements in the control group. We believe these unexpected findings indicate that the level of oxidative stress in the CKD group (as reflected by endothelial cell protein expression and circulating markers) was too great to be overcome by the ascorbic acid infusion, despite a substantial rise in plasma ascorbic acid levels. The improvement in the control group comprised of healthy middle-aged and older adults is consistent with previous literature demonstrating an improvement in age-associated impairment in FMD_{BA} in healthy middle-aged and older adults.(24, 29) Acute infusion of ascorbic acid has also been shown to improve conduit artery or microvascular endothelium-dependent dilation in individuals with diabetes,(30), hypertension,(31) and smokers.(32) Additionally, we recently demonstrated that our
ascorbic acid infusion protocol improved $\text{FMD}_{\text{BA}}$ in adults with early-stage autosomal dominant polycystic kidney disease and preserved kidney function.\(^{20}\)

An acute ascorbic acid infusion previously failed to improve radial artery FMD; however, in this study kidney disease was severe (eGFR $<20$ ml/min/1.73m\(^2\)) and a different artery was assessed.\(^{15}\) Additionally, oral ascorbic acid has failed to improve $\text{FMD}_{\text{BA}}$ in adults with CKD,\(^{5}\) but oral administration does not raise plasma ascorbic acid levels (which were not assessed in this study) nearly as much as an acute supraphysiological infusion.\(^{24}\) A recent small study including both CKD and peritoneal dialysis patients showed no change in $\text{FMD}_{\text{BA}}$ following an ascorbic acid infusion.\(^{16}\) However, microvascular endothelium dependent dilation in the cutaneous microvasculature is improved to the level of healthy controls in adults with moderate-to-severe CKD following local ascorbic acid administration, indicating potential differences across vascular beds.\(^{14}\) Overall, these data support that CKD may have extensive oxidative stress that is not overcome by ascorbic acid, and this should be taken into account when testing future anti-oxidant therapies in patients with kidney disease.

We observed a 35% lower $\text{FMD}_{\text{BA}}$, in the CKD group, reiterating the presence of impaired endothelium-dependent dilation in CKD.\(^{5-7}\) Of interest, the CKD group also demonstrated a longer duration to peak dilation than the control group following cuff release. Time to peak dilation has also been shown to be delayed in older sedentary versus young healthy adults,\(^{33}\) individuals with the metabolic syndrome\(^{34}\) and type 2 diabetes mellitus\(^{35}\) as compared to healthy controls, as
well as adults with moderate versus low cardiovascular risk. This has not been reported previously in CKD and may be an additional reflection of vascular dysfunction. Suggested mechanisms that may contribute to impaired time to peak dilation include reduced arterial wall compliance, changes in enzyme rate production, and free radicals interacting with endothelium-derived vasodilators.

Shear rate is produced by the hyperemic blood flow response to the cuff deflation and is the mechanical stimuli that promotes vasodilation. Notably, peak shear rate has been shown to differ according to Framingham risk factors, as well as the presence of the metabolic syndrome, diabetes, and advanced age. Peak shear rate has typically not been quantified in previous CKD studies, although hyperemic blood flow or peak velocity have been reported to be similar to controls. In the Framingham Heart study, inclusion of shear rate attenuated the association between cardiovascular risk factors and FMD, suggesting that impaired FMD in the presence of cardiovascular risk factors may represent an attenuated hyperemic stimulus rather than brachial endothelium dysfunction. However, it has also been proposed that shear rate should be presented rather than corrected for when comparing FMD between groups. We observed a difference in shear rate between the CKD and control group, and the difference in FMD was no longer significant after adjustment for shear rate, suggesting at the minimum an importance of the hyperemic stimulus when evaluating FMD in participants with CKD.
In addition to reduced FMD_{BA}, we observed impaired brachial artery dilation to the NO donor nitroglycerin, suggesting that there is also smooth muscle cell impairment (i.e., impaired endothelium independent dilation) in non-dialysis dependent CKD. Previous literature has demonstrated mixed results regarding the presence of impaired brachial artery dilation to nitroglycerin.(5-7)

Carotid-femoral PWV was 46% greater in participants with moderate-to-severe CKD compared to healthy controls. The results are consistent with previous literature demonstrating greater large elastic artery stiffness in non-dialysis CKD.(8-10) The group difference remained highly significant after adjustment for mean arterial pressure, suggesting structural changes contributing to increased arterial stiffness. Additionally, individuals with CKD in the present study had elevated carotid systolic BP compared to controls, consistent with higher brachial systolic BP (although still controlled according to guidelines at the time). Evidence on local arterial compliance, such as the carotid artery, has been much less reported, but our results of increased β-stiffness index is also consistent with limited available evidence.(9)

Circulating markers of increased oxidative stress or reduced antioxidant defenses,(7, 15) as well as increased inflammation(43, 44) were previously shown to be altered in moderate-to-severe CKD. We have provided the first direct evidence that oxidative stress is increased at the level of the vascular endothelium in humans with CKD. This was observed despite a lack of difference in oxidized LDL, a circulating marker of oxidative damage. Notably, consistent with previous evidence, circulating markers of inflammation were elevated in the CKD group.
Increased oxidative stress and inflammation are both likely promoters of a
decline in NO bioavailability. Reduced NO is a contributing mechanism common to both
large-elastic artery stiffness and endothelial dysfunction. However, no difference in
endothelial cell PeNOS protein expression was observed in CKD participants compared
to healthy controls.

The major strength of this study is that we employed novel methodology to
evaluate physiological mechanisms contributing to vascular dysfunction in CKD - the
most comprehensive assessment to date. We have extended existing literature
indicating circulating markers of oxidative stress in CKD by collecting vascular
endothelial cells to providing direct evidence of vascular oxidative stress. We also
assessed FMD_{BA} following acute inhibition of oxidative stress. Given the
comprehensive nature of these assessments, these measurements were performed in a
relatively large number of CKD participants.

This study also has several notable limitations. Given that the CKD participants
also had other comorbidities, it is difficult to separate the contributions of these factors
from other contributing mechanisms. Differences between the two groups besides the
presence of CKD may have contributed to the observed results, beyond the primary
disease process alone. For example, ages weren’t precisely matched and there were
more males in the CKD group, as trial 2 was a Veterans Affairs funded trial.
Importantly, our findings are still clinically meaningful, despite any residual group
differences. The results are cross-sectional and cannot provide insight into changes in
vascular function and associated mechanisms over time. Additionally, we recognize
that the sample size was smaller than the overall cohort for endothelial cell protein expression, due limitations in the technique (e.g., i.v. failure, inadequate cell recovery) and lack of remaining slides from CKD trial 1, which may have introduced selection bias or increased the likelihood of a type I error in the comparison of NAD(P)H oxidase protein expression between groups.

In conclusion, we have provided initial evidence that oxidative stress may be a physiological mechanism contributing to vascular dysfunction in moderate-to-severe CKD. Our results also reiterate that vascular dysfunction is present in CKD, prior to the initiation of dialysis. Future research should follow changes in vascular function and associated mechanisms longitudinally. Additionally, physiological mechanisms contributing to vascular oxidative stress and inflammation should continue to be delineated, including how targeting these processes influence vascular function. Interventions to reduce oxidative stress in individuals with moderate-to-severe CKD could potentially reduce the risk of cardiovascular events and mortality in patients with CKD.
Disclosures

The authors have nothing to disclose.

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Author Contributions

Kristen Nowak: Conceptualization; Data curation; Formal analysis; Funding acquisition; Investigation; Methodology; Resources; Supervision; Writing - original draft
Anna Jovanovich: Conceptualization; Data curation; Funding acquisition; Investigation; Methodology; Resources; Supervision; Writing - review and editing
Heather Farmer-Bailey: Data curation; Formal analysis; Writing - review and editing
Nina Bispham: Data curation; Formal analysis; Writing - review and editing
Taylor Struemph: Data curation; Writing - review and editing
Mikaela Malaczewski: Data curation; Writing - review and editing
Wei Wang: Data curation; Formal analysis; Methodology; Writing - review and editing
Michel Chonchol: Conceptualization; Funding acquisition; Investigation; Methodology; Resources; Validation; Writing - review and editing

All authors approved the final version of the manuscript.
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### Table 1: Demographics and Clinical Characteristics of Chronic Kidney Disease and Control Participants

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<th>Variable</th>
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<th>Control (n=17)</th>
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<tr>
<td><strong>Diabetes, %</strong> *</td>
<td>56%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>ACEi/ARB, %</strong> *</td>
<td>75%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Diuretic, %</strong> *</td>
<td>56%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Beta Blocker, %</strong> *</td>
<td>55%</td>
<td>0%</td>
</tr>
<tr>
<td><strong>Calcium Channel Blockers, %</strong> *</td>
<td>41%</td>
<td>0%</td>
</tr>
<tr>
<td>Variable</td>
<td>CKD (n=64)</td>
<td>Control (n=17)</td>
</tr>
<tr>
<td>----------------------------------</td>
<td>------------</td>
<td>----------------</td>
</tr>
<tr>
<td>Statin, % *</td>
<td>69%</td>
<td>12%</td>
</tr>
<tr>
<td>Antidepressant or Antianxiety</td>
<td>27%</td>
<td>18%</td>
</tr>
<tr>
<td>Medication, %</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Thyroid Medication, %</td>
<td>20%</td>
<td>6%</td>
</tr>
</tbody>
</table>

Data are mean±S.D. or n (%). * p<0.05 by Chi-square or Fisher’s exact tests for categorical data and independent sample t-test for continuous variables. CKD, chronic kidney disease; BMI, body-mass index; BP, blood pressure (seated position); eGFR; estimated glomerular filtration rate (by the Modification of Diet in Renal Disease (MDRD) Study equation for the chronic kidney disease group and by the Chronic Kidney Disease Epidemiology Collaboration equation for the control group); LDL, low density lipoprotein; HDL, high density lipoprotein; ACEi, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blocker.
Table 2: Vascular Parameters in Chronic Kidney Disease and Control Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>CKD (n=64)</th>
<th>Control (n=17)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>FMD(_{BA}), %</td>
<td>3.5±2.8</td>
<td>5.5±3.2</td>
<td>0.02</td>
</tr>
<tr>
<td>FMD(_{BA}), mm</td>
<td>0.14±0.10</td>
<td>0.20±0.11</td>
<td>0.04</td>
</tr>
<tr>
<td>Baseline FMD diameter, mm</td>
<td>4.1±0.7</td>
<td>3.7±0.6</td>
<td>0.02</td>
</tr>
<tr>
<td>Resting shear rate, s(^{-1})</td>
<td>132±47</td>
<td>121±38</td>
<td>0.39</td>
</tr>
<tr>
<td>Hyperemic shear rate, s(^{-1})</td>
<td>644±225</td>
<td>831±271</td>
<td>0.01</td>
</tr>
<tr>
<td>Time to peak FMD(_{BA})</td>
<td>57±22</td>
<td>44±16</td>
<td>0.03</td>
</tr>
<tr>
<td>Brachial artery dilation to nitroglycerin, %</td>
<td>18.2±9.7</td>
<td>26.0±7.3</td>
<td>0.01</td>
</tr>
<tr>
<td>Baseline nitroglycerin diameter, mm</td>
<td>4.1±0.7</td>
<td>3.8±0.6</td>
<td>0.13</td>
</tr>
<tr>
<td>Carotid-femoral PWV, cm/sec</td>
<td>1071±336</td>
<td>732±128</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carotid-radial PWV, cm/sec</td>
<td>1003±246</td>
<td>955±165</td>
<td>0.46</td>
</tr>
<tr>
<td>Carotid artery compliance, (mm/mmHg) x 10(^{-1})</td>
<td>0.76±0.32</td>
<td>0.78±0.18</td>
<td>0.65</td>
</tr>
<tr>
<td>Carotid β-stiffness index, A.U.</td>
<td>11.8±4.6</td>
<td>8.7±2.0</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Carotid IMT, mm</td>
<td>0.67±0.19</td>
<td>0.58±0.09</td>
<td>0.04</td>
</tr>
<tr>
<td>Carotid Systolic BP, mmHg</td>
<td>134±19</td>
<td>116±17</td>
<td>0.001</td>
</tr>
<tr>
<td>Brachial Mean Arterial BP, mmHg</td>
<td>90±14</td>
<td>87±10</td>
<td>0.41</td>
</tr>
</tbody>
</table>

pulse-wave velocity; IMT, intimal medial thickness; BP, blood pressure. n=14 controls and n=49 CKD participants were administered nitroglycerin. n=56 CKD and all (n=17) control participants had measurements of carotid artery compliance, β-stiffness index, and n=64 CKD and all (n=17) control participants had measurement of carotid IMT. N=62/N=63 CKD participants and n=16/n=17 control participants had successful measurements of CFPWV and CRPWV, respectively. All vascular parameters were assessed in the supine position.
Table 3: Circulating Markers of Oxidative Stress, Inflammation, and Nitric Oxide Production in Chronic Kidney Disease and Control Participants

<table>
<thead>
<tr>
<th>Variable</th>
<th>CKD (n=57-58)</th>
<th>Control (n=17)</th>
<th>P-Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>CRP, mg/L</td>
<td>2.60 (1.08, 6.15)</td>
<td>0.70 (0.50, 3.53)</td>
<td>0.01</td>
</tr>
<tr>
<td>IL-6, pg/mL</td>
<td>1.39 (0.94, 2.23)</td>
<td>0.71 (0.43, 0.96)</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>oxLDL, mU/L</td>
<td>62302 (49508, 79326)</td>
<td>71213 (57265, 84198)</td>
<td>0.18</td>
</tr>
</tbody>
</table>

Data are median (interquartile range). P-values are independent t-test comparisons between groups using log-transformed variables. CKD, chronic kidney disease; CRP, C-reactive protein; IL-6, interleukin-6; oxLDL, oxidized low density lipoprotein.
Legends to Figures

Figure 1. Brachial artery flow-mediated dilation (FMD) following an acute infusion of saline (black bars) and ascorbic acid (gray bars) in participants with chronic kidney disease (CKD) and healthy controls. Infusions were performed in all control participants (n=17) and n=60 CKD participants. Ascorbic acid significantly improved FMD<sub>BA</sub> in control participants (saline: 5.5±0.8%; ascorbic acid: 6.8±0.9%); as compared to CKD participants (saline: 3.5±0.4%; ascorbic acid: 3.6±0.4%) (P-interaction (group*condition) = 0.04). Values are mean±S.E.

Figure 2. Protein expression of NAD(P)H oxidase (Panel A; CKD: 1.48±0.05; Control: 1.25±0.11; p=0.05), interleukin-6 (IL-6; Panel B; CKD: 0.94±0.02; Control: 0.98±0.05; p=0.43), nuclear factor κ B (NFκB; Panel C; CKD: 0.78±0.02; Control: 0.67±0.08; p=0.19), and phosphorylated endothelial cell nitric oxide synthase (PeNOS; Panel D; CKD: 1.34±0.04; Control 1.23±0.10; p=0.34) in vascular endothelial cells collected from a peripheral vein of participants with chronic kidney disease (CKD; black bars) compared to healthy controls (white bars). Expression is relative to human umbilical vein endothelial cell (HUVEC) control, with representative images shown below (quantitative immunofluorescence). Values are mean±S.E. * p≤0.05.
Figure 1.
Figure 2.