NADPH oxidase-derived reactive oxygen species contribute to impaired cutaneous microvascular function in chronic kidney disease

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DuPont JJ, Ramick MG, Farquhar WB, Townsend RR, Edwards DG. NADPH oxidase-derived reactive oxygen species contribute to impaired cutaneous microvascular function in chronic kidney disease. Am J Physiol Renal Physiol 306: F1499–F1506, 2014. First published April 23, 2014; doi:10.1152/ajprenal.00058.2014.—Oxidative stress promotes vascular dysfunction in chronic kidney disease (CKD). We utilized the cutaneous circulation to test the hypothesis that reactive oxygen species derived from NADPH oxidase and xanthine oxidase impair nitric oxide (NO)-dependent cutaneous vasodilation in CKD. Twenty subjects, 10 stage 3 and 4 patients with CKD (61 ± 4 yr; 5 men/5 women; eGFR: 39 ± 4 ml·min−1·1.73 m−2) and 10 healthy controls (55 ± 2 yr; 4 men/6 women; eGFR: >60 ml·min−1·1.73 m−2) were instrumented with 4 intradialysis fibers for the delivery of J Ringer solution (Control), 2) 10 μM tempol (scavenge superoxide), 3) 100 μM apocynin (NAD(P)H oxidase inhibition), and 4) 10 μM allopurinol (xanthine oxidase inhibition). Skin blood flow was measured via laser-Doppler flowmetry during standardized local heating (42°C). N-nitro-l-arginine methyl ester (l-NAME; 10 mM) was infused to quantify the NO-dependent portion of the response. Cutaneous vascular conductance (CVC) was calculated as a percentage of the maximum CVC achieved during sodium nitroprusside infusion at 43°C. Cutaneous vasodilation was attenuated in patients with CKD (77 ± 3 vs. 88 ± 3%, P = 0.01), but augmented with tempol and apocynin (tempol: 88 ± 2 (P = 0.03), apocynin: 91 ± 2% (P = 0.001). The NO-dependent portion of the response was reduced in patients with CKD (41 ± 4 vs. 58 ± 2%, P = 0.04), but improved with tempol and apocynin (tempol: 58 ± 3 (P = 0.03), apocynin: 58 ± 4% (P = 0.03). Inhibition of xanthine oxidase did not alter cutaneous vasodilation in either group (P > 0.05). These data suggest that NAD(P)H oxidase is a source of reactive oxygen species and contributes to microvascular dysfunction in patients with CKD.

Endothelial dysfunction is a nontraditional risk factor for CVD and has been shown to predict future CVD events (29, 72) and contribute to the progression of renal disease (65). Endothelial dysfunction is characterized by a decreased production and/or bioavailability of nitric oxide (NO). Endothelial-derived NO is important for maintaining vascular health, promotes vasodilation, and prevents leukocyte adhesion, smooth muscle cell proliferation, and platelet aggregation. Endothelial dysfunction is characteristic of CKD (17, 64, 82) and specifically, in patients with renal failure, several studies have observed impairments in microvascular function (2, 70). We have previously shown that oxidative stress plays a role in impairing NO-mediated cutaneous vasodilation in patients with stage 3 and 4 CKD via local delivery of ascorbic acid (21). It is likely that ascorbic acid effectively restored microvascular function through scavenging of superoxide. Elevated superoxide production has been responsible for a large portion of the NO deficit in animal models of hypertension (25, 59). Superoxide may also contribute to endothelial dysfunction in CKD. However, ascorbic acid is nonspecific and may have resulted in improvements in vasodilation via the stabilization of NO synthase (NOS) cofactor tetrahydrobiopterin (BH4) (76) or inhibition of the arginine pathway that competes for NO precursor l-arginine (63).

Despite the efficacy of acute ascorbic acid, chronic antioxidant therapy has generally been ineffective in the prevention of CVD as well as in hemodialysis patient outcomes (14, 26, 28, 38, 35). However, several studies have reported improvements in cardiovascular outcomes and endothelial function in renal failure (5) and in patients with mild to moderate CKD (56) with chronic vitamin E supplementation. Chronic antioxidants may not be effective due to the nonspecific scavenging of reactive oxygen species (ROS) and extremely localized production of ROS. Targeting the specific enzymatic sources of ROS may offer a more effective treatment in the management of cardiovascular and renal outcomes in CKD. NADPH oxidases have been identified as major sources of ROS in numerous pathologies: rodent renal disease (3, 78), rodent hypertension (59), human atherosclerosis (67), and coronary heart disease (68). Another potential source of ROS in patients with CKD may be xanthine oxidase, which has been shown to be a source of superoxide in coronary artery disease (68) and heart failure (43). The potential roles of NADPH oxidases and XO in microvascular dysfunction in patients with CKD are currently unknown.

The cutaneous circulation provides an accessible vascular bed for the assessment of in vivo human microcirculatory function that represents systemic vascular function (1, 37, 61). Impairments in cutaneous microvascular function are related to indices of vascular function in the coronary bed (41). The use of laser-Doppler flowmetry coupled with intraderal microdialysis allows for the simultaneous local delivery of pharmacological substances and measurement of skin blood flow during local heating. Local heating of the skin results in a biphasic...
vasodilatory response; the first phase consists of an initial peak due to an axon reflex followed by a secondary plateau phase that is primarily (~60%) mediated by NO (39, 53) and has been shown to be endothelial derived (8, 40). Therefore, assessing the skin blood flow response to local heating is a useful method to assess NO-mediated microcirculatory function as well as mechanisms of dysfunction in NO-dependent processes.

The purpose of this investigation was to determine the roles of NADPH oxidase, xanthine oxidase, and ROS derived from these enzymes in reducing NO-mediated cutaneous vasodilation in response to local heating in patients with moderate to severe CKD. We hypothesized that pharmacological inhibition of tempol-sensitive ROS, NADPH oxidase, and XO would improve cutaneous vasodilation in response to local heating in patients with moderate to severe CKD.

METHODS

Subjects. Ten individuals (5 men, 5 women) with stages 3–4 CKD and 10 age- and sex-matched apparently healthy individuals (healthy control; HC) were studied. Stage 3 and 4 CKD is defined as an estimated glomerular filtration rate (eGFR) of <60 and >15 ml·min⁻¹·1.73 m⁻². GFR was estimated using the Modification of Diet in Renal Disease (MDRD) equation based on serum creatinine, age, sex, and race (46). All apparently healthy individuals were required to have an estimated creatinine clearance of >90 ml/min, which was calculated using the Cockcroft-Gault equation, since the MDRD equation has not yet been validated in apparently healthy individuals (16). Participants with a history of myocardial infarction, angina, heart failure, lung disease, liver disease, cancer, autoimmune disease, and current tobacco use were excluded from study participation. We excluded the listed CVD because we aimed to study vascular dysfunction in CKD as it relates to the development of CVD. Patients with CKD were on a variety of antihypertensive medications, statins, insulin, etc. Participants continued use of medications throughout study participation; however, they were instructed to delay taking medications other than those for the treatment of diabetes on screening and testing days until after all testing had been completed.

Experiments were approved by the Institutional Review Board at the University of Delaware and conformed to the guidelines set forth by the Declaration of Helsinki. All participants gave verbal and written consent before study participation.

Participants arrived for screening after an overnight fast. Blood and urine samples were collected to assess liver enzymes, blood lipid profiles, renal function, hemoglobin, hematocrit, glucose, and urinary albumin-to-creatinine ratio. A medical history form was also completed along with resting brachial blood pressure, a resting 12-lead electrocardiogram, height, and weight.

Subject instrumentation. Participants arrived at the laboratory and were instrumented with four microdialysis (MD) fibers (MD 2000, Bioanalytical Systems; 10-mm, 30-kDa cutoff membrane) in the ventral side of the nondominant forearm as previously described (20, 21, 30). A 25-gauge needle was inserted into the dermis after a 10-min application of ice to the skin surface to provide short-term local anesthesia. The entry and exit points of each needle were ~2.5 cm apart. The MD fibers were then threaded through the lumen of the needle, which was removed once the semipermeable membrane of the fiber was in place. The MD fibers were taped down, and Ringer solution was perfused through all sites for 60–90 min or until the local hyperemia associated with fiber insertion subsided.

Skin blood flow was measured as cutaneous red blood cell (RBC) flux from 1.5 mm² of skin with a multifiber laser-Doppler probe placed in a local heater (MoorLAB, Temperature Monitor SH02, Moor Instruments, Devon, UK). Each local heater was placed directly on the skin above each MD site. Brachial blood pressure was also measured every 10 min on the contralateral arm by an automated oscillometric sphygmomanometer (Dinamap Dash 2000, GE Medical Systems).

Experimental protocol. A standardized nonpainful local heating protocol was utilized to induce NO-dependent cutaneous vasodilation (39, 53) (Fig. 1). Once the local hyperemic response subsided, the four MD sites were randomly assigned to receive 1) Ringer solution (control site); 2) 10 μM tempol (to scavenge superoxide; Calbiochem, San Diego, CA); 3) 100 μM apocynin (NADPH oxidase inhibition; Sigma-Aldrich, St. Louis, MO); and 4) 10 μM allopurinol (XO inhibition; Sigma-Aldrich). All solutions were perfused at a rate of 2 μl/min. For baseline measurements, local heaters were set to 33°C, and RBC flux was recorded for ~30 min. Following baseline, local temperature was increased to 42°C at a rate of 0.1°C/s and remained at 42°C for the duration of the local heating protocol. Local heating of the skin results in a biphasic response; an initial peak occurs within the first 10 min of heating and is primarily mediated by an axon reflex. The axon reflex consists of the release of neurotransmitters from afferent sensory nerves via the activation of TRPV-1 channels and also includes a small NO component through the activation of NOS by the neurotransmitters (53, 79).

The initial peak was followed by a secondary plateau that is predominately mediated by NO (39, 53), which has been shown to be endothelial derived in the forearm (8, 40). The remaining 40% of the plateau phase is mediated by endothelial-derived hyperpolarizing factors, specifically through the activation of K⁺Ca channels and epoxyeicosatrienoic acids (9). Once a stable secondary plateau was achieved, 10 mM N⁶-nitro-l-arginine methyl ester (l-NAME; Sigma-Aldrich) was perfused through all MD fibers to quantify NO-dependent vasodilation at all MD sites. Following a stabilized post-l-NAME plateau, local heaters were set to 43°C and 28 mM sodium nitroprusside (SNP; Nitropress, Hospira, Lake Forest, IL) was perfused through all MD fibers to cause maximal cutaneous vasodilation (51). Concentrations of 10 mM l-NAME and 28 mM SNP were chosen because these dosages have been shown to sufficiently inhibit NO synthase and elicit maximal vasodilation in the skin (53). Additionally, 100 μM apocynin, 10 μM allopurinol, and 10 μM tempol have been shown to sufficiently inhibit NADPH oxidase, xanthine oxidase, and ROS in a previous study (51).

Blood analyses. Venous blood samples were obtained after a 12-h fast. All samples were collected in tubes containing EDTA, immediately centrifuged at 4°C, and plasma was frozen at −70°C until analysis. Plasma nitrotyrosine, protein carbonyl, and 4-hydroxynonenal (4-HNE) levels were each measured using a competitive ELISA (Cell Biolabs, San Diego, CA). Plasma asymmetric dimethylarginine (ADMA) levels were analyzed using immunoassay. ADMA was measured with enzyme immunoassay (Cell Biolabs, San Diego, CA). Highly sensitive plasma t-arginine levels were also measured using a competitive ELISA (Cell Biolabs, San Diego, CA)

![Fig. 1. Original recording of the skin blood flow response to local heating. Baseline measurements are made taken for ~30 min at a local temperature of 33°C. An initial peak occurs upon increasing the local temperature to 42°C, followed by a sustained plateau that is primarily mediated by nitric oxide (NO). Once a stable plateau has been established, N⁶-nitro-l-arginine methyl ester (l-NAME) is delivered through the microdialysis fiber to inhibit NO synthase (NOS). Following a stable post-l-NAME plateau, sodium nitroprusside is delivered through the fiber and the local temperature is increased to 43°C to cause a maximal dilation response.](http://ajprenal.physiology.org/)

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(ADMA) and l-arginine levels were also measured by competitive ELISA (Eagle Biosciences, Nashua, NH).

Data and statistical analyses. RBC flux data were collected at 40 Hz using the PL3516 PowerLab data-acquisition system and LabChart software (ADInstruments, Colorado Springs, CO). Brachial blood pressure was used to calculate cutaneous vascular conductance (CVC) as RBC flux divided by mean arterial pressure. Baseline and plateau CVC were obtained over a stable 10-min period. Initial peak CVC was calculated by averaging the highest value achieved over a stable 60-s period. Post-l-NAME plateau CVC was obtained over a stable 10-min period. To assess the contribution of NO to cutaneous vasodilation, we subtracted the post-L-NAME plateau from the NO plateau in each site. All data are expressed as a percentage of the maximal CVC obtained during SNP infusion (%CVCmax). A 2 × 4 ANOVA was performed to determine the effect of group, treatment, and group × treatment interaction of %CVCmax for baseline, initial peak, plateau, post-l-NAME plateau, NO contribution, and absolute maximal CVC. Tukey’s post hoc testing was completed to determine between- and within-group differences. Student’s paired t-tests were performed to compare subject characteristics and blood markers between groups. The level of significance was set at $P < 0.05$.

RESULTS

Subject characteristics. The baseline characteristics of the CKD and control groups can be found in Table 1. By design, medications (no. of patients)

<table>
<thead>
<tr>
<th>Medications (no. of patients)</th>
<th>Control</th>
<th>CKD</th>
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<tbody>
<tr>
<td>Antihypertensives</td>
<td>0</td>
<td>9</td>
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<tr>
<td>ACE inhibitors</td>
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<td>3</td>
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<td>ANG II-receptor antagonists</td>
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<td>2</td>
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<td>Beta-blockers</td>
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<td>3</td>
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<td>Calcium channel blockers</td>
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<td>3</td>
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<tr>
<td>Diuretics</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Statins</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Insulin</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>0</td>
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Values are means ± SE. expressed as red blood cell flux/mmHg.

Table 2. Absolute maximal cutaneous vascular conductance

<table>
<thead>
<tr>
<th></th>
<th>HC</th>
<th>CKD</th>
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<tbody>
<tr>
<td>Ringer</td>
<td>1.82</td>
<td>2.06</td>
</tr>
<tr>
<td>Tempol</td>
<td>2.01</td>
<td>1.73</td>
</tr>
<tr>
<td>Apocynin</td>
<td>1.86</td>
<td>1.67</td>
</tr>
<tr>
<td>Allopurinol</td>
<td>1.38</td>
<td>2.02</td>
</tr>
</tbody>
</table>

Values are means ± SE. expressed as red blood cell flux/mmHg.

measurements of renal function (serum creatinine, blood urea nitrogen, and eGFR) were significantly different between groups. Patients with CKD had significantly higher systolic blood pressure and HbA1c than the control group ($P < 0.05$). Patients with CKD also had significantly lower hemoglobin and hematocrit than the control group ($P < 0.05$). Five of 10 patients with CKD had type 2 diabetes. None of the control group participants were taking medications. The patients with CKD were on a variety of medications, including antihypertensives and statins (Table 1).

Microvascular function. There were no significant differences in baseline %CVCmax between groups or across MD sites ($P > 0.05$). Absolute maximal CVC was not significantly different between groups across all sites (Table 2), $P > 0.05$, indicating that the maximal dilatory capacity of the skin vasculature was not significantly different between groups. Initial peak %CVCmax was significantly impaired in the patients with CKD at the Ringer site ($P = 0.003$) and was significantly augmented by tempol and apocynin ($P = 0.03$) (Fig. 2). The plateau phase of the cutaneous hyperemia response was significantly impaired in the patients with CKD at the Ringer site ($P = 0.01$) and augmented by the local delivery of tempol ($P = 0.03$) and apocynin ($P = 0.001$) (Fig. 3A). Post-l-NAME plateau responses were not significantly different between groups or across MD sites ($P > 0.05$) (Fig. 3B). The NO contribution to cutaneous thermal hyperemia was significantly decreased in the patients with CKD ($P = 0.04$) and was significantly improved with the local delivery of tempol ($P = 0.03$) and apocynin ($P = 0.04$) (Fig. 3C). The inhibition of xanthine oxidase with allopurinol did not alter the cutaneous vasodilation responses in either group ($P > 0.05$).

Blood analyses. There were no significant differences in plasma protein carboxyl and plasma 4-HNE levels between groups ($P > 0.05$). Plasma nitrotyrosine levels were increased in the patients with CKD and trended toward statistical significance ($P = 0.08$) (Table 3). Plasma ADMA was significantly higher in the patients with CKD vs. HC ($P = 0.036$). Plasma l-arginine was not significantly different between groups ($P > 0.05$); however, the l-arginine/ADMA ratio was significantly lower in patients with CKD ($P = 0.001$) (Fig. 4).

DISCUSSION

We have demonstrated that NO-mediated cutaneous vasodilation in response to local heating is impaired in patients with stage 3–4 CKD compared with age- and sex-matched apparently healthy individuals, as we have previously described (21). The novel findings of this study are that 1) local scavenging of tempol-sensitive ROS and inhibition of NADPH oxidase augment NO-dependent cutaneous vasodilation in response to local heating in patients with stage 3–4 CKD and that 2) local inhibition of xanthine oxidase had no effect on NO-
dependent cutaneous vasodilation in response to local heating in patients with stage 3–4 CKD. These findings suggest that NADPH oxidase is a source of ROS and impairs microvascular function in patients with CKD.

Endothelial dysfunction is characteristic of CKD and considered to be a contributing factor to the increased risk of CVD in patients with CKD (17, 64, 82). We observed impairments in the plateau phase of the skin blood flow response to local heating as well as a reduced contribution of NO to the response in the patients with CKD. These findings are consistent with previous studies of vascular function in the population with CKD. Several studies have reported impairments in conduit artery function in kidney failure and in patients with mild to moderate CKD, as assessed by brachial artery flow-mediated dilation, a measure of endothelium-dependent dilation (17, 27, 42). In patients with renal failure, several studies have observed impairments in microvascular function via venous occlusion plethysmography (2) and a local heating stimulus (70). Additional studies have reported impaired relaxation responses to acetylcholine in subcutaneous resistance vessels from predialysis and dialysis patients with CKD (55).

We have previously reported that local delivery of ascorbic acid improves the local heating response in patients with stage 3–4 CKD, indicating that oxidative stress is a mechanism of impaired microvascular function in these patients (21). Ascorbic acid has also been shown to improve endothelial function in predialysis patients with renal failure (17) as well as patients on hemodialysis (27). While ascorbic acid is a useful tool for assessing the presence of oxidative stress, it is a nonspecific scavenger of ROS. Thus it does not offer the mechanistic insight necessary to determine which oxidants are increased and what their specific sources may be (36). In the present study, we utilized the superoxide dismutase (SOD) mimic...
tempol to specifically scavenge superoxide while measuring changes in skin blood flow. Tempol restored the plateau phase of the skin blood flow response to local heating as well as increasing the contribution of NO to the response. These data suggest that superoxide may be increased and play a role in reducing NO bioavailability in patients with CKD. Superoxide limits NO bioavailability through its rapid reaction with NO to form peroxynitrite, a cell-damaging oxidant. Peroxynitrite can oxidize critical NO cofactor BH4 (44), which leads to the uncoupling of endothelial NOS (eNOS), causing eNOS to produce superoxide instead of NO (73). Peroxynitrite has also been shown to inactivate endogenous antioxidant defense enzymes such as glutathione reductase and superoxide dismutase (70). However, tempol has also been shown to metabolize H2O2, OH-, and peroxynitrite (62). Thus our data indicate a role for tempol-sensitive ROS but may not necessarily be specific to superoxide, although it is plausible that tempol acted on superoxide in addition to the aforementioned ROS. Other studies have reported increases in superoxide in rodent renal disease (78), rodent hypertension (59), human atherosclerosis (67), and coronary artery disease (68). Thus superoxide is a major contributor to reducing NO bioavailability in a variety of pathologies related to cardiovascular disease.

Our current data also demonstrate that NADPH oxidases are a source of ROS and contribute to microvascular dysfunction in patients with stage 3–4 CKD. The local administration of apocynin, an inhibitor of NADPH oxidase, restored the plateau phase and increased the NO contribution to the cutaneous thermal hyperemia response in the patients with CKD similarly to tempol. NADPH oxidase has been shown to be a source of ROS in rodent models of renal disease (78) and hypertension (59), as well as human atherosclerosis (67) and coronary artery disease (68). Apocynin inhibits oxidase assembly by preventing the association of p47phox with the membrane-bound heterodimer; thus it does not specify which NOX (NADPH oxidase) isoform(s) may be upregulated and contributing to increases in ROS. Indeed, isoform specificity is a limitation of the use of apocynin (reviewed in Ref. 15); however, evidence supports its mode of action of inhibiting the association of p47phox with the membrane-bound heterodimer (65, 71, 74). It is plausible that increased NOX2 activity is a major source of superoxide, as NOX2-derived superoxide is thought to be primarily responsible for the endothelial injury and reductions in NO signaling that are characteristic of the development of vascular disease (7, 52). The current study did not investigate the mechanism(s) of increased NADPH oxidase activity; thus we can only speculate as to what the mechanism(s) may be. Angiotensin II is a potent stimulus of NADPH oxidase activity and expression through its action on the cell surface AT1 receptor (32, 34). Stimulation of the AT1 receptor results in protein kinase C-mediated phosphorylation of the p47phox subunit, which activates NADPH oxidase (49). Thus activation of the renin-angiotensin-aldosterone system (RAAS), as occurs in hypertension and patients with CKD, may contribute to increases in ROS production by NADPH oxidase. However, half of the patients with CKD in the current study were taking RAAS-targeting medications, which may suggest that the activation of NADPH oxidase is independent of RAAS activation, or alternatively, that the drug therapy is not adequate to inhibit NADPH oxidase activation by RAAS at the level of the cutaneous circulation. Furthermore, NADPH oxidase activity and expression has also been shown to be regulated by inflammatory cytokines such as TNF-α (31). TNF-α levels have been associated with the progression of renal disease and, thus, may have contributed to increases in NADPH oxidase activity in the present study (69).

In addition to inhibiting oxidase assembly, apocynin is also a scavenger of hydrogen peroxide (66, 71). The NOX4 isoform is also known to produce hydrogen peroxide instead of superoxide (18). Therefore, it is plausible that hydrogen peroxide may be increased and also contributes to reductions in NO bioavailability in patients with CKD. Yet, data on the role of hydrogen peroxide in vascular disease are mixed; some evidence supports that hydrogen peroxide can impair endothelial NO production through the phosphorylation of a tyrosine residue on eNOS (47). In contrast, several studies have demonstrated that hydrogen peroxide increases eNOS expression and activity (19, 75) and acts as a vasodilator in some vascular beds (50, 57, 58). Future studies are warranted to explore the potential role of hydrogen peroxide in microvascular function in patients with CKD.

In the present study, we found that local inhibition of xanthine oxidase via allopurinol did not improve NO-dependent cutaneous vasodilation in patients with stage 3–4 CKD. Our findings are in agreement with several other studies in humans. Acute xanthine oxidase inhibition via allopurinol did not improve brachial artery flow-mediated dilation in older adults (22). In the same study, vascular endothelial protein expression of xanthine oxidase was not different in young and older adults (22). Additionally, xanthine oxidase inhibition did not improve endothelium-dependent dilation in patients with essential hypertension (11). In contrast, xanthine oxidase inhibition has been shown to improve endothelium-dependent dilation in other pathologies such as hypercholesterolemia (11), congestive heart failure (23), and diabetes (10). Thus it appears that xanthine oxidase may not be mechanistically linked to impairments in endothelial function in all oxidative-stress-associated pathologies.

In addition to the alterations in NO contribution that we observed in the patients with CKD, we also observed an attenuation of the initial peak portion of the skin blood flow response to local heating. These findings are similar to what we have previously reported (21). In that study, the local delivery of ascorbic acid normalized the initial peak in the patients with CKD (21). In the present study, the initial peak was improved by the local delivery of tempol and apocynin, which suggests that increases in tempol-sensitive ROS contribute to the impairment we observed in patients with CKD. The initial peak is primarily mediated by an axon reflex; however, a portion of the reflex can be attenuated by NOS antagonism (53, 54). It is likely that increases in oxidative stress, specifically, tempol-sensitive ROS play a role in attenuating the NOS-mediated portion of the axon reflex. However, tempol has also been shown to decrease sympathetic nervous system activity independently of its effects on NO (80). Thus it is possible that the improvements observed in the initial peak with tempol administration may be independent of NO synthesis. Further investigation is warranted to determine the contributions of neural vs. NOS portions of the initial peak in patients with CKD and whether increases in oxidative stress have additional effects on the neural portion of the axon reflex.
In agreement with our previous findings (21) and others (77), our current data show that plasma ADMA levels are increased in patients with CKD. We also observed no significant differences in plasma L-arginine levels; however, when we calculated the ratio of L-arginine to ADMA the ratio was significantly lower in patients with CKD, indicating a deficit of L-arginine relative to increases in ADMA. The progression of CKD to end-stage renal disease and the occurrence of cardiovascular events in predialysis patients with CKD have been shown to be predicted by plasma ADMA levels (24, 60, 83). ADMA production depends on type 1 protein arginine methyltransferases (PRMTs) (12, 13), whereas ADMA clearance is dependent on dimethylarginine dimethylaminohydrolases (DDAH2) in the endothelium (45). PRMTs and DDAHs are redox sensitive and, as such, their activity is affected by increases/decreases in oxidative stress. PRMT1 stimulation causes increased ADMA synthesis that is associated with oxidative stress (6). Uremia-related oxidative stress and uremic toxins such as homocysteine and advanced glycation end-products decrease DDAH activity, which increases ADMA levels (6).

Thus it is likely that the ADMA-associated deficit of L-arginine in patients with CKD is related to increases in oxidative stress. Therapies that lower ADMA levels and/or PRMT1 expression may be an effective treatment strategy in patients with CKD. Interventions that lower oxidative stress in patients with CKD may also be an effective strategy for lowering ADMA levels due to the redox-sensitive state of PRMT and DDAH. We also measured plasma markers of oxidative stress, including nitrotyrosine, protein carbonyls, and 4-HNE. There were no significant differences in the level of these markers between the two groups; however, nitrotyrosine tended to be increased in the patients with CKD ($P = 0.08$). Oxidative stress markers in the plasma may not fully represent what is occurring at the tissue level, thus these measurements may not be an accurate representation of what is occurring in the cutaneous microvasculature. Nonetheless, our in vivo data clearly demonstrates a role for oxidative stress in microvascular dysfunction in patients with CKD.

**Limitations.** There were several differences in patient populations in addition to the presence of CKD that are known to influence endothelial function such as blood pressure, HbA1c, and hemoglobin. The differences in blood pressure, HbA1c, and hemoglobin cannot be ruled out as potential factors that may have influenced the results of the current study. In addition, the patients with CKD were on a variety of antihypertensive medications that are vasoactive. However, our data demonstrate that despite chronic treatment with a variety of antihypertensive medications, impairments in microvascular function are still present in patients with CKD. Importantly, there were no differences in blood pressure throughout the local heating protocol within and between groups. Thus the baseline difference in systolic blood pressure may not have played a substantial role in the functional differences observed. The patients with CKD were on average older than the HC subjects; however, both groups of subjects included a broad range of ages (CKD: 31–77 yr, HC: 45–67 yr), and there was no significant difference in age between groups ($P = 0.125$).

Recent tissue trauma from the placing of the catheter at the measurement site may have affected our results; however, the experimental protocol allows for the resolution of the hyperemia that occurs upon the placement of the microdialysis fiber before baseline measurements are recorded. We also acknowledge the small number of participants in our study, which may have contributed to the failure to detect significant differences in plasma oxidative stress measurements.

In conclusion, the current study identified an in vivo role for NADPH oxidase-derived ROS in microvascular dysfunction in patients with stage 3–4 CKD. These data also suggest that xanthine oxidase is not a major source of ROS in microvascular dysfunction in patients with CKD. These findings are novel in that they are the first to identify, in vivo, the specific enzymatic sources of ROS in patients with moderate to severe CKD. The identification of NADPH oxidases as a major source of ROS in CKD may offer a novel therapeutic target in improving cardiovascular and renal outcomes in the population with CKD. Future intervention studies are needed to identify methods of reducing the contribution of NADPH oxidase-derived ROS to microvascular dysfunction in patients with CKD.

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**DISCLOSURES**

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

**AUTHOR CONTRIBUTIONS**

Author contributions: J.J.D., W.B.F., R.R.T., and D.G.E. provided conception and design of research; J.J.D., M.G.R., and D.G.E. performed experiments; J.J.D., M.G.R., and D.G.E. analyzed data; J.J.D., M.G.R., W.B.F., R.R.T., and D.G.E. interpreted results of experiments; J.J.D. and D.G.E. prepared figures; J.J.D. drafted manuscript; J.J.D., M.G.R., W.B.F., R.R.T., and D.G.E. edited and revised manuscript; J.J.D., M.G.R., W.B.F., R.R.T., and D.G.E. approved final version of manuscript.

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