Endothelial cell transfusion ameliorates endothelial dysfunction in 5/6 nephrectomized rats

Maricica Pucurari,1 Dongqi Xing,1,2 Rob H. P. Hilgers,1 Yuan Yuan Guo,1,2 Zhengqin Yang,1 and Fadi G. Hage1,2
1Vascular Biology and Hypertension Program, Division of Cardiovascular Disease, Department of Medicine, The University of Alabama at Birmingham, Birmingham, Alabama; and 2Section of Cardiology, Birmingham Veteran’s Administration Medical Center, Birmingham, Alabama

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Endothelial cell transfusion ameliorates endothelial dysfunction in 5/6 nephrectomized rats. Am J Physiol Heart Circ Physiol 305: H1256–H1264, 2013. First published August 16, 2013; doi:10.1152/ajpheart.00132.2013.—Endothelial dysfunction is prevalent in chronic kidney disease. This study tested the hypothesis that transfusion of rat aortic endothelial cells (ECs) ameliorates endothelial dysfunction in a rat model of chronic kidney disease. Male Sprague-Dawley rats underwent sham surgery or 5/6 nephrectomy (Nx). Five weeks after Nx, EC (1.5 × 10^6 cells/rat) or vehicle were transfused intravenously. One week later, vascular reactivity of mesenteric artery was assessed on a wire myograph. Sensitivity of endothelium-dependent relaxation to acetylcholine and maximum vasodilation were impaired by Nx and improved by EC transfusion. Using selective pharmacological nitric oxide synthase isoform inhibitors, we demonstrated that the negative effect of Nx on endothelial function and rescue by EC transfusion are, at least in part, endothelial nitric oxide synthase mediated. Plasma asymmetric dimethylarginine was increased by Nx and decreased by EC transfusion, whereas mRNA expression of dimethylarginine dimethylaminohydrolases 1 (DDAH1) was decreased by Nx and restored by EC transfusion. Immunohistochemical staining confirmed that local expression of DDAH1 is decreased by Nx and increased by EC transfusion. In conclusion, EC transfusion attenuates Nx-induced endothelial-dependent vascular dysfunction by regulating DDAH1 expression and enhancing endothelial nitric oxide synthase activity. These results suggest that EC-based therapy could provide a novel therapeutic strategy to improve vascular function in chronic kidney disease.

endothelial cell; chronic kidney disease; vascular reactivity; 5/6 nephrectomy; nitric oxide synthase

APPROXIMATELY 14% OF the general population of the United States has chronic kidney disease (CKD), and the prevalence of CKD in the United States has chronic kidney disease (CKD), and the prevalence of CKD increases with increasing age (35% in those older than 60 yr) (30a). CKD increases risk for cardiovascular morbidity and mortality; e.g., among Medicare enrollees aged 66 and older, acute myocardial infarction (9.1 vs. 2.2%), heart failure (31.8 vs. 7.3%), and peripheral arterial disease (26.3 vs. 9.3%) are more prevalent among CKD than non-CKD persons, and mortality associated with cardiovascular disease is higher in those with CKD (15, 30a). Despite these worrisome statistics and although current guidelines recommend that patients with CKD be considered in the highest-risk group for subsequent cardiovascular events, few interventions have been shown to reduce cardiovascular disease risk in this patient population (24). There is, therefore, an urgent need to develop novel therapeutic strategies to improve cardiovascular health in CKD.

The relationship between CKD and cardiovascular pathology is bi-directional and likely involves alterations in multiple pathways (e.g., traditional cardiovascular risk factors, inflammation, oxidative stress, calcium-phosphorus balance, anemia, and accumulation of uremic toxins) (15). It has been suggested that endothelial dysfunction is the central link that leads to cardiovascular disease in patients with CKD (28, 32). Endothelial dysfunction is a precursor of increased arterial stiffness, atherosclerosis, restenosis after percutaneous coronary intervention, and stenosis of venous bypasses of coronary arteries and of arteriovenous grafts used for dialysis. Studies examining flow-mediated dilation of brachial arteries (35) and vascular reactivity of ex vivo resistance arteries mounted on a wire myograph (26, 29) have demonstrated impaired endothelial function in arteries of patients with CKD.

Endothelial dysfunction in CKD is thought to be related to reduced nitric oxide release secondary to endothelial nitric oxide synthase (eNOS) inhibition by asymmetric dimethylarginine (ADMA) (41). Levels of ADMA, an endogenous low-molecular weight product of protein hydrolysis, are elevated in CKD because of a combination of decreased renal clearance and decreased degradation by dimethylarginine dimethylaminohydrolases (DDAH) (6). An additional contributor to endothelial dysfunction in the setting of CKD is impairment of endothelial repair due to decrease in endothelial progenitor cell count and function (19). We have recently reported the successful use of endothelial cell (EC) transfusion to inhibit inflammation and neointima formation in balloon-injured rat carotid arteries (38). In the present study we tested the hypothesis that systemic transfusion of rat aortic ECs improves endothelial function by increasing eNOS activity in a rat model of CKD.

MATERIALS AND METHODS

Animals and procedures. Male Sprague-Dawley rats were obtained from Charles River Breeding Laboratories, maintained at constant humidity (60 ± 5%), temperature (24 ± 1°C), and light cycle (6 AM to 6 PM), and fed a standard rat pellet diet ad libitum. All protocols were approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham and were consistent with the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health.

CKD was induced via renal mass reduction by performing 5/6 nephrectomy (Nx) in two stages. Eleven-week-old rats were anesthetized with 80 mg/kg ketamine and 5 mg/kg xylazine and the upper and lower thirds of the left kidney were surgically resected without renal artery ligation (17). One week later, the right kidney was completely

Address for reprint requests and other correspondence: F. G. Hage, Zeigler Research Bldg., 1024, 703 19th St. S, Birmingham, AL 35294-0007 (e-mail: fadihage@uab.edu).

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resected. Sham-operated rats served as controls (Sham). Pilot data showed that serum creatinine progressively increases for 3 wk after Nx and then plateaus at approximately twofold baseline values. Five weeks after Nx, rats were transfused intravenously with normal saline (Nx + Veh) versus 1.5 × 10^6 rat aortic ECs (Nx + EC).

ECs used in the present study were previously characterized (38). Rat aortic ECs and the EC growth medium MCDB-131C were purchased from VEC Technologies (catalog No. MCDB-131C). No other growth factors were added to the medium. Passage 5 to 6 ECs were transduced with adenoviral vector containing green fluorescent protein (GFP) using the AdEasy Adenoviral Vector System (Strategene) and cultured in 100-mm culture dishes until >80% of cells expressed GFP (1.5 × 10^6 cells per dish). Cells were washed with 0.9% saline, collected with a cell scraper, dispersed by gentle pipetting, and then concentrated with centrifugation at 100 g and resuspended in 1.5 ml normal saline. The ECs used in this study were shown to express eNOS and DDAH by Western blot analysis. Rats assigned to Nx + EC were transfused through a femoral venous catheter with a total of 1.5 × 10^6 cells/1.5 ml divided into three doses (0.5 × 10^6 cells/dose), each separated by 2 h.

Immunohistochemistry. To determine the presence and location of transfused ECs, fresh frozen optimal cutting temperature (OCT)-embedded sections of mesenteric arteries were analyzed for detection of GFP and von Willebrand factor (vWF) using a VECTASTAIN ABC kit (Vector, Burlingame, CA). Sections were treated according to the manufacturer’s instructions using specific antibody against GFP or vWF (Abcam, Cambridge, MA), diluted 1:300 and 1:200, respectively, in 5% normal goat serum/PBS and incubated overnight at 4°C. Sections were then incubated with biotinylated secondary antibody for 30 min followed by incubation with the enzyme conjugate for 30 min, both at room temperature. Immunodetection was determined using a VECTA NovaRED peroxidase substrate, and the development of reaction product was monitored under a microscope. After color development, sections were washed, counterstained with hematoxylin, and mounted. The chromogen produces a red/brown reaction product at immunopositive sites, whereas cell nuclei stain blue. To quantify the contribution of transfused ECs versus native ECs to the mesenteric artery endothelium, random sections from mesenteric arteries of seven Nx + EC rats were stained for GFP, and the number of GFP-positive and -negative ECs was counted (total number of counted cells = 999). To evaluate the effect of Nx and EC transfusion on the local expression of DDAH1 in the endothelium of the mesenteric artery, sections were stained using an anti-DDAHI antibody (Abcam), diluted 1:300 in conjunction with the VECTASTAIN ABC kit with no counterstaining. Three readers blinded to treatment independently graded sections of arteries from Sham, Nx + Veh, and Nx + EC rats with respect to DDAH1 staining of the endothelium. The grades were averaged for all readers and presented on an arbitrary scale.

Fluorescent imaging. Tissue fresh frozen OCT-embedded sections from mesenteric arteries, liver, lung, spleen, kidney, and heart were examined using a fluorescent microscope imaging system (Nikon TE2000U) with filters set for GFP emission. To detect whether GFP colocalizes with ECs in the mesenteric artery, indirect immunofluorescence staining was carried out, as previously described (12). The sections were blocked with 10% normal goat serum and then incubated with anti-GFP, anti-CD31 (EC marker), or normal mouse/rabbit IgG at 4°C overnight. The slides were incubated with a fluorescein-conjugated anti-mouse secondary antibody (1:100, catalog No. Fi-2000; Vector) and a Texas-red-conjugated anti-rabbit secondary antibody (1:100, catalog No. Ti-1000; Vector Laboratories) for 1 h at room temperature. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI) (50 ng/ml) in PBS for 15 min. Coverslips were washed, mounted with 90% glycerol, and visualized by fluorescence microscopy (×400).

Vascular reactivity. Seven days after EC transfusion, blood pressure was measured in the carotid artery of conscious rats using a polyethylene cannula. Rats were then anesthetized and terminated by open thoracotomy. Blood samples were obtained by cardiac puncture, and the mesentry was collected and placed immediately in ice-cold Krebs-Ringer buffer (KRB) consisting of 118.5 NaCl, 4.7 KCl, 2.5 CaCl2, 1.2 MgSO4, 1.2 KH2PO4, 25.0 NaHCO3, and 5.5 glucose. Segments of first-order mesenteric artery were collected by pinning down the mesentry on a 90-mm glass petri dish coated with black Silgard presoaked in cold KRB. Under a dissecting microscope, the mesenteric arteries were gently cleaned of adipose and connective tissue and cut into segments of 2 mm in length. The mesenteric artery segments were mounted in a wire myograph (model 610M, Danish Myotechnology, Aarhus, Denmark) and placed in myograph chamber filled with 5 ml KRB, maintained at 37°C, and continuously aerated with 95% O2-5% CO2 to measure vascular reactivity as previously described (18). With the use of LabChart7 software (ADInstruments, Colorado Springs, CO), each segment was progressively stretched to the diameter at which the largest contractile response to 10 μM norepinephrine could be obtained (referred to as optimal diameter and set to 100% contraction). After a 20-min washout period, a cumulative concentration-response curve (CCR) to phenylephrine (0.03–30 μM) was obtained. After a second 20-min washout period, the segments were precontracted with phenylephrine (10 μM) until a stable contraction was obtained, followed by a CCR to the endothelium-dependent muscarinic vasodilator acetylcholine (ACH, 0.001–10 μM). The relaxation responses were recorded in the absence of any pharmacological inhibitors.

To assess the contribution of nitric oxide synthase (NOS) isoforms to endothelial relaxation, mesenteric artery segments were incubated for 30 min with NOS isoform inhibitors, with 30-min washout and resting periods, followed by relaxation responses to ACh (0.001–10 μM) as described above. First, segments were incubated with neuronal NOS inhibitors (nNOS, Nω-propyl-l-arginine, l-NPA, 2 μM (40) and inducible NOS inhibitors [iNOS, Nω-[3-(amimomethyl)phenyl]methyl]l-ethanamidimide dihydrochloride, 1400W, 10 μM] (10) inhibitors. Second, segments were incubated with eNOS inhibitors [N5′-(1-iminomethyl)-l-ornithine HCl, l-nitro, l-NIO, 10 μM] (31) and iNOS (1400W) inhibitors. Finally, segments were incubated with the nonselective NOS inhibitor (Nω-nitro-l-arginine methyl ester, l-NAM, 100 μM) (31), the soluble guanylate cyclase inhibitor (1H[1,2,4]oxadiazolo[4,3,-a]quinazolin-1-one, ODQ, 10 μM), and the cyclooxygenase inhibitor (indomethacin, 1 μM).

To study the effect of exogenous ADMA on vascular reactivity, ACh-induced relaxation (0.001–10 μM) of mesenteric arteries from unoperated male Sprague-Dawley rats was assessed in the presence of 100 μM ADMA (Sigma-Aldrich, St. Louis, MO) as previously described (20). Endothelium-independent relaxation was then tested with the nitric oxide donor sodium nitroprusside (0.001–10 μM). ACh, phenylephrine, norepinephrine, l-NPA, l-NIO, 1400W, and l-NAM (Sigma-Aldrich) were dissolved in double distilled water. ODQ (Calbiochem, EMD Millipore, Billerica, MA) was dissolved in DMSO. Indomethacin (Sigma-Aldrich) was dissolved in ethanol. The concentrations of DMSO and ethanol did not exceed 0.1% in the organ bath.

Quantitative real-time polymerase chain reaction. To test the hypothesis that EC transfusion ameliorates CKD-induced endothelial dysfunction via the DDAH-ADMA-eNOS axis, we measured mesenteric artery DDAHI and eNOS mRNA expression and serum ADMA levels in Sham, Nx + Veh, and Nx + EC rats.

Mesenteric arteries were cleaned of adipose and connective tissue. RNA was extracted using an Ambion PureLink RNA kit (Life Technologies, Grand Island, NY) and quantitated by Nanodrop 2000 spectrophotometer (Thermo Fisher Scientific). Good quality RNA (1 μg) having a ratio of A260/280 > 1.9 was reverse transcribed to cDNA using an Invitrogen SuperScript III kit (Life Technologies), amplified by polymerase chain reaction using Sybr Green master mix (Applied Biosystems) with specific primers for rat DDAHI (5′-ACCGTGACCCAGCCACGCT-3′ and 5′-ACTCTCGGGGACTGAGG-3′, Invitrogen) and eNOS (5′GCTCGGGGATCCAGTCAAC-3′ and 5′-GGCTGTGACTCTGGAGGAGG-3′, Invitrogen) and eNOS (5′-ACATCGATTGAGTTGAGTCT-3′ and 5′-TGGGATCCGATCTGCTCTG-3′, Invitrogen).
CAACG-3' and 5'-GCAGGTCAAGGACCAGG-3') and quantified with the iCycler (Applied Biosystems). Levels of specific mRNAs were normalized by use of reference genes ribosomal protein S9 and 18S, as previously described (5, 14).

Serum creatinine and ADMA and plasma advanced oxidative protein products. Serum creatinine concentration was measured using an liquid chromatography-tandem mass spectrometry (LC-MS/MS) [Waters 2795 separation module (LC)/Micromass Quatro micro API (MS/MS), Analytical Direct, Durham, CT] instrument according to the method described by Young et al. (39). ADMA concentrations were measured using an ELISA kit (MyBioSource, San Diego, CA). Since Nx has been associated with increased oxidative stress (22, 34) and since oxidative stress and eNOS uncoupling are known to induce vascular dysfunction (30), we measured the plasma concentrations of advanced oxidative protein products (AOPPs) that are generated during oxidative stress using the OxiSelect AOPP Assay Kit (Cell Biolab, San Diego, CA).

Data analysis. Contractile responses are expressed as a percentage of the maximal contractile response to 10 μM norepinephrine, whereas relaxation responses are expressed as a percentage relative to maximal contractile response to 10 μM phenylephrine. Individual CCRCs were fitted to a nonlinear sigmoid regression curve (GraphPad Prism 5.0, GraphPad Software, San Diego, CA), and sensitivity (pEC50) and maximal effect (Emax) were calculated. Results are expressed as means ± SE without transformation. Comparisons among experimental groups were performed with one-way ANOVA, followed by Newman-Keuls multiple comparison test when the overall F-test result of ANOVA was significant. Statistical significance was set at P < 0.05.

RESULTS

Renal mass reduction by Nx in male Sprague-Dawley rats resulted in renal dysfunction as assessed by serum creatinine rise. At 6 wk after surgery, serum creatinine increased almost twofold in both Nx groups but remained unchanged from baseline in the sham-operated group (Fig. 1A, P < 0.05). Mean arterial pressure, measured with an invasive carotid artery catheter in conscious rats, was slightly but significantly increased following Nx (118 ± 3 vs. 108 ± 2 mmHg for Nx vs. Sham, Fig. 1B, P < 0.05). EC transfusion, at 5 wk after Nx, had no significant effect on serum creatinine or blood pressure measured 1 wk later.

Transfused ECs attach to the endoluminal surface of mesenteric arteries and are integrated into the arterial endothelium. At 7 days after intravenous transfusion of GFP-labeled ECs or saline, tissues were harvested, embedded fresh frozen in OCT, and sectioned. On fluorescent microscopy, GFP was not detected in sections from the liver, lung, or heart, but small numbers of GFP-positive cells were seen in the spleen and kidney of Nx + Veh rats. GFP was detected on the endoluminal surface of mesenteric arteries of Nx + EC rats and colocalized with CD31 (Fig. 2A). No GFP was detected in sections from Sham or Nx + Veh rats.

Immunostaining for GFP was negative in Sham and Nx + Veh rats. Nx + EC rats showed positive GFP staining in the endothelium of the mesenteric artery (Fig. 2B). vWF staining confirmed that these cells are ECs (Fig. 2C). Sections from large caliber vessels (carotid arteries) showed similar staining (data not shown). Quantitative analysis showed that GFP-positive cells constitute ~13% of ECs of the mesenteric arteries.

EC transfusion ameliorates CKD-induced endothelial dysfunction. Vascular function of segments of first order mesenteric arteries from Sham, Nx + Veh, and Nx + EC rats was assessed using wire myography. There was no significant effect of renal mass reduction or EC transfusion on the contractile reactivity to phenylephrine. The maximal contraction (Emax) to 30 μM phenylephrine [111 ± 3, 117 ± 7, and 112 ± 5% for Sham, Nx + Veh, and Nx + EC, respectively; *P < 0.05 by ANOVA for both graphs. *P < 0.05 vs. Sham].

Mesenteric arteries from Nx + Veh rats had severe impairment in endothelial-dependent relaxation in response to ACh compared with Sham controls, with a rightward shift of the CCRC curve (Fig. 4). Sensitivity to 0.001–10 μM ACh (pEC50) averaged 7.21 ± 0.10 in Nx + Veh rats and 8.02 ± 0.06 in Sham controls (Fig. 4, top, right, P < 0.05). Furthermore, mesenteric artery maximal relaxation (Emax) to 10 μM ACh was significantly lower in Nx + Veh rats (79 ± 7%) than in Sham controls (96 ± 2%, Fig. 4, bottom, right, P < 0.05). EC transfusion significantly increased ACh-induced endothelium-dependent vasorelaxation. ACh pEC50 in the Nx + EC group averaged 7.56 ± 0.05, significantly higher than 7.21 ± 0.10 in Nx + Veh group (Fig. 4, top, right, P < 0.05). Emax to 10 μM ACh was also significantly increased in Nx + EC (93 ± 3%, P < 0.05, Fig. 4, bottom, right) versus Nx + Veh (79 ± 7%). Importantly, EC transfusion in Nx rats completely normalized Emax to 10 μM ACh, with no significant difference between Nx + EC and Sham control. Sensitivity to ACh (pEC50) in Nx + EC was significantly lower than in Sham control.

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The effect of EC transfusion on CKD-induced endothelial dysfunction is mediated by eNOS. To elucidate the mechanism by which EC transfusion ameliorates CKD-induced endothelial dysfunction, we examined the contributions of the three NOS isoforms to endothelium-dependent ACh-induced relaxation (0.001–10 μM) of mesenteric arteries (Fig. 5). In the Sham control group (Fig. 5, A and B), inhibition of nNOS (with 2 μM L-NPA) and iNOS (with 10 μM 1400W) did not significantly change $E_{\text{max}}$ to 10 μM ACh. In contrast, inhibition of eNOS (with 10 μM ML-NIO) and iNOS (with 10 μM 1400W) resulted in a significant decrease in $E_{\text{max}}$ compared with 10 μM ACh alone (65 ± 7% vs. 95 ± 2%, $P < 0.05$, Fig. 5B). Inhibition of nitric oxide-dependent ACh-induced relaxation using the non-selective NOS inhibitor L-NAME (100 μM) and the soluble guanylate cyclase inhibitor ODQ (10 μM) together with prostacyclin inhibition using the cyclooxygenase inhibitor indomethacin (10 μM) further blunted maximal relaxing responses to ACh (41 ± 7%, $P < 0.05$ vs. ACh alone and vs. l-NIO + 1400W).

In arteries from Nx + Veh animals, the maximal relaxation response to 10 μM ACh was significantly reduced compared with those from Sham control animals in the presence of nNOS and iNOS inhibition (l-NPA + 1400W) (46 ± 5 vs. 82 ± 4%, $P < 0.05$, Fig. 5C). EC transfusion abrogated the effect of Nx on the maximal relaxation response to 10 μM ACh ($E_{\text{max}}$ vs. Sham, 82 ± 4%). In contrast, when eNOS and iNOS were inhibited (l-NIO + 1400W), Nx and EC transfusion did not significantly change $E_{\text{max}}$ to 10 μM.

**Fig. 2.** ECs are incorporated in the mesenteric artery endothelium of Nx rats at 7 days after systemic transfusion. A: representative micrograph of cross-section of mesenteric artery from a 5/6 Nx rat transfused with ECs labeled with green fluorescent protein (GFP) 7 days before harvest. Double immunofluorescent staining for GFP (green) and CD31 (red) indicates the presence of transfused cells in the endothelium and demonstrates that the GFP-positive cells are ECs. B: representative GFP immunohistochemically stained micrographs (×1000) of mesenteric artery from 5/6 Nx rats transfused with ECs. GFP-labeled ECs are seen in the endothelium of Nx + EC rats at 7 days after EC transfusion. C: staining for the EC marker, von Willebrand factor (vWF), confirms that these cells are ECs.

**Fig. 3.** Nx does not alter vascular contractility of mesenteric arteries. Vascular contractility of mesenteric arteries from Sham, Nx + Veh, and Nx + EC rats. Left: cumulative concentration-response curves to phenylephrine (Phe; 0.03–30 μM) for the 3 groups. $pEC_{50}$ (top, right) and $E_{\text{max}}$ (bottom, right) are shown. The numbers above the bars indicate the number of mesenteric arteries analyzed in each group. There was no statistical difference between the groups via ANOVA.
ACh (Fig. 5E). Thus Nx induced and EC transfusion ameliorated endothelial dysfunction only when eNOS was not inhibited, suggesting that the negative effect of Nx on endothelial function and the rescue by EC transfusion are eNOS mediated.

**EC transfusion reverses the effects of CKD on the DDAH-ADMA axis and on oxidative stress.** Nx and EC transfusion did not significantly alter mRNA expression of eNOS in mesenteric arteries (1.00 ± 0.08, 0.98 ± 0.14, and 0.90 ± 0.09 for Sham, Nx + Veh, and Nx + EC, respectively; ANOVA, P = NS). Serum ADMA concentration was significantly increased at 6 wk after Nx (0.40 ± 0.06 vs. 0.25 ± 0.03 μM for Nx + Veh vs. Sham, respectively; Fig. 6A, P < 0.05). EC transfusion in Nx rats decreased serum ADMA significantly to a level not different from Sham control (0.21 ± 0.01 μM, P < 0.05 vs. Nx + Veh, P = NS vs. Sham). Mesenteric artery DDAH1 mRNA expression was significantly decreased by Nx and restored to normal levels by EC transfusion (1.00 ± 0.24, 0.41 ± 0.06, and 1.24 ± 0.15 for Sham, Nx + Veh, and Nx + EC, respectively; P < 0.05 for Nx + Veh vs. Sham and for Nx + EC vs. Nx + Veh and NS for Nx + EC vs. Sham). Immunohistochemical staining for DDAH1 confirmed that local expression of DDAH1 protein is decreased in Nx + Veh versus Sham (Fig. 6B). EC transfusion increased DDAH1 protein expression compared with NX + Veh, but this level remained lower than that seen in sham-operated rats. To confirm that ADMA impairs endothelial function, we studied the effect of exogenous ADMA administration on ACh-induced mesenteric artery relaxation (Fig. 6C). Endothelium-dependent relaxation was inhibited by pretreatment with 100 μM ADMA, whereas endothelium-independent relaxation remained intact.

AOPP levels, a reflection of oxidative stress, were significantly elevated in Nx + Veh versus Sham (333 ± 48 vs. 211 ± 23 μM, P < 0.05, Fig. 6D). In Nx rats, EC transfusion significantly reduced AOPP levels compared with vehicle (231 ± 12 vs. 333 ± 48 μM for Nx + EC vs. Nx + Veh, respectively; P < 0.05). Importantly, EC transfusion normalized AOPP levels, resulting in no significant difference in levels between Nx + EC versus Sham control rats (Fig. 6D, P = NS for Nx + EC vs. Sham).

**DISCUSSION**

This study showed for the first time that rat aortic ECs transfused intravenously incorporate into the arterial endothelium of Nx rats and improve small vessel endothelial function. The findings reported here support our hypothesis that trans- fusion of ECs replenishes the damaged endothelium in Nx rats and normalizes elevated systemic ADMA levels, likely by delivering DDAH-1 to the endothelium, releasing inhibition of eNOS, and therefore reversing CKD-induced endothelial dysfunction. This study demonstrates the potential of EC-based therapy as a novel therapeutic strategy to improve vascular function in CKD.

The association between CKD and cardiovascular disease is well established, but the mechanism for this association is not fully understood. Since cardiovascular risk is evident even with mild reductions in renal function (11), the pathophysiological link is presumed to occur at an early stage in the disease process. The endothelium plays a crucial role in maintaining vascular health by regulating vascular tone, hemostasis, inflammatory cell adhesion, and migration into the vessel wall, platelet activity, and angiogenesis (37). Studies have demonstrated endothelial dysfunction in arteries of patients with both early and advanced-stage CKD, but these studies were often confounded by increases in traditional risk factors, which are independently associated with abnormalities in endothelial function (e.g., diabetes mellitus and hypertension) (28). Studies that have demonstrated endothelial dysfunction in animal models of CKD have also been confounded by the development of severe hypertension in the setting of CKD (36). We used a CKD renal mass reduction model that avoids renal infarction by directly resecting renal tissue. Consistent with previous studies that used this model (1), we observed only slight elevations in systemic blood pressure (mean, 10 mmHg), and the blood pressure of Nx animals remained in the normotensive range.
range. In the absence of hypertension, CKD was associated with endothelial dysfunction with severe impairment in ACh-induced endothelium-dependent relaxation. Furthermore, we have demonstrated that CKD-induced endothelial dysfunction is dependent on eNOS (Fig. 5). In sham-operated control rats, pharmacological inhibition of nNOS and iNOS did not inhibit endothelium-dependent maximum relaxation, whereas inhibition of eNOS did. Moreover, EC transfusion rescued the endothelial dysfunction caused by Nx when nNOS and iNOS were inhibited (but eNOS was not) but had no effect on endothelial function when eNOS was inhibited. Interpretation of these data is limited by the incomplete selectivity of the inhibitors of the NOS isoforms. For example, although L-NIO is considered a selective inhibitor of eNOS, it inhibits to a lesser degree both nNOS and iNOS (3). Furthermore, there was a statistically nonsignificant trend toward inhibition of ACh-induced vasodilation with L-NPA, and L-NIO + 1400W did not completely inhibit ACh-induced vasodilation. This suggests that nNOS may also be involved in this process, as recently shown (33, 34).

Patients with CKD have markedly decreased numbers of circulating endothelial progenitor cells compared with healthy volunteers (7, 8, 23). Endothelial progenitor cells contribute to vascular integrity by homing to sites of vascular injury and incorporating into the endothelium (9). In addition to their decreased number, progenitor cells derived from CKD patients have functional impairment, including impaired migratory function in response to vascular endothelial growth factor, decreased adherence to fibronectin-coated diureido-pyrimidinone-polycaprolactone, decreased incorporation into EC vasculogenic structures in culture (Matrigel tube formation assay), reduced endothelial outgrowth potential, and reduced antithrombogenic function (7, 23). A recent study by Lorenzen et al. (25) of 265 patients with end-stage CKD demonstrated that reduction in functionally active endothelial progenitor cells is an independent predictor of incident cardiovascular events over a median follow-up period of 36 mo. Taken together, these studies suggest that CKD is associated with impairment of endogenous capacity to repair the vasculature. When combined with increased endothelial injury in CKD, this results in an imbalance that favors endothelial dysfunction (19). Local delivery of endothelial progenitor cells to balloon-injured arteries with denuded endothelium has been shown to accelerate endothelialization and improve endothelium-dependent vasoreac-

**Fig. 5.** Nx induced vascular dysfunction is mediated by endothelial nitric oxide synthase. Vascular relaxation of mesenteric arteries from Sham, Nx + Veh, and Nx + EC rats in the presence of nitric oxide synthase inhibitors. Cumulative concentration-response curves to ACh (0.001–10 μM; A) and E\text{max} (B) for mesenteric arteries from sham-operated animals that were preincubated with L-NPA (2 μM), N\text{5}-\{1-iminoethyl\}-L-ornithine HCl (L-NIO; 10 μM) + 1400W (10 μM), or N\text{5}-nitro-L-arginine methyl ester (L-NAME; 100 μM) + 1H[1,2,4]oxadiazolo[4,3-d]quinoxalin-1-one (ODQ; 10 μM) + indomethacin (Ind; 10 μM) for 30 min. P < 0.05 by ANOVA. *P < 0.05 vs. Veh; #P < 0.05 vs. L-NPA + 1400W; $P < 0.05 vs. L-NIO + 1400W. ACh E\text{max} for mesenteric arteries from Sham, Nx + Veh, and Nx + EC rats preincubated with L-NPA + 1400W (C), L-NIO + 1400W (D), and L-NAME + ODQ + Ind (E) for 30 min. The numbers above the bars indicate the number of mesenteric arteries analyzed in each group. P < 0.05 by ANOVA for C. *P < 0.05 vs. Sham; #P < 0.05 vs. Nx + Veh.
Our current data show that systemically transfused, functionally active ECs integrate into the endothelium of CKD rats (Fig. 2) and improve endothelial function of their mesenteric arteries 7 days after their introduction (Fig. 4). The effect of cell therapy on vascular function in human CKD has not been evaluated. Furthermore, the mode of delivery is a critical factor in clinical cell therapy, and systemic administration, the strategy used in these experiments, is attractive for future clinical applications (38).

ADMA, a natural inhibitor of eNOS, is a metabolic byproduct of the hydrolysis of methylated proteins that has been implicated in the pathogenesis of cardiovascular disease in CKD (41). Elevated levels of ADMA in CKD were originally attributed to decreased renal clearance of the protein, but it is now evident that the majority of ADMA is catabolized by DDAH into inactive metabolites. Animal experiments have demonstrated that genetic manipulation of DDAH can modulate circulating ADMA levels, which in turn can alter cardiovascular physiology (6). For example, DDAH overexpression using adenovirus-mediated gene transfer in Nx rats lowered circulating ADMA levels and prevented the elevation of blood pressure seen with Nx (27). Recently, Kajimoto et al. (20) showed that in transgenic mice overexpressing DDAH1, circulating ADMA levels did not increase with Nx and were 50% lower than in wild-type sham-operated mice. Importantly, unlike the aortic rings from wild-type Nx mice, those from transgenic Nx mice did not exhibit impaired endothelium-dependent relaxation, suggesting that an increase in DDAH1 can reverse Nx-induced endothelial dysfunction. Our current data show that the administration of EC to Nx rats replenishes DDAH1 levels in the endothelium. The increased local expression of DDAH1 was associated with systemic decreases in ADMA levels and oxidative stress and improvement in endothelial function despite a stable level of expression of eNOS. It has been shown that ADMA can uncouple eNOS in the vascular endothelium, resulting in endothelial dysfunction due to decreased bioavailability of nitric oxide and increased vascular oxidative stress (2, 21, 30). These observations offer an explanation for the elevated levels of AOPPs and the decreased activity of eNOS in our Nx + Veh rats as well as the restoration of vascular function and decreased AOPPs associated with decreased ADMA levels after the administration of ECs.

In conclusion, the studies described here provide the framework for novel therapeutic interventions that use cell therapy that homes to the endoluminal surface of the vasculature to deliver DDAH1 locally to the endothelium to reverse CKD-induced endothelial dysfunction.

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Present address of M. Pacurari: College of Science, Engineering, and Technology, Jackson State University, Jackson, MS.

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Fig. 6. EC transfusion increases local dimethylarginine dimethylaminohydrolases 1 (DDAH1) expression in vasculature of Nx rats leading to decrease in asymmetric dimethylarginine (ADMA) levels in circulation. Serum ADMA level (4) and local expression of DDAH1 by immunohistochemistry (IHC, protein) (8) in Sham, Nx + Veh, and Nx + EC are shown. C: exogenous ADMA (100 µM) inhibits ACh-induced relaxation (0.001–10 µM) of mesenteric arteries but not endothelium-independent relaxation tested with the nitric oxide donor sodium nitroprusside (SNP; 0.001–10 µM). D: oxidative stress, measured as serum level of advanced oxidative protein products (AOPPs) in rats at 7 days after transfusion of ECs or vehicle indicates that Nx is associated with increased oxidative stress and that EC transfection lowers AOPP levels. The numbers above the bars indicate the number of mesenteric arteries analyzed in each group. P < 0.05 by ANOVA for all graphs. *P < 0.05 vs. Sham; #P < 0.05 vs. Nx + Veh. AU, arbitrary units.
DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

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