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Selective Inhibition of Arginase-2 in Endothelial Cells but not Proximal Tubules Reduces Renal Fibrosis

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Running headline: Role of Arginases in Kidney Fibrosis

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Abstract

Fibrosis is the final common pathway in the pathophysiology of most forms of chronic kidney disease (CKD). As treatment of renal fibrosis still remains largely supportive, a refined understanding of the cellular and molecular mechanisms of kidney fibrosis and the development of novel compounds are urgently needed. Whether arginases play a role in development of fibrosis in CKD is unclear. We hypothesize that endothelial-arginase-2 (Arg2) promotes the development of kidney fibrosis induced by unilateral ureteral obstruction (UUO).

Arg2 expression and arginase activity significantly increased following renal fibrosis. Pharmacological blockade or genetic deficiency of Arg2 conferred kidney protection following renal fibrosis as reflected by a reduction in kidney interstitial fibrosis and fibrotic markers. Selective deletion of Arg2 in endothelial cells (Tie2\textsuperscript{Cre}/Arg2\textsuperscript{flox/flox}) reduced the level of fibrosis after UUO. In contrast, selective deletion of Arg2 specifically in proximal tubular cells (Ggt1\textsuperscript{Cre}/Arg2\textsuperscript{flox/flox}) failed to reduce renal fibrosis after UUO. Furthermore, arginase inhibition restored kidney nitric oxide (NO) levels, oxidative stress, and mitochondrial function following UUO.

These findings indicate that endothelial-Arg2 plays a major role in renal fibrosis via its action on NO and mitochondrial function. Blocking Arg2 activity or expression could be a novel therapeutic approach for prevention of CKD.

Key Words: Kidney fibrosis, arginase, Nitric oxide, Mitochondrial function.
Introduction

Novel therapeutic interventions for preventing or attenuating renal fibrosis remain a focus of significant interest. Fibrosis is the final common pathway in the pathophysiology of most forms of chronic kidney disease (CKD), and involves glomerular sclerosis and/or interstitial fibrosis that ultimately leads to end-stage renal failure (1-3). A major hallmark of renal tubulointerstitial fibrosis is the accumulation of myofibroblasts and extracellular matrix proteins (4-7). Unilateral ureteral obstruction (UUO) is an established experimental model of progressive renal tubulointerstitial fibrosis (8). Ureteral obstruction results in marked renal hemodynamic and metabolic changes, followed by tubular injury and cell death by apoptosis or necrosis, with interstitial macrophage infiltration (9). Currently, no effective interventions are available to alleviate renal fibrosis in clinical practice. Therefore, exploring the molecular mechanism of renal fibrosis as well as identifying new therapeutic modalities have great significance for delaying CKD progression.

Endothelial cell dysfunction is a central pathophysiological mechanism that contributes to renal fibrosis, mainly via undergoing endothelial–mesenchymal transition and apoptosis (10, 11). The importance of endothelial cells and pericytes as a major source of renal collagen-producing cells following UUO has only recently been recognized, which shifts attention from the tubular epithelial cell to the renal vasculature as a focus for renal fibrotic injury (12). In addition, endothelial dysfunction promotes vascular permeability and leukocyte recruitment/adhesion, leading to further changes in renal perfusion and O2 delivery and thus to inflammation (13, 14). Importantly, dietary arginine supplementation improved renal fibrosis, apoptosis, and macrophage infiltration after UUO (15) by increasing the expressions of endothelial nitric oxide synthase-3
(NOS3) and enhancing arginine availability for NOS3 activity to NO in lieu of producing superoxide generator (16). Therefore, targeting therapeutic interventions to promote endothelial/epithelial function may be an effective strategy to reduce renal fibrosis.

Dramatic alterations in arginine metabolism occur in endothelial injury (17-19) due to changes in the activity and/or expression of nitric oxide (NO) synthases (NOS) and arginases. Arginase catalyzes hydrolysis of L-arginine to L-ornithine and urea and thus competes with NOS for the common substrate L-arginine (20). Depending on the stimulus, either or both arginases may be expressed and induced in macrophages, endothelial cells, and other cell types (21, 22). Arginase 2 (Arg2) is constitutively expressed and also inducible in kidney cells such as endothelial cells (17-19) and tubular epithelial cells (23-25). Arg2 is localized in the mitochondria with the highest expression in the kidney (21, 23). We showed that arginase inhibition or deficiency not only prevents the development but also the progression of diabetic nephropathy in animal models of diabetes (25, 26) via an NOS3-dependent mechanism (27). However, the role of arginases in the pathogenesis of renal fibrosis is not known.

In the current study, we tested the hypothesis that arginases are a critical determinant of renal fibrosis. Toward this goal, we employed several approaches such as arginase inhibition in conjunction with full body Arg2 deficient mice, and cell specific Arg2 knockout in renal endothelial and proximal tubular cells in a well-established UUO fibrosis model. Our results indicate that targeting endothelial Arg2 activity or expression may be a novel therapeutic intervention to prevent kidney fibrosis and to reduce the incidence of kidney failure associated with CKD.
Results

Increased arginase expression and activity in renal UUO fibrosis model.

Male wild-type (WT) mice were subjected to UUO for 7 days, then kidney tissues were removed and analyzed. Arg1 mRNA and protein expressions were virtually undetectable in sham mice (Fig. 1A and C) but were increased in the obstructed kidney. Whole kidney Arg2 mRNA and protein expressions increased significantly after UUO (Fig. 1B and C), paralleling the increase in kidney arginase activity (Fig. 1D) compared to sham. This raises the possibility of an effect of Arg1 and/or Arg2 to mediate renal fibrosis.

Inhibition of arginase reduces kidney fibrosis and fibrotic markers after renal UUO.

WT mice were subjected to UUO and treated with either vehicle or the non-selective arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC) (2.3 mg/kg/day) via osmotic minipump for 7 days beginning at the time of surgery. Vehicle-treated UUO mice displayed an increase in percent of fibrosis (using ImageJ) in Masson’s trichrome (Fig. 2A-B) and Sirius red (Fig. 2C-D) stained sections 7 days after UUO injury, which was reduced by treatment with the arginase inhibitor BEC. Similarly, real-time PCR analysis show marked increases in kidney fibronectin (Fig. 2E) and smooth muscle actin (Fig. 2F) following UUO. Inhibition of arginases by BEC significantly blunted the increase in these fibrosis markers.

Deficiency of Arg2 reduces kidney fibrosis and fibrotic markers after renal UUO.

WT and Arg2−/− mice were subjected to UUO and treated with either vehicle or BEC via osmotic pump for 7 days. Whereas WT mice displayed an increase in percent of fibrosis
in Masson’s trichrome (Fig. 3A-B) and Sirius red (Fig. 3C-D) stained sections 7 days after UUO injury, deletion of Arg2 or inhibition of arginase in WT mice reduced the level of fibrosis after UUO. Similarly, real-time PCR analysis show marked reduction in whole kidney fibronectin (Fig. 3E) and smooth muscle actin (Fig. 3F) after deletion of Arg2 or inhibition of arginase in WT mice.

**Deficiency of Arg2 does not reduce kidney macrophage infiltration after renal UUO.**
Both WT and Arg2−/− mice showed a marked increase in infiltrating kidney macrophages following UUO (Fig. 4A-B) compared to sham controls. Interestingly, BEC treatment did not reduce macrophage infiltration after UUO in either WT or Arg2−/− mice.

**Selective deficiency of Arg2 in endothelial cells, but not proximal tubular cells reduces kidney fibrosis and fibrotic markers after renal UUO.**
We next sought to determine the effects of cell specific arginase deletion in proximal tubular and endothelial cells using Ggt1Cre/Arg2floxflox and Tie2Cre/Arg2floxflox mice; respectively, following UUO. We first generated and confirmed Arg2floxflox mice with two LoxP site (Supplemental Fig. 1). Arg2floxflox mice were crossed with commercially available Ggt1Cre and Tie2Cre mice to obtain Arg2 specific deletion in proximal tubular and endothelial cells (Supplemental Fig. 2A). Tubular epithelial and endothelial cells were isolated from kidneys to confirm Arg2 deletion from Ggt1Cre/Arg2floxflox (Supplemental Fig. 2B) and Tie2Cre/Arg2floxflox (Supplemental Fig. 2C) mice; respectively. As expected, Arg2floxflox mice (controls) displayed an increase in percent of fibrosis in Masson’s trichrome (Fig. 5A-B) and Sirius red (Fig. 5C-D) stained sections 7 days after UUO injury.
In contrast, deletion of Arg2 specifically in endothelial cells (Tie2\textsuperscript{Cre}/Arg2\textsuperscript{floxfloxflox}) reduced the level of fibrosis after UUO. Interestingly, deletion of Arg2 specifically in proximal tubular cells (Ggt1\textsuperscript{Cre}/Arg2\textsuperscript{floxfloxflox}) failed to reduce renal fibrosis and was similar to vehicle treated UUO in Arg2\textsuperscript{floxfloxflox} mice. Similarly, real-time PCR analysis of Arg2\textsuperscript{floxfloxflox} mice (controls) showed marked increases in whole kidney smooth muscle actin (Fig. 5E) following UUO. In contrast, deletion of Arg2 specifically in endothelial cells (Tie2\textsuperscript{Cre}/Arg2\textsuperscript{floxfloxflox}); but not in proximal tubular cells (Ggt1\textsuperscript{Cre}/Arg2\textsuperscript{floxfloxflox}) reduced whole kidney \(\alpha\)-smooth muscle actin after UUO.

**Inhibition of arginases restores kidney NO and mitochondrial function after renal UUO.**

Whereas renal UUO reduced total kidney NOx (Fig. 6A) and increased kidney thibarbituric acid reactive substances (TBARS) as an indicator for oxidative stress (Fig. 6B), arginase inhibition significantly restored total kidney NOx and TBARS levels after UUO. In addition, mice subjected to UUO had a marked decrease in kidney levels of mitochondrial ATP (Fig. 6C) and complex I activity (Fig. 6D), and this was reversed with BEC treatment. In contrast, vehicle-treated UUO mice had a significant increase in kidney Mitochondrial Ca\(^{2+}\) uniporter complex (MCU) protein expression compared to sham after 7 days of UUO (Fig. 6E). UUO mice treated with arginase inhibition restored MCU complex to normal levels.
Discussion

Fibrosis is the final common pathway in the pathophysiology of most forms of CKD (1-3). Currently, no available treatment modalities exist for renal fibrosis. Arginases have a well-established role to alter endothelial function in cardiovascular diseases (17-19), yet their role in renal fibrosis has not previously been determined. To examine the direct role of arginases in renal fibrosis along with its cellular target and mechanism of action, we employed several approaches in genetically altered mice in a well-established UUO fibrosis model. First, we show increased Arg2 expression and arginase activity following renal fibrosis. Second, we show that pharmacological blockade or genetic deficiency of Arg2 mediates renal tissue protection following renal fibrosis, as reflected by preservation of kidney interstitial fibrosis and fibrotic markers. Our results confirm previous report showing arginase inhibition protects the kidney from structural damage in the 5/6 renal mass ablation/infarction model of CKD (28). Third, we showed using our newly developed Arg2\(^{flox/flox}\) mice that selective deletion of Arg2 specifically in endothelial cells, but not in proximal tubular epithelial cells, reduced the level of fibrosis after UUO. Our isolated endothelial and proximal tubule cells showed high but not total Arg2 knockdown, but the deficiency was enough to be considered relevant for our studies and conclusions. Fourth, we investigated the possible mechanism(s) of action of Arg2 and found that Arg2 effects could be mediated via restoring NO, oxidative stress, and mitochondrial function following UUO fibrosis model. Our study however reflects the short-term nature of UUO and that additional longer-term studies are needed. Previous reports showed that treatment with arginase inhibitors reduced fibrosis and collagen deposition in aorta (29, 30), heart (30), lung (31-33), intestine (34), and peritoneal membrane (35). Since the liver has a very high
amount of Arg1, enormous amounts of an inhibitor would be required to inhibit liver arginase sufficiently for any inhibition of the urea cycle to become apparent. Importantly, the reactions of the urea cycle are not diffusion-controlled but are tightly coupled, such that arginine generated within the urea cycle and used by arginase does not exchange with the free arginine pool within the cell (36). Consequently, a competitive arginase inhibitor will not exchange with arginine generated within the urea cycle and thus should have little to no effect on the urea cycle. Furthermore, competitive arginase inhibitors have been used in many animal studies, and hyperammonemia or other adverse effects have not been reported in any of them. Our findings therefore, reveal an important role for Arg2 in the pathogenesis of renal fibrosis and provide evidence for arginase inhibition as potential new therapeutic modality for treating CKD patients.

Arg2 is highly expressed in kidney cells such as endothelial cells (17-19), and tubular epithelial cells (23-25). Unlike Arg2, Arg1 is undetectable at the protein level under normal conditions (our data and (25)), yet both isoforms were elevated following renal fibrosis indicating a possible role of Arg2 and/or Arg1 to mediate renal tissue injury following UUO. Arg1 and Arg2 are encoded by different genes, differ with regard to their tissue distribution and subcellular localization, and are independently regulated (21, 22). The fact that Arg1 protein was elevated in renal fibrosis suggests a possible contribution of Arg1 to the development and progression of renal fibrosis, possibly via its expression in infiltrating macrophages. Additional experiments are needed to explore this possibility. The fact that pharmacological blockade or genetic deficiency of Arg2 reduced renal fibrosis indicates that Arg2 is the primary target for arginase inhibition. Increased expression of Arg1 and Arg2 in endothelial cells stimulated conversion of arginine to proline (37) suggesting that
this may be one mechanism whereby elevated arginase contributes to increased fibrosis in UUO.

Endothelial dysfunction, characterized by reduced bioavailability of NO and increased oxidative stress, is a hallmark of renal fibrosis, mainly via capillary loss and consequent renal ischemia and hypoxia (10, 11). NO is produced from arginine by NOS. Under conditions of low arginine availability or hypoxia, endothelial NOS (NOS3) is uncoupled, producing reactive oxygen species (ROS) and oxidative stress in lieu of NO (38, 39). Our data show that the protective effect of arginase inhibition to reduce renal fibrosis could be mediated by restoring NO levels and oxidative stress. The mechanism by which arginase inhibition reduces kidney oxidative stress could be due to decreased immune response, or increased availability of arginine to NOS3. Endothelial cells have been recently recognized as a pivotal target for renal fibrosis (12). Similarly, dysfunction and loss of tubular epithelial cells play a central role in renal fibrosis. Renal tubules are very sensitive to oxygen deprivation or nephrotoxic substances (40, 41); likely due to reduced ATP production and/or function, and generation of ROS (42). Renal tubular cells can also produce a number of proinflammatory cytokines, including TNFA, IL6, TGFB, and chemokines such as RANTES, MCP1, ENA78, GroA, and IL8 (43, 44). Therefore, targeting therapeutic interventions to promote endothelial/epithelial function may be an effective strategy to reduce renal fibrosis. Toward this goal, we further defined the contribution of proximal tubule and endothelial cell arginases in kidney injury using tissue-specific deletion of Arg2 in proximal tubule and endothelial cells. Our data show that selective deletion of Arg2 specifically in endothelial cells blunted the increase in renal fibrosis after UUO indicating a possible direct role of endothelial cells-Arg2 in the
pathogenesis of renal fibrosis. It is important to note that Tie2cre may result in Arg2 being knocked out both in endothelial and immune cells (45). Arg2 is expressed in bone-marrow (BM)-derived monocyte/macrophages, and activation of M1 macrophages by LPS exclusively induces Arg2 but not Arg1 expression in murine and human macrophages (46). Conversely, silencing Arg2 expression in human monocyte/macrophage cell lines or macrophages from Arg2−/− mice decreases pro-inflammatory cytokine levels (46). We however speculate that endothelial cells are the primary trigger for Arg2 effect. This conclusion is based on our finding demonstrating increased macrophages infiltration after UUO in both Arg2 wild type and Arg2 knockout mice compared to sham. Arginase inhibition failed to reduce the elevated macrophage infiltration after UUO indicating that the protective effect of arginase deficiency and arginase inhibition in UUO model is independent of macrophages infiltration. Interestingly, while deletion of Arg2 specifically in proximal tubules failed to reduce renal fibrosis indicating that proximal tubular epithelial cells-Arg2 is not important to mediate renal tissue injury, however arginase inhibition also failed to reduce renal tissue fibrosis in Ggt1Cre/Arg2fl/fl after UUO indicating that part of the effect of arginase inhibition could be mediated through Arg1 and/or non-proximal tubular epithelial cells. Although in vivo inhibition of arginases improved high blood pressure (47), yet our data show no differences in blood pressure between Tie2Cre/Arg2fl/fl and Ggt1Cre/Arg2fl/fl compared to Arg2fl/fl (Supplemental Table 1). Similarly, our previous reports (25-27, 48) showed that the beneficial effects of arginase inhibition were not due to reductions in blood pressure in diabetic nephropathy model.
Mitochondrial dysfunction has emerged as a new therapeutic target in renal injury (49-58). In the kidney, proximal tubules and endothelial cells are especially vulnerable to mitochondrial dysfunction (53, 59-63) and contribute to oxidative stress, persistent energy depletion, impairment of energy-dependent repair mechanisms, and cell death in kidney injury (59, 60). Mitochondrial Ca\(^{2+}\) (\(m\text{Ca}^{2+}\)) uptake is driven by the electrochemical gradient across the inner mitochondrial membrane and facilitated by the highly selective \(m\text{Ca}^{2+}\) uniporter (MCU) (64-66). The MCU complex comprises multiple functional domains with the MCU as the central pore-forming subunit (67). MCU is a multimeric complex that mediates the rapid uptake of cytosolic Ca\(^{2+}\) from intracellular store release (68-76). We have shown that endothelial cells mechanotransduction-mediated regulation of MCU activity in the context of vascular physiology (77). MCU has been shown to be dysregulated in hypoxia and ischemic-reperfusion injury; and that restoration of MCU function prevented mitochondrial dysfunction and cell death (78). Importantly, \(\text{Arg}2\) is localized in the mitochondria, with the highest expression in the kidney and endothelial cells (21, 24). However, the role of \(\text{Arg}2\) in kidney mitochondrial dysfunction and/or MCU complex in renal fibrosis has not been investigated. Our data show that arginase inhibition restored the reduction in kidney mitochondrial ATP, complex 1 activity, and MCU complex. To our knowledge this is the first study linking MCU with renal fibrosis. Our results, therefore could support a role for \(\text{Arg}2\) in mitochondrial biogenesis following renal fibrosis. Additional studies are needed to confirm a cause-effect relationship.

In conclusion, our study demonstrates for the first time that \(\text{Arg}2\) plays an essential role in the development of renal fibrosis, mainly by targeting \(\text{Arg}2\) expressed in endothelial cells. The mechanism of renal tissue protection using arginase inhibition in renal fibrosis
could be mediated by its effect on endothelial cells and mitochondrial function. Results of our study may ultimately result in novel therapeutic interventions designed to attenuate arginase activity or signaling that regulates Arg2 expression in the treatment of renal fibrosis.
Materials and Methods

**UUO mouse model:** Experiments were conducted in male 8-week-old C57BL/6J and Arg2−/− mice (The Jackson Laboratory, Bar Harbor, ME). For UUO model, mice were anesthetized, and a midline incision was made as described previously (79). The left ureter was tied off twice with a non-absorbable silk surgical suture. Osmotic pumps (Durect Corporation, Cupertino, CA) containing either PBS (Vehicle) or BEC (2.3mg/kg/day; Cayman Chemical, Ann Arbor, MI) were implanted subcutaneously at the time of surgery as we described (25, 26). Sham surgeries were conducted in a similar manner, except the ureter was not tied off. These sham mice were used as controls throughout the study. Following surgery mice were placed in a warm cage and observed until they recovered. Kidney tissue was collected 7 days post UUO surgery. Mouse systolic blood pressure were recorded using CODA Non-invasive Blood Pressure system (Kent Scientific Corporation, Torrington CT) as we described previously (25). Mice were acclimated for 10 minutes at 26 °C before readings began. Readings were taken at the same time of day for all groups to prevent any diurnal variations.

**Generation of Arg2 specific knockout mice:** Arg2flox/flox was developed by Cyagen (Santa Clara, CA). The mouse Arg2 gene (GenBank accession number: NM_009705.3), encoded by 8 exons, is located on mouse chromosome 12. Exon 3 was selected as conditional knockout region as its deletion will result in the loss of function of the Arg2 gene. To engineer the targeting vector, homology arms and conditional KO (cKO) region were generated by PCR using BAC clone RP24-73D13 and RP23-110C12 from the C57BL/6J library as template. In the targeting vector, the Neo cassette was flanked by Frt
sites, and cKO region was flanked by LoxP sites (Supplemental Fig. 1). Several pups with the desired KO construct were identified by multiple PCR screening. Homozygous Arg2\(^{flox/flox}\) mice were crossed with commercially available \(Ggt1^{Cre}\) and \(Tie2^{Cre}\) mice (Jackson Laboratory, catalog # 008863 and 12841; respectively) to obtain Arg2 specific knockout in proximal tubular and endothelial cells (Supplemental Fig. 2A).

**Kidney derived cell isolation:** Proximal tubular epithelial and endothelial cells were isolated from kidneys to confirm Arg2 deletion from \(Ggt1^{Cre}/Arg2^{flox/flox}\) (Supplemental Fig. 2B) and \(Tie2^{Cre}/Arg2^{flox/flox}\) (Supplemental Fig. 2C) mice; respectively as described previously (80-83). For endothelial cells, kidneys were removed on ice, stripped of their outer capsules, and cortexes minced before being placed in 1 mg/ml collagenase (Sigma-Aldrich, St. Louis, MO) solution at 37°C with shaking for 45 minutes. Tissues were then passed through a 70 µM filter twice, centrifuged at 400 x g for 5 mins at 4°C, and endothelial cells were separated using CD31 magnetic beads (Miltenyi Biotec, Bergisch Gladbach, Germany). Cells were then grown on coated plates with RPMI-1640 (Thermo Scientific, Waltham, MA) + 10% fetal bovine serum for 7-10 days before being used for experiments. For proximal tubules isolation, we used a modified method as described previously (84). Specifically, mice were perfused with 0.5% iron III oxide (Sigma-Aldrich, St Louis, MO), then kidney cortexes isolated and minced before being digested in 1 mg/ml collagenase at 37°C with shaking for 30 mins and filtered with 250 and 70 µM cell strainers. Supernatants were then centrifuged at 50 x g for 5 mins, then the iron coated pellet was grown on plates pre-coated with 20 mM acetic acid and 5 µg collagen type 1 (Thermo Scientific, Waltham, MA) in RPMI-1640 + 10% fetal bovine serum overnight.
Media was then collected next day, centrifuged at 50 x g for 4 mins, then pellets suspended in fresh growth media and returned to the original plate. Proximal tubule cells were grown to confluency for 4-7 days, then used for experiments.

**Western Blotting:** Kidney tissue was homogenized in RIPA buffer containing 0.1% Triton X-100 supplemented with protease inhibitors (Roche Diagnostics, Indianapolis, IN) and cleared by centrifugation at 10,000 x g for 10 mins at 4°C. Cells were scraped from vessels using cell scrapers, centrifuged at 10,000 x g for 10 mins at 4°C, and supernatant collected. Protein concentration was determined by Bicinchonnic Acid (BCA) assay (Thermo Scientific, Waltham, MA). Fifty μg of kidney or cell lysate were separated on a 4-12% Bis-Tris gel (Life Technologies, Carlsbad, CA), and transferred onto PVDF membranes before blocking with 5% dry milk. Western blots were performed using the following antibodies: arginase-1 (Catalog # sc-271430, 0.4 μg/ml, Santa-Cruz Biotechnology, Dallas, TX), arginase-2 (Catalog # sc-374420, 0.4 μg/ml, Santa-Cruz Biotechnology, Dallas, TX), Tomm20 (Catalog # 42406, 1:1000, Cell Signaling, Danvers, MA), MCU (Catalog # 14997, 1:1000, Cell Signaling, Danvers, MA), GAPDH (Catalog # 5174, 1:2000, Cell Signaling, Danvers, MA), and β-actin (Catalog # 4970, 1:1000, Cell Signaling, Danvers, MA) antibodies. Western blots were quantitated using ImageJ software (NIH, Bethesda, MD) and normalized to loading control protein expression.

**RNA Isolation and Real-Time PCR:** RNA was isolated by Trizol extraction from whole kidney sections and reverse-transcribed to cDNA using the Bio-Rad (Hercules, CA) iScript cDNA synthesis kit. A 1:50 dilution of the cDNA was prepared and used for real-
time PCR analysis using SYBR Green Master Mix (Thermo Scientific, Rockford IL), as previously described (25). Primers and sequences used are fibronectin (forward: GTC CTG TGG GAG GGG TGT TTG A, reverse: TGC TTT CTT TTG CCA TCT GAC CTG), smooth muscle actin (forward: CTG CCG TTT TCC CCC TTC CTC T, reverse: TTG CTT CCT CCT CCT TTG G), and β-actin (forward: DCT GTT TGT GTA AGG TAA GGT GTC G, reverse: GAG GGG GTT GAG GTG TTG AGG). Relative levels of mRNA were calculated as previously described (25, 85).

**Histology and Immunohistochemistry**: Sections of left kidney tissues were fixed in 10% neutral-buffered formalin, embedded in paraffin, and 3 μm sections were cut. Sections were then stained with Masson Trichrome or Sirius Red. To determine percent area fibrosis Masson Trichrome or Sirius Red, pictures were obtained at 10x, saved as jpeg files, and analyzed in Image J to measure percent area of fibrosis. F4-80 macrophage staining was performed on sections similar to methods previously described (86) using F4-80 antibody (Catalog # sc-59171, 1:400, Santa Cruz Biotechnology, Dallas TX). Images were taken on a Nikon Eclipse E600 scope using a Nikon Digital Camera DYM1200.

**Measurement of Total Nitrate and Nitrite**: Fresh kidney homogenates were prepared in 1x PBS, pH 7.4, and clarified supernatants were used to determine protein concentration and total nitrate/nitrite using the Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI) as previously described (26, 87, 88).
**TBARS Assay:** Proteins were precipitated from kidney homogenates using 10% trichloroacetic acid, and subsequent supernatant was used for thiorbituric acid reactive substances (TBARS) assay as described (26, 89).

**Arginase Activity Assay:** Kidney lysates were assessed for arginase activity as previously described (25, 87).

**Mitochondria Isolation and Function:** Mitochondria were isolated from kidney tissue using a mitochondria isolation kit (Thermo Scientific, Rockford IL). ATP levels were assessed using a luciferase-based assay (Promega, Madison, WI) following manufacturer’s instructions. Complex I activity was measured as previously described by Birch-Machin and Turnbull (90).

**Statistics:** Comparisons between groups were conducted using GraphPad Prism software (version 7.04, San Diego, CA). Results are expressed as mean ± SEM. Unpaired t-test was used for comparison between two groups. One-way ANOVA was used to compare significance between more than two groups. A *p* value of < 0.05 represented significant difference.

**Study approval:** All animal studies were approved by the Penn State University College of Medicine and University of Texas Health Science Center at San Antonio Institutional Animal Care and Use Committees.
Author contributions

AA: Designed research studies.

MW, KS, WW, SM: conduct experiments, acquiring data, analyzing data.

AA, MM: analyzed data.

MW, AA: Writing manuscript.

MW, KS, WW, SM, MM, WBR, AA: Reviewed and edited manuscript.

MM, WBR: Provided critical advice.
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Figure 1: Arginases expression and activity increased following UUO fibrosis model. The left ureter was ligated and kidneys were collected after 7 days. Arg1 (A and C) and Arg2 (B and C) protein levels were determined using Western blot (n=4 each group). Kidney arginase activity were determined as described in materials and methods (D) (n=5 each group). Values are means ± SEM. *p < 0.05, **p < 0.01 compared to sham using unpaired t-test.
Figure 2: Arginase inhibition reduces kidney fibrosis following UUO. Representative images and quantitation of Masson’s Trichrome (A-B) and Sirius Red (C-D) stained left kidney sections. RT-PCR analysis of left kidneys from indicated mice subjected to UUO with or without BEC treatment for fibronectin (E) or smooth muscle actin (F). Values are means ± SEM. *p < 0.05, **p < 0.001 compared to vehicle sham; #p < 0.05 compared to vehicle UUO using One-Way ANOVA (n=3-7 each group).
Figure 3: Arg2 deficiency or arginase inhibition reduces kidney fibrosis and fibrotic markers following UUO. Representative images and quantitation of Masson’s Trichrome (A-B) and Sirius Red (C-D) stained left kidney sections. RT-PCR analysis of left kidneys from indicated mice for fibronectin (E) or smooth muscle actin (F). Values are means ± SEM. *p < 0.05, **p < 0.01 compared to WT sham; #p < 0.05, ##p < 0.01 compared to WT UUO using One-Way ANOVA (n=4-7 each group).
Figure 4: Arg2 deficiency or arginase inhibition does not reduce kidney macrophage infiltration following UUO. Representative images and quantitation of F4/80 stained left kidney sections (A-B). Values are means ± SEM. *p < 0.05 compared to WT sham using One-Way ANOVA (n=4-8 each group).
Figure 5: Arg2 deficiency specifically in endothelial cells; but not proximal tubular epithelial cells reduces kidney fibrosis and fibrotic markers following UUO. Representative images and quantitation of Masson's Trichrome (A-B) and Sirius Red (C-D) stained left kidney sections. RT-PCR analysis of left kidneys from indicated mice for smooth muscle actin (E). Values are means ± SEM. *p < 0.05 compared to Arg2<sup>flox/flox</sup> sham; #p < 0.05 compared to Arg2<sup>flox/flox</sup> UUO using One-Way ANOVA (n=5-9 each group).
Figure 6: Arginase inhibition restored kidney NO and restored mitochondrial functions following UUO. (A) Levels of nitrate & nitrite were determined from whole left kidney tissue 7 days after UUO. (B) TBARS assay following UUO in whole kidney tissue. (C) Levels of mitochondrial ATP from indicated left kidney tissue. (D) Complex I activity levels from indicated left kidney tissue. (E-F) Protein was isolated from left kidney sections for wild type mice subjected to UUO for 7 days. Western blotted for MCU normalized to Tomm20 as a mitochondrial marker while beta-actin show a comparable protein loading. Values are means ± SEM. *p < 0.05 compared to vehicle sham; #p < 0.05 compared to vehicle UUO using One-Way ANOVA (n=5-6 each group).
Supplemental Table 1: Blood pressure measurement on transgenic mice.

| Mouse type               | Systolic blood pressure (mmHg) |
|--------------------------|---------------------------------|
| Arg2^{flox/flox}         | 104.99 ± 15.95                  |
| Ggt1^{Cre/Arg2^{flox/flox}} | 89.54 ± 4.02                    |
| Arg2^{flox/flox}         | 116.83 ± 3.15                   |
| Tie2^{Cre/Arg2^{flox/flox}} | 117.14 ± 4.02                   |

Systolic blood pressure was measured on Arg2^{flox/flox}, Ggt1^{Cre/Arg2^{flox/flox}}, and Tie2^{Cre/Arg2^{flox/flox}} mice. (n=4 each group). Values are means ± SEM, analyzed using unpaired t-test.
Supplemental Fig. 1: Generation of Arg2\textsuperscript{flox/flox} mice. (A) Design for insertion of LoxP sites into Arg2 gene. (B) Embryonic stem (ES) cells were implanted into a chimeric mouse and crossed with flp delete mouse to get Arg2\textsuperscript{flox/WT} offspring. (C) Arg2\textsuperscript{flox/WT} was confirmed by PCR analysis of 2 flox region deletion sites at 427 (mutant) and 283 (WT) bp.
Supplemental Fig. 2: Generation and confirmation of Ggt1^{Cre}/Arg2^{floxed/floxed} and Tie2^{Cre}/Arg2^{floxed/floxed} mice. (A) Arg2^{floxed/floxed} mice were cross-bred with Ggt1^{Cre} and Tie2^{Cre} mice for 2 generations to obtain Ggt1^{Cre}/Arg2^{floxed/floxed} and Tie2^{Cre}/Arg2^{floxed/floxed}, respectively. (B-C) Western blot analysis of Arg2 expression in isolated proximal tubules and endothelial cells from Ggt1^{Cre}/Arg2^{floxed/floxed} and Tie2^{Cre}/Arg2^{floxed/floxed}. Black stars represent the Arg2^{floxed/floxed} status.