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Arginase inhibition mediates renal tissue protection in diabetic nephropathy by a nitric oxide synthase 3-dependent mechanism

Hanning You¹, Ting Gao¹, Timothy K. Cooper², Sidney M. Morris Jr.³, and Alaa S. Awad¹

¹Department of Medicine, Division of Nephrology, Penn State University College of Medicine, Hershey, PA

²Department of Comparative Medicine, Penn State University College of Medicine, Hershey, PA

³Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, PA

Abstract

Recently we showed that pharmacological blockade or genetic deficiency of arginase-2 confers kidney protection in diabetic mouse models. Here we tested whether the protective effect of arginase inhibition is nitric oxide synthase-3 (eNOS)-dependent in diabetic nephropathy. Experiments were conducted in eNOS knockout and their wild type littermate mice using multiple low doses of vehicle or streptozotocin and treated with continuous subcutaneous infusion of vehicle or the arginase inhibitor S-(2-Boronoethyl)-L-cysteine by an osmotic pump. Inhibition of arginases for 6 weeks in diabetic wild type mice significantly attenuated albuminuria, the increase in plasma creatinine and blood urea nitrogen, histopathological changes, kidney fibronectin and TNF- α expression, kidney macrophage recruitment, and oxidative stress compared to vehicle-treated diabetic wild type mice. Arginase inhibition in diabetic eNOS knockout mice failed to affect any of these parameters but reduced kidney macrophage recruitment and kidney TNF- α expression compared to vehicle-treated diabetic eNOS knockout mice. Furthermore, diabetic wild type and eNOS knockout mice exhibited increased kidney arginase-2 protein, arginase activity and ornithine levels. Thus, arginase inhibition mediates renal tissue protection in diabetic nephropathy by an eNOS-dependent mechanism and has an eNOS-independent effect on kidney macrophage recruitment.

Introduction

Diabetic nephropathy (DN) is the leading cause of end stage kidney disease, responsible for over 40% of all cases in the US and this number is likely to increase unabated.¹ Thus it is important to identify the mechanisms involved in the development of diabetic kidney disease. Early alterations in diabetic kidneys include the development of glomerular

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Address reprint requests: Alaa S. Awad, M.D., M.Sc., FSAN, Assistant Professor of Medicine and Cellular & Molecular Physiology, Penn State University, Hershey Medical Center College of Medicine, Division of Nephrology, H040, 500 University Drive, P.O. Box 850, BMR Building, C5830, Hershey, PA 17033, Ph: 717-531-1713, Fax: 717-531-6776, asa17@psu.edu.

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hyperfiltration and hypertrophy, followed by thickening of the glomerular basement membrane (GBM), mesangial matrix accumulation, increased urinary albumin excretion (UAE) rate and ultimately progression to glomerular sclerosis and end-stage renal failure.

Endothelial cell dysfunction is a central pathophysiological mechanism that contributes to diabetes and DN. Endothelial dysfunction, characterized by reduced bioavailability of nitric oxide (NO) and increased oxidative stress, is a hallmark characteristic in diabetes² and DN.³ NO is produced from L-arginine by NO synthases (NOS). Under conditions of low arginine level or hyperglycemia, eNOS is uncoupled, producing reactive oxygen species in lieu of NO.^{4,5} Recently, low or lack of eNOS has been shown to exacerbate DN;^{6,7} thus, elucidating the basis for vascular dysfunction in DN is critical.⁸

The metabolism of L-arginine is of great interest because it involves a wide range of physiological and pathophysiological conditions and plays important roles in many different kind of diseases.⁹ Dramatic alterations in arginine metabolism occur in endothelial injury¹⁰⁻¹² due to changes in the activity and/or expression of arginases. Arginase-2 is constitutively expressed and also inducible in endothelial cells¹⁰⁻¹² as well as in kidney cells^{13,14} and, when elevated, can inhibit NOS activity/expression and induce endothelial NOS uncoupling, thus reducing NO bioavailability and inhibiting the NO/cGMP pathway. In contrast, arginase-1 is not normally expressed in the kidney.^{15,16} *In vivo* inhibition of arginases improved vascular function and high blood pressure,¹⁷ allergen-induced airway obstruction,^{18,19} liver ischemia/reperfusion injury,^{20,21} autoimmune encephalitis,²² erectile function²³ and DN.¹⁶

Our recent publication¹⁶ showed that pharmacological blockade of arginases or genetic deficiency of arginase-2 confers kidney protection in diabetic kidney disease. However, its mechanism(s) of action was not clear at that time. Because arginases can modulate NO production by catabolizing arginine, we hypothesized that the protective effect of arginase inhibition is eNOS-dependent in DN. In the current study, we found that arginase inhibition mediates renal tissue protection in DN via an eNOS-dependent mechanism. In contrast, arginase inhibition has an eNOS-independent effect on kidney macrophage recruitment.

Results

Arginase inhibition reduces characteristics of DN in eNOS^{+/+} but not eNOS^{-/-} mice

To assess the possible role of endothelial NOS in the mechanism(s) of arginase effects in diabetic mice kidney, we infused continuously the arginase inhibitor BEC or vehicle into diabetic eNOS^{-/-} mice and their wild-type eNOS^{+/+} for 6 weeks. As shown in Table 1, vehicle-treated diabetic eNOS^{+/+} mice had increased blood glucose levels, decreased body weight, increased kidney weight/body weight ratio and reduced fluid composition compared to normal eNOS^{+/+} mice. Arginase inhibition of diabetic eNOS^{+/+} mice significantly increased body weight and reduced kidney weight/body weight ratio without affecting other measurements. In contrast, arginase inhibition of diabetic eNOS^{-/-} mice failed to affect any measurements. As expected, blood pressure was significantly elevated in eNOS^{-/-} groups compared to eNOS^{+/+} groups.

Inhibition of arginases reduces albuminuria, plasma creatinine and blood urea nitrogen in diabetic eNOS^{+/+} but not eNOS^{-/-} mice

We next measured 24-hr UAE, plasma creatinine and blood urea nitrogen (BUN) as indicators of renal injury in diabetic eNOS^{+/+} and eNOS^{-/-} mice with and without BEC treatment. BEC-treated diabetic eNOS^{+/+} mice significantly reduced the increase in UAE (Fig. 1A), albumin/creatinine ratio (Fig. 1B), plasma creatinine and BUN (Table 1) compared to vehicle-treated diabetic eNOS^{+/+} mice following 6 weeks of STZ- induced diabetes. These parameters were increased also in diabetic eNOS^{-/-} mice but, in marked contrast to the effects of BEC in diabetic eNOS^{+/+} mice, BEC treatment of diabetic eNOS^{-/-} mice failed to reduce the increases in any of these parameters.

Inhibition of arginases decreases renal histopathological changes and fibronectin expression in diabetic eNOS^{+/+} but not eNOS^{-/-} mice

Periodic acid-Schiff (PAS) staining of kidney sections showed increased glomerular cellularity and mesangial expansion in vehicle-treated diabetic vs. normal eNOS^{+/+} mice (score: 0.99±0.09 vs. 0.19±0.01, p<0.01) after 6 weeks of diabetes (Fig. 2). Inhibition of arginases in diabetic eNOS^{+/+} mice resulted in significantly reduced glomerular changes (scores: 0.6±0.06; p<0.01) compared to vehicle-treated eNOS^{+/+} mice. In contrast, inhibition of arginases in diabetic eNOS^{-/-} mice failed to reduce glomerular changes, which remained similar to those in vehicle-treated eNOS^{-/-} mice (score: 1.3±0.08 vs. 1.1±0.06); respectively (Fig. 2). Similar results were obtained using RT-PCR for kidney fibronectin expression as a fibrotic marker. As shown in Fig. 3A, vehicle-treated eNOS^{+/+} mice showed a 3.5-fold increase in fibronectin mRNA (p<0.001) compared with normal eNOS^{+/+} mice, an effect attenuated by BEC treatment (p<0.05 to vehicle-treated diabetic mice). In contrast, inhibition of arginases in diabetic eNOS^{-/-} mice failed to reduce fibronectin mRNA expression.

Inhibition of arginases decreases macrophage recruitment and TNF- α expression in diabetic eNOS^{+/+} and eNOS^{-/-} mice

To determine whether arginase is critical for macrophage infiltration in DN and whether that effect is dependent on endothelial NOS, we show the distribution and quantitation of macrophages in kidneys by immunohistochemistry (Mac-2 positive macrophages) (Fig. 4). As expected, vehicle-treated eNOS^{+/+} mice showed significant increases in glomerular macrophages (2.4±0.2 macrophages/glomerulus; p<0.01) compared with normal eNOS^{+/+} mice (0.9±0.1 macrophages/glomerulus). Inhibition of arginases in diabetic eNOS^{+/+} mice resulted in significantly reduced glomerular macrophage recruitment (1.2±0.03 macrophages/glomerulus; p<0.01) compared to vehicle-treated eNOS^{+/+} mice following 6 weeks of diabetes. Similarly, vehicle-treated eNOS^{-/-} mice showed significant increases in glomerular macrophages (2.4±0.2 macrophages/glomerulus; p<0.01) compared with normal eNOS^{-/-} mice (0.9±0.06 macrophages/glomerulus). Interestingly, inhibition of arginases in diabetic eNOS^{-/-} mice still reduced glomerular macrophage recruitment (1.3±0.08 macrophages/glomerulus; p<0.01) compared to vehicle-treated eNOS^{-/-} mice following 6 weeks of diabetes. Similar results were obtained using RT-PCR for kidney TNF- α expression as an inflammatory marker. As shown in Fig. 3B, vehicle-treated eNOS^{+/+} mice

showed a 2.5-fold increase in TNF- α mRNA ($p < 0.001$) compared with normal eNOS^{+/+} mice, an effect completely reduced by BEC treatment ($p < 0.005$) compared to vehicle-treated diabetic mice. Interestingly, inhibition of arginases in diabetic eNOS^{-/-} mice still reduced TNF- α mRNA ($p < 0.05$) compared to vehicle-treated eNOS^{-/-} mice following 6 weeks of diabetes.

Inhibition of arginases increases eNOS protein expression in diabetic eNOS^{+/+} mice

To determine whether arginase inhibition has an effect on kidney eNOS protein expression, we used western blot analysis for total kidney homogenates of eNOS in eNOS^{+/+} mice. Our data show significant increase in eNOS expression using arginase inhibitor compared to normal and vehicle-treated diabetic kidneys (Fig. 5A).

Inhibition of arginases decreases urinary TBARS in diabetic eNOS^{+/+} but not eNOS^{-/-} mice

We next assessed urinary TBARS as an indicator for oxidative stress. Urinary TBARS levels were significantly elevated in diabetic eNOS^{+/+} mice, but BEC-treatment completely prevented this increase in urinary TBARS (Fig. 5B). In contrast, urinary TBARS levels were not significantly different between all groups in eNOS^{-/-} mice and also did not differ from TBARS levels in normal eNOS^{+/+} mice.

Inhibition of arginases decreases kidney ornithine levels in diabetic eNOS^{+/+} and eNOS^{-/-} mice

To determine whether arginase inhibition was effective in both diabetic eNOS^{+/+} and eNOS^{-/-} mice, we measured kidney ornithine levels (Fig. 6) as an index of arginase activity. Kidney ornithine levels were significantly increased in both vehicle-treated diabetic eNOS^{+/+} and eNOS^{-/-} mice. BEC-treated diabetic eNOS^{+/+} and eNOS^{-/-} mice had significantly reduced kidney ornithine levels following 6 weeks of STZ-induced diabetes.

Arginase-2 expression in diabetic eNOS^{+/+} and eNOS^{-/-} mice

Our previous study¹⁶ showed increased arginase-2 expression in diabetic kidneys. Whether inhibition of arginase has an effect on kidney arginase-2 expression in diabetic mice was not clear at that time. Here we confirm increased arginase-2 protein expression in both diabetic eNOS^{+/+} and eNOS^{-/-} mice (Fig. 7). Interestingly, inhibition of arginase was associated with a further increase in arginase-2 protein expression in diabetic eNOS^{+/+} mice but not in diabetic eNOS^{-/-} mice.

Kidney arginase activity in diabetic eNOS^{+/+} and eNOS^{-/-} mice

Similar to kidney arginase-2 protein expression measured by Western blot (Fig. 7), kidney arginase activity measured in vitro was significantly increased in both diabetic eNOS^{+/+} and eNOS^{-/-} mice (Fig. 8). Inhibition of arginase was associated with a further increase in kidney arginase activity in diabetic eNOS^{+/+} mice but not in diabetic eNOS^{-/-} mice. It is important to emphasize that in vitro measurements of arginase activity only demonstrate that levels of arginase-2 protein correlate with levels of enzymatically competent arginase-2. Owing to the 40-fold dilution of kidney protein and metabolites during tissue extraction and in the activity assay, plus the very high concentration of arginine in the assay (50 mM), the

in vivo inhibition of arginase activity in animals that are receiving the competitive arginase inhibitor BEC will not be apparent in the *in vitro* assay.

Discussion

Our previous publication demonstrated that pharmacological blockade or genetic deficiency of arginase-2 confers kidney protection in diabetic mouse models.¹⁶ However the exact mechanism by which the inhibition of arginase mediates these effects was not clear. The current study shows that arginase inhibition mediates renal tissue protection in DN indirectly via eNOS. In addition, arginase inhibition has an eNOS-independent effect on kidney macrophage recruitment. Whether the effect of arginase inhibition is mediated strictly via eNOS expressed in endothelial cells or via eNOS expressed in non-endothelial cells such as mesangial and tubular cells cannot be determined from the present study. Additional studies using mice with cell-specific knockouts of eNOS expression are needed to explore this.

Alteration in endothelial function is a common underlying event for vascular abnormalities observed in patients with diabetes, insulin resistance, glucose intolerance, hypertension, and peripheral vascular disease. The role of endothelial cell dysfunction in DN is well established (for review;²⁴) and is mainly due to alteration of NO bioavailability and/or action. NO is produced from arginine by NOS. Recently, low or lack of eNOS has been shown to exacerbate DN;^{6,7} thus, elucidating the basis for vascular dysfunction in DN is critical.⁸ Arginase catalyzes hydrolysis of L-arginine to L-ornithine and urea and thus can compete with NOS for the common substrate L-arginine.²⁵ Depending on stimulus, either or both arginases may be expressed and induced in macrophages, endothelial cells, and other cell types.^{9,26}

To examine the mechanism of arginase inhibition in DN, we investigated the effects of inhibiting arginases on kidney dysfunction, glomerular histopathological changes and macrophage recruitment using BEC in *eNOS*^{-/-} and their wild type littermate diabetic mouse model. BEC significantly ameliorated diabetic albuminuria and was associated with reduced glomerular pathology and kidney fibronectin expression in diabetic *eNOS*^{+/+} mice, confirming our previous finding.¹⁶ In contrast to the above findings, BEC treatment failed to affect renal functional, histological changes or kidney fibronectin expression in diabetic *eNOS*^{-/-} mice. Increased expression of fibronectin is an early event in the pathogenesis of diabetic renal disease and its accumulation in the kidney is thought to lead to the development of glomerulosclerosis.^{27,28} In diabetes, both TGF- β and fibronectin stimulate matrix production and block matrix degradation.^{29,30} Taken together, our results indicate a direct interaction between arginase blockade and eNOS-derived NO and support the concept that arginase inhibition mediates renal tissue protection through its effect on eNOS.

We further investigated the effect of arginase inhibition on kidney macrophage recruitment and TNF- α expression. Macrophages/monocytes are a heterogeneous population that plays a pivotal role in different stages of inflammation in multiple organs. For instance, macrophages play an important role in inducing renal injuries through potent cytokines such as MCP-1,³¹⁻³³ TNF α ,³⁴ and IL-1.³⁵ Our data show that the renal protective effect of BEC correlates with a significant reduction of kidney macrophage infiltration and TNF- α .

expression in diabetic eNOS^{+/+} mice. Interestingly, BEC treatment still correlates with a significant reduction of kidney macrophage infiltration and TNF- α expression in diabetic eNOS^{-/-} mice despite absence of effect on renal functional and histological changes, indicating an effect of arginase inhibition on macrophage recruitment that is independent of endothelial NOS. TNF- α is mainly produced by monocytes/macrophages and has been associated with increased vascular endothelial permeability in diabetes. A recent report³⁶ supports our data and indicates that targeted disruption of arginase-2 in mice directly suppresses macrophage proinflammatory responses through mitochondrial ROS production, reduction of monocyte/macrophage adhesion activity to endothelial cells, and reduction of TNF- α mRNA and protein expression.

We have shown previously that renal interstitial fluid of NO is significantly reduced in diabetic rats.³⁷ The reduction in NO during diabetes could be due to NO quenching,³⁸ inhibition of both Ca²⁺-dependent and -independent NOS activities,³⁹ ROS production,^{5,40,41} a decrease in NOS activity,⁴² NO capture by glucose,⁴³ and/or reduction in angiotensin AT₂ receptor.⁴⁴ An increase in arginase activity could be another reason for reduced NO during diabetes. Therefore, we questioned if arginase inhibition regulates eNOS expression in DN. Our data show increased renal eNOS protein expression using arginase inhibitor in diabetic eNOS^{+/+} mice. The effects of arginase activity and arginase inhibition on NO production were evaluated by measuring kidney nitric oxide end products (nitrate + nitrite) in nondiabetic and in BEC- and vehicle-treated diabetic mice. STZ treatment resulted in a significant reduction in kidney nitrate + nitrite, but this reduction was largely prevented by arginase inhibition (Supplemental Fig. 1). Moreover, a recent report provided evidence for eNOS uncoupling and increased ROS and oxidative stress in kidneys of diabetic spontaneously hypertensive (SHR) rats.⁴¹ Whether arginase inhibition has an effect on eNOS uncoupling is not completely clear. However, our urinary TBARS data suggests that arginase inhibition reduces oxidative stress in DN via an eNOS-dependent mechanism. This conclusion is consistent with published data indicating that L-NAME has no significant effect on TBARS levels in endothelial cells derived from eNOS^{-/-} mice.⁴⁵

Our previous publication showed that arginase-2 but not arginase-1 is expressed and induced in diabetic mouse kidney.¹⁶ In addition, we and others have shown that overall arginase activity in the kidney is due almost entirely to arginase-2.^{16,46} Our data demonstrate increased kidney arginase-2 expression and kidney arginase activity in both diabetic eNOS^{+/+} and eNOS^{-/-} mice. BEC treatment in both diabetic eNOS^{+/+} and eNOS^{-/-} mice did not reduce arginase-2 expression as measured by Western blot or *in vitro* activity assay. This result was not unexpected because BEC is a competitive inhibitor of arginase activity, not an inhibitor of arginase expression or an irreversible inhibitor of arginase activity. However, the fact that BEC treatment in diabetic eNOS^{+/+} but not in eNOS^{-/-} mice was associated with further increases in arginase-2 expression suggests a possible regulatory role of eNOS-derived NO “either directly or indirectly” on kidney arginase-2 expression. We speculate that eNOS activity can affect kidney arginase activity and protein expression in diabetes. Importantly, the fact that BEC reduced kidney ornithine levels in both in both diabetic eNOS^{+/+} and diabetic eNOS^{-/-} mice is consistent with inhibition of renal arginase activity *in vivo*. However, because kidney is the primary site for the first step of creatine

synthesis, which also produces ornithine, we cannot conclusively rule out the possibility that changes in creatine synthesis rate also may contribute to the observed changes in kidney ornithine levels.

In conclusion, our study demonstrates the mechanism of renal tissue protection using an arginase inhibitor as well as its important interaction with NO in DN. This conclusion is based on two novel observations: First, we demonstrated that the protective effect of arginase inhibition in DN is mediated via endothelial NOS. Second, we showed that arginase inhibition has an eNOS-independent effect on kidney macrophage recruitment.

Materials and Methods

Diabetic mouse model

All the animal experiments were approved by the Penn State University College of Medicine Institutional Animal Care and Use Committee. Endothelial NOS deficient (eNOS^{-/-}, stock # 2684 on C57BL/6J background) and wild type (eNOS^{+/+}) mice (6–8 weeks old) were purchased from Jackson laboratory (Bar Harbor, MN, USA). Diabetes was induced by streptozotocin (STZ, Sigma, St. Louis, MO) injection intraperitoneally (50mg/kg body weight) for 5 consecutive days. S-(2-Boronoethyl)-L-cysteine (BEC) was administered by continuous subcutaneous infusion via a mini-osmotic pump (Alzet, Durect Corporation, Cupertino, CA) after confirming high blood glucose levels in diabetic mice (5 days after the last dose of STZ injection) as described previously.¹⁶ After 6 weeks of diabetes, the mice were sacrificed, kidneys were removed and plasma was collected for further studies.

Blood pressure measurement

Systolic blood pressure was measured using Coda blood pressure system (Kent Scientific Corp, Torrington, Connecticut) as previously described.^{16,27} Mice were habituated to the blood pressure measurement for 5 days before the day of the experiment and then were allowed to rest quietly for 15 minutes at room temperature. All measurements were performed at the same time for all groups to prevent any diurnal variations.

Histology and immunohistochemistry

Mouse kidneys were fixed in 4% paraformaldehyde, embedded in paraffin, and 3- μ m sections were cut. Sections were stained with periodic acid Schiff (PAS). All glomeruli within the section (between 50–107 glomeruli) were scored individually at 40 \times in a blinded manner and then averaged (n=4–8 mouse in each group; Table 1). All images were taken with an Olympus BX51 microscope and DP71 digital camera using cellSens Standard 1.6 imaging software (Olympus America, Center Valley, PA). Images were obtained with 100 \times (oil) objective with a total magnification of 1000 \times . Semiquantitative scores (0–4+) were assigned based on the masked readings. The mesangial matrix expansion or sclerosis scoring was performed as described previously.^{16,27} Briefly, each glomerulus on a single section was graded from 0 to 4+, where 0 represents no lesion, and 1, 2, 3, and 4+ represent mesangial matrix expansion or sclerosis, involving 25, 25 to 50, 50 to 75, or >75% of the glomerular tuft area, respectively. Immunohistochemistry for macrophages was performed

using rat anti-mouse Mac-2 antibody (clone M3/38; Cedarlane, Burlington, NC) on paraffin sections as described previously.^{16,27}

Quantitative real-time PCR

Total RNA was extracted from mouse kidneys using Tri reagent (Molecular research center, Inc, Cincinnati, OH) per manufacturer's instructions. Single-strand cDNA was synthesized using iScript cDNA Synthesis Kits (Bio-Rad, Hercules, CA) for two-step real-time RT-PCR. Quantitative PCR assays were performed using an ABI-Prism 7900HT PCR system, TaqMan Fast Universal PCR master mixture (Applied Biosystems, Foster City, CA), and a TaqMan Gene Expression Assay Mix comprising *Fnl* (Mm01256744_m1) and *Tnf* (Mm00443260_g1). A mouse *Gapdh* (Mm99999915_g1) assay mix served as an endogenous control. Data was analyzed using SDS Software 2.2.2.

Determination of kidney ornithine levels

Kidney tissues were weighed, homogenized in 300 μ l 3M perchloric acid (Fisher Scientific, Fair Lawn, NJ), and supernatants were transferred to a fresh tube after centrifugation at 14000rpm at 4°C for ornithine assay. Kidney tissue extracts were spiked with isotope-labeled internal ornithine standard (Cambridge Isotope Laboratories, INC, Andover, MA) and purified with solid phase extraction column. The purified sample was then analyzed by a LC-MS system (Waters, Milford, MA) in the MS core facility at Penn State College of Medicine. UPLC was performed at a flow rate of 0.4 mL/min. The injection volume was 3 μ L. The standard curve ranged from 1 μ M to 200 μ M for ornithine (Sigma Aldrich, St. Louis, MO). The Synapt Q-TOF mass spectrometer was operated with an electrospray ionization positive mode. Ornithine and its isotope-labeled internal standard were detected by daughter scan parent compound and the most abundant daughter fragment was used for quantification (Supplemental Fig. 2).

Analytical methodology

Urine albumin concentration was measured by ELISA using an Albuwell M kit (Exocell, Philadelphia, PA) as described elsewhere.^{16,27} Creatinine was determined using a Creatinine Liquid Reagents Assay kit (Diazyme Laboratories, Poway, CA) as described previously.^{16,27} Blood urea nitrogen (BUN) was determined using Vitros DT6011 chemistry slides (Ortho-Clinical Diagnostics, Rochester, NY) as previously described.^{16,27} Body composition was measured using LF90 Minispec Time Domain Nuclear Magnetic Resonance Spectrometer (Burker Optics, Billerica, MA) as described previously.^{16,27} Urinary thiobarbituric acid reactive substance (TBARS) levels, which are recommended by the AMDCC (<http://www.amdcc.org/shared/showFile.aspx?doctypeid=3&docid=33>) as an index of oxidative stress in DN, were determined as described previously.⁴⁷

Western blot

Fifteen [g of kidney lysates were electrophoresed on SDS-polyacrylamide gels (12% Mini-PROTEAN® TGX™ Precast Gel; Bio-Rad, Hercules, CA) and electroblotted onto PVDF membranes (Millipore, Billerica, MA). Membranes were probed with antibodies for arginase-2 (Santa Cruz Biotechnology, Cat #: Sc-20151), eNOS (Abcam, Cat #: ab76198)

and beta-actin (Sigma-Aldrich, Cat #: A5441), and immunoblots were developed with appropriate secondary antibodies and enhanced chemiluminescence. Densitometric analysis of autoradiograms was performed using Image J software (NIH, <http://rsbweb.nih.gov/ij/index.html>).

Arginase activity assay

Kidney lysates were prepared by homogenization in lysis buffer (50 mM Tris-HCl, pH 7.5, 0.1 mM EDTA and protease inhibitors) at 4°C, followed by centrifugation for 20 min at 14,000 × g at 4°C. The supernatants of kidney lysates were assayed for arginase activity as previously described.^{16,48}

Statistic analysis

Comparisons between groups were examined by using the SPSS version 19.0 software for Windows (SPSS, Chicago, IL) program. Data are expressed as mean ± SEM. One-way ANOVA was used when more than two groups were compared, and significance of observed differences among the groups was evaluated with a least significant difference post hoc test. Statistical significance was identified at $p < 0.05$.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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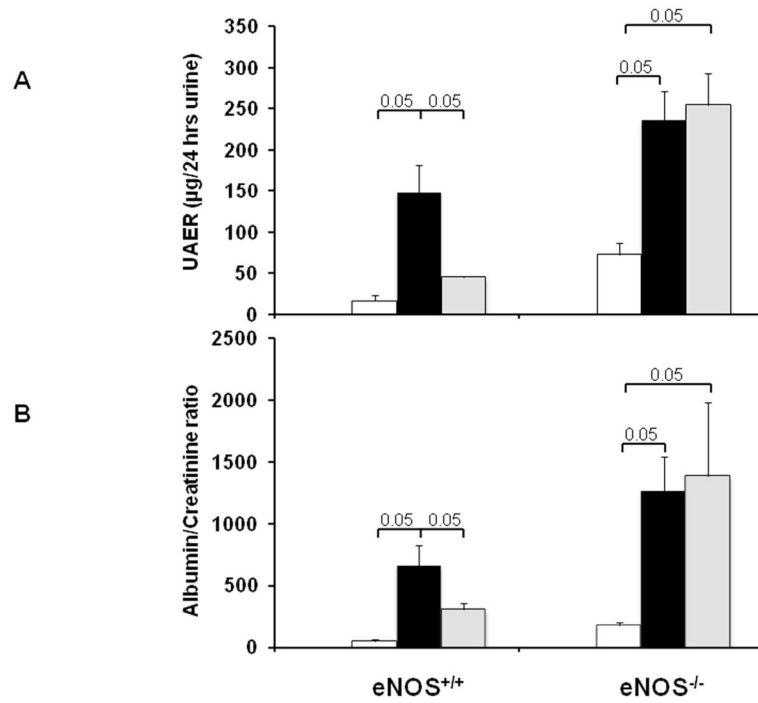


Figure 1. Arginase inhibition reduces increases in urinary albuminuria in diabetic eNOS^{+/+}; but not eNOS^{-/-} mice

eNOS^{-/-} and their wild type littermate mice were treated with BEC or vehicle via osmotic minipump for 6 weeks. Urine was collected for measurement of UAE (A) or albumin/creatinine ratio (B). Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, BEC-treated groups. Results are means ± SEM.

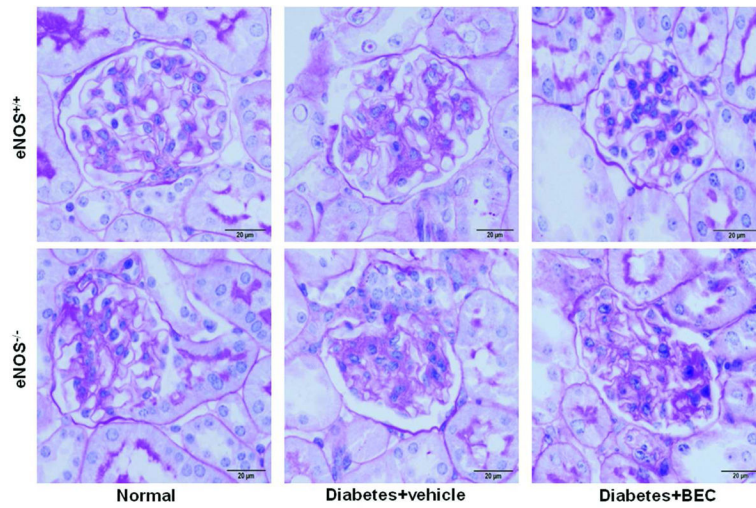


Figure 2. Arginase inhibition reduces histological changes in diabetic eNOS^{+/+}; but not eNOS^{-/-} mice

Sections were stained with PAS and images were taken with 100x (oil) objective with a total magnification of 1000x. Images are representative of 4–8 mice in each group.

Semiquantitative scores (0–4+) were assigned based on the masked readings, where 0 represents no lesion, and 1, 2, 3, and 4+ represent mesangial matrix expansion or sclerosis, involving 25, 25 to 50, 50 to 75, or >75% of the glomerular tuft area, respectively.

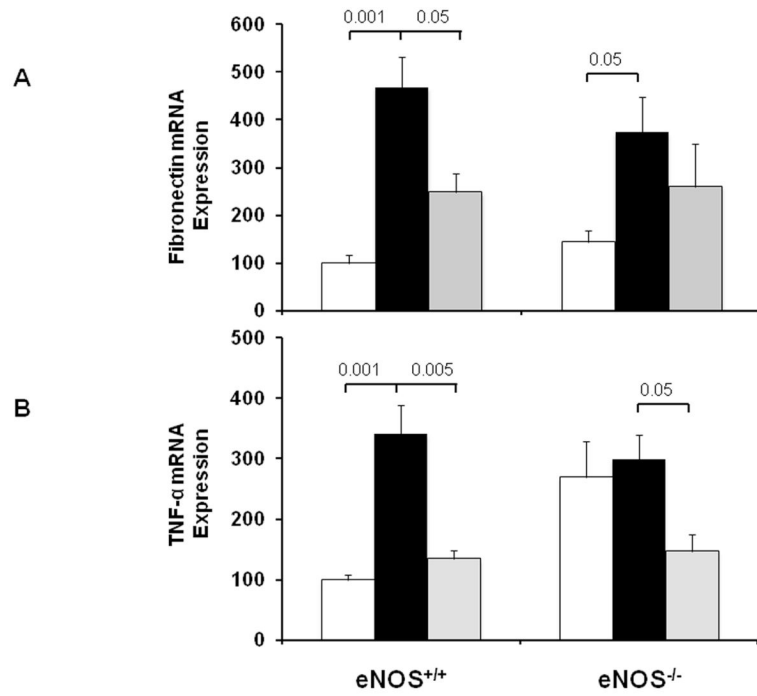


Figure 3. Effect of arginase inhibition on kidney fibronectin and TNF- α expression in diabetic eNOS^{+/+} and eNOS^{-/-} mice

RT-PCR was performed on whole mouse kidney total RNA after 6 weeks following diabetes. Fibronectin (A) and TNF- α (B) mRNA expression were normalized with GAPDH mRNA. Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, BEC-treated groups. Results are means \pm SEM.

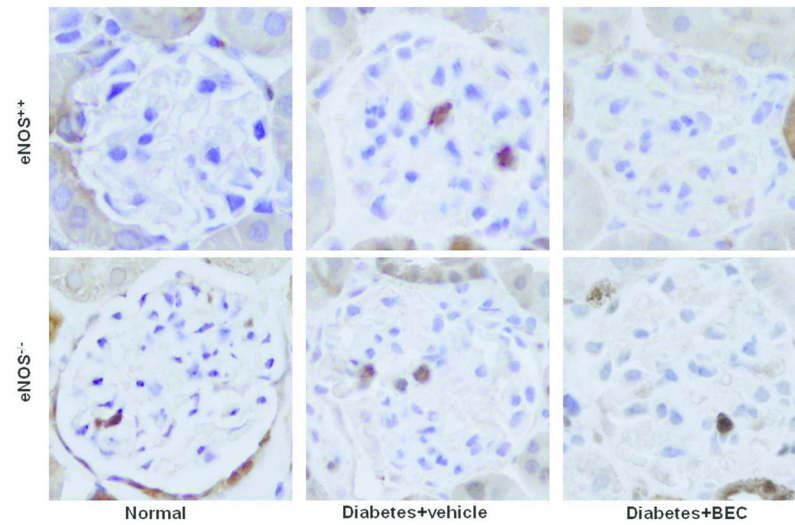


Figure 4. Arginase inhibition reduces macrophage infiltration in diabetic eNOS^{+/+} and eNOS^{-/-} mice

Mac-2-positive macrophages in glomeruli were identified by immunohistochemical staining after 6 weeks following diabetes. Images are representative of 40 fields from 4–8 mice in each group.

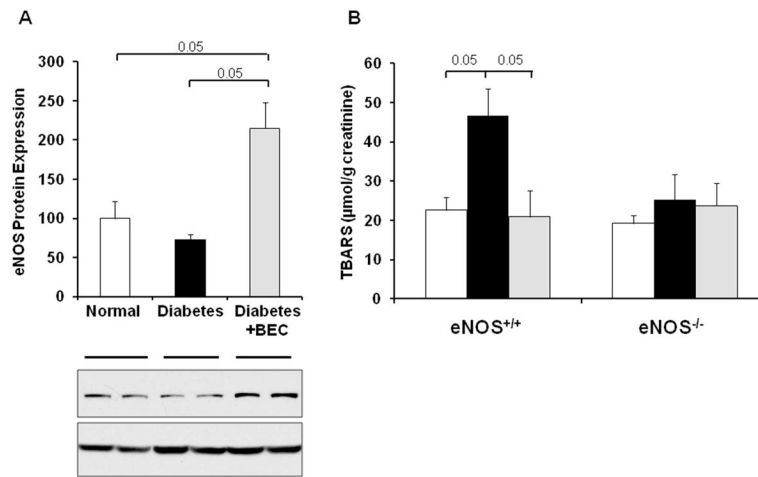


Figure 5. Effect of arginase inhibition on kidney eNOS protein expression and urinary TBARS in diabetic mice

A) Western-blot was performed to evaluate eNOS expression in kidney lysates at 6 weeks following diabetes in eNOS^{+/+} mice. β -actin was used as a loading control. Quantification of eNOS expression was performed by densitometry followed by normalization to β -actin B) Urine was collected for measurement of TBARS levels. Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, BEC-treated groups. Results are means \pm SEM.

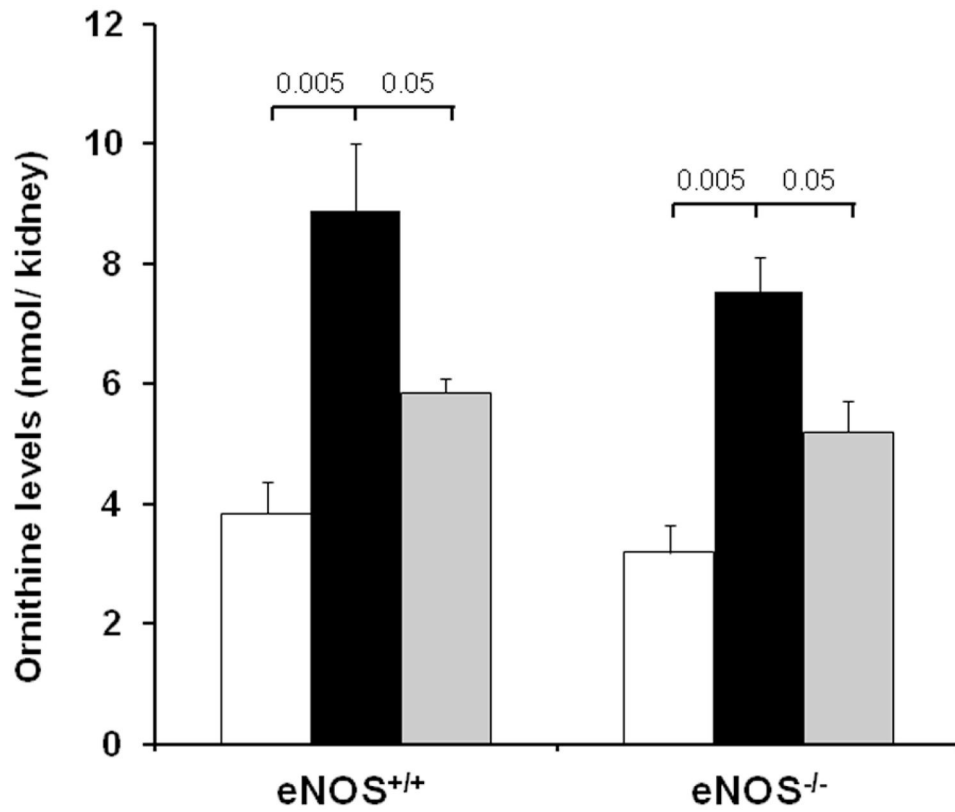


Figure 6. Arginase inhibition reduces kidney ornithine levels in diabetic eNOS^{+/+} and eNOS^{-/-} mice

Kidney ornithine levels were assayed in kidney lysates prepared at 6 weeks following STZ-induced diabetes using a LC-MS system. Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, BEC-treated groups. Results are means \pm SEM.

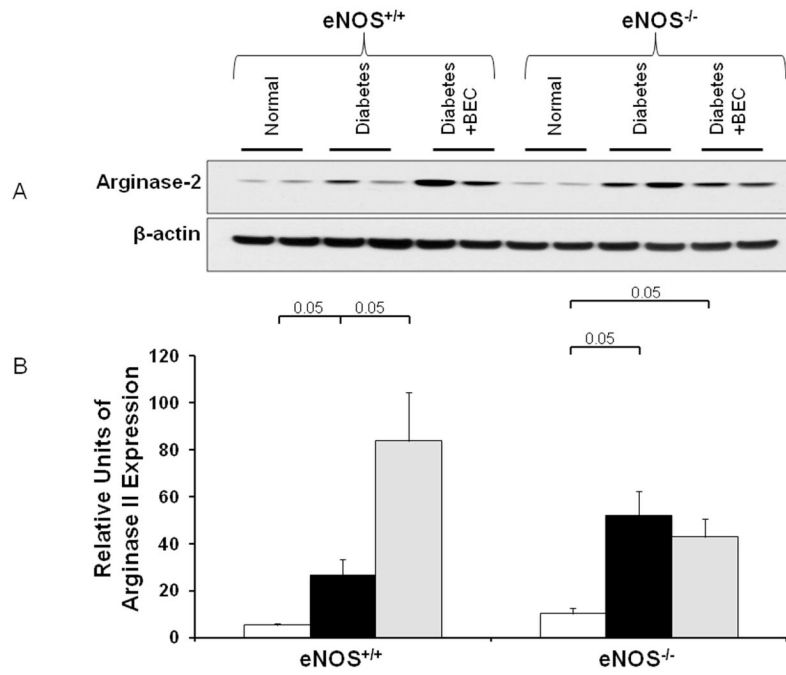


Figure 7. Arginase-2 expression is induced in diabetic $eNOS^{+/+}$ and $eNOS^{-/-}$ mice
 Western-blot was performed to evaluate arginase-2 expression in kidney lysates from indicated groups of mice at 6 weeks following STZ-induced diabetes. β -actin was used as a loading control (A). Quantification of arginase-2 expression was performed by densitometry followed by normalization to β -actin (B). Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, BEC-treated groups. Results are means \pm SEM.

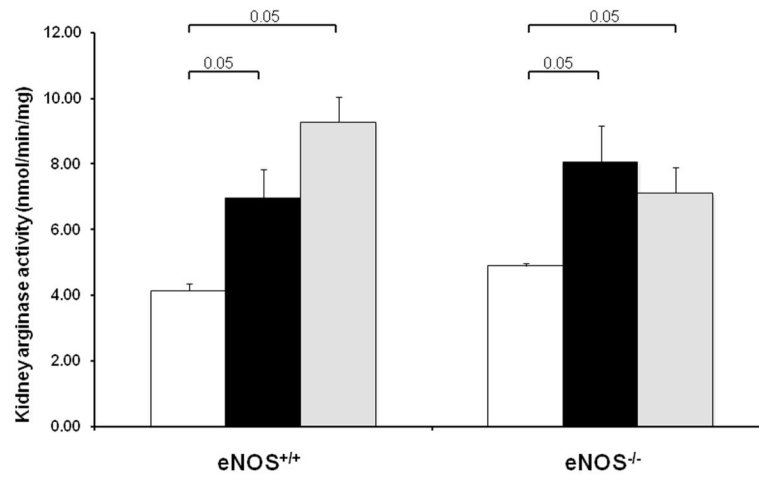


Figure 8. Kidney arginase activity is induced in diabetic eNOS^{+/+} and eNOS^{-/-} mice
Arginase activities were assayed in kidney lysates prepared at 6 weeks following STZ-induced diabetes. Open bar, normal group; black-filled bar, vehicle-treated diabetic groups; grey-filled bar, BEC-treated groups. Results are means \pm SEM.

Table 1

General characteristics of eNOS^{+/+} and eNOS^{-/-} mice

Treatment	Week 6								
	eNOS ^{+/+}				eNOS ^{-/-}				
	Normal	Diabetes+vehicle	Diabetes+BEC	Normal	Diabetes+vehicle	Diabetes+BEC	Normal	Diabetes+vehicle	Diabetes+BEC
Animal #	5	6	8	4	8	8	4	8	8
Body Weight (g)	25±1	20±1 ^a	25±1	28±1	21±1 ^b	23±1	28±1	21±1 ^b	23±1
Blood Glucose (mg/dL)	149±2 ^c	499±17	434±27	198±14 ^d	483±12	473±23	198±14 ^d	483±12	473±23
SBP (mmHg)	136±3	130±4	135±7	148±4.227 ^e	150±4	147±5	148±4.227 ^e	150±4	147±5
Kidney/Body Weight (mg/g)	0.59±0.02	0.97±0.09 ^f	0.66±0.06	0.58±0.05 ^g	0.82±0.07	0.72±0.05	0.58±0.05 ^g	0.82±0.07	0.72±0.05
% Fluid	7.04±0.02	5.95±0.17	6.69±0.80	6.63±0.19	5.56±0.271	5.54±0.22	6.63±0.19	5.56±0.271	5.54±0.22
Plasma BUN (mg/dL)	23±3.02	33.83±4.74 ^h	26±1.08	30.25±1.65 ⁱ	43.22±4.51	41.25±3.83	30.25±1.65 ⁱ	43.22±4.51	41.25±3.83
Plasma Creatinine (mg/dL)	0.14±0.02	0.29±0.02 ^j	0.12±0.021	0.17±0.09 ^k	0.35±0.04	0.41±0.11	0.17±0.09 ^k	0.35±0.04	0.41±0.11

Data are mean±SEM; SBP: Systolic blood pressure; BUN: Blood urea nitrogen

^a *P*<0.01 compared to eNOS^{+/+} normal and diabetes+BEC groups.

^b *P*<0.01 compared to eNOS^{-/-} normal group.

^c *P*<0.01 compared to eNOS^{+/+} diabetes+vehicle and diabetes+BEC groups.

^d *P*<0.01 compared to eNOS^{-/-} diabetes+vehicle and diabetes+BEC groups.

^e *P*<0.05 compared to eNOS^{+/+} normal group.

^f *P*<0.05 compared to eNOS^{+/+} normal and diabetes+BEC groups.

^g *P*<0.05 compared to eNOS^{-/-} diabetes+vehicle and diabetes+BEC groups.

^h *P*<0.05 compared to eNOS^{+/+} normal and diabetes+BEC groups.

ⁱ *P*<0.05 compared to eNOS^{-/-} normal and diabetes+BEC groups.

^j *P*<0.05 compared to eNOS^{+/+} normal and diabetes+BEC groups.

^k *P*<0.05 compared to eNOS^{-/-} normal and diabetes+BEC groups.