Arginase inhibition: a new treatment for preventing progression of established diabetic nephropathy

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1Department of Medicine, Division of Nephrology, Penn State University College of Medicine, Hershey, Pennsylvania; 2Department of Comparative Medicine, Penn State University College of Medicine, Hershey, Pennsylvania; and 3Department of Microbiology and Molecular Genetics, University of Pittsburgh, Pittsburgh, Pennsylvania

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You H, Gao T, Cooper TK, Morris SM, Jr, Awad AS. Arginase inhibition: a new treatment for preventing progression of established diabetic nephropathy. Am J Physiol Renal Physiol 309: F447–F455, 2015. First published June 3, 2015; doi:10.1152/ajprenal.00137.2015.—Our previous publication showed that inhibition of arginase prevents the development of diabetic nephropathy (DN). However, identification of targets that retard the progression of established DN—which is more clinically relevant—is lacking. Therefore, we tested the hypothesis that arginase inhibition would prevent the progression of established DN. Effects of arginase inhibition were compared with treatment with the angiotensin-converting enzyme inhibitor captopril, a current standard of care in DN. Experiments were conducted in Ins2Akita mice treated with the arginase inhibitor S-(2-boronoethyl)-L-cysteine (BEC) or captopril starting at 6 wk of age for 12 wk (early treatment) or starting at 12 wk of age for 6 wk (late treatment). Early and late treatment with BEC resulted in protection from DN as indicated by reduced albuminuria, histological changes, kidney macrophage infiltration, urinary thiobarbituric acid-reactive substances, and restored nephrin expression, kidney nitrate/nitrite, kidney endothelial nitric oxide synthase phosphorylation, and renal medullary blood flow compared with vehicle-treated Ins2Akita mice at 18 wk of age. Interestingly, early treatment with captopril reduced albuminuria, histological changes, and kidney macrophage infiltration without affecting the other parameters, but late treatment with captopril was ineffective. These findings highlight the importance of arginase inhibition as a new potential therapeutic intervention in both early and late stages of diabetic renal injury.

arginase; established diabetic nephropathy; nitric oxide; ACEi

PATHOGENESIS OF DIABETIC NEPHROPATHY (DN) is a multifactorial process and not completely understood. DN has become the leading cause of end-stage renal disease (ESRD) in the United States (13). DN is a chronic disorder initiated early by the development of glomerular hyperfiltration/hypertrophy, followed by thickening of the glomerular basement membrane, increased urinary albumin excretion (UAE) rate, often within 5–10 yr of its detection, and ultimately progression to ESRD. Among the pathogenic factors are hyperglycemia, increased systemic and glomerular pressure, increased activity of the renin-angiotensin-aldosterone system, endothelial dysfunction, and stimulation of several cytokines and growth factors by metabolic and hemodynamic factors (14). Several therapeutic interventions targeting these mechanisms have been developed and implemented with various degrees of success. Current therapies, including blood pressure and glucose control and other life style changes, have been only modestly successful in delaying the progression of renal failure (1). Unfortunately, the majority of current research is focused on drug discovery to reduce the development of DN rather than the progression of DN, which is more clinically relevant. Therefore, more effective approaches to reduce the progression of DN are urgently needed. Angiotensin-converting enzyme (ACE) inhibitors (ACEi) are recognized as the standard of care in DN and have been shown to postpone the development or slow down the progression of DN (1). However, this therapy does not produce a full reversal or even halting of renal function deterioration (23). Thus, it is important to identify pharmacotherapeutics that will halt the development and/or reverse the progression of diabetic kidney disease.

Decreased nitric oxide (NO) bioavailability—due in part to reduced endothelial NO synthase (eNOS) activity or expression—and endothelial dysfunction play key roles in the pathophysiology of diabetes and DN (9, 10, 19, 29, 32, 38). As l-arginine is the substrate for arginases as well as all NO isoforms, changes in arginase activity can have a significant impact on NO production via substrate competition (39). Arginases are expressed as two distinct isoforms (arginase-1 and arginase-2), and arginase-2 is almost exclusively the isoform expressed in the kidney (16, 27, 28, 36). In this regard, our previous work demonstrated that pharmacological blockade or genetic deficiency of arginase-2 confers kidney protection in diabetic mouse models (28). We further showed that arginase inhibition mediates renal tissue protection in DN via an eNOS-dependent mechanism (42). As those studies focused only on early changes of nephropathy, the role of arginase inhibition in progressive DN, including glomerulosclerosis and tubulointerstitial disease, was unknown. We therefore proceeded to test the hypothesis that arginase inhibition will also prevent the progression of established DN.

The current study used a pharmacologic approach to evaluate the efficacy of arginase inhibition in a model of established DN compared with treatment with the ACE inhibitor captopril. Overall, our results with delayed treatment of arginase inhibition in established diabetes will address a clinically relevant question. Our results indicate that arginase inhibition may be a promising treatment for late-stage DN.

MATERIALS AND METHODS

Diabetic mouse models. Experiments were conducted in male D2.B6-Ins2Akita/MatbJ and their wild-type littermate mice (DBA/2J background; Jackson Laboratory, stock number 007562) starting at 6 wk of age (~3 wk of diabetes). Ins2Akita mice, recommended by the Animal Models of Diabetes Complications Consortium (AMDCC) as a model of DN (6, 7), develop hyperglycemia at 3 wk of age. Mice with blood glucose levels >350 mg/dl were considered diabetic. Mice

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were provided ad libitum access to food and water. Urine collections were obtained by housing mice in metabolic cages for 18 h with free access to water while food was withheld to prevent any contamination. Animal experiments were approved by the Penn State University College of Medicine Institutional Animal Care and Use Committee.

**Drug delivery.** Vehicle (PBS) or S-(2-Boronoethyl)-L-cysteine (BEC; 2.3 mg·kg⁻¹·day⁻¹) was administered by continuous subcutaneous infusion via a mini-osmotic pump (Alzet, Durect, Cupertino, CA) as described previously (28, 42). Captopril (Sigma) was provided daily in drinking water (24 mg/L). Treatments were started at 6 wk of age (early treatment) or at 12 wk of age (late treatment) until 18 wk of age in Ins2Akita mice. At the end of experiments (18 wk of age), mice were euthanized; kidneys were removed and plasma was collected for further studies.

**Analytical methodology.** The following methods were employed as described in the indicated references: measurement of urine albumin by ELISA using an Alburell M kit (Exocell, Philadelphia, PA) (2, 28, 41); urine creatinine was determined using an enzymatic assay (Crystal Chem, Downers Grove, IL); assay of urinary thiorbituric acid-reactive substances (TBARS) (22, 42); and measurement of renal medullary blood flow by a Laser Doppler Flow Meter (model BLE-21D, Transonic Systems, Ithaca, NY) (28). A Coda blood pressure system (Kent Scientific, Torrington, CT) was used to measure systolic blood pressure (41, 42); mice were allowed to rest quietly for 15 min at room temperature before measurement. All blood pressure measurements were performed at the same time for all groups to prevent any diurnal variations.

**Kidney nitrate and nitrite assay.** Kidney tissues were homogenized in 1× PBS, pH 7.4, and centrifuged at 10,000 g for 20 min at 4°C. Supernatants were transferred to a clean tube and were used to determine protein concentration and nitrate/nitrite using a Nitrate/Nitrite Colorimetric Assay Kit (Cayman Chemical, Ann Arbor, MI, catalog no. 7800001) as we described previously (43).

**Western blot.** Kidney tissues were homogenized in 0.1% Triton X-100 supplemented with protease inhibitor cocktail tablets (Roche Diagnostics, Indianapolis, IN) and sodium orthovanadate (Sigma). Tissue homogenates were centrifuged at 20,000 g for 10 min. Fifteen-microgram kidney lysates were separated on a 4–12% NuPAGE Bis-Tris gel (Invitrogen), and the separated proteins were transferred onto a polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA). After blocking in 5% nonfat milk, membranes were incubated with primary antibodies overnight, followed by 70% ethanol at room temperature. Primary antibodies and dilutions were as follows: anti-nephrin antibody (1:500, catalog no. 20R-NP002, Fitzgerald Industries International, Acton, MA), anti-phospho-eNOS at S1177 antibody (1:1,000, catalog no. ab76198, Abcam, Cambridge, MA), anti-eNOS antibody (1:1,000, catalog no. 5174S, Cell Signaling Technology, Danvers, MA), and anti-GAPDH antibody (1:1,000, catalog no. 5174S, Cell Signaling Technology). Enhanced chemiluminescence detection (Thermo Fisher Scientific) was used to develop membranes after final washing, followed by exposure to X-ray films. Densitometry was performed using Image J (National Institutes of Health, http://rsbweb.nih.gov/ij/index.html). Nephrin abundance was expressed relative to the average ratio in nondiabetic mice.

**Histology and immunohistochemistry.** Mouse kidneys were fixed in 10% neutral buffered formalin overnight, followed by 70% ethanol wash and paraffin embedment. Periodic acid Schiff (PAS) staining was performed on 3-μm tissue sections. All glomeruli (between 51–110 glomeruli) in a single transverse section for each mouse were examined under a microscope at ×400 total magnification and scored individually in a blinded manner and then averaged. All images were obtained with an Olympus BX51 microscope and DP71 digital camera using cellSens Standard 1.6 imaging software (Olympus America, Center Valley, PA). Images were taken with ×100 (oil) objective with a total magnification of ×1,000. Semiquantitative scores (0–4+) were assigned based on the masked readings as previously described (28, 41, 42).

**Immunohistochemistry for macrophages** was performed using anti-mouse Mac-2 antibody (clone M3/38; Cedaraus, Burlington, NC) on paraffin sections. The number of glomerular macrophages was counted in 40 glomeruli per section (number of macrophages in glomeruli divided by the number of glomeruli) in blinded fashion under ×40 magnification and averaged as described previously (28, 42).

**Statistical analysis.** Comparisons between groups were examined by using SPSS version 21.0 software for Windows (SPSS, Chicago, IL). Data are expressed as means ± SE. 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.

## RESULTS

**Arginase inhibition reduces the progression of established DN in Ins2Akita mice.** To assess the significance of arginase inhibition in DN, we treated Ins2Akita mice with vehicle or BEC via osmotic minipump, or captopril (24 mg/meal in drinking water), beginning at either 6 wk of age until 18 wk of age (early treatment) or at 12 wk of age until 18 wk of age (late treatment). As shown in Table 1, Ins2Akita vehicle-treated mice had increased blood glucose level, and decreased body weight, compared with nondiabetic mice. Early or late treatment with either BEC or captopril did not reduce blood glucose levels or blood pressure.

We next measured urine albumin excretion (UAE) as an indicator of diabetic kidney injury (Fig. 1). At 6 wk of age (3 wk after diabetes; before start of treatment), all groups of Ins2Akita mice had a significant increase in UAE compared with nondiabetic mice. At 12 wk of age (6 wk after early treatment), early treatment of captopril—but not early treatment with BEC—significantly reduced albuminuria compared with vehicle-treated mice. At 18 wk of age, early or late treatment of BEC significantly reduced albuminuria (Fig. 1) and urine albumin/creatinine ratio (Fig. 2) compared with vehicle-treated mice. In contrast, only early but not late treatment with captopril reduced albuminuria (Fig. 1) and urine albumin/creatinine ratio

### Table 1. Effects of early and late treatment with arginase inhibitor or captopril in diabetic Ins2Akita mice at 18 wk of age

<table>
<thead>
<tr>
<th>Mouse number</th>
<th>Nondiabetic</th>
<th>Ins2Akita + Vehicle</th>
<th>Ins2Akita + BEC Early</th>
<th>Ins2Akita + BEC Late</th>
<th>Ins2Akita + Captopril Early</th>
<th>Ins2Akita + Captopril Late</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>3.5 ± 0.8</td>
<td>24.6 ± 1*</td>
<td>24.3 ± 0.5†</td>
<td>24.7 ± 0.39</td>
<td>20.3 ± 1.2*</td>
<td>24.2 ± 0.7*</td>
</tr>
<tr>
<td>9</td>
<td>31.5 ± 3</td>
<td>494 ± 4†</td>
<td>500 ± 1†</td>
<td>500 ± 1†</td>
<td>493 ± 3†</td>
<td>489 ± 9†</td>
</tr>
<tr>
<td>9</td>
<td>120 ± 3</td>
<td>112 ± 2</td>
<td>124 ± 4</td>
<td>119 ± 2</td>
<td>119 ± 5</td>
<td>115 ± 5</td>
</tr>
</tbody>
</table>

Data are means ± SE. BEC, S-(2-boronoethyl)-L-cysteine; BP, blood pressure. *P < 0.001; †P < 0.0001 compared with nondiabetic. ‡P < 0.01 compared with Ins2Akita + captopril early.

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dysfunction, characterized by reduced NO bioavailability, is
that of vehicle-treated mice.

Arginase inhibition restores kidney NO production and eNOS phosphorylation in established DN. Since endothelial dysfunction, characterized by reduced NO bioavailability, is a central pathophysiological mechanism that contributes to diabetes and DN (9, 10, 19, 29, 32, 38), we first measured kidney nitrate + nitrite as an indicator of NO status at 18 wk of age. Kidney nitrate + nitrite level was significantly reduced in vehicle-treated mice compared with nondiabetic mice (Fig. 3A). Early or late treatment of BEC significantly restored kidney nitrate + nitrite level at 18 wk of age compared with vehicle-treated mice. In contrast, neither early nor late treatment with captopril affected kidney nitrate + nitrite levels, which were comparable with levels in vehicle-treated mice.

We also measured eNOS phosphorylation using Western blot analysis since eNOS plays a critical role in DN (9, 10, 19, 29, 32, 38, 47). Phosphorylation of eNOS at serine 1177 upregulates eNOS activity (8). Our data demonstrated that eNOS phosphorylation was significantly reduced in vehicle-treated mice compared with nondiabetic mice (Fig. 3B). Early or late treatment of BEC significantly restored eNOS phosphorylation at 18 wk of age compared with vehicle-treated mice. In contrast, neither early nor late treatment with captopril affected eNOS phosphorylation, which was comparable in levels with vehicle-treated mice.

Arginase inhibition restores renal medullary blood flow in established DN. We next measured renal medullary blood flow to evaluate the downstream effect of endothelial dysfunction in DN. As shown in Fig. 4, renal medullary blood flow was significantly reduced in vehicle-treated mice compared with nondiabetic mice. As expected, early and late treatment with BEC significantly restored renal medullary blood flow at 18 wk of age compared with vehicle-treated mice. In contrast, neither early nor late
treatment with captopril affected renal medullary blood flow, which was comparable with vehicle-treated Ins2Akita mice.

Arginase inhibition reduces urinary TBARS in established DN. Levels of urinary TBARS, which is an indicator of oxidative stress, were significantly elevated in vehicle-treated Ins2Akita mice compared with nondiabetic mice (Fig. 5). Early or late treatment with BEC significantly reduced urinary TBARS levels at 18 wk of age compared with vehicle-treated mice. Similarly, early or late treatment with captopril significantly reduced urinary TBARS levels at 18 wk of age compared with vehicle-treated mice.

Arginase inhibition decreases glomerular macrophage infiltration in established DN. Our previous data demonstrated that early BEC treatment resulted in significantly reduced glomerular macrophage infiltration in diabetic mice (28, 42). In this study (Fig. 6), we showed that vehicle-treated Ins2Akita mice had significantly increased glomerular macrophage infiltration compared with nondiabetic mice (P < 0.0001). Early or late treatment with BEC significantly decreased glomerular macrophage infiltration at 18 wk of age (P < 0.05) compared with vehicle-treated mice. In contrast, only early but not late treatment with captopril significantly decreased glomerular macrophage infiltration (P < 0.05) to a similar extent as early...
arginase inhibition not only prevents the development but also the progression of DN in the Ins2Akita mice. First, we show that early or late treatment with BEC reduces albuminuria, histopathological changes, and kidney macrophage infiltration in DN. Second, early or late treatment with BEC restores NO production/action as indicated by increased kidney nitrite + nitrate, eNOS phosphorylation, renal blood flow, and reduced urinary TBARS in DN. Third, arginase inhibition results in restoration of renal nephrin expression in DN. Taken together, our data indicate that arginase inhibition could be a potentially new therapeutic tool for treating diabetic patients.

Reduced NO bioavailability and increased oxidative stress are hallmark characteristics of insulin resistance, diabetes mellitus, hypertension, and DN (9, 10, 19, 29, 32, 38, 47). Specifically, endothelial dysfunction plays a critical role in DN (30); thus, elucidating the basis for vascular dysfunction in DN is critical (3). NO is produced from L-arginine, which is a common substrate of NOS enzymes and arginases. Reduced eNOS expression (38) or genetic depletion of eNOS (47) exacerbates DN (38, 47), indicating the critical role of eNOS in DN. Interestingly, arginase inhibition prevents DN via an eNOS-dependent mechanism (42); thus, restoring NO could be an important therapeutic tool in the prevention and treatment of DN.

Although a significant role for eNOS in DN is well-established, we appreciate that neuronal (n)NOS or inducible (i)NOS may also play a role in DN. In fact, expression of nNOS, which is expressed especially in the collecting duct and macula densa (17, 24), was reduced in several models of progressive chronic kidney disease, including DN (5, 20, 40). On the other hand, some investigators have reported increased nNOS expression in DN (12, 31). Although the current study did not evaluate the impact of diabetes or arginase inhibition on renal nNOS or iNOS expression, we showed previously that diabetes resulted in increased renal expression of iNOS but not of nNOS (43). It is important to note that whether the expression of NOS isoforms changes in a consistent pattern in DN remains a matter of controversy, with various studies demon-

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**Fig. 4.** Late treatment with arginase inhibitor restores renal medullary blood flow in Ins2Akita mice at 18 wk of age. Renal medullary blood flow was measured in Ins2Akita mice at 18 wk of age, and expressed relative to the mean value for nondiabetic mice, which was arbitrarily set at 100. Results are means ± SE. *P < 0.05, **P < 0.01 compared with nondiabetic. #P < 0.05, ##P < 0.01 compared with vehicle-treated Ins2Akita mice. †P < 0.01 compared with Ins2Akita mice treated with captopril early. ‡P < 0.01 compared with Ins2Akita mice treated with captopril late.

**Fig. 5.** Late treatment with arginase inhibitor reduces urinary thiobarbituric acid-reactive substances (TBARS) in Ins2Akita mice at 18 wk of age. Urine was collected for measurement of TBARS levels. Results are means ± SE. *P < 0.01, **P < 0.001 compared with nondiabetic. #P < 0.05 compared with vehicle-treated Ins2Akita mice.
strating an increase, no change, or a decrease in their expression in diabetic rats (for a review, see Ref. 21). The variable results likely reflect differences in animal species or strains, methods of inducing diabetes, the duration of diabetes (early or late), and the methods used to evaluate NOS expression.

In addition to NOS restoration, inhibition of arginase could reduce arginase-dependent production of some downstream metabolites, such as polyamines (26). Increased plasma levels of polyamines have been shown in chronic kidney disease such as DN (18). In our study, arginase inhibition resulted in less mesangial expansion and glomerular hypercellularity in diabetes, indicating a possible contribution of arginases to the initiation and/or progression of diabetic renal fibrosis. Additionally, inhibition of arginase reduced kidney macrophage infiltration and restored nephrin expression. Macrophages play a pivotal role in inducing renal injuries through potent cytokines such as monocyte chemoattractant protein-1 (15, 33, 37), TNF-α (34), and IL-1 (35), while nephrin expression is a very important protein in the pathophysiology of proteinuria (45).

The present study addressed a clinically relevant question: does arginase inhibition prevent late stage of established DN? The current study not only confirms our previous results that early treatment with arginase inhibition prevents the development of DN (28) but, importantly, extends these observations by demonstrating that arginase inhibition is also effective in treating the progression of established DN.

A second issue addressed in the current study was the efficacy of arginase inhibition compared with treatment with captopril (an ACEi), which is the gold standard for treating diabetic patients. Our data show that early treatment with captopril was almost as effective as early treatment with arginase inhibition in reducing albuminuria, kidney macrophage infiltration, and histopathological changes along with reduced urinary oxidative stress. However, the effect of early treatment with captopril in reducing albuminuria was immediate (within 6 wk of treatment and was maintained thereafter) while the effect of early treatment with arginase inhibition in reducing albuminuria was delayed. Early treatment with cap-

Fig. 6. Late treatment with arginase inhibitor reduces macrophage infiltration in Ins2Akita mice at 18 wk of age. Mac-2-positive macrophages in glomeruli were identified by immunohistochemical staining at 18 wk of age in nondiabetic (A), vehicle-treated Ins2Akita (B), early BEC-treated (C), late BEC-treated (D), early captopril-treated (E), or late captopril-treated (F) Ins2Akita mice. G: summary data for macrophages/glomerulus. Results are means ± SE. *P < 0.05, **P < 0.001, ***P < 0.0001 compared with nondiabetic. #P < 0.05 compared with vehicle-treated Ins2Akita mice.
topril, however, lacked any effect on nephrin expression or kidney nitrate/nitrite levels, eNOS phosphorylation, or renal blood flow. Thus, the effect of early treatment with captopril to ameliorate DN is eNOS-independent. This conclusion is supported by reports that captopril remarkably reduced albuminuria in low-dose streptozotocin in eNOS-deficient mice (44) and remarkably reduced glomerular and tubulointerstitial injury in db/db eNOS-deficient mice (46). Interestingly, late treatment with captopril lacked any effect to ameliorate diabetic renal injury. Additional studies to investigate the efficacy of combined BEC and captopril treatments will be needed in the future.

Data from urinary TBARS were not surprising. Early or late treatment with captopril reduced the elevated urinary TBARS associated with DN despite the lack of their effect on NO. A previous report concluded that sulphydryl group containing ACEi such as captopril may exhibit antioxidant properties (4).

The fact that late treatment of captopril failed to protect the kidney in DN despite its effect to reduce urinary TBARS indicates that oxidative stress is a possible consequence rather than a cause of diabetic injury. Captopril was also reported to have anti-inflammatory activity (25). Indeed, early treatment with captopril reduced glomerular macrophage infiltration to a similar extent as early treatment with BEC.

In the current study, we administered the arginase inhibitor BEC systemically via an osmotic mini-pump. It is appropriate to consider whether systemic administration of arginase inhibitors may adversely affect the hepatic urea cycle. Currently available arginase inhibitors are competitive and are not isozyme-specific. As the liver contains a very high amount of arginase 1, enormous amounts of an inhibitor would be required to inhibit liver arginase sufficiently for any inhibition of the urea cycle to become apparent. More importantly, however, the reactions of the urea cycle are not diffusion-controlled but.
A representative Western blot is shown. Results are means ± SE. *P < 0.05 compared with nondiabetic. †P < 0.05 compared with vehicle-treated Ins2Akita mice. ‡P < 0.05 compared with Ins2Akita mice treated with captopril late.

Fig. 8. Late treatment with arginase inhibitor restores renal nephrin expression in Ins2Akita mice at 18 wk of age. Kidney nephrin expression was evaluated using Western blot at 18 wk of age. Quantification was performed by densitometry followed by normalization to GAPDH. The mean nephrin/GAPDH value for nondiabetic mice was arbitrarily set to 100. Tryptophan metabolism followed by normalization to GAPDH. The mean nephrin/GAPDH value for nondiabetic mice was arbitrarily set to 100. The mean nephrin/GAPDH value for nondiabetic mice was arbitrarily set to 100. The mean nephrin/GAPDH value for nondiabetic mice was arbitrarily set to 100.

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 (11). Consequently, a competitive arginase inhibitor will not exchange with arginine generated within the urea cycle and thus should have little or no effect on the urea cycle. It should be noted that competitive arginase inhibitors have been used in many animal studies, and hyperammonemia or other adverse effects have not been reported in any of them. Even mild hyperammonemia would become apparent via altered behavior, but no such effects have been noted.

In conclusion, our study demonstrates that treatment with an arginase inhibitor not only prevents the development but also the progression of established DN. Our data therefore are clinically relevant since identification of targets that retard the progression of established DN is lacking. Results of this study may ultimately lead to novel therapeutic interventions using arginase inhibition in the treatment of diabetic kidney disease.

REFERENCES


