Arginase-2 Mediates Diabetic Renal Injury

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OBJECTIVE—To determine *1*) whether renal arginase activity or expression is increased in diabetes and *2*) whether arginase plays a role in development of diabetic nephropathy (DN).

RESEARCH DESIGN AND METHODS—The impact of arginase activity and expression on renal damage was evaluated in spontaneously diabetic *Ins2*^{Akita} mice and in streptozotocin (STZ)-induced diabetic Dilute Brown Agouti (DBA) and arginase-2–deficient mice (*Arg2*$^{-/-}$).

RESULTS—Pharmacological blockade or genetic deficiency of arginase-2 conferred kidney protection in *Ins2*^{Akita} mice or STZ-induced diabetic renal injury. Blocking arginases using *S*-(2-boronoethyl)-L-cysteine for 9 weeks in *Ins2*^{Akita} mice or 6 weeks in STZ-induced diabetic DBA mice significantly attenuated albuminuria, the increase in blood urea nitrogen, histopathological changes, and kidney macrophage recruitment compared with vehicle-treated *Ins2*^{Akita} mice. Furthermore, kidney arginase-2 expression increased in *Ins2*^{Akita} mice compared with control. In contrast, arginase-1 expression was undetectable in kidneys under normal or diabetes conditions. *Arg2*$^{-/-}$ mice mimicked arginase blockade by reducing albuminuria after 6 and 18 weeks of STZ-induced diabetes. In wild-type mice, kidney arginase activity increased significantly after 6 and 18 weeks of STZ-induced diabetes but remained very low in STZ-diabetic *Arg2*$^{-/-}$ mice. The increase in kidney arginase activity was associated with a reduction in renal medullary blood flow in wild-type mice after 6 weeks of STZ-induced diabetes, an effect significantly attenuated in diabetic *Arg2*$^{-/-}$ mice.

CONCLUSIONS—These findings indicate that arginase-2 plays a major role in induction of diabetic renal injury and that blocking arginase-2 activity or expression could be a novel therapeutic approach for treatment of DN. ***Diabetes*** **60:3015–3022, 2011**

Diabetes is a global health problem. The prevalence of diagnosed and undiagnosed diabetes in the U.S. is progressively increasing (e.g., from 7.8% in 2007 to 14.5% in 2010) and is expected to rise between 24.7 and 32.8% in 2050 (1). Diabetes is the most common cause of end-stage renal disease (ESRD), responsible for more than 40% of all cases in the U.S. (2,3), and this number is likely to continue to increase unabated. Diabetic nephropathy (DN) is a chronic, progressive disorder leading to a rapid decline and ESRD, frequently in <5 years. Over the last decade, the incidence of ESRD due to diabetes has doubled. Current therapy, including blood pressure and glucose control and other life style changes, has been modestly successful in delaying the progression of renal failure. Early alterations in diabetic kidneys include the development of glomerular hyperfiltration and hypertrophy, followed by thickening of the glomerular basement membrane, endothelial dysfunction, mesangial matrix accumulation, increased urinary albumin excretion (UAE) rate (UAER), and ultimately progression to glomerular sclerosis and end-stage renal failure. Thus, it is important to identify the mechanisms involved in the development of diabetic kidney disease.

Dramatic alterations in arginine metabolism can result in vascular dysfunction due to increased activity of arginases (4–7), including vascular dysfunction in diabetes (8–11). The latter studies raised the possibility that arginases may play a role also in the pathogenesis of DN. Mammalian arginase exists as two isozymes (arginase-1 and arginase-2) that are encoded by different genes differ with regard to tissue distribution, subcellular localization, and immunologic reactivity and are independently regulated (12,13). Arginase-2 is the predominant isoform normally expressed in kidney (14–16). Arginase catalyzes hydrolysis of L-arginine to L-ornithine and urea and thus competes with nitric oxide (NO) synthases (NOS) for the common substrate L-arginine (17). Depending on stimulus, either one or both of the arginases may be expressed and induced in macrophages, endothelial cells, and other cell types (12,13). With rare exceptions (12,13), arginase activities correlate with arginase protein levels, which, in turn correlate with arginase mRNA levels, primarily reflecting transcriptional regulation. Although potent arginase inhibitors have been developed, none of them are specific for an individual isozyme (12,13). Thus, selectively ablating expression of a specific isozyme by molecular biology techniques is the only way to evaluate functions of individual arginases in vivo and in cultured cells.

The current study tested the hypothesis that arginases are a critical determinant of progressive DN. We found that *Ins2*^{Akita} mice and streptozotocin (STZ)-induced diabetic wild-type mice are protected from albuminuria and display reduced histopathological changes associated with DN and reduced macrophage infiltration into kidney when treated with a potent arginase inhibitor or when arginase-2 expression was genetically abolished. Furthermore, kidney arginase-2 expression increased in diabetic mice and was associated with a reduction in renal medullary blood flow, whereas arginase-1 expression was undetectable in the kidneys under normal or diabetes conditions. These results indicate that targeting arginase-2 activity or expression may be a novel therapeutic intervention in the treatment of DN.

RESEARCH DESIGN AND METHODS

Diabetic mouse models. Experiments were conducted in male D2.B6-*Ins2*^{Akita}/MatbJ and their wild-type littermate mice (Dilute Brown Agouti [DBA]/2J background; The Jackson Laboratory, stock number 007562) starting at 5 weeks of age (2 weeks of diabetes) until 14 weeks of age (11 weeks of diabetes). *Ins2*^{Akita} mice, recommended by the Animal Models of Diabetes Complications Consortium as an optimal model of DN (18,19), developed

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hyperglycemia at 3 weeks of age. Additional experiments were conducted in male 6-week-old DBA/2J mice (The Jackson Laboratory, stock number 000671) and in male arginase-2–deficient ($Arg2^{-/-}$) mice on C57BL/6J background (provided by B. Lee, Baylor College of Medicine, Houston, TX) using multiple low doses of vehicle (lactated Ringer's solution) or STZ (Sigma, St. Louis, MO; 50 mg/kg body wt dissolved in lactated Ringer's solution) via intraperitoneal injection. All animal studies were approved by the Penn State University College of Medicine Institutional Animal Care and Use Committee.

Drug delivery. *S*-(2-Boronoethyl)-L-cysteine (BEC), a potent arginase inhibitor (2.3 mg/kg/day; Cayman Chemical, Ann Arbor, MI), or vehicle (phosphate-buffered saline) was administered by continuous subcutaneous infusion for 9 weeks (in $Ins2^{Akita}$ experiments) or for 6 weeks (in STZ-DBA mice experiments) via a mini-osmotic pump (Alzet; Durect Corporation, Palo Alto, CA) as described previously (20). The osmotic pump was incubated in phosphate-buffered saline for 60 h at 37°C before implantation and was implanted dorsally between the shoulders. The condition of mice and body weight were monitored daily after the pump implantation.

Blood pressure measurement. Systolic blood pressure was measured using Coda blood pressure (Kent Scientific Corp, Torrington, CT) (21). Mice were allowed to rest quietly for 10 min at 26°C. All measurements were performed at the same time for all groups to prevent any diurnal variations.

Renal histochemistry and immunohistochemistry. Kidneys were fixed in 4% paraformaldehyde and embedded in paraffin, and 3-μm sections were cut. Sections were stained with periodic acid Schiff (PAS) stain, and all glomeruli were examined at 400× in a blinded manner. All images were obtained with an Olympus BX51 microscope and DP71 digital camera using MicroSuite Basic 2.6 imaging software. Images were taken with 100× (oil) objective with a total magnification of 1,000×. Semiquantitative scores (0–4+) were assigned based on the masked reading, as previously described (22). In brief, 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, 25–50, 50–75, or >75% of the glomerular tuft area, respectively.

Immunohistochemistry for macrophages was performed using rat anti-mouse Mac-2 monoclonal antibody (clone M3/38; Cedarlane, Burlington, NC) on paraffin sections. Sections were incubated with primary antibody (50 μg/mL) followed by a biotinylated goat IgG anti-rat (Vector Laboratories, Burlingame, CA) secondary antibody according to the manufacturer's protocol. Sections were viewed using an Olympus BX51 microscope and DP71 digital camera using MicroSuite Basic 2.6 imaging software. Images were taken with 100× (oil) objective with a total magnification of 1,000×. The number of glomerular macrophages was counted in 20 glomeruli per section (number of macrophages in glomeruli divided by the number of glomeruli) in blinded fashion under 40× magnification and averaged.

Analysis of kidney macrophage content by fluorescence-activated cell sorter. We used flow cytometry to analyze kidney macrophage content ($CD11b^{+}F4/80^{low}$) at the end of the experiments as described previously (23,24). In brief, kidneys were extracted, minced, digested, and then passed through a filter and a cotton wool column. Fresh kidney suspensions were incubated with anti-mouse CD45-fluorescein isothiocyanate (30-F11; eBioscience, San Diego, CA) for 30 min on ice. Kidney macrophages were then identified using allophycocyanin-labeled rat anti-mouse F4/80 (BM8; eBioscience) and phycoerythrin-labeled rat anti-mouse CD11b (M1/70; eBioscience). All samples were treated with anti-mouse CD16/CD32 (2.4G2) to block nonspecific FcR binding and 7-AAD to eliminate dead cells (Invitrogen, Carlsbad, CA). Counting beads (Caltag, Carlsbad, CA) were used to determine the total number of $CD45^{+}$ cells per gram of kidney tissue. Subsequent flow cytometry data acquisition was performed on FASCalibur (Becton Dickinson, San Jose, CA). Data were analyzed by Flowjo software 6.4 (Tree Star, Ashland, OR). All the antibodies were purchased from eBioscience.

Quantitative real-time PCR. Total RNA was extracted from kidneys using RNeasy Mini Kit (Qiagen, GmbH, Hilden, Germany). Single-strand cDNA was synthesized using iScript cDNA Synthesis Kits (Bio-Rad, Hercules, CA) for two-step real-time RT-PCR. Gene-specific primers for arginase-1 and arginase-2 were designed using Beacon Designer Probe/Primer Design Software (Premier Biosoft International, Palo Alto, CA). Specificity of the PCR products was verified by melting curve analysis. Quantitative real-time PCR was performed using Bio-Rad CFX96 system. Reactions were performed in duplicate, and threshold cycle numbers were averaged. Samples were calculated with normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (25).

Analytical methods. UAE was measured by ELISA using Albuwell M (Exocell, Philadelphia, PA) as described previously (25). Blood urea nitrogen (BUN) was measured using VITROS DT60II chemistry slides (Ortho-Clinical Diagnostics, Rochester, NY). Body composition was determined using LF90 Minispec Time Domain Nuclear Magnetic Resonance Spectrometer (Bruker Optics, Billerica MA).

Arginase activity assay. Kidney lysates were prepared by homogenization in lysis buffer (50 mmol/L Tris-HCl, pH 7.5, 0.1 mmol/L 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 and plasma samples were assayed for arginase activity as previously described (26).

Western blot. Plasma (5-μL aliquots) was electrophoresed on SDS-polyacrylamide gels (12% Mini-PROTEAN TGX Precast Gel; Bio-Rad) and electroblotted onto polyvinylidene fluoride membranes (Millipore, Billerica, MA). Arginase-1 was visualized by immunoblotting as previously described (12,13).

Renal blood flow. A midline abdominal incision was made. Renal medullary blood flow was monitored by using a laser flow meter (model ALF-21D; Transonic Systems, Inc., Ithaca, NY).

Statistical 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 $P < 0.05$.

RESULTS

Arginase inhibition reduces characteristics of DN in $Ins2^{Akita}$ mice. To assess the possible clinical significance of arginases in diabetic mice, we continuously infused the arginase inhibitor BEC or vehicle into $Ins2^{Akita}$ mice and their wild-type DBA littermates for 9 weeks, beginning at 5 weeks of age. As shown in Table 1, $Ins2^{Akita}$ vehicle-treated mice had increased blood glucose levels, decreased body weight, increased kidney weight, increased kidney weight/body weight ratio, reduced fat and fluid composition, and increased lean composition compared

TABLE 1
Effects of BEC on diabetic $Ins2^{Akita}$ mice versus wild-type littermate controls at 14 weeks of age

	Wild type		$Ins2^{Akita}$	
	Vehicle	BEC	Vehicle	BEC
Glucose (mg/dL)	167 ± 8	142 ± 7	466 ± 15***	441 ± 17+++
BW (g)	33 ± 2	32 ± 1	27 ± 1**	27 ± 1++
KW (mg)	271 ± 7	262 ± 4	322 ± 6***	269 ± 10###
KW/BW (mg/g)	8.4 ± 0.4	8.1 ± 0.1	12 ± 0.3***	10 ± 0.3++##
BUN (mg/dL)	22 ± 2	28 ± 3	47 ± 8*	28 ± 3#
Systolic BP (mmHg)	112 ± 7	118 ± 10	127 ± 1	134 ± 2
Fat (%)	13.3 ± 0.9	12.4 ± 0.9	6.8 ± 0.3***	6.7 ± 0.6+++
Lean (%)	66.7 ± 1.1	66.3 ± 0.5	70.7 ± 0.8**	67.6 ± 1.2
Fluid (%)	7.9 ± 0.2	7.9 ± 0.1	7.1 ± 0.4	6.4 ± 0.1+

Data are mean ± SEM for n = 6–8 per group. BW, body weight; KW, kidney weight; BP, blood pressure. *$P < 0.005$ vs. control + vehicle group. **$P < 0.001$ vs. control + vehicle group. ***$P < 0.0001$ vs. control + vehicle group. +$P < 0.01$ vs. control + BEC group. ++$P < 0.005$ vs. control + BEC group. +++$P < 0.0001$ vs. control + BEC group. #$P < 0.05$ vs. $Ins2^{Akita}$ + vehicle group. ##$P < 0.001$ vs. $Ins2^{Akita}$ + vehicle group. ###$P < 0.0001$ vs. $Ins2^{Akita}$ + vehicle group.

with control mice. Arginase inhibition of *Ins2*^{Akita} mice significantly reduced kidney weight and kidney weight/body weight ratio without affecting other measurements. It is noteworthy that treatment with BEC did not reduce blood glucose levels or blood pressure.

Inhibition of arginases reduces albuminuria and BUN in *Ins2*^{Akita} mice. To determine whether arginases contribute to diabetic renal injury, we measured 24-h UAE and BUN as indicators of renal injury in *Ins2*^{Akita} with and without BEC treatment. Vehicle-treated *Ins2*^{Akita} mice had a significant increase in albuminuria compared with controls at 5 and 14 weeks of age (Fig. 1). Albuminuria was significantly reduced in *Ins2*^{Akita} mice treated with BEC at 14 weeks of age. Similarly, BUN was significantly increased in vehicle-treated *Ins2*^{Akita} mice compared with other groups (Table 1).

Arginase-2, but not arginase-1, is expressed in the kidney and induced in *Ins2*^{Akita} mice. We assessed whether both arginases are expressed in the kidneys under normal and diabetes conditions. Arginase-2, but not arginase-1, mRNA was detectable in the kidney (Fig. 2*A*). This result supports the view that the effect of BEC in the kidney is mainly mediated via inhibition of arginase-2. Both vehicle-treated and BEC-treated *Ins2*^{Akita} mice had increased arginase-2 mRNA levels (Fig. 2*B*). Thus, diabetes is associated with increased kidney arginase-2 expression. Although BEC inhibits arginase activity, it did not downregulate arginase-2 mRNA in diabetes.

Inhibition of arginases decreases macrophage recruitment in *Ins2*^{Akita} mice. To determine whether arginase is critical for macrophage infiltration in DN, we show the distribution and quantitation of macrophages in kidneys by immunohistochemistry (Mac-2–positive macrophages) (Fig. 3*A*). Vehicle-treated *Ins2*^{Akita} mice showed significant increases in glomerular macrophages (2.95 ± 0.14 macrophages/glomerulus; $P < 0.0001$) compared with vehicle-treated control (0.34 ± 0.04 macrophages/glomerulus). Both BEC-treated control and *Ins2*^{Akita} mice had significantly reduced glomerular macrophage recruitment (0.29 ± 0.03 and 0.9 ± 0.14 macrophages/glomerulus; $P < 0.0001$), respectively, compared with vehicle-treated *Ins2*^{Akita} mice at 14 weeks of age.

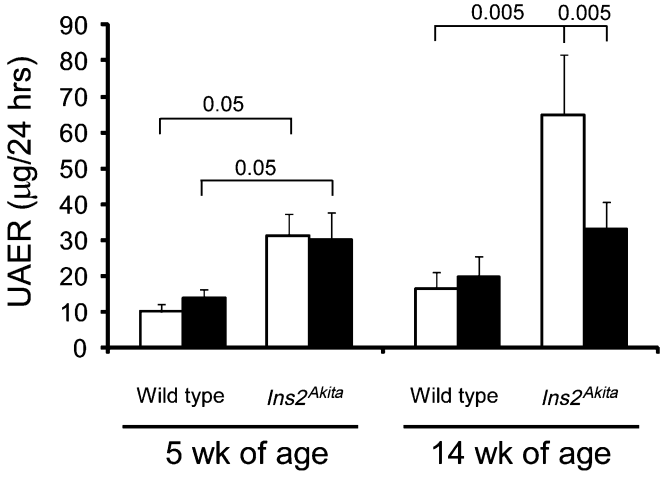


FIG. 1. Arginase inhibition reduces increases in UAE in diabetic *Ins2*^{Akita} mice. *Ins2*^{Akita} and wild-type littermate mice were treated with BEC or vehicle via osmotic minipump for 9 weeks. Urine was collected for measurement of UAE before treatment (5 weeks of age) and after treatment (14 weeks of age). Open bar, vehicle-treated groups; filled bar, BEC-treated groups. Results are means ± SEM for *n* = 6–8 mice in each group.

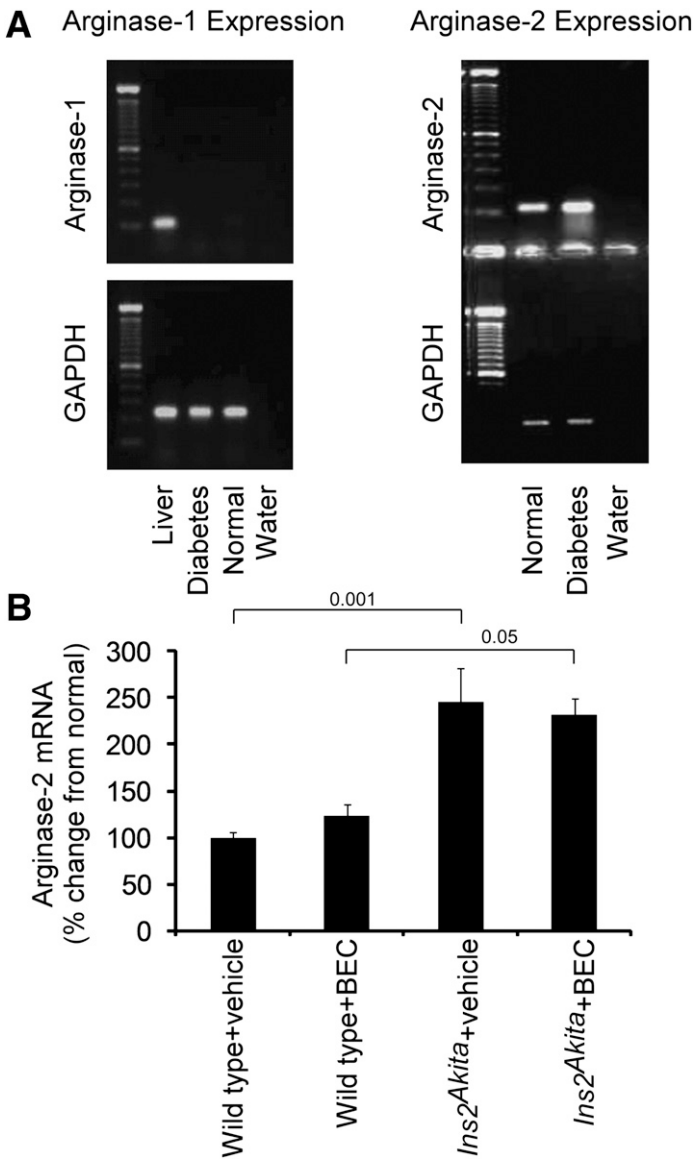


FIG. 2. Abundance of arginase-2 mRNA is increased in kidneys of diabetic *Ins2*^{Akita} mice. *A*: RT-PCR was performed on RNA isolated from mouse kidney. GAPDH mRNA was used as an internal control for all RNA samples. Liver was used as a positive control for expression of arginase-1. *B*: Quantitative RT-PCR was performed on kidney RNA isolated at 14 weeks of age. Arginase-2 mRNA levels were normalized to GAPDH mRNA and expressed relative to levels in control DBA mice (set at 100%).

Similar results were obtained when kidney macrophages (identified as CD11b^{+}F4/80^{low}) were detected by fluorescence-activated cell sorter (Fig. 3*B*). Kidneys of vehicle-treated *Ins2*^{Akita} mice had a significantly greater number of macrophages compared with vehicle-treated control (3.6 ± 0.1 vs. 2.1 ± 0.3 macrophages/gram kidney tissue; $P < 0.005$). In contrast, kidneys of BEC-treated *Ins2*^{Akita} mice had significantly reduced numbers of macrophages (1.6 ± 0.3 macrophages/gram kidney tissue; $P < 0.0001$) compared with vehicle-treated *Ins2*^{Akita} mice at 14 weeks of age.

Inhibition of arginases reduces albuminuria in STZ-induced diabetic mice. To determine whether the effect of arginase inhibition is specific to *Ins2*^{Akita} mice, we used

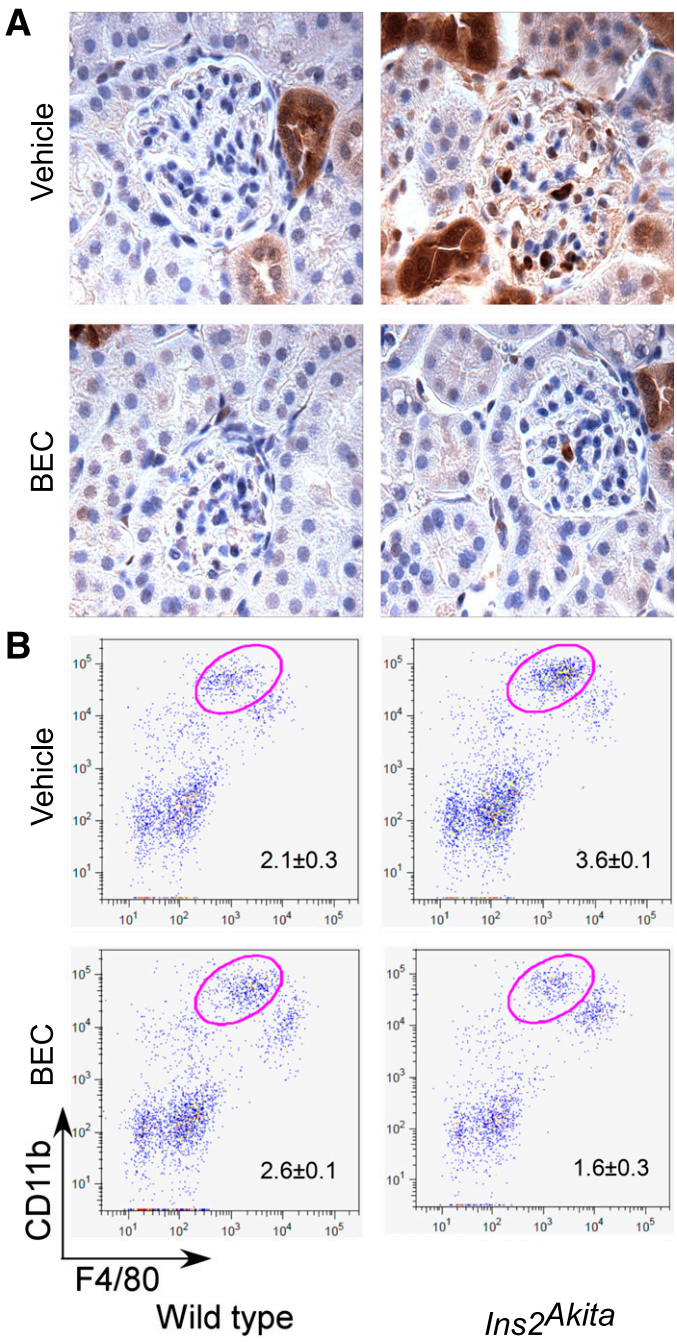


FIG. 3. Arginase inhibition reduces macrophage infiltration in diabetic *Ins2*^{Akita} mice. *A*: Mac-2–positive macrophages in glomeruli were identified by immunohistochemical staining at 14 weeks of age in wild-type DBA mice treated with vehicle, *Ins2*^{Akita} mice treated with vehicle, wild-type mice treated with BEC, and *Ins2*^{Akita} mice treated with BEC. Images are representative of 20 fields from six to eight mice in each group. *B*: Experimental groups were as described in (*A*). Kidneys were harvested at 14 weeks of age and processed for fluorescence-activated cell sorter analysis as described in RESEARCH DESIGN AND METHODS. Macrophages were identified as CD11b^{high}F4/80^{low}. Graphs show representative contour plots. Numbers of CD11b^{high}F4/80^{low} macrophages per gram kidney tissue are expressed as means ± SEM for *n* = 6–8 mice in each group. (A high-quality digital representation of this figure is available in the online issue.)

another type 1 diabetic mouse model (DBA/2J) using STZ to induce diabetes for a 6-week study period. We measured 24-h UAE as indicators of renal injury with and without BEC treatment. Vehicle-treated, STZ-induced diabetic mice had a significant increase in albuminuria compared with controls after 6 weeks, an effect that was significantly reduced with BEC treatment in diabetic mice despite comparable blood glucose levels (Fig. 4). We also show an increase in kidney macrophage recruitment using Mac-2 staining in vehicle-treated, STZ-induced diabetic mice (1.9 ± 0.1 macrophages/glomerulus; $P < 0.0001$) compared with control (0.6 ± 0.05 macrophages/glomerulus). In contrast, BEC-treated, STZ-diabetic mice had a significant reduction in glomerular macrophage recruitment (1.2 ± 0.06 macrophages/glomerulus; $P < 0.0001$) compared with vehicle-treated STZ-diabetic mice.

Inhibition of arginases decreases renal histopathological changes in STZ-induced diabetic mice. PAS staining of kidney sections showed increased glomerular cellularity and mesangial expansion (score, 1.9 ± 0.05 vs. 1.1 ± 0.09; $P < 0.005$) after 6 weeks of diabetes in vehicle-treated, STZ-diabetic mice versus controls (Fig. 5*A* and *B*). Inhibition of arginases in STZ-induced diabetic mice exhibited significantly reduced glomerular changes (scores, 1.6 ± 0.08; $P < 0.05$) compared with vehicle-treated, STZ-induced mice (Fig. 5*C*). As expected for a period of induced diabetes of only 6 weeks, there was little or no indication of fibrosis by Masson's trichrome or the periodic acid methenamine silver (aka Jones stain) staining, which was not detectably different between any of the groups (data not shown).

Deficiency of arginase-2 reduces albuminuria in diabetic mice. We next assessed whether a genetic lack of arginase-2 will mimic the changes observed in *Ins2*^{Akita} mice after pharmacologic arginase inhibition. Wild-type and *Arg2*$^{-/-}$ mice were treated with STZ and killed at either 6 or 18 weeks after STZ-induced diabetes. Wild-type mice displayed a significant increase in UAE after 6 weeks and after 18 weeks of STZ-induced diabetes (Fig. 6). In contrast, the increase in UAE was significantly reduced in *Arg2*$^{-/-}$ mice in a similar manner to BEC treatment in

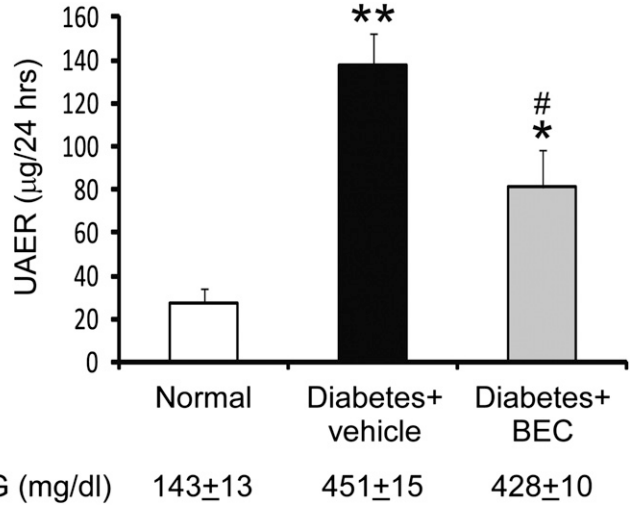
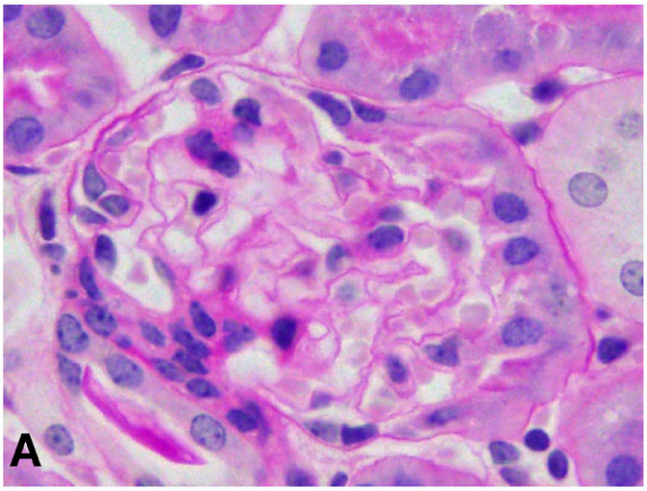
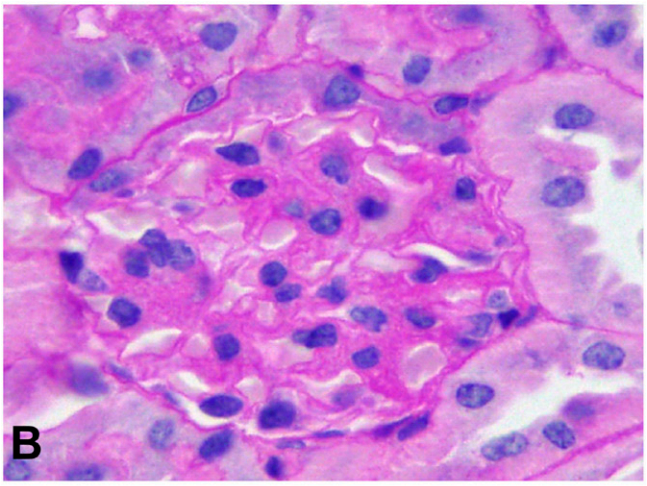


FIG. 4. Arginase inhibition reduces increases in UAE in STZ-diabetic mice. DBA mice were given multiple intraperitoneal injections of vehicle or STZ as described in RESEARCH DESIGN AND METHODS. Mice were treated with BEC (2.3 mg/kg/day) or vehicle via osmotic minipump for 6 weeks, at which time urine was collected for measurement of UAE. Blood glucose (BG) levels and UAER values are means ± SEM for *n* = 5–6 mice in each group. **P* < 0.05; ***P* < 0.0001, compared with normal; #*P* < 0.05, compared with diabetes + vehicle group.





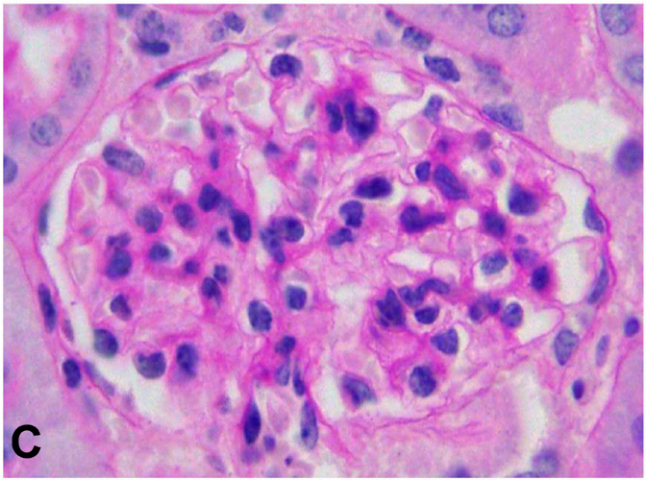
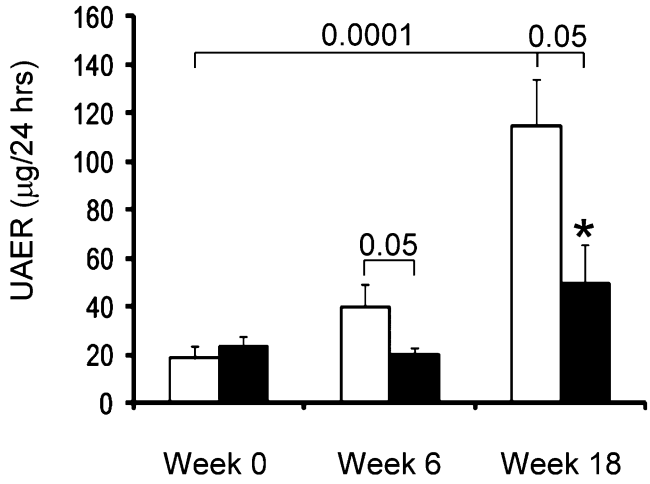


FIG. 5. Arginase inhibition reduces histological changes in STZ-diabetic mice. Sections were stained with PAS, and all glomeruli were graded individually at 400× magnification. Images were taken with 100× (oil) objective with a total magnification of 1,000×. Images are representative of six to eight mice in each group. *A*: Representative PAS section from normal mouse showing morphologically normal glomerulus with delicate PAS-positive basement membranes and minimal PAS staining of mesangial matrix. Adjacent tubular basement membranes and brush-border stain PAS-positive as well. *B*: Representative PAS section from vehicle-treated, STZ-diabetic mouse showing glomerular expansion in which PAS-positive material occupies 25–50% of the mesangial matrix within the tuft. *C*: Representative PAS section from BEC-treated, STZ-diabetic mouse showing glomerular expansion in which PAS-positive material occupies <25% of the mesangial matrix. (A high-quality digital representation of this figure is available in the online issue.)



Blood Glucose (mg/dl)			
Wild type:	178±8	499±2	488±6
Arg2$^{-/-}$:	180±5	392±30	497±2

FIG. 6. Arginase-2 deficiency prevents increases in UAE in STZ-diabetic mice. Urine was collected for measurement of UAER in wild-type and *Arg2*$^{-/-}$ mice before (week 0) and after weeks 6 and 18 of STZ-induced diabetes. Data are means ± SEM for *n* = 6–15 mice in each group. Open bars are wild-type mice and filled bars are *Arg2*$^{-/-}$ mice. **P* < 0.05 vs. *Arg2*$^{-/-}$ at week 0. Values of blood glucose (BG) are also shown as means ± SEM for *n* = 6–15 mice in each group.

Ins2^{Akita}. It is noteworthy that arginase-2 deficiency did not alter hyperglycemia in the diabetic mice (Fig. 6).

Arginase activity is increased in kidney and plasma of diabetic mice. Diabetes was associated with a significant increase in kidney arginase activity at 6 and 18 weeks in wild-type mice after STZ-induced diabetes (Fig. 7*A*). These data were confirmed in vehicle-treated *Ins2*^{Akita} mice after 9 weeks of diabetes, with increased kidney arginase activity versus control mice (22 ± 1 vs. 12 ± 1 nmol/min/mg). In contrast, *Arg2*$^{-/-}$ mice had minimal kidney arginase activity at all time points, confirming previous studies showing that overall arginase activity in the kidney is due almost entirely to arginase-2 (27). The small amount of kidney arginase activity in *Arg2*$^{-/-}$ mice under normal and diabetes conditions may be due to kidney-resident leukocytes that express arginase-1 or to trace arginase-1 contamination from plasma.

Plasma arginase activity, which is due almost exclusively to arginase-1 (28), was increased in both wild-type and *Arg2*$^{-/-}$ mice after 18 weeks of STZ-induced diabetes and correlated well with increased arginase-1 protein by Western blot (Fig. 7*B*). These data suggest a possible minor contribution of elevated plasma arginase-1 to the development and progression of DN.

Deficiency of arginase-2 restores renal medullary blood flow in diabetic mice. Because arginases and NOS can compete for L-arginine, we assessed whether changes in levels of arginase-2 would correlate with changes in renal blood flow. Whereas increased kidney arginase activity in diabetic mice (Fig. 7*A*) was associated with reduced renal medullary blood flow (Fig. 7*C*), medullary blood flow was not reduced in *Arg2*$^{-/-}$ mice, suggesting that renal arginase-2 modulates NO production by catabolizing arginine and demonstrating that elevated plasma arginase-1 had no impact on medullary blood flow.

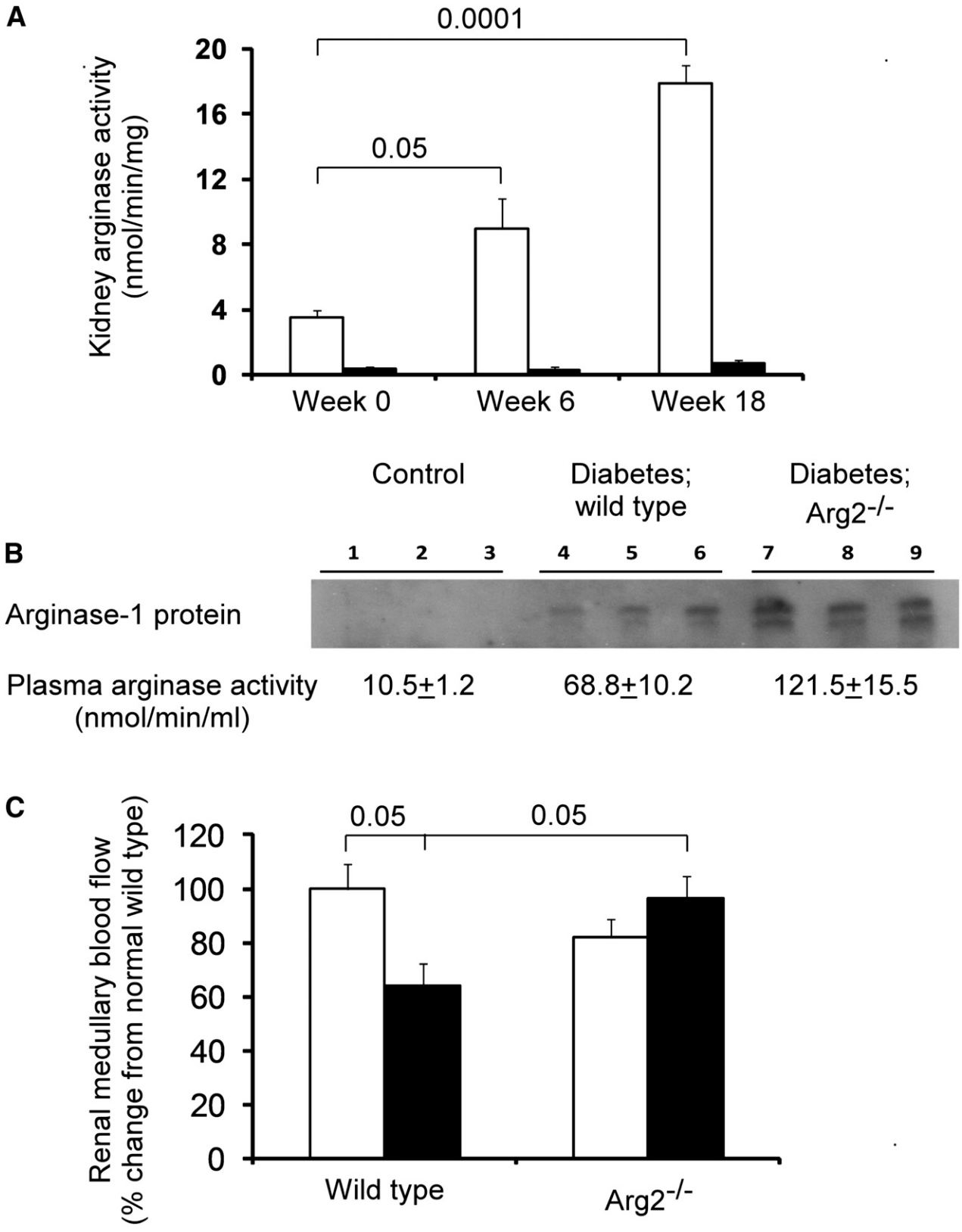


FIG. 7. Kidney arginase activity is induced and associated with a reduction in renal medullary blood flow in diabetic wild-type mice but not in diabetic *Arg2*$^{-/-}$ mice. *A*: Arginase activities were assayed in kidney lysates prepared at the indicated times after STZ-induced diabetes. Open bars are wild-type mice and filled bars are *Arg2*$^{-/-}$ mice. Results are means ± SEM for n = 3–6 mice in each group. *B*: Plasma samples were obtained from wild-type controls and from wild-type and *Arg2*$^{-/-}$ mice after 18 weeks of STZ-induced diabetes. Arginase activities and arginase-1 protein levels were determined as described in RESEARCH DESIGN AND METHODS. Plasma arginase activities are expressed as means ± SEM for each group of individual mice depicted in the Western blot. *C*: Renal medullary blood flow was measured after 6 weeks of STZ-induced diabetes. Open bars are normal mice and filled bars are diabetic mice. Results are means ± SEM for n = 6–10 mice in each group.

DISCUSSION

Arginases have a well-established role to alter endothelial function in cardiovascular diseases, yet their role in diabetic kidney injury has not previously been determined. This study shows that pharmacological blockade or genetic deficiency of arginase-2 mediates renal tissue protection as proven by a reduction in albuminuria, BUN, histopathological changes, and kidney macrophage recruitment during diabetes. We speculate that the effect of arginase inhibition in the kidney is mainly mediated via inhibition of arginase-2 because arginase-1 expression was not detectable in the kidney. These findings reveal an important role for arginase-2 in the pathogenesis of DN and provide evidence for arginase inhibition as potential therapeutic modality for treating diabetic patients.

To examine the role of arginases in DN, we used two approaches (use of a potent arginase inhibitor and use of *Arg2*$^{-/-}$ mice) in two different models of type 1 diabetes: *Ins2*^{Akita} diabetic mice and STZ-induced diabetic mice. We investigated the effects of inhibiting arginases on kidney dysfunction, glomerular histopathological changes, and macrophage recruitment using BEC in *Ins2*^{Akita} mice and the STZ-induced diabetic model. BEC significantly ameliorated diabetic albuminuria and was associated with reduced kidney macrophage infiltration and glomerular pathology. Similar to the BEC treatment, lack of arginase-2 expression ameliorated renal function by normalizing UAE in diabetes, even though these mice had elevated blood glucose levels comparable to diabetic wild-type mice. Taken together, our results provide support for blocking arginase as a therapeutic modality for the treatment of DN.

The expression of arginase-2 rather than arginase-1 in the kidney suggests that BEC effects are largely, but perhaps not entirely, mediated via inhibition of arginase-2. Because BEC inhibits both arginase-1 and -2, it is possible that arginase-1 also may contribute somewhat to DN. Currently, there are no isoform-specific arginase inhibitors. The fact that plasma arginase activity and arginase-1 protein were significantly increased in both diabetic wild-type and *Arg2*$^{-/-}$ mice suggests a possible contribution of plasma arginase-1 to reductions in arginine bioavailability and to the development and progression of DN. Additional experiments are needed to explore this possibility.

The renal protective effect of BEC correlates with a significant reduction of kidney macrophage infiltration. Whether the reduction in macrophage recruitment is mediated directly by arginase inhibition or indirectly by reducing diabetic renal injury is not clear at this time. Additional studies are required to elucidate the role of arginases on kidney macrophage infiltration.

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. Previous immunohistochemical studies have identified arginase-2 expression in proximal straight tubules and inner medullary collecting duct (29,30). The expression of arginase-2 in glomerular cells, such as podocytes, and/or glomerular endothelial cells will be important to investigate.

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. Endothelial dysfunction, characterized by reduced bioavailability of NO and increased oxidative stress, is a hallmark of diabetes (31) and DN (32). NO is produced from arginine by NOS. Under conditions of low arginine level or hyperglycemia, endothelial NOS can become uncoupled, producing reactive oxygen species in lieu of NO (33,34). Recently, low or lack of endothelial NOS has been shown to exacerbate DN (22,35); thus, elucidating the basis for vascular dysfunction in DN is critical (36). Arginase-2 is constitutively expressed and also inducible in endothelial cells (4–6) as well as in kidney cells (29,30). When elevated, arginase-2 can inhibit NOS activity and expression and thus induce endothelial NOS uncoupling, thereby reducing NO bioavailability and inhibiting the NO/cGMP pathway as well as increasing oxidative stress. Previous studies have shown that vascular dysfunction in rodent models of diabetes (8,10,11) or diabetic patients (9) is associated with increased arginase-1 (9,11) or arginase-2 (8) expression and that vascular function is normalized by inhibition of arginase activity or genetic ablation of arginase-2 expression (8,10). The current study is the first to evaluate a possible role for arginase-2 in vascular dysfunction specifically in the kidney. Our data showing reduced renal medullary blood flow in diabetic wild-type mice and its restoration in arginase-2–deficient mice are consistent with these findings, indicating a possible role of arginase-2 in regulating NO bioavailability in the kidney.

In conclusion, our study demonstrates for the first time that arginase-2 plays an essential role in the development of DN. This conclusion is based on two novel observations. First, we demonstrated a beneficial effect of arginase inhibition in animal models of DN. Second, we showed that deficiency specifically of arginase-2 ameliorates diabetic renal injury. Results of this study may ultimately result in novel therapeutic interventions designed to attenuate arginase activity or signaling that regulates arginase-2 expression in the treatment of diabetic kidney disease.

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