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

Hanning You[1], Ting Gao[1], Timothy K. Cooper[2], Sidney M. Morris Jr[3] and Alaa S. Awad[1]

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

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 with 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 with 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.

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Correspondence: *Alaa S. Awad, Division of Nephrology, Department of Medicine, Penn State University College of Medicine, H040, 500 University Drive, PO Box 850, BMR Building, C5830, Hershey, Pennsylvania 17033, USA. E-mail: asa17@psu.edu*

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Diabetic nephropathy (DN) is the leading cause of end-stage kidney disease, responsible for over 40% of all cases in the United States, 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 hyperfiltration and hypertrophy, followed by thickening of the glomerular basement membrane, mesangial matrix accumulation, increased urinary albumin excretion 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 diabetes[2] and DN.[3] NO is produced from L-arginine by NO synthases (NOS). Under conditions of low arginine level or hyperglycemia, endothelial nitric oxide synthase (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 has important roles in many different kinds of diseases.[9] Marked alterations in arginine metabolism occur in endothelial injury[10–12] owing to changes in the activity and/or expression of arginases. Arginase-2 is constitutively expressed and also inducible in endothelial cells[10–12] and 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 function,[23] and DN.[16]

Our recent publication[16] 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. As 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$^{+/+}$ mice but not in 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 S-(2-boronoethyl)-L-cysteine (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 with 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 with eNOS$^{+/+}$ groups.

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

We next measured 24-h urinary albumin excretion, plasma creatinine, and blood urea nitrogen 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 urinary albumin excretion (Figure 1a), albumin/creatinine ratio (Figure 1b), plasma creatinine, and blood urea nitrogen (Table 1) compared with vehicle-treated diabetic eNOS$^{+/+}$ mice after 6 weeks of streptozotocin (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$^{+/+}$ mice but not in eNOS$^{-/-}$ mice

Periodic acid–Schiff staining of kidney sections showed increased glomerular cellularity and mesangial expansion in vehicle-treated diabetic versus normal eNOS$^{+/+}$ mice (score: 0.99 ± 0.09 vs. 0.19 ± 0.01, $P<0.01$) after 6 weeks of diabetes (Figure 2). Inhibition of arginases in diabetic eNOS$^{+/+}$ mice resulted in significantly reduced glomerular changes (scores: 0.6 ± 0.06; $P<0.01$) compared with 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 (Figure 2). Similar results were obtained using reverse transcriptase PCR for kidney fibronectin expression as a fibrotic marker. As shown in Figure 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

Table 1 | General characteristics of eNOS$^{+/+}$ and eNOS$^{-/-}$ mice

	Week 6					
	eNOS$^{+/+}$			eNOS$^{-/-}$		
Treatment	Normal	Diabetes + vehicle	Diabetes + BEC	Normal	Diabetes + vehicle	Diabetes + BEC
No of animals	5	6	8	4	8	8
Body weight (g)	25 ± 1	20 ± 1^{a}	25 ± 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
SBP (mm Hg)	136 ± 3	130 ± 4	135 ± 7	148 ± 4.227^{e}	150 ± 4	147 ± 5
Kidney/body weight ratio	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
% 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
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
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

Abbreviations: BEC, S-(2-boronoethyl)-L-cysteine; BUN, blood urea nitrogen; SBP, systolic blood pressure.
Data are mean ± s.e.m.
[a] $P<0.01$ compared with eNOS$^{+/+}$ normal and diabetes + BEC groups.
[b] $P<0.01$ compared with eNOS$^{-/-}$ normal group.
[c] $P<0.01$ compared with eNOS$^{+/+}$ diabetes + vehicle and diabetes + BEC groups.
[d] $P<0.01$ compared with eNOS$^{-/-}$ diabetes + vehicle and diabetes + BEC groups.
[e] $P<0.05$ compared with eNOS$^{+/+}$ normal group.
[f] $P<0.05$ compared with eNOS$^{+/+}$ normal and diabetes + BEC groups.
[g] $P<0.05$ compared with eNOS$^{-/-}$ diabetes + vehicle and diabetes + BEC groups.
[h] $P<0.05$ compared with eNOS$^{+/+}$ normal and diabetes + BEC groups.
[i] $P<0.05$ compared with eNOS$^{-/-}$ normal and diabetes + BEC groups.
[j] $P<0.05$ compared with eNOS$^{+/+}$ normal and diabetes + BEC groups.
[k] $P<0.05$ compared with eNOS$^{-/-}$ normal and diabetes + BEC groups.