Arginase Inhibition Improves Microvascular Endothelial Function in Patients With Type 2 Diabetes Mellitus

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Objectives: The development of microvascular complications in diabetes is a complex process in which endothelial dysfunction is important. Emerging evidence suggests that arginase is a key mediator of endothelial dysfunction in type 2 diabetes mellitus by reciprocally regulating nitric oxide bioavailability. The aim of this prospective intervention study was to test the hypothesis that arginase activity is increased and that arginase inhibition improves microvascular endothelial function in patients with type 2 diabetes and microvascular dysfunction.

Design: Microvascular endothelium-dependent and -independent dilatation was determined in patients with type 2 diabetes (n = 12) and healthy age-matched control subjects (n = 12) with laser Doppler flowmetry during iontophoretic application of acetylcholine and sodium nitroprusside, respectively, before and after administration of the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (120 min). Plasma ratios of amino acids involved in arginase and nitric oxide synthase activities were determined. The laser Doppler flowmetry data were the primary outcome variable.

Results: Microvascular endothelium-dependent dilatation was impaired in subjects with type 2 diabetes ($P < .05$). After administration of N^{ω}-hydroxy-nor-L-arginine, microvascular endothelial function improved significantly in patients with type 2 diabetes to the level observed in healthy controls. Endothelium-independent vasodilatation did not change significantly. Subjects with type 2 diabetes had higher levels of ornithine and higher ratios of ornithine/citrulline and ornithine/arginine ($P < .05$), suggesting increased arginase activity.

Conclusion: Arginase inhibition improves microvascular endothelial function in patients with type 2 diabetes and microvascular dysfunction. Arginase inhibition may represent a novel therapeutic strategy to improve microvascular endothelial function in patients with type 2 diabetes. **(*J Clin Endocrinol Metab* 101: 3952–3958, 2016)**

Patients with diabetes are at high risk of developing cardiovascular complications (1). These include microvascular dysfunction that contributes to clinically important complications such as retinopathy, nephropathy, and neuropathy for which specific therapeutic targets remain to be identified. The mechanisms underlying microvascular dysfunction are complex and incompletely understood but include genetic (2) and metabolic (3, 4) changes of the vascular structure (5, 6). Endothelial dysfunction is a central feature in the development of vascular

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Abbreviations: Ach, acetylcholine; BMI, body mass index; CRP, C-reactive protein; eNOS, endothelial NOS; HbA1C, glycosylated hemoglobin; LDF, laser Doppler flowmetry; NO, nitric oxide; Nor-NOHA, N^{ω}-hydroxy-nor-L-arginine; NOS, NO synthase; SNP, sodium nitroprusside.

complications associated with diabetes (7), and microvascular complications in type 2 diabetes mellitus have been linked to impaired endothelial function (8–10). Reduced bioavailability of nitric oxide (NO) is a key factor behind endothelial dysfunction and the progression of cardiovascular disease due to its importance in normal vascular homeostasis (11). NO is produced from L-arginine by endothelial NO synthase (eNOS) with citrulline as a coproduct. Arginase has emerged as a key regulator of NO bioavailability and endothelial function by hydrolyzing L-arginine to ornithine and urea (12, 13). Thus, increased arginase activity may reduce the production of NO and increase production of reactive oxygen species due to substrate deficiency for eNOS (14). Interestingly, vascular arginase has been demonstrated to be up-regulated in both animal models and patients with type 2 diabetes (15–17). Blockade of arginase improves resistance and conduit artery endothelial function in patients with type 2 diabetes via a NO synthase (NOS)-dependent action (16, 18), supporting an important role of arginase in endothelial dysfunction in type 2 diabetes. Further data suggest that arginase is involved in microvascular dysfunction in rats with type 2 diabetes, identifying this enzyme as a potential target for the treatment of microvascular complications in type 2 diabetes (15). However, the efficacy of arginase inhibition to improve microvascular function in patients with type 2 diabetes has not been investigated. In the present study, we therefore tested the hypothesis that inhibition of arginase improves microvascular function in patients with type 2 diabetes and microvascular complications.

Subjects and Methods

Subjects

Patients were recruited from the Department of Endocrinology and Diabetology and the Department of Cardiology, Karolinska University Hospital, in Stockholm in 2014–2015. Twelve patients with type 2 diabetes and microvascular dysfunction and 12 healthy controls were included after giving their written informed consent. Subjects were classified as having type 2 diabetes if fasting plasma glucose exceeded 7.0 mmol/L on two different occasions, if plasma glucose exceeded 11.1 mmol/L 2 hours after oral administration of 75 g of glucose, or if they had a history of type 2 diabetes. Microvascular dysfunction was defined as urine albumin-creatinine ratio > 3.0 mg/mmol or the presence of retinopathy determined by retinal photography. Exclusion criteria were age > 80 years, myocardial infarction or unstable angina within the last 3 months, changed dose of any vasodilator drug during the preceding 6 weeks, ongoing treatment with warfarin, or concomitant disease that may have interfered with the possibility for the patients to comply with or complete the study protocol. The control subjects were matched for age, were free of medication, had no medical history of any cardiovascular disease, and had a normal oral glucose tolerance test. All subjects gave their oral and written consent to participate. The study was approved by the local ethics committee and was conducted in accordance with the Declaration of Helsinki.

Experimental protocol

Subjects arrived at the laboratory in the morning after overnight fasting. They were instructed not to take their morning medication and to refrain from caffeine- or nicotine-containing products before the experiment. Subjects on long-acting insulin were instructed to take 50% of their ordinary dose the evening before the investigation. After collection of basal blood samples, the subjects were given a standardized breakfast. All investigations were performed with the subject in the supine position in a dimly lit room with a temperature of 23°C. A catheter was introduced in the brachial artery of the nondominant arm (left in all subjects) after local anesthesia (2 mL of carbocain 10 mg/mL) for drug administration. Cutaneous microvascular flow was determined by laser-Doppler flowmetry (LDF; Periflux 5000; Perimed AB) connected to drug delivery chambers. Two different

Table 1. Basal Characteristics

	Controls	Type 2 Diabetes
n	12	12
Age, y	66 ± 4	66 ± 6
No. of males	12	9
BMI, kg/m^2	24 ± 1	34 ± 11[b]
W/H	0.98 ± 0.06	1.02 ± 0.06
Duration of type 2 diabetes, y	NA	21.4 ± 9.8
No. of smokers	0	0
SBP, mm Hg	136 ± 16	145 ± 21
DBP, mm Hg	81 ± 7	76 ± 14
Urine albumin/creatinine, mg/mmol	—	20.4 ± 20.2
Fasting glucose, mmol/L	5.6 ± 0.4	9.7 ± 3.5[c]
HbA1C, mmol/mol	36 ± 2	66 ± 14[c]
HbA1C, %	5.4 ± 0.14	8.2 ± 1.3[c]
CRP, mg/L	1.2 ± 0.4	2.3 ± 1.4[a]
Creatinine, mmol/L	81 ± 7	107 ± 43
Triglycerides, mmol/L	1.1 ± 0.6	1.3 ± 0.8
Total cholesterol, mmol/L	5.8 ± 1.2	3.2 ± 0.7[c]
HDL cholesterol, mmol/L	1.7 ± 0.4	1.2 ± 0.3[b]
LDL cholesterol, mmol/L	3.6 ± 0.9	1.5 ± 0.5[c]
Medications, no. of patients		
ACE inhibitors/ARB	0	10
Aspirin	0	12
Lipid-lowering drugs	0	9
β-blockers	0	5
Calcium channel blockers	0	5
Long-acting nitrates	0	2
Insulin	0	10
Metformin	0	7
GLP-1 analogs	0	2
DDP-4 inhibitors	0	2
Sulfonylurea	0	1

Abbreviations: ACE, angiotensin-converting enzyme; ARB, angiotensin receptor blocker; DBP, diastolic blood pressure; DDP-4, dipeptidyl peptidase-4; GLP-1, glucagon-like peptide-1; HbA1C, glycated hemoglobin; HDL, high-density lipoprotein; LDL, low-density lipoprotein; NA, not available; SBP, systolic blood pressure; W/H, waist/hip ratio. Data are expressed as mean ± SD, unless specified otherwise.

[a–c] Significant differences between groups are shown: [a] $P < .05$; [b] $P < .01$; [c] $P < .001$.

chambers (approximately 0.2 mL) were filled with acetylcholine (Ach; 20 mg/mL) and sodium nitroprusside (SNP; 20 mg/mL) for the determination of endothelium-dependent and endothelium-independent microvascular dilatation, respectively (19, 20). The chambers were placed 5 cm apart on the forearm and connected to a power source (Perilont Power Supply; Perimed AB) for drug delivery by iontophoresis. The attachment sites were cleaned with saline before placement. The probes were heated to 32°C. The coefficient of variation of the iontophoretic method is 19% (19).

During an intra-arterial infusion of saline (1 mL/min), baseline LDF was recorded for 3 minutes. Endothelium-dependent and -independent microvascular dilatation was determined by three stimulations of 0.08, 0.14, and 0.20 mA for 60 seconds each (equivalent to 4.8, 8.4, and 12 millicoulombs). The response to each stimulation was recorded for 15 minutes to ensure that the maximum responses to Ach and SNP were obtained before the next stimulation started. Thereafter, an intrabrachial artery infusion of the arginase inhibitor N^{ω}-hydroxy-nor-L-arginine (nor-NOHA; 0.1 mg/min) was started. The dose of nor-NOHA was based on a previous study (16). After 120 minutes, endothelium-dependent and -independent dilatation was reevaluated. Data were continuously recorded on a laptop with optimized software (Perisoft, version 2.5.5).

Plasma analysis

Blood samples were drawn in EDTA tubes. Routine blood samples were analyzed at the Department of Clinical Chemistry, Karolinska University Hospital. Additional samples were centrifuged at 4°C and 2000 *g* for 15 minutes. The supernatant was stored frozen at −80°C. Amino acids arginine, ornithine, and citrulline reflecting arginase and NOS activities (see Supplemental Figure 1) were analyzed by liquid chromatography tandem mass spectrometry (21). The maximum intra-assay coefficient of variation for the amino acid analysis was 5.4%.

Substances

Ach (Bausch & Lomb Nordic AB) and SNP (Hospira Inc.) were diluted in sterile saline to a concentration of 20 mg/mL. Nor-NOHA (Bachem) was dissolved in double-distilled water to a concentration of 10 mg/mL, sterile filtrated through a Millipore filter, tested for bacterial toxins and sterility, and stored frozen at −80°C. On the study day, nor-NOHA was diluted in sterile saline.

Calculations and statistical analysis

Data are presented as mean ± standard deviation unless stated otherwise. Based on a previous interventional study of microvascular function of comparable design (22), we estimated that 12 individuals were sufficient to detect a significant improvement in endothelium-dependent dilatation. Data on LDF were exported as mean values during 30 seconds. The responses to Ach and SNP were calculated as the area under the curve of the increase from baseline flow during the first 14 minutes after electrical stimulation. All LDF data were analyzed off-line by a person blinded to the group identification. Amino acids are expressed in absolute concentrations and as ratios to reflect arginase and NOS activities. Changes in endothelium-dependent and -independent vasodilatation within and between the groups were analyzed with repeated measures two-way ANOVA, whereas differences concerning clinical characteristics and amino acid concentrations were analyzed with unpaired Student's *t* test. Normal distribution of data was tested using D'Agostino-Pearson normality test. A $P < .05$ was considered statistically significant. All statistical analyses were performed using GraphPad Prism 6 (GraphPad Software, Inc).

Results

Subject characteristics

Patient characteristics are presented in Table 1. The subjects with type 2 diabetes had significantly higher body mass index (BMI), fasting glucose, glycosylated hemoglobin (HbA1C), and C-reactive protein (CRP), but lower total, high-density lipoprotein, and low-density lipoprotein cholesterol compared to the control subjects. No subject was a current smoker. All subjects tolerated the study protocol well, and no adverse effects were observed.

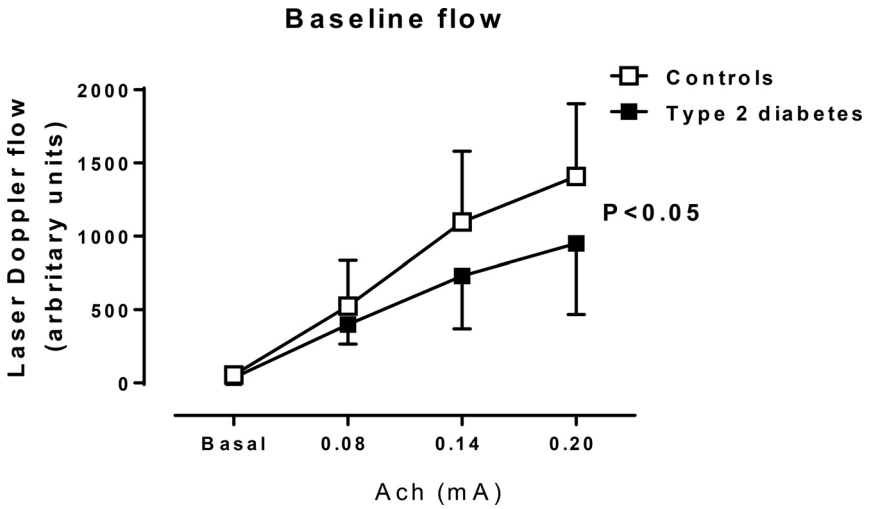

Figure 1. Endothelium-dependent microvascular flow induced by Ach in control subjects and patients with type 2 diabetes at baseline. Data are expressed as means ± SD. Significant differences between the groups are depicted. $P < .05$ (two-way ANOVA). Ach, acetylcholine; mA, milliampere.

Endothelium-dependent and -independent vasodilatation

Basal LDF did not differ between the study groups. Baseline endothelium-dependent microvascular dilatation was significantly impaired in the type 2 diabetes group compared to the control group (Figure 1). Endothelium-dependent dilatation improved significantly in patients with type 2 diabetes after administration of nor-NOHA (Figure 2). By contrast, endothelium-dependent dilatation was not affected by nor-NOHA in control subjects (Figure 2).

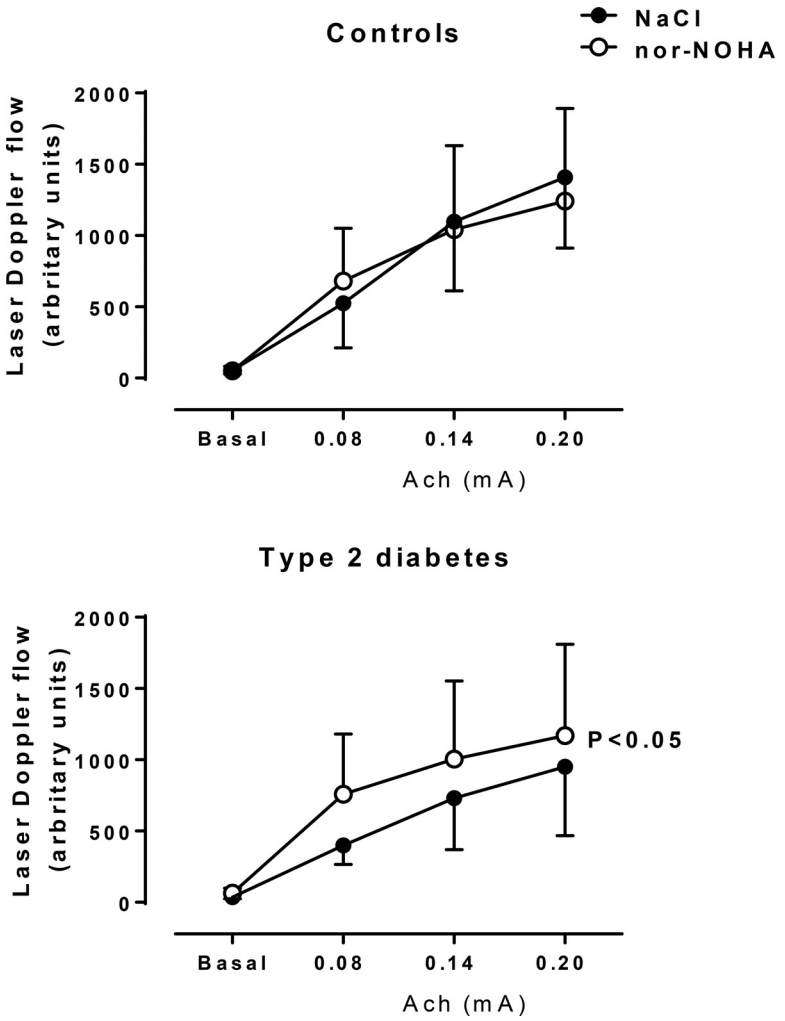

Figure 2. Endothelium-dependent microvascular flow induced by Ach in control subjects (upper panel) and patients with type 2 diabetes (lower panel) at baseline (saline) and after a 2-hour infusion of the arginase inhibitor nor-NOHA. Data are expressed as means ± SD. Significant differences between groups are depicted. $P < .05$ (two-way ANOVA). Ach, acetylcholine; mA, milliampere; nor-NOHA, N(omega)-hydroxy-nor-L-arginine.

Accordingly, endothelium-dependent microvascular dilatation was similar in patients with type 2 diabetes and controls after arginase blockade (Figure 3). Endothelium-independent microvascular dilatation induced by SNP did not differ between the groups, and it did not change significantly ($P = .1$) in response to the intervention in any of the study groups (Figure 4).

Amino acid analysis

Subjects with type 2 diabetes had significantly higher plasma levels of ornithine than controls (Table 2). The ratios of the substrate (arginine) and products of arginase (ornithine) and NOS (citrulline) were calculated as a reflection of the relative activities of arginase and NOS. Subjects with type 2 diabetes had higher ratios of ornithine/citrulline and ornithine/arginine than the control group (Table 2), indicating increased arginase activity.

Discussion

The main results of this study are: 1) arginase inhibition improves microvascular endothelial function in patients with type 2 diabetes; and 2) patients with type 2 diabetes and microvascular complications have increased ornithine/citrulline and ornithine/arginine ratios. These observations indicate up-regulation of arginase activity that has important functional implications for microvascular dysfunction in patients with type 2 diabetes.

Microvascular complications are common clinical problems in patients with type 2 diabetes for which specific therapeutic targets remain to be identified. Several lines of evidence suggest that endothelial dysfunction is a key underlying cause of microvascular complications in type 2 diabetes (8, 9, 23). Our present results showing a reduction of endothelium-dependent microvascular dilatation in patients with type 2 diabetes are in line with these observations. Despite the fact that endothelial dysfunction occurs early, there is no treatment that specifically targets the cause of it. Emerging evidence suggests that arginase is of central importance for the development of endothelial dysfunction in diabetes (12). Arginase is up-regulated in experimental models of diabetes (15) and in arterioles from patients with diabetes (17) resulting in impaired NO bioavailability. Furthermore, we have previously shown that arginase inhibition improves skeletal muscle resistance artery endothelial function in patients with type 2 diabetes and coronary artery disease via a NOS-dependent mechanism (16). Based on these observations, we hypothesized that arginase inhibition would improve microvascular function in patients with type 2 diabetes. Accordingly, administration of the arginase inhibitor nor-NOHA significantly improved endothelium-dependent microvascular dilatation, but did not affect endothelium-independent dilatation in the patient group. Arginase inhibition did not affect microvascular function in the control group, demonstrating a specific effect in the diabetes group, in which it was able to normalize endothelium-dependent microvascular dila-

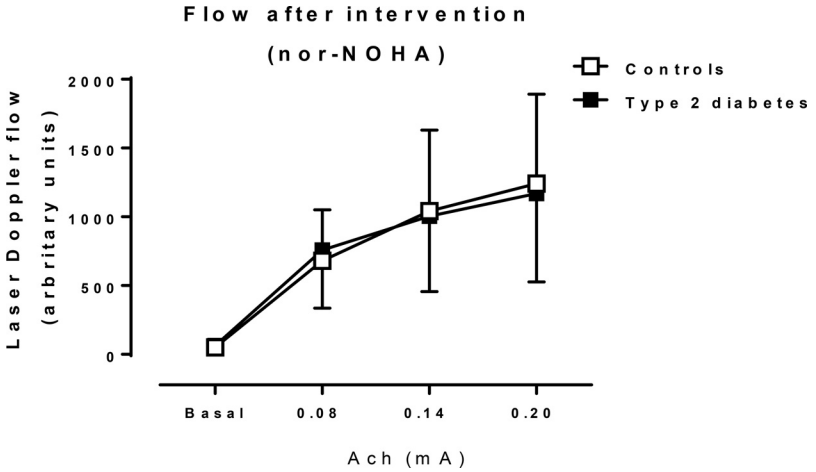

Figure 3. Endothelium-dependent microvascular flow induced by Ach in control subjects and patients with type 2 diabetes after administration of the arginase inhibitor nor-NOHA. Data are expressed as means ± SD.

tation to the level observed in the control group. This interesting observation suggests that arginase is a critical mediator of microvascular endothelial dysfunction in patients with type 2 diabetes. Thus, arginase may be a promising target for therapy in patients with type 2 diabetes and microvascular complications.

Arginase is believed to impair endothelial function through multiple mechanisms. Because arginase and NOS metabolize the same substrate, it is reasonable to believe that increased arginase activity in diabetes reduces the availability of arginine for NOS to produce NO (12, 24). Accordingly, a previous study demonstrated that the improvement in endothelial function after arginase inhibition in patients with type 2 diabetes was blocked by NOS inhibition (16), clearly demonstrating its dependence on NO production. An additional mechanism may be that NOS produces superoxide during substrate deficiency (25, 26), a phenomenon referred to as eNOS uncoupling (14), which further reduces NO bioavailability. Arginase inhibition may reduce superoxide formation in diabetes by reversing eNOS uncoupling (25).

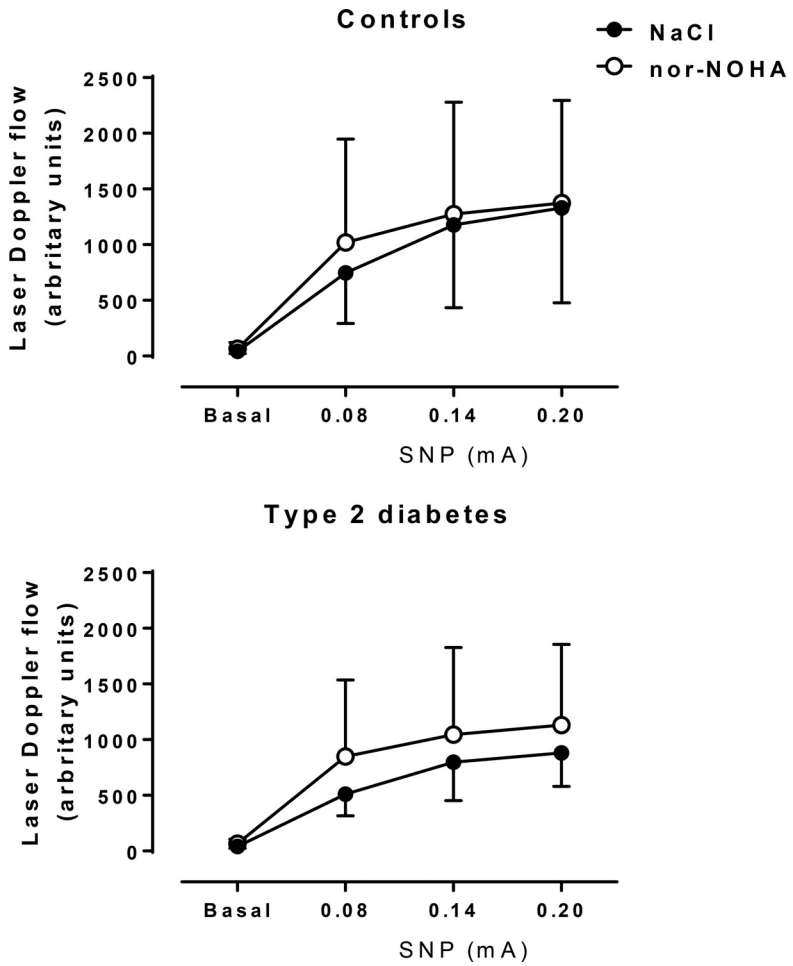

Figure 4. Endothelium-independent microvascular flow induced by SNP in control subjects (upper panel) and patients with type 2 diabetes (lower panel) at baseline (saline) and after a 2-hour infusion of the arginase inhibitor nor-NOHA. Data are expressed as means ± SD.

In order to estimate the relative activities of arginase and NOS, the ratios between their amino acid products ornithine (arginase) and citrulline (NOS) and substrate (arginine) were calculated (as illustrated in Supplemental Figure 1). Ornithine levels were increased in patients with type 2 diabetes, resulting in increased ratios of ornithine/citrulline and ornithine/arginine, supporting the theory of increased arginase activity. One may expect citrulline levels to be reduced in the group of patients with diabetes as a result of impaired NOS activity. However, citrulline may also be formed from ornithine by ornithine carbamoyl transferase and converted to arginine by aminosuccinate synthase and aminosuccinate lyase (27), which complicates the interpretation of citrulline plasma levels. The present data suggesting increased arginase activity are supported by previous observations in plasma (28) as well as in corpus cavernosum in patients with type 2 diabetes (29). Several

Table 2. Amino Acid Levels and Ratios

	Controls (n = 12)	Type 2 Diabetes (n = 12)
Amino acids, μmol/L		
Arginine	78.8 ± 14.4	79.2 ± 18.0
Ornithine	50.0 ± 8.2	71.3 ± 28.1[a]
Citrulline	37.9 ± 10.7	38.4 ± 13.7
Ratios		
Ornithine/Arginine	0.65 ± 0.16	0.91 ± 0.28[a]
Ornithine/Citrulline	1.41 ± 0.38	1.99 ± 0.73[a]
Citrulline/Arginine	0.49 ± 0.13	0.49 ± 0.18

Data are expressed as mean ± SD. Significant differences between groups are shown: [a] $P < .05$.

mechanisms may explain increased arginase activity in diabetes, including elevated glucose, reactive oxygen species, and proinflammatory cytokines (24). Our study is, to our knowledge, the first to link increased arginase activity to microvascular endothelial dysfunction in patients with type 2 diabetes.

Certain limitations need to be acknowledged. The study was performed on a limited sample size. Despite this, significant differences were detected in baseline endothelial function and in the efficacy of arginase inhibition between the groups. These promising results need to be confirmed in a larger randomized controlled study. The investigations were not blinded, but all evaluation was performed off-line by personnel blinded to group identification. There are differences in basal characteristics that might affect endothelial function. The diabetes group had lower low-density lipoproteins and higher CRP and BMI, all of which may affect baseline endothelial function and arginase activity (12, 25). Furthermore, baseline medication that affects baseline endothelial function in the group of diabetes patients may have been a confounder in the study. However, it is of interest that arginase inhibition improved endothelial function despite ongoing medication including angiotensin-converting enzyme inhibitors, statins, metformin, and incretins that all improve endothelial function per se (30, 31). This suggests that arginase inhibition exerts beneficial effects on microvascular function beyond these established medications.

In conclusion, the present study demonstrates that arginase inhibition improves microvascular endothelial function in patients with type 2 diabetes and microvascular complications. Arginase inhibition may represent a future therapeutic strategy targeting microvascular endothelial dysfunction in patients with type 2 diabetes.

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References

1. **Moreno PR, Fuster V.** New aspects in the pathogenesis of diabetic atherothrombosis. *J Am Coll Cardiol*. 2004;44:2293–2300.
2. **Seaquist ER, Goetz FC, Rich S, Barbosa J.** Familial clustering of diabetic kidney disease. Evidence for genetic susceptibility to diabetic nephropathy. *N Engl J Med*. 1989;320:1161–1165.
3. The effect of intensive treatment of diabetes on the development and progression of long-term complications in insulin-dependent diabetes mellitus. The Diabetes Control and Complications Trial Research Group. *N Engl J Med*. 1993;329:977–986.
4. **Meyer MF, Schatz H.** Influence of metabolic control and duration of disease on microvascular dysfunction in diabetes assessed by laser Doppler anemometry. *Exp Clin Endocrinol Diabetes*. 1998;106:395–403.
5. **Jaap AJ, Shore AC, Stockman AJ, Tooke JE.** Skin capillary density in subjects with impaired glucose tolerance and patients with type 2 diabetes. *Diabet Med*. 1996;13:160–164.
6. **Rayman G, Malik RA, Sharma AK, Day JL.** Microvascular response to tissue injury and capillary ultrastructure in the foot skin of type I diabetic patients. *Clin Sci (Lond)*. 1995;89:467–474.
7. **Brunner H, Cockcroft JR, Deanfield J, et al.** Endothelial function and dysfunction. Part II: association with cardiovascular risk factors and diseases. A statement by the Working Group on Endothelins and Endothelial Factors of the European Society of Hypertension. *J Hypertens*. 2005;23:233–246.
8. **Nguyen TT, Shaw JE, Robinson C, et al.** Diabetic retinopathy is related to both endothelium-dependent and -independent responses of skin microvascular flow. *Diabetes Care*. 2011;34:1389–1393.
9. **Schmiedel O, Schroeter ML, Harvey JN.** Microalbuminuria in type 2 diabetes indicates impaired microvascular vasomotion and perfusion. *Am J Physiol Heart Circ Physiol*. 2007;293:H3424–H3431.
10. **Sogawa K, Nagaoka T, Tanano I, et al.** Association between diabetic retinopathy and flow-mediated vasodilation in type 2 DM. *Curr Eye Res*. 2012;37:446–451.
11. **Münzel T, Heitzer T, Harrison DG.** The physiology and pathophysiology of the nitric oxide/superoxide system. *Herz*. 1997;22:158–172.
12. **Pernow J, Jung C.** Arginase as a potential target in the treatment of cardiovascular disease: reversal of arginine steal? *Cardiovasc Res*. 2013;98:334–343.

13. **Durante W, Johnson FK, Johnson RA.** Arginase: a critical regulator of nitric oxide synthesis and vascular function. *Clin Exp Pharmacol Physiol*. 2007;34:906–911.
14. **Förstermann U, Sessa WC.** Nitric oxide synthases: regulation and function. *Eur Heart J*. 2012;33:829–837, 837a–837d.
15. **Grönros J, Jung C, Lundberg JO, Cerrato R, Ostenson CG, Pernow J.** Arginase inhibition restores in vivo coronary microvascular function in type 2 diabetic rats. *Am J Physiol Heart Circ Physiol*. 2011; 300:H1174–H1181.
16. **Shemyakin A, Kövamees O, Rafnsson A, et al.** Arginase inhibition improves endothelial function in patients with coronary artery disease and type 2 diabetes mellitus. *Circulation*. 2012;126:2943–2950.
17. **Beleznai T, Feher A, Spielvogel D, Lansman SL, Bagi Z.** Arginase 1 contributes to diminished coronary arteriolar dilation in patients with diabetes. *Am J Physiol Heart Circ Physiol*. 2011;300:H777–H783.
18. **Kövamees O, Shemyakin A, Pernow J.** Effect of arginase inhibition on ischemia-reperfusion injury in patients with coronary artery disease with and without diabetes mellitus. *PLoS One*. 2014;9: e103260.
19. **Jörneskog G, Kalani M, Kuhl J, et al.** Early microvascular dysfunction in healthy normal-weight males with heredity for type 2 diabetes. *Diabetes Care*. 2005;28:1495–1497.
20. **Morris SJ, Shore AC, Tooke JE.** Responses of the skin microcirculation to acetylcholine and sodium nitroprusside in patients with NIDDM. *Diabetologia*. 1995;38:1337–1344.
21. **Hezel MP, Liu M, Schiffer TA, et al.** Effects of long-term dietary nitrate supplementation in mice. *Redox Biol*. 2015;5:234–242.
22. **Settergren M, Pernow J, Brismar K, Jörneskog G, Kalani M.** Endothelin-A receptor blockade increases nutritive skin capillary circulation in patients with type 2 diabetes and microangiopathy. *J Vasc Res*. 2008;45:295–302.
23. **Yamamoto-Suganuma R, Aso Y.** Relationship between post-occlusive forearm skin reactive hyperaemia and vascular disease in patients with type 2 diabetes–a novel index for detecting micro- and macrovascular dysfunction using laser Doppler flowmetry. *Diabet Med*. 2009;26:83–88.
24. **Pernow J, Jung C.** The emerging role of arginase in endothelial dysfunction in diabetes. *Curr Vasc Pharmacol*. 2016;14:155–162.
25. **Romero MJ, Platt DH, Tawfik HE, et al.** Diabetes-induced coronary vascular dysfunction involves increased arginase activity. *Circ Res*. 2008;102:95–102.
26. **Kim JH, Bugaj LJ, Oh YJ, et al.** Arginase inhibition restores NOS coupling and reverses endothelial dysfunction and vascular stiffness in old rats. *J Appl Physiol (1985)*. 2009;107:1249–1257.
27. **Romero MJ, Platt DH, Caldwell RB, Caldwell RW.** Therapeutic use of citrulline in cardiovascular disease. *Cardiovasc Drug Rev*. 2006; 24:275–290.
28. **Kashyap SR, Lara A, Zhang R, Park YM, DeFronzo RA.** Insulin reduces plasma arginase activity in type 2 diabetic patients. *Diabetes Care*. 2008;31:134–139.
29. **Bivalacqua TJ, Hellstrom WJ, Kadowitz PJ, Champion HC.** Increased expression of arginase II in human diabetic corpus cavernosum: in diabetic-associated erectile dysfunction. *Biochem Biophys Res Commun*. 2001;283:923–927.
30. **Hadi HA, Suwaidi JA.** Endothelial dysfunction in diabetes mellitus. *Vasc Health Risk Manag*. 2007;3:853–876.
31. **Hamilton SJ, Watts GF.** Endothelial dysfunction in diabetes: pathogenesis, significance, and treatment. *Rev Diabet Stud*. 2013;10:133–156.