JIM Original Article

doi: 10.1111/joim.12785

Arginase inhibition improves endothelial function in patients with type 2 diabetes mellitus despite intensive glucose-lowering therapy

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Abstract. Mahdi A, Kövamees O, Checa A, Wheelock CE, von Heijne M, Alvarsson M, Pernow J (Karolinska Institutet, Karolinska University Hospital, Stockholm, Sweden). Arginase inhibition improves endothelial function in patients with type 2 diabetes mellitus despite intensive glucose-lowering therapy. *J Intern Med* 2018; **284**: 388–398.

Background. Arginase is implicated in the pathogenesis behind endothelial dysfunction in type 2 diabetes mellitus (T2DM) by its inhibition of nitric oxide formation. Strict glycaemic control is not sufficient to improve endothelial function or cardiovascular outcomes in patients with T2DM, thus other treatment strategies are needed. We hypothesized that arginase inhibition improves endothelial function beyond glucose-lowering therapy following glucose optimization in patients with poorly controlled T2DM.

Methods and results. Endothelial function was evaluated in 16 patients with poorly controlled T2DM (visit 1) and 16 age-matched controls using venous occlusion plethysmography. T2DM patients were re-evaluated (visit 2) after intensive glucose-lowering regimen. Endothelium-dependent (EDV) and -independent (EIDV) vasodilatations were evaluated before and after 120 min intra-arterial infusion of the arginase inhibitor N(ω)-hydroxy-nor-L-arginine (nor-NOHA). HbA1c was reduced from 87 ± 17 (visit 1) to 65 ± 11 mmol mol^{-1} (visit 2, $P < 0.001$). Basal EDV, but not EIDV, was significantly lower in patients with T2DM than in healthy subjects ($P < 0.05$). EDV and EIDV were unaffected by glucose-lowering regimen in patients with T2DM. Arginase inhibition enhanced EDV in T2DM patients both at visit 1 and visit 2 ($P < 0.01$). There was no difference in improvement in EDV between the two occasions. EIDV was unaltered by nor-NOHA in T2DM at visit 1, but was slightly improved at visit 2.

Conclusions. Arginase inhibition improves endothelial function in patients with poorly controlled T2DM, which is maintained following glucose optimization. Thus, arginase inhibition is a promising therapeutic target beyond glucose lowering for improving endothelial function in T2DM patients.

Keywords: arginase, endothelial dysfunction, glucose regulation, nitric oxide, plethysmography, type 2 diabetes.

Introduction

The prevalence of type 2 diabetes mellitus (T2DM) is increasing worldwide and it is estimated that ~7% of the adult population will suffer from the disease by 2030 [1]. T2DM is associated with multiple complications of which cardiovascular disease carries the biggest burden [2]. One of the most prominent feature in T2DM is hyperglycaemia, which triggers and drives the development of several of these complications, including coronary artery disease, retinopathy and nephropathy [3]. However, existing data from large clinical trials indicate that intensive glucose-lowering therapy does not convincingly reduce cardiovascular complications or mortality [4–7]. Therefore, improved insights into the mechanisms behind cardiovascular complications are needed to develop and evaluate novel treatments specifically targeting these mechanisms.

Several factors interact and contribute to the complex pathogenesis of cardiovascular complications in T2DM. Endothelial dysfunction (ED)

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characterized by reduced bioavailability of the vasodilator nitric oxide (NO) is an early manifestation [8–10]. It is suggested that chronic hyperglycaemia is a factor that links diabetes to ED by evoking pathological responses in the endothelium, resulting in NO deregulation [11]. NO is produced by the enzyme endothelial NO synthase (eNOS) from the substrate L-arginine with citrulline as a by-product [12]. The metalloenzyme arginase has generated considerable interest in the regulation of NO production [13]. Arginase competes with eNOS for their common substrate L-arginine, which suggests that excessive arginase activity reciprocally inhibits NO production [13, 14]. In addition, substrate limitation may result in eNOS uncoupling, a state during which eNOS produces superoxide instead of NO [13, 14]. Interestingly, reduced bioavailability of NO and increased production of reactive oxygen species (ROS) as well as ED induced by hyperglycaemia is maintained even after restoration of normoglycaemia [15, 16]. This phenomenon, referred to as hyperglycaemic memory, is an area under intensive investigation and could be part of the explanation why glucose-lowering treatment in clinical trials fails to improve cardiovascular outcomes in patients with T2DM [4–6, 17, 18]. It is therefore an unmet need for new therapeutic strategies, which act beyond the glucose-lowering effect of current drugs, for preventing cardiovascular complications in T2DM.

Emerging evidence suggests that arginase is implicated in the pathogenesis of vascular complications in T2DM [19–22]. We have previously shown that arginase inhibition markedly improves *in vivo* endothelial function in patients with coronary disease and T2DM, but does not affect endothelial function in healthy age-matched individuals [20]. Moreover, arginase inhibition also improves microvascular endothelial function in patients with T2DM [23]. It has also been shown that arginase activity is increased by glucose and ROS in endothelial cells *in vitro* [14, 24]. These findings suggest that hyperglycaemia triggers activation of arginase which contributes to ED in T2DM. It is unknown to what extent arginase is driven by hyperglycaemia in patients with T2DM-induced ED *in vivo*.

Based on the activation of arginase in T2DM and the observation that arginase inhibition improves endothelial function in patients with T2DM, we investigated the effect of arginase inhibition on endothelial function before and after intensive glucose-lowering regimen in patients with poorly controlled T2DM to answer the question whether arginase inhibition improves endothelial function beyond intensive glucose-lowering therapy.

Materials and methods

Study population

We recruited 16 patients with T2DM from the Endocrinology Department at Karolinska University Hospital and Danderyd's Hospital, Stockholm, Sweden between 2015 and 2017. Patients were defined as having T2DM if fasting blood glucose exceeded 7.0 mmol L^{-1} on at least two occasions or blood glucose was >11.0 mmol L^{-1} 2 h after an oral glucose loading (75 g). Inclusion criterion for patients was poor glycaemic control defined as mean daily blood glucose ≥12 mmol L^{-1} from 7 measurements or HbA1c ≥70 mmol mol^{-1}. Exclusion criteria were any cardiovascular event including stroke and acute coronary syndrome in the past 6 months, on-going treatment with warfarin or new oral anticoagulants or age <20 or >80 years. In addition, we also recruited 16 healthy subjects for characterization of endothelial function. The healthy subjects were recruited during the same time period and were matched for age and sex, were free of medication and had no medical history of cardiovascular disease. T2DM was excluded in the healthy population by fasting blood glucose ≤6.1 mmol L^{-1} and a blood glucose <8.9 mmol L^{-1} 2 h after an oral glucose tolerance test (75 g). Participants were informed of the nature, purpose and possible risk involved in the study. Oral and written informed consent were obtained from all the participants prior to inclusion in the study. The study was conducted according to the declaration of Helsinki and approved by the regional ethics committee.

Study protocol

The patients with T2DM underwent examination of endothelial function including response to arginase inhibition using forearm plethysmography (see below) on two different occasions: one at inclusion during poor glycaemic control as defined above (visit 1) and one after optimization of glycaemic control (visit 2). After visit 1, the patients started an intensive glucose-lowering regimen at the Diabetes Day Care at the Department of Endocrinology according to clinical routine with the aim to reach a mean daily blood glucose of <9 mmol L^{-1} within a minimum of 8 weeks. The subjects underwent a 4-day educational

programme in groups, which included lifestyle advices, optimization of medication and regulation of food intake and were equipped with glucose monitoring devices. All drug combinations were allowed and antidiabetic medications where withdrawn only if there was any adverse event. The subjects also underwent a thorough investigation of cardiovascular complications including neuropathy, nephropathy and retinopathy. They were also followed-up by phone or visit to a physician or a diabetes nurse a few weeks after this programme started for further adjustments of medication. The patients were also instructed to report their mean daily blood glucose 4 weeks after their visit in the Department of Endocrinology. If this value was >9 mmol L^{-1}, the glucose-lowering therapy was further intensified. Thereafter, the patients were planned for visit 2. Subjects underwent the examination at visit 2 only if a mean daily blood glucose had reached 9 mmol L^{-1} or if additional glucose reduction was considered not feasible. Therefore, the mean follow-up time at visit 2 was in several cases extended beyond 8 weeks.

Forearm blood flow measurements

The subjects arrived to the laboratory at 8 AM and were instructed to refrain from caffeine and nicotine-containing products on the study day. The patients were instructed not to take any medication in the morning on the study day. Patients on long-acting insulin were instructed to take 50% of their ordinary dose the evening before the investigation. After collection of baseline blood samples by venous puncture, a light breakfast was served. The investigations were performed in a quiet and temperature-controlled room with the subjects in a supine position. The brachial artery in the non-dominant arm was catheterized in the proximal direction under local anaesthesia and sterile conditions. Forearm blood flow (FBF) was measured simultaneously in both arms by venous occlusion plethysmography, using an indium-gallium strain gauge applied around the widest part of the forearm. FBF was determined by obstruction of the venous outflow from the forearm by inflating a cuff placed around the upper arm to supra-venous pressure (50 mmHg) in repeated cycles of 10 s. The hand circulation was excluded by inflation of a wrist cuff to 30 mmHg above systolic blood pressure. Basal FBF was measured during a continuous intra-arterial infusion of sodium chloride (NaCl 0.9%) at a rate of 1 mL min^{-1}. Endothelium-dependent (EDV) and -independent (EIDV) vasodilatations were examined by intra-arterial infusions of serotonin (21, 70 and 210 ng min^{-1}) and sodium nitroprusside (SNP; 1, 3 and 10 μg min^{-1}), respectively. Each dose was given for 2 min at a rate of 2.5 mL min^{-1} with 1 min washout period between each dose. It has previously been demonstrated that serotonin induce NO-dependent vasodilatation in the human forearm of healthy subjects and patients with T2DM [20]. After determination of basal EDV and EIDV, an intra-arterial infusion of the arginase inhibitor N(ω)-hydroxy-nor-L-arginine (nor-NOHA) was started at a dose of 0.1 mg min^{-1} (1 mL min^{-1}) and maintained for 120 min. It has been demonstrated that this dose of nor-NOHA does not affect eNOS activity and is highly specific for arginase [25]. We have previously demonstrated that co-infusion of a NOS inhibitor completely blocks the improvement of EDV induced by nor-NOHA in patients with T2DM, suggesting that the effect of nor-NOHA is NOS-dependent [20]. All patients with T2DM underwent identical examinations at visits 1 and 2.

For determination of baseline endothelial function, a control group of 16 healthy age- and sex-matched subjects underwent one examination for characterization of their EDV and EIDV as above. We have previously shown that arginase inhibition does not improve endothelial function in the same age range as the subjects included in the current study [20].

Plasma sampling and analyses

Blood samples from the cubital vein of all subjects were drawn in EDTA tubes at arrival on the experiment day. Routine blood samples were analysed at the department of clinical chemistry, Karolinska University Hospital. For estimation of arginase activity, samples were also collected for analyses of the arginase substrate arginine and the product ornithine. Samples were centrifuged at 1500 ***g*** for 15 min at 4 °C, and the supernatant was stored at −80 °C. Amino acids were extracted from 25 μL of plasma using 2-propanol as previously described [26].

Amino acid LC-MS/MS measurements

Urea cycle amino acid analyses were performed on an ACQUITY UPLC System from Waters Corporation (Milford, MA, USA) coupled to a Waters Xevo® TQ-S triple quadrupole system equipped with an Electrospray Ion Source. Separation was carried out on a SeQuant® ZIC®-HILIC (100 × 2.1 mm,

3.5 μm, 100 Å) column equipped with a SeQuant® ZIC®-HILIC guard column (20 × 2.1 mm), both from Merck (Solna, Sweden). Mobile phases consisted of 0.1% formic acid in water (aqueous) and 0.1% formic acid in acetonitrile (organic). The elution gradient used was as follows: 0.0, 95% B; time range 0.0–0.5 min, 95 → 80% B (linear decrease); time range 0.5–6.0 min, 80 → 40% B (linear decrease); time range 6.0–8.0 min, 40 → 25% B (linear decrease); time range 8.0–9.5 min, 25% B (isocratic step); time range 9.5–9.7 min, 25 → 95% B (linear increase) and 9.7–12.2 min, 95% B (isocratic column conditioning). The flow rate was set at 300 μL min^{-1}, the injection volume was 2.5 μL and the column oven was maintained at 27 °C. Detection was performed in positive ionization mode. A quantifier and qualifier SRM were acquired for all compounds and described in Table S1. For additional confirmation, the ion ratios between the quantifier and qualifier ions (maximum deviation = 15%) were calculated.

Chemicals and reagents

Nor-NOHA (Bachem, Bubendorf, Switzerland) and serotonin (Sigma-Aldrich, Schnelldorf, Germany) were dissolved in double-distilled water, sterile filtrated through a Millipore filter and tested for bacterial toxins and sterility and stored frozen at −80 °C. SNP (Abbot, Chicago) was stored at room temperature. All substances were diluted to the proper concentrations in 0.9% NaCl on the day of the experiment. For LC-MS/MS analysis, formic acid, acetonitrile and isopropanol (all Optima®-LC/MS grade) were purchased from Fisher-Scientific (Loughborough, UK).

Statistical analyses and calculations

Data are presented as means ± standard errors of the means (SEM), unless otherwise stated. Basal FBF and the responses to serotonin and SNP were calculated as the mean of the highest four inflow recordings during 2 min. Basal FBFs are expressed as absolute values. EDV and EIDV are presented as absolute change in FBF in response to serotonin or SNP, respectively. In our previous study on patients with coronary artery disease and T2DM, we observed an improvement in endothelium-dependent vasodilatation by a mean of 18 mL min^{-1}/1000 mL [20]. Assuming an improvement of 12 mL min^{-1}/1000 mL and an SD of 13 in the current population, we estimated that 16 individuals would be sufficient to detect a significant effect at the level of 0.05 and at a power of 0.8. Differences in FBF during infusions of serotonin or SNP were assessed by two-way analysis of variance (ANOVA) with repeated measures. Differences in basal characteristics of the healthy subjects T2DM subjects were analysed using unpaired *t*-test or Mann–Whitney test, depending on if the data sets were normally distributed or not and differences between visits 1 and 2 for the T2DM subjects were assessed using paired *t*-test or Wilcoxon test, depending on normality. Normality check was performed with D'Agostino-Pearson normality test. All the statistical analyses in the present study were performed with Prism 7.0, GraphPad, San Diego, CA, USA. $P < 0.05$ was considered as statistically significant.

Results

Subjects

Characteristics of the study subjects are presented in Table 1. The subjects were matched for age and sex. The T2DM group had higher body mass index and waist–hip ratio than the control group. As expected, patients with T2DM had higher fasting glucose. Blood pressure did not differ between the groups.

Table 1 *Basal characteristics of study subjects*

Variables	Healthy $n = 16$	T2DM $n = 16$
Age (years)	63 ± 7	64 ± 10[a]
Males/females, *n*	13/3	12/4
Years since diagnosis	–	13 ± 9
Years on glucose-lowering medication	–	11 ± 6
BMI, kg m^{-2}	26.1 ± 3.0	31.4 ± 4.6***,[a]
Blood pressure, mmHg		
Systolic	137 ± 16	146 ± 19[a]
Diastolic	82 ± 10	83 ± 15[b]
W/H ratio	0.94 ± 0.05	0.98 ± 0.05***,[a]
Smokers, *n*	0	5
Haemoglobin, g L^{-1}	146 ± 10	142 ± 15[a]
Creatinine, μmol L^{-1}	84 ± 13	81 ± 19[a]

BMI, body mass index; W/H, waist–hip.
Data are presented as means ± SD. ***$P < 0.001$, compared to healthy. [a]Unpaired Student's *t*-test, from normally distributed data sets. [b]From Mann–Whitney test for data sets that were not normally distributed.

Change in clinical variable between Visits 1 and 2

Key differences between visit 1 and visit 2 for patients with T2DM are shown in Table 2. The mean time between visits 1 and 2 was 17 ± 5 weeks (range: 10–28 weeks). Indices of glycaemic control were markedly improved between visit 1 and visit 2. Fasting glucose was reduced from 13.8 ± 2.8 to 9.3 ± 3.2 mmol L^{-1} ($P < 0.01$) and HbA1c was reduced from 88 ± 17 to 65 ± 11 mmol mol^{-1} ($P < 0.001$). The T2DM subjects were instructed to report a mean daily blood glucose of seven measurements at visit 1, 4 weeks later and prior to visit 2. Mean daily blood glucose

Table 2 *Characteristics of T2DM group before (visit 1) and after (visit 2) glucose optimization*

Variables	T2DM visit 1 $n = 16$	T2DM visit 2 $n = 16$
Triglycerides, mmol L^{-1}	2.1 ± 0.9	1.6 ± 0.8[a]
Total cholesterol, mmol L^{-1}	4.3 ± 1.2	3.5 ± 0.7*,[b]
HDL, mmol L^{-1}	1.1 ± 0.3	1.1 ± 0.3[b]
LDL, mmol L^{-1}	2.2 ± 1.1	1.6 ± 0.6*,[a]
hs-CRP	1.9 ± 2.6	3.6 ± 7.7[b]
HbA1c, mmol mol^{-1}	88 ± 17	65 ± 11***,[b]
Comorbidities, *n*		
CAD	4	4
Retinopathy	0	0
Neuropathy	1	1
Nephropathy	1	1
Leg ulcer	1	1
General medication, *n*		
ACEi/ARB	9	12
Aspirin	6	6
Lipid lowering	12	13
b-blockers	7	7
Calcium channel *i*	5	6
Glucose-lowering medication, *n*, daily dose		
Insulin	8, 40U	10, 43U
Metformin	14, 2.0 g	14, 1.9 g
GLP1	4, 1.05 mg	9, 1.13 mg
DDP-4i	4, 88 mg	4, 113 mg
SU	4, 1.75 mg	5, 3.2 mg
SGLT2i	1, 10 mg	2, 10 mg
Combination of glucose-lowering drugs, *n*		
0	1	0
1	3	2
2	4	3
3	8	8
>3	0	3

ACEi angiotensin-converting enzyme; ARB, angiotensin II receptor blocker; CAD, coronary artery disease; DPP-4i, dipeptidyl peptidase-4 inhibitor; g, gram; GLP1, glucagon like peptide 1 analogue; HbA1c, glycated haemoglobin; HDL; high density lipoprotein, hs-CRP, high sensitivity C-reactive protein; LDL, low-density lipoprotein; mg, milligram; SGLT2i, sodium-glucose co-transporter 2 inhibitor; U, units. [a]Paired Student's *t*-test, from normally distributed data sets. [b]From Wilcoxon test for data sets that were not normally distributed. *$P < 0.05$, ***$P < 0.001$, compared to visit 1.

was reduced by ~4 units between visit 1 and 4 weeks later, and it was maintained at the same level until visit 2 (Fig. 1). Total cholesterol and LDL cholesterol were significantly reduced between visits 1 and 2. The main differences in medications between the visits were that several patients received GLP1-agonists and slightly higher doses of GLP-1 agonists, DDP-4 inhibitors and sulfonylurea (Table 2).

Endothelium-dependent and -independent vasodilatation

Baseline FBF (mL min^{-1} × 1000 mL) during infusion of NaCl was 25 ± 9 in the healthy control group, 34 ± 12 in patients with T2DM at visit 1 and 31 ± 11 at visit 2. No significant differences in baseline FBF between the three groups were observed.

Baseline EDV was significantly lower in the diabetic group than in the healthy control group (Fig. 2a), whereas EIDV did not differ between the groups (Fig. 2b). Within the group of patients with T2DM, there was no difference in EDV between visit 1 and visit 2 (Fig. 2a). Two hours infusion of the arginase inhibitor nor-NOHA did not affect baseline FBF (Table 3) but induced a significant improvement in EDV in the T2DM group with poor glycaemic control (visit 1, Fig. 3a). A similar magnitude of improvement in EDV induced by nor-NOHA was observed also after intensive glucose-lowering regimen (visit 2, Fig. 3c). Nor-NOHA did not affect EIDV at visit 1 (Fig. 3b), but induced a slight, but significant, increase in EIDV after glucose-lowering treatment at visit 2 (Fig. 3d).

Amino acid analysis

Ratios of plasma ornithine (product of arginase) and arginine (substrate for arginase) as a reflection of arginase activity were significantly higher in patients with T2DM at visit 1 as compared to healthy subjects (Fig. 4a), indicating increased arginase activity in patients with T2DM. There was no change in the ratio of ornithine/arginine in patients with T2DM at visit 1 compared to visit 2 (Fig. 4b).

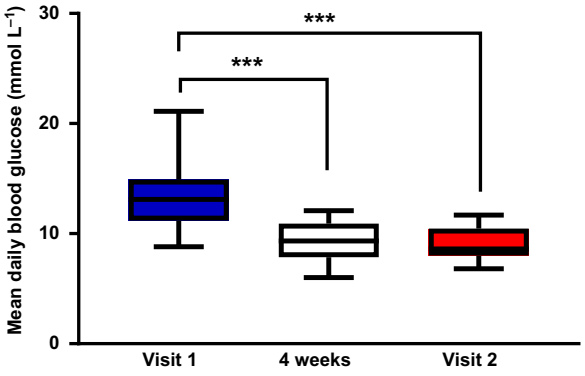

Fig. 1 *Mean daily blood glucose of seven measurements of patients with type 2 diabetes (*n = *16 in each group) at visit 1, 4 weeks after inclusion and at visit 2. Data are presented as median and interquartile ranges. Significant differences with one-way analyses of variance with repeated measures and Tukey's post hoc test are shown.* ***P < *0.001. No significant difference between 4 weeks and visit 2 (*P = *0.74).*

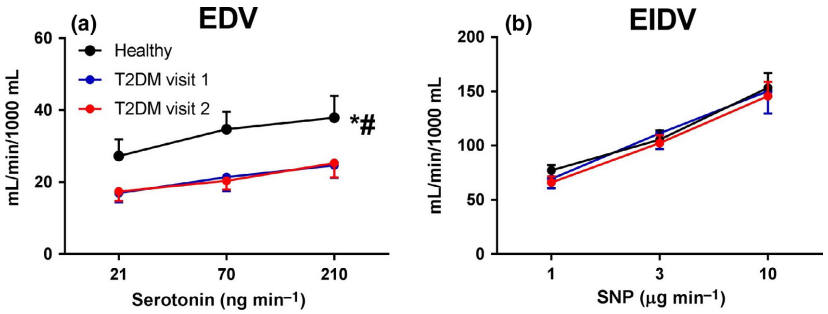

Fig. 2 *Basal (i.e. during infusion of NaCl) endothelium-dependent vasodilatation induced by serotonin (a), and endothelium-independent vasodilatation induced by SNP (b) in healthy subjects (*n = *16) and patients with type 2 diabetes mellitus (T2DM) both at poor glycaemic control (T2DM visit 1,* n = *16) and after glucose optimization treatment (T2DM visit 2,* n = *16). Significant differences with two-way analyses of variance with repeated measures are shown.* *P < *0.05 vs T2DM visit 1,* #P < *0.05 vs T2DM visit 2.*

Discussion

The main findings of the current study are that (i) intensive glucose-lowering therapy failed to improve EDV in patients with T2DM, despite a large reduction in HbA1c and glucose levels; (ii) arginase inhibition improves EDV in patients with T2DM despite intensive glucose-lowering therapy and (iii) the ratio of ornithine/arginine, reflecting arginase activity, was unchanged following glucose-lowering therapy in patients with T2DM. These observations suggest that optimized glucose-lowering therapy is not sufficient to improve EDV, but that arginase inhibition improves EDV beyond glucose-lowering therapy.

Table 3 *Baseline FBF (mL min^{-1}/1000 mL) during infusion of NaCl at baseline and after 120 min of nor-NOHA infusion on the two visits of T2DM patients*

	T2DM visit 1	T2DM visit 2
NaCl	34 ± 12	31 ± 11
Nor-NOHA	38 ± 16	33 ± 11

Data are presented as means ± SD. Paired student *t*-test was used to analyse differences before compared to after nor-NOHA treatment and differences between the visits. No significant differences using paired *t*-test were observed. All data sets were normally distributed.

T2DM is a well-known risk factor for the development of vascular complications for which impaired endothelial function is an early manifestation and a key factor for disease progression. ED is known to be the result of reduced bioavailability of NO and increased ROS production [8]. Arginase is a central enzyme in the regulation of NO by reciprocally inhibiting its production through metabolism of the substrate L-arginine which in turn leads to eNOS uncoupling and thus increased oxidative stress [13, 14]. Experimental evidence suggests that glucose-induced oxidative stress is important for the regulation of arginase [14]. Furthermore, it has been shown that arginase is correlated to glucose levels in patients with T2DM and that an insulin clamp *in vivo* reduces arginase activity [22]. Based on these observations, we hypothesized that glycaemic dysregulation is a trigger for arginase activity resulting in ED, and consequently that arginase inhibition improves EDV in patients with T2DM. Indeed, we observed that administration of the arginase inhibitor nor-NOHA markedly improved EDV but not EIDV in patients with

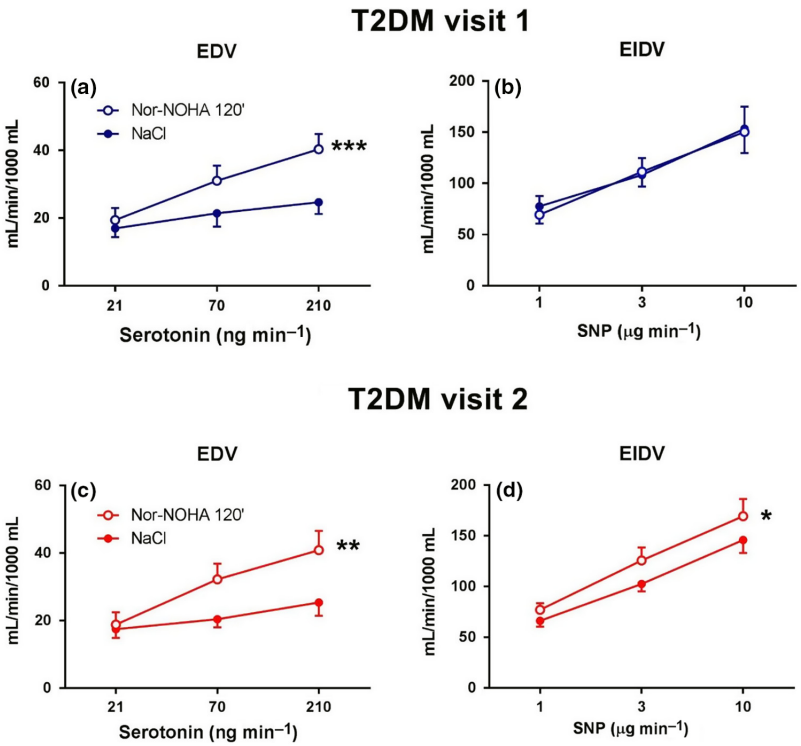

Fig. 3 *Change in endothelium-dependent vasodilatation (EDV; a and c) and endothelium-independent vasodilatation (EIDV; b and d) from baseline during infusion of NaCl and after 2 h infusion of nor-NOHA in patients with type 2 diabetes mellitus (T2DM) with poor glycaemic control (T2DM visit 1,* n = *16) and after glucose optimization (T2DM visit 2,* n = *16). Significant differences with two-way analyses of variance with repeated measures are shown.* *P < *0.05,* **P < *0.01,* ***P < *0.001 vs NaCl.*

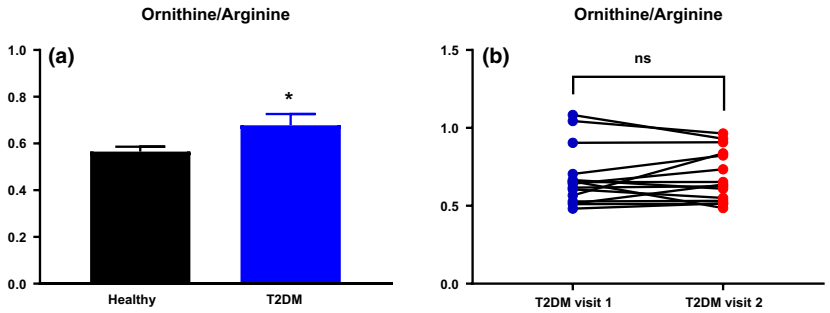

Fig. 4 *Ratio of plasma ornithine/arginine measured with liquid tandem mass spectrometry in healthy subjects (*n = *14) vs. T2DM patients (*n = *15) (a) presented as means ± SEM, and in T2DM patients at visits 1 and 2 presented as a paired dot-plot (*n = *15 each) (b).* *P < *0.05 vs. healthy with unpaired* t*-test,* P = *0.75 with paired* t*-test for T2DM visit 1 vs. visit 2.*

T2DM and poor glycaemic control. Interestingly, this improvement was not affected by intensive glucose-lowering therapy. Neither was plasma arginase activity, as reflected by the ornithine/arginine ratio, changed between the visits. These observations are important additions to our previous observations on the effect of arginase inhibition on endothelial function in patients with coronary artery disease and on microvascular function in patients with T2DM for several reasons [20, 27]. Here, we show that the improvement in EDV induced by arginase inhibition is independent of glucose levels, suggesting that arginase could be a future potential target in addition to glucose-lowering therapies in patients with T2DM for the improvement in vascular function. Moreover, arginase inhibition is able to improve EDV in a diabetic cohort without pre-specified coronary artery disease.

Others and we have shown in both experimental and clinical studies that increased arginase activity in T2DM is associated with ED [14, 20, 23, 27]. Moreover, mechanistic studies have revealed the importance of glucose in the regulation of arginase. Romero *et al.* [14] have shown that high glucose induces up-regulation of arginase 1 in bovine coronary endothelial cells via a mechanism involving NADPH oxidase-derived ROS and up-regulation of Rho kinase, which in turn reduces NO bioavailability. Moreover, it has been shown that ED induced by *ex vivo* exposure to high glucose/palmitate was completely reversed by acute administration of an arginase inhibitor [24]. Collectively, these data suggest an intriguing interaction between glucose and arginase in T2DM in the regulation of endothelial function. In line with these observations, we were able to demonstrate that arginase inhibition improves endothelial function in T2DM. However, it may appear surprising that glucose-lowering therapy did not reduce the beneficial effect of arginase on endothelial function or plasma arginase activity. It is possible that the increase in arginase activity is initially driven by hyperglycaemia, but that this effect cannot be reversed by improved glycaemic control. Also, in contrast to this finding, Kashyap *et al.* showed that arginase is correlated to the degree of hyperglycaemia and that insulin reduces plasma arginase activity in patients T2DM [22]. However, the administration of insulin in their study was maintained for a short period, whereas the patients in the current study underwent a long-term glucose-lowering regimen, not strictly limited to insulin, which could explain the different outcomes.

Unexpectedly, we observed that arginase inhibition was able to improve EIDV following glucose optimization. We have previously observed that agents that improve endothelial function in T2DM may also improve EIDV to some extent [20, 28]. The mechanism behind this is unclear and the present observation may indicate that this effect could be related to glycaemic control.

The observation that the ornithine/arginine ratio was higher in patients with T2DM compared to healthy controls, suggesting increased arginase activity, is in accordance with previous findings [23, 27]. The present study, in addition, demonstrates that arginase activity was unchanged following glucose optimization, which is in agreement with the finding that arginase inhibition improves EDV by a similar magnitude in T2DM during poor glycaemic control and following glucose optimization. This further supports the conclusion that

targeting arginase in T2DM improves endothelial function beyond glycaemic control.

In line with previous data, we observed a difference in baseline EDV between healthy subjects and patients with T2DM [16, 20, 29]. As expected, we did not observe a difference in EDV before compared to after intensive glucose-lowering therapy in the T2DM group, in line with other studies on the subject. Constantino *et al.* [16] showed that a reduction of HbA1c by 13% (from 71 to 62 mmol mol^{-1}) failed to improve endothelial function in patients with T2DM. Despite a reduction by 27% (from 88 to 65 mmol mol^{-1}) and a higher initial HbA1c in the present study, we were also unable to show an improvement in endothelial function. This is further supported by Valenzuela-Garcia *et al.* [29] who failed to show a difference in endothelium-dependent coronary microvascular function between T2DM patients with optimal vs. suboptimal glycaemic control. Interestingly, acute administration of glucose induces ED in healthy subjects, which is attributable to an acute deficit in NO and increase in ROS [30, 31]. Also, co-administration of glucose and L-arginine or antioxidants restores vascular haemodynamic alterations induced by hyperglycaemia alone, suggesting that the NO-deficit is reversible in the acute setting of hyperglycaemia [31, 32]. These observations further support the notion that glucose may be a trigger for vascular dysfunction via ROS and NO-dependent mechanisms. However, the long-term effects of hyperglycaemia in T2DM are different for several reasons. A growing body of evidence on molecular and epidemiological levels suggest that changes induced by hyperglycaemia are difficult to reverse by normoglycaemia [6, 15]. It has been suggested that a phenomenon referred to as metabolic (hyperglycaemic) memory is one of the key reasons behind the observation that exposure to high glucose levels results in persistent ED. This is proposed to be mediated by irreversible epigenetic alterations, including changes in promoter regions that encode proteins of importance for eNOS activity and ROS formation [15]. Moreover, ED is sustained even after normoglycaemia, suggesting that dysglycaemia is the trigger of ROS-associated glycaemic memory [15]. The present findings, that the improvement in EDV by arginase inhibition and arginase activity are unaltered following glucose optimization, extend these observations by pointing out arginase as an additional key factor in the complex pathophysiology of ROS-associated memory. Another theory that has been proposed is that hyperglycaemia and oxidative stress results in formation of advanced glycation end-products (AGEs) and the interaction with its receptor (RAGEs) [33]. This further leads to an inflammatory cascade including activation of (NF)-κB resulting in functional and structural changes ultimately leading to atherosclerosis in T2DM [34]. Arginase inhibition reduces eNOS uncoupling and therefore inhibit ROS and increases NO production. Our results suggest that this effect is unaltered on both high and low glucose levels, which suggest that arginase inhibition can potentially be added to glucose-lowering medication for the improvement of vascular function in patients with T2DM. However, the exact mechanisms by which arginase is involved in metabolic memory has yet to be explored.

The major differences between healthy subjects and patients with T2DM in the current study, beyond indices of glucose, are BMI and waist/hip ratio. Previous studies have pointed out that arginase plays a central role in vascular dysfunction in obesity, which suggest that obesity could confound our results [24, 35]. However, El Assar *et al.* [35] demonstrated that arginase-induced endothelial dysfunction in isolated arteries from obese individuals was only observed in arteries from insulin-resistant individuals, suggesting that the involvement of arginase was related more to insulin resistance than obesity *per se.* The extent by which arginase contributes to endothelial dysfunction in obese subjects in the absence of T2DM *in vivo* remains to be established.

Some limitations of the current study need to be acknowledged. The current study was conducted in a relatively small cohort and may therefore only be valid for patients with similar characteristics to those included in the current study. Extrapolation to the diabetic group in general requires larger study groups. The lack of blinding and randomization is another limitation although personnel blinded to the order of the visits performed all evaluations of the blood flow measurements. Although we evaluated the patients after a significant decrease in HbA1c and blood glucose, the lack of improvement in EDV following glucose lowering may be due to the limited time interval between the visits. Hence, it cannot be excluded that long-term hyperglycaemic control with additional reduction in glucose levels and HbA1c may affect baseline endothelial function and affect the improvement induced by arginase inhibition.

Conclusion

In conclusion, the present study demonstrates that the ability of arginase inhibition to improve endothelial function in patients with T2DM is maintained also following optimization of glycaemic control. The results suggest that arginase inhibition is a promising therapeutic target beyond glucose lowering for improving endothelial function in patients with T2DM.

Conflict of interest

None to declare.

Acknowledgement

The technical assistance of David Ersgård and Ann Lindström is gratefully acknowledged.

Funding

This study was supported by the Swedish Research Council Medicine (10857), the Swedish Heart and Lung Foundation (20160239), the Stockholm County Council (ALF, 20160084), Söderberg Foundation (M60/15), Karolinska Institutet/Stockholm County Council Strategic Cardiovascular Programme (20120741) and the Tripartite Immunometabolism Consortium (TrIC) – Novo Nordisk Foundation (NNF15CC0018486 & NNF15OC0015992).

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Table S1. Summary of retention times (RT), selected reaction monitoring (SRM) transitions and internal standards employed for the detection urea cycle amino acids LC-MS/MS quantification.