Nitric oxide modulation of renal and cardiac hemodynamics in type 2 diabetes

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\textbf{Abstract}

\textbf{Objective}: To evaluate endothelial function in type 2 diabetic patients with and without diabetic nephropathy.

\textbf{Methods}: We studied the effects of systemic infusion of the nitric oxide (NO) synthase inhibitor N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA) on cardiovascular and renal hemodynamics in six type 2 diabetic patients with microalbuminuria (D2-MA), six type 2 diabetic patients with normoalbuminuria (D2-NA) and five control subjects. Both type 2 diabetic patients and control subjects had mild arterial hypertension.

\textbf{Results}: L-NMMA infusion decreased the cardiac index in all groups. A reduction in glomerular filtration rate (GFR) and an increase in filtration fraction were observed only in controls. Renal plasma flow decreased in controls and D2-NA patients and renal vascular resistance increased in all groups.

\textbf{Conclusions}: The effect of L-NMMA on cardiac output was similar in controls and type 2 diabetic patients with and without diabetic nephropathy. In contrast, the effect on GFR was impaired in both diabetic groups, suggesting that glomerular NO homeostasis is altered in type 2 diabetes. Moreover, the discrepancy, in diabetic patients, between cardiac and renal effects during L-NMMA infusion suggests that the modulation of glomerular hemodynamics is independent from NO-regulated cardiac output.

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\textbf{Introduction}

One of the endothelial factors implicated in the regulation of vascular tone is nitric oxide (NO), a highly reactive molecule synthesized in the endothelium from L-arginine by endothelial NO synthase enzyme (eNOS), a member of the NOS isoform family. This family has two groups: constitutive NOS, including brain NOS and eNOS isoforms, both calcium and calmodulin dependent, and inducible NOS (iNOS) implicated in conditions of inflammation, sepsis and shock (1); iNOS is calcium independent. These different NOS isoforms are present in different organs (1).

NO acts as a messenger molecule, inhibits platelet aggregation and adhesion to the endothelium, modulates leukocyte chemotaxis and adhesion and mediates vascular relaxation (2). Moreover, it has been suggested that NO plays a role in modulating systemic and renal circulation (3–7).

Previous studies have shown that abnormalities of endothelial function may be present in a large number of pathological conditions: defects in the bioactivity of NO are present in essential hypertension (8, 9) and in hypercholesterolemia (10). Conflicting findings have been reported with regard to NO synthesis and release in type 1 and 2 diabetes. Indeed, some studies have shown that endothelium-dependent vasodilatation in forearm resistance vessels is impaired in type 1 and type 2 diabetic patients (11–13). However, other authors did not find evidence of a disturbance of NO production (14–16) or even observed an increased NO synthesis in type 2 diabetic patients with normal albumin excretion rate (17). Conflicting data have been also reported with regard to the role of NO in the pathogenesis of diabetic nephropathy. Elliott et al. (18) found that there was a reduced vasoconstrictive response to N\textsuperscript{G}-monomethyl-L-arginine (L-NMMA), a compound capable of markedly inhibiting...
NO synthesis by endothelium (2) in type 1 diabetic patients with microalbuminuria, and suggested that endothelial dysfunction was present in these patients. More recently, it has been hypothesized that early NO excess may be responsible for the development of glomerular hyperfiltration in the early stages of diabetic nephropathy, whilst an impaired NO synthesis occurs in more advanced stages of diabetic renal disease (2, 19). The conflicting data available might, in part, be explained by the fact that different vascular areas have been evaluated and by the short-term infusion of L-NMMA. We adopted a research protocol encompassing a prolonged and systemic administration in vivo of L-NMMA to overcome putative drawbacks related to the short life of such a compound in forearm vessels. We also investigated the relationships between the effects of L-NMMA simultaneously at cardiovascular and renal levels. To evaluate the role of NO in the pathogenesis of diabetic nephropathy, we studied type 2 diabetic patients with microalbuminuria and biopsy-proven diabetic glomerulopathy and patients with normal albumin excretion rate as well as non-diabetic subjects.

Materials and methods

Subjects

Seventeen Caucasian subjects were studied: six type 2 diabetic patients with microalbuminuria and biopsy-proven diabetic glomerulopathy (D2-MA) (20, 21), six type 2 diabetic patients with normoalbuminuria (D2-NA) and five controls. Diabetic patients were diagnosed as having type 2 diabetes when onset was after the age of 40 years, when they were treated with diet alone or in association with oral hypoglycemic agents and/or insulin and when they did not receive insulin in the first 2 years after diagnosis. Insulin-treated patients with normal body weight had a glucagon test performed to confirm the diagnosis of type 2 diabetes (when C-peptide levels were normal). Patients were defined as microalbuminuric when albumin excretion rate (AER) was 20–200 µg/min in at least two of three consecutive sterile 24-h urine collections. Background (three patients) and proliferative (three patients) retinopathy was present in D2-MA patients. Diabetic retinopathy was absent in D2-NA patients. Body mass index (BMI) was calculated using the formula: weight (kg)/height$^2$(m$^2$).

All the subjects studied were males, had mild arterial hypertension (≥140/90 mmHg) (22) and were treated with angiotensin-converting enzyme inhibitors, which were withdrawn 5 days before and during the study. All participants gave their written informed consent before the study. The study protocol was approved by the Ethics Committee of the University of Padova.

Procedures and calculations

After overnight fast, an antecubital vein was catheterized bilaterally for separate blood sampling and infusion. A priming dose of a solution containing 50 µCi $^{51}$Cr ethylenediaminetetra-acetic acid ($^{51}$Cr-EDTA; Amersham Sorin Radiofarmaci, Milan, Italy), for the measurement of glomerular filtration rate (GFR), and 0.7 g cold 20% para-aminohippuric acid (PAH) (Jacopo Monico, Venice, Italy) for the measurement of renal plasma flow (RPF) was infused, followed by a continuous infusion of 200 µCi $^{51}$Cr-EDTA and 3.2 g PAH in 53 ml saline solution during the entire study (rate of infusion: 0.11 ml/min for both). After the first 2 h of equilibration, blood specimens were drawn every 15 min between 120 and 240 min for baseline measurements.

After 4 h, infusion of L-NMMA (Inalco, Milan, Italy) was started, with a priming dose of 3 mg/kg body weight, followed by continuous infusion of 3 mg/kg per h for 2 h. Blood samples were withdrawn every 15 min throughout the study. During the study, blood pressure was recorded at 15-min intervals and the values represent the mean of these repeated measurements expressed as mean blood pressure (MBP) (diastolic blood pressure +1/3 pulse pressure). At baseline and during L-NMMA infusion, we measured cardiac index (CI) and heart rate (HR) by two-dimensional Doppler pulsed and color Doppler echocardiographic examination (Toshiba Sonolayer SSA-270 A, Tokyo, Japan). CI was obtained by multiplying stroke volume by heart rate and the values represent the mean of repeated measurements, normalized for body surface area (BSA). Stroke volume was calculated as the product of the velocity time integral (VTI) and cross-sectional area of the left ventricular outflow tract. VTI was registered by pulsed Doppler with the sample volume 1 cm below the level of the aortic leaflets; the left ventricular outflow area was measured from the two-dimensional echocardiogram, with the transducer located in the parasternal long axis view during early systole, as the distance between the hinge points of the anterior and posterior aortic cups (23). This method has been previously validated using as control standard the Fick method (24) and thermodilution technique (25). The coefficient of variation for repeated measurements in the same subjects was 3±1%. GFR and RPF were obtained by the steady-state plasma clearance of $^{51}$Cr-EDTA and PAH (26, 27). Thus, $^{51}$Cr-EDTA and PAH were measured in the infusate, then by multiplying the infusion rate for the concentration in the infusate and dividing for plasma concentration we obtained the values that were normalized for BSA. $^{51}$Cr radioactivity was measured in duplicate 1 ml aliquots of plasma in a gamma counter (Cobra-5002 CPM; Camberra Packard, Milan, Italy) and PAH was measured in duplicate 1 ml plasma specimens by an immunoturbidimetric
method (Spectrophotometer Cobas Mira Plus, Basel, Switzerland). The coefficient of variation was 2.7±0.2% for GFR and 3.8±0.4% for RPF. Filtration fraction (FF) was calculated by dividing GFR by RPF. Renal blood flow (RBF) by dividing RPF by (1−hematocrit) and renal vascular resistance (RVR) was calculated by dividing mean blood pressure by RBF (26, 27).

Statistical analysis

Data are expressed as means±S.E.M. Values for AER, not normally distributed, are expressed as median and range. For comparison among the groups we used one-way ANOVA and then Bonferroni t-test. For repeated measurements Wilcoxon matched-pairs signed-ranks test and for correlations between the parameters bivariate correlations procedure (Spearman’s rho correlation coefficient) were used. A value of \( P < 0.05 \) was considered significant.

Results

L-NMMA infusion was well tolerated and no adverse effects due to its administration were observed. Table 1 shows the clinical features of the subjects. Glycemic control was similar in type 2 diabetic patients with and without abnormalities in albumin excretion rate (D2-MA and D2-NA) and also remained similar during L-NMMA infusion (8.9±1.2 mmol/l in D2-MA, 8.6±1.4 in D2-NA and 4.7±0.4 in controls). No significant differences were observed in BMI and age between controls, D2-MA and D2-NA subjects, although controls tended to be younger than diabetic patients. Duration of diabetes was longer in D2-MA than in D2-NA patients (\( P < 0.03 \)). Tables 2 and 3 show the cardiovascular and renal parameters at baseline and during L-NMMA infusion in D2-MA and D2-NA patients and in controls. Cardiovascular and renal parameters at baseline were similar in the three groups.

Table 1 Clinical features of type 2 diabetic patients with microalbuminuria (D2-MA) and with normoalbuminuria (D2-NA) and in controls. Values are means±S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>D2-MA</th>
<th>D2-NA</th>
<th>Controls</th>
</tr>
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<tbody>
<tr>
<td>Number</td>
<td>6</td>
<td>6</td>
<td>5</td>
</tr>
<tr>
<td>Age (years)</td>
<td>56±4</td>
<td>55±3</td>
<td>45±4</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>27±2</td>
<td>27±1</td>
<td>25±2</td>
</tr>
<tr>
<td>Known diabetes duration (years)</td>
<td>15±3*</td>
<td>6±1</td>
<td>—</td>
</tr>
<tr>
<td>HbA1c (%)</td>
<td>8±1†</td>
<td>8±1†</td>
<td>5±0.1</td>
</tr>
<tr>
<td>Fasting plasma glucose (mmol/l)</td>
<td>9.7±1†</td>
<td>9.3±1†</td>
<td>4.9±0.1</td>
</tr>
<tr>
<td>AER (µg/min) (median and range)</td>
<td>93 (20−200)</td>
<td>7 (4−19)</td>
<td>5 (4−6)</td>
</tr>
<tr>
<td>Total serum cholesterol (mmol/l)</td>
<td>5.3±0.3</td>
<td>5.2±0.8</td>
<td>4.9±0.3</td>
</tr>
</tbody>
</table>

\* \( P < 0.03 \), D2-MA vs D2-NA, \( \dagger P < 0.05 \), D2-MA and D2-NA vs controls. HbA1c, hemoglobin A1c.

Controls

L-NMMA infusion caused a significant decrease in CI (\( P < 0.05 \) vs baseline) (Table 2 and Fig. 1), which remained stable during the 2 h of infusion of L-NMMA. MBP tended to increase (\( P = 0.06 \) vs baseline) and HR did not change significantly from baseline values (Table 2).

The effects of L-NMMA on kidney function are summarized in Table 3 and Figs 2, 3 and 4. GFR significantly decreased during L-NMMA infusion (\( P < 0.05 \) vs baseline) (Table 3 and Fig. 2) and a marked decrease was also observed in RPF (\( P < 0.05 \) vs baseline) (Table 3 and Fig. 3); FF increased from baseline (\( P < 0.05 \) vs baseline) (Table 3 and Fig. 4). During L-NMMA infusion we also observed a marked increase in RVR (\( P < 0.05 \) vs baseline) (Table 3).

D2-NA patients

L-NMMA infusion also decreased CI (\( P < 0.05 \) vs baseline) in D2-NA patients (Table 2 and Fig. 1), while MBP and HR did not change (Table 2). GFR did not change significantly (Table 3 and Fig. 2); RPF significantly decreased from baseline (\( P < 0.03 \)) (Table 3 and Fig. 3); RVR increased during L-NMMA infusion.

Table 2 Cardiovascular parameters in type 2 diabetic patients with D2-MA and with D2-NA and in controls at baseline, and during L-NMMA administration. Values are means±S.E.M.

<table>
<thead>
<tr>
<th></th>
<th>CI (ml/min per 1.73m²)</th>
<th>MBP (mmHg)</th>
<th>HR (beats/min)</th>
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</thead>
<tbody>
<tr>
<td>Baseline</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>D2-MA</td>
<td>2948±120</td>
<td>114±3</td>
<td>70±4</td>
</tr>
<tr>
<td>D2-NA</td>
<td>3248±289</td>
<td>113±4</td>
<td>69±5</td>
</tr>
<tr>
<td>Controls</td>
<td>2837±154</td>
<td>112±8</td>
<td>65±3</td>
</tr>
<tr>
<td>L-NMMA</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>D2-MA</td>
<td>2699±69*</td>
<td>126±3†</td>
<td>67±3</td>
</tr>
<tr>
<td>D2-NA</td>
<td>2687±157*</td>
<td>119±4</td>
<td>64±4</td>
</tr>
<tr>
<td>Controls</td>
<td>2521±147*</td>
<td>118±10</td>
<td>62±3</td>
</tr>
</tbody>
</table>

\* \( P < 0.05 \), \( \dagger P < 0.03 \) vs baseline. CI, cardiac index; MBP, mean blood pressure; HR, heart rate.
(P < 0.03 vs baseline) and FF did not change (Table 3 and Fig. 4).

**Discussion**

This study demonstrates that systemic and prolonged inhibition of NO synthesis, induced by primed and sustained L-NMMA infusion, results in a fall in cardiac output, in both control subjects and type 2 diabetic patients, irrespective of the presence of microalbuminuria. The fall in cardiac output cannot be explained by changes in blood pressure or heart rate since a significant increase in blood pressure was observed.
only in patients with diabetic nephropathy. In contrast, a significant decrease in GFR in response to inhibition of NO synthesis and release in vivo during systemic L-NMMA infusion was observed only in controls. These findings suggest that NO synthesis or response to L-NMMA is impaired in type 2 diabetic patients at the renal level. Evaluation of endothelial NO function in type 2 diabetic patients generated conflicting results.
Studies have shown that both endothelium-dependent and independent vasodilatation are impaired in type 2 diabetic patients (28). Other authors have found no difference in basal (29) and acetylcholine-stimulated NO generation and endothelium-dependent relaxation in patients without chronic diabetic complications (15). It has also been suggested that endothelium-dependent vasoreactivity changes during the course of the disease (30). These apparently conflicting results can be the consequence of the influences of different conditions, such as age, duration of diabetes, blood glucose control, circulating cholesterol levels, variation in the forearm length, experimental use of different muscarinic receptor agonists (acetylcholine, carbachol, metacholine) and the study of different arterial beds, which might have different responses to different vasodilating agents.

We adopted, for the first time in type 2 diabetic patients, the experimental approach of prolonged and systemic administration of L-NMMA to obtain an effective inhibition of endothelial NO synthesis and release both at cardiovascular and renal levels. This approach overcomes potential drawbacks related to variations of forearm length between subjects as well as the inadequate effects of L-NMMA on NOS activity when this compound is available within the endothelium for too short a period. Although we do not have a direct measurement of the inhibition of NO synthesis (circulating metabolites), the dose of L-NMMA used should have been adequate to inhibit NO synthesis as previously reported (31). Unfortunately the rapid metabolism and short half-life of NO poses a considerable obstacle for its analytical assessment in humans; nitrite and nitrate (NO$_2^-$ + NO$_3^-$) plasma concentration, the circulating metabolites of NO metabolism, can be measured, although they are influenced by several NOS-independent factors. We are aware that the number of patients studied was small; however, this methodological approach is extremely demanding for the patients and the operators, and involves high costs related to the large amounts of L-NMMA. Since all the diabetic patients in our study were hypertensive, we selected control subjects with hypertension; thus the only difference between diabetics and controls was the presence of diabetes. Moreover, we investigated two different groups of type 2 diabetic patients, who differed in the duration of the disease and in renal and retinal complications. We observed a clear cardiac effect of L-NMMA infusion in both diabetic and control subjects; thus we can conclude that the inhibition of NO synthesis by L-NMMA resulted in a similar cardiac response in diabetic and control subjects, suggesting that this particular metabolic pathway is not impaired in the heart in type 2 diabetes.

In contrast, the hemodynamic response to L-NMMA infusion at glomerular level was abnormal in both diabetic groups.

In fact, GFR decreased sharply and FF rose in control subjects during L-NMMA infusion, whereas no change was observed in both D2-MA and D2-NA patients. This different hemodynamic behavior at renal level in comparison with controls also occurred in RPF in diabetic patients.
patients with microangiopathy in whom no significant change in RPF occurred in response to L-NMMA administration; in contrast RPF significantly decreased in controls and in diabetic patients without microangiopathy. The NO pathway might also be modulated by the duration of diabetes; however, in our patients, we did not find any correlation between duration of diabetes, the baseline parameters and the degree of response to L-NMMA infusion. Thus we can exclude the possibility that different durations of diabetes in D2-MA and D2-NA patients may have influenced cardiovascular and renal response to L-NMMA infusion.

These abnormalities in glomerular hemodynamics may be explained by two alternative or concomitant mechanisms. First, it can be postulated that NO synthesis and release is increased or alternatively decreased in type 2 diabetic patients at baseline, therefore accounting for an impaired glomerular response to L-NMMA administration. Two recent reports found an increased rate of excretion of NO$_2$ + NO$_3$ in type 1 diabetes (16, 19). In contrast, no evidence of a disturbance in basal or stimulated NO production has been observed by other authors in type 2 diabetes (15) and in type 1 diabetes (14). Secondly, disorders in the glomerular regulation by other hormonal factors such as prostaglandins, angiotensin II, dopamine etc. may explain the impaired glomerular response to L-NMMA infusion (1, 32). More particularly, inhibition of endogenous P-450 metabolites of arachidonic acid, such as 20-hydroxyeicosatetraenoic acid as well as other eicosanoids fully reverses the reduction in GFR by NO$^\circ$-nitro-l-arginine-methyl-ester (L-NAME), a compound capable of inhibiting NO synthesis (33). Moreover, eicosanoids contribute to the regulation of renal hemodynamics of several vasoactive peptides, such as endothelin 1 and angiotensin II (34). Eicosanoid synthesis and release have been shown to be altered in diabetes (35–37).

Whatever the mechanism responsible for the abnormality in glomerular function in D2-MA and D2-NA patients, this renal hemodynamic disturbance probably occurs early in type 2 diabetes, even before the development of microvascular complications. The slightly more pronounced impairment of glomerular hemodynamics in type 2 diabetic patients with microangiopathy may suggest that the defect of NO regulation of glomerular function worsens progressively with the occurrence of renal complications. These findings are in keeping with the observations of Dogra et al. (38) who have shown that endothelial dysfunction is present in normoalbuminuric type 1 diabetic patients, and is more marked in microalbuminurics.

It is still unclear if the L-NMMA-induced renal effects are independent from those simultaneously observed at cardiovascular level. It is possible that systemic administration of NO blockers produces increases in blood pressure which, in turn, have effects on the kidney. Thus a substantial part of the renal vasoconstriction observed during L-NMMA infusion might be secondary to the L-NMMA-induced cardiac changes.

However, this hypothesis is not in keeping with previous reports showing that systemic infusion of low doses of NO inhibitors, not affecting blood pressure, causes renal vasoconstriction in animals and in healthy subjects (39, 40). Moreover, it has been described that pressor and renal responses to NO inhibition are separately regulated (40, 41). Thus the fall in GFR and RPF during L-NMMA infusion does not seem to be a mere consequence of the reduction in cardiac output secondary to L-NMMA administration.

In the present study, the L-NMMA induced decrease in cardiac output was accompanied by changes in renal hemodynamics only in controls. In contrast, in type 2 diabetic patients, especially in those with microangiopathy, the fall in cardiac output was not accompanied by changes in renal hemodynamics. These findings suggest that NO synthesis and release at glomerular level are impaired in patients with type 2 diabetes and that renal and cardiac hemodynamics are separately regulated.

In conclusion, this study, using for the first time a systemic and prolonged infusion of L-NMMA in type 2 diabetic patients, demonstrates that sustained, systemic inhibition of NO synthesis by L-NMMA induces similar cardiac effects in controls and type 2 diabetic patients. On the contrary, the response of GFR and FF to L-NMMA was significantly impaired in diabetic patients with and without microangiopathic complications, indicating that the regulation of glomerular hemodynamics by NO is altered in type 2 diabetes.

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References


