Hyperfiltration and the effect of nitric oxide inhibition on renal and endothelial function in humans with uncomplicated type 1 diabetes mellitus

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ABSTRACT:

Objective: Studies of experimental diabetes mellitus (DM) suggest that increased nitric oxide (NO) bioactivity contributes to renal hyperfiltration. However, the role of NO in mediating hyperfiltration has not been fully elucidated in humans. Our aim was to examine the effect of NO synthase inhibition on renal and peripheral vascular function in normotensive subjects with uncomplicated type 1 DM.

Methods: Renal function and brachial artery flow-mediated vasodilatation (FMD) were measured before and after an IV infusion of the NO synthase inhibitor L-NMMA in 21 healthy control and 37 type 1 DM patients. Measurements in DM participants were made under clamped euglycemic conditions. The effect of L-NMMA on circulating and urinary NO metabolites (NOₓ) and cGMP and on urinary prostanoids was also determined.

Results: Baseline characteristics were similar in the 2 groups. For analysis, the DM patients were divided into those with hyperfiltration (DM-H, n=18) and normal GFR levels (DM-N, n=19). Baseline urine NOₓ and cGMP were highest in DM-H. L-NMMA led to a decline in GFR in DM-H (152±16 to 140±11 ml/minute/1.73 m²) but not DM-N or healthy control participants. The decline in effective renal plasma flow in response to L-NMMA (806±112 to 539±80 ml/minute/1.73 m²) in DM-H was also exaggerated compared to the other groups (repeated measures ANOVA, p<0.05), along with declines in urinary NOₓ metabolites and cGMP. Baseline FMD was lowest in DM-H compared with the other groups and did not change in response to L-NMMA. L-NMMA reduced FMD and plasma markers of NO bioactivity in the healthy control and DM-N groups.
**Conclusions:** In patients with uncomplicated type 1 DM, renal hyperfiltration is associated with increased NO bioactivity in the kidney and reduced NO bioactivity in the systemic circulation, suggesting a paradoxical state of high renal and low systemic vascular NO bioactivity.
INTRODUCTION:

Glomerular hyperfiltration is an early renal hemodynamic change in animal and human studies of DM, and may help to predict the risk for the subsequent development of diabetic nephropathy (38). The pathogenesis of hyperfiltration is complex and involves both tubuloglomerular feedback and hemodynamic abnormalities. Renal hemodynamic changes associated with hyperfiltration include afferent vasodilatation and efferent constriction. Hyperfiltration is, however, only partially corrected after selective cyclooxygenase-2 inhibition (predominant afferent constriction) or renin angiotensin system (RAS) blockade (predominant efferent vasodilatation) in humans with uncomplicated type 1 DM (8, 54). These findings suggest the presence of non-prostaglandin, non-RAS- dependent hemodynamic mechanisms that perpetuate the hyperfiltration state (60).

Functional expression studies in DM models have determined that endothelial nitric oxide synthase (eNOS) expression is consistently upregulated and localized to the afferent arteriole, renal cortex and medulla (32). In addition to eNOS, macula densa neuronal NOS (nNOS) activation reduces afferent arteriolar tone and influences glomerular capillary pressure and tubuloglomerular feedback mechanisms that have been implicated in hyperfiltration (31, 62). Conversely, inducible NOS (iNOS) expression studies have demonstrated less consistent results (32). Therefore, it is generally accepted that eNOS and nNOS expression are upregulated in experimental models of DM leading to increased nitric oxide (NO) bioactivity, particularly in the early adaptive stages, but the functional significance of this upregulation in humans has not been fully elucidated (3).

In contrast with renal microvascular data suggesting increased NO bioactivity, studies examining systemic macrovascular function have suggested an opposite and paradoxical state of NO suppression. For example, animal models of type 1 DM have demonstrated a consistent state
of NO-mediated renal hyperfiltration, despite evidence of decreased NO activity and endothelial dysfunction in large vessels (6, 37). This paradoxical state of high renal versus low macrovascular NO bioactivity may also exist in humans, since type 2 DM patients exhibit a positive association between urinary NO excretion and creatinine clearance in observational cohort studies, despite evidence of macrovascular dysfunction and low NO bioactivity (25, 26). While similar associations have been made in humans with type 1 DM (37), previous work has not, to our knowledge, used simultaneous gold-standard measures of renal microvascular and large vessel function to study this apparent renal-systemic NO paradox.

Accordingly, we hypothesized that NOS inhibition with L-NMMA would result in a greater reduction in GFR in hyperfiltrating patients with type 1 DM (DM-H) compared to DM patients with normal GFR (DM-N) and healthy control subjects due to maximal renal NO stimulation in DM-H. Second, we hypothesized that paradoxically, impaired brachial artery FMD associated with renal hyperfiltration (9) would be associated with decreased responsiveness to L-NMMA in DM-H vs. DM-N and healthy controls.

METHODS:

MATERIALS and METHODS

Subjects

Twenty-one healthy controls and thirty-seven participants with uncomplicated type 1 DM (19 DM-N and 18 with DM-H) participated in this study (Table 1). Hyperfiltration was defined using the usual definition of a GFR ≥135 ml/min/1.73m² (38). Inclusion criteria were: duration of type 1 DM ≥5 years, age ≥ 18 years, blood pressure < 140/90, no history of renal disease or macrovascular disease and participants could not be taking any regular medications other than
insulin and had to be normoalbuminuric on a 24 hour urine collection. We aimed to study female subjects during the early follicular phase of the menstrual cycle, determined by cycle day and measurement of 17ß-estradiol levels. None were using oral contraceptive medication. The local Research Ethics Board at the University Health Network (Toronto, Canada) approved the protocol and all subjects gave informed consent.

**Experimental Design**

In order to maintain suppression of endogenous RAS activity, subjects adhered to a high sodium (>140 mmol/day) and moderate protein (<1.5 g/kg/day) diet during the 7-day period before each experiment, as described previously (Table 1, Figure 1). In patients with DM, clamped euglycemic (4-6 mmol/L) conditions were maintained for approximately 6 hours preceding and during all investigations, a period of time previously demonstrated to be sufficient to influence vascular function (8). In all phases of the experiment, blood glucose was maintained by a modified glucose clamp technique, as described previously (8). A 16-gauge peripheral venous cannula was inserted into the left antecubital vein for infusion of glucose and insulin and a second cannula was inserted for blood sampling more distally. Blood glucose was measured every 5-10 minutes and the insulin infusion was adjusted to maintain euglycemia. In healthy control subjects, studies were performed on a single day during normoglycemic conditions. All experiments were performed in the same warm (25°C), temperature-controlled room and in a dark, quiet environment after 10 minutes of rest in the supine position.

After the desired level of clamped euglycemia was achieved, baseline measures of FMD were taken as described below and baseline blood samples were collected for the following assays: inulin and paraaminohippurate (PAH) blank, baseline angiotensinogen, plasma renin
concentration (PRC), plasma renin activity (PRA), angiotensin (ANG) II and aldosterone. To assess biochemical makers of NO bioactivity, plasma NO metabolites (NO\textsubscript{2} and NO\textsubscript{3} or NO\textsubscript{x}) and cyclic guanosine monophosphate (cGMP) were also measured. Baseline urine samples were also collected for NO\textsubscript{x} and cGMP excretion (corrected for creatinine concentration). Due to the important effect of NO blockade on prostanoids (58), we also measured the urinary excretion of 2,3-dinor-6-keto PGF1-alpha, PGF1-alpha TXB2, PGE2 and PGEM. Next, baseline values of renal function were then obtained using inulin and PAH clearance techniques to obtain 2 baseline clearance periods, which were then averaged (8). L-NMMA (250 mg/vial, Clinalpha, Laüfelfingen, Switzerland) was then administered intravenously to direct drug to the renal circulation at incremental doses, first as a bolus of 1 mg/kg bolus in 5 minutes followed by a 1 mg/kg infusion over 25 minutes using established, standard dosing and infusion protocols (17, 19, 53, 61). The dose was then increased, with a 3 mg/kg bolus IV in 5 minutes, followed by an infusion of 3 mg/kg over 25 minutes. Renal function parameters (inulin and PAH clearances) were assessed at the end of each L-NMMA infusion period, at the same time that brachial artery vasodilatory responses were measured. Forearm blood flow and endothelial dependent vasodilatation were measured starting at the midpoint of each 30 minute interval after starting the L-NMMA infusion. At this 30 minute time point, blood was also drawn for circulating NO\textsubscript{x} and cGMP levels. At the end of the high dose L-NMMA infusion, we also measured urine NO\textsubscript{x}, cGMP and prostanoid excretion. Arterial pressure and heart rate were measured by an automated sphygmanomanometer over the right brachial artery (DINAMAP\textsuperscript{®} sphygmomanometer, Critikon, Florida, USA) at 30 minute intervals throughout the study and at 5 minute intervals during the L-NMMA infusion.
Assessment of Endothelial Function

Brachial artery endothelial function was determined by recording diameter changes in the brachial artery in response to increased blood flow generated during reactive hyperemia (flow-mediated dilatation) at baseline and in response to the graded infusion of L-NMMA. Briefly, the right brachial artery was scanned 2 to 5 cm above the antecubital fossa using high resolution B-mode vascular ultrasound (Vivid i, 7-15 MHz linear-array transducer, GE/Vingmed, WI, USA). Longitudinal, ECG-gated, end-diastolic images were acquired over 6 cardiac cycles, and the brachial arterial diameter was determined for each image using integrated software and the results were averaged (9). Diameter measurements were taken from the anterior to the posterior interface between the media and adventitia. After baseline images were recorded, the blood pressure cuff was inflated around the forearm distal to the elbow to >200 mmHg for 5 minutes. After cuff deflation, the increase in blood flow was measured (reactive hyperemia) along with the change in vessel diameter (endothelium-dependent dilatation), which was measured for a further 5 minutes. FMD was defined and reported as the maximal percentage changes in vessel diameter after reactive hyperemia (9). The intra-observer variability for repeated measurements of arterial diameters at flow mediated vasodilatation was 0.01±0.005 mm (absolute diameter) or 0.26±0.01% (% absolute value of brachial artery at flow mediated vasodilatation), which is similar to that previously reported (24, 30).

Assessment of Renal Parameters

Following the assessment of endothelial function, a third intravenous line was inserted into the right arm and was connected to a syringe infusion pump for administration of inulin and paraaminohippurate (PAH). After collecting blood for inulin and PAH blank, a priming infusion
containing 25% inulin (60 mg/kg) and 20% PAH (8 mg/kg) was administered. Thereafter, inulin and PAH were infused continuously at a rate calculated to maintain their respective plasma concentrations constant at 20 and 1.5 mg/dl. After a 90 minute equilibration period, blood was collected for inulin, PAH and hematocrit (HCT). Blood was further collected every 30 minutes for 60 minutes for inulin and PAH, and glomerular filtration rate (GFR) and effective renal plasma flow (ERPF) were estimated by steady state infusion of inulin and PAH, respectively (8).

Sample Collection and Analytical Methods:

Blood samples collected for inulin and PAH determinations were immediately centrifuged at 3000 rpm for 10 minutes at 4° C. Plasma was separated, placed on ice, and then stored at -70° C before the assay. Inulin and PAH were measured in serum by colorimetric assays using anthrone and N- (1-naphthy) ethylenediamine respectively (14, 22, 29). The mean of 2 baseline clearance periods represent GFR and ERPF, expressed per 1.73 m². Renal blood flow (RBF) was derived using ERPF / (1-hematocrit) and renal vascular resistance (RVR) was derived by dividing the MAP by the RBF. All renal hemodynamic measurements were adjusted for body surface area (14, 22).

Plasma renin concentration was measured by two-site immunoradiometric assay where 2 monoclonal antibodies to human active renin were used (Renin III Generation RIA Kit, Bio-Rad Laboratories, #79986). One antibody was coupled to biotin while the second was radiolabeled for detection. The sample containing active renin was incubated simultaneously with both antibodies to form a complex. The radioactivity of this complex was directly proportional to the amount of immunoreactive renin present in the sample. Plasma Renin Activity (PRA) was measured with a radioimmunoassay kit (GammaCoat® Plasma Renin Activity ¹²⁵I RIA Kit, CA-
Plasma Ang II, aldosterone and angiotensinogen were also measured using previously described methods (7, 8).

To assess NO formation (40), plasma NO$_x$ and cGMP levels were measured at baseline and 30 minutes after the 1 mg/kg and 3 mg/kg doses of L-NMMA. Urine NO$_x$ and cGMP levels were also measured at baseline and at the end of the 3 mg/kg L-NMMA infusion. The assay for cGMP is based on the competition between cGMP in the standards or samples and a cGMP-acetylcholinesterase (AChE) conjugate (cGMP tracer) for a limited number of cGMP-specific rabbit antibody binding sites. The rabbit antibody-cGMP complex (either free or tracer) binds to the mouse monoclonal antibody IgG that is coated to the well. The plate is washed to remove the unbound reagent and then Ellman’s Reagent (acetylthiocholine and 5,5’-dithiobis-2-nitrobenzoic acid, a substrate to AchE) is added to the well. The product of this enzymatic reaction, 5-thio-2-nitrobenzoic acid, has a distinct yellow color and absorbs strongly at 412 nm. The intensity of the color is proportional to the amount of cGMP tracer bound to the well, which is inversely proportional to the amount of free cGMP present in the standards or sample. Plasma samples are extracted with EtOH prior to analysis. For NO$_x$ levels (NO, R&D Systems, Minneapolis, MN - total NO/Nitrite/Nitrate assay kit, Cat No.KGE001) the assay determines NO concentration based on the enzymatic conversion of nitrate to nitrite by nitrate reductase. The reaction is followed by colorimetric detection of nitrite as an azo dye product of the Griess Reaction. The Griess Reaction is based on the two-step diazotation reaction in which acidified nitrite produces a nitrosating agent which reacts with sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-(1-naphthyl)ethylenediamine to form the chromophoric azo-derivative which absorbs light at 540-570 nm.

Urinary prostanoids were also measured in all participants before and after the L-NMMA infusion, since the vascular effects of NO inhibition depend in part on prostanoid bioactivity (59).
Following recommended purification protocols, urinary prostaglandins (PGE2, 6-keto-PGF1alpha, TXB2) and their metabolites (PGEM, 2,3-dinor-6keto-PGF1alpha) were measured by competitive enzyme immunoassays (Cayman Chemical). Quantification is based on a colorimetric reaction catalyzed by acetylcholinesterase. PGEM is a measure of both renal and systemic PGE2, while 2,3-dinor-6-keto PGF1-alpha corresponds to systemic prostacyclin production and subsequent urinary excretion (52). All samples were corrected for creatinine and expressed as pg/mg creatinine.

Urinary albumin excretion rate was determined from three timed overnight urine collections. Urinary albumin concentration was determined by immunoturbidimetry. Hemoglobin A1C was measured by high-performance liquid chromatography, and plasma insulin levels were measured in patients with DM using standard techniques (9).

**Statistical Analysis:**

Descriptive statistics were used to compare baseline clinical and demographic characteristics. *Between group* comparisons in baseline parameters in diabetic vs. healthy control subject groups were made using analysis of variance (ANOVA). Changes in renal and systemic vascular function in response to L-NMMA were assessed using a mixed factorial 2 way (group x time) repeated measures ANOVA. All statistical analyses were performed using the statistical package SAS Version 9.2, and expressed as mean±SD. The vascular data were obtained and analyzed by a single observer (D.Z.I.C.) who was blinded to the renal measurements.

**RESULTS:**

**Baseline Characteristics:**
Healthy control, DM-N and DM-H participants were similar with respect to gender distribution, age, BMI, albumin excretion and dietary parameters (Table 1). Although studies were scheduled to correspond to the early follicular phase of the menstrual cycle, plasma estrogen levels were variable and mean values were more consistent with the mid-late follicular cycle. Despite the variability, estrogen levels were similar in women in the three groups. In addition, the DM-N and DM-H groups exhibited similar plasma insulin levels (64±30 vs. 74±17 pmol/L, respectively), HbA1C and diabetes duration parameters. None of the participants experienced adverse effects from the L-NMMA or other study procedures. In DM patients, mean venous blood glucose was 4.6±0.6 mmol/L at the beginning of the vascular studies, indicating that the desired level of clamped euglycemia was achieved. In control subjects, mean venous blood glucose was 4.5±0.2 mmol/L (range 4.2-5.4 mmol/L), again indicating normoglycemia.

Urinary cGMP excretion was significantly higher in DM-H vs. DM-N and control participants and urinary NOx excretion was higher in DM-H vs. DM-N; between-group baseline differences in urinary prostanoids were not significant (Figure 2 panels a and b, Table 3).

Baseline plasma NOx levels were significantly higher in healthy control vs. the DM-N and DM-H groups (Figure 2 panel c). Baseline plasma cGMP levels were numerically lower in DM-H compared with the DM-N and control groups, although these differences were not significant (Figure 2 panel c, p=0.07); urinary excretion of 2,3-dinor-6-keto PGF1-alpha was similarly lower in DM-H vs. the other groups but between-group differences were not significant (Table 3).

Circulating aldosterone, ANG II, plasma renin concentration and plasma renin activity were higher in control vs. DM-H patients; only between-group differences for aldosterone and ANG II were significant for the healthy control vs. DM-N group comparison (Table 1).
Numerical differences in DM-N vs. DM-H patients for baseline circulating RAAS mediators did not reach significance (Table 1).

**Renal Function Responses to L-NMMA**

Baseline values for blood pressure and heart rate were similar in the control and DM groups (Table 2). As expected from our previous work (8, 54), DM-H patients exhibited higher ERPF and GFR and lower RVR measurements compared to the healthy control participants and the DM-N group.

In response to L-NMMA, SBP and DBP increased in all three groups (Table 2). *Between-group* differences in blood pressure responses were not significant. For renal parameters, L-NMMA-associated declines in ERPF and RBF, and increases in FF and RVR were observed in all groups but were exaggerated in DM-H patients (Table 2). Furthermore, L-NMMA administration was associated with a decrease in GFR in the DM-H only (Table 2, ANOVA p=0.002). Despite a decline in GFR, the mean GFR in the DM-H group after L-NMMA remained in the hyperfiltration range.

In DM-H patients, exaggerated renal hemodynamic responses were associated with significant declines in the urinary excretion of NOx and cGMP (Figure 2, panels a and b). Similar trends in healthy control and DM-N did not reach significance. After L-NMMA, urinary 6-keto-PGF1-alpha, PGE2 and PGEM excretion generally declined in the 3 groups (*within group* effects p>0.05), and after L-NMMA 6keto-PGF1-alpha, TXB2 and PGE2 excretion rates were lower in DM-N vs. healthy control participants. In addition, the decrease in urinary TXB2 was significant in the DM-H group vs. the response in healthy control group (Table 3).

**Endothelial Function Responses to L-NMMA**
Baseline FMD was significantly higher in healthy control and DM-N compared to DM-H patients (Figure 3, ANOVA p=0.009). FMD responses remained significantly lower in the DM-H group throughout the rest of the study (repeated measures ANOVA p≤0.01). In response to the L-NMMA infusion, FMD declined significantly in the healthy control and DM-N groups but did not change in the DM-H group. Between-group differences in the response to L-NMMA were significant at the higher dose for DM-H patients vs. the healthy control and DM-N.

Blunted FMD responses to L-NMMA in DM-H were associated with lower plasma levels of NOx throughout the L-NMMA infusion (Figure 2 panel c). Declines in plasma NOx levels were greatest in healthy controls, with more modest declines in the DM groups. Plasma cGMP levels decreased significantly in response to L-NMMA in the healthy control group but within-group changes in DM-N and DM-H were not significant (Figure 2 panel d). Urinary excretion of 2,3-dinor-6-keto PGF1-alpha did not change in response to L-NMMA in any of the groups (Table 3).

**DISCUSSION:**

Data from experimental models of types 1 and 2 DM suggest that increased expression and activity of NO is associated with renal hyperfiltration (27, 35). The contribution of increased NO bioactivity to early, pre-clinical changes in renal function in humans remains unclear. Paradoxically the hyperfiltration state has also been associated with impaired systemic endothelial function and changes in arterial stiffness, suggesting, at least in part, a state of generalized vascular dysfunction (9, 10). The simultaneous co-existence of potentially opposite levels of NO activity in humans with DM has not previously been established. Our major observations were: 1) declines in GFR, ERPF and urinary excretion of renal vasodilators in
response to L-NMMA during clamped euglycemia were exaggerated in DM-H vs. healthy controls and DM-N patients, suggesting a state of high renal NO bioactivity in DM-H; 2) baseline FMD and plasma vasodilators were generally suppressed in DM-H subjects. L-NMMA suppressed FMD in DM-N and healthy control subjects but not in DM-H, suggesting a state of low systemic NO bioactivity in DM-H.

Our first major observation was that similar to effects observed in diabetic animals, NOS blockade with L-NMMA during clamped euglycemia was associated with a decline in GFR in DM-H patients, suggesting a state of high renal vascular NO bioactivity (55). The 12 ml/min/1.73 m² decline in GFR was modest and GFR remained in the hyperfiltration range after L-NMMA. Moreover, L-NMMA reduced ERPF to a greater degree compared to GFR, resulting in an exaggerated 27% rise in FF, which was greater than the expected 10-13% ΔFF after L-NMMA which is typical of healthy non-DM humans (2, 13). These effects on GFR were not observed in DM-N patients or healthy controls. The effect of L-NMMA on GFR suggests that NO bioactivity contributes to the pathogenesis of hyperfiltration in humans with uncomplicated type 1 DM. While we cannot precisely anatomically localize differences in segmental resistance in human studies, the exaggerated rise in FF with L-NMMA suggests that intraglomerular pressure increases to mitigate the renal vasoconstriction and resulting fall in ERPF associated with NOS inhibition.

Studies in normoalbuminuric patients with type 1 DM measuring urinary ¹⁵N nitrate excretion after an intravenous injection of radiolabeled L-arginine demonstrate increased whole-body NO synthesis compared to healthy controls (45). Urinary ¹⁵N nitrate excretion has also been positively associated with creatinine clearance, suggesting a role for NO bioactivity in the pathogenesis of glomerular hyperfiltration (45). In the present study, urinary NOₓ and cGMP
excretion rates were highest in DM-H participants, and L-NMMA significantly reduced urinary NO\textsubscript{x} and cGMP in this group. Combined with greater renal hemodynamic responses to L-NMMA assessed on the basis of gold-standard inulin clearances, biochemical changes in DM-H suggest a state of high baseline NO production which contributes to hyperfiltration in this subset of type 1 DM patients.

Functional effects of L-NMMA may also be mediated through changes in prostanoid bioactivity (11, 23, 28, 36, 39). We therefore measured urinary excretion rates of prostanoids, since these factors are important regulators of renal vascular tone and have been implicated in the pathogenesis of hyperfiltration (8, 12, 18). For example, TXB2 inhibition reduces microalbuminuria, possibly through post-glomerular vasodilatory effects (20, 43). TXB2 suppression by L-NMMA has been attributed to IL-1\textbeta in \textit{in vivo} animal models and in \textit{in vitro} studies using human tissue but this interaction has not, to our knowledge, been studied in \textit{in vivo} human studies (11, 23, 28, 36, 39). Our results extend these previous biochemical observations by demonstrating for the first time that the L-NMMA-induced reduction in urinary TXB2 excretion was associated with a decline in GFR in DM-H. While we cannot determine causality, changes in urinary TXB2 excretion may have contributed to the renal hemodynamic effects of L-NMMA that we observed through changes in the renal microcirculation. Our results also suggest that PGE2 and prostacyclin do not contribute to L-NMMA responsiveness in humans with early type 1 DM.

Endothelial dysfunction is associated with an increased risk of diabetic nephropathy and vascular disease (44, 46, 51). The hyperfiltration state is associated with changes in macrovascular function in patients with uncomplicated type 1 DM, including higher nocturnal blood pressures, impaired FMD and differences in arterial compliance (9, 10, 47). We have also
previously reported that abnormal FMD responsiveness in DM-H depend at least in part on vasodilatory prostanoids (9). To further elucidate physiologic mechanisms linking early renal and systemic vascular dysfunction in early type 1 DM, we obtained simultaneous brachial artery endothelial function measurements with renal function measurements at each time point before and during a graded infusion of L-NMMA. Consistent with our previous work in a separate younger cohort of patients with type 1 DM, baseline FMD was suppressed in the DM-H group, (9). Circulating NO\textsubscript{x} and cGMP levels were consistent with FMD responses, in that values of these vasodilators were generally lowest in the DM-H group. This finding suggests a state of systemic vasodilator suppression in DM-H, a conclusion that was further reinforced by diminished FMD responsiveness to L-NMMA, and blunted changes in circulating NO\textsubscript{x} and cGMP in response to L-NMMA in the DM-H group.

The discrepancy between NO bioactivity in the renal microcirculation and systemic macrovasculature has been the subject of considerable discussion, and there are several possible explanations for this finding (48). First, although NO metabolites and NOS mRNA expression are increased in the renal microcirculation, NO activity in conduit arteries may be blunted through a variety of pathways, including superoxide anion free radical-mediated quenching of NO (48). Second, the role of NO may differ between vascular beds and other factors influencing endothelium-dependent vasodilatation, such as endothelium-derived hyperpolarizing factor and reactive oxygen species, may differ between large conduit arteries compared with the renal microcirculation (16). Third, a number of factors such as glycemic control, age and differences in experimental design including approaches used to measure NO bioactivity may lead to inconsistent reports (31, 48). We tried to avoid some of these limitations by including a uniform, well-characterized cohort of subjects and used methods established in our laboratory which control for potential important confounders such as volume status, ambient glycemia and diet.
Further, we used a direct NO inhibitor to assess dynamic, simultaneous changes in renal and systemic vascular function in study participants who were examined at a uniform stage of the natural history of disease.

A final mechanism that may contribute to the dissociation between renal and systemic vascular function relates to the RAS. Previous work has demonstrated a consistent suppression of circulating RAS mediators in DM patients, despite evidence of RAS activation at the tissue level, referred to as the paradox of the low-renin state in DM (49). The observation that circulating RAS mediators were higher in healthy control vs. DM-N patients, with even lower values in DM-H patients is novel and may suggest that hyperfiltration is associated with the greatest disparity between circulating RAS mediators and RAS activation at the tissue level. Interestingly, the RAS does exert a suppressive effect on NOS isoforms through complex negative feedback loops in different renal compartments (1, 15). Whether upregulation of the tissue RAS in DM-H subjects was in part responsible for the low NO bioactivity observed in the systemic vasculature is an intriguing possibility and should be further investigated. Furthermore, if baseline renal RAS activity is highest in DM-H, as we have previously suggested, then, similar to observations in animals, NOS inhibition may have permitted unopposed RAS activity leading to enhanced renal vasoconstrictive and autoregulatory effects of ANG II (54, 56).

We attempted to minimize the effect of the small sample size in our study by using homogeneous study groups and by using a careful pre-study preparation phase with a focus on known factors that influence neurohormonal activation. We also scheduled studies in female participants to coincide with the early follicular phase of the menstrual cycle to avoid confounding effects of estrogen on vascular function. Although plasma estrogen levels were somewhat higher than expected and more consistent with the mid to late follicular phase, estrogen concentrations were similar in the three groups. In addition, we decreased variability by
using a study design that allowed each subject to act as his/her own control. Nevertheless, the small sample size may have limited our ability to detect between-group differences in certain parameters such as urinary NOx. Next, because NO, cGMP and prostanoids are widely expressed in different anatomical compartments of renal tissues, we were unable to determine the specific origin of these urinary factors in this intact human study (1, 5, 33, 34). In addition, tubuloglomerular feedback is likely an important factor leading to renal hyperfiltration and may have been influenced by L-NMMA. Due to the existing complexity of this set of experiments, tubular factors were not assessed and should be examined in future protocols. Next, although differences were not statistically significant, the rise in BP in response to L-NMMA was numerically greater in the DM-H group. We therefore cannot rule out the possibility that autoregulatory responses may have contributed to the greater RVR increase in the DM-H group. Next, subjects were not placed on a controlled nitrate diet, and this may have limited our ability to detect some between-group differences in plasma and urine NOx. A final limitation of this work is that changes in shear rate (area under the curve) have the potential to contribute to reductions in FMD using systemic infusions of L-NMMA (50). Future work should determine if differences in shear rate played a role in the observed suppression of FMD in the DM-H group.

In conclusion, renal hyperfiltration is associated with simultaneously increased renal NO bioactivity and impaired endothelial function in patients with uncomplicated type 1 DM, suggesting that a state of paradoxical high renal and low systemic vascular NO activity exists in this group. Further work is required to determine the physiological basis for this differential regulation of vascular function by NO.

**PERSPECTIVES AND SIGNIFICANCE:**
DM remains the most cause of end-stage renal disease in developed countries, which ultimately requires dialysis or renal transplantation. Unfortunately, current therapies do not fully protect patients against renal disease progression, possibly because RAS blockade-based strategies do not abolish hyperfiltration (21, 54). While our results suggest that NO may contribute to hyperfiltration, targeted renal NOS inhibitors are not available and may be associated with risk due to systemic hypertensive effects. On the other hand, we have recently demonstrated that NO contributes to tubuloglomerular feedback pathways leading to afferent vasodilatation in patients with uncomplicated type 1 DM (42). Future work should assess the effect of increasing distal tubular sodium delivery with novel agents including sodium-glucose co-transport 2 inhibitors which reduce afferent vasodilatation and hyperfiltration in animals, in part via effects on pre-glomerular NO bioactivity (4, 57).
CONTRIBUTIONS:

D.Z.I.C., S.J., R.H., V.L., R.L.H., R.N. and E.B.S. researched data, wrote the manuscript. H.N.R., J.W.S. contributed to discussion, reviewed/edited manuscript.

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CONFLICT OF INTEREST:

The results presented in this paper have not been published previously in whole or in part.
REFERENCES:


**FIGURE LEGEND:**

**Figure 1:** Flow diagram

**Figure 2:** Plasma and Urine NO and cGMP Responses to L-NMMA During Clamped Euglycemia in Healthy Controls and in Type 1 Diabetes Patients and Either Normofiltration or Hyperfiltration (mean±SD)

* p<0.05 for mean value in DM-H vs. DM-N groups
† p<0.05 for mean value in healthy control vs. DM-H
‡ p<0.05 for mean value in healthy control vs. DM-N
§ p<0.05 for within group effect of L-NMMA

**Figure 3:** FMD Responses to L-NMMA in Healthy Control, DM-N and DM-H Patients During Clamped Euglycemia (mean±SD)

* p<0.05 for *between-group* baseline differences for DM-H subjects vs. DM-N and healthy controls
† p<0.05 vs. *within group* baseline value in healthy control and DM-N patients
‡ p<0.05 for *between-group* change in response to L-NMMA vs. DM-N patients
§ p<0.05 for *between group* change in response to L-NMMA vs. healthy control subjects
Table 1: Baseline characteristics and biochemistry (mean±SD)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Healthy Control Group (n=21)</th>
<th>Normofiltration group (n=19)</th>
<th>Hyperfiltration group (n=18)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Baseline demographic parameters</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Males</td>
<td>12/20 (60%)</td>
<td>11/19 (58%)</td>
<td>11/18 (61%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td>24±4</td>
<td>23±5</td>
<td>22±4</td>
</tr>
<tr>
<td>Diabetes Duration (years)</td>
<td>N/A</td>
<td>18±7</td>
<td>17±6</td>
</tr>
<tr>
<td>Weight (kg)</td>
<td>71±8</td>
<td>72±12</td>
<td>73±11</td>
</tr>
<tr>
<td>Height</td>
<td>173±9</td>
<td>173±9</td>
<td>172±9</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>24±1</td>
<td>24±1</td>
<td>25±1</td>
</tr>
<tr>
<td><strong>Baseline biochemistry</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HbA1C - % (mmol/mol)</td>
<td>N/A</td>
<td>8.8±0.4 (73±4)</td>
<td>8.8±0.5 (72±5)</td>
</tr>
<tr>
<td>Estrogen (pmol/L - in women)</td>
<td>198±130</td>
<td>220±40</td>
<td>204±135</td>
</tr>
<tr>
<td>Sodium excretion (mmol/24 hr)</td>
<td>168±20</td>
<td>173±20</td>
<td>180±32</td>
</tr>
<tr>
<td>Protein intake (gram/kg/day)</td>
<td>0.92±0.26</td>
<td>0.90±0.31</td>
<td>0.91±0.24</td>
</tr>
<tr>
<td><strong>Baseline circulating RAS mediators</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aldosterone (ng/dl)</td>
<td>11.6±9.5</td>
<td>1.73±0.68*</td>
<td>1.05±0.43†</td>
</tr>
<tr>
<td>ANG II (pg/ml)</td>
<td>10.2±6.9</td>
<td>3.1±2.2*</td>
<td>2.5±1.5†</td>
</tr>
<tr>
<td>Renin (pg/ml)</td>
<td>10.3±5.6</td>
<td>7.6±5.3</td>
<td>5.5±2.0†</td>
</tr>
<tr>
<td>Plasma renin activity (ng/mL/h)</td>
<td>1.83±1.0</td>
<td>1.05±0.67</td>
<td>0.71±0.36†</td>
</tr>
<tr>
<td>Angiotensinogen (ng/ml)</td>
<td>1195±94</td>
<td>1290±184</td>
<td>1593±366</td>
</tr>
</tbody>
</table>

*p<0.05 in normofiltering subjects vs. healthy controls
†p<0.05 in hyperfiltering subjects vs. healthy controls
Table 2: Hemodynamic Responses to a Graded Infusion of L-NMMA During Clamped Euglycemia in Healthy Controls and in Type 1 Diabetes Patients and Either Normofiltration or Hyperfiltration (mean±SD)

<table>
<thead>
<tr>
<th>Blood pressure</th>
<th>Healthy Control Subjects</th>
<th>Normofiltering Type 1 DM Subjects</th>
<th>Hyperfiltering Type 1 DM Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate (beats/minute)</td>
<td>61±7 56±8 55±6</td>
<td>63±11 57±10 57±9</td>
<td>68±10 62±6 59±5</td>
</tr>
<tr>
<td>SBP (mmHg)</td>
<td>110±10 112±10 119±12†</td>
<td>112±7 114±7 120±10†</td>
<td>112±10 117±13† 124±15†</td>
</tr>
<tr>
<td>DBP (mmHg)</td>
<td>64±8 67±8 74±9†</td>
<td>60±6 66±7† 73±8§</td>
<td>62±3 69±4† 77±5†</td>
</tr>
</tbody>
</table>

Renal hemodynamic function

| ERPF (ml/minute/1.73 m²) | 586±116 565±66 513±76† | 634±107 564±101† 483±80† | 809±112* 660±95†§ 539±80†§ |
| GFR (ml/minute/1.73 m²) | 120±16 123±16 121±14 | 113±12 113±14 114±15 | 152±16* 149±18 140±11†§ |
| FF | 0.21±0.05 0.22±0.02 0.24±0.02 | 0.18±0.03 0.20±0.03 0.23±0.03† | 0.19±0.04 0.22±0.03 0.26±0.04‡§ |
| RBF (ml/minute/1.73 m²) | 943±177 896±95 813±126† | 1006±174 891±171† 764±136† | 1281±182 1080±196‡§ 889±153‡§ |
| RVR (mmHg/L/minute) | 0.079±0.039 0.088±0.015 0.107±0.022† | 0.080±0.012 0.095±0.015† 0.110±0.031† | 0.061±0.016 0.079±0.020† 0.101±0.032† |

SBP = systolic blood pressure; DBP = diastolic blood pressure; ERPF = effective renal plasma flow = ml/minute/1.73 m²; GFR = glomerular filtration rate; RBF = renal blood flow; RVR = renal vascular resistance. *p<0.05 for between-group baseline differences for hyperfiltering subjects vs. normofiltering and healthy controls †p<0.05 vs. within group baseline value ‡p<0.05 for between-group change in response to L-NMMA vs. normofiltering patients §p<0.05 for between group change in response to L-NMMA vs. healthy control subjects
Table 3: Urine Prostanoid Responses to L-NMMA During Clamped Euglycemia in Healthy Controls and in Type 1 Diabetes Patients and Either Normofiltration or Hyperfiltration (mean±SD)

<table>
<thead>
<tr>
<th></th>
<th>Healthy Control Subjects</th>
<th>Normofiltering Type 1 DM Subjects</th>
<th>Hyperfiltering Type 1 DM Subjects</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Baseline L-NMMA 3 mg/min</td>
<td>Baseline L-NMMA 3 mg/min</td>
<td>Baseline L-NMMA 3 mg/min</td>
</tr>
<tr>
<td>Urine 2,3dinor6kPGF1α (pg/mg creatinine)</td>
<td>395±93</td>
<td>330±41</td>
<td>369±69</td>
</tr>
<tr>
<td>Urine PGF1α (pg/mg creatinine)</td>
<td>344±84</td>
<td>289±33‡</td>
<td>207±34</td>
</tr>
<tr>
<td>Urine TXB2 (pg/mg creatinine)</td>
<td>135±32</td>
<td>191±20†‡</td>
<td>95±18</td>
</tr>
<tr>
<td>Urine PGE2 (pg/mg creatinine)</td>
<td>285±70</td>
<td>219±20‡</td>
<td>151±21</td>
</tr>
<tr>
<td>Urine PGEM (pg/mg creatinine)</td>
<td>205±45</td>
<td>152±12</td>
<td>134±18</td>
</tr>
</tbody>
</table>

†p<0.05 for mean value in healthy control vs. DM-H
‡p<0.05 for mean value in healthy control vs. DM-N
§p<0.05 for response to L-NMMA in DM-H vs. healthy controls
Figure 1: Study outline

Pre-Study Preparation (controlled sodium and protein diet for 7 days), and collect 24-hour urine (protein, sodium, urea, creatinine)

↓

0800 hr: Admit to Renal Physiology Unit

↓

Euglycemia (4-6 mmol/L) for 6 hours

↓

1400 hr: Baseline flow-mediated vasodilatation

↓

1400 hr: Blood for baseline estradiol/progesterone (in women), angiotensinogen, plasma renin concentration, plasma renin activity, angiotensin I, angiotensin II, aldosterone NO and cGMP and urine for NO, cGMP and prostanoids

↓

1530 hr: Inulin and PAH infusion, clearance measures (MAP, GFR, ERPF, FF, RBF, RVR - 2 baseline measurements)

↓

1600 hr: L-NMMA infusion (1 mg/kg) for 30 minutes. Repeat measures of MAP, GFR, ERPF, FF, RBF, RVR and FMD.

↓

1630 hr: L-NMMA infusion (3 mg/kg) for 30 minutes. Repeat measures of MAP, GFR, ERPF, FF, RBF, RVR and FMD.

↓

End of Study
A.

![Graph showing Urine NO (μmol/mg creatinine) for Control, DM-N, and DM-H groups with Baseline and 3 mg/kg L-NMMA conditions.](image-url)
D.

![Bar chart showing plasma cGMP concentrations](image)

- **Baseline**
- **1 mg/kg L-NMMA**
- **3 mg/kg L-NMMA**

**X-axis:** Control, DM-N, DM-H

**Y-axis:** Plasma cGMP (pmol/L)