Increased Circulating Nitric Oxide in Young Patients With Type 1 Diabetes and Persistent Microalbuminuria

Relation to Glomerular Hyperfiltration

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Hyperglycemia has been causally linked to vascular and glomerular dysfunction by a variety of biochemical mechanisms, including a glucose-dependent abnormality in nitric oxide (NO) production and action. NO is a candidate for mediating hyperfiltration and the increased vascular permeability induced by diabetes. Serum nitrite and nitrate (NO_2^-+ NO_3^-) concentrations were assessed as an index of NO production in 30 adolescents and young adults with type 1 diabetes, 15 with and 15 without microalbuminuria (albumin excretion rate [AER] between 20 and 200 µg/min), compared with a well-balanced group of healthy control subjects. In all subjects, glomerular filtration rate (GFR) was determined by radionuclide imaging. Our study showed that NO_2^- + NO_3^- serum content and GFR values were significantly higher in microalbuminuric diabetic patients than in the other 2 groups. GFR was significantly and positively related to AER levels (r^2 = 0.75, P < 0.0001), whereas NO_2^- + NO_3^- serum content was independently associated with both AER and GFR values (β = 2.086, P = 0.05, β = 1.273, P = 0.0085, respectively), suggesting a strong link between circulating NO, glomerular hyperfiltration, and microalbuminuria in young type 1 diabetic patients with early nephropathy. Interestingly, mean HbA_{1c} serum concentration was significantly higher in microalbuminuric than in normoalbuminuric diabetic subjects (P < 0.05) and was independently associated with AER values, suggesting a role for chronic hyperglycemia in the genesis of diabetic nephropathy. Moreover, HbA_{1c} serum concentration was significantly and positively related to NO_2 + NO_3 serum content (r^2 = 0.45, P = 0.0063) and GFR values (r^2 = 0.57, P = 0.0011), suggesting that chronic hyperglycemia may act through a mechanism that involves increased NO generation and/or action. In conclusion, we suggest that in young type 1 diabetic patients with early nephropathy, chronic hyperglycemia is associated with an increased NO biosynthesis and action that contributes to generating glomerular hyperfiltration and persistent microalbuminuria. *Diabetes* 49:1258–1263, 2000

Early diabetic nephropathy in children and adolescents is caused predominantly by microangiopathy, representing functional and structural abnormalities in the microvascular system leading to microalbuminuria (1–3). Microvascular disease carries a substantive morbidity in young patients with type 1 diabetes (3,4). Long duration of diabetes and poor glycemic control have been shown to be the most important risk factors for the development of microvascular disease in these patients (5). A considerable body of evidence in humans indicates that microalbuminuria is strictly associated with a generalized endothelial vascular dysfunction (2,6). In this regard, a glucose-dependent abnormality in nitric oxide (NO) production and action has become an attractive hypothesis for the pathogenesis of early diabetic nephropathy (7–11). In fact, vasodilation due to increased NO generation or action has recently been implicated in the pathogenesis of glomerular hyperfiltration (7,8,12–14) and in the enhanced permeability to macromolecules that leads to microalbuminuria (12–14). In a rat model of streptozotocin-induced diabetes, the plasma and urinary excretion levels of stable products of NO oxidation were considerably higher than in normal rats, suggesting a generalized increase in NO synthesis throughout the body (14).

It is well known that NO decomposes very rapidly in biological solutions into nitrite (NO_2^-) and nitrate (NO_3^-) (15). These stable compounds can be analyzed in serum, and NO_2^- + NO_3^- levels are now considered an indicator of NO activity in vivo (16).

Aims of the present study were *1*) to evaluate serum NO_2^- + NO_3^- levels in diabetic adolescents with persistent microalbuminuria as compared with diabetic adolescents without microalbuminuria and healthy control subjects; *2*) to clarify whether serum NO_2^- + NO_3^- levels are influenced by persistent hyperglycemia in diabetic patients; and *3*) to assess

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AER, albumin excretion rate; CV, coefficient of variation; GFR, glomerular filtration rate; NO, nitric oxide; NO_2^-, nitrite; NO_3^-, nitrate; NOS, nitric oxide synthase; ^{99m}Tc-DTPA, ^{99m}technetium dimetiltriamino pentacetic acid.

whether NO plays a pivotal role in the development of glomerular hyperfiltration and persistent microalbuminuria in young patients with type 1 diabetes and early nephropathy.

RESEARCH DESIGN AND METHODS

Patient selection. The study was carried out in 30 young type 1 diabetic adolescents—15 with (group 1) and 15 without (group 2) microalbuminuria—and in 15 healthy control subjects (group 3). The 3 groups were matched for age, sex, BMI, blood pressure, and serum lipids. Moreover diabetic groups were also matched for duration of the disease and insulin requirements (Table 1). All subjects in the study were pubertal as defined by Tanner stage 4–5 (17). Clinical characteristics of the 3 groups are summarized in Table 1. Persistent microalbuminuria was defined as albumin excretion rate (AER) between 20 and 200 μg/min in 2 of 3 overnight urine collections performed over 6 months. None of the diabetic patients was affected by other complications, such as retinopathy (evaluated by stereoscopic fundus photography) or neuropathy (evaluated by nervous conduction velocity and autonomic tests), or was in treatment with other drugs except insulin. All patients included in the study were nonsmokers; none was taking antioxidant supplements or drugs with known antioxidant activity.

The institutional review committee approved the study. All patients included in this study gave informed written consent.

Laboratory procedures. Blood samples were collected in fasting condition and immediately centrifuged at 4°C. HbA_{1c} concentrations were measured every 3 months using a high-performance liquid chromatography method (Diamat analyzer; BioRad, Richmond, CA). The normal range was 4.2–6.0%, with an interassay coefficient of variation (CV) of 3%. All the patients had at least 3 determinations per year, and the mean of these determinations was used for statistical analysis. The total protein concentration in each serum sample was determined by the Biuret method (Boehringer Mannheim, Mannheim, Germany), and its CV was <2%. Urinary albumin concentration was measured by double antibody radioimmunoassay (Pharmacia, Uppsala, Sweden) with a sensitivity of 0.5 mg/l, an intra-assay CV of 4.5%, and an inter-assay CV of 5.3% in the range of 10–50 mg/l.

NO assay. NO is rapidly converted to NO_2^- and NO_3^- in typical oxygenated aqueous solutions such as human serum. Because an excellent colorimetric reagent (the Griess reagent) exists for the determination of NO_2^-, it is common practice to use enzymatic or chemical reduction to convert all NO_3^- to NO_2^- in samples and measure total NO_2^- as an indicator of overall NO production (18).

In this study, serum NO_2^-/NO_3^- levels were measured in triplicate by conversion of NO_3^- to NO_2^- by a commercially available kit based on the Griess reaction (OXIS International, Portland, OR) following the manufacturer's instructions. In particular, to exclude an effect of interfering substances (such as glycated hemoglobin or albumin) on the assay in samples with high protein content, we achieved a deproteinization of the samples with $ZnSO_4$ before the measurement. Guevara et al. (18) have shown that deproteinization does not influence the sensitivity of NO detection in biological fluids. This method has been previously validated (19) and successfully applied for determination of circulating NO levels in diabetic patients (20).

Briefly, each serum sample (10–50 μl) was adjusted to 190 μl with water, and then 10 μl of 30% (wt/vol) $ZnSO_4$ solution was added. The samples were then mixed by vortexing, incubated at room temperature for 15 min, and centrifuged (4,000 rpm) for 5 min. One hundred microliters of the resulting supernatants were then transferred to microcentrifuge tubes containing 0.5 g granulated cadmium each as chemical reducent and incubated at room temperature overnight with agitation. After that, the samples were recentrifuged (4,000 rpm) at room temperature and the supernatants were applied to a 96-well microtiter plate, followed by 100 μl of Griess reagent (1 g/l sulfanilamide, 25 g/l phosphoric acid, and 0.1 g/l *N*-1-naphthylethylenediamine dihydrochloride). After 10 min of color development at room temperature, the absorbance was measured on a microplate reader (Titertek Multiskan MCC/340; Flow Lab, McLean, VA) at a wavelength of 540 nm. Results are reported as $NO_2^- + NO_3^-$ and expressed as micromoles per liter (16).

Glomerular filtration rate assessment. Glomerular filtration rate (GFR) was assessed using radionuclide imaging studies with ^{99m}technetium dimetiltriamino pentacetic acid (^{99m}Tc-DTPA), as previously described (21). In brief, after breakfast (without tea, coffee, meat, or milk) and the usual morning insulin dose, the patient lay on the scanning couch, with the γ-camera placed posteriorly and interfaced to a digital computer system; a bolus injection of ^{99m}Tc-DTPA (100 μCi/kg) was given intravenously. The computer data acquisition was started soon after the completion of the radionuclide injection, and the total acquisition period was 20 min. The values for individual renal uptake were calculated from the normalized time/activity curve for each kidney within 2–3 min postinjection. The pre- and postinjection static 1-min syringe counts were determined, and the net administered count was calculated by subtraction. The percentage of total kidney uptake of DTPA was calculated using depth correction to compensate for γ-ray attenuation by the soft tissues interposed between the kidney center and the γ-camera. The percentage total uptake ranged from 2 to 12%. Tonnesen's formula was used to determine the renal depth in centimeters for normally positioned kidneys (22). The corrected renal counts were then determined, and the percentage renal uptake was calculated (23). Finally, to correct the GFR for body surface area, the following linear regression equation was used: GFR ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$) = 11.6065 –5.2975 (total renal uptake, 11.6065; regression coefficient, –5.2975 intercept).

Statistical analysis. Data are reported as means ± SD. Differences between the 3 groups were analyzed by 1-way analysis of variance followed by the Scheffe's test for multiple comparison between groups. As a consequence of this comparison, further analyses were performed for each group separately. Simple linear regression was used for a preliminary testing of the association between the variables of interest (HbA_{1c}, GFR, AER, $NO_2^- + NO_3^-$). Multiple regression analysis was used to confirm significant associations at the univariate analysis after controlling for potential confounders. The statistical significance was defined as a P value <0.05. Statistical analyses were performed using the STATVIEWS 4.5 software (Abacus Concepts, Berkeley, CA) for Macintosh Performa 5300 computer (Cupertino, CA).

TABLE 1
Clinical characteristics of type 1 diabetic adolescents and young adults with (group 1) and without (group 2) microalbuminuria and of healthy control subjects (group 3)

	Group 1	Group 2	Group 3
n	15	15	15
Age (years)	18.6 ± 4.1	18.5 ± 3.9	18.4 ± 4.0
Sex (F/M)	7/8	8/7	7/8
BMI	24.2 ± 3.9	24.7 ± 4.7	23.8 ± 3.9
Diabetes duration (years)	12.5 ± 2.5	11.2 ± 3.0	—
HbA_{1c} (%)	9.6 ± 1.4*†	7.3 ± 0.93*	5.4 ± 0.6
Insulin requirement ($U \cdot kg^{-1} \cdot day^{-1}$)	1.2 ± 0.3	1.0 ± 0.4	—
Systolic blood pressure (mmHg)	116 ± 13.5	114 ± 13.7	111 ± 12.2
Diastolic blood pressure (mmHg)	68 ± 6.4	68 ± 8.6	65 ± 7.9
Glycemia (mmol/l)	10.2 ± 2.4*†	7.7 ± 2.5*	5.1 ± 0.8
AER (μg/min)	84.2 ± 34.4†	10.4 ± 0.4	—
GFR ($ml \cdot min^{-1} \cdot 1.73\ m^{-2}$)	173 ± 14.0*†	126 ± 6.4	114 ± 7.4
Cholesterol (mmol/l)	4.2 ± 1.2	4.0 ± 1.3	3.9 ± 1.1
HDL cholesterol (mmol/l)	1.3 ± 0.4	1.1 ± 0.5	1.0 ± 0.4
Triglycerides (mmol/l)	1.3 ± 0.4	1.2 ± 0.3	1.1 ± 0.4

Data are *n* or means ± SD. *P < 0.05 vs. group 3; †P < 0.05, group 1 vs. group 2.

RESULTS

Clinical characteristics. The 3 groups did not significantly differ in blood pressure, serum lipids and lipoproteins, insulin requirements, or duration of the disease (Table 1). Mean HbA_{1c} values were significantly higher in microalbuminuric than in normoalbuminuric diabetic subjects (9.6 ± 1.4 vs. 7.3 ± 0.93%, $P < 0.05$) (Table 1). Fasting glucose levels were also significantly higher in the microalbuminuric group than in the normoalbuminuric one (10.2 ± 2.4 vs. 7.7 ± 2.5 mmol/l, $P < 0.05$).

Serum NO level. Serum $NO_2^- + NO_3^-$ concentrations were higher in group 2 than in group 3, but the difference was not statistically significant (32.2 ± 6.1 vs. 25.4 ± 4.2 µmol/l, NS) (Fig. 1). The microalbuminuric group had significantly higher $NO_2^- + NO_3^-$ serum concentrations than did the other 2 groups (group 1 vs. group 2, 46.7 ± 7.9 vs. 32.2 ± 6.1 µmol/l, $P < 0.05$; group 1 vs. group 3, 46.7 ± 7.9 vs. 25.4 ± 4.2 µmol/l, $P < 0.05$) (Fig. 1).

GFR values. GFR values (Fig. 2) were significantly higher in microalbuminuric than in normoalbuminuric diabetic patients (173 ± 14.0 vs. 126 ± 6.4 ml · min^{-1} · 1.73 min^{-2}, $P < 0.0001$). No significant difference was found between normoalbuminuric diabetic subjects and healthy control subjects (Fig. 2).

Associations. The significant increase in $NO_2^- + NO_3^-$ serum content among microalbuminuric diabetic subjects compared with the other 2 groups strongly suggested that high levels of NO_3^- might play a role in increasing albumin excretion. To provide confirmatory evidence for this hypothesis, further analyses were performed for each of the 3 groups separately.

Group 1. In the univariate analysis, HbA_{1c} was significantly associated with $NO_2^- + NO_3^-$ serum content ($r^2 = 0.45$, $P = 0.0063$) (Fig. 3). Moreover, HbA_{1c} concentration was also positively related to GFR and AER ($r^2 = 0.57$, $P = 0.0011$ and $r^2 = 0.74$, $P < 0.0001$, respectively). In the same group, serum $NO_2^- + NO_3^-$ content and GFR were significantly and positively associated with AER ($r^2 = 0.74$, $P < 0.0001$ and $r^2 = 0.75$, $P < 0.0001$, respectively) (Fig. 4). The $NO_2^- + NO_3^-$ serum content was also significantly and positively related to GFR ($r^2 = 0.69$, $P < 0.0001$) in microalbuminuric patients (Fig. 4).

No significant correlation between the duration of diabetes and the studied parameters was found in either diabetic group. However, in microalbuminuric patients, diabetes duration was inversely but not significantly associated with serum $NO_2^- + NO_3^-$ content ($r^2 = 0.28$, $P = 0.08$) and GFR ($r^2 = 0.21$, $P = 0.09$).

When the multivariate analysis was performed, HbA_{1c} and $NO_2^- + NO_3^-$ serum content were both independently associated with AER in microalbuminuric diabetic patients ($\beta = 10.428$, $P = 0.02$; and $\beta = 2.086$, $P = 0.05$, respectively), whereas only serum $NO_2^- + NO_3^-$ content was independently associated with GFR values ($\beta = 1.273$, $P = 0.0085$).

Group 2. In the univariate analysis, HbA_{1c} was significantly associated with $NO_2^- + NO_3^-$ serum content ($r^2 = 0.36$, $P = 0.0190$). Moreover, $NO_2^- + NO_3^-$ was also positively and significantly correlated with GFR ($r^2 = 0.52$, $P = 0.0024$). No other significant association was found.

When the multivariate analysis was performed, only the serum $NO_2^- + NO_3^-$ content was independently associated with GFR ($\beta = 0.822$, $P < 0.02$).

Group 3. In the univariate analysis, no significant correlation was found between the variables of interest (HbA_{1c}, $NO_2^- + NO_3^-$, GFR, AER).

DISCUSSION

NO may increase both blood flow and vascular permeability and thus is a candidate for mediating the vascular changes that have been described in diabetes with early nephropathy (24).

Our study supports the hypothesis that glomerular hyperfiltration due to high circulating NO level may be operative in young patients with type 1 diabetes and persistent microalbuminuria. In fact, we show that $NO_2^- + NO_3^-$ serum content, as well as GFR, was significantly higher in microalbuminuric than in normoalbuminuric diabetic subjects. Moreover, in the multivariate analysis, $NO_2^- + NO_3^-$ serum content was positively and independently associated with GFR values, suggesting that NO has a crucial role in determining the hyper-

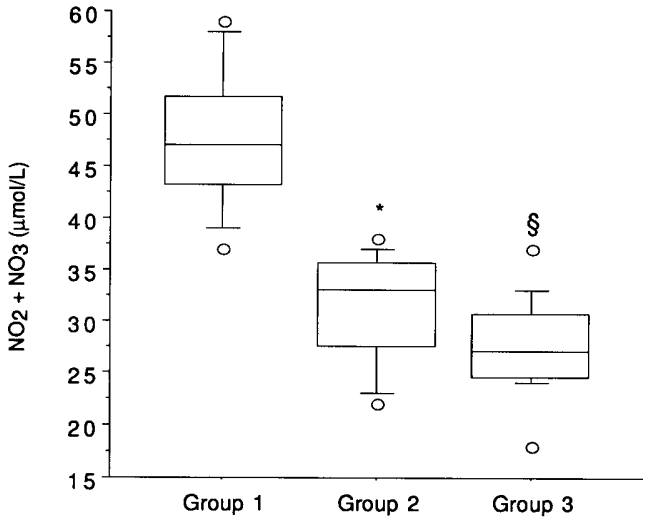

FIG. 1. Serum $NO_2^- + NO_3^-$ concentrations in 15 microalbuminuric (group 1) and 15 normoalbuminuric (group 2) diabetic subjects and in 15 healthy controls (group 3). Data are means ± SD. *$P < 0.05$ for group 2 vs. group 1; §$P < 0.05$ for group 3 vs. group 1.

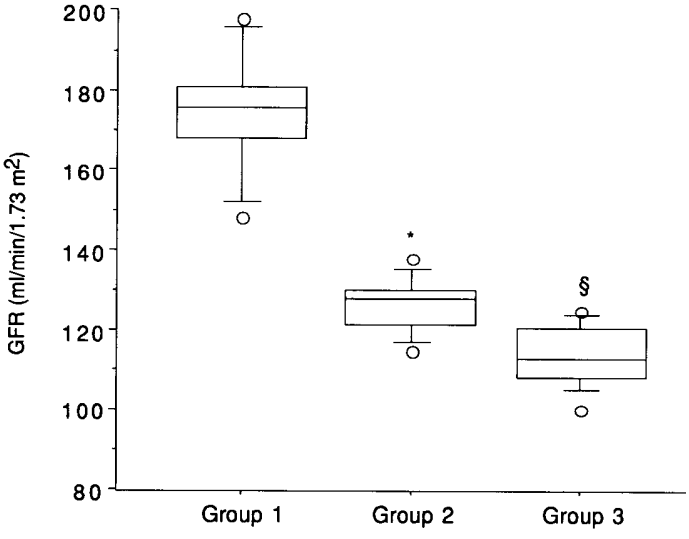

FIG. 2. GFR values in 15 microalbuminuric (group 1) and 15 normoalbuminuric (group 2) diabetic subjects and in 15 healthy control subjects (group 3). Data are means ± SD. *$P < 0.05$ for group 2 vs. group 1; §$P < 0.05$ for group 3 vs. group 1.

filtration found in microalbuminuric patients. In light of this, it has recently been reported that NO is important in the regulation of renal hemodynamics and, in particular, in maintaining the normal state of vascular tone (12,25). In agreement with our data, urinary excretion and plasma $NO_2^- + NO_3^-$ content have been found to be enhanced in diabetic animals (14,25,26). In this regard, an increased renal expression of endothelial cell NO synthase (NOS) and inducible NOS proteins has been found after 1 week in streptozotocin-induced diabetic rats (27). This increased expression could be responsible, at least in part, for the attenuated tubuloglomerular feedback and the glomerular hyperfiltration observed in early diabetes. Furthermore, recent data have shown enhanced NO synthesis by endothelial cell NOS in afferent arterioles and glomerular endothelial cells in streptozotocin-induced diabetic rats, suggesting a pivotal role of NO in preferential afferent arteriolar dilatation, glomerular enlargement, and functional glomerular hyperfiltration in the early stages of diabetic nephropathy (28). Recently, treatment with inhibitors of constitutive NO synthesis has been shown to attenuate renal

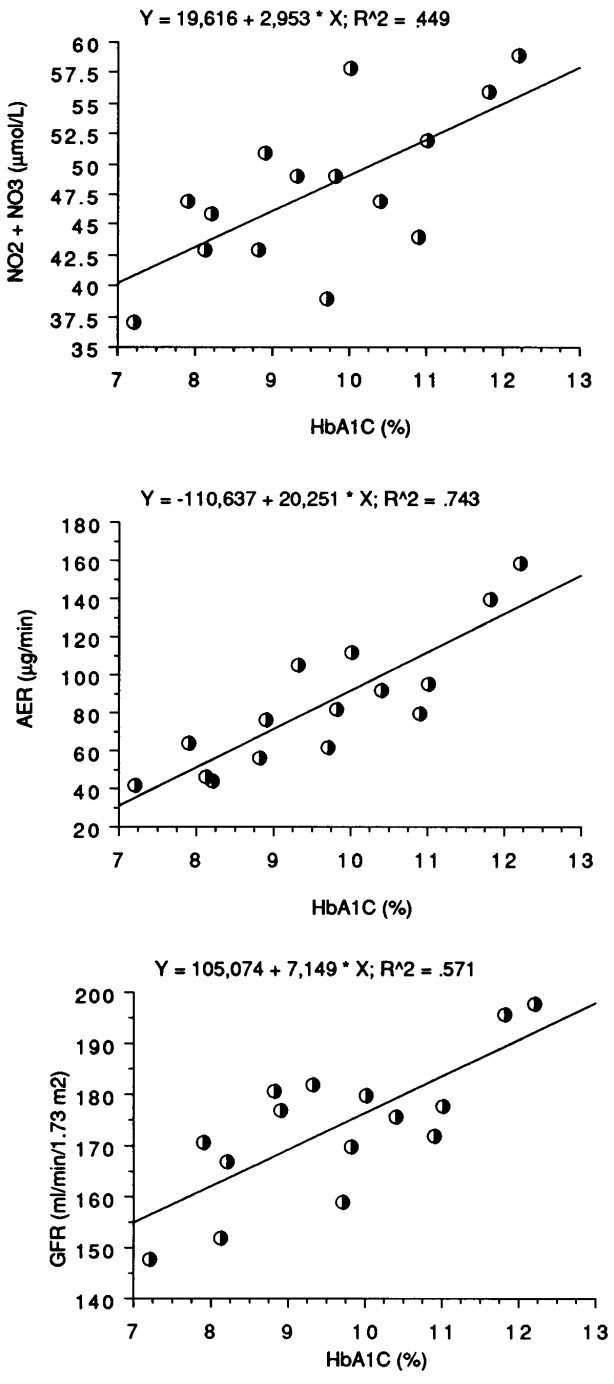

FIG. 3. Relations between mean value of HbA_{1c} and serum $NO_2^- + NO_3^-$ concentration, GFR, and AER in 15 young microalbuminuric patients with type 1 diabetes.

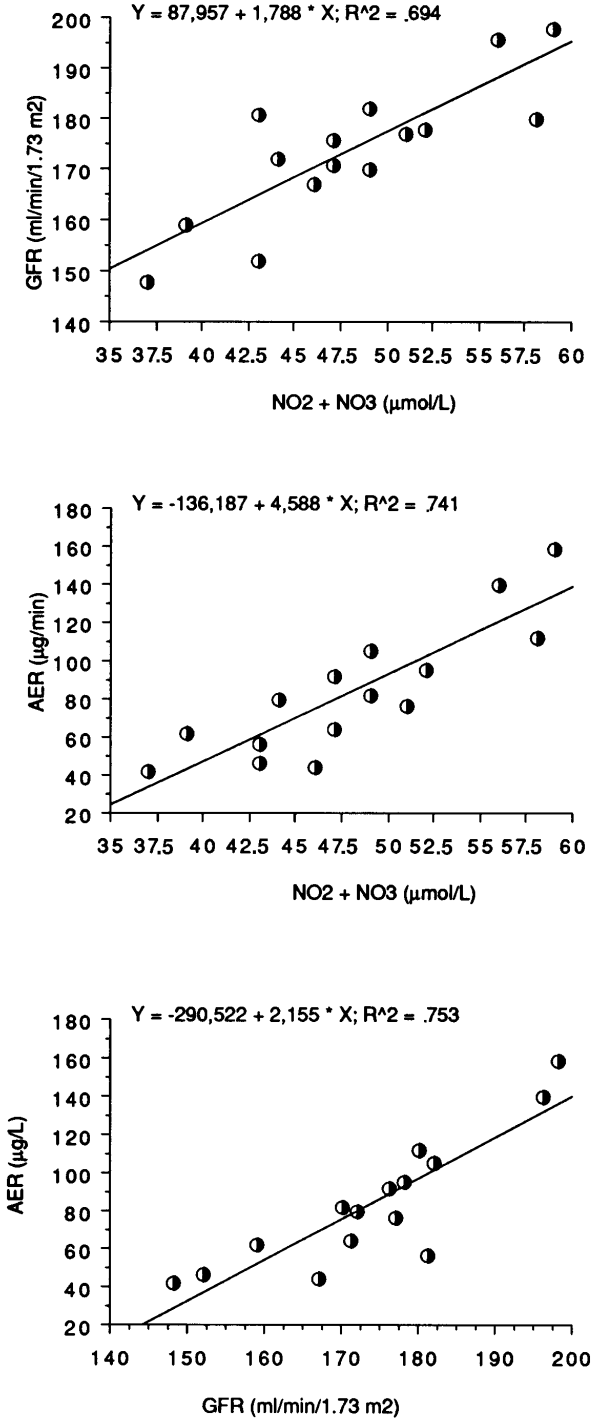

FIG. 4. Relations between serum $NO_2^- + NO_3^-$ concentration, GFR, and AER in 15 young microalbuminuric patients with type 1 diabetes.

hyperfiltration and block elevation in regional albumin permeation in diabetic rats (7,29–31), and 8-week treatment with an experimental NO scavenger seems to prevent diabetes-induced endothelial dysfunction (32).

The significant positive association between GFR and AER further suggests that glomerular hyperfiltration may have contributed to the increased albumin excretion found in microalbuminuric diabetic patients with early nephropathy in this study. Accordingly, it has recently been shown that persistent glomerular hyperfiltration is a risk factor for the development of microalbuminuria and incipient nephropathy in type 1 diabetic adolescents and young adults (33–35). Thus, NO overproduction caused by altered renal hemodynamics could increase renal perfusion and glomerular filtration, favoring the development of microalbuminuria.

In our opinion, the observed significant increase in circulating NO may result from a generalized synthesis throughout the body, confirming that microalbuminuria is associated with a diffuse endothelial dysfunction. Accordingly, it has recently been observed that endothelium regulation of coronary flow is progressively impaired in diabetic rats in the presence of increased expression of constitutive NOS protein, mRNA, and enzyme activity (36).

Reportedly, the development of diabetic nephropathy closely correlates with the duration and the magnitude of the antecedent hyperglycemia (5). A recent study showed that poor glycemic control was directly related to hyperfiltration and renal hyperperfusion (37). Accordingly, our microalbuminuric patients showed higher serum levels of mean HbA_{1c} and GFR than did normoalbuminuric diabetic subjects, and in the univariate analysis, serum HbA_{1c} concentrations were positively associated with GFR and AER. Furthermore, serum $NO_2^- + NO_3^-$ content was significantly associated with HbA_{1c}, GFR, and AER in microalbuminuric patients and with HbA_{1c} and GFR in normoalbuminuric ones, thus suggesting a relevant relationship between glycemic control and NO biosynthesis, glomerular hyperfiltration, and persistent microalbuminuria.

On the contrary, the duration of the disease seems not to affect the studied variables in our patients. Nonetheless, an interesting but not statistically significant inverse relationship between duration of the disease and NO serum levels or GFR values was observed. This fact could indicate that the level of NO as well as renal vascular function is a time-course phenomenon in patients with diabetes and early nephropathy.

Hyperglycemia has been causally linked to vascular and glomerular dysfunction by a variety of biochemical mechanisms (38). Recently, a glucose-dependent increase in NO production and action has become an attractive hypothesis for the pathogenesis of early diabetic nephropathy (7–10,27). The significant correlation between HbA_{1c} and serum $NO_2^- + NO_3^-$ content found in the present study confirms that poor glycemic control may directly influence NO synthesis. This correlation was observed in both normoalbuminuric and microalbuminuric adolescents, suggesting that NO production is increased early in the natural course of diabetes and independently of the presence of microvascular complications. However, the strong correlation between serum $NO_2^- + NO_3^-$ content and GFR and AER observed only in microalbuminuric patients supports the hypothesis that long-term poor metabolic control leads to glomerular hyperfiltration and persistent microalbuminuria, possibly by NO-mediated changes in glomerular vascular tone.

In addition, it has recently been demonstrated that the prolonged exposure of endothelial cells to high glucose increases both NO and superoxide anion production (8–10). It is well known that expression of NO action largely depends on its relative levels and on its interaction with superoxide anions (39). Thus, we hypothesize that in our patients with early nephropathy, NO production overcomes NO degradation by superoxide anions and this, in turn, favors renal vasodilatation. However, it has recently been shown that when peroxynitrite—a potent cytotoxic species generated by the interaction of NO with superoxide—is present in small amounts, it is able to induce vasodilation via thiol-dependent formation of NO (40).

In conclusion, we suggest that in type 1 diabetic adolescents and young adults with early nephropathy, chronic hyperglycemia is associated with increased NO biosynthesis and action that may crucially contribute to glomerular hyperfiltration and persistent microalbuminuria.

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