

# L-Arginine–Nitric Oxide Kinetics in Normal and Type 2 Diabetic Subjects

## A Stable-Labelled $^{15}\text{N}$ Arginine Approach

Angelo Avogaro,<sup>1</sup> Gianna Toffolo,<sup>2</sup> Edward Kiwanuka,<sup>1</sup> Saula Vigili de Kreutzenberg,<sup>1</sup> Paolo Tessari,<sup>1</sup> and Claudio Cobelli<sup>2</sup>

Defective endothelium is a key event in the development of atherosclerosis in diabetes: alteration of the L-arginine–nitric oxide (NO) pathway has been suggested. We propose a modeling approach of the L-arginine–NO pathway in vivo in both control and type 2 diabetic subjects based on the intravenous bolus injection of L- $^{15}\text{N}$ arginine and subsequent noncompartmental and compartmental model analysis of L- $^{15}\text{N}$ arginine in plasma and  $^{15}\text{N}$ nitrate in the urine. No differences in arginine kinetics were observed between normal subjects and diabetic patients.  $^{15}\text{N}$ nitrate were detectable up to 48 h from the L- $^{15}\text{N}$ arginine administration; no differences were found in the tracer-to-tracee ratio in each urine collection. However, the NO synthesis in plasma from arginine was lower ( $P = 0.05$  for the noncompartmental and 0.1208 for the compartmental analysis, by Mann-Whitney test) in diabetic patients than in control subjects when expressed both in absolute terms (50% decrease) and as percentage of NO turnover (30% decrease). This new modeling approach of L-arginine–NO pathway provides a detailed picture of arginine kinetics and nitrate metabolism. From our data, it appears that noncomplicated type 2 diabetic patients have a decreased conversion of arginine to NO. *Diabetes* 52:795–802, 2003

**E**ndothelial dysfunction is a possible culprit of the accelerated atherosclerosis in diabetic patients. Several different mechanisms negatively act on endothelial function (1). The assessment of endothelial function in humans can be achieved by the measurement of 1) morphological and mechanical characteristics of the vascular wall, 2) circulating levels of soluble markers, and 3) the endothelium-dependent regulation of vascular tone (2). Most of these approaches provide indirect evidence of an impaired nitric oxide (NO)

production, and none is specific for the measurement of NO synthesis.

Castillo and colleagues (3,4) proposed a method to determine the rate of conversion of arginine to citrulline and NO in vivo; they assessed the formation of NO from arginine.

Forte et al. and Katz et al. (5–8) assessed NO production by measuring the 24-h urinary nitrate and  $^{15}\text{N}$ nitrate excretions, after the administration of L- $^{15}\text{N}$ arginine. However, they were only able to provide a percentage of labeled nitrate excretion derived from labeled L-arginine without making any physiological inference on the L-arginine–NO pathway in vivo in humans.

We now propose a modeling approach of the L-arginine–NO pathway in vivo in both control and type 2 diabetic subjects based on the intravenous bolus injection of L- $^{15}\text{N}$ arginine and subsequent analysis of L- $^{15}\text{N}$ arginine in plasma and  $^{15}\text{N}$ nitrate in the urine.

### RESEARCH DESIGN AND METHODS

**Patients.** Six men with type 2 diabetes with no clinical or laboratory evidence of atherosclerotic cardiovascular disease were studied (Table 1). Current treatments included diet ( $n = 4$ ) and diet plus sulfonylureas ( $n = 2$ ). Antidiabetic treatment was stopped 7 days before the study. Informed consent was obtained after the study was approved by the Research Ethics Committee of the University of Padova. Healthy volunteers were recruited from local staff in the hospital and university. All subjects underwent a full health screen. There was no evidence of autonomic neuropathy according to bedside cardiovascular reflexes. Subjects with a urinary albumin excretion rate  $>20$   $\mu\text{g}/\text{min}$  were excluded. None had diabetic retinopathy. To rule out preexisting known risk factors, only subjects with no history of hypertension, dyslipidemia, cerebrovascular disease, ischemic heart disease, or peripheral artery disease were recruited. In each diabetic patient, regional atherosclerotic manifestations were excluded by Doppler ultrasound of the carotid arteries and of the femoropopliteal axis arteries. Coronary artery disease was also excluded on the basis of stress dipiridamole echocardiography and by the World Health Organization questionnaire.

All participants received a limited-nitrate diet of  $\sim 9,211$  kJ/day containing 15% of proteins for 24 h before and 48 h after the administration of labeled  $^{15}\text{N}$ -arginine. They were asked to avoid all vegetables, salads, tomato-based products such as spaghetti/pizza sauce, potatoes, cured meats, seafood, and cheese. The estimated daily dietary arginine intake with this diet was  $\sim 5$  g/day (28.7 mmol daily).

**Experimental protocol.** All subjects were studied between 0800 and 0830 in fasting state. They were first asked to void urine present in their bladder and then to lie supine on a couch. An 18-gauge butterfly needle was inserted into an antecubital vein. L- $^{15}\text{N}_2$ -arginine (Mass Trace, Woburn, MA), 300 mg dissolved in 20 ml of 0.9% saline, was then infused over 2 min. Blood samples were collected before the L- $^{15}\text{N}$ arginine bolus injection for glucose,  $\text{HbA}_{1c}$ , insulin, C-peptide, L-arginine, and nitrate concentrations. Baseline urine samples were taken. After the L- $^{15}\text{N}$ arginine bolus injection, blood samples were obtained at 2, 3, 4, 5, 6, 8, 10, 12, 15, 20, 25, 30, 35, 40, 60, 80, and 100 min for the determination of L- $^{15}\text{N}$ arginine. After completion of the infusion,

From the <sup>1</sup>Department of Metabolic Disease, University of Padova, Padova, Italy; and the <sup>2</sup>Department of Information Engineering, University of Padova, Padova, Italy.

Address correspondence and reprint requests to Angelo Avogaro, MD, Cattedra di Malattie del Metabolismo, Via Giustiniani 2, 35128 Padova, Italy. E-mail: angelo.avogaro@unipd.it.

Received for publication 18 August 2002 and accepted in revised form 2 December 2002

Additional information for this article can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

FSR, fractional synthetic rate; IRMS, isotope ratio mass-spectrometry.

TABLE 1  
Clinical characteristics of the study subjects

	Type 2 diabetic patients	Control subjects
<i>n</i>	6	5
Age (years)	41 ± 6	38 ± 8
Duration of diabetes (years)	9 ± 6	
BMI (kg/m <sup>2</sup> )	27 ± 4	25 ± 5
Systolic blood pressure (mmHg)	120 ± 12	118 ± 11
Diastolic blood pressure (mmHg)	73 ± 8	70 ± 7
Waist circumference (cm)	91 ± 5	87 ± 5
Fasting plasma glucose (mg/dl)	176 ± 42	84 ± 16*
Plasma cholesterol (mg/dl)	195 ± 26	185 ± 22
Plasma HDL cholesterol (mg/dl)	52 ± 11	54 ± 14
Plasma triglycerides (mg/dl)	139 ± 42	128 ± 42
HbA <sub>1c</sub> (%)	8.0 ± 1.8	5.1 ± 0.8*

Data are means ± SE. \*Statistically significant difference vs. type 2 diabetic patients ( $P < 0.001$ ).

blood pressure was determined with a sphygmomanometer in the nondominant arm in the sitting position. Instructions were given to collect all urine for the following 48 h. At home, patients completed urine collections in pre-washed 2 l polypropylene bottles containing 5 ml of 5 mol/l sodium hydroxide to prevent reduction of nitrate for the periods 0–6, 6–12, 12–24, and 24–48 h after dosing. The urine volume was measured and a sample from each collection stored at  $-80^{\circ}\text{C}$  until analysis for nitrate. Blood samples were stored at  $-80^{\circ}\text{C}$  until analysis.

**Analytical measurements.** Plasma glucose was measured with a glucose oxidase method (Glucose II analyzer; Beckman, Brea, CA), whereas HbA<sub>1c</sub> was determined by a chromatographic technique (normal range 4–6.5%). Insulin and C-peptide were measured by conventional radioimmunoassay.

Labeled nitrates were assayed according to the method of Green et al. (9). Then, 500  $\mu\text{l}$  plasma, 1 ml benzene, and 1 ml  $\text{H}_2\text{SO}_4$  were added. Samples were vortexed for 15 min, centrifuged, and the organic phase transferred and filtered through Silica Sep-Pack Cartridges (Water Associates, Milan, Italy). The eluates were then dried under nitrogen stream until a final amount of 100  $\mu\text{l}$  was obtained. This allows the analysis of [<sup>15</sup>N]-to-[<sup>14</sup>N]nitrobenzene. The residue was injected onto a gas chromatograph isotope ratio mass spectrometry (IRMS) (Delta Plus; Finnigan, San José, CA). The gas chromatographic separation was on a Hewlett Packard 5MS capillary column (length 30 m, internal diameter 0.25 mm) using a carrier flow of helium of 25 ml/min and an oven temperature program from 60 to 120 $^{\circ}$  at 16 $^{\circ}$ /min. Retention time was 4.8 min. [<sup>15</sup>N] and [<sup>14</sup>N]nitrobenzene were eluted and converted to nitrogen gas by combustion at 1,000 $^{\circ}\text{C}$  and analyzed by continuous-flow gas IRMS. The precision of the <sup>15</sup>N-to-<sup>14</sup>N ratio measurement was  $\pm 0.0003\%$ . A calibration curve was prepared by spiking plasma containing a known amount of <sup>14</sup>N nitrates with various amounts of <sup>15</sup>N nitrate. Unlabelled nitrates were assayed as previously described using a chromatographic system (10). All determinations were done in duplicate. The concentrations were obtained using a calibration curve with increasing amount of nitrates.

L-<sup>15</sup>N]arginine in plasma has been determined according to the method of Castillo et al. (11). The spectrometric analysis was accomplished in chemical ionization mode at 314, 315, 316, 317, 318, and 319 m/z. Total arginine content was assayed with high-performance liquid chromatography.

**Data analysis.** The tracer-to-tracee ratios of plasma arginine ( $ttr_1$ ) and urinary nitrate ( $ttr_3$ ) were evaluated from ion current ratios (13). From  $ttr_3$ , urinary nitrate concentration  $C_3$  (tracer + tracee) and urinary volume  $V_3$  (in milliliters), the amount of tracer ( $q_3$ , in micromoles per liter) and tracee ( $Q_3$ , in micromoles per liter) in the urine collected at different sampling times were evaluated using the following equations.

$$q_3 = \frac{V_3 \cdot C_3 \cdot ttr_3}{1 + ttr_3} \quad Q_3 = \frac{V_3 \cdot C_3}{1 + ttr_3} \quad (1)$$

**Arginine kinetics.** A noncompartmental analysis was applied to quantify arginine kinetics (13). Briefly, a multiexponential model was fitted to plasma arginine  $ttr$ , as follows:

$$ttr_1(t) = \sum_{i=1}^N A_i e^{-\alpha_i t} \quad (2)$$

We estimated arginine mass in the accessible compartment  $Q_1$ , distribution volume of the accessible arginine compartment  $V_1$ , rate of arginine appear-

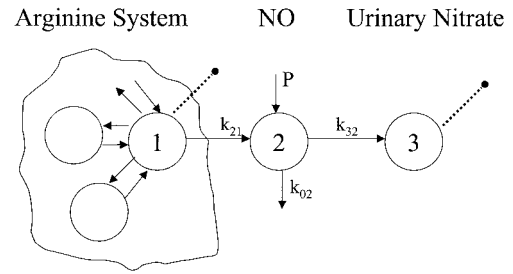


FIG. 1. Model of arginine-NO system. Compartment 1 denotes the accessible arginine pool, compartment 2 the plasma NO pool, and compartment 3 the urinary nitrate pool. P is the rate of NO appearance in plasma from sources other than arginine, and  $k_{21}$ ,  $k_{32}$ , and  $k_{02}$  are rate constants. The dashed line with bullet denotes measurements.

ance in the accessible compartment, and arginine plasma clearance rate according to conventional tracer kinetic method (13).

**Arginine-NO system.** A schematic map of the arginine-NO system is shown in Fig. 1, including the accessible arginine pool (compartment 1), the plasma NO pool (compartment 2), and the accessible urinary nitrate pool (compartment 3). Plasma NO derives either from plasma arginine or from other sources, e.g., diet, and is lost either by excretion in the urine or via alternate pathways (14).

**The conventional formula.** The flux of NO synthesis from arginine,  $k_{21}Q_1$ , was first quantified by adapting the formula (originally proposed for a constant tracer arginine infusion [3]) to the arginine tracer experiment. According to this formula,  $k_{21}Q_1$  can be estimated by multiplying the total amount of tracer NO measured in the urine collected up to the end of the experiment,  $q_3(T)$ , normalized to arginine tracer dose by arginine rate of appearance, as follows:

$$k_{21}Q_1 = \frac{q_3(T)}{0.6 \cdot D} \cdot Ra = \frac{1.67q_3(T)}{D} \cdot Ra \quad (3)$$

where the factor 1.67 accounts for the incomplete urinary recovery due to alternate NO losses.

In the Appendix, equation 3 has been derived based on a noncompartmental analysis of the arginine-NO system. This analysis highlights the assumptions underlying the formula and provides formulas to quantify: urinary NO excretion  $k_{32}Q_2$  (in micromoles per hour), NO appearance rate in plasma from sources other than arginine P (in micromoles per hour), total NO appearance rate in plasma  $k_{21}Q_1 + P$  (in micromoles per hour), and NO synthesis from arginine normalized to total NO appearance rate  $k_{21}Q_1\%$ . The quantification of  $k_{21}Q_1$  and P requires a priori knowledge of the percentage of urinary loss, whereas that of  $k_{32}Q_2$  and  $k_{21}Q_1\%$  does not.

**Compartmental modeling approach.** The above formula to derive  $k_{21}Q_1$  assumes that the underlying structure (Fig. 1) is valid, without testing it on the data. A more robust approach to estimate model parameters is to fit the compartmental model of Fig. 1 on all available experimental data.

Tracer model equations are as follows:

$$\begin{aligned} \dot{q}_2(t) &= k_{21}q_1(t) - (k_{32} + k_{02})q_2(t) \\ \dot{q}_3(t) &= k_{32}q_2(t) \\ y_1(t) &= q_3(t) \end{aligned} \quad (4)$$

where overdot denotes derivative with respect to time;  $q_1(t)$ , the amount of arginine tracer in compartment 1, is the known input;  $q_1(t) = ttr_1(t)Q_1$ ,  $y_1$  is the measured amount of tracer in the urine; and  $k_{21}$ ,  $k_{32}$ , and  $k_{02}$  are unknown model parameters.

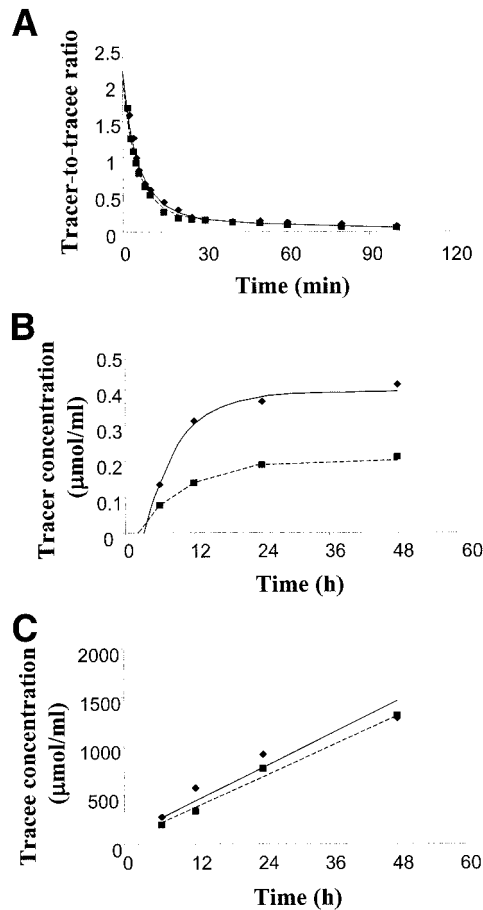
Tracee model equations are as follows:

$$\begin{aligned} \dot{Q}_2(t) &= 0 = k_{21}Q_1 - (k_{32} + k_{02})Q_2 + P \\ \dot{Q}_3(t) &= k_{32}Q_2 \\ y_2(t) &= Q_3(t) \end{aligned} \quad (5)$$

where  $y_2$  is the measured amount of tracer in the urine.

The tracer-tracee model is a priori nonidentifiable (13). Only some parameter combinations are uniquely identifiable, namely  $k_{32}k_{21}$ ,  $k_{32} + k_{02}$  (from tracer model), and  $k_{32}Q_2$  (from tracee model). From these combinations and knowledge of  $Q_1$ , the percentage NO synthesis from arginine  $k_{21}Q_1\%$ , the nitrate residence time  $\tau$  (h) in plasma and the fractional synthetic rate (FSR) (in hours<sup>-1</sup>) of arginine to NO can be quantified, as follows:

$$k_{21}Q_1(\%) = \frac{k_{21}Q_1}{k_{21}Q_1 + P} = \frac{k_{21}Q_1}{(k_{32} + k_{02})Q_2} = \frac{k_{32}k_{21}}{(k_{32} + k_{02})k_{32}Q_2} Q_1 \quad (6)$$



**FIG. 2.** *A:* Average plasma arginine tracer-to-tracee ratio data versus two-exponential model prediction in normal subjects (◆ vs. —) and diabetic subjects (■ vs. —). *B:* Average urinary tracer data versus compartmental model prediction in normal subjects (◆ vs. —) and diabetic subjects (◆ vs. —). *C:* Average urinary tracee data versus compartmental model prediction in normal subjects (◆ vs. —) and diabetic subjects (◆ vs. —).

$$\tau = \frac{1}{k_{32} + k_{02}} \quad (7)$$

$$FSR = \frac{k_{21}Q_1}{Q_2} = \frac{k_{32}k_{21}}{k_{32}Q_2} Q_1 \quad (8)$$

Identification of other model parameters requires the use of a priori knowledge, i.e., to assume that NO urinary excretion accounts for 60% of total NO loss. Under this assumption,  $k_{21}$ ,  $k_{32}$ ,  $k_{02}$ , and  $Q_2$  are uniquely identifiable. Then,  $P$  can be calculated from equation 5.

**TABLE 2**  
The two-exponential model and noncompartmental parameters of arginine kinetics

	$A_1$ (%)	$A_2$ (%)	$\alpha_1$ ( $\text{min}^{-1}$ )	$\alpha_2$ ( $\text{min}^{-1}$ )	$Q_1$ ( $\mu\text{mol}/\text{kg}$ )	$V_1$ ( $\text{l}/\text{kg}\%$ )	$R_a$ ( $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ )	PCR ( $\text{l} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ )
Control subjects								
Mean	91 (10)*	9 (31)	0.194 (13)	0.012 (37)	10.9	17.2	54.4	0.87
SE	2	2	0.045	0.001	0.6	1.4	10.7	0.19
Min-max	86-99	1-14	0.110-0.340	0.008-0.017	8.9-12.8	12.8-20.3	26.8-83.8	0.40-1.33
Type 2 diabetic patients								
Mean	89 (13)	11 (21)	0.205 (17)	0.012 (64)	11	14.6	46.3	0.62
SE	2	2	0.029	0.003	2	2.5	7.7	0.10
Min-Max	81-95	5-19	0.110-0.290	0.007-0.026	5.4-17.9	8.1-22.1	21.4-72.6	0.30-0.94

\*Mean of precision of parameter estimate expressed as CV%, i.e., if  $\pi$  denotes the parameter estimate, one has  $CV(\pi) = SD(\pi)/\pi \times 100$ .

**Calculations.** A one-, two-, and three-exponential model were fitted to arginine tr data by using SAAM II software (15). Weights were chosen optimally, i.e., equal to the inverse of the measurement errors, assumed to be Gaussian, independent with zero mean, and with a constant coefficient of variation, which has been estimated a posteriori. The best exponential model was selected on the basis of criteria such as the evaluation of residual errors, the precision of parameter estimates, and the principle of parsimony (13).

The arginine-NO compartmental model was fitted to urinary nitrate tracer and tracee data by using SAAM II (15). Weights were chosen as before, and the selected multiexponential model of arginine trr was used to specify the tracer model input, which was assumed to be noise-free.

**Statistics.** All values are stated as the mean  $\pm$  SD. Comparison between groups was performed by the Mann-Whitney test. A two-tailed probability value  $<0.05$  was considered statistically significant.

**RESULTS**

Fasting plasma unlabelled arginine concentration was  $64 \pm 4 \mu\text{mol}/\text{l}$  in control subjects and  $73 \pm 6$  in diabetic patients (NS). No differences were observed in arginine trr between the two groups (Fig. 2). In all subjects, the one exponential model failed to described data. Both the two- and three-exponential model provided a reasonable fit, yet the former was superior in terms of both model parsimony and parameter precision. Thus, it was selected as the best representation of arginine data. Its average predictions are shown in Fig. 2, superimposed to the data. Values of its parameters, together with arginine kinetic parameters (Table 2), indicate no difference between the two groups. As shown in Table 3,  $[^{15}\text{N}]$  nitrates were detectable in the urine up to 48 h from the tracer administration; no significant differences were found in all parameters of Table 3 between groups, even if tracer masses tend to be lower in diabetic patients.

Based on the model of Fig. 1, noncompartmental and compartmental analyses were applied to these data. As shown in Fig. 2B and C, the compartmental model was able to fit both tracer and tracee data reasonably, provided that a delay was introduced between arginine tracer disappearance from plasma and tracer nitrate appearance in the urine. The delay, estimated in each individual, averaged  $\sim 3$  h in control subjects (1-5 h) and  $\sim 2$  h in diabetic patients (0-4 h).

Values of NO synthesis from arginine ( $k_{21}Q_1$ ), urinary NO excretion ( $k_{32}Q_2$ ), and NO appearance in plasma from sources other than arginine ( $P$ ) were similar when estimated by the two approaches (Tables 4 and 5).

$k_{21}Q_1$  was lower ( $P = 0.05$  for the noncompartmental and 0.1208 for the compartmental analyses) in diabetic patients than in control subjects when expressed both in

TABLE 3  
Urinary nitrate data

	0–6 h	6–12 h	12–24 h	24–48 h
Control subjects				
Urinary volume (ml)	306 ± 38	416 ± 57	1,080 ± 186	1,070 ± 235
Nitrate concentration (μmol/l)	1,044 ± 509	699 ± 268	367 ± 96	415 ± 106
Tracer-to-tracee ratio (%)	0.047 ± 0.012	0.057 ± 0.013	0.016 ± 0.002	0.012 ± 0.005
Tracee mass (μmol)	274 ± 131	297 ± 122	346 ± 91	376 ± 73
Tracer mass (μmol)	0.135 ± 0.064	0.177 ± 0.085	0.053 ± 0.012	0.048 ± 0.026
Type 2 diabetic patients				
Urinary volume (ml)	392 ± 42	488 ± 48	1,600 ± 150	1,517 ± 180
Nitrate concentration (μmol/l)	491 ± 253	296 ± 57	278 ± 56	354 ± 91
Tracer-to-tracee ratio (%)	0.047 ± 0.008	0.043 ± 0.007	0.011 ± 0.005	0.004 ± 0.001
Tracee mass (μmol)	199 ± 117	137 ± 27	431 ± 84	545 ± 175
Tracer mass (μmol)	0.077 ± 0.036	0.063 ± 0.017	0.049 ± 0.019	0.021 ± 0.019

Data are means ± SE.

absolute terms (50% decrease) and as percentage of NO turnover (30% decrease). Differences in  $k_{21}Q_1$  are due to a decreased rate constant  $k_{21}$  (Table 5) since arginine mass  $Q_1$  is similar in the two groups (Table 2).  $k_{32}Q_2$  and  $P$  are similar in the two groups (Tables 4 and 5), as well as compartmental parameters  $k_{32}$ ,  $Q_2$ , and  $\tau$  (Table 5), whereas the FSR is lower in diabetic patients than in control subjects, although not significantly. The parameters for each subject can be found in an online appendix at <http://diabetes.diabetesjournals.org>.

## DISCUSSION

We proposed for the first time a noncompartmental and compartmental modeling analysis of the arginine-NO pathway in vivo in humans. With this approach, relevant parameters of the arginine-NO system can be identified, as well as the conversion of arginine to nitrates, the nitrate rate of appearance in plasma from sources other than arginine, the nitrate rate of appearance of in the urinary compartment, and the residence time of plasma nitrates. This approach is valuable because the total body nitrate pool is replenished by different sources (14). Different L-[<sup>15</sup>N]arginine input formats have been used, i.e., 24-h, 10-min, and 3-h infusion, respectively, and different parameters have been estimated. In any case, the proposed method relies on a simple precursor product relationship and only uses the determination of total [<sup>15</sup>N]nitrate excretion in the urine. Moreover, a correction factor equal to 1.67 was used by Castillo et al. (3), but not by Forte et al. (5) to account for the incomplete [<sup>15</sup>N]nitrate recovery in the urine.

TABLE 4  
Noncompartmental parameters of the NO system

	$k_{21}Q_1$ (%)	$k_{32}Q_2$ (μmol · h <sup>-1</sup> · kg <sup>-1</sup> )	$k_{21}Q_1$ (μmol · h <sup>-1</sup> · kg <sup>-1</sup> )	$P$ (μmol · h <sup>-1</sup> · kg <sup>-1</sup> )
Control subjects				
Mean	2.8	0.373	0.019	0.603
SE	0.4	0.096	0.007	0.154
Min-max	1.7–3.8	0.146–0.667	0.006–0.042	0.238–1.073
Type 2 diabetic patients				
Mean	1.6*	0.311	0.009	0.511
SE	0.4	0.096	0.002	0.159
Min-max	0.3–2.7	0.165–0.787	0.001–0.016	0.270–1.299

\* $P < 0.05$  vs. control subjects.

A noncompartmental model analysis was first applied to L-[<sup>15</sup>N]arginine data to derive parameters of arginine kinetic. The conventional approach to estimate arginine conversion to NO was then revisited in the noncompartmental model framework. This allowed us to clarify the underlying assumptions and to estimate a number of noncompartmental parameters by coupling total nitrate and [<sup>15</sup>N]nitrate excretion in the urine with the available information on arginine kinetics. Finally, we moved to the compartmental analysis of the arginine-NO system, which uses all of the available measurements and provides a more detailed description of the system.

**Arginine kinetics.** The noncompartmental analysis of plasma arginine trr was performed by adopting a two-exponential model (Fig. 2A). Hence, arginine kinetic parameters were estimated. Its average plasma appearance was  $54.4 \pm 10.7 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  in control subjects and  $46.3 \pm 7.8 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  in diabetic patients, similar to values obtained by Castillo et al. (3,11), but lower than those obtained by Wever et al. (16). The description of its kinetic by a two-exponential model testifies the presence of a compartmentalization of this amino acid at both organ and subcellular levels (17).

**Noncompartmental analysis of the arginine-NO system.** With this approach, we have first derived the simple formula to calculate NO synthesis from arginine ( $k_{21}Q_1$ ), originally proposed (3) for the tracer continuous infusion. The derivation of the formula in a noncompartmental framework highlights the underlying assumptions regarding the extent to which the tracer is irreversibly lost before reaching the urine pool. Consistently with Castillo et al.

TABLE 5  
Compartmental parameters of the NO system

	$k_{21}Q_1\%$	FSR (%/h <sup>-1</sup> )	$\tau$ (h)	$k_{32}Q_2$ ( $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ )	$k_{21} \cdot 10$ (h <sup>-1</sup> )	$k_{32}$ (h <sup>-1</sup> )	$k_{02}$ (h <sup>-1</sup> )	$k_{21}Q_1$ ( $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ )	$Q_2$ ( $\mu\text{mol}/\text{kg}$ )	P ( $\mu\text{mol} \cdot \text{h}^{-1} \cdot \text{kg}^{-1}$ )
Control subjects										
Mean	2.61 (9)	0.47 (10)	7.0 (17)	0.391 (12)	0.016 (9)	0.111 (17)	0.074 (17)	0.018 (9)	4.14 (26)	0.633 (2)
SE	0.45	0.09	1.9	0.110	0.005	0.026	0.017	0.006	1.05	0.178
Min-max	1.74-4.25	0.13-0.64	3.5-13.9	0.149-0.727	0.005-0.032	0.043-0.173	0.029-0.115	0.006-0.04	0.88-5.7	0.243-1.187
Type 2 diabetic patients										
Mean	1.97 (15)	0.33 (19)	9.0 (31)	0.295 (11)	0.010 (15)	0.126 (31)	0.084 (31)	0.009 (15)	4.18 (43)	0.482 (1)
SE	0.41	0.13	2.8	0.078	0.004	0.045	0.03	0.002	1.59	0.129
Min-max	0.27-2.80	0.12-0.94	1.9-17.9	0.175-0.678	0.001-0.027	0.034-0.308	0.022-0.205	0.001-0.014	0.64-9.74	0.284-1.116

\*Mean of precision of parameter estimate expressed as CV%, i.e., if pi denotes the parameter estimate, one has CV% = SD(pi)/pi × 100.

(3,18), we have assumed that only 60% of plasma nitrates reaches the urine, 40% being lost via alternative pathways. Moreover, our analysis evidenced that the amount of [<sup>15</sup>N]nitrate must be quantified in the urine collected for a time long enough to guarantee that the measurement well approximates its maximum level, theoretically reached at time infinity. Urine was collected up to 48 h following the tracer bolus, when tracer mass well approximates its plateau level (Fig. 2B).

With this analysis, additional parameters can be estimated: NO production from sources other than arginine (P), total NO turnover ( $k_{21}Q_1 + P$ ), and urinary NO excretion ( $k_{32}Q_2$ ). Hence, NO synthesis from arginine can then be expressed as a percentage of total NO turnover,  $k_{21}Q_1\% = k_{21}Q_1/(P + k_{32}Q_2)$ . Our analysis reveals that  $k_{32}Q_2$  and  $k_{21}Q_1\%$  can be expressed directly as a function of the measurements (Appendix equations A4 and A5), apart from any assumption on the ratio between urinary and non-urinary nitrate loss, which is conversely necessary to evaluate  $k_{21}Q_1$  and P (Appendix equations A6 and A9).

$k_{21}Q_1$  was  $0.019 \pm 0.007 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  in control subjects and  $0.009 \pm 0.002 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  in diabetic patients and accounts for 0.035 and 0.019%, respectively, of arginine plasma turnover. Previous figures (3,6,17) corroborate the finding that only a small percentage of total arginine pool is converted to NO. This study supports the finding of Castillo et al. (3), who showed that NO synthesis represents only 1.2% of the flux of plasma arginine in adults. The fraction of NO synthesis from arginine agrees also in terms of daily amino acid intake; the same group given 28 mmol arginine per day, a figure similar to our estimated intake, found a fractional conversion of labeled arginine to nitrates of 1.95% (18). They found that the contribution of arginine to NO based on intravenous [<sup>15</sup>N]arginine infusion is lower than that obtained with intragastric infusion (18). This dissimilarity may be explained by the significant compartmentation of this amino acid (19). Another possible cause for the lower conversion of arginine to NO observed in diabetic patients could be the presence of a competitive inhibitor of NO synthase. It was found that the asymmetric dimethylarginine is increased in type 2 diabetic patients (20).

Total NO turnover (Appendix equation A8) is  $0.62 \pm 0.16 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  in control subjects and  $0.52 \pm 0.16 \mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$  in diabetic subjects, not dissimilar from values obtained by others in normal volunteers, e.g., Castillo et al. (3) with  $0.95 \pm 0.1$  and Forte et al. (6) with urinary NO output 32-35  $\mu\text{mol}/\text{h}$ , which, after corrected by 1.67 to account for the nonurinary nitrate loss and expressed as  $\mu\text{mol} \cdot \text{kg}^{-1} \cdot \text{h}^{-1}$ , gives an estimate of total NO turnover equal to 0.75.

**Compartmental analysis of the arginine-NO system.** This approach required postulating the model structure of Fig. 1. Tracer and tracee mass balance in the various compartments is expressed by differential equations (equations 4-5), where the rate constants among compartments and tracee mass in compartment 2 are model parameters to be estimated from the data. The multiexponential model of arginine trr data was used to specify the tracer model input and to avoiding the difficult task of postulating the compartmental structure of arginine kinetics. Tracee

input is the arginine flux to NO and NO production, occurring in plasma. In reality, NO can be synthesized also in the kidney; the proximal tubule produces nitrates because at this level there is a substantial reabsorption of these compounds (14). However, this production appears to be trivial because the measured nitrate enrichment is similar to the urinary plasma enrichment (data not shown).

Model parameters were estimated by using all of the available data, e.g., cumulative tracer and tracee masses in the urine at the various collection times. Figure 2A and B indicate that the model is able to reproduce the data. A delay, estimated equal to  $\sim 3$  h in normal subjects and  $\sim 2$  h in diabetic patients, was introduced between arginine tracer disappearance from plasma and tracer nitrate appearance in the urine. Among compartmental parameters, summarized in Table 5,  $k_{21}Q_1\%$ , FSR,  $\tau$ , and  $k_{32}Q_2$  can be expressed as a function of uniquely identifiable parameter combinations.  $k_{21}Q_1\%$  and  $k_{32}Q_2$  have the same meaning and assume similar values as their noncompartmental counterparts. FSR is a parameter widely used to characterize the synthesis of a product from a precursor and measures the rate of NO synthesis from arginine per unit of NO mass. Its value indicates that only a small fraction of NO mass,  $0.47 \pm 0.09\%$  in normal subjects and  $0.33 \pm 0.13\%$  in diabetic patients, is derived per hour from arginine. The estimated residence time  $\tau$  of plasma nitrates obtained from our model was  $7.0 \pm 1.9$  h in control subjects and  $9.0 \pm 2.8$  h in diabetic patients, figures higher than those reported from Zeballos et al. (21) in dogs but similar to those obtained by Jurgenstein et al. (22) in humans. These discrepancies may suggest specie differences.

The estimation of the remaining parameters in Table 5 required that urinary loss to 60% of total loss be fixed. In addition to parameters such as  $k_{21}Q_1$  and P, which can also be recovered via noncompartmental analysis, rate constant parameters related to arginine conversion to NO ( $k_{21}$ ), urinary excretion ( $k_{32}$ ), and non-urinary loss ( $k_{02}$ ) were estimated in each subject, as well as the nitrate pool size ( $Q_2$ ) equal to  $\sim 4$   $\mu\text{mol/kg}$  both in control subjects and diabetic patients.

**Noncompartmental versus compartmental approaches.** The noncompartmental and compartmental analysis yield similar values of the four parameters, which can be estimated by both approaches, i.e.,  $k_{21}Q_1$ ,  $k_{21}Q_1\%$ ,  $k_{32}Q_2$ , and P. Both approaches are based on the model of Fig. 1. The noncompartmental approach is based on a single determination of nitrate and [ $^{15}\text{N}$ ]nitrate in the urine and uses simple formulas. In contrast, the compartmental model approach is more demanding because it requires multiple determinations in the urine and the use of parameter estimation. However, it gives a more detailed description of the system and provides evidence for model validity, due to the agreement found between data and model prediction. Moreover, it has a more general validity, whereas the noncompartmental model approach can be applied provided that urine is collected with enough time in humans to guarantee that the cumulative tracer measurement has reached the plateau value, the compartmental model does not require this assumption and can be used even in situations where this criterion is not satisfied. The good agreement between the results obtained with the

two approaches indicates that with the protocol adopted in this study—bolus injection of L-[ $^{15}\text{N}$ ]arginine and 48-h urine collection—the noncompartmental analysis allows a reliable quantification of the four parameters,  $k_{21}Q_1$ ,  $k_{21}Q_1\%$ ,  $k_{32}Q_2$ , and P. If a more detailed description of the system is desired, the compartmental analysis must be adopted.

**NO synthesis in diabetic versus normal subjects.** Our results showed that in the diabetic patients the fraction of arginine converted to NO is lower than that in control subjects. The relationship between diabetes and endothelial dysfunction is complex because of the interfering presence of other risk factors that are known to negatively affect NO production (23) in these patients. We have shown that in type 2 diabetic patients with no evidence of atherosclerotic vascular disease and without other confounding risk factors have normal endothelial function (11). The discrepancy between the traditional techniques and the present approach may be partly explained by the capability of the former to produce complex hemodynamic interactions in the vascular milieu (24).

Our study has limitations. This technique cannot differentiate among the different enzyme of NO synthase and assesses only the basal level of this enzyme. Moreover, other metabolic pathways of L-arginine, which include its incorporation into creatine and putrescine and other polyamines (25), were not assessed.

In conclusion we have proposed a new modeling approach of the L-arginine-NO pathway in normal and non-complicated type 2 diabetic patients based on the bolus injection of L-[ $^{15}\text{N}$ ]arginine. This approach not only provides information on arginine kinetics, but also allows relevant insights into nitrate metabolism. From our data, it appears that noncomplicated type 2 diabetic patients have an altered basal L-arginine-NO pathway.

## APPENDIX

Noncompartmental analysis of the arginine-NO system (Fig. 1) is based on the tracer mass balance equation in compartments 2 and 3 (Fig. 1), as follows:

$$k_{21} \int_0^{\infty} q_1(t) dt = (k_{32} + k_{02}) \int_0^{\infty} q_2(t) dt$$

$$k_{32} \int_0^{\infty} q_2(t) dt = q_3(\infty) \quad (\text{A1})$$

where  $q_1$ ,  $q_2$ , and  $q_3$  (in micromoles) denote tracer amount of tracer in compartments 1, 2, and 3, and  $k_{21}$ ,  $k_{32}$ , and  $k_{02}$  (in  $\text{hours}^{-1}$ ) are the rate constants of arginine to NO conversion, urinary NO loss, and NO loss via alternative pathways, respectively.

The mass balance equation for the tracee in compartment 2, assumed to be in steady state during the experiment, allows for the following:

$$k_{21}Q_1 + P = (k_{32} + k_{02})Q_2 \quad (\text{A2})$$

where  $Q_1$  and  $Q_2$  (in micromoles) denote the amount of tracee arginine and NO in compartments 1 and 2,  $k_{21}Q_1$  (in micromoles per hour) is the flux of NO synthesis from

arginine, and P (in micromoles per hour) is NO appearance rate in plasma from sources other than arginine. Because  $Q_2$  is assumed to be constant during the experiment, the amount ( $Q_3$ ) (in micromoles) of tracee nitrate in the urine increases linearly with time, as follows:

$$Q_3(t) = k_{32}Q_2t \quad (A3)$$

From equation A3, the flux of urinary NO excretion,  $k_{32}Q_2$ , equals the amount of nitrate measured in the urine collected for a time interval T divided by T, as follows:

$$k_{32}Q_2 = \frac{Q_3(T)}{T} \quad (A4)$$

By combining equations A1–A3, a formula can be derived for  $k_{21}Q_1$  normalized to total NO turnover, as follows:

$$k_{21}Q_1(\%) = \frac{k_{21}Q_1}{k_{21}Q_1 + P} = \frac{q_3(\infty)/Q_3(T)}{T \int_0^{\infty} ttr_1(t)dt} \approx \frac{q_3(T)/Q_3(T)}{T \int_0^{\infty} ttr_1(t)dt} = \frac{ttr_3(T)}{T \int_0^{\infty} ttr_1(t)dt} \quad (A5)$$

The correct implementation of equation A5 requires that urine is collected for a time (T) long enough (48 h in the present experiment) to guarantee that the measured tracer amount [ $q_3(T)$ ] well approximates the total amount reached at time infinity [ $q_3(\infty)$ ].

To quantify  $k_{21}Q_1$ , let's use for  $k_{21}$  an expression derived from equation A1, as follows:

$$k_{21}Q_1 = \frac{(k_{32} + k_{02}) \int_0^{\infty} q_2(t)dt}{\int_0^{\infty} q_1(t)dt} Q_1 = \frac{(k_{32} + k_{02})}{k_{32}} \frac{q_3(\infty)}{T \int_0^{\infty} ttr_1(t)dt} \quad (A6)$$

If NO urinary excretion is assumed to account for 60% of total NO loss (14), namely  $k_{32}/(k_{32} + k_{02}) = 0.6$  or equivalently  $(k_{32} + k_{02})/k_{32} = 1.67$ , and if  $q_3(\infty)$  is well approximated by  $q_3(T)$ , then equation A6 reduces to the conventional formula, equation 3, rewritten here as:

$$k_{21}Q_1 = \frac{1.67q_3(\infty)}{D/Ra} \approx \frac{1.67q_3(T)}{D/Ra} \quad (A7)$$

By using the above assumption into equation A2, an expression can be derived for the total NO turnover, as follows:

$$k_{21}Q_1 + P = (k_{32} + k_{02})Q_2 = \frac{k_{32} + k_{02}}{k_{32}} k_{32}Q_2 = 1.67 \frac{Q_3(T)}{T} \quad (8)$$

Finally, from equation A7–A8, P can be derived, as follows:

$$P = 1.67 \frac{Q_3(T)}{T} - \frac{q_3(T)}{D/Ra} \quad (A9)$$

In summary, the quantification of  $k_{21}Q_1$  (equation A6) and P (equation A9) requires a priori knowledge of the percentage of urinary loss, whereas the quantification of  $k_{32}Q_2$  (equation A4) and  $k_{21}Q_1\%$  (equation A5) does not.

### ACKNOWLEDGMENTS

This study has been supported by Regione Veneto Grant Invecchiamento dell' Anziano.

We thank Monica Vettore and Diana Valbusa for their excellent technical support.

### REFERENCES

1. Hsueh WA, Quinones MJ, Creager MA: Endothelium in insulin resistance and diabetes. *Diabetes Rev* 5:343–352, 1997
2. Verma S, Anderson TJ: Fundamentals of endothelial function for the clinical cardiologist. *Circulation* 105:546–549, 2002
3. Castillo L, Beaumier L, Ajami AM, Young VR: Whole body nitric oxide synthesis in healthy men determined from [ $^{15}N$ ]arginine-to-[ $^{15}N$ ]citrulline labeling. *Proc Natl Acad Sci U S A* 93:11460–11465, 1996
4. Lau T, Owen W, Yu YM, Noviski N, Lyons J, Zurakowski D, Tsay R, Ajami A, Young VR, Castillo L: Arginine, citrulline, and nitric oxide metabolism in end-stage renal disease patients. *J Clin Invest* 105:1217–1225, 2000
5. Forte P, Smith LM, Milne E, Benjamin N: Measurement of nitric oxide synthesis in humans using L-[ $^{15}N$ ]arginine. *Methods Enzymol* 301:92–98, 1999
6. Forte P, Copland M, Smith LM, Milne E, Sutherland J, Benjamin N: Basal nitric oxide synthesis in essential hypertension. *Lancet* 349:837–842, 1997
7. O'Byrne S, Forte P, Roberts LJ 2nd, Morrow JD, Johnston A, Anggard E, Leslie RD, Benjamin N: Nitric oxide synthesis and isoprostane production in subjects with type 1 diabetes and normal urinary albumin excretion. *Diabetes* 49:857–862, 2000
8. Katz SD, Khan T, Zeballos GA, Mathew L, Potharlanka P, Knecht M, Whelan J: Decreased activity of the L-arginine-nitric oxide metabolic pathway in patients with congestive heart failure. *Circulation* 99:2113–2117, 1999
9. Green LC, Wagner DA, Glogowski J, Skipper PL, Wishnok JS, Tannenbaum SR: Analysis of nitrate, nitrite, and [ $^{15}N$ ]nitrate in biological fluids. *Anal Biochem* 126:131–138, 1982
10. Avogaro A, Piarulli F, Valerio A, Miola M, Calveri M, Pavan P, Vicini P, Cobelli C, Tiengo A, Calo L, Del Prato S: Forearm nitric oxide balance, vascular relaxation, and glucose metabolism in NIDDM patients. *Diabetes* 46:1040–1046, 1997
11. Castillo L, Ajami A, Branch S, Chapman TE, Yu YM, Burke JF, Young VR: Plasma arginine kinetics in adult man: response to an arginine-free diet. *Metabolism* 43:114–122, 1994
12. Cobelli C, Toffolo G, Foster DM: Tracer-to-tracee ratio for analysis of stable isotope tracer data: link with radioactive kinetic formalism. *Am J Physiol* 262:E968–E975, 1992
13. Cobelli C, Foster D, Toffolo G: *Tracer Kinetic in Biomedical Research: From Data to Model*. New York, Kluwer Academic/Plenum Publishers, 2000
14. Baylis C, Vallance P: Measurement of nitrite and nitrate levels in plasma and urine—what does this measure tell us about the activity of the endogenous nitric oxide system? *Curr Opin Nephrol Hypertens* 7:59–62, 1998
15. Barret PHR, Bell BM, Cobelli C, Golde H, Schumitzky A, Vicini P, Foster D: SAAM II: simulation, analysis and modeling software for tracer and pharmacokinetics studies. *Metabolism* 47:484–492, 1998
16. Wever R, Boer P, Hijmering M, Stroes E, Verhaar M, Kastelein J, Versluis K, Lagerwerf F, van Rijn H, Koomans H, Rabelink T: Nitric oxide production is reduced in patients with chronic renal failure. *Arterioscler Thromb Vasc Biol* 19:1168–1172, 1999
17. Wu G, Morris SM Jr: Arginine metabolism: nitric oxide and beyond. *Biochem J* 336:1–17, 1998
18. Castillo L, deRojas TC, Chapman TE, Vogt J, Burke JF, Tannenbaum SR, Young VR: Splanchnic metabolism of dietary arginine in relation to nitric oxide synthesis in normal adult man. *Proc Natl Acad Sci U S A* 90:193–197, 1993
19. Jungermann K, Katz N: Functional specialization of different hepatocyte populations. *Physiol Rev* 69:708–764, 1989
20. Fard A, Tuck CH, Donis JA, Sciacca R, Di Tullio MR, Wu HD, Bryant TA, Chen NT, Torres-Tamayo M, Ramasamy R, Berglund L, Ginsberg HN,

- Homma S, Cannon PJ: Acute elevations of plasma asymmetric dimethyl-arginine and impaired endothelial function in response to a high-fat meal in patients with type 2 diabetes. *Arterioscler Thromb Vasc Biol* 20:2039–2044, 2000
21. Zeballos GA, Bernstein RD, Thompson CI, Forfia PR, Seyedi N, Shen W, Kaminiski PM, Wolin MS, Hintze TH: Pharmacodynamics of plasma nitrate/nitrite as an indication of nitric oxide formation in conscious dogs. *Circulation* 91:2982–2988, 1995
22. Jungersten L, Edlund A, Petersson AS, Wennmalm A: Plasma nitrate as an index of nitric oxide formation in man: analyses of kinetics and confounding factors. *Clin Physiol* 16:369–379, 1996
23. Harrison DG: Cellular and molecular mechanisms of endothelial cell dysfunction. *J Clin Invest* 100:2153–2157, 1997
24. Farouque HM, Meredith IT: The assessment of endothelial function in humans. *Coron Artery Dis* 12:445–454, 2001
25. Beaumier L, Castillo L, Yu YM, Ajami AM, Young VR: Arginine: new and exciting developments for an old amino acid. *Biomed Environ Sci* 9:296–315, 1996