L-Arginine Transport in the Human Coronary and Peripheral Circulation

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Background—Nitric oxide synthase (NOS) uses arginine for the production of nitric oxide (NO). High intracellular concentrations of arginine suggest that NOS activity should be independent of plasma arginine supply. However, under certain conditions, increased plasma arginine concentrations appear to be associated with increased NOS activity. The purpose of this study was to explore arginine transport within the human coronary and peripheral circulation.

Methods and Results—Mass-labeled $^{15}$N$_2$-arginine was infused to steady state before cardiac catheterization in 31 patients. After diagnostic angiography, a catheter was placed in the coronary sinus. The transcardiac concentration gradient (aorta−coronary sinus) of $^{15}$N$_2$-arginine was used as a measure of arginine transport at baseline and during infusions of acetylcholine and $N^\omega$-monomethyl-L-arginine (L-NMMA). No gradient was detected at rest. During the infusion of acetylcholine, a significant gradient was detected (2.5±1.2 $\mu$mol/L, P=0.01) corresponding to a fractional extraction of 11.7±7.5%. This is consistent with in vitro studies that suggest that stimulation of NOS induces arginine transport. During the infusion of L-NMMA, the concentration of $^{15}$N$_2$-arginine increased in the coronary sinus, producing a gradient of −3.9±1.3 $\mu$mol/L (P=0.0002), corresponding to a fractional production of 20.5±5.0%. This is consistent with in vitro studies that suggest that L-NMMA induces the efflux of arginine from the cell to the extracellular space via transporter-mediated transstimulation.

Conclusions—The use of steady-state $^{15}$N$_2$-arginine to examine transorgan L-arginine gradients represents a novel tool for the study of L-arginine transport and the mechanisms of endothelial and NOS dysfunction. (Circulation. 2004;109:1278-1283.)

Key Words: nitric oxide synthase • coronary disease • endothelium

Endothelial nitric oxide synthase (NOS) synthesizes nitric oxide (NO) from L-arginine. The intracellular concentration of L-arginine (100 to 800 $\mu$mol/L) is several orders of magnitude greater than the half-saturating concentrations ($K_m$, 3 $\mu$mol/L) for NOS and is substantially higher than concentrations found in plasma. In addition, endothelial cells have the ability to recycle L-arginine from L-citrulline. These observations suggest that NOS activity should be independent of plasma arginine concentrations. However, a number of studies have shown that L-arginine supplementation improves endothelium-dependent vasomotor responses. This response to elevated extracellular L-arginine concentrations despite (presumably) high intracellular L-arginine concentrations has been called the “arginine paradox.”

The arginine paradox would suggest that under some conditions, NOS activity is linked to L-arginine transport. This hypothesis is supported by histochemical studies that suggest that NOS is colocalized to specialized caveolae with a known L-arginine transporter. Additional in vitro studies suggest that NOS activation is associated with increased L-arginine transport. In vivo evidence linking L-arginine transport to NO production is limited. However, Kaye et al documented a decrease in forearm and cardiac L-arginine transport in patients with congestive heart failure, a condition known to be associated with impaired endothelium-dependent vasomotor responses.

Using mass-labeled L-arginine ($^{15}$N$_2$-arginine) infused to steady state before cardiac catheterization, this study investigated L-arginine transport in the human peripheral and coronary circulation.

Methods

The study was reviewed and approved by the Human Subjects Review Committee of the University of Toronto, Toronto, Ontario, Canada, and written informed consent was obtained in all cases.

Patient Selection

Eligible patients were approached in the clinic setting, and the study protocol was explained. Patients were eligible if they were >18

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years of age, had normal left ventricular function, and had been referred for cardiac catheterization because of suspected coronary artery disease (CAD). Exclusion criteria included congestive heart failure, clinically significant valvular heart disease, and childbearing potential.

Baseline Variables
Historical variables were determined during the initial clinic visit. Fasting blood for cholesterol and creatinine was drawn on the day of the cardiac catheterization.

15N2-Arginine Infusions
Patients were admitted to the hospital at 6:30 AM the morning of the study. The protocol for 15N2-arginine infusion to achieve steady state has been published previously. An intravenous catheter was placed in an antecubital vein, and a bolus of 15N2-arginine (6 μmol/kg over a period of 10 minutes) was administered. Subsequently, the 15N2-arginine was infused at 6 μmol/kg per hour. Patients were taken to the cardiac catheterization laboratory after 5 hours of continuous infusion. The infusion was stopped at the conclusion of the study.

Catheterization Technique and Intracoronary Infusions
Cardiac medications (including β-blockers, calcium channel blockers, ACE inhibitors, nitrates, and diuretics) were withheld for at least 48 hours before cardiac catheterization. Arterial and venous sheaths (8F and 7F, respectively) were placed in the femoral artery and vein, respectively. Coronary angiography was performed by the standard Judkins technique. The presence of obstructive CAD was defined as the presence of a >50% diameter obstruction in a major epicardial vessel as determined by quantitative coronary angiography. Patients were defined as either having (CAD+) or being free of (CAD−) obstructive CAD on this basis.

After diagnostic catheterization, a catheter was placed in the coronary sinus (CS) via the femoral or brachial vein. After the placement of the CS catheter, 50 to 70 units/kg of intravenous heparin was administered. Intracoronary infusions were administered through the diagnostic Judkins catheter directly into the left main coronary artery. Intracoronary infusions included 5% dextrose in water (DSW), acetylcholine (ACh: 10−4 mol/L at 1.25 mL/min), and 2 doses of N°-monomethylL-arginine (L-NMMA: 4 μmol/min and 46.4 μmol/min). For those patients with severe CAD in whom ACh infusion was potentially unsafe, only DSW and L-NMMA were infused. The duration of each infusion was 6 minutes.

Blood Sampling for Mass-Labeled L-Arginine
Arterial and femoral vein blood samples were drawn at the beginning of the case before diagnostic angiography. Arterial and CS bloods were drawn 4 minutes into each intracoronary infusion. All blood samples were immediately transferred to EDTA tubes and placed on ice. The blood was then centrifuged for 10 minutes to obtain plasma within 30 minutes of the end of the case before diagnostic angiography. All blood samples are outlined in the Table. Of these, 29 had baseline transfemoral blood samples and 25 had baseline transcardiac blood samples. In 2 patients, transfemoral blood samples were lost. In 6 patients, a CS catheter was not placed because of patient preference or technical difficulties. Within the group with transfemoral blood work, 14 received intracoronary ACh, 18 received L-NMMA, and 13 received both ACh and L-NMMA. In 6 patients, no intracoronary infusions were performed after CS catheter insertion because of the presence of severe CAD.

Steady-State 15N2-Arginine Concentrations
Previously published data suggest that steady state is achieved within 4 hours of the initiation of intravenous infusion of labeled L-arginine. Accordingly, patients were brought to the catheterization suite after 4 to 5 hours of 15N2-arginine infusion. The longest procedures occurred in the 13 patients who received both ACh and L-NMMA infusions, with a mean procedure time of 125±5 minutes. The mean arterial plasma 15N2-arginine concentration before the diagnostic procedure was 14.6±2.8 μmol/L. At the beginning of the research protocol, the mean arterial 15N2-arginine concentration was 14.4±2.5 μmol/L, and by the end of the procedure, the mean concentration was 13.4±1.8 μmol/L (P=NS).

Steady-State 15N2-Arginine Gradients
In 29 patients, the mean transfemoral 15N2-arginine gradient at baseline was 0.56±1.9 μmol/L. This represents a mean fractional extraction of 1.8±7% (P=NS).
In 25 patients, the mean transcardiac $^{15}$N$_2$-arginine gradient was $0.1\pm0.6$ mmol/L. This represents a mean fractional extraction of $-0.2\pm3.5\%$ ($P=\text{NS}$).

Effect of ACh Infusion on the Transcardiac $^{15}$N$_2$-Arginine Gradient

Fourteen patients received ACh. At baseline, the transcardiac $^{15}$N$_2$-arginine gradient was $-0.3\pm0.5$ mmol/L. During ACh infusion, a significant transcardiac gradient was noted ($2.5\pm1.2$ mmol/L; $P=0.01$), corresponding to a fractional extraction of $11.7\pm7.5\%$. During the recontrol period, the mean transcardiac gradient was $-1.3\pm1.2$ mmol/L ($P=\text{NS}$; Figure 1).

Effect of L-NMMA on Transcardiac l-Arginine Gradient

In the 18 patients who received L-NMMA, the baseline transcardiac $^{15}$N$_2$-arginine gradient was $-1.3\pm1.0$ mmol/L. Sequential infusions with L-NMMA 4 and 46 mmol/min resulted in progressive changes in the transcardiac gradient: $-1.6\pm1.1$ and $-3.9\pm1.3$ mmol/L, respectively. The latter represents a significant increase in the CS l-arginine concentration relative to the aortic $^{15}$N$_2$-arginine concentration ($P=0.0002$; Figure 2). The corresponding fractional production during infusion with L-NMMA 46 mmol/min was $20.5\pm5.1\%$.

Effect of CAD on Transorgan Gradients

Of the 29 patients with transfemoral blood samples, 18 had significant CAD, and 11 were free of obstructive CAD. In those with CAD, the mean transfemoral $^{15}$N$_2$-arginine gradient was $-1.5\pm1.4$ mmol/L, compared with $3.9\pm1.7$ mmol/L in patients without CAD (unpaired t test; CAD$-\text{vs}\text{-CAD}+: P=0.02$; Figure 3).

At baseline, no difference was seen in the transcardiac gradient in patients with or without CAD. Of the 14 patients who received ACh, 10 were free of significant CAD, and 4 had significant CAD. ACh infusion provoked a significant transcardiac gradient only in those without CAD ($3.1\pm1.6$ mmol/L; $P=0.01$), whereas no significant gradient was seen in the 4 patients with CAD ($1.0\pm1.2$ mmol/L; $P=\text{NS}$; Figure 3).

Discussion

The role of l-arginine transport in the maintenance of endothelial function and NOS activity is unclear. The l-arginine paradox would suggest that under certain conditions, intracellular l-arginine supply might be insufficient for optimal NOS activity. This could occur in the setting of decreased intracellular l-arginine bioavailability, in the presence of naturally occurring competitive inhibitors, or in the setting of increased demand because of NOS stimulation. In these settings, there may be an important relationship between l-arginine transport and NOS activity. The existence of such a relationship is supported by the colocalization of NOS with l-arginine transporters within specialized caveo-
Although the true nature of the relationship between l-arginine transport and nitric oxide production remains undefined, l-arginine transport may represent both a unique measure of endothelial health and a target for therapeutic intervention.

That these relationships remain speculative is because of the current paucity of in vivo evidence, particularly as it relates to the coronary circulation. A reduction in l-arginine transport has been demonstrated in patients with congestive heart failure.7,8 Given that heart failure is associated with abnormal endothelial-dependent vasomotor responses,9 these findings would be consistent with the hypothesis that l-arginine transport is important for the maintenance of adequate NO bioavailability.

Under steady-state conditions, the extent to which measures of l-arginine transport correlate with NOS activity depends on a number of variables. It should be recalled that l-arginine contributes to protein synthesis and participates in several metabolic pathways, including the urea cycle. The extent to which these pathways contribute to the uptake and utilization of l-arginine would determine their impact on transorgan arterial and venous l-arginine concentrations and diminish the correlation between l-arginine uptake and NO production accordingly. In an organ system in which arginine is extensively catabolized, measures of arginine transport would tend to overestimate NOS activity.

In the present study, no transcardiac gradient was found at baseline, suggesting that within the coronary circulation, l-arginine transport is not supporting these alternative pathways in a measurable way. This is consistent with previous work that suggests that endothelial cells lack a fully functional urea cycle.11 This may not be true for other organ systems, such as the kidney or liver.

Of greater concern is the potential to underestimate NOS activity. The caveolar colocalization of l-arginine transport proteins and NOS should ensure a steady-state relationship between the plasma compartment and the intracellular l-arginine compartment used by NOS.7 If other pathways do not catabolize or recycle l-arginine and if the intracellular concentration of l-arginine remains stable, then NOS activity would be directly dependent on l-arginine transport. In this model, the transcardiac gradient would directly reflect NOS activity (Figure 4A). However, NOS also tends to colocalize with l-arginine recycling enzymes. If the activity of the recycling enzymes (l-arginine production) equalled NOS activity (l-arginine catabolism), no transorgan l-arginine gradient would be detected despite ongoing NOS activity (Figure 4B). The intracellular regeneration of l-arginine would thus tend to negate the ability of the transcardiac l-arginine gradient to reflect NOS activity.

This problem of l-arginine regeneration can be overcome with the use of mass-labeled 15N2-arginine, as was used in the present study. This compound is labeled on the guanidino-terminal with 2 mass-labeled 15N atoms. It is from this terminal that nitrogen is cleaved by NOS to produce 15N1-citrulline. Because there should be no other source of 15N within the cell, regeneration enzymes would produce 15N2-arginine rather than 15N1-arginine. Even if the activity of the recycling enzymes equal NOS activity, a net transport of 15N2-arginine into the cell would have to occur to maintain steady-state 15N2-arginine concentrations (Figure 4C). Thus, if the cell is in steady state with the plasma compartment, the transcardiac gradient of 15N2-arginine should not underestimate NOS activity.

Finally, measures of l-arginine transport will not correlate with NOS activity if NOS is confined to a completely separate l-arginine compartment that is not in steady state with the plasma and thus independent of l-arginine transport mechanisms.14 This would allow for NOS activity independent of l-arginine transport (Figure 4D).

At baseline, we were unable to demonstrate a transcardiac gradient of 15N2-arginine. This might support the model illustrated in Figure 4D, in which NOS uses an intracellular l-arginine compartment that is not in steady state with the plasma compartment. However, l-arginine transporters are known to colocalize with NOS,5 and the present observation that ACh-induced stimulation of NOS produces a significant transcardiac 15N2-arginine gradient suggests an important relationship between the NOS activity and l-arginine transport. The failure to demonstrate a transcardiac l-arginine gradient at baseline is more likely explained by the fact that a low basal NOS activity is below the level of detection of the analytical methods used for this study. This would suggest that the NOS-dependent maintenance of basal vasomotor tone is not associated with the transport of large quantities of plasma l-arginine, a finding that is consistent with clinical studies in which l-arginine supplementation does not affect resting vasomotor tone.4

The correlation between l-arginine transport and NOS activity becomes more complicated in the absence of steady state. This could occur if inadequate time has been allotted to tracer infusion. In this setting, movement of the tracer from the plasma to the cellular compartment might represent movement down the concentration gradient of the tracer rather than net l-arginine transport. Acute, short-term perturbations such as the intracoronary infusions of ACh or L-NMMA performed in this study may not allow for steady-state conditions to be reached by the time that repeat blood sampling was performed. Alterations to the balance of transporter activity (influx versus efflux) may initially produce net movement of arginine into (influx) or out of (efflux) the cell as the system moves toward a new steady state. In this study, no attempt was made to document a new steady state during intracoronary infusions, and thus, any observations must be viewed as directional (qualitative) rather than quantitative.

ACh infusion was associated with the development of a significant transcardiac 15N2-arginine gradient. Activation of NOS may produce increased l-arginine catabolism, with a resultant decline in intracellular l-arginine, thus precipitating increased l-arginine transport. This would imply a direct correlation between NOS activity and l-arginine transport. Alternatively, it should be noted that transporter-mediated l-arginine transport is voltage-dependent.15 Hyperpolarization increases the influx Vm (the maximal rate of l-arginine transport into the cell) and decreases the Km (half saturating concentration) for l-arginine influx (movement from the plasma into the cell) while increasing the Km for l-arginine efflux (movement out of the cell into the plasma). Thus, any
stimulus that hyperpolarizes the endothelial cell could induce L-arginine transport independent of NOS activity. Of note, NOS stimulation and the resulting increase in NO bioavailability leads to endothelial cell hyperpolarization, and in this way, NOS stimulation is closely linked to increased L-arginine transport. Although we cannot deduce the mechanism, the findings of the present study are consistent with the results of in vitro studies that suggest that activation of NOS is associated with increased L-arginine transport. This is also consistent with in vivo studies that report that L-arginine supplementation improves stimulated endothelium-dependent vasomotion.

The observation that L-NMMA infusion results in an efflux of L-arginine across the coronary circulation is of
particular interest. Because there is no external source of $^{15}$N$_2$-arginine between the aorta and CS, this “additional” $^{15}$N$_2$-arginine must come from a nonplasma source within the coronary circulation. Efflux from the endothelial intracellular compartment is an attractive explanation. This may be simply because of the inhibition of L-arginine influx, resulting in unopposed L-arginine efflux. Alternatively, this phenomenon may reflect “transstimulation.” Transstimulation occurs when a compound on one side of a membrane stimulates transport on the other side of the membrane. In 1996, Bogle et al reported that L-NMMA transstimulated the efflux of L-arginine from the intracellular space into the culture medium. At the same time, L-NMMA would be transported into the cell via the same y$^+$ transporter system. This transporter-mediated exchange could be called “countertransport.” Whether the observed data represent transstimulation or simply the unopposed efflux of L-arginine, the acute effect of L-NMMA infusion appears to include both the introduction of an NOS inhibitor and the reduction of the intracellular concentration of L-arginine.

The failure to demonstrate significant extraction of L-arginine across the peripheral circulation at rest is inconsistent with previously published data. This discrepancy may be a result of baseline differences in the study populations. Alternative explanations may rest in the different vascular beds studied, different sensitivities of the analytic techniques used, or the ability to achieve intracellular steady state with different infusion protocols. As noted in the Results section, we believe that our model of prolonged infusion allows for the development of intracellular steady state with labeled $^{15}$N$_2$-arginine, something that may not be achieved with shorter infusion protocols. Without the development of true steady-state conditions, the apparent uptake of a tracer may simply represent transport down a concentration gradient of the tracer and not net L-arginine uptake.

Limitations to this study include the small sample size and the inclusion of only subjects with or at high risk for CAD. Larger studies are needed to examine differences between patients with and without documented CAD. Although the present data are insufficient to draw important conclusions, the preliminary association between the presence of CAD and impaired arginine transport bears further study. Observed gradients are assumed to be a result of L-arginine transport between plasma and endothelial cells but may represent transport between plasma and other blood-borne cells. The speculative nature of the relationship between arginine transport and NOS activity could be strengthened with improved biochemical techniques. When $^{15}$N$_2$-arginine passes through NOS, the mass-labeled nitrogens will pass to $^{15}$NO (and thus $^{15}$N-nitrate/nitrite) and single-labeled $^{15}$N-citrulline. The development of sensitive assays for these compounds will eventually provide for increased insights into the correlation between L-arginine transport and NOS activity. Similar assays for $^{15}$N-urea will help to control for the influence of the urea cycle on transorgan gradients.

Despite these issues and independent of its correlation to NOS activity, L-arginine transport represents a novel measure of endothelial health. Transport is dependent on membrane-bound proteins that would be susceptible to a variety of insults. Thus, in its most basic interpretation, L-arginine transport should represent a unique measure of endothelial integrity.

**Conclusion**

The use of steady-state $^{15}$N$_2$-L-arginine to examine transorgan L-arginine gradients represents a novel tool for the study of L-arginine transport and the mechanisms of endothelial and NOS dysfunction.

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