In Vivo and In Vitro Evidence for Impaired Arginine Transport in Human Heart Failure

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Background—The clinical features of congestive heart failure (CHF) result from a complex interaction between reduced ventricular function, neurohormonal activation, and impaired endothelial function. Although endothelial dysfunction has been well documented, the mechanisms that contribute to this abnormality remain unknown. Recent studies, however, indicate a potential therapeutic role for supplemental L-arginine, suggesting the presence of an underlying disorder of L-arginine metabolism.

Methods and Results—We used 2 complementary approaches to assess L-arginine transport in control subjects and patients with CHF. During a steady-state intra-arterial infusion of \([^{3}H]\)L-arginine (100 nCi/min), forearm clearance of \([^{3}H]\)L-arginine was significantly reduced in CHF patients compared with forearm kinetics in control subjects (64 ± 2 versus 133 ± 14 mL/min, \(P=0.002\)). In conjunction with this, \([^{3}H]\)-arginine uptake by peripheral blood mononuclear cells (PBMCs) was also substantially reduced in heart failure patients compared with controls (\(V_{\text{max}} 10.1 ± 1.3 \text{ vs } 49.8 ± 7.1 \text{ pmol/10}^5 \text{ cells per 5 minutes}, P<0.001\)). In association with this finding, we observed a 76% (\(P<0.01\)) reduction in mRNA expression for the cationic amino acid transporter CAT-1, as assessed by ribonuclease protection assay.

Conclusions—These data document both in vivo and in vitro evidence for a marked depression of L-arginine transport in human CHF and therefore provide an explanation for the restorative actions of supplemental L-arginine on vascular function in CHF. (Circulation. 2000;102:2707-2712.)

Key Words: amino acids ■ nitric oxide ■ radioisotopes ■ endothelium ■ cells

The systemic vasoconstriction that occurs in heart failure is widely considered to represent the net effect of activation of neurohormonal vasoconstrictor systems in the presence of endothelial dysfunction. Numerous studies have consistently demonstrated the presence of an attenuated response to endothelium-dependent vasodilators, most typically acetylcholine, in the peripheral and coronary circulation in human and experimental models of heart failure.1–6 Furthermore, these observations have been confirmed by use of a more physiological stimulus such as exercise and the hyperemia induced by ischemia.7,8

Although the evidence for reduced endothelial function in congestive heart failure (CHF) is compelling, the mechanisms responsible for this phenomenon remain unclear. In broad terms, potential mechanisms include reduced expression of muscarinic cholinergic receptors on endothelial cells, altered intracellular signaling, reduced nitric oxide (NO) production, increased NO degradation, or an attenuated response by the intracellular targets of NO or cGMP.7 Recently, several groups have demonstrated an improvement in endothelial function in patients with heart failure in response to supplemental oral or intravenous L-arginine.10,11 This strategy has also been shown to improve endothelial function in other clinical paradigms that are characterized by endothelial dysfunction, most notably atherosclerosis.12–14

The observations that L-arginine supplementation can improve endothelial function may provide some insight into the mechanisms responsible for this phenomenon. One possible explanation is a reduction in the rate of transport of L-arginine by endothelial cells, leading to relative intracellular substrate depletion for NO synthase. In the present study, we aimed to test the hypothesis that L-arginine transport is reduced in human heart failure using a novel in vivo approach in the forearm, in conjunction with an in vitro assessment of the kinetics of L-arginine transport by isolated peripheral blood mononuclear cells (PBMCs).

Methods

Subjects

The study population included 16 patients with CHF (mean±SEM age 52±3 years) recruited from the Heart Failure Service, Alfred Hospital, Melbourne, Australia, and 21 healthy control volunteers (mean±SEM age 41±2 years). The cause of heart failure was nonischemic in 10 patients and myocardial ischemia in 6 patients.
All patients were treated with an ACE inhibitor and diuretics, 10 received digoxin, and 1 received a β-blocker. Left ventricular ejection fraction was 23±4% (mean±SEM), and all patients had New York Heart Association class III symptoms of CHF. All patients participated after giving informed consent, and the study was conducted with the approval of the Alfred Hospital Ethics Review Committee.

Forearm Vascular Function and \([^{3}H]\) L-Arginine Kinetics

In 7 healthy control subjects and 5 heart failure patients, a 3F cannula was inserted into the brachial artery of the nondominant arm under local anesthesia for the infusion of radiolabeled arginine and acetylcholine as indicated below. A 5F cannula was inserted percutaneously into a deep antecubital forearm vein, as previously described, for venous blood sampling. Forearm blood flow was measured by venous occlusion plethysmography, as previously described.

After a stabilization period of 20 minutes, resting forearm blood flow was measured and intra-arterial infusion of radiolabeled arginine was begun. After an initial priming bolus of 1 μCi of \([4,5-{^{3}H}]\) arginine (ICN Pharmaceuticals, specific activity 98 to 106 Ci/mmol) in 2 mL of 0.9% NaCl, a continuous intra-arterial infusion of 100 nCi/min of \([4,5-{^{3}H}]\) arginine was begun. Deep venous blood samples were drawn at 20, 40, and 80 minutes after commencement of the infusion and immediately transferred to ice-chilled tubes containing EGTA, which were stored on ice until completion of the study.

After deep venous sampling and forearm blood flow estimation at 80 minutes, acetylcholine (BDH Chemicals) was infused into the brachial artery at doses of 9.25 and 37 μg/min at a flow rate of 2 mL/min for 5 minutes, as previously described. After completion of the study, blood samples were centrifuged at 4°C, and plasma was stored at −70°C. The plasma concentration of \([^{3}H]\) arginine was determined by ion-exchange chromatography, essentially as described previously. In brief, plasma proteins were removed from 750 μL of plasma by the addition of 250 μL of 20% trichloroacetic acid, followed by cooling on ice and subsequent removal of the precipitated proteins by centrifugation. Samples were then extracted at least 5 times in ether to remove trichloroacetic acid and combined in equal volume with 20 mmol/L HEPES, pH 6. Samples were then applied to a Dowex 50W-X8 column that had been pre-equilibrated and then lysed in 0.1% Triton X for subsequent liquid scintillation spectroscopy. Recovery of \([^{3}H]\) arginine from standard plasma samples was typically 90% to 95%.

To obtain an index of arginine uptake in the forearm, the clearance rate of \([^{3}H]\) arginine was calculated when the plasma concentration of \([^{3}H]\) arginine in deep venous plasma had achieved steady state, according to the formula

\[
\text{Forearm} \left[ ^{3}H \right] \text{arginine clearance (mL/min)} = \frac{\text{[} ^{3}H \text{]arginine infusion rate (dpm/min)}}{\text{[} ^{3}H \text{]arginine concentration (dpm/mL)}}
\]

\([^{3}H]\) L-Arginine Transport in PBMCs

To obtain a complemental index of arginine transport in healthy subjects and patients with heart failure, we investigated the kinetics of \([^{3}H]\) arginine transport by PBMCs. In 10 heart failure patients and 14 healthy control subjects, 30 mL of peripheral blood was collected into tubes containing EGTA and diluted in equal volume with PBS. PBMCs were then isolated by Ficoll Paque (Pharmacia) density gradient centrifugation according to the manufacturer’s instructions. The resultant lymphocyte-rich band typically contained at least 90% lymphocytes (as assessed by fluorescence-activated cell sorter analysis; data not shown), and ≥90% of the cells were viable (as assessed by trypan blue exclusion). The PBMC band was resuspended in 17 mL of Krebs-Henseleit buffer and divided into 32 aliquots of 500 μL. Cell numbers were determined manually by hemocytometer counting.

For uptake studies, PBMCs were incubated in balanced salt solution containing l-arginine in concentrations ranging from 1 to 300 μmol/L, which included 100 nmol/L \([^{3}H]\)-arginine, for a period of 5 minutes at 37°C. Uptake studies were performed in duplicate. For each subject, additional parallel uptake studies were performed in the presence of 10 mmol/L l-lysine, a specific competitor for transport by the cationic amino acid transport system. At the conclusion of the incubation period, the incubation tubes were rapidly cooled on ice. Cells were washed twice in ice-cold Krebs-Henseleit buffer and then lysed in 0.1% Triton X for subsequent liquid scintillation spectroscopy. Arginine transport by cationic amino acid transporter(s) was calculated as the difference between uptake in the absence and presence of 10 mmol/L lysine in the uptake solution.

Molecular Biology

In conjunction with the above protocol, PBMCs were isolated from peripheral blood as described above for studies of cationic amino acid transporter (CAT) mRNA expression in 7 healthy subjects and 5 patients with heart failure. After collection of PBMCs from 30 mL of peripheral blood, total cellular RNA was isolated according to the method of Chomczynski and Sacchi. Reverse transcription–polymerase chain reaction (RT-PCR) amplification of total RNA from mononuclear cells was used to construct cDNA fragments of CAT-1 and CAT-2B, with species- and isoform-specific sequences as follows: CAT-1 sense 5′-CCCCGCGGTCGCTGATGAAA-3′ and antisense 5′-TTTACAGCCTAATGGGGTCG-3′ (GenBank accession number x59155); CAT-2B sense 5′-GATCCATTTCCAAAATGCT-3′ and antisense 5′-GGTGCAGTGGCAGTTTGAT-3′ (GenBank accession number u76369). PCR amplification was performed for 40 cycles, 30 seconds at 95°C denaturation, 30 seconds at 63°C annealing, and 1 minute at 72°C extension with Perkin-Elmer Taq polymerase and 1.5 mmol/L MgCl2. The amplified cDNA was subsequently cloned into pGEM-T vector (Promega). The identities of the cloned PCR products were confirmed by sequencing.

The cloned CAT-1 and CAT-2B fragments were then used as templates to generate \(^{32}P\)-labeled riboprobes for use in ribonuclease protection assays, as previously described. RNA loading was assessed with a riboprobe specific for human GAPDH. The protected fragments were then separated and analyzed on a 5% denaturing polyacrylamide/urea gel. Radioactive signals were measured with a PhosphorImager (Fuji BAS-1000).

Statistical Analysis

Data are presented as mean±SEM. Between-group comparisons were performed by Student’s t test. ANCOVA was also performed to determine the potential contribution of differences in blood flow to the between-group differences in \([^{3}H]\)arginine clearance. The Mann-Whitney test was used for comparison of group data that were not normally distributed. A P value <0.05 was considered significant.

Results

Forearm Blood Flow and Arginine Transport

In response to a continuous intra-arterial infusion of \([^{3}H]\) arginine, there was a progressive increase in the deep venous concentration of the radiotracer, which reached steady-state levels in plasma after ~40 minutes (Figure 1, top). At 80 minutes, the deep venous plasma concentration of \([^{3}H]\) arginine was consistently higher in patients with CHF (Figure 1, bottom). Consistent with previous studies, the resting forearm blood flow of patients with CHF was somewhat lower than that in control subjects, although the difference was not statistically significant (Figure 2, top). Mean arterial pressures were significantly lower in CHF patients (72±3 versus 90±3 mm Hg, P<0.01), with similar forearm vascular...
resistance in the 2 groups (CHF versus controls: 35±3 versus 35±6 resistance units, \( P = \text{NS} \)).

As reflected by the higher plasma \( [\text{H}]\text-L-\text{arginine} \) concentration in deep venous blood samples from patients with heart failure, calculation of the \( [\text{H}]\text-L-\text{arginine} \) clearance rate from the forearm revealed a significant reduction in the rate of uptake of \( [\text{H}]\text-L-\text{arginine} \) across the forearm in heart failure (Figure 2, bottom). Although the forearm blood flow was lower, albeit nonsignificantly, we further examined the role of this difference as a potential confounding factor. By ANCOVA, the difference in forearm arginine clearance between heart failure patients and healthy volunteers remained statistically significant \( (P < 0.01) \) when forearm blood flow was included as a covariate. In conjunction with the findings of reduced arginine clearance, we also observed a significant depression in the forearm vascular response to the endothelium-dependent vasodilator acetylcholine (Figure 3). A modest positive relationship between the maximal vasodilator response to acetylcholine and the forearm arginine clearance was observed \( (r = 0.58, P = 0.06) \).

**Mononuclear Cell Arginine Transport in Heart Failure**

Arginine transport mediated by CAT(s) was examined in PBMCs obtained from patients with heart failure and healthy control subjects. In preliminary experiments (Figure 4A), incubation times of up to 10 minutes were observed to fall within the period of maximal transport velocity, and accordingly incubation times of 5 minutes were chosen for the remainder of the study.

In both healthy volunteers and heart failure patients, accumulation of \( [\text{H}]\text-L-\text{arginine} \) was readily detectable over the physiological range (Figure 4B). However, the rate of accumulation of arginine by PBMCs obtained from individuals with heart failure was consistently substantially lower than that in healthy subjects. Furthermore, in heart failure patients, the maximal rate of transport \( (V_{\text{max}}) \) was signifi-
cantly lower than that seen in healthy individuals (10.1±1.3 versus 49.8±7.1 pmol/10^5 cells per 5 minutes, P<0.001), whereas no significant change in K_m was apparent (CHF patients versus controls, 107±43 versus 185±37 μmol/L).

**CAT mRNA Expression**

To examine the molecular basis for the reduction in the rate of arginine accumulation by PBMCs in patients with heart failure, we used ribonuclease protection analysis to examine the abundance of mRNA for CAT-1 and CAT-2B. We did not specifically examine the expression of CAT-2A, because preliminary studies using RT-PCR were barely able to detect CAT-2A mRNA expression in PBMCs. As shown in Figure 5, we observed a significant (76%) reduction in mRNA expression for CAT-1 that was readily detected by ribonuclease protection assay. Expression of CAT-2B in mononuclear cells, however, was negligible in both control subjects and heart failure patients (data not shown).

**Discussion**

Over the past decade, there has been an exponential accumulation of data highlighting the crucial role that the endothelium plays in regulating vascular function in both health and disease. In this context, a key finding in a wide range of conditions, including heart failure, atherosclerosis, diabetes, and hypertension, has been a significant reduction in the response to endothelium-dependent vasodilators such as acetylcholine. In human heart failure, depressed vasodilator responses to acetylcholine have been observed in multiple vascular beds, including the forearm, lower limb, coronary circulation, and isolated resistance arteries.

The finding of a beneficial effect of L-arginine supplementation on endothelial function suggests that a relative deficiency of L-arginine, the key substrate for NO synthesis, may exist in heart failure. However, these observations do not distinguish whether the deficiency is extracellular or intracellular. In the present study, we tested the hypothesis that an impairment of cellular arginine transport was present in human heart failure. Using a novel in vivo approach in the human forearm, we have demonstrated for the first time a substantial reduction in the rate of clearance of [^3]H-l-arginine from the circulation, consistent with reduced endothelial arginine transport. In conjunction, we have also demonstrated the presence of endothelial dysfunction in our heart failure cohort, as reflected by the presence of a significantly attenuated response to intra-arterial infusions of acetylcholine. To provide a more detailed evaluation of arginine transport in the context of human heart failure, we also examined the kinetics of [^3]H-l-arginine uptake by freshly isolated PBMCs from healthy subjects and CHF patients. In keeping with the findings of the in vivo radiotracer study, we observed a substantial diminution in the rate of [^3]H-l-arginine accumulation by mononuclear cells over a range of physiologically relevant concentrations.

Our findings would therefore suggest that in heart failure, a reduction in arginine transport by endothelial cells could lead to a relative deficiency of intracellular arginine, thereby affecting NO synthesis. Supplementation with L-arginine would be expected to deliver increased substrate intracellularly, given that the normal plasma concentration of L-arginine is well below the saturating range for CAT-mediated transport, and therefore an increase in the L-arginine concentration would be expected to result in an increased rate of transport. Other workers have investigated the possibility that the beneficial actions of L-arginine supplementation represent the expected response to a deficiency in the plasma concentration of this amino acid, although this remains controversial. Alternatively, other investigators have examined the potential role of circulating inhibitors of L-arginine transport, such as asymmetric dimethyl arginine (ADMA) and N^G-monomethyl-l-arginine (L-NMMA). Recently, studies in human and experimental heart failure have reported low, micromolar-range plasma concentrations for both ADMA and L-NMMA. However, it appears unlikely that either compound would exert a significant effect on either L-arginine transport or endothelial function in the range of reported concentrations.

Furthermore, in the present study, the abnormality of L-arginine transport was detected in both the forearm circulation and isolated PBMCs, making it unlikely that accumulation of potential transport inhibitors provides a major mechanism for this apparent defect. Alternatively, our in vivo findings could possibly be explained by the presence of antifailure medications, although such therapy has generally been shown to improve endothelial function.

Despite the intracellular concentration of L-arginine being far in excess of the K_m for NO synthase, limitation of L-arginine availability has been shown to reduce NO generation by both NOS2 and NOS3. Furthermore, the term “L-arginine paradox” has been applied to the converse observation in which L-arginine supplementation augments NO production. On the basis of these observations, it might be expected that the attenuation of arginine transport ob-
served in the present study could result in a diminution of substrate delivery to NO synthase sufficient to reduce NO generation. To the best of our knowledge, the intracellular concentration of L-arginine in the setting of heart failure has only been examined in erythrocytes and surprisingly was found to be 100 μmol/L, whereas it was undetectable in red cells from healthy volunteers. The significance of this observation is uncertain given that in the same study, the intracellular concentration of another cationic amino acid, L-lysine, was not different from that in control subjects, and also that erythrocytes do not express NO synthase.

At physiological concentrations of L-arginine, a single functional transport system (y\(^+\)) accounts for 60% to 80% of the total carrier-mediated uptake activity. Cloning studies have identified 4 CAT proteins. CAT-1 and CAT-2B are both high-affinity, low-capacity transporters, thus resembling system y\(^+\). In contrast, CAT-2A is a low-affinity, high-capacity transporter with limited distribution. CAT-3, a recently identified high-affinity transporter, has limited distribution, confined largely to the brain. To identify the mechanism responsible for the observed reduction in both the V\(_{\text{max}}\) for transport in isolated PBMCs and forearm clearance of \(^{15}\text{N}\)L-arginine, we examined the mRNA expression of CAT-1 and CAT-2B in PBMCs. Using RNase protection analysis, we observed a significant reduction in the expression of CAT-1 mRNA, whereas expression of CAT-2B was barely detectable. Although the precise mechanism responsible for the down-regulation of CAT-1 mRNA expression remains unclear, our findings could be explained by elevated levels of circulating cytokines that are known to be present in heart failure. In particular, it has recently been shown that inflammatory mediators may downregulate CAT-1 mRNA in macrophages. In the present study, we did not discontinue antifailure medication because of concerns about the severity of the patients’ degree of left ventricular dysfunction. Therefore, we cannot exclude the possibility that our observations were influenced by the presence of antifailure medications, although these drugs have not been shown to influence CAT expression.

In the present study, we did not aim to pharmacologically characterize the precise nature of the L-arginine–mediated transport in PBMCs, other than to identify the L-lysine–sensitive component. Although the y\(^+\) system accounts for the major proportion of arginine transport, it is possible that changes in expression of other arginine transporting systems, including the b\(^0\)\(^+\) system, could explain our findings. However, this would seem unlikely given the fact that heart failure was associated with up to an 80% reduction in arginine transport, suggesting the involvement of the predominant arginine transport system.

In summary, for the first time, we demonstrate an impairment of L-arginine transport in patients with CHF. This observation provides one explanation for the well-documented impairment of endothelial function in human heart failure and for the observed beneficial actions of supplemental L-arginine.

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References


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