Reduced Myocardial and Systemic L-Arginine Uptake in Heart Failure

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Abstract—Altered nitric oxide (NO) bioavailability has been ascribed an important role in the pathophysiology of congestive heart failure (CHF). In the peripheral vasculature, we recently demonstrated a depression of L-arginine transport in association with pharmacological evidence of reduced endothelial function. In contrast, increased myocardial NO generation has been proposed to account for a component of the reduced myocardial contractility in CHF, although this remains controversial. We determined the whole body clearance rate and cardiac fractional extraction of L-arginine during a steady-state intravenous infusion of [3H]L-arginine (300 nCi/min) in 9 healthy control subjects and 7 patients with moderate to severe CHF. In patients with CHF, there was a 30% reduction in the transcardiac extraction of [3H]L-arginine compared with controls (P<0.05), which was accompanied by a trend toward reduced [3H]L-citrulline release (P=0.06). In conjunction, the systemic clearance rate of [3H]L-arginine was significantly lower in patients with CHF (778±148 versus 1278±144 mL/min, P<0.05). In association with these biochemical indices, we observed a 38% reduction (P<0.05) in the mRNA expression of the cationic amino acid transporter CAT-1 in ventricular myocardial samples from patients with CHF compared with healthy unused donor myocardium, whereas myocardial NOS enzymatic activity and NOS protein were unchanged. These data indicate the presence of a significant reduction in the myocardial uptake of L-arginine in patients with CHF. Furthermore, this abnormality seems to be part of a systemic downregulation of L-arginine transport. (Circ Res. 2002;91:1198-1203.)

Key Words: heart failure • nitric oxide • amino acids • radioisotopes

Congestive heart failure (CHF) is a common cardiovascular disorder characterized by considerable morbidity and mortality. The pathophysiology of CHF is increasingly being appreciated as highly complex, representing the result of interactions between multiple processes, including maladaptive ventricular remodeling, activation of neurohormonal and cytokine systems, and alterations in vascular and skeletal muscle function.

The well-documented process of progressively worsening cardiac performance in CHF1,2 has been ascribed to multiple processes. These include primary alterations in myocardial structure, including geometric considerations, the number of cardiomyocytes (resulting from necrosis and apoptosis), and the presence of myocardial fibrosis.3 In conjunction, fundamental changes in cardiomyocyte cellular and molecular biology that alter contractile function are also well recognized. These include changes in calcium-handling proteins, contractile protein expression, and the β-adrenoceptor signaling pathway, as recently reviewed.2 Within this context, considerable emphasis has been placed on the potential negative inotropic effects of several autocrine/paracrine and circulating factors, such as nitric oxide (NO)4,5 and tumor necrosis factor α.6

Although several investigators have documented increased expression of the inducible isoform of NO synthase (NOS) in the failing heart,7,8 this finding is not uniform,9 and there remains little direct biochemical evidence of increased NO generation within the failing myocardium.10,11 At a functional level, reduced coronary endothelial vasodilator function is well documented in CHF,12 whereas the consequences of NOS overexpression in the myocardium are controversial.13–15 In the present study, we hypothesized that the apparent paradox of increased NOS expression in the face of reduced NO production could be explained by a relative limitation in the intracellular availability of L-arginine attributable to downregulation of the relevant L-arginine transport system, as we have recently observed in the forearm circulation and mononuclear cells of patients with CHF.16

Materials and Methods

Clinical Study

Study Population

The present study included 9 healthy volunteers and 7 patients with CHF (mean age, 57±4 versus 56±2 years; P=NS). CHF patients with a left ventricular ejection fraction (LVEF) of <35% were recruited from the Heart Failure Clinic, Alfred Hospital, Melbourne.
The mean LVEF of the CHF cohort was 17±2%, and the average New York Heart Association heart failure class was 2.9±0.1, consistent with moderate to severe heart failure. All patients with CHF received angiotensin-converting enzyme inhibitors, diuretics, and carvedilol. Three patients also received 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitors. The cause of CHF was ischemic cardiomyopathy in 2 patients and nonischemic dilated cardiomyopathy in the remaining 5 patients. All patients gave written informed consent, and the study was performed with the approval of the Alfred Hospital Ethics Review Committee.

**Catheterization Protocol**
All studies were performed in the morning, and antifailure medications were continued to avoid hemodynamic instability. A balloon-tipped thermodilution catheter (7F Arrow, Arrow International) was inserted via an introducer sheath placed in the right internal jugular vein or antecubital vein for the determination of pulmonary arterial pressures, wedge pressure, and cardiac output. A right radial arterial line was placed for arterial blood pressure measurement and blood sampling. After the hemodynamic assessment, a coronary sinus thermodilution catheter (Webster Laboratories) was positioned in the coronary sinus under fluoroscopic control. The tip of the catheter was positioned at least 2 cm proximal to the orifice of the coronary sinus, as confirmed by injection of radiographic contrast. Coronary sinus blood flow was estimated by thermodilution, and an average was determined from at least 2 measurements.

**Radiotracer Measurement of Systemic and Cardiac Arginine Uptake**
To determine the rate of cardiac and total systemic L-arginine clearance, a steady-state intravenous infusion of [14C]-labeled L-arginine was administered. After an initial priming bolus of 6 μCi of [4,5-3H]L-arginine (ICN Pharmaceuticals, specific activity 98 to 106 Ci/mmol) in 2 mL of 0.9% NaCl, a continuous intravenous infusion of 300 nCi/min of [4,5-3H]L-arginine was delivered. Arterial blood samples were drawn at 10, 20, and 40 minutes (to confirm the presence of a steady-state plasma arterial [3H]-arginine concentration). On collection, samples were immediately transferred to ice-chilled tubes containing EGTA and stored on ice until the completion of the study. After a period of at least 40 minutes of intravenous infusion of [3H]-arginine, simultaneous arterial and coronary sinus blood samples were drawn.

After the completion of the study, blood samples were centrifuged at 4°C and plasma was stored at −70°C. The plasma concentration of [3H]-arginine and [3H]-citrulline was determined using ion-exchange chromatography, essentially as previously described.16,17 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 50-WX8 column that had been preequilibrated with 20 mmol/L HEPES, pH 6. [3H]-citrulline was identified in the initial column flow through and water elution, whereas [3H]-arginine was eluted from the column using 1 N NaOH. Radioactivity was determined by liquid scintillation spectrometry.

The fractional extraction (FEx) of [3H]-arginine across the myocardium was calculated as follows:

\[ \text{Cardiac } [3H]\text{-arginine FEx} = \frac{(C_{\text{Art}} - C_{\text{CS}})}{C_{\text{Art}}} \]

The transcardiac flux of [3H]-citrulline was calculated as follows:

\[ \text{Cardiac } [3H]\text{-citrulline flux} = (C_{\text{CS}} - C_{\text{art}}) \times \text{coronary sinus plasma flow} \]

Total systemic clearance rate was calculated using the following equation:

\[ \text{Systemic } [3H]\text{-arginine clearance rate} = \frac{[3H]\text{-arginine infusion rate}}{C_{\text{Art}}} \]

where \( C_{\text{art}} \) and \( C_{\text{CS}} \) are the arterial and coronary sinus plasma concentrations of [3H]-arginine or [3H]-citrulline in disintegrations per minute per milliliter, where relevant.

**Myocardial Expression of Cationic Amino Acid Transporters**
To complement the clinical biochemical approach to assessing the cardiac extraction rate of L-arginine, the expression of the cationic amino acid transporter (CAT) mRNA in the myocardium was also examined. Myocardial tissue samples were obtained from 5 unused donor hearts and from the explanted hearts of 7 patients undergoing cardiac transplantation. Total cellular RNA was isolated according to standard methodology.18 Cloned human-specific CAT1 and CAT2B fragments, as previously described,16 were then used as templates to generate 32P-labeled riboprobes for use in ribonuclease protection assays. RNA loading was assessed using a riboprobe specific for human GAPDH. The protected fragments were then separated and analyzed on a nondenaturing polyacrylamide gel. Radioactive signals were quantitated with a phosphorimager (Fuji BAS-1000).

**Measurement of NOS Activity and Protein Expression**
NOS enzymatic activity in myocardial homogenates was determined by measuring the rate of conversion of [3H]-arginine to [3H]-citrulline, as previously described.19 In brief, myocardial homogenates were obtained from 4 unused donor hearts and from the explanted hearts of 5 patients undergoing cardiac transplantation, and total NOS activity was determined in the presence excess substrate and cofactors. Expression of endothelial NOS (eNOS) and inducible NOS (iNOS) protein expression was performed by Western blot using appropriate monoclonal antibodies (Transduction Laboratories), as previously described.20 Protein content was determined by a modification of the Lowry method (BioRad).

**Statistical Analysis**
Data are presented as mean±SEM. Between-group differences were compared using an unpaired Student’s t test. Analysis of covariance was also performed to determine the potential contribution of confounding hemodynamic differences between the two study groups. \( P<0.05 \) was considered statistically significant.

**Results**

**Subject Characteristics**
Consistent with the clinical (New York Heart Association class) and functional (LVEF) indices of the population with CHF, the physiological data reflected a significantly impaired hemodynamic profile compared with the healthy control subjects (Table).
Cardiac and Systemic $[\text{H}]$-Arginine Kinetics
During the intravenous infusion of $[\text{H}]$-arginine, there was a rapid, progressive increase in the arterial plasma $[\text{H}]$-arginine concentration, reaching steady-state values after $\approx 20$ minutes (Figure 1A). Furthermore, the rate of clearance of $[\text{H}]$-arginine from plasma in patients with heart failure was significantly lower than that observed in healthy control subjects ($1278 \pm 144 \text{ mL/min}$ versus $777 \pm 148 \text{ mL/min}$, $P=0.03$), as shown in Figure 1B. Given the potential contribution of different hemodynamics on the systemic clearance rate for $[\text{H}]$-arginine, we examined the relationship between cardiac output, arterial blood pressure, and systemic $[\text{H}]$-arginine clearance. In this analysis, there was no apparent direct correlation between cardiac output and $[\text{H}]$-arginine clearance across the entire study population. However, a significant inverse relationship between cardiac output and $[\text{H}]$-arginine clearance was evident in the control group ($r=-0.80$, $P<0.01$, data not shown). Although a significant difference in plasma triglycerides was present between the two study groups, no relationship between any lipid parameter and systemic $[\text{H}]$-arginine clearance was evident.

In conjunction with the determination of total systemic $[\text{H}]$-arginine clearance, we determined the extent of extraction of $[\text{H}]$-arginine during passage through the coronary circulation. As illustrated in Figure 2, the extraction of $[\text{H}]$-arginine was significantly lower in patients with heart failure compared with controls ($P=0.03$). There was no statistically significant relationship between resting coronary sinus blood flow and the transcardiac extraction of $[\text{H}]$-arginine.

NOS Expression and $[\text{H}]$-Citrulline Generation
To complement the biochemical measures of transcardiac arginine extraction, we determined the relative expression of CAT1 and CAT2B. Using RNAse protection analysis, we observed a significant reduction (38%, $P<0.05$) in the level of CAT1 mRNA in the failing human heart (Figures 3A and 3B). In contrast, we did not observe any significant difference in the expression of CAT2B (Figure 3C) in subjects with heart failure compared with unused nonfailing donor hearts. In conjunction with these studies, we also investigated the level of NOS enzymatic activity and protein expression in control and failing myocardium. In these studies, we observed similar levels of total NOS activity (Figure 4A) and NOS protein expression (Figure 4B) between the two groups. To examine the combined effects of impaired l-arginine uptake in the presence of maintained NOS expression, we determined the rate of transcardiac $[\text{H}]$-citrulline generation. Although the net transcardiac gradient was low, patients
with CHF tended to have a lower rate of transcardiac \(^{3}H\) L-citrulline release than healthy controls (Figure 5).

**Discussion**

Although the initiating cardiac injury that triggers the onset of CHF is often (although not always) appreciated, the mechanisms that contribute to ongoing reduction in cardiac performance seem to be highly complex. Although early studies demonstrated that NO exerted a negative inotropic effect\(^{4,21}\) in addition to its classical vasodilator effect, the role of this molecule in CHF pathophysiology has remained controversial. The demonstration, in some studies, of increased myocardial expression of NOS2 mRNA and protein\(^{7,8}\) or reduced NOS3 mRNA and protein\(^{9,22}\) has been taken by some investigators to indicate that excess myocardial NO generation might play a role in the pathophysiology of CHF. Specifically, increased myocardial NO generation has been implicated in the contractile dysfunction, whereas reduced local vascular NO generation could account for the associated endothelial dysfunction. Despite these data, however, direct biochemical evidence for altered NO production is lacking. Previously, we measured the transcardiac concentration gradient for nitrate/nitrite in both healthy volunteers and patients with heart failure and could not demonstrate any difference between the two groups\(^{10}\). Furthermore, Recchia et al\(^{11}\) showed that the cardiac production of NO declined during the development of heart failure in the rapid pacing canine model of CHF.

In conjunction with the contrasting molecular biological and biochemical studies of the role of NO in the failing heart, physiological and pharmacological studies have also yielded conflicting data. Extensive studies of endothelial function, both in the peripheral and coronary circulation, in clinical and experimental CHF have consistently shown evidence of diminished endothelium-dependent vasodilator function.\(^{12,23-26}\) To examine the functional consequences of NO production within the myocardium, investigators have generally taken the approach of inhibiting endogenously produced NO or infusion of NO donors. In patients with heart failure, inhibition of NOS seems to have little direct effect on contractility.\(^{5,27}\) However, during \(\beta\)-adrenoceptor stimulation, endogenously generated NO does seem to exert a modulatory effect. Infusion of NO donors, in contrast, largely seems to modify diastolic function.\(^{28}\) Taken together, these data seem to indicate that although NOS may be expressed in the failing heart, there is conflicting data as to the generation and bioavailability of NO within the heart and, accordingly, its role in CHF pathophysiology.

By extending a recently developed method for studying the kinetics of regional L-arginine transport,\(^{16}\) we showed in the present study that the fractional extraction of \(^{3}H\) L-arginine during passage through the coronary circulation was significantly reduced in patients with CHF. To complement this finding, we also found that the expression of mRNA for the cationic amino acid transporter, CAT1, was significantly reduced in myocardial samples obtained from patients with heart failure compared with healthy unused donor myocardium. In separate studies, we showed that the levels of NOS enzymatic activity and NOS protein expression were not significantly different between normal and failing heart. Clearly, the molecular biological studies were not performed in the same group of CHF and healthy subjects that underwent assessment of arginine kinetics. Furthermore, the catheterization studies were performed on a separate group of patients with CHF from those in which explanted tissue samples were obtained, and accordingly the degree of CHF was relatively milder than in the former group, although the patients clearly had significantly elevated filling pressures and reduced cardiac index. To integrate our findings of reduced arginine transport with unchanged NOS expression, we determined the rate of citrulline flux from the heart. Consistent with our hypothesis that reduced expression of the CAT1 transporter leads to NOS substrate depletion, we observed a trend to reduced myocardial citrulline generation. However, neither the biochemical nor molecular biological

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**Figure 4.** A, Bar graph representing NOS enzymatic activity in failing and unused donor myocardial samples. B, Western blots showing expression of eNOS and iNOS in control and CHF samples. + indicates positive control samples.

**Figure 5.** Bar graph showing transcardiac flux of \(^{3}H\) L-citrulline in control patients and patients with CHF.
studies can precisely identify which cell types are characterized by reduced arginine transport capacity, nor can any direct correlation between individual cellular arginine transport and NOS enzyme activity be inferred.

The present data do provide a unique explanation for the seemingly paradoxical biochemical findings of reduced myocardial NO production,\textsuperscript{10,11} despite reports of increased myocardial iNOS and possibly eNOS expression. Our study would be consistent with a situation in which limited availability of intracellular L-arginine diminished the production of NO within the myocardium or coronary endothelium, given that limitation of L-arginine supply has been shown to reduce NO production in other contexts.\textsuperscript{29,30} The resultant relative deficiency of myocardial NO generation could then provide an explanation for several of the biochemical and physiological changes within the myocardium known to be associated with acute NOS inhibition and also with CHF. Among these, the metabolic derangements seen in both heart failure and NOS inhibition include increased myocardial oxygen consumption,\textsuperscript{11,31,32} increased glucose utilization,\textsuperscript{33} and reduced fatty acid consumption.\textsuperscript{11} Functional studies of cardiac NOS inhibition or endocardial removal include impaired diastolic relaxation,\textsuperscript{34,35} which is commonly observed in CHF.

Under conditions of L-arginine depletion, numerous investigators have demonstrated that NOS is capable of generating superoxide radicals.\textsuperscript{36–39} Furthermore, Xia and Zweier\textsuperscript{38} demonstrated that production of peroxynitrite was markedly augmented in macrophages in which iNOS had been induced, under conditions of L-arginine depletion. These observations, taken together with our own, could thus be consistent with a recent study by Ferdinandy et al.,\textsuperscript{40} who showed that peroxynitrite was a major contributor to the myocardial depression observed in a cytokine model of myocardial depression, although L-arginine transport was not specifically characterized in this study. More directly, Ide et al.\textsuperscript{41} also recently showed that production of superoxide, hydrogen peroxide, and the hydroxyl radical was significantly elevated in failing myocardium obtained from dogs that had undergone rapid ventricular pacing. The enzymatic source of superoxide, however, was not determined in this study.

In keeping with our previous study that demonstrated reduced L-arginine clearance in the forearm circulation and impaired L-arginine uptake by peripheral blood mononuclear cells,\textsuperscript{16} we observed in the present study that there was a significant reduction in the total systemic clearance rate for L-arginine. This abnormality seemed to be independent of the potential contribution of between-group differences in cardiac output. This finding is consistent with previous studies by Katz et al.\textsuperscript{42} that demonstrated reduced urinary excretion of \textsuperscript{15}N-nitrate after an infusion of \textsuperscript{15}N-L-arginine. In this study, although the creatinine clearance was significantly lower in patients with CHF, the urinary excretion of \textsuperscript{15}N-nitrate remained substantially lower in patients with CHF, even when normalized to urinary creatinine excretion, suggesting that differences in renal function or renal blood flow were probably not responsible for the observed differences. Although the investigators interpreted their data as reflecting reduced eNOS activity, it is possible that their findings did reflect diminished arginine transport, although the excretion of urea was not changed. Our study is potentially confounded by the use of a range of drug therapy in the patients with CHF. However, to our knowledge, expression or activity of the cationic amino acid transporters is not influenced by these drugs, and in the present study, we did not observe differences in NOS expression or activity in patients with CHF compared with controls.

Taken together, in conjunction with our previous studies in the forearm vasculature and isolated peripheral blood mononuclear cells,\textsuperscript{16} our data indicate the presence of a generalized abnormality of L-arginine transport in CHF. Although some investigators have proposed that circulating factors such as asymmetric dimethyl arginine may be major inhibitors of L-arginine transport and NOS,\textsuperscript{43} this remains controversial given their relatively low plasma concentration compared with the plasma L-arginine concentration. In contrast with this potential mechanism, our molecular biological studies point to a generalized regulation of CAT1 mRNA. The mechanism responsible for these observations is not clear from our study. Previous experimental studies have shown that inflammatory cytokines may lead to the upregulation of CAT1 mRNA in some, although not all, cell types.\textsuperscript{44–46} However, at present, little additional data regarding the effect of other factors of relevance to CHF on CAT1 mRNA expression are available.

In conclusion, our data provide evidence for cardiac and systemic impairment of L-arginine transport. These findings provide a mechanistic explanation for the impairment of endothelial function that typically accompanies CHF. Additional investigation into the cellular and molecular basis for the alteration of cationic amino acid transporter expression is warranted in this important clinical condition.

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