Increased L-arginine uptake and inducible nitric oxide synthase activity in aortas of rats with heart failure

PETER B. STATHOPULOS, XIANGRU LU, JI SHEN, JEREMY A. SCOTT,
JAMES R. HAMMOND, DAVID G. McCORMACK,
J. MALCOLM O. ARNOLD, AND QINGPING FENG
*Cardiology Research Laboratory, Departments of Medicine, Pharmacology, and Toxicology, London
Health Sciences Centre, University of Western Ontario, London, Ontario, Canada N6A 4G5*

Stathopulos, Peter B., Xiangru Lu, Ji Shen, Jeremy A. Scott, James R. Hammond, David G. McCormack, J. Malcolm O. Arnold, and Qingping Feng. Increased L-arginine uptake and inducible nitric oxide synthase activity in aortas of rats with heart failure. *Am J Physiol Heart Circ Physiol* 280: H859–H867, 2001.—L-Arginine crosses the cell membrane primarily through the system y^+ transporter. The aim of this study was to investigate the role of L-arginine transport in nitric oxide (NO) production in aortas of rats with heart failure induced by myocardial infarction. Tumor necrosis factor-α levels in aortas of rats with heart failure were six times higher than in sham rats ($P < 0.01$). L-Arginine uptake was increased in aortas of rats with heart failure compared with sham rats ($P < 0.01$). Cationic amino acid transporter-2B and inducible (i) nitric oxide synthase (NOS) expression were increased in aortas of rats with heart failure compared with sham rats ($P < 0.05$). Aortic strips from rats with heart failure treated with L-arginine but not D-arginine increased NO production ($P < 0.05$). The effect of L-arginine on NO production was blocked by L-lysine, a basic amino acid that shares the same system y^+ transporter with L-arginine, and by the NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME). Treatment with L-lysine and L-NAME in vivo decreased plasma nitrate and nitrite levels in rats with heart failure ($P < 0.05$). Our data demonstrate that NO production is dependent on iNOS activity and L-arginine uptake and suggest that L-arginine transport plays an important role in enhanced NO production in heart failure.

cationic amino acid transporter; vascular biology; myocardial dysfunction

L-ARGININE IS A CATIONIC and dibasic amino acid that is semiessential in the mammalian diet. Nitric oxide synthase (NOS) uses L-arginine as its substrate during the production of nitric oxide (NO). NO is produced from the guanidino group of L-arginine in an NADPH-dependent reaction catalyzed by a family of NOS enzymes (23). There are at least three distinct isoforms of NOS, derived from separate genes: neural NOS (nNOS or NOS I), inducible NOS (iNOS or NOS II), and endothelial NOS (eNOS or NOS III). Although eNOS and nNOS are calcium-dependent enzymes, iNOS is usu-ally induced by cytokines, and its enzyme activity is calcium independent.

L-Arginine is transported into cells from the extracellular space primarily through the specific amino acid transport system y^+, now known as the cationic amino acid transporter (CAT; see Ref. 5). System y^+ is a facilitative process that is Na^+ independent and pH insensitive. Four members of the CAT family in system y^+ have recently been cloned and identified. CAT-1 is constitutively expressed and is present in a wide range of tissues (19, 29). CAT-2A and CAT-2B are more limited in their tissue distribution and have been reported to be physiologically regulated. CAT-3 is a brain tissue-specific CAT. It is of interest that analogs of L-arginine have potent inhibitory effects on system y^+ (2). For instance, L-homoarginine, differing from L-arginine by the presence of a CH_2 group, is a strong competitive inhibitor of system y^+ action. Other cationic amino acids such as L-lysine also competitively inhibit L-arginine transport via system y^+.

Heart failure is associated with increases in peripheral resistance of the vasculature. This augmented vascular resistance is detrimental and may contribute to the high mortality of heart failure in the late phases of the disease. Studies have demonstrated that, although endothelium-dependent, NO-mediated vasodilation is decreased (7, 9), basal NO production is increased in heart failure (8, 13). The increased basal NO production may be due to increased iNOS activity in the myocardium and vasculature of heart failure (14, 16). Consequently, it is possible that consumption of intracellular L-arginine may be increased as a result of increased NO production in heart failure. However, it is not known if L-arginine transport is increased in the vasculature of heart failure. Cytokines such as TNF-α are increased in patients with heart failure (14, 21). Recent studies have demonstrated that cytokines induce CAT-2 gene expression in cardiac myocytes and vascular smooth muscle cells (11, 26). It is not known if CAT-2 expression is increased in heart failure. We hypothesized that CAT-2 expression and L-arginine

uptake are increased and contribute to the increased NO production in aortas of heart failure. To test this hypothesis, we employed a rat model of heart failure induced by myocardial infarction and investigated the role of L-arginine transport in NO production in the aortas of rats with heart failure.

MATERIALS AND METHODS

Animals. Experiments were initially conducted on male Sprague-Dawley rats (175–200 g). All animals were maintained on normal rat chow and were given water ad libitum in a 12:12-h light-dark cycle. The animals were caged individually after the initial surgical operation. This study protocol was approved by the Council on Animal Care at the University of Western Ontario.

Induction of heart failure. Heart failure was induced by left coronary artery ligation, as described previously (8, 9). Experiments were performed 8 wk after this surgery. Hemodynamic measurements were made under pentobarbital sodium anesthesia (50 mg/kg ip), as previously described (8, 9). Briefly, left ventricular end-diastolic pressure and left ventricular contractility were assessed by inserting a heparinized polyethylene catheter (PE-50; Becton-Dickson, Sparks, MD) in the left ventricle via the right carotid artery. The maximal rates of pressure development and relaxation were assessed with a pressure signal connected to a differentiator. Mean arterial pressure was evaluated using the same catheter in the right carotid artery, after removal from the left ventricle. All pressures and heart rates were measured with a Stratham pressure transducer (model P10 EZ) connected to a pressure monitor and were recorded on a Gould recorder (model 2400 S). All measurements were made in the anesthetized state after an equilibration period (20–30 min) for stabilization after completion of cannulations. At the end of the experiments, the animal was killed, and the heart weight, left ventricular volume and infarct size were measured as we have described previously (8, 9).

Tumor necrosis factor-α ELISA. Tumor necrosis factor-α (TNF-α) levels in aortas were measured using a solid-phase sandwich ELISA (Rat TNF-α Ultrasensitive Immunoassay Kit; Biosource International, Camarillo, CA) according to the manufacturer's instructions. Briefly, excised aortas were placed in ice-cold PBS (pH 7.4) containing protease inhibitors (0.25 mM phenylmethylsulfonyl fluoride, 4.5 μM leupeptin, and 5 μM aprotinin) and mechanically homogenized for 1 min (Polytron Homogenizer; Pro Scientific, Monroe, CT). Homogenates were subsequently centrifuged (10,000 g; 10 min) at 4°C. After the determination of protein concentration using the Lowry method, aliquots (100 μl) of lysates corresponding to 200 μg of total protein were added to microtiter plates coated with anti-TNF-α antibody. Next, a biotinylated antibody specific for TNF-α was added. Subsequently, streptavidin-peroxidase was added; the streptavidin-peroxidase binded to the biotinylated antibody. Finally, a stabilized chromogen was added for color development, and optical density was measured at 450 nm. The amount of TNF-α in each sample was determined from a linearized standard curve and was expressed as picograms per milligram of total protein.

L-Arginine uptake assay. Uptake of radioactive L-arginine in the rat aorta was measured according to previously described methods with modifications (1, 12). Immediately after the rat was killed, the aorta was carefully excised from the left renal artery in the abdomen to the aortic valve ring and placed in ice-cold Na^+-free, choline-containing Krebs bicarbonate. After the removal of all adherent adventitial tissue, the vessel was cut into six segments ~10–15 mm in length. Each segment was cut longitudinally in half, blotted dry, and weighed. In some experiments, the aortic tissues were incubated at 37°C for 3 h to deplete intracellular L-arginine (zero-*trans* conditions) before the uptake assay (15). To measure total uptake, aortic segments were incubated with 2 ml of L-[³H]arginine (10–500 μM) with specific activities of 0.5 μCi/ml. For nonspecific uptake measurements, aortic segments were incubated with L-[³H]arginine and 10 mM L-homoarginine, which competitively inhibits L-arginine uptake by system y^+ (12, 15). In some experiments, L-[³H]arginine uptake was competitively inhibited using L-lysine (10–800 μM). In other experiments, L-[³H]arginine was replaced with L-[³H]lysine, maintaining a constant specific activity of 0.5 μCi/ml, and L-[³H]lysine uptake was in turn competitively inhibited using L-arginine (10–800 μM). The aortic segments were incubated in an atmosphere of 5% CO_2 and 95% O_2 with continuous shaking at 37°C. After a 40-min incubation period, the segments were removed from the radioactive buffer and washed three times with 1-ml aliquots of ice-cold Na^+-free, choline-containing Krebs bicarbonate solution. Subsequently, each segment of the aorta was placed in a tube containing 2% (vol/vol) SDS solution and was heated at 100°C for 1 h. Radioactivity of the ambient incubation buffer and SDS solutions was measured by β-scintillation counting (Beckman, Mississauga, ON).

Determination of NOS activity. The activity of the NOS was determined through the conversion of L-[³H]arginine to L-[³H]citrulline based on modifications of previously described methods (25). The protein content of the tissue was measured by the method of Bradford (3). The activity of the

Table 1. *Primer sequences, Genbank accession numbers, target product sizes, annealing temperatures employed, and cycle numbers used*

Target Gene	Size, bp	Annealing Temperature, °C	No. of Cycles	Primer Sequence
CAT-1 (U70476)	529	64	24	Forward 5′-GATCCCACCAAGGGGAAAGG-3′ Reverse 5′-TTGAAGGGAAGGCATGGGAA-3′
CAT-2B (M62838)	510	66	35	Forward 5′-CTATGGCGGAGGATGGGTTG-3′ Reverse 5′-GGACAAGGAACAGGGCGAGA-3′
iNOS (D14051)	523	65	38	Forward 5′-AGCTCCCCATTCTGAAGCCC-3′ Reverse 5′-TGGAGCACGCTGAACACCTC-3′
GAPDH (M17701)	297	64/66	19	Forward 5′-AAAGGGCATCCTGGGCTACA-3′ Reverse 5′-CAGTGTTGGGGGGCTGAGTTG-3′

CAT, cationic amino acid transporter; iNOS, inducible nitric oxide synthase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase. Nos. in parentheses are accession nos.
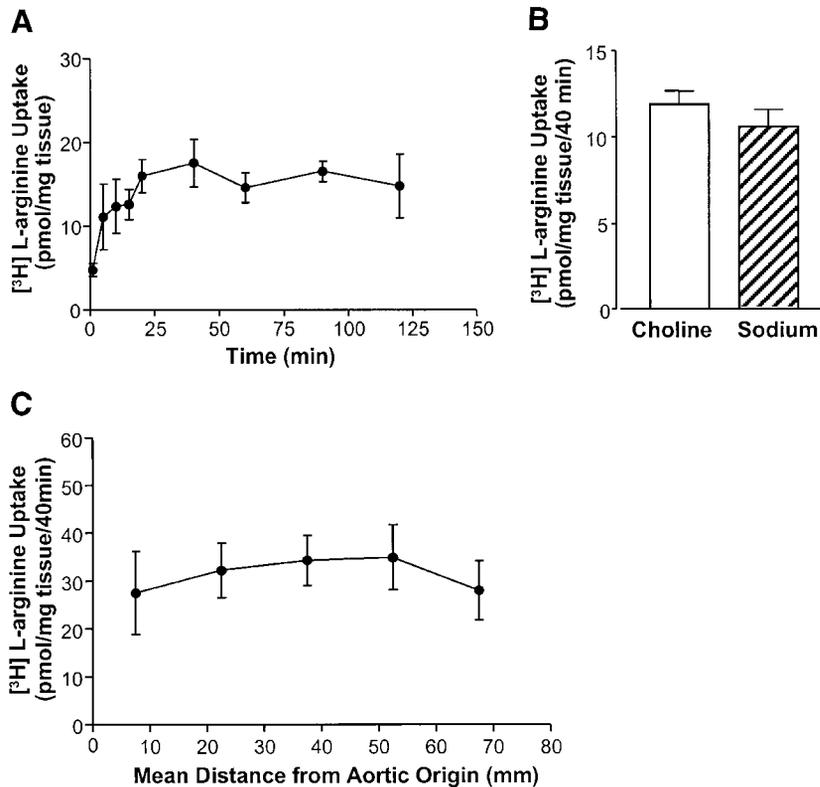
Fig. 1. Characterization of L-arginine uptake in normal rat aortas. *A*: time course for the uptake of 100 μM L-[³H]arginine (n = 4–5 experiments/time point). *B*: comparison of 100 μM L-[³H]arginine uptake using Na⁺-containing (n = 4) and Na⁺-free (choline-substituted; n = 5) incubation buffers. *C*: uptake of 200 μM L-[³H]arginine in different regions of aortas (n = 4/point).

calcium-dependent NOS was determined from the difference between the L-[³H]citrulline produced from samples containing calmodulin/calcium and those containing EDTA/EGTA. The activity of the calcium-independent NOS was determined as the total L-[³H]citrulline production from the aforementioned samples containing EDTA/EGTA.

RT-PCR. Total RNA isolated from fresh aortic tissue with Trizol reagent was reverse transcribed into first-strand cDNA with the utilization of the Moloney murine leukemia virus RT system. Evaluation of CAT-1 and CAT-2B mRNA expression was performed based on a "primer-dropping" method of PCR (30). To minimize competition for PCR substrates, iNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were amplified independently. Target gene accession numbers, reaction primer sequences, cycle numbers, and annealing temperatures are summarized in Table 1. Samples were then electrophoresed in gels containing ethidium bromide, illuminated, and subsequently analyzed via computer densitometry. All densitometric data were standardized to GAPDH mRNA.

iNOS Western blot analysis. iNOS protein expression in aortas was determined by Western blot analysis. Total proteins were extracted from aortas as described for the TNF-α ELISA. To each sample, SDS was added to a final concentration of 0.1%. Protein concentration was determined by the method of Lowry. Aliquots of lysate corresponding to 100 μg of total protein were mixed with loading buffer (1×) and boiled for 5 min. The samples were separated by SDS-PAGE (6% acrylamide/bisacrylamide). Proteins were transferred to nitrocellulose membranes (Hybond; Amersham Life Sciences, Oakville, ON) in the presence of glycine/methanol transfer buffer using the Mini Protean II Transfer System (Bio-Rad). Membranes were blocked in 5% nonfatty dry milk powder in PBS (pH 7.6) overnight at 4°C. Subsequently, the membranes were exposed to anti-mouse iNOS (Transduction Laboratories, Franklin Lakes, NJ) at a concentration of 1:500 in 2.5% milk in PBS (2 h; room temperature). The membranes were washed with PBS (pH 7.6) and 0.05% Tween 20 and exposed 1:2,000 to horseradish peroxidase-conjugated anti-mouse IgG in 2.5% milk in PBS (2 h; room temperature). After the wash, enhanced chemiluminscence detection reagents were employed to visualize peroxidase reaction products. iNOS was detected as a 120-kDa band.

Nitrate/nitrite measurements. Plasma nitrate/nitrite (NOₓ) levels were measured using *Aspergillus* nitrate reductase and the Griess reagent as we have described previously (8). The Griess reagent (2% sulfanilamide and 0.2% naphthylenediamine both in 5% phosphoric acid, 1:1 mixture) was used to react with nitrite in the sample, and the absorbance was determined at 540 nm with a spectrophotometer. The concentrations were calculated from a linearized standard curve derived from solutions of NaNO₃ (5–100 μmol/l). All animals were fasted for 18 h before the collection of plasma to eliminate any effects that rat chow nitrates and nitrites could potentially have on these experiments. Animals were given water containing 10% D-glucose during this fasting period.

In vitro NO production in aortas. The in vitro aortic NO production was measured according to a previously described method with modifications (28). Briefly, fresh thoracic aortas from rats with heart failure were isolated and placed in sterilized ice-cold PBS (pH 7.4). Under sterile conditions, aortas were cleared of adventitial tissue and washed three times in PBS. Subsequently, the aortas were cut longitudinally into four segments. After each segment was weighed individually, 1 ml of MEM culture medium alone or supplemented with 1 mM L-arginine, 1 mM L-arginine plus 10 mM L-lysine, 1 mM L-arginine plus 1 mM N^G-nitro-L-arginine methyl ester (L-NAME), or 1 mM D-arginine was separated into aliquots into four culture dishes. Aortic segments were transferred to the culture dishes and incubated at 37°C

Fig. 2. Effects of L-lysine and endothelium on L-arginine uptake in normal rat aortas. *A*: inhibition of 200 μM L-[³H]arginine uptake by increasing concentrations of L-lysine (*n* = 3). *B*: inhibition of 200 μM L-[³H]lysine uptake by increasing concentrations of L-arginine (*n* = 3). *C*: concentration-dependent uptake of L-[³H]arginine in aortas with intact (E+) and disrupted (E−; *n* = 4/group) endothelium. *D*: uptake of 200 μM L-[³H]arginine in aortas with intact and disrupted endothelium after 3 h of incubation in L-arginine-free medium to deplete intracellular L-arginine (*n* = 6/group). *P < 0.05.
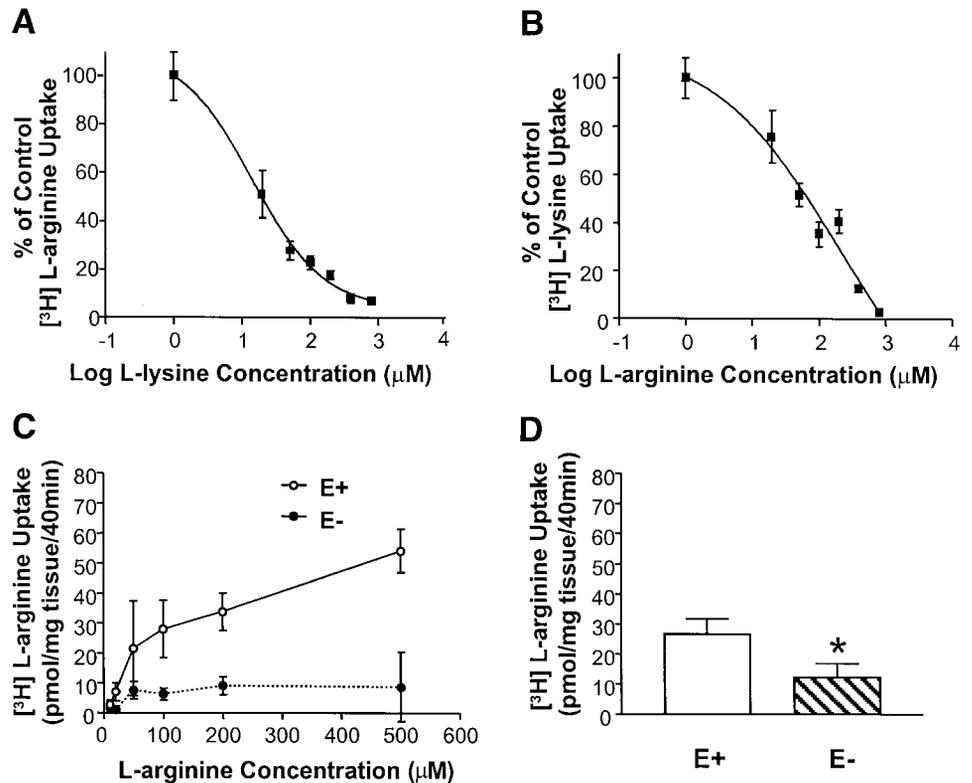
under atmospheric conditions of 5% CO_2 and 95% air. After a 24-h incubation time, the culture medium of each dish was collected in separate microcentrifuge tubes. The medium of each tube was subsequently dried (model SVC100H; Savant Speed Vacuum Concentrator), and the resulting pellets were redissolved with 100 μl of distilled water. Total nitrite and nitrate contained in each sample was measured using Griess reagent as described above.

Reagents. The *Aspergillus* nitrate reductase was purchased from Boehringer Mannheim (Laval, QU), and the 550° silicone oil was obtained from Dow Corning (London, ON). The L-[2,3,4,5-³H]arginine was purchased from Amersham Life Sciences. The L-[3,4-³H]lysine was from ICN Pharmaceuticals (Irvine, CA). MEM culture medium without phenyl red and without L-glutamine, TRIzol reagent, Moloney murine leukemia virus, and *Taq* polymerases were purchased from GIBCO-BRL (Burlington, ON). All other chemicals were purchased from Sigma (Oakville, ON).

Statistical analysis. Data are expressed as means ± SE. Differences between heart failure and sham control groups were compared using unpaired Student's *t*-test or Mann-Whitney rank sum test. For multigroup comparisons, ANOVA followed by the Student-Newman-Keuls test was performed (Sigma Stat version 1.1; Jandel). A two-tailed *P* value <0.05 was considered statistically significant.

RESULTS

Characterization of L-arginine uptake in normal rat aortas. Incubation of normal rat aortas with L-[³H]arginine resulted in a time- and concentration-dependent increase in L-arginine uptake that was Na^+ independent (Fig. 1, *A* and *B*). Peak uptake of L-[³H]arginine was ~40 min after incubation. Hence, a 40-min incubation time was used for all subsequent experiments.

Uptake of L-[³H]arginine was similar in different segments from the aortic root to the abdominal aorta, suggesting that there is no regional difference in L-arginine uptake in aortas (Fig. 1*C*). L-[³H]arginine uptake was inhibited completely by L-lysine in a concentration-dependent manner, and vice versa uptake of L-[³H]lysine was inhibited completely by increasing

Table 2. *Basal hemodynamic characteristics in rats with heart failure and sham operation*

	Heart Failure	Sham	*P*
Weight			
Body weight, g	511 ± 11(23)	520 ± 12(26)	NS
Heart, g	1.81 ± 0.06(23)	1.40 ± 0.03(26)	<0.01
Heart-to-body ratio, mg/g	3.57 ± 0.11(23)	2.71 ± 0.05(26)	<0.01
Left ventricle, g	1.15 ± 0.03(23)	0.99 ± 0.02(26)	<0.01
Right ventricle, g	0.41 ± 0.03(23)	0.27 ± 0.01(26)	<0.01
Left ventricle volume, μl	196 ± 44(9)	58 ± 10(7)	<0.05
MAP, mmHg	97 ± 5(10)	109 ± 3(10)	<0.05
HR, beats/min	387 ± 19(10)	395 ± 12(10)	NS
LVEDP, mmHg	21 ± 2(10)	4 ± 2(10)	<0.01
CI, ml·min⁻¹·100 g⁻¹	17 ± 2(10)	29 ± 2(10)	<0.01
+dP/d*t*, mmHg/s	7,964 ± 413(9)	10,383 ± 252(7)	<0.01
−dP/d*t*, mmHg/s	6,821 ± 326(9)	10,562 ± 482(7)	<0.01
Infarct size, %	42 ± 2(23)	0 (26)	

Values are means ± SE; no. of rats in parentheses. MAP, mean arterial pressure; HR, heart rate; LVEDP, left ventricular end-diastolic pressure; CI, cardiac index; dP/d*t*, first derivative of the left ventricular pressure with plus (+) indicating maximum rate and minus (−) indicating relaxation. Significance indicates comparisons between the two groups by unpaired Student's *t*-test. NS, not significant.
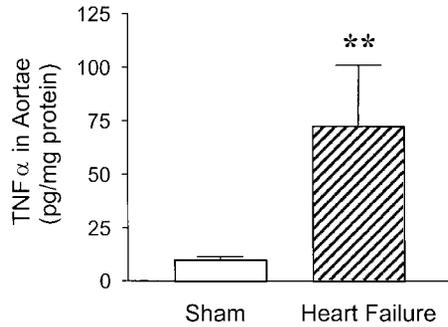
Fig. 3. Tumor necrosis factor-α (TNF-α) in aortas of rats with heart failure ($n = 5$) and sham-operated rats ($n = 5$). TNF-α protein content in aortic tissue was determined by ELISA. Aortas from rats with heart failure had markedly higher TNF-α levels vs. sham controls. **$P < 0.01$.

Fig. 4. L-Arginine uptake in aortas of rats with heart failure ($n = 7$) and sham-operated rats ($n = 6$). Uptake of 200 μM L-[^{3}H]arginine in aortas was carried out after 3 h of incubation in L-arginine-free medium to deplete intracellular L-arginine. **$P < 0.01$. Removal of endothelium from aortas of rats with heart failure ($n = 5$) significantly reduced L-[^{3}H]arginine uptake. *$P < 0.05$.

concentrations of L-arginine in rat aortas (Fig. 2, *A* and *B*).

To assess the contribution of the endothelium to L-arginine uptake in rat aortas, the vessel endothelium was disrupted by scraping with a scalpel blade. A concentration-dependent increase in L-[^{3}H]arginine uptake was observed in normal rat aortas with intact endothelium. However, L-arginine uptake was decreased significantly in endothelium-denuded vessels ($P < 0.05$; Fig. 2*C*). Preincubation of aortas in Krebs buffer for 3 h to deplete intracellular amino acids did not significantly alter L-[^{3}H]arginine uptake at 200 μM

concentration of L-arginine in either endothelium-intact or -denuded aortas. However, a significant difference in L-[^{3}H]arginine uptake was still present between endothelium-intact and endothelium-denuded aortas ($P < 0.05$; Fig. 2*D*).

General characteristics of heart failure. The infarct size of coronary artery-ligated rats was 42 $\pm$ 2% ($n =$
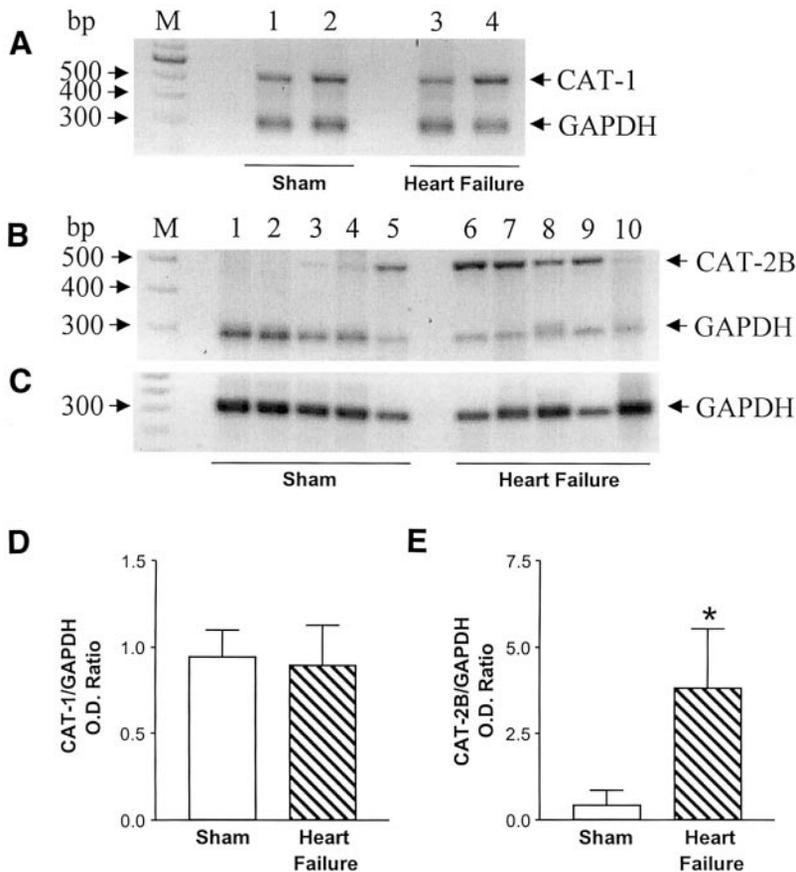
Fig. 5. Expression of cationic amino acid transporter (CAT)-1 and CAT-2B mRNA in aortas of rats with heart failure by RT-PCR. *A*: representative gel of CAT-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) coamplification. The PCR products from rats with heart failure and sham rats ($n = 2$/group) were electrophoresed through a 1.5% agarose gel. M, molecular marker. *B*: representative gel of CAT-2B and GAPDH coamplification. *C*: amplification of GAPDH alone. *D*: CAT-1-to-GAPDH optical density (OD) ratio. No significant difference was observed between heart failure ($n = 4$) and sham ($n = 4$) aortas. *E*: CAT-2B-to-GAPDH optical density ratio ($n = 5$/group). *$P < 0.05$.

23) of the left ventricle. No myocardial infarction was observed in sham-operated control rats ($n = 26$). Rats with heart failure had signs of cardiac dilatation, pulmonary and hepatic congestion, pleural effusion, and ascites. In heart failure, the weight of the whole heart and left and right ventricles was increased significantly compared with sham-operated rats ($P < 0.01$), whereas body weight was similar between the two groups [P = not significant (NS); Table 2]. Ten heart failure and 10 sham-operated rats were chosen randomly for hemodynamic measurements. Cardiac index was decreased, whereas left ventricular end-diastolic pressure was significantly increased in rats with heart failure ($P < 0.01$; Table 2). These hemodynamic changes in coronary artery-ligated rats were characteristic of clinical heart failure.

TNF-α levels in aortas of rats with heart failure. To determine that heart failure was associated with altered cytokine levels, aortic TNF-α was assessed by ELISA. Indeed, average levels of TNF-α in the aortic tissue of rats with heart failure were six times higher than in sham-operated rats ($P < 0.01$; Fig. 3).

L-Arginine uptake in aortas of rats with heart failure. To examine whether the functional transport of L-arginine is altered in aortas of rats with heart failure, uptake studies of L-[³H]arginine were carried out. Uptake experiments were performed with 200 μM L-arginine, a concentration reflective of the basal L-arginine plasma level in normal rats as we demonstrated in our previous studies (8). The data from endothelium-intact aortas showed a 40% increase in L-[³H]arginine uptake in rats with heart failure compared with sham-oper-

ated controls ($P < 0.01$; Fig. 4). Removal of endothelium from aortas of rats with heart failure significantly reduced L-[³H]arginine uptake ($P < 0.05$; Fig. 4).

CAT mRNA expression in aortas of rats with heart failure. PCR reactions were carried out at linear amplifications for CAT-1, CAT-2B, and GAPDH. With the use of a primer-dropping method, two distinct bands coamplified from equal aliquots of cDNA were observed for each aortic sample (Fig. 5, *A* and *B*). CAT-1-to-GAPDH density ratios revealed no significant differences between rats with heart failure and sham rats (P = NS; Fig. 5D). However, the CAT-2B-to-GAPDH density ratio was significantly increased in aortas of heart failure compared with sham-operated controls ($P < 0.05$; Fig. 5E). Interestingly, GAPDH levels seem to vary with the amount of CAT-2B present, where greater amounts of CAT-2B amplification seem to compete with GAPDH coamplification. To confirm that equal amounts of cDNA were being amplified in each sample, GAPDH was amplified alone for 19 cycles. Indeed, it was demonstrated that similar amounts of cDNA were present in each sample undergoing the coamplification procedure (Fig. 5C).

Expression of iNOS protein and activity in aortas of rats with heart failure. The cDNA of iNOS and GAPDH from aortas was amplified by PCR. PCR products of iNOS and GAPDH are shown in Fig. 6. The density ratios of iNOS/GAPDH were increased significantly in heart failure compared with sham controls ($P < 0.05$; Fig. 6D). Additionally, marked iNOS protein expression was observed in aortas from rats with heart failure compared with sham controls (Fig. 6C). Calcium-
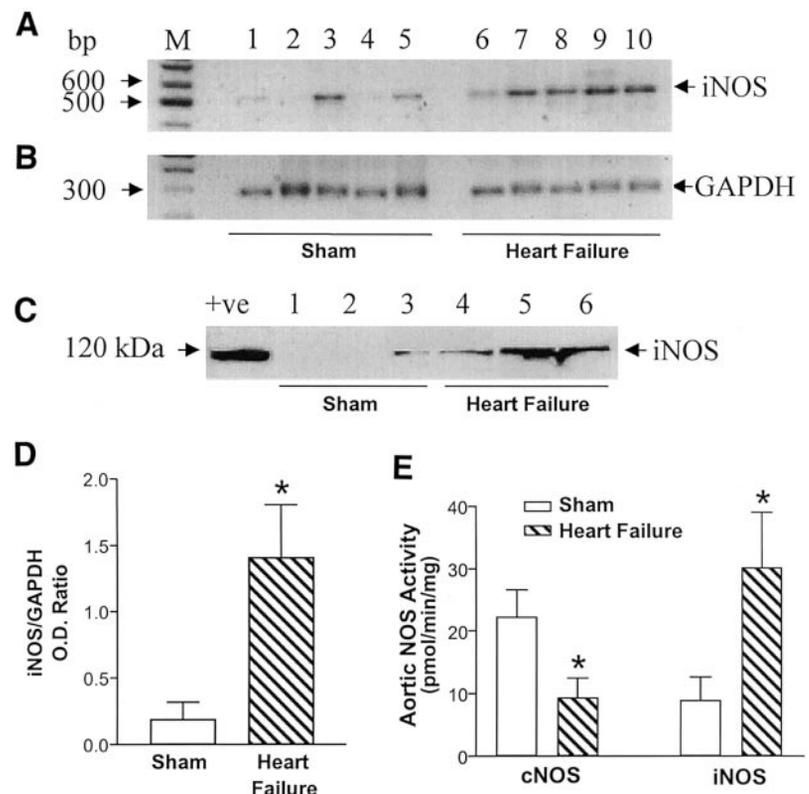
Fig. 6. Expression of inducible nitric oxide synthase (iNOS) protein and activity in aortas of rats with heart failure. The RT-PCR products from rats with heart failure and sham rats were electrophoresed through a 1.5% agarose gel. *A*: iNOS amplification. *B*: GAPDH amplification. *C*: iNOS protein expression in aortas of rats with heart failure and sham-operated controls ($n = 3$/group). *D*: iNOS-to-GAPDH optical density ratio ($n = 5$/group). *E*: calcium-dependent and -independent nitric oxide synthase (NOS) activity in the aorta of rats with heart failure and sham rats. Aortas from 2–4 rats were used for each assay. Eight assays were performed in each group. cNOS, calcium-dependent NOS. *$P < 0.05$ vs. sham.

dependent and -independent NOS activities were determined in aortas by the L-arginine-L-citrulline conversion assay. Calcium-dependent NOS activity was decreased in heart failure ($P < 0.05$), whereas calcium-independent NOS activity was increased in heart failure compared with sham-operated rats ($P < 0.05$; Fig. 6E).

Effects of L-arginine transport on NO production in heart failure. To examine the role of L-arginine uptake in NO production, aortic segments from rats with heart failure were treated in culture with supplemental L-arginine, or with L-arginine plus L-lysine, a competitive inhibitor of L-arginine uptake through system y^+. Segments treated with D-arginine or in the culture medium alone were used as controls. Culture medium from the segments containing supplemental L-arginine demonstrated a significant increase in NO_x levels compared with control segments (278 ± 21 vs. 82 ± 24 pmol/mg aortic tissue, $P < 0.05$; Fig. 7). This effect of L-arginine was inhibited completely by L-lysine and L-NAME. D-Arginine is not a substrate of NOS nor is it transported through system y^+; moreover, this enantiomer did not alter NO_x levels in the medium compared with the control segments ($P = $ NS; Fig. 7). When the endothelium was removed, L-arginine did not increase NO_x production in the aortas of rats with heart failure ($P = $ NS; Fig. 7).

Plasma nitrate and nitrite levels in heart failure. Plasma NO_x levels were measured in rats with heart failure and sham-operated controls to assess total NO production. To eliminate the effects of diet on NO_x measurements, both rats with heart failure and sham rats were fasted for 18 h before blood samples were taken. During the fasting period, 10% glucose as drinking water was accessible to all rats. There was a greater than twofold increase in plasma NO_x levels in rats with heart failure compared with sham-operated
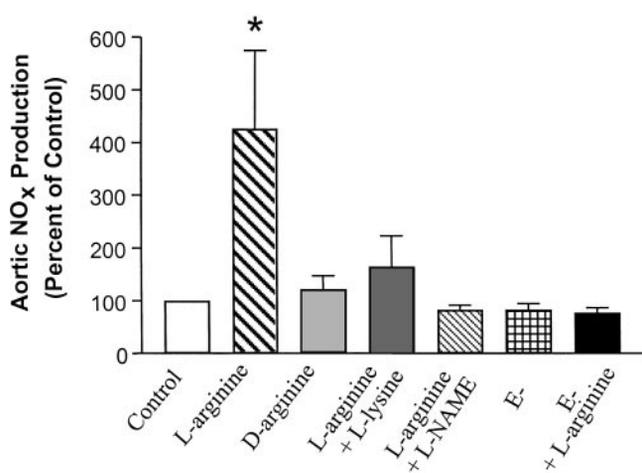
Fig. 7. Effects of L-arginine uptake on nitrate/nitrite (NO_x) production (%control) in aortas of rats with heart failure. Treatment with L-arginine (1 mM) significantly increased NO_x production (*$P < 0.05$). This effect was inhibited by 10 mM L-lysine or 1 mM N^G-nitro-L-arginine methyl ester (L-NAME). D-Arginine (1 mM) had no effect. Removal of endothelium abolished the effects of L-arginine. Control, MEM medium with no amino acid supplementation ($n = 4$–6/group).
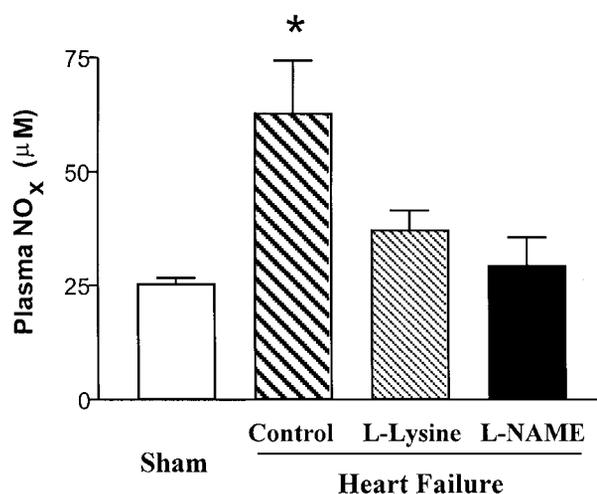
Fig. 8. Plasma NO_x concentrations in sham rats and rats with heart failure. All rats were fasted for 18 h and were treated with 10% D-glucose alone or with 9.1 g/l (50 mmol/l) L-lysine or 100 mg/l L-NAME added to their 10% D-glucose drinking water ($n = 5$–8/group). *$P < 0.05$ vs. all groups.

controls ($P < 0.05$; Fig. 8). Treatment with L-lysine (9.1 g/l) or L-NAME (100 mg/l) in 10% glucose drinking water during the 18-h fasting period significantly decreased plasma NO_x levels in rats with heart failure ($P < 0.05$; Fig. 8).

DISCUSSION

In the present study, we demonstrated for the first time that functional L-arginine uptake and CAT-2B expression are significantly increased in aortas of rats with heart failure. We also demonstrated that NO production and iNOS expression are increased in aortas of rats with heart failure. Furthermore, selective inhibition of L-arginine uptake decreases NO production in aortas and plasma NO_x levels in heart failure. These results suggest that upregulation of both L-arginine uptake and iNOS activity is required for increased NO production in rats with heart failure.

Uptake of cationic amino acids in mammalian cells occurs primarily through system y^+ (5). Studies have shown that anywhere from 70 to 90% of the measurable uptake of L-arginine in pulmonary endothelial cells occurs via system y^+ (12). In the present study, the incubation buffers containing Na^+ or free of Na^+ demonstrated similar uptake in rat aortas. L-Arginine uptake was inhibited competitively by increasing concentrations of another basic amino acid, L-lysine, which is transported via system y^+ but does not interfere with metabolic pathways of L-arginine (2, 12). Furthermore, L-arginine uptake was decreased markedly after removal of the endothelium. The results suggest that L-arginine uptake in rat aortas is through system y^+ and that the endothelium is the major site of uptake. In rats with heart failure, aortic L-arginine uptake was increased. When the endothelium was removed, L-arginine uptake was decreased, suggesting that endothelium contributes significantly to the increased L-arginine uptake in the aorta of heart failure.

Transporters responsible for L-arginine uptake have recently been cloned and identified. CAT-1 mRNA expression has been demonstrated to be ubiquitous in mammalian tissue, whereas CAT-2 mRNA expression has been reported to be physiologically regulated (11, 22). Furthermore, CAT-1 and CAT-2B isoforms are high-affinity transporters of system y^+, whereas CAT-2A is a low-affinity transporter (6). This investigation evaluated mRNA expression of the high-affinity CAT-1 and CAT-2B transporters. We demonstrated that both CAT-1 and CAT-2B transporters were present in the rat aortas. Although CAT-1 mRNA expression was not altered, CAT-2B mRNA expression was significantly increased in the aortas of rats with heart failure. These data suggest that increased expression of CAT-2B may contribute to the increased L-arginine uptake in the aortas of rats with heart failure.

Interestingly, Hanssen et al. (15) have recently demonstrated that L-arginine uptake is increased in erythrocytes of patients with heart failure. Although eNOS protein has been demonstrated to be present in erythrocytes by some studies, the significance of NO production by erythrocytes is still not clear (4). In a separate study, we also demonstrated an increased L-arginine uptake in erythrocytes of rats with heart failure (unpublished data). Thus changes of L-arginine uptake in erythrocytes seem to parallel those of aortas in heart failure. However, the biochemical pathways of L-arginine, especially the contribution of the L-arginine/NO pathway in erythrocytes, remains to be determined.

Heart failure is associated with dysfunction of the endothelium. Decreased endothelium-dependent relaxation has been documented in the peripheral arteries of a pacing-induced heart failure dog model (20) and in thoracic aortas and hindquarter resistance arteries in the infarction-induced heart failure rat model by numerous investigators, including ourselves (7–9). In the present study, calcium-dependent NOS activity was also decreased in the aortas of these rats with heart failure, suggesting that the attenuated vasodilation is due to a decrease of eNOS activity. Paradoxically, plasma nitrate and nitrite, stable metabolites of NO, were increased significantly, suggesting increased basal NO production. We further demonstrated that iNOS mRNA and protein expression and iNOS activity were increased in aortas of rats with heart failure. Our data suggest that the increased iNOS expression and activity contribute to increased NO production in this heart failure rat model.

The role of L-arginine transport in NO production in the aortas of heart failure has not been demonstrated previously. In the present study, aortic segments from rats with heart failure were treated with D-arginine and L-arginine with and without L-lysine, a competitive inhibitor of L-arginine uptake through system y^+. A novel finding of our study is that competitive inhibition of L-arginine uptake by L-lysine decreases NO production in aortas of rats with heart failure. The results indicate that the iNOS enzyme relies on a continuous L-arginine supply from the extracellular compartment,

and L-arginine transport is essential for the enhanced NO production as a result of increased iNOS expression. L-NAME inhibits NOS activity without any effects on L-arginine transport (2). We further demonstrated that L-NAME and L-lysine independently inhibited NO production in aortas in vitro and decreased plasma nitrate and nitrite levels in vivo in rats with heart failure. These data strongly suggest that increased activities of both iNOS and L-arginine uptake are required for increased NO production in the aortas of rats with heart failure.

Mechanisms of the increased iNOS and CAT-2B expression in heart failure are still not fully understood. Cytokines such as TNF-α have been implicated in the pathogenesis of many heart diseases, including acute myocardial infarction (18). In patients with heart failure, circulating levels of TNF-α are elevated markedly (21). Furthermore, strong immunoreactivity for TNF-α colocalizes with iNOS in myocardial tissue of patients with dilated cardiomyopathy, suggesting that TNF-α is associated with iNOS expression (14). We recently showed that TNF-α induces iNOS expression in cultured neonatal cardiomyocytes (27). Studies have demonstrated that expression of CAT-2 mRNA is increased in vascular smooth muscle cells, cardiac myocytes, and activated macrophages upon treatment with cytokines (11, 26). In the present study, we demonstrated for the first time that TNF-α levels were increased in the aortas of rats with heart failure compared with sham controls. Our data suggest that increased production of cytokines may play an important role in iNOS and CAT-2B induction in the aortas of heart failure. Other factors may also be involved in the complex regulation of iNOS and CAT-2B induction in heart failure. Among these, activation of ANG II and α-adrenergic receptors, activation of protein kinase C isoforms, and increases in cAMP, all of which are important components of the neurohumoral activation characteristic of heart failure, may promote both iNOS and CAT-2B expression (17, 22, 24). Infiltration of macrophages expressing iNOS represents another mechanism whereby iNOS activity may be increased in heart failure (10). Thus increased cytokines and the associated promoting factors and activated macrophages may contribute significantly to the increased iNOS and CAT-2B expression in heart failure.

In summary, the present study demonstrated that in rats with heart failure TNF-α levels, functional L-arginine uptake, and molecular expression of CAT-2B are increased in aortas. We also demonstrated that basal NO production and aortic iNOS expression are increased. Furthermore, selective inhibition of L-arginine uptake decreases NO production in aortas and plasma NO_x levels. These results suggest that upregulation of both L-arginine uptake and iNOS activity is associated with increased NO production in rats with heart failure. Because increased NO production is dependent on increased L-arginine uptake, we conclude that L-arginine transport plays an important role in enhanced NO production in heart failure.

REFERENCES

1. **Abbott RE and Schachter D.** Regional differentiation in rat aorta: L-arginine metabolism and cGMP content in vitro. *Am J Physiol Heart Circ Physiol* 266: H2287–H2295, 1994.
2. **Bogle RG, Moncada S, Pearson JD, and Mann GE.** Identification of inhibitors of nitric oxide synthase that do not interact with the endothelial cell L-arginine transporter. *Br J Pharmacol* 105: 768–770, 1992.
3. **Bradford MM.** A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* 72: 248–254, 1976.
4. **Chen LY and Mehta JL.** Evidence for the presence of L-arginine-nitric oxide pathway in human red blood cells: relevance in the effects of red blood cells on platelet function. *J Cardiovasc Pharmacol* 32: 57–61, 1998.
5. **Christensen HN.** Role of amino acid transport and countertransport in nutrition and metabolism. *Physiol Rev* 70: 43–77, 1990.
6. **Closs EI, Lyons CR, Kelly C, and Cunningham JM.** Characterization of the third member of the MCAT family of cationic amino acid transporters. Identification of a domain that determines the transport properties of the MCAT proteins. *J Biol Chem* 268: 20796–20800, 1993.
7. **Drexler H and Lu W.** Endothelial dysfunction of hindquarter resistance vessels in experimental heart failure. *Am J Physiol Heart Circ Physiol* 262: H1640–H1645, 1992.
8. **Feng Q, Fortin AJ, Lu X, and Arnold JMO.** Effects of L-arginine on endothelial and cardiac function in rats with heart failure. *Eur J Pharmacol* 376: 37–44, 1999.
9. **Feng Q, Lu XR, Fortin AJ, Pettersson A, Hedner T, Kline RL, and Arnold JMO.** Elevation of an endogenous inhibitor of nitric oxide synthesis in experimental congestive heart failure. *Cardiovasc Res* 37: 667–675, 1998.
10. **Fukuchi M, Hussain SNA, and Giaid A.** Heterogeneous expression and activity of endothelial and inducible nitric oxide synthases in end-stage human heart failure. *Circulation* 98: 132–139, 1998.
11. **Gill DJ, Low B, and Grigor MR.** Interleukin-1b and tumor necrosis factor-a stimulate the CAT-2 gene of the L-arginine transporter in cultured vascular smooth muscle cells. *J Biol Chem* 271: 11280–11283, 1996.
12. **Greene B, Pacitti AJ, and Souba WW.** Characterization of L-arginine transport by pulmonary artery endothelial cells. *Am J Physiol Lung Cell Mol Physiol* 264: L351–L356, 1993.
13. **Habib F, Dutka D, Crossman D, Oakley CM, and Cleland JG.** Enhanced basal nitric oxide production in heart failure: another failed counter-regulatory vasodilator mechanism? *Lancet* 344: 371–373, 1994.
14. **Habib FM, Springall DR, Davies GJ, Oakley CM, Yacoub MH, and Polak JM.** Tumor necrosis factor and inducible nitric oxide synthase in dilated cardiomyopathy. *Lancet* 347: 1151–1155, 1996.
15. **Hanssen H, Brunini TM, Conway M, Banning AP, Roberts NB, Mann GE, Ellory JC, and Mendes Ribeiro AC.** Increased L-arginine transport in human erythrocytes in chronic heart failure. *Clin Sci (Lond)* 94: 43–48, 1998.
16. **Haywood GA, Tsao PS, Von LHE, Mann MJ, Keeling PJ, Trindade PT, Lewis NP, Byrne CD, Rickenbacher PR, Bishopric NH, Cooke JP, McKenna WJ, and Fowler MB.** Expression of inducible nitric oxide synthase in human heart failure. *Circulation* 93: 1087–1094, 1996.
17. **Ikeda U, Murakami Y, Kanbe T, and Shimada K.** Alpha-adrenergic stimulation enhances inducible nitric oxide synthase expression in rat cardiac myocytes. *J Mol Cell Cardiol* 28: 1539–1545, 1996.
18. **Irwin MW, Mak S, Mann DL, Qu R, Penninger JM, Yan A, Dawood F, Wen WH, Shou Z, and Liu P.** Tissue expression and immunolocalization of tumor necrosis factor-a in postinfarction dysfunctional myocardium. *Circulation* 99: 1492–1498, 1999.
19. **Kim JW, Closs EI, Albritton LM, and Cunningham JM.** Transport of cationic amino acids by the mouse ecotropic retrovirus receptor. *Nature* 352: 725–728, 1991.
20. **Kiuchi K, Sato N, Shannon RP, Vatner DE, Morgan K, and Vatner SF.** Decreased β-adrenergic receptor- and endothelium-mediated vasodilation in conscious dogs with heart failure. *Circ Res* 73: 1013–1023, 1993.
21. **Levine B, Kalman J, Mayer L, Fillit HM, and Packer M.** Elevated circulating levels of tumor necrosis factor in severe chronic heart failure. *N Engl J Med* 323: 236–241, 1990.
22. **Low BC and Grigor MR.** Angiotensin II stimulates system y+ and cationic amino acid transporter gene expression in cultured vascular smooth muscle cells. *J Biol Chem* 270: 27577–27583, 1995.
23. **Moncada S, Palmer RM, and Higgs EA.** Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 43: 109–142, 1991.
24. **Oddis CV, Simmons RL, Hattler BG, and Finkel MS.** cAMP enhances inducible nitric oxide synthase mRNA stability in cardiac myocytes. *Am J Physiol Heart Circ Physiol* 269: H2044–H2050, 1995.
25. **Scott JA, Machoun M, and McCormack DG.** Inducible nitric oxide synthase and vascular reactivity in rat thoracic aorta: effect of aminoguanidine. *J Appl Physiol* 80: 271–277, 1996.
26. **Simmons WW, Closs EI, Cunningham JM, Smith TW, and Kelly RA.** Cytokines and insulin induce cationic amino acid transporter (CAT) expression in cardiac myocytes. Regulation of L-arginine transport and no production by CAT-1, CAT-2A, and CAT-2B. *J Biol Chem* 271: 11694–11702, 1996.
27. **Song W, Lu X, and Feng Q.** Tumor necrosis factor-α induces apoptosis in cardiac myocytes via inducible nitric oxide synthase. *Cardiovasc Res* 45: 595–602, 2000.
28. **Wang BY, Ho HK, Lin PS, Schwarzacher SP, Pollman MJ, Gibbons GH, Tsao PS, and Cooke JP.** Regression of atherosclerosis: role of nitric oxide and apoptosis. *Circulation* 99: 1236–1241, 1999.
29. **Wang H, Kavanauhg MP, North RA, and Kabat D.** Cell-surface receptor for ecotropic murine retroviruses is a basic amino-acid transporter. *Nature* 352: 729–731, 1991.
30. **Wong H, Anderson WD, Cheng T, and Riabowol KT.** Monitoring mRNA expression by polymerase chain reaction: the "primer-dropping" method. *Anal Biochem* 223: 251–258, 1994.