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Research Article

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Increased Angiotensin-I Converting Enzyme Gene Expression in the Failing Human Heart

Quantification by Competitive RNA Polymerase Chain Reaction

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Abstract

Local activation of the components of the renin angiotensin system in the heart is regarded as an important modulator of cardiac phenotype and function; however, little is known about their presence, regulation, and potential activation in the human heart. To investigate the gene expression of major angiotensin-II–forming enzymes in left ventricles of normal ($n = 9$) and failing human hearts ($n = 20$), we established a competitive RNA–polymerase chain reaction (PCR) for mRNA quantification of angiotensin-I converting enzyme (ACE) and human heart chymase. For each gene, competitor RNA targets with small internal deletions were used as internal standards to quantify the original number of transcripts and to control reverse transcription and PCR. In PCR, each target and the corresponding competitor were amplified by competing for the same primer oligonucleotides. The variability of ACE RNA-PCR was 11% indicating a high reproducibility of this method. In addition, ACE mRNA levels obtained by competitive RNA-PCR correlated favorably with traditional slot blot hybridization ($r = 0.69$, $n = 10$; $P < 0.05$). Compared with nonfailing hearts, the number of ACE transcripts referred to 100 ng of total RNA was increased threefold in patients with chronic heart failure (4.2 ± 2.5 vs. $12.8 \pm 6 \times 10^5$; $P < 0.0005$). In contrast, no significant difference was found in chymase gene expression between normal and failing hearts. Thus, the expression of the cardiac ACE but not of human heart chymase is upregulated in failing human heart indicating an activation of the cardiac renin–angiotensin system in patients with advanced heart failure. (*J. Clin. Invest.* 1994. 94:301–310.)

Key words: angiotensin-I converting enzyme mRNA • human heart chymase mRNA • dilated cardiomyopathy • coronary artery disease • cardiac renin angiotensin system

Introduction

Evidence has been presented for an endogenous tissue renin angiotensin system in the heart by demonstrating the cardiac gene expression of all components of this system (for review see references 1–3), including angiotensinogen (4–6), angiotensin-II receptors (7, 8), renin (9–11), angiotensin-I converting enzyme (ACE) (12, 13) and other angiotensin-II forming enzymes such as the human heart chymase (14–16). Recent studies have shown that the cardiac expression of angiotensinogen and ACE is increased in experimental heart failure (12, 13, 17, 18). Cardiac ACE activity as well as the rate of angiotensin-II production is enhanced in hypertrophied rat hearts (12) and associated with altered diastolic properties possibly contributing to the vulnerability of diastolic function in hypertrophy during hypoperfusion (19). Other potential actions of the cardiac renin angiotensin system include a trophic effect, as suggested by identifying angiotensin-II as a growth-promoting factor in cardiac muscle cells (20–22). This hypothesis is supported by recent data demonstrating that ACE inhibitors given in very low doses not affecting blood pressure prevented the development of cardiac hypertrophy in pressure-overloaded rats (23, 24). These experimental observations raise the possibility that the beneficial effects of ACE inhibitors in patients with heart failure may not only be due to neuroendocrine and hemodynamic modulation, but also to the interference with an activated tissue cardiac renin angiotensin system. In this regard, Foult et al. (25) have shown that the intracoronary infusion of an ACE inhibitor in patients with dilated cardiomyopathy resulted in a fall of coronary vascular resistance and a reduction in left ventricular diastolic pressure, consistent with the hypothesis that an activated cardiac tissue renin–angiotensin system may modulate coronary perfusion and diastolic function in humans.

An alternative pathway for the conversion of angiotensin-I to angiotensin-II in the human heart is achieved by the human heart chymase (26) which has been purified and cloned recently (14, 15). This chymotrypsin-like serine proteinase is the most efficient and specific angiotensin-II–forming enzyme described and not inhibited by ACE inhibitors (14, 16). Recently, Urata et al. (27) have delineated localization and expression of human heart chymase in the human myocardium, suggesting that mRNA level is unchanged in human heart failure. In contrast, little is known about the regulation and potential activation of the components of the renin–angiotensin system in the human heart. Recently, the expression of the angiotensinogen gene and its localization in the human heart has been reported, yet without reference to its abundance in diseased myocardium (28).

In this study we investigated the gene expression of ACE and human heart chymase in failing and nonfailing human

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1. *Abbreviations used in this paper:* ACE, angiotensin-I converting enzyme; ANF, atrial natriuretic factor; CHF, congestive heart failure; RAS, renin–angiotensin system.

hearts by using the quantitative method of competitive RNA-PCR (29–31). By adding a known quantity of competitor RNA as an internal standard to the reaction, this method allowed the detection and quantification of low abundant transcripts (31). Using this method, we demonstrate that the gene expression of cardiac ACE is upregulated in failing human hearts while in the same tissues the gene expression of chymase remains unchanged.

Methods

Patients

Failing human hearts were obtained from patients undergoing heart transplantation including 13 patients with dilated cardiomyopathy and 7 patients with coronary artery disease (see Table I). All patients were in New York Heart Association (NYHA) functional class IV heart failure and demonstrated abnormal pretransplant hemodynamics (see Table I). Nonfailing control hearts (see Table II) were obtained from organ donors whose hearts could not be used for transplantation because of the lack of a suitable recipient. Tissue samples of the left ventricular free wall were taken at the time of explantation, immediately frozen in liquid nitrogen and stored at −80°C until use. Care was taken not to take scarred, fibrotic, or adipose tissue, endocardium, epicardium, or great vessels. This study was approved by the Ethical Committee of the University of Freiburg.

RNA preparation

Frozen left ventricular tissue was dismembrated in a Mikro-Dismembrator II (B. Braun Melsungen Inc., Melsungen, FRG). Total cellular RNA was isolated from dismembrated frozen tissue by the method of acid guanidinium thiocyanate/phenol/chloroform extraction (32) and stored in diethyl pyrocarbonate-treated water at −20°C until use. The amount of RNA was evaluated by absorption at 260 nm, using a DU-65 spectrophotometer (Beckman Instruments Ltd., Munich, FRG). The ratio of absorption (260:280 nm) of all preparations was between 1.8 and 2.0. Finally, all samples were subjected to gel electrophoresis and stained with ethidium bromide to prove the integrity and the amount of the 18 and 28 S rRNA by densitometric scanning (Personal densitometer; Molecular Dynamics Ltd., Krefeld, FRG) of a Polaroid 665 negative film (Polaroid Ltd., Offenbach, FRG).

Isolation of poly(A)$^+$ RNA

Poly(A)$^+$ RNA was purified from total RNA by an oligo (dT)-cellulose column. The column material was prepared according to the supplier's recommendation (Boehringer, Mannheim, FRG). Samples of total RNA were first heat-denatured (65°C), then adjusted to an appropriate salt concentration (0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA), and incubated with the column material for two hours at room temperature by rotation. Then, the whole mixture was filled into a Poly-Prep chromatography column (Bio-Rad Ltd., Munich, FRG). Unbound RNA was removed by several washes with high-salt buffer (0.5 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA) followed by several washes with low-salt buffer (0.1 M NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM EDTA). The poly(A)$^+$ RNA was recovered by using elution buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA) prewarmed to 65°C.

Slot blot analysis

Poly(A)$^+$ RNA samples were prepared according to standard protocols (33) and spotted to a nylon membrane (Amersham Buchler Ltd., Braunschweig, FRG) using a slot blot apparatus (Serva Ltd., Heidelberg, FRG). The RNA was fixed to the membrane by UV irradiation. Hybridization was first done with the human ACE cDNA and then with a $(dT)_{25}$-oligonucleotide to control for the quantity of mRNA.

To obtain a radioactive-labeled ACE cDNA probe, the human ACE cDNA clone pB35-19 (34) was cut with EcoRI and BglII to yield a 1.6- and a 1.7-kb insert. Both fragments were separated from the Bluescript plasmid vector on a low melting point Sea plaque agarose gel (Biozym, Hameln, FRG) and labeled with [alpha^{32}P]dCTP (Amersham Buchler Ltd., Braunschweig, FRG) by using a Multiprime DNA labeling kit, according to the supplier's recommendation (Amersham Buchler Ltd., Braunschweig, FRG). 5′ end labeling of the $(dT)_{25}$-oligonucleotide with [gamma^{32}P]dATP (Amersham Buchler Ltd., Braunschweig, FRG) was performed with T4 polynucleotide kinase (Amersham Buchler Ltd.) according to Schäfer et al. (35).

For ACE cDNA hybridization, the membrane was prehybridized for ~ 4 h at 65°C in hybridization buffer containing 2× SSC (0.3 M NaCl, 0.03 M Na-Citrate, pH 7.0), 10× Denhardt's solution (0.2% polyvinylpyrrolidone, 0.2% bovine serum albumin, 0.2% Ficoll), 0.1% SDS, 0.1% pyrophosphate, 2 mM EDTA, 2.5% dextran sulfate, and 50 μg/ml sonicated, denatured *E. coli* DNA (Sigma Ltd., Deisenhofen, FRG). Hybridization was performed at 65°C for at least 16 h in fresh hybridization buffer completed with the radioactive labeled probe in a concentration of ~ 1 × 10^6 cpm/ml. After hybridization, the membrane was successively washed in 2× SSC/0.1% SDS, 1× SSC/0.1% SDS, and 0.5× SSC/0.1% SDS for 30 min at 60°C, respectively.

For $(dT)_{25}$-oligonucleotide hybridization, the membrane was prehybridized for 1 h at 45°C in hybridization buffer containing 5× SSPE (0.75 M NaCl, 50 mM NaH_2PO_4, 5 mM EDTA, pH 7.0), 5× Denhardt's solution, 0.1% SDS, and 10 μg/ml sonicated, denatured *E. coli* DNA (Sigma Ltd.). Hybridization was done in the same solution completed with the radioactive labeled oligonucleotide in a concentration of ~ 1 × 10^6 cpm/ml at 45°C for an additional 4 h. The hybridization temperature (T_m − 5°C) was calculated according to Suggs et al. (36). Membranes were washed three times for 30 min in 6× SSC at room temperature and for 1 min in 6× SSC at 45°C.

After washing procedure, the membranes were air dried and exposed to X-OMAT AR x-ray films (Kodak Inc., Stuttgart, FRG) with Quanta III intensifying screens (Siemens, Stuttgart, FRG). Relative amounts of ACE mRNA were evaluated by densitometric scanning (Personal densitometer; Molecular Dynamics Ltd.) of the band densities and expressed in relation to those of total mRNA, obtained by $(dT)_{25}$ oligonucleotide hybridization.

Northern blot analysis

10 μg of total RNA was prepared and subjected to electrophoresis according to Rosen et al. (37), transferred to a nylon membrane (Amersham Buchler Ltd.) by overnight capillar blotting and fixed to the membrane by UV irradiation. The blot membrane was successively hybridized with a 0.58-kb rat atrial natriuretic factor (ANF) PstI cDNA fragment (38) which is 85% identical to the human ANF cDNA sequence (39) and with a oligonucleotide (5′-GGTATCTGATCGTCTTCGAA-3′) deduced from the rat ribosomal 18 S RNA (18 S rRNA) sequence (40). Labeling of the cDNA and the oligonucleotide, prehybridization, hybridization, washing, and exposure of Northern blots were performed as described for slot blot analysis with the following exceptions: Prehybridization and hybridization with 18 S rRNA oligonucleotide was performed at 53°C. After hybridization, membranes were washed three times for 30 min in 6× SSC at room temperature and for 1 min in 6× SSC at 53°C.

Relative amounts of ANF mRNA were evaluated by densitometric scanning (Personal densitometer; Molecular Dynamics Ltd.) of the band densities and expressed in relation to those of 18 S rRNA.

Competitive RNA-PCR for quantification of ACE and chymase mRNA

Quantification of ACE and human heart chymase mRNA was performed by reverse transcription followed by polymerase chain reaction (PCR) in the presence of a defined concentration of either a shortened ACE or human heart chymase competitor RNA that served as an internal standard. Consequently, competitor RNA and target mRNA were reverse transcribed in the same reaction tube and coamplified by the same

primer oligonucleotides to avoid sample to sample variations in reverse transcription and PCR.

Reverse transcription. For first-strand cDNA synthesis equal amounts of total RNA (normally 625 ng) were mixed with increasing quantities of either ACE (50 to 0.5 × 10^6 molecules) or chymase competitor RNA (200 to 2.5 × 10^4 molecules) in 1× reverse transcription buffer (50 mM Tris-HCl, pH 8.3, 75 mM KCl, 3 mM $MgCl_2$) completed with 0.5 mM dNTPs (Pharmacia Biosystems Ltd., Freiburg, FRG) and 250 pmol of random hexanucleotide primers. These mixtures were heated to 72°C for 3 min. Then, dithiothreitol (10 mM), RNase inhibitor (2 U/100 ng total RNA; Life Technologies Ltd., Eggenstein, FRG) and Moloney murine leukemia virus reverse transcriptase (10 U/100 ng total RNA; Life Technologies Ltd.) were added to the reverse transcription reaction to a total volume of 25 μl and incubated at 42°C for 60 min.

PCR amplification. Duplicate samples of PCR reaction were performed in a total volume of 50 μl, respectively, each containing 10 μl of reverse transcription reaction, 35 μl of a PCR master mix (20 mM Tris-HCl, pH 8.3, 50 mM KCl, 0.5 mM $MgCl_2$, 20 pmol of sense and antisense primer) and 2.5 U of *Thermus aquaticus* DNA polymerase (Amersham Buchler Ltd.). The mixture was overlaid with mineral oil (Sigma Ltd.) and then subjected to 30 cycles of PCR amplification using a Gene ATAQ Controller (Pharmacia Biosystems Ltd.). The cycle profile included denaturation for 1 min at 94°C, annealing for 2 min at 65°C (ACE template) or 63°C (chymase template) and extension for 3 min at 72°C. As a negative control, no amplification product occurred if reverse transcriptase or total RNA was omitted in the first-strand cDNA reaction. The PCR products of ACE and chymase mRNA were found to be of the expected size as shown by gel electrophoresis. In addition, the specificity of the amplified sequences of ACE and chymase was confirmed by restriction enzyme analysis and by hybridization with specific internal oligonucleotide probes.

Quantitative analysis. The amplification products of 10 μl of each PCR reaction were separated by electrophoresis on a 1.5% agarose gel, stained with ethidium bromide, visualized by UV irradiation and photographed (Polaroid 665 negative film, Polaroid Ltd.). The negative film was taken to evaluate the band densities by integration over the whole area of extension using a laser densitometer and a computer based imaging system (Personal densitometer; Molecular Dynamics Ltd.). To correct for differences in molecular weight, the band densities of the ACE competitor template were multiplied by 1.2 (776:650 bp), those of the chymase competitor template were multiplied by 1.3 (622:482 bp). The mean value of duplicate samples (variation between duplicate samples ≤ 5%) was plotted as logarithm of the ratio of competitor to gene target PCR products versus the logarithm of the known number of competitor molecules ($r \geq 0.94$, $P \leq 0.0005$). At the competition equivalence point (log ratio = 0) the original number of target mRNAs corresponds to the initial number of competitor RNA molecules used.

In separate control studies, for each tissue sample and for both the ACE and the chymase mRNA template, the optimal range of total RNA amount was determined in which the output of RNA-PCR was linear to the original amount of total RNA used. To ensure that quantitative analysis at 30 cycles is performed during the exponential phase of PCR amplification, in previous studies each gene target was subjected to 25 to 36 cycles of amplification. Furthermore, when target and corresponding competitor RNA of each gene was subjected to serial dilutions, this resulted in parallel amplification curves indicating similar amplification efficiencies for each gene target and the corresponding competitor cDNA.

Selection and synthesis of the PCR primers

Appropriate sense and antisense primer oligonucleotides were selected from the human cDNA sequences of ACE (sense primer: 1656–1675 5′-GCAAGGAGGCAGGCTATGAG-3′, antisense primer: 2412–2431 5′-CGGGTAAAACTGGAGGATGG-3′) (34) and heart chymase (sense primer: 30–50 5′-TCTCCCCCTGCTGCTCTTTCT-3′, antisense primer: 631–651 5′-CACCCCAGCACACAGAAGAGG-3′) (15) by computer analysis using the Oligo program (National Biosciences Inc., Plymouth, MN) and were synthesized on a DNA synthesizer (Applied Biosystems Inc., Foster City, CA). To ensure that no genomic DNA contamination was present in the RNA solution the chosen primer oligonucleotides spanned several splice junctions (41, 15).

Construction and in vitro transcription of the competitor templates

For construction of internal standard competitor RNAs, shortened fragments of the human cDNAs of ACE and chymase were made and transcribed into RNA. The ACE competitor template was obtained from a 951-bp (1481–2431) cDNA fragment which has been amplified with the sense primer 5′-CGCTACAACTTCGACTGGTGG-3′ and the antisense primer 5′-CGGGTAAAACTGGAGGATGG-3′ by using the human ACE cDNA (34) as template in PCR reaction. The amplified cDNA fragment was phosphorylated, blunted and ligated into a blunt-ended, dephosphorylated Bluescript SK (−) vector (Stratagene Ltd., Heidelberg, FRG). A fragment of 126 bp was released by digestion with the restriction enzymes PpuMI and BglII. Another PpuMI cleavage site within this sequence was blocked by overlapping dcm (DNA cytosine methylase) methylation. The shortened cDNA fragment was separated on a low melting point agarose gel, blunted, and religated. To obtain the chymase competitor template, a fragment of 140 bp was deleted from the human heart chymase cDNA clone (cloning vector: Bluescript KS [−]) (15) using the restriction enzymes BsmI and StyI. Again, the shortened cDNA fragment was separated on a low melting point agarose gel, blunted and religated. For in vitro transcription, both of the shortened cDNA clones were first linearised with the restriction enzyme EcoRI. Then, 1 μg of each of the digested cDNA templates were transcribed into RNA by using a T3/T7-RNA polymerase in vitro transcription kit according to the supplier's recommendation (Amersham Buchler Ltd.). Subsequently, the DNA template was removed by addition of 15 U of RNase-free DNase (Life Technologies Ltd.) and incubation for 30 min at 37°C. The competitor RNA templates were purified by phenol extraction, precipitated and quantitated by absorption at 260 nm. Different concentrated stocks of each competitor RNA template were prepared by serial dilutions, stabilized by adding 30 μg/ml of yeast tRNA (Life Technologies Ltd.) and stored at −20°C until use. There was no remaining DNA detectable when each competitor RNA was subjected to RNA-PCR omitting reverse transcriptase in the first-strand cDNA reaction.

Statistical analysis

All data are presented as mean±SD. Statistical differences were calculated using the unpaired Student's *t* test or the Mann-Whitney test as appropriate. The correlation between PCR and slot blot data were examined by linear regression analysis. Significance was accepted at the level of $P < 0.05$.

Results

As a stringent internal control of reverse transcription and PCR, and for quantification of the original number of transcripts, competitor RNA targets with small internal deletions were prepared from the human cDNA sequences of ACE and chymase (Fig. 1). After reverse transcription into cDNA, each target and the corresponding competitor cDNA were amplified by competing for the same primer oligonucleotides (Fig. 1) which were selected from the human cDNA sequences of ACE and chymase. Figs. 2 and 3 show representative competitor titration experiments that use a constant amount of total RNA and a different number of either ACE or chymase competitor RNA molecules. The PCR products were separated by gel electrophoresis, stained and evaluated by laserdensitometric scanning of their band densities (Figs. 2 and 3, top). To obtain the original

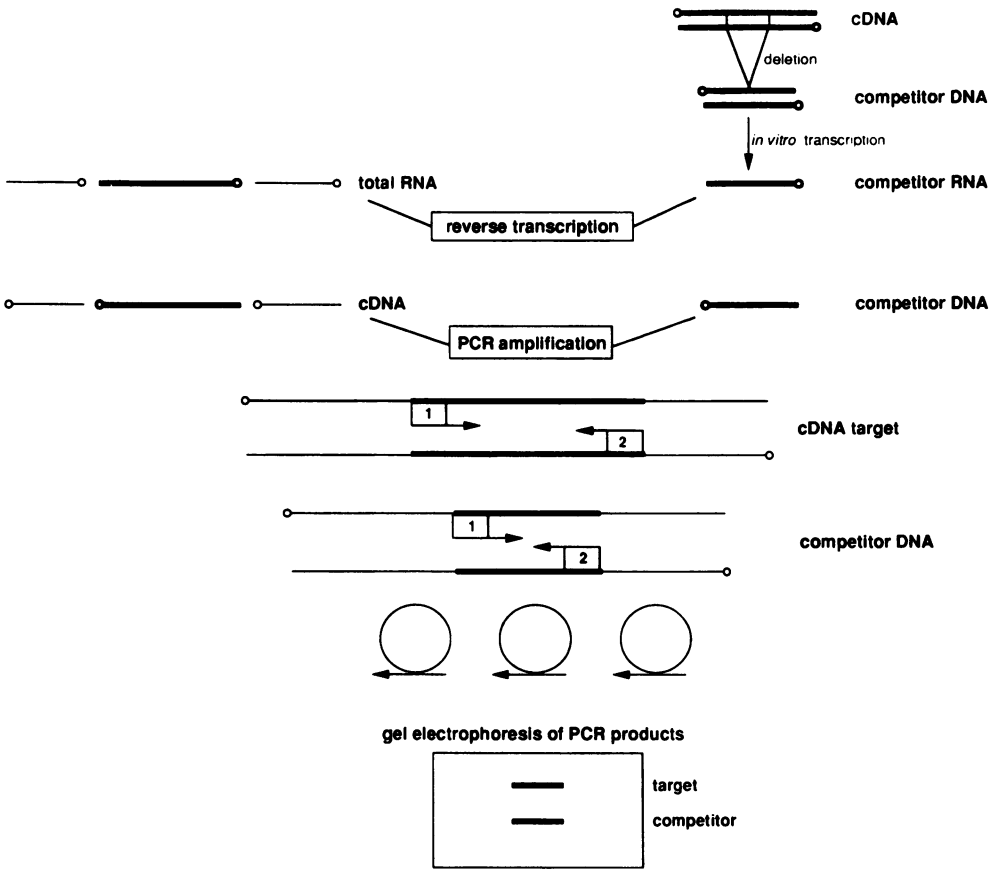

Figure 1. mRNA quantification by competitive RNA-PCR. Competitor RNAs with small internal deletions were used as internal standards for quantification of human ACE and human heart chymase transcripts. Competitor RNA and target mRNA were reverse transcribed and amplified in the same reaction tube to avoid sample to sample variations in reverse transcription and PCR. In PCR, competitor and target cDNA were coamplified in competing for the same primer oligonucleotides. The PCR products were separated by gel electrophoresis and visualized by ethidium bromide staining.

number of either ACE or chymase transcripts the logarithm of the ratio of competitor to gene target PCR products were plotted versus the logarithm of the number of competitor RNA molecules (Figs. 2 and 3, bottom). At the point where equal molar amounts of competitor and gene target PCR products were generated (log ratio = 0) the original number of target mRNAs is equivalent to the initial number of competitor RNA molecules used.

Using this technique of competitive RNA-PCR, we quantified the mRNA levels of ACE and chymase in left ventricular myocardium of nonfailing heart donors and patients with chronic congestive heart failure. As shown in Fig. 4 *A*, the number of ACE transcripts referred to 100 ng of total RNA was increased ($P < 0.0005$) in failing human hearts ($12.8 \pm 6 \times 10^5$; $n = 20$) compared to nonfailing control hearts ($4.2 \pm 2.5 \times 10^5$; $n = 9$) with no significant differences between dilated cardiomyopathy ($3.5 \pm 6.2 \times 10^5$, $n = 13$) and coronary artery disease ($11.4 \pm 5.8 \times 10^5$, $n = 7$). Similarly, no significant differences in ACE gene expression were observed in patients with ACE-inhibitor therapy ($13.6 \pm 6.2 \times 10^5$, $n = 13$) versus patients without any ACE-inhibitor therapy before transplantation ($11.2 \pm 5.7 \times 10^5$, $n = 7$). As shown in Fig. 4 *B*, chymase gene expression was not significantly different in failing and nonfailing human hearts ($6.6 \pm 9.4 \times 10^4$, $n = 20$ vs. $3.1 \pm 4.7 \times 10^4$, $n = 9$). The higher mean value of failing hearts is attributed to only two heart samples which were very high in chymase gene expression whereas the majority of failing and nonfailing hearts had similar values. There was no significant difference in chymase gene expression between patients with dilated cardiomyopathy and patients with coronary artery disease ($5.3 \pm 7.8 \times 10^4$, $n = 13$ vs. $9 \pm 12.1 \times 10^4$, $n = 7$). In comparison to the ACE gene expression the mean values of chymase mRNA levels were lower in normal as well as failing human hearts (factor 13 and 19, respectively).

As shown in Table I, most patients demonstrated severely abnormal pretransplant hemodynamics. Furthermore, we investigated ANF gene expression by Northern blot analysis demonstrating a single band at 0.95 kb both in failing and nonfailing heart samples (data not shown). The ANF gene expression was upregulated in failing hearts while the ANF gene expression was very low in nonfailing donor hearts (1.70 ± 1.75 vs 0.02 ± 0.02; $P < 0.001$; Tables I and II). There was no significant correlation between ANF and ACE gene expression.

To test the variability of competitive RNA-PCR, total RNA from three patients was taken to determine ACE mRNA levels in three independently performed competitor titration experiments, respectively. The variability of the three measurements averaged 11% indicating a high reproducibility of this method. In 10 patients sufficient myocardial tissue and, consequently, RNA was available to compare the results obtained by competitive RNA-PCR with ACE mRNA levels determined by slot blot analysis of poly(A)$^+$ RNA (Fig. 5). There was a significant correlation between both methods ($r = 0.69$, $P < 0.05$) (Fig. 5 *A*), indicating that competitive RNA-PCR is suitable for mRNA quantification. In accordance to the PCR results, the ACE mRNA levels as determined by slot blot analysis and expressed

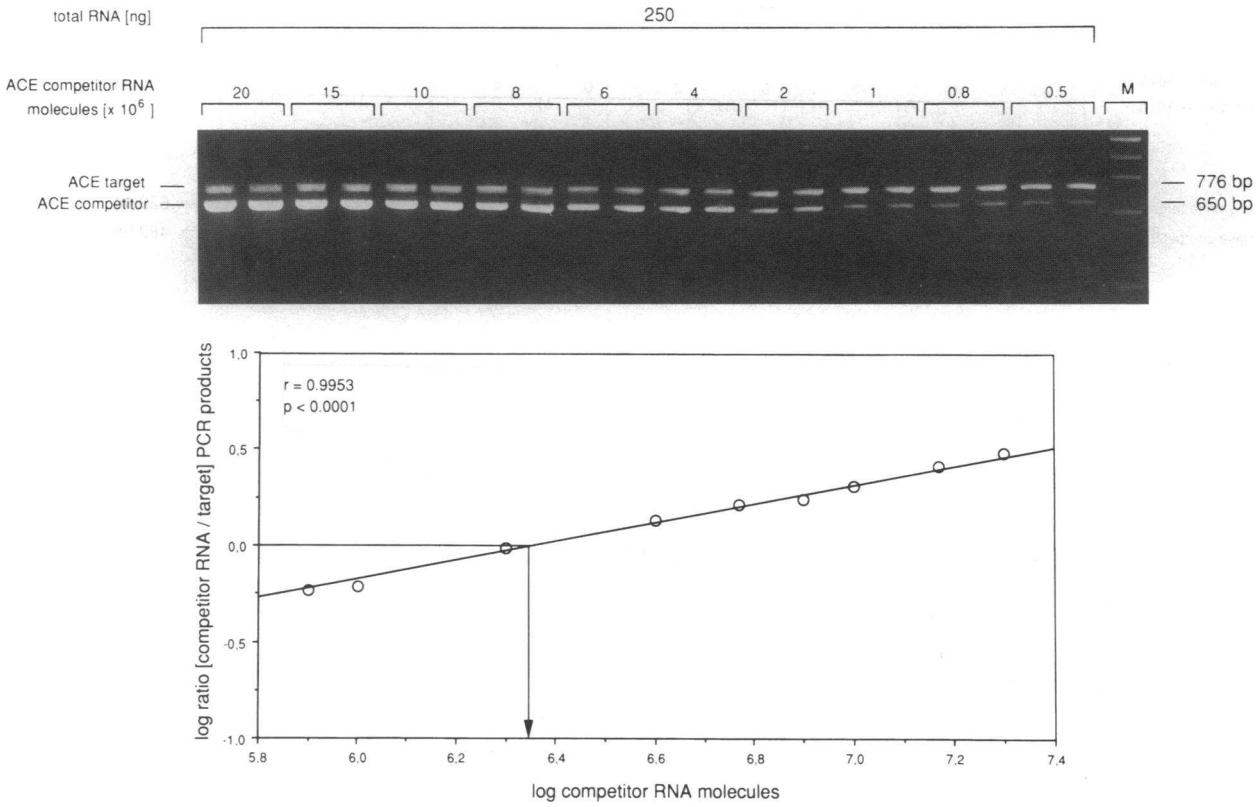

Figure 2. ACE mRNA quantification by competitive RNA-PCR. (*Top*) A constant amount of total RNA was mixed with an increasing number of ACE competitor RNA molecules, reverse transcribed into cDNA and amplified in duplicate samples by PCR (variation between duplicate samples $\leq 5\%$). The PCR products, indicated as ACE target (*776 bp*) and ACE competitor (*650 bp*), were separated by gel electrophoresis, stained with ethidium bromide and visualized by UV irradiation. On the right-hand side, a molecular weight DNA standard (*M*) was loaded. (*Bottom*) The band densities of the ACE target and competitor DNA were evaluated using a laserdensitometer and a computer based imaging system. The mean value of duplicate samples were plotted as logarithm of the ratio of competitor to gene target PCR products versus the logarithm of the known number of competitor molecules. At the competition equivalence point (log ratio = 0) the original number of target mRNAs corresponds to the initial number of competitor RNA molecules used. In this case, 250 ng of total RNA contained 2.19×10^6 ACE transcripts.

in relation to total mRNA (obtained by $[dT]_{25}$ oligonucleotide hybridization) were increased ($P < 0.01$) in failing human hearts (1.5 ± 0.3, $n = 7$ vs. 0.6 ± 0.5, $n = 3$ in normal hearts) (Fig. 5 *B*).

Discussion

This study demonstrates that left ventricular ACE gene expression is significantly enhanced in the failing human myocardium, regardless of the underlying etiology of congestive heart failure. In contrast, the expression of the human heart chymase, another enzyme that converts angiotensin-I to angiotensin-II, is not significantly different in failing and nonfailing human hearts.

A substantial variation in the number of ACE and chymase transcripts was observed in both failing and nonfailing myocardium, similar to the wide range of ventricular gene expression reported recently for ANF or sarcoplasmatic reticulum ATPase (42, 43). Because of the high reproducibility of competitive RNA-PCR, it appears unlikely that this variation resulted from methodological limitations. Notably, handling of tissue samples during removal, freezing, transport, and assay was standardized in all cases and RNA gels obtained from all samples indicated no RNA degradation. Importantly, comparison of RNA-PCR data with ACE mRNA levels determined by traditional slot blot hybridization yielded a close correlation. Indeed, the ACE mRNA levels as assessed by slot blot analysis were significantly increased in failing hearts supporting the principle finding of the quantitative RNA-PCR. Consistent with other investigators (42, 44, 45) the ANF gene expression was upregulated only in failing but not in nonfailing human hearts. These findings are consistent with the patients clinical characteristics supporting the presence of advanced heart failure. Indeed, ANF is considered to represent a well-defined marker gene of cardiac hypertrophy whose gene expression is upregulated in advanced chronic heart failure (44–46). Conversely, the very low expression of ANF in our nonfailing tissues support the notion that the samples of these 'donors' were indeed normal. The lack of changes in chymase expression in our heart samples with increased ACE mRNA levels may suggest a "selective" upregulation of ACE in relation to the human heart chymase.

Similar to the inter-individual variation of myocardial ACE gene expression, the level of plasma ACE is highly variable between individuals (47). An insertion/deletion polymorphism within the ACE gene locus was found to account for 47% of the variance of plasma ACE levels in normal

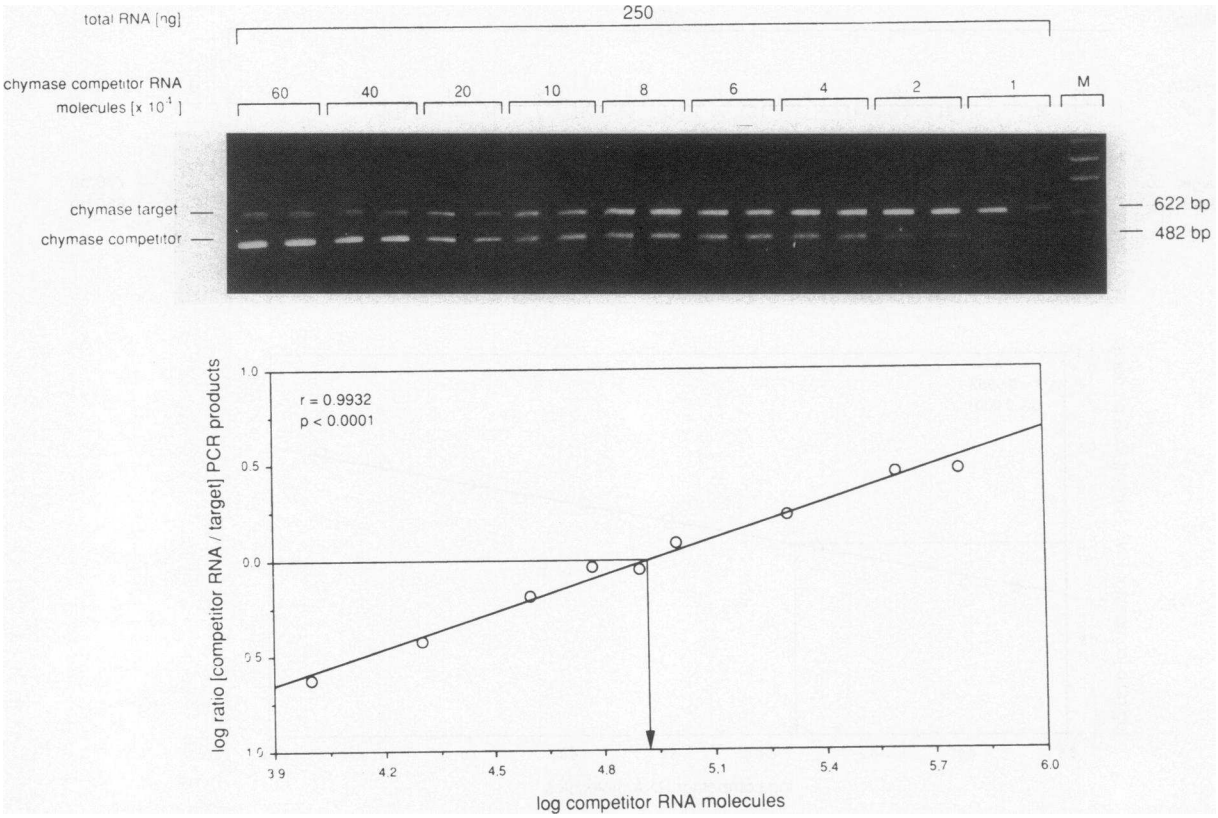

Figure 3. Chymase mRNA quantification by competitive RNA-PCR. (*Top*) A constant amount of total RNA was mixed with an increasing number of chymase competitor RNA molecules, reverse transcribed into cDNA and amplified in duplicate samples by PCR (variation between duplicate samples $\leq$ 5%). The PCR products, indicated as chymase target (*622 bp*) and chymase competitor (*482 bp*), were separated by gel electrophoresis, stained with ethidium bromide and visualized by UV irradiation. On the right-hand side, a molecular weight DNA standard (M) was loaded. (*Bottom*) The band densities of the chymase target and competitor DNA were evaluated using a laserdensitometer and a computer based imaging system. The mean value of duplicate samples were plotted as logarithm of the ratio of competitor to gene target PCR products versus the logarithm of the known number of competitor molecules. At the competition equivalence point (log ratio = 0) the original number of target mRNAs corresponds to the initial number of competitor RNA molecules used. In this case, 250 ng of total RNA contained 8.81×10^4 chymase transcripts.

individuals (48) and seems to be a potent risk factor of coronary artery disease (49, 50). It is likely that the genetic control of serum ACE level is exerted at the transcriptional level, although the polymorphism itself may not play a direct part in controlling ACE transcription but act more likely as a marker of strong linkage disequilibrium with regulatory elements of the ACE gene (48). Extending this notion to tissue ACE expression the variation in the number of ACE transcripts that we observed in failing and nonfailing human myocardium may, in part, be explained by an inter-individual different genetic control of ACE gene expression. The number of tissue samples in the present study was too small to investigate whether cardiac ACE gene expression is associated with ACE gene insertion/deletion polymorphism. However, Raynolds et al. (51) have recently reported that the ACE D/D genotyp is increased in ischemic and dilated cardiomyopathy. It should be noted, however, that a genetic predisposition to higher ACE gene expression does not exclude that other factors (i.e., increased wall stress) are contributing to the upregulation of cardiac ACE gene expression in the failing heart. In rats with abdominal aortic banding, an enhanced ACE expression and ACE activity is found only in the overloaded left ventricle, but not in the right ventricle (12, 52). On the other hand, with pulmonary banding, the ACE activity is enhanced only in the right ventricle (own unpublished observations). ACE expression and ACE activity is enhanced in noninfarcted left ventricular myocardium of normotensive rats with large left ventricular infarcts (13, 52) which have been shown to be associated with increased wall stress (53). Similarly, the cardiac expression of angiotensinogen is confined to the left ventricle, both in the normotensive rat infarct model (18) and the pressure overload rat model (54). Interestingly, the expression of angiotensinogen in human hearts was more prominent in subendocardial layers (28) following the pattern observed for the expression of atrial natriuretic factor (55). Taken together, these observations would be consistent with the idea, that the activation of the intracardiac renin angiotensin system is related to an enhanced myocardial wall stress rather than to an increased coronary perfusion pressure.

The enhanced ventricular ACE expression in the failing human heart is consistent with previous observations in different rat models of cardiac hypertrophy and failure (12, 13). The markedly enhanced ventricular ACE expression in

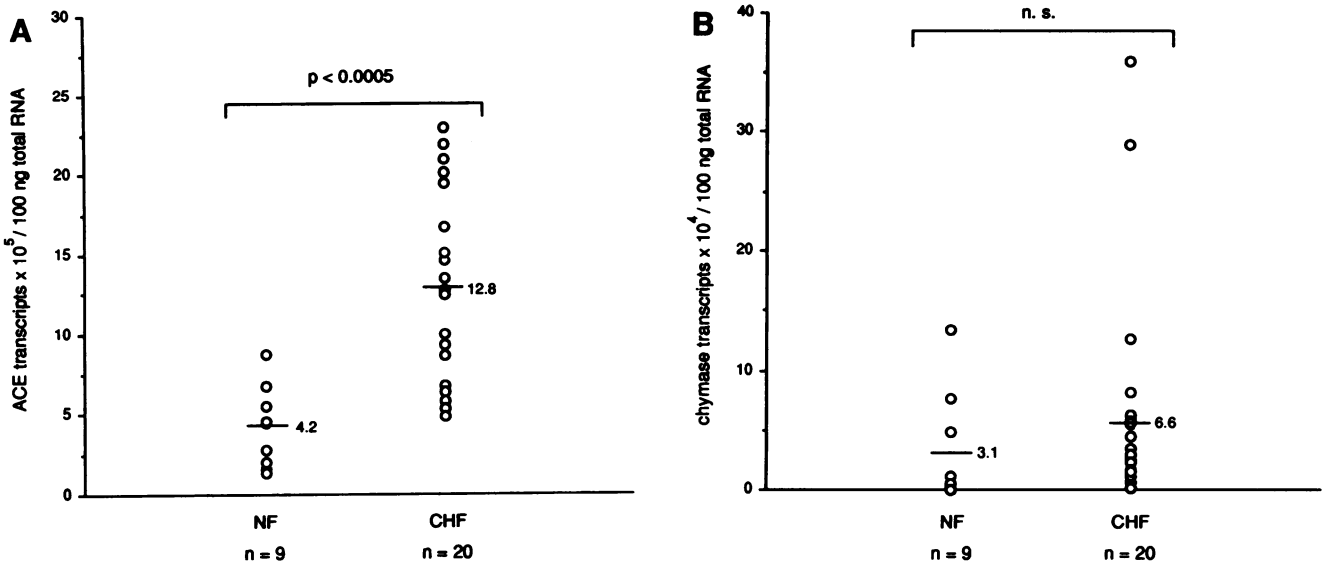

Figure 4. Number of transcripts of ACE (*A*) and heart chymase (*B*) in left ventricular myocardium of 9 nonfailing heart donors (*NF*) and 20 patients with chronic congestive heart failure (*CHF*).

experimental cardiac hypertrophy and failure is associated with an increased tissue ACE activity (12, 13) and enhanced angiotensin-II formation from angiotensin-I perfusate in vitro (12). It has been proposed, therefore, that this enhanced angiotensin conversion may contribute to the vulnerability of diastolic function of hypertrophied hearts during hypoperfusion (12), probably due to angiotensin-II effects on intracellular free Ca^{2+} in the ischemic myocytes (19). Conversely, intracoronary infusion of an ACE inhibitor avoiding systemic effect of the drug resulted in a decrease in coronary vascular

Table I. Hemodynamic Data, Cardiac ANF Gene Expression, and Drug Treatments of Patients with Heart Failure

							Drug treatment			
Pat. No.	Sex	Age	PCW	EF	CI	ANF mRNA*	Digoxin	ACE-I	Diuretics	Ca^{2+}-blocker
		yr	*mmHg*	*%*	*l/min·m²*					
1	M	49	25	—	3.1	—	+	−	+	−
2	M	46	19	16	2.1	5.02	+	+	+	−
3	M	61	18	21	2.4	4.68	+	+	+	−
4	M	52	24	18	3.1	1.38	+	−	+	+
5	F	38	11	15	1.7	1.34	−	+	+	−
6	M	47	13	16	2.1	0.74	+	+	+	−
7	M	43	34	10	2.5	0.28	−	−	−	−
8	M	54	21	13	1.6	0.13	+	+	+	−
9	F	54	23	26	1.3	0.23	+	+	+	−
10	M	39	15	29	3.6	2.46	+	+	+	−
11	M	30	28	30	2.0	2.46	+	+	+	−
12	M	64	31	—	2.3	0.02	+	+	+	−
13	M	57	29	—	2.7	1.22	+	+	+	−
14	M	55	27	23	2.7	0.22	+	−	+	−
15	M	53	35	17	1.7	0.04	+	+	+	−
16	M	53	10	40	—	2.77	+	+	+	+
17	M	52	8	17	2.6	0.02	+	−	+	−
18	M	53	10	—	2.1	2.73	+	+	+	−
19	M	64	27	20	2.8	—	+	−	+	−
20	M	51	6	43	3.4	4.86	−	−	−	+
Mean		50.8	20.7	22.1	2.4	1.70				
± SD		8.5	9.0	9.4	0.6	1.75				

Dilated cardiomyopathy: patients 1–13; coronary artery disease: patients 14–20. *PCW*, pulmonary wedge pressure; *EF*, ejection fraction; *CI*, cardiac index; *ACE-I*, ACE-inhibitor. * ANF mRNA hybridization signal was normalized to the hybridization signal of 18S rRNA.

Table II. Clinical Characteristics and Cardiac ANF Gene Expression of Nonfailing Heart Donors

Code	Sex	Age	Cause of death	Other diagnosis	Drugs at HT	ANF mRNA*
	yr					
1	M	43	CB	Fatty liver, obesity	DA 50 mg/h	0.01
2	M	53	Cerebral venous thrombosis, cerebral edema	Suspicion of cerebral cancer	DA 50 mg/h Dexamethasone 20 mg bolus	0.01
3	M	56	Polytrauma, CB	Arteriosclerosis	DA 50 mg/h	—
4	F	46	CA, CB	Obesity, hypertension, mild arteriosclerosis	DA 6 mg/h	0.01
5	M	36	CA, CB	Arteriosclerosis, nicotine abuse	DA 6 mg/h	0.04
6	F	29	Polytrauma	None	DA 15 mg/h NA 0.08 mg/h	0.01
7	F	24	CB	Suspicion of atrial myxom	None	0.06
8	F	54	SAB	None	None	0.01
9	F	45	CB	None	None	0.01
Mean		42.9				0.02
± SD		11.2				0.02

HT, heart transplantation; *CB*, intracerebral bleeding; *SAB*, subarachnoidal bleeding; *CA*, aneurysm of cerebral artery; *DA*, dopamine; *NA*, noradrenalin.
* ANF mRNA hybridization signal was normalized to the hybridization signal of 18 S rRNA.

resistance and left ventricular filling pressures in patients with dilated cardiomyopathy (25).

The cell types synthesizing ACE in the myocardium are not elucidated. Early immunohistochemical analysis detected ACE only in endothelial cells (56). In the rat, ACE has been detected by radioactive labeling in several regions of the heart, yet to a variable extent. For example, ACE concentrations were shown to be high in coronary arteries and low in endocardium and ventricular myocardium (57); however, the cellular distribution was not identified by this study. More recently, ACE as well as renin and angiotensinogen has been detected immunohistochemically in cultured cardiomyocytes and fibroblasts from ventricles of neonatal rats (58). It remains to be determined whether ACE is also expressed in adult rat and human myocytes. Moreover, further studies should elucidate which cell type(s) contribute to the increased expression of ACE in the pressure overload or failing heart. However, regardless of the cell types contributing to the increased cardiac expression of ACE, an increased local synthesis of angiotensin II may act in an autocrine and paracrine fashion within the myocardium as suggested previously (59).

There were no significant differences in the cardiac ACE gene expression of patients treated with ACE inhibitors and patients not treated with ACE inhibitors. Short-term administration of ACE inhibitors for 3 d have been reported to exert feedback upregulation of ACE gene expression in rat lung (60). In contrast, preliminary data from our laboratory indicate that long-term administration of ACE-inhibitors for several weeks

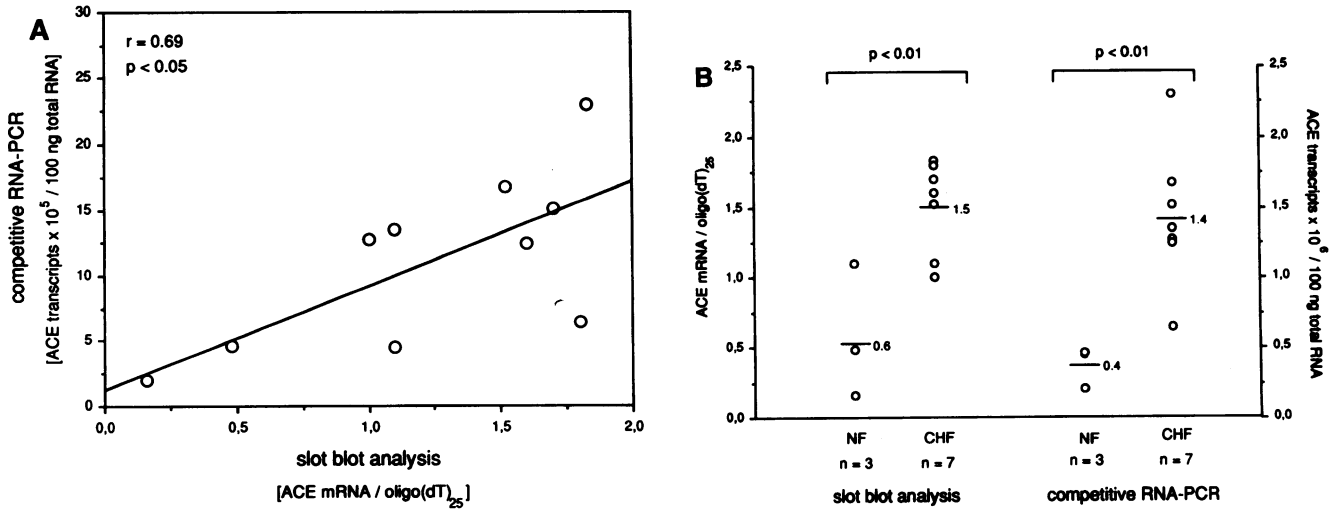

Figure 5. Correlation (*A*) and comparison (*B*) of the ACE mRNA levels in left ventricular myocardium of 3 nonfailing heart donors (*NF*) and 7 patients with chronic congestive heart failure (*CHF*) obtained by competitive RNA-PCR and by slot blot analysis.

is not associated with induction of left ventricular ACE mRNA levels in rat postinfarction reactive hypertrophy (unpublished observations). The latter observations are consistent with the present findings that chronic ACE inhibitor treatment is not associated with sustained induction of left ventricular ACE transcription in patients with end-stage heart failure. However, this does not exclude that a transient upregulation of cardiac ACE expression occurs early after administration of ACE inhibitors.

The human heart chymase was described as the major enzyme pathway for angiotensin-II formation in the human myocardium (26), although our data demonstrated that the chymase is less abundantly expressed in myocardium than ACE. However, this could be explained by the higher specificity and efficiency of the human heart chymase in the conversion of angiotensin-I to angiotensin-II (14, 16). Furthermore, turnover of chymase enzyme seems to be much slower than ACE turnover (27). Consistent with recent observations (27), this study revealed no significant differences in left ventricular chymase mRNA levels of failing and nonfailing human hearts. In addition, Urata et al. (27) observed no significant differences in chymase-like immunoreactivity and activity between normal and failing human hearts. Using in situ hybridization and EM-immunocytochemical techniques, these investigators (27) have shown that the human heart chymase is not exclusively expressed and stored in human cardiac mast cells as expected from other mammalian chymases but also in cardiac endothelial cells and actively dividing fibroblasts of the cardiac interstitium. In addition, high levels of chymase-like immunoreactivity are localized in the cardiac interstitium and are likely to be associated with the interstitial extracellular matrix (27). However, it is not known which stimuli are responsible for the release of the chymase enzyme from its intracellular storages. Hence, until specific inhibitors of human heart chymase are available, the functional significance of the human heart chymase for angiotensin-II generation in normal and failing human hearts remains uncertain.

In summary, the present study provides evidence for the intracardiac expression of ACE in the human heart and for its activation in end-stage heart failure. In contrast, there was no difference in human heart chymase gene expression between failing and nonfailing human hearts. By taking advantage of quantitative RNA-PCR to assess the cardiac expression of ACE and other components of the renin–angiotensin system, future studies should now be directed to elucidate the role of the cardiac tissue renin angiotensin system in patients in vivo.

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