Local stress, not systemic factors, regulate gene expression of the cardiac renin-angiotensin system in vivo: A comprehensive study of all its components in the dog (heart/heart hypertrophy/gene regulation/heart failure)

YOUNG-AE LEE*, CHANG-SENG LIANG†, MIN-AE LEE*, and KLAUS LINDPAINTER**†

*Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, and Department of Cardiology, Children's Hospital, Harvard Medical School, Division of Biological Sciences, Harvard School of Public Health, Boston, MA 02115; and †Division of Cardiology, Department of Medicine, Strong Memorial Hospital, University of Rochester, Rochester, NY 14624

Communicated by David E. Housman, Massachusetts Institute of Technology, Cambridge, MA, May 30, 1996 (received for review December 7, 1995)

ABSTRACT Cardiac hypertrophy is associated with altered expression of the components of the cardiac renin-angiotensin system (RAS). While in vitro data suggest that local mechanical stimuli serve as important regulatory modulators of cardiac RAS activity, no in vivo studies have so far corroborated these observations. The aims of this study were to (i) examine the respective influence of local, mechanical versus systemic, soluble factors on the modulation of cardiac RAS gene expression in vivo; (ii) measure gene expression of all known components of the RAS simultaneously; and (iii) establish sequence information and an assay system for the RAS of the dog, one of the most important model organisms in cardiovascular research. We therefore examined a canine model of right ventricular hypertrophy and failure (RVHF) in which the right ventricle (RV) is hemodynamically loaded, the left ventricle (LV) is hemodynamically unloaded, while both are exposed to the same circulating milieu of soluble factors. Using specific competitive PCR assays, we found that RVHF was associated with significant increases in RV mRNA levels of angiotensin converting enzyme and angiotensin II type 2 receptor, and with significant decreases of RV expression of chymase and the angiotensin II type 1 receptor, while RV angiotensinogen and renin remained unchanged. All components remained unchanged in the LV. We conclude that (i) dissociated regional regulation of RAS components in RV and LV indicates modulation by local, mechanical, not soluble, systemic stimuli; (ii) components of the cardiac RAS are independently and differentially regulated; and (iii) opposite changes in the expression of angiotensin converting enzyme and chymase, and of angiotensin II type 1 and angiotensin II type 2 receptors, may indicate different physiological roles of these RAS components in RVHF.

The renin-angiotensin system (RAS) contributes importantly to the maintenance of acute and chronic hemodynamic homeostasis by affecting the function as well as the structure of the cardiovascular system. In addition to the classically described endocrine (circulating) RAS, a number of organs and tissues have in recent years been found to express components of the RAS, leading to the concept of "local" or "tissue-resident" RASs acting, presumably, via paracrine, autocrine, or intracrine pathways. The existence of an endogenous RAS in the heart has been confirmed by the demonstration of expression of the genes coding for angiotensinogen, renin (1), angiotensin converting enzyme (ACE), and angiotensin II receptors (2, 3), and by immunohistochemical demonstration of intracardiac angiotensin I and II (4). A second pathway for angiotensin II formation via a highly specific and potent serine protease, chymase, has recently been described in the human and canine heart (5, 6).

Angiotensin II has been reported to modulate adaptive growth patterns in cardiac hypertrophy via autocrine or paracrine pathways. Angiotensin II stimulates protein synthesis in cardiomyocytes (7) and in cultured aortic vascular smooth muscle cells (8, 9), and appears to be required for the rapid growth of the neonatal heart (10). Upregulation of left ventricular angiotensinogen and ACE has been described in association with pressure-overload hypertrophy (11) and tachypacing-induced heart failure (12). ACE inhibitors are more effective in reversing or preventing cardiac hypertrophy associated with systemic hypertensive states (13, 14) than other agents with equal hypotensive effects (15). Recent in vitro data indicate that activation of the cardiomyocyte RAS is induced by mechanical stress, and that angiotensin II is an essential mediator of stretch-induced cardiac myocyte hypertrophy (16). So far, no corresponding information assessing the role of mechanical stress versus that of circulating soluble factors in modulating RAS expression in vivo has been presented. Also, a comprehensive assessment of cardiac RAS gene expression in vivo and in vitro has been precluded because no study so far has examined all components of the cardiac RAS. Thus, the goals of the present work were to study the possible role of local mechanical versus systemic soluble factors in the modulation of cardiac RAS gene expression, and to conduct a comprehensive assessment of cardiac gene expression of all known components of the RAS. We studied a canine model of combined pressure- and volume-overload-induced right ventricular hypertrophy and failure (RVHF). In contrast to left ventricular failure, which invariably also results in some hemodynamic embarrassment and, thus, mechanical stress of the right ventricle (RV) transmitted via the pulmonary vascular bed, this model selectively stresses the RV, while the left ventricle (LV) is effectively unloaded; meanwhile, both ventricles remain exposed to the same circulating neurohumoral milieu. Therefore, if cardiac RAS modulation is mediated through mechanical stress, it should only affect gene expression patterns in the RV, whereas a putative RAS-modulating role of soluble factors would be expected to result in parallel changes in both ventricles.

Abbreviations: RAS, renin-angiotensin system; ACE, angiotensin converting enzyme; RVHF, right ventricular hypertrophy and failure; RV, right ventricle; LV, left ventricle; AT1 and AT2, angiotensin II type 1 and 2, respectively; RT, reverse transcription.

Data deposition: The sequences reported in this paper have been deposited in the GenBank data base (accession nos. U67197–U67202).

†To whom reprint requests should be addressed at: Cardiovascular Division, Department of Medicine, Brigham and Women's Hospital, 75 Francis Street, Thorn 1103, Boston, MA 02115-6195. e-mail: kll@calvin.bwh.harvard.edu.
The results of our experiments strongly suggest that, similar to the in vitro situation, local, mechanical factors are the predominant modulations of cardiac RAS gene expression in vivo. Our study represents a comprehensive assessment of gene expression of all components of the cardiac RAS and demonstrates that hypertrophy and failure are associated with specific, differential modulation of gene expression.

MATERIALS AND METHODS

Animal Model. RVHF was induced in adult mongrel dogs (n = 11) by tricuspid valve avulsion and progressive pulmonary artery constriction as described (17, 18). A separate group of dogs (n = 9) underwent identical surgical procedures; however, neither tricuspid valve avulsion nor pulmonary artery constriction were performed. The animals studied in the present experiments have been the subject of previous reports (17, 18).

Experimental Protocol. For the hemodynamic studies, animals were placed in a lateral decubitus position, and the pulmonary artery occluders were deflated. The previously implanted intravascular catheters were connected to pressure transducers (Statham Instruments, Oxnard, CA) and a multichannel recorder (Brush model 480; Gould, Cleveland) for measuring heart rate, aortic, and right and left atrial pressures. The micromanometer (Konigsberg Instruments, Pasadena, CA) was connected to the Brush recorder for determining LV pressure and its first derivative (dP/dt) with an electronic differentiator. Right ventricular pressure and its peak dP/dt were measured by a transducer-tipped catheter (Millar Instruments, Houston) inserted through an external jugular vein under local anesthesia. Cardiac output was determined by injecting indocyanine green (Hyson, Westcott & Dunning, Baltimore) in the pulmonary artery and sampling arterial blood for dye concentration with a cardiac output system (Gilford). Resting hemodynamic measurements were made in triplicate at 5-min intervals at least 1 h after insertion of the Millar catheter. Averages of the triplicate tests were used for statistical analysis. The animals were sacrificed by a lethal injection of pentobarbital, and the hearts were excised rapidly. RV and LV were separated and weighed, and tissue specimens were stored in liquid nitrogen.

RNA Extraction from Tissues. Poly(A) RNA was isolated directly from frozen canine right and left ventricular tissue using the PolyATract System 1000 (Promega). Genomic DNA was removed by incubation with RNase-free DNase (Promega) in 1X buffer (40 mM Tris-HCl, pH 7.9/10 mM NaCl/6 mM MgCl2/10 mM CaCl2) at 37°C for 20 min. The mRNA was phenol/chloroform/isooamyl alcohol extracted, twice chloroform extracted, ethanol precipitated, and resuspended in nuclease-free water (Promega). The concentration of mRNA was determined spectrophotometrically and samples were stored at −135°C until use.

Cloning of Canine cDNA of the β-Actin, Angiotensinogen, Renin, ACE, Angiotensin II Type 1 (AT1) and Angiotensin II Type 2 (AT2) Receptor Genes, and Construction of Reference Plasmids for Competitive PCR. Canine cDNA regions of the β-actin, angiotensinogen, renin, ACE, and AT1 and AT2 receptor genes were amplified by PCR using primers designed by homology to highly conserved regions of the coding sequence of each gene from cDNA prepared by reverse transcription (RT) of canine mRNA. Amplification products were cloned into the pCRII vector using the TA Cloning Kit (Invitrogen) and sequenced using the Taq Dye Deoxy Terminator Cycle Sequencing Kit on an Applied Biosystems model 373A automatic sequencer. All newly determined canine sequence domains have been deposited with the GenBank data base. Appropriate sense and reverse primers designed to span intronic sequences were identified (except for the AT1 and AT2 receptor coding sequences that are intronless). Inverse PCR was used to generate deletion mutants of each canine cDNA (19). Briefly, a 3' and a 5' primer were designed in juxtapositioned to each other, resulting in amplification of the entire vector and insert such that 18- to 30-bp deletions, corresponding to about 10% of the length of the amplicon used for competitive PCR quantification, were produced in the insert.

Preparation of Internal Standard for Calibration of RT Efficiency. A vector (pSP64T; Promega) containing portions of the Xenopus laevis 5'- and 3'-untranslated β-globin sequences (20) was used to calibrate the efficiency of RT. An insertion mutant was constructed by ligating a 22-bp oligonucleotide (5'-gatcgaattccggggggctgcag-3') into a BglII restriction site within the insert. (All restriction enzymes were obtained from New England Biolabs.) The β-globin mutant plasmid was linearized using the restriction enzymes ScaI and NheI. The resulting 533-bp fragment containing the SP6 site and the globin insert was isolated from a 1.5% agarose gel after electrophoretic size fractionation, and the DNA was recovered using a silica adsorption method (Qiagen, Chatsworth, CA). In vitro RNA was generated by transcription from 360 ng DNA, DNase digestion, and purification using the MEGAscript kit (Ambion, Austin, TX) according to the manufacturer's protocol. Integrity and quantity of the RNA was assessed by formaldehyde agarose gel electrophoresis and spectrophotometric analysis.

Reverse Transcription. One microgram of each canine mRNA sample was reverse transcribed along with 1 pg of mutant β-globin RNA in 1X RT buffer (50 mM Tris-HCl, pH 8.3/75 mM KCl/3 mM MgCl2), in the presence of 10 mM DTT, 0.5 mM dNTP, 1.0 µg oligo(dT)12-18, and 50 ng random hexanucleotide primers using Superscript II RNase H- Reverse Transcriptase (Life Technologies, Gaithersburg, MD) at 42°C for 60 min. The reaction was terminated by incubation at 70°C for 15 min.

Quantification of mRNAs Encoding Canine β-Actin, Angiotensinogen, Renin, ACE, Chymase, AT1, and AT2 Receptor, and X. laevis β-Globin Using Competitive PCR. All PCR-related samples and reagents were diluted in nuclease-free water (Promega) and stored in sterile nuclease-free microcentrifuge tubes (Sarstedt). Aerosol barrier pipet tips were used exclusively for all sample manipulations.

Competitive PCR was performed in 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM MgCl2, 0.1% Triton X-100, 200 µM dNTPs, 1.0 µM of each primer in the presence of 1.67 µCi [α-32P]dCTP (New England Nuclear, 1 Ci = 37 GBq) and 0.625 unit of Taq polymerase (Promega) in a total reaction volume of 25 µl under mineral oil (Sigma). Constant amounts of canine cDNA were mixed with eight (three for renin) stepwise increasing quantities of the appropriate reference template linearized by restriction digest with BglII or HindIII. Thus, eight (three for renin) separate PCRs were performed for each sample and each gene of interest. The amplification protocol entailed an initial denaturation step of 3 min at 94°C and a final elongation step of 7 min at 72°C, as well as a variable number of amplification cycles consisting of a 30-s denaturation step at 94°C, a 45-s annealing step at a gene-specific annealing temperature, and an extension step of 45 s at 72°C. The amounts of reference template and the cycle numbers used for each gene are listed in Table 1.

Following PCR, 5 µl of formamide containing 0.01 M EDTA (pH 8.0), 0.1% bromophenol blue, and 0.1% xylene cyanol were added to the reaction. PCR products were then denatured at 94°C for 5 minutes, snap cooled on ice, and size fractionated on 5.5% denaturing polyacrylamide gels (Appligene, Strasbourg, France). The gels were fixed in 10% acetic acid and 25% methanol and dried using a heated vacuum slab gel dryer.

Quantitative and Statistical Analysis. To determine the amounts of each mRNA in the experimental samples, signals from PCR bands corresponding to β-globin, canine β-actin,
angiotensinogen, renin, ACE, chymase, the AT1 and AT2 receptors, and their respective competitors (reference constructs) were quantified by using phosphorimaging (Molecular Dynamics). The signal generated by reference standards was corrected appropriately for dCTP incorporation according to the number of cytosine residues present in wild-type and reference sequences. The logarithms of the ratio of signal obtained from wild type and reference in each reaction were plotted against the logarithm of the amount of reference DNA used (21). At the competition equivalence point [log (wild type/control) = 0] the original number of target cDNA molecules corresponds to the initial number of competitor DNA molecules present (Fig. 1). By adjusting for the RT-specific efficiency coefficient determined by cotranscription of β-globin mRNA, the actual number of target mRNA molecules present in the tissue extract was derived. All data are presented as mean ± SD. Pearson correlations were calculated to correlate mRNA expression with physiologic variables. Statistical differences were calculated using a two-way ANOVA to account for disease status (RVHF or sham-operated) and tissue source (RV or LV). Statistical significance was defined as P < 0.05. Linear regression and statistical analysis were performed using the MICROSOFT EXCEL 5.0 (Microsoft) and the STATITICA 4.0 (Statsoft, Tulsa, OK) software packages. RT–PCR-derived measurements with a correlation coefficient (r2) for ln(wt/ctr) versus ln(ctr) of less than 0.90 were rejected and the respective competitive PCRs repeated.

RESULTS

Cardiac and Hemodynamic Parameters. Dogs were divided into two experimental groups represented by 9 sham-operated and 11 RVHF dogs. RVHF was associated with an increase in absolute RV mass, in the ratio of RV to LV mass, and in the ratio of RV to body mass when compared with sham-operated controls (mean ± SD: 2.14 ± 0.33 versus 1.55 ± 0.15 g/kg, P < 0.0001). RVHF was associated with significant increases of right atrial pressure (10.6 ± 1.5 versus 4.3 ± 1.9 mmHg, P < 0.00001) and heart rate (127 ± 25 versus 95 ± 12 beats per min, P < 0.003). In contrast, mean aortic pressure (101.5 ± 4.8 versus 114.7 ± 10.9 mmHg, P < 0.002) was decreased, as was cardiac output (2.79 ± 0.43 versus 4.14 ± 1.02 liter/min, P < 0.002). In addition, left atrial pressure and resting values of RV dP/dt, LV dP/dt, and dP/dt/P were lower in RVHF animals than in sham-operated controls (Table 2).

RT–PCR. Specificity of all PCR assays was initially determined by dyeode sequencing of the amplified products. Prior to quantitative analysis, titration assays were performed for each gene to determine the appropriate relative amounts of cDNA and internal reference construct to be used. Quantitative analysis was restricted to the exponential phase of PCR amplification. The RT and competitive PCR assays for each of the RAS genes were performed using a single dilution of reference construct and a single master mixture preparation for all RV and LV samples from RVHF and sham-operated dogs (40 samples, 320 reactions).

To calibrate efficiency of individual RT reactions, nonmammalian DNA containing parts of the X. laevis 5' and 3' untranslated β-globin sequences was transcribed in vitro and a known amount subsequently reverse-transcribed along with each canine mRNA sample. Amplification of canine cDNA in the presence of X. laevis β-globin primers, but in the absence of β-globin DNA, did not yield a PCR product. Therefore, the number of β-globin cDNA copies determined after RT cor-

<table>
<thead>
<tr>
<th>Parameters</th>
<th>RVHF (n = 11)</th>
<th>Sham (n = 9)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td>127 ± 25</td>
<td>95 ± 12</td>
<td>&lt;0.003</td>
</tr>
<tr>
<td>Right atrial pressure, mmHg</td>
<td>10.6 ± 1.5</td>
<td>4.3 ± 1.9</td>
<td>&lt;1.0^-6</td>
</tr>
<tr>
<td>Mean aortic pressure, mmHg</td>
<td>101.5 ± 4.8</td>
<td>114.7 ± 10.9</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Cardiac output, liter/min</td>
<td>2.79 ± 0.43</td>
<td>4.14 ± 1.02</td>
<td>&lt;0.002</td>
</tr>
<tr>
<td>Left atrial pressure, mmHg</td>
<td>7.3 ± 2.0</td>
<td>9.2 ± 2.5</td>
<td>NS</td>
</tr>
<tr>
<td>RV dP/dt, mmHg/sec</td>
<td>413 ± 147</td>
<td>601 ± 82</td>
<td>&lt;10^-3</td>
</tr>
<tr>
<td>LV dP/dt, mmHg/sec</td>
<td>2055 ± 351</td>
<td>3130 ± 445</td>
<td>&lt;10^-5</td>
</tr>
<tr>
<td>LV dP/dt, sec^-1</td>
<td>33.7 ± 4.2</td>
<td>42.8 ± 6.9</td>
<td>&lt;10^-3</td>
</tr>
<tr>
<td>LV wt/body wt, g/kg</td>
<td>3.82 ± 0.60</td>
<td>4.28 ± 0.28</td>
<td>NS</td>
</tr>
<tr>
<td>RV wt/body wt, g/kg</td>
<td>2.14 ± 0.33</td>
<td>1.55 ± 0.15</td>
<td>&lt;10^-4</td>
</tr>
</tbody>
</table>

Cardiac and hemodynamic parameters of RVHF and sham-operated animals. Values are mean ± SD. RV, right ventricular; LV, left ventricular; wt, weight.

Table 2. Physiologic parameters
responded to that fraction of \textit{in vitro} transcribed \(\beta\)-globin RNA that was reverse transcribed during the reaction. To validate the approach of calibrating RT efficiency using an artificial construct, we determined if the presence of \textit{in vitro} transcribed \(\beta\)-globin RNA in the RT reaction affects RT efficiency for the genes of interest and the accuracy of RT efficiency measurement.

Canine mRNA was reverse transcribed in the absence and in the presence of \textit{in vitro} transcribed \(\beta\)-globin RNA at 50 times the quantity that was later used for calibration of RT efficiency. The two reactions were otherwise performed as exact duplicates of each other, using single, split preparations of reaction components. After reverse transcription expression levels of the AT\(_1\) receptor were determined by competitive PCR. Each of the two reactions was performed in duplicate. No significant difference in the amount of AT\(_1\) mRNA reverse transcribed in the presence or absence of the \(\beta\)-globin construct were discernable (3.22 ± 0.37 versus 3.45 ± 0.10 fmol/mg mRNA, \(P = 0.260\)).

Next, two mRNA samples from the same tissue, but set up as completely separate reactions, were reverse transcribed along with \(\beta\)-globin RNA according to the protocol described above. Each of the reactions was performed in duplicate. RT efficiency was determined for each sample by quantification of the fraction of \(\beta\)-globin RNA reverse transcribed. AT\(_1\) mRNA levels were measured by quantitative PCR by correcting for RT efficiency determined. When corrected for sample-specific \(\beta\)-globin recovery, AT\(_1\) mRNA levels in the two reactions were found to be within 2% of each other.

RT efficiencies were found to vary between extremes of 11% and 81% in separate assays, although the variance among samples processed in parallel was considerably less (42% ± 16%, mean ± SD). These results indicate the importance of performing this standardization for the determination of absolute amounts.

**RVHF and Gene Expression of Components of the RAS.** For all components of the cardiac RAS, comparisons were carried out among sham-operated dogs and RVHF, and between RV and LV in each group. Angiotensinogen mRNA levels were significantly higher in RVs than in LVs in both sham-operated and RVHF animals (158.5 ± 69.7 versus 52.2 ± 38.7 fmol/nmol \(\beta\)-actin, \(P < 0.003\)) and no significant change in expression level was observed in RVHF as compared with sham-operated animals (Fig. 2A).

Renin mRNA was detected in all samples at very low levels. No significant differences in renin gene expression was found when comparing RVHF versus sham-operated animals (Fig. 2B).

ACE and chymase were expressed at similar levels in both ventricles. Chymase mRNA levels were about fourfold higher than those of ACE. RVHF was not associated with any changes of ACE and chymase expression in the LV. However, in the RV a significant increase of ACE mRNA by about 90% was observed (0.85 ± 0.40 versus 0.47 ± 0.19 pmol/nmol \(\beta\)-actin, \(P < 0.03\); Fig. 2C), while chymase mRNA levels markedly decreased (0.83 ± 0.40 versus 2.18 ± 1.28 pmol/nmol \(\beta\)-actin, \(P < 0.004\); Fig. 2D). ACE mRNA levels showed significant correlation with right atrial pressure \((r^2 = 0.76, P < 0.005)\).

Fig. 2E and F show the expression of the AT\(_1\) and AT\(_2\) receptor genes in the LVs and RVs of sham-operated and RVHF animals. AT\(_1\) mRNA was found to be expressed more abundantly than that of the AT\(_2\) receptor. In sham-operated animals, no significant differences between expression levels in the LVs and RVs were discernible for either receptor. In RVHF dogs, right ventricular AT\(_1\) mRNA levels were significantly decreased compared with sham-operated controls (1.86 ± 0.83 versus 3.30 ± 1.29 pmol/nmol \(\beta\)-actin, \(P < 0.01\)), while a concomitant trend toward increased expression of the AT\(_1\) message was observed in the LV. AT\(_1\) expression showed significant correlation with RV weight \((r^2 = -0.66, P < 0.03)\).

**DISCUSSION**

Angiotensin II has been described as a potent direct stimulator of cardiac myocyte and fibroblast growth (22), and a significant role in the mediation and modulation of cardiac growth and hypertrophy has been proposed for the cardiac RAS. Recent \textit{in vitro} work suggests that induction of the cardiac RAS is mediated via mechanical (stretch) stimuli (16), highlighting the importance of a clearer understanding of the regulatory biology of this system in health and disease. The present study was aimed at testing whether \textit{in vivo}, in the face of systemic activation of neurohumoral effector systems, the same is true, or whether under these conditions soluble factors modulate cardiac RAS gene expression. In addition, to gain a more comprehensive understanding of cardiac RAS gene regulation, we deemed it important to examine simultaneously, and in all tissues, gene expression of all presently known components of the RAS. Lastly, we considered it important to develop the molecular tools for the study of the canine cardiac...
RAS: despite the wide use of dogs as a model organism in cardiology research, none of the genes coding for RAS components (with the exception of chymase) had been cloned in this species, and no information about the canine RAS on the level of gene expression has so far been presented. To distinguish load-dependent from load-independent changes in the canine heart, we examined a model of right ventricular pressure and volume overload. In contrast to LV overload models of hypertrophy/failure, which are inevitably associated with some degree of hemodynamic embarrassment (i.e., mechanical stress) of the RV, the effective unloading experienced by the LV in this model renders it an ideal nonstressed control. Because the LV is still exposed to the same circulating neurohumoral milieu, this model allows the distinction between the two putative mechanisms affecting cardiac RAS gene expression. In pursuing this line of investigation, we have (i) provided evidence indicating that local, mechanical stress rather than soluble factors modulate cardiac RAS gene expression in vivo, (ii) generated new comprehensive information on gene expression of all components of the cardiac RAS concomitantly, and (iii) established partial sequence information and a set of competitive PCR assays for all components of the canine RAS.

We used a model of ventricular hypertrophy that had progressed to the stages of failure since observations in rats indicated that changes in the expression of RAS components become progressively more pronounced during the transition from compensated hypertrophy to frank heart failure (M. Lee and K.L., unpublished data). Indeed, marked increases in the expression of ACE were observed, in keeping with previous observations in various forms of (left) ventricular hypertrophy, consistent with an activation of the cardiac RAS (23, 24). Meanwhile, chymase expression was significantly decreased in the hypertrophic RV, possibly indicating differential roles of the two enzymes in health and disease. Apart from man, marmoset, and hamster, the dog is the only known species in which this alternative, highly specific pathway of angiotensin II formation has been described (5). Previous studies in human hearts did not report any changes in chymase expression in heart failure (24); however, these studies were conducted on surgical specimens from patients with various cardiac disorders such as coronary artery disease and dilated cardiomyopathies, and from patients who were, in part, treated with ACE inhibitors.

At least two main receptor subtypes for angiotensin II, termed AT₁ and AT₂, have been identified based on binding to non-peptide antagonists (25, 26). Almost all known cardiovascular functions of angiotensin II, such as vasconstriction, positive inotropy and chronotropy, and in vitro-induced cardiomyocyte hypertrophy (22) appear to be mediated by the G-protein-coupled AT₁. In contrast, the function of the AT₂ receptor, which is preferentially expressed during fetal development and downregulated after birth (27, 28), remains largely unknown, although recent data support a possible role in behavior, blood pressure, and apoptosis (29–31). AT₁ and AT₂ receptors are present in the myocardium, and increased binding to both receptors has been reported in cardiac hypertrophy due to renovascular and genetic hypertension in the rat (32). A recent report (33) indicates that the AT₂ receptor may mediate angiotensin II stimulated collagen synthesis in human cardiac fibroblasts. We show that in the adult dog, the AT₁ receptor is predominantly expressed. The discordant changes in expression levels of the AT₁ and AT₂ receptor seen in RVHF illustrate selective, differential modulation of cardiac RAS component gene expression. The observed pattern is commensurate with a switch toward a fetal gene expression program that has previously been described as typical for cardiac hypertrophy (34). Our findings are in agreement with receptor binding studies in cardiac hypertrophy in the rat where a similar reversal of the AT₁/AT₂ receptor ratio has been reported (35) and in wound healing (36) that are consistent with the notion that the AT₁/AT₂ receptor ratio may critically influence the trophic effects of angiotensin peptides.

We found no changes in angiotensinogen mRNA expression in RVHF, in contrast to previous reports of a transient increase in the expression of cardiac angiotensinogen in rat models of afterload-induced and postmyocardial infarction LV hypertrophy (11, 37). This discrepancy may be due to differences between rat and dog, between volume and pressure overload hypertrophy, between compensated hypertrophy and frank failure, between RV and LV, between acute and chronic states of cardiac hypertrophy, or may represent a time course-dependent phenomenon. Comparative studies in other models of canine RVHF, presently underway, will provide answers to this question. Similarly, the role of renin, so far least explored due to the difficulty measuring the low concentrations present in the heart, can be successfully examined using the assay system established for the present study.

The RVHF model examined allowed us to compare changes in expression of the RAS components in the presence or absence of hemodynamic stress, a comparison not possible in models of left ventricular hypertrophy and failure. Markedly different modulation of gene expression in RV and LV, both exposed to the same circulating milieu, but to significantly different loads of mechanical stress (increased in the RV, reduced in the LV) suggests strongly that expression of the components of the RAS in the intact animal, much like in in vitro experiments, is modulated by local, mechanical rather than by systemic, humoral factors. Although the ratio of right to left coronary flow, and thus potentially the exposure to soluble factors that may modulate gene expression, changes in the model studied, differential coronary flow is unlikely to account for our observations as baseline differential flow (left > right) is not associated with differential gene expression for those RAS components that we found to be affected by RVHF (38–40). Opposite changes observed for ACE and chymase, and for AT₁ and AT₂ receptor expression, support not only specific physiological roles of the different components of the cardiac RAS in cardiac hypertrophy, but also the existence of a complex and differentiated messenger system that translates mechanical stimuli into specific transcriptional regulatory signals.

We chose to develop a set of competitive PCR assays to measure canine RAS gene expression for several reasons. (i) The low expression of some components, particularly renin, renders alternative approaches such as Northern blotting and RNase protection assays useless; (ii) the ability to perform measurements on very small tissue samples opens the possibility for future studies with high longitudinal (time course) or spatial (myocardial regions) resolution using biopsy specimens; and (iii) the method provides a means of determining absolute amounts of messenger RNA. To ensure reproducibility and to allow for absolute quantitation, we used specific strategies and rigorous quality control to assess efficiency of both RT and PCR reactions, enabling us to express levels of expression in moles, normalized only for the expression of a housekeeping gene, with an accuracy of ±5%. While glycolaldehyde 3-phosphate dehydrogenase (GAPDH) is currently the gene most commonly used for this purpose, the presence of multiple GAPDH pseudogenes in the dog prohibited its use. We are aware that β-actin, the alternative we chose, has been shown to be induced to some extent in cardiac hypertrophy. Such an induction would result in a bias toward underestimating the increased expression of the AT₂ receptor and ACE genes we observed, and toward a potential overestimation of the decreases in expression found for the AT₁ and chymase. However, the extent to which these genes were found to be at variance from control values clearly exceeds previously observed modest changes of β-actin mRNA in cardiac hypertrophy (41). Also, comparisons among our RVHF and control
tissues showed only minimal, statistically not significant differences in β-actin mRNA expression (0.136 ± 0.064 versus 0.106 ± 0.058 pmol/mg mRNA, P = 0.283). Our method provides robust and powerful means to maximize the information that can be gained from rare and valuable tissue samples by allowing the measurement of multiple mRNA species in one sample without the need for repeated RT reactions.

Our initial concerns about possible heterogeneity of gene sequences among mongrel dogs were found unsubstantiated based on sequencing of cDNAs prepared from several individual animals which showed no dissimilarities. This is not surprising since the regions selected for amplification were known to be highly conserved.

A limitation of the present study is its restriction to assessing transcriptional modulation. Functional studies measuring local peptide/protein synthesis (a task well recognized for its difficulty due to spillover of circulating RAS components and sequestration in tissues), enzyme activities, and receptor binding will be necessary to incorporate an assessment of post-transcriptional regulation. In addition, the present data provide no information regarding the cardiac cell populations—among which cardiomyocytes and fibroblasts are the most important candidates—that are the source of individual transcripts. Also, the present study addresses only the state of RVHF, and allows no direct extrapolation regarding less advanced stages of RV overload. Lastly, the present investigation was not aimed at delineating a proposed causative or contributory role of altered cardiac RAS gene expression to the process of ventricular hypertrophy, remodeling, or failure. The powerful methodology established with this work, however, provides the means for the study of this and other questions in the future.

In summary, the present experiments established a system for the study of RAS gene expression in the dog, a prominent model system in cardiovascular research. Importantly, we provide evidence that local mechanical factors, rather than systemically circulating humoral factors, regulate expression changes of RAS components in vitro, validating previous in vivo data as physiologically relevant. The present work represents the first investigation of a tissue RAS in which all components of the system were measured simultaneously, providing a previously unavailable, comprehensive view of its overall regulation. We expect that the reagents and methods developed will prove helpful to the future study of the canine cardiac RAS in a variety of pathophysiological conditions.

We are grateful to Dr. Detlev Ganten for helpful discussions and advice. K.L. was supported by a Harcourt General Charitable Foundation Young Investigator’s Award and by a Research Career Development Award from the National Heart, Lung, and Blood Institute. Y.-A.L. was supported by a Scholarship Award of the German Academic Exchange Service.