Angiotensin II formation from ACE and chymase in human and animal hearts: methods and species considerations

EDUARDO BALCELLS, QING C. MENG, WALTER H. JOHNSON, J. R., SUZANNE OPARIL, AND LOUIS J. DELL’ITALIA

Birmingham Veteran Affairs Medical Center and Division of Cardiovascular Disease, Department of Medicine, Vascular Biology and Hypertension Program, and Division of Pediatric Cardiology, Department of Pediatrics, University of Alabama, Birmingham, Alabama 35294

The presence of chymase in the heart has raised important questions regarding the origin of ANG II and the mechanism of action of ACE inhibitors, not only in the human heart but also in animal models of hypertension and heart failure. ANG II formation from ACE and chymase has not been systematically compared in the human heart and in the hearts of various animal species. Thus the purpose of the current investigation was to quantitatively measure the capacities of ACE and chymase in heart tissue from human, dog, rat, mouse, and rabbit.

ANG II formation from ACE and chymase in human and animal hearts: methods and species considerations. Am. J. Physiol. 273 (Heart Circ. Physiol. 42): H1769–H1774, 1997.—The current study examined the contributions of angiotensin-converting enzyme (ACE) vs. chymase to angiotensin II (ANG II) generation in membrane preparations from left ventricles of humans, dogs, rabbits, and rats and from total heart of mice. ACE and chymase activity were measured in membrane preparations extracted with low or high detergent (LD and HD, respectively) concentrations. We hypothesized that ACE, which is membrane bound in vivo, would be preferentially localized to the HD preparation, whereas chymase, which is localized to the cytoplasm and cardiac interstitium, would be localized to the LD preparation. In human heart, ACE activity was 16-fold higher in the HD than in the LD preparation, whereas chymase activity was 15-fold higher in the LD than in the HD preparation. Total ANG II formation was greater in human heart (15.8 ± 3.4 SE µmol ANG II·g⁻¹·min⁻¹) than in dog, rat, rabbit, and mouse hearts (3.90 ± 0.35, 0.41 ± 0.02, 0.61 ± 0.07, and 1.16 ± 0.08 SE µmol ANG II·g⁻¹·min⁻¹, respectively. P < 0.05, by analysis of variance). ANG II formation from ACE was higher in mouse heart (1.09 ± 0.05 µmol ANG II·g⁻¹·min⁻¹, P < 0.001) than in rabbit, human, dog, and rat hearts (0.55 ± 0.06, 0.34 ± 0.01, 0.32 ± 0.06, and 0.31 ± 0.02 µmol ANG II·g⁻¹·min⁻¹, respectively). In contrast, chymase activity was higher in human heart (15.3 ± 3.4 µmol ANG II·g⁻¹·min⁻¹) than in dog, rat, rabbit, and mouse hearts (3.59 ± 0.29, 0.10 ± 0.01, 0.06 ± 0.01, and 0.07 ± 0.01 µmol ANG II·g⁻¹·min⁻¹, respectively). Our results demonstrate important species differences in the pathways of intracardiac ANG II generation. Chymase predominated over ACE activity in human heart, accounting for extremely high total ANG II formation in human heart compared with dog, rat, rabbit, and mouse hearts.

Components of the renin-angiotensin system (RAS) have been demonstrated in the heart by biochemical, immunohistochemical, and molecular biologic techniques (12) and have been shown to be upregulated in hearts that have developed pressure and volume overload-induced hypertrophy and failure (5, 9, 17, 22). Thus there is increasing evidence that angiotensin II (ANG II) formation in the heart is mediated by a local RAS, acts independently of the circulating RAS, and is upregulated by hemodynamic stress. However, most research on the intracardiac RAS in the basal state and under conditions of stress has been performed in rodent models, and the relevance to humans has been questioned, especially since there are multiple ANG II-forming pathways in cardiac tissue that vary among species (4, 16). In particular, a serine protease with extremely high affinity for ANG I, “chymostatin-sensitive angiotensin-generating enzyme,” has recently been identified in the human (25), dog (1, 3), and baboon (6) heart, but not in the rodent heart (7).

Human “heart chymase” has been purified, cloned, and sequenced from the human heart (26). It is insensitive to angiotensin-converting enzyme (ACE) inhibition, and the catalytic activity for conversion of ANG I to ANG II is 20-fold higher for chymase than for ACE (8). However, there has been controversy regarding the ANG II-forming capacity of chymase vs. ACE in human heart tissue extracts. Urata and co-workers (25) reported that this enzyme represents >90% of the ANG II-forming capacity in human heart tissue extracts solubilized with low detergent concentration (0.01% Triton), suggesting that ACE is not the major ANG II-forming enzyme in the human left ventricle in vitro. In contrast, Zisman and co-workers (29) demonstrated >80% ANG II formation from ACE in human heart tissue extracts solubilized with high detergent concentration (0.6% Triton) and dialysis. However, Wolny and co-workers (27) recently showed that solubilization with high concentrations of detergent and dialysis result in a major loss of chymase activity during sample preparation, thus providing a possible explanation for underestimated chymase-mediated ANG II-forming activity in the study of Zisman and co-workers. Taken together, these studies demonstrate that ANG II-forming capacities of ACE and chymase in cardiac tissue in vitro differ according to the method used to process the tissue.

The controversy over the predominance of ACE- vs. chymase-induced ANG II formation in heart tissue extracts may be related to the localization of chymase and ACE in the heart. ACE is bound to cell membranes of endothelial cells (10), whereas chymase is stored in vesicles in the intracellular compartment of mast cells and other types of interstitial cells in the heart (24). The presence of chymase in the heart has raised important questions regarding the origin of ANG II and the mechanism of action of ACE inhibitors, not only in the human heart but also in animal models of hypertension and heart failure. ANG II formation from ACE and chymase has not been systematically compared in the human heart and in the hearts of various animal species. Thus the purpose of the current investigation was to quantitatively measure the capacities of ACE and chymase in heart tissue from human, dog, rat, mouse, and rabbit. We hypothesized that ACE,
which is membrane bound in vivo, would be preferentially localized to the preparation with the high detergent concentration, whereas chymase, which is localized to the cytoplasm and cardiac interstitium, would be localized to the preparation with the low detergent concentration.

METHODS

Tissue Procurement

Normal human donor hearts (n = 5) not suitable for transplantation were obtained at the time hearts were harvested for organ donation at the University of Alabama at Birmingham. All hearts were kept in cold cardioplegia solution from the time of removal, and tissue from the left ventricular free wall was frozen in liquid nitrogen within 5–10 min. Hearts from adult mongrel dogs (n = 5, 20–25 kg body wt) and rabbits (n = 3, 3–4 kg body wt; New Zealand White, Myrtle, Memphis, TN) were obtained after a deep surgical plane of anesthesia was induced with isoflurane inhalation anesthesia for the thoracotomy. Hearts were arrested with a lethal dose of KCl, removed from the chest, rapidly cooled in ice-cold phosphate buffer, and placed on a stainless steel tray on ice. All animal hearts were dissected free of major blood vessels, cardiac valves, atria, and right ventricle. Tissue from the left ventricular midwall was frozen in liquid nitrogen within 2–3 min and stored at −80°C. Male Sprague-Dawley rats (n = 8, mean weight 300 g) and male CD-1 mice (n = 6, mean weight 25 g) were purchased from Charles River Breeding Laboratories (Wilmington, MA) and decapitated before removal of the heart. Rat hearts were dissected free of cardiac valves, atria, and right ventricle. The left ventricular free wall was frozen in liquid nitrogen within 2–3 min and stored at −80°C. Mouse hearts were frozen in toto in liquid nitrogen within 2–3 min after decapitation and stored at −80°C. The protocol was approved by the Institutional Review Board for Human Use of the University of Alabama at Birmingham and by the Animal Services Committees at the University of Alabama at Birmingham.

Cardiac Membrane Preparation

Low detergent concentration. Membranes were prepared at 4°C in a manner similar to that previously described in our laboratory (1, 3) and by Urata and co-workers (25). Frozen heart tissue was homogenized with a Polytron (Fisher Scientific, Pittsburgh, PA) for 60 s in 100 mM potassium phosphate buffer (PBS), pH 7.4 in a 10:1 volume ratio, then centrifuged for 30 min at 44,000 g. The tissue suspension was then centrifuged at 40,000 g for 30 min, and the supernatant and pellet fractions were collected as fractions supernatant 1 (S1) and pellet 1 (P1), respectively (Fig. 1). ANG II-forming activity in P1 was obtained from an aliquot of the resuspended tissue pellet in 100 mM PBS (pH 8.3).

High detergent concentration. The pellet fraction (P1), prepared as described above, was resuspended in 1 ml of PBS buffer (pH 8.3) with 0.6% Triton X-100, vortexed for 3 min, and mechanically agitated for 4 h at 4°C. The tissue suspension was then centrifuged at 40,000 g for 30 min, and the supernatant and pellet fractions were collected as fractions S2 and P2, respectively. ANG II-forming activity in P2 was obtained from an aliquot of the resuspended tissue pellet in 100 mM PBS (pH 8.3), whereas an aliquot of the S2 fraction was assayed without further processing.

ANG II-Forming Activity From ACE and Heart Chymase

Aliquots (10 µl) were taken from each of the above fractions and preincubated for 30 min at room temperature with an

enzyme inhibitor solution specific to chymase-like or ACE activity assays. For ANG II-forming chymase activity assays, the inhibitor solution contained 2 mM EDTA, 100 µM captopril (Sigma Chemical, St. Louis, MO), 1 mM o-phenanthroline, and 20 µM aprotinin with and without 100 µM chymostatin. For ANG II-forming ACE activity assays, the inhibitor solution contained 1 mM o-phenanthroline, 20 µM aprotinin, and 100 µM chymostatin with and without 100 µM captopril. Samples were then incubated for 60 min at 37°C with 600 µM ANG I (Sigma Chemical) in 100 mM phosphate buffer (pH 8.3) solution containing 300 mM NaCl and 10−4 M ZnCl2 (omitted from chymase activity assays) to a final total volume of the reaction assay of 250 µl. Reactions were terminated by addition of ice-cold ethanol in a 1:3 (vol/vol) sample-to-ethanol ratio.

Generated ANG II was quantitated using a reverse-phase Alltech 5-µm phenyl-high-performance liquid chromatography column (Alltech Associates, Deerfield, IL), as previously performed in our laboratory (1, 3). The peak area corresponding to a synthetic ANG II standard was integrated to calculate absolute ANG II formation. ANG II-forming activity from ACE was defined as the captopril-inhibitable ANG II formed, whereas chymase activity was defined as the chymostatin-inhibitable ANG II formed. ACE and chymase-like activities are expressed as moles of ANG II formed per gram of protein per minute and protein content was determined by the method of Lowry and co-workers (14).

Statistics

Values are means ± SE. Analysis of variance with Newman-Keuls post hoc comparison was used to compare ANG II-forming capacity from ACE and chymase in S1, S2, and P2 of HD and LD cardiac membrane preparations and total ANG II formation across species. P < 0.05 was required to reject the null hypothesis.

RESULTS

ANG II Formation From ACE and Chymase in the Human Heart

ANG II formation from chymase was higher in the low-detergent membrane fractions (P1 and P2: 15.3 ± 3.4 and 13.3 ± 2.9 µmol·g protein−1·min−1) than in the high-detergent membrane fraction (S2: 0.96 ± 0.21 µmol·g protein−1·min−1, P < 0.001; Fig. 2A). In con-
trast, ACE activity was higher in the high-detergent membrane fraction (S2: 0.339 ± 0.008 μmol·g protein⁻¹·min⁻¹) than in the low-detergent membrane fractions (P1 and P2: 0.022 ± 0.022 and 0.087 ± 0.056 μmol·g protein⁻¹·min⁻¹, P < 0.01; Fig. 2B). Nevertheless, total ANG II formation from chymase predominated over ANG II formation from ACE not only in low-detergent membrane fractions (P1 and P2: 15.3 ± 3.4 and 13.3 ± 2.9 vs. 0.022 ± 0.022 and 0.087 ± 0.056 μmol·g protein⁻¹·min⁻¹) but also in the high-detergent membrane fraction (S2: 0.96 ± 0.21 vs. 0.339 ± 0.008 μmol·g protein⁻¹·min⁻¹).

Species Differences in ANG II Formation

Total intracardiac ANG II formation from ACE and chymase was determined utilizing the HD (S2) and LD (P1) membrane preparations as follows:

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\text{total ANG II formation (μmol/g/min)} = [\text{chymase activity}]^1 + [\text{ACE activity}]^2
\]

The percent ANG II formed from ACE was determined from ACE activity in the high-detergent membrane fraction (S2) divided by total ANG II formation as defined above, whereas the percent ANG II formed from heart chymase was determined from chymase activity in the low-detergent membrane fraction (P1) divided by total ANG II formation as defined above.

Figure 3 demonstrates that total ANG II formation from ACE and chymase, as defined by the equation above, was highly variable across species. Total ANG II formation was greater in human and dog hearts (15.8 ± 3.4 and 3.90 ± 0.35 μmol·g protein⁻¹·min⁻¹, respectively) than in rat, rabbit, and mouse hearts (0.41 ± 0.02, 0.61 ± 0.07, and 1.16 ± 0.08 μmol·g protein⁻¹·min⁻¹, respectively), and this difference was due to greater chymase activity (Fig. 4A). In contrast, total ANG II formation from ACE did not differ among human, dog, rat, and rabbit hearts (0.34 ± 0.01, 0.32 ± 0.06, 0.31 ± 0.02, and 0.55 ± 0.06 μmol·g protein⁻¹·min⁻¹, respectively) but was higher in the mouse heart (1.09 ± 0.05 μmol·g protein⁻¹·min⁻¹, P < 0.001; Fig. 4B). Taken together, percent ANG II formation from chymase predominated over ACE in human (97.5 ± 0.2% vs. 2.5 ± 0.4%) and dog (92.3 ± 3.0% vs. 7.7 ± 2.3%) hearts, whereas ACE predominated over chymase in rat (75.6 ± 1.5% vs. 24.4 ± 0.4%), rabbit (90.2 ± 5.7% vs. 9.8 ± 5.7%), and mouse hearts (94.0 ± 0.6% vs. 6.0 ± 0.6%; Fig. 5).

DISCUSSION

The major finding of the current study was that ACE activity was 16-fold higher in high- than in low-detergent preparations, whereas chymase activity was 15-fold higher in low- than in high-detergent human cardiac membrane preparations. By use of combined high- and low-detergent membrane preparations, chymase predominated over ACE activity in human and dog hearts, whereas ACE was the predominant ANG II-forming mechanism in mouse, rat, and rabbit hearts. The presence of chymase accounted for the extremely high total ANG II formation in human heart compared with other species. Whether enhanced ANG II formation from chymase in the human heart is of physiological or pathophysiological significance is a source of controversy.

Studies in animals known to express chymase have yielded contradictory results regarding the roles of ACE vs. chymase in ANG II formation. In conscious baboons, intravenous infusion of the chymase-specific substrate [Pro11-D-Ala12]ANG I resulted in increased left ventricular systolic and diastolic pressures consistent with arterial vasoconstriction (6). These effects were antagonized by the ANG II receptor antagonist losartan but not by an ACE inhibitor, thus demonstrating the in vivo contribution of chymase to ANG II generation. In patients with peripheral vascular disease, maximal walking distance and subjective symptoms were improved by nafamostat, a serine protease...
inhibitor (23). Isolated vascular ring preparations of the human gastroepiploic arteries (16) and coronary arteries (27) demonstrated that 40–90% of contraction was a result of chymase-related ANG I conversion. The high tissue ANG II-forming capacity in our samples is consistent with the important functional effect of chymase-mediated ANG I conversion in the vasculature of humans with peripheral vascular disease (23) and in the human vascular ring preparations cited above (16, 27).

In contrast, other in vivo studies in the dog have demonstrated the predominance of ACE-mediated ANG II formation across the coronary circulation. We previously showed in the normal dog that >60% of ANG II formation across the myocardial vascular bed was inhibited by intracoronary infusion of captopril, whereas only 15% was inhibitable by intracoronary infusion of chymostatin (1). In a similar fashion, the fractional conversion of ANG I to ANG II across the myocardial circulation in orthotopic heart transplant recipients was reduced by 89% during intracoronary infusion of enalaprilat (29). Taken together, circulatory ANG I conversion appears to be predominantly ACE mediated because of the location of ACE in endothelial cell membranes, with its catalytic site exposed to the luminal surface, and the presence of ACE in the plasma. In contrast, there is minimal chymase-mediated ANG I conversion in the circulation, because 1) chymase is located in the interstitium, within mast cells, and within endothelial cells with secretion in the basolateral direction and 2) chymase is not present in the plasma (25).

There has also been controversy regarding the ANG II-forming capacity of chymase vs. ACE in human heart tissue extracts. Urata and co-workers (25) reported that chymase represents ~90% of the ANG II-forming capacity in human heart tissue extracts solubilized with low detergent concentration (0.01% Triton), suggesting that ACE is not the major ANG II-forming enzyme in human left ventricle in vitro. In contrast, Zisman and co-workers (29) demonstrated >80% ANG II formation from ACE in human heart tissue extracts solubilized with high detergent concentration (0.6% Triton) and dialysis. Our results demonstrated that ACE activity is 16-fold higher in high- than in low-detergent cardiac membrane preparations, suggesting that the discordant results of these two studies may be related to experimental design. Because ACE is bound to cell membranes of endothelial cells, accessibility of ANG I substrate to ACE may be decreased in low-detergent membrane preparations. Indeed, Kinoshita and co-workers (11) demonstrated that initial detergent extraction with 0.1% Triton X-100 during purification of ACE from human lung tissue increased ACE activity 3.6-fold compared with the nonsolubilized tissue extract. Furthermore, Wolny and co-workers (27) demonstrated that solubilization and dialysis during membrane preparation, as utilized in the study by Zisman and co-workers, resulted in a >70% loss of chymase-mediated ANG II generation in human heart tissue. Thus methods of handling during biochemical assays can clearly affect enzymatic activity. Nevertheless, our results demonstrated that ANG II formation from chymase was threefold higher than ANG II formation from ACE, even in the high-detergent cardiac membrane preparations (0.96 ± 0.21 vs. 0.339 ± 0.008 µmol·g protein⁻¹·min⁻¹; Fig. 2) and is consistent with the greater activity of chymase than of ACE in converting ANG I to ANG II (8).

In contrast to previous reports that the rodent heart lacks chymase-like activity, our study demonstrated that 24% of ANG II-forming capacity of rat heart was inhibited by chymostatin. Recent phylogenetic evidence indicates that mammalian chymases occur as three distinct enzyme groups, α and β (2), which differ in their substrate specificity. α-Chymases include human, dog, and rat chymase-3, which convert ANG I to ANG II by cleaving the Phε⁸-His² bond in ANG I (21). The ANG II generated is not further degraded, because its Tyr⁴-Ile¹ bond is resistant to cleavage by the α-chymases. β-Chymases (rat chymase-1 and -2 and mouse chymase-1, -2, -3, and -L) are angiotensinases, because...
they readily cleave the Tyr^4-Ile^5 and Phe^8-His^9 bonds in angiotensins, thus inactivating them. The action of rat chymase-3 could account for the chymostatin-inhibitable ANG II formation in our rat heart assays. This may, in part, explain the failure of ACE inhibitor therapy to attenuate pressure (15, 18, 28) and volume (19) overload-induced left ventricular hypertrophy in the rat, which has been observed in some studies. These findings suggest incomplete inhibition of ANG II formation by ACE inhibitors and/or additional ANG II-forming pathways. In addition, stretch of neonatal rat myocytes has been shown to induce release of ANG II that is independent of an increase in ACE activity and unaffected by prior treatment with ACE inhibitor (20). Taken together, in vivo and in vitro data suggest that there are non-ACE-dependent pathways of ANG II formation in the rat heart that may be attributed to rat chymase-3.

Differences among species in the relative contributions of ACE and chymase to ANG II formation in heart reflect the limitations of extrapolating from animal models of heart failure and cardiac hypertrophy to the human. Use of ACE inhibitors in the routine treatment of heart failure makes this issue even more important, since inhibition of intracardiac ACE with these drugs would increase ANG I levels and shunt this substrate to ANG II formation in vitro should be assessed using a combined approach that optimizes the conditions for membrane-bound (ACE) and intracellular and interstitial (chymase) location of these enzymes.

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Address for reprint requests: L. J. Dell’Italia, Div. of Cardiology, Dept. of Medicine, University of Alabama at Birmingham, 310 Lyons Harrison Research Bldg., 701 South 19th St., Birmingham, AL 35294.

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