Primary role of angiotensin-converting enzyme-2 in cardiac production of angiotensin-(1–7) in transgenic Ren-2 hypertensive rats

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Submitted 1 November 2006; accepted in final form 12 February 2007

Trask AJ, Averill DB, Ganten D, Chappell MC, Ferrario CM. Primary role of angiotensin-converting enzyme 2 in cardiac production of angiotensin-(1–7) in transgenic Ren-2 hypertensive rats. Am J Physiol Heart Circ Physiol 292: H3019–H3024, 2007. First published February 16, 2007; doi:10.1152/ajpheart.01198.2006.—Angiotensin-converting enzyme-2 (ACE2) converts angiotensin II (ANG II) to angiotensin-(1–7) [ANG-(1–7)], and this enzyme may serve as a key regulatory juncture in various tissues. Although the heart expresses ACE2, the extent that the enzyme participates in the cardiac processing of ANG II and ANG-(1–7) is equivocal. Therefore, we utilized the Langendorff preparation to characterize the ACE2 pathway in isolated hearts from male normotensive Sprague-Dawley [Tg(−/ −)] and hypertensive [mRen2(27) Tg(+/ +)] rats. During a 60-min recirculation period with 10 nM ANG II, the presence of ANG-(1–7) was assessed in the cardiac effluent. ANG-(1–7) generation from ANG II was similar in both the normal and hypertensive hearts [Tg(−/ −): 510 ± 55 pm, n = 20 vs. Tg(+/ +): 497 ± 63 pm, n = 14] with peak levels occurring at 30 min after administration of the peptide. ACE2 inhibition (MLN-4760, 1 μM) significantly reduced ANG-(1–7) production by 83% (P < 0.01, n = 7) in the Tg(+/ +) rats, whereas the inhibitor had no significant effect in the Tg(−/ −) rats (285 ± 53 pm, P > 0.05, n = 10). ACE2 activity was found in the effluent of perfused Tg(−/ −) and Tg(+/ +) hearts, and it was highly associated with ACE2 protein expression (r = 0.78). This study is the first demonstration for a direct role of ACE2 in the metabolism of cardiac ANG II in the hypertrophic heart of hypertensive rats. We conclude that predominant expression of cardiac ACE2 activity in the Tg(+/ +) may be a compensatory response to the extensive cardiac remodeling in this strain.

angiotensin II; hypertension; isolated heart

The involvement of the renin-angiotensin system (RAS) in the development of hypertension, cardiac hypertrophy, and subsequent transition to heart failure is without question. However, newer studies suggest that the hypertrophic and profibrotic actions of angiotensin II (ANG II) may be facilitated by a reduced counterbalancing action of angiotensin-(1–7) [ANG-(1–7)] since infusion of the heptapeptide attenuated the cardiac remodeling and the decrease in left ventricular pumping ability produced by myocardial infarction in rats (14). ANG-(1–7) may act to counterbalance the actions of ANG II by reducing vascular resistance (2), improving vascular endothelial function (10), reversing cardiac hypertrophy (24), and cardiac collagen deposition (17), as well as ischemia-induced cardiac arrhythmias (8). Indeed, Tallant et al. (27) demonstrated that ANG-(1–7) attenuated the ANG II-dependent activation of mitogen-activated protein kinase kinase in isolated cardiomyocytes. Moreover, the protective actions of ANG-(1–7) were blocked by oligonucleotide-directed inhibition of the mas pro-

tein, providing further evidence that the cardioprotective actions of ANG-(1–7) are mediated by the mas receptor (23, 27).

Angiotensin-converting enzyme 2 (ACE2), a newly discovered enzyme member of the RAS, links the two functionally opposing arms of the RAS: the ANG II pressor-hypertrophic path to the depressor-antiproliferative actions of ANG-(1–7) (14). Zisman et al. (32) recently showed that exogenous ANG-(1–7) formation in the human heart tissue was dependent on ANG II. In ACE2 knockout mice, Crackower et al. (6) reported significantly higher levels of cardiac ANG II that were associated with cardiac dilatation. Given the importance that the new studies suggest in terms of the mechanisms that regulate cardiac performance and may mitigate the hypertrophic actions of ANG II, the current study investigated the role of ACE2 in the production ANG-(1–7) from ANG II in intact hearts from both normotensive and transgenic hypertensive rats.

Materials and Methods

Animals. Twenty 10- to 12-wk-old male Sprague-Dawley [Tg(−/ −)] and female aged-matched [mRen2(27) Tg(+/ +)] rats bred from our colony at the Hypertension & Vascular Research Center were housed in individual cages (12:12-h light-dark cycle) with ad libitum access to rat chow and tap water. Procedures were approved by the Wake Forest University School of Medicine Animal Care and Use Committee.

Langendorff procedure. Rats were weighed, placed under deep halothane (2.5–3%) anesthesia, and given heparin (300 USP units) via a catheter inserted into the jugular vein. The chest was opened down the midline, and the heart was excised and immediately placed in ice-cold Krebs buffer (in mM: 118 NaCl, 4.7 KCl, 3.28 MgSO4, 1.18 KH2PO4, 2.52 CaCl2, 25 NaHCO3, 5.55 glucose, and 4.0 Na-pyruvate). Rat hearts were then perfused at a constant flow (10–12 ml/min) on a Langendorff isolated heart perfusion apparatus (model IH-SR, Harvard Apparatus, Holliston, MA). Perfusion pressures were measured with an ISOTEC force transducer (Harvard Apparatus, Holliston, MA), and flow rates were monitored using a TS420 flowmeter (Transonic Systems, Ithaca, NY), both of which were connected to the Biopac MP100A-CE hardware (Biopac Systems, Goleta, CA). Measurements were recorded using the Biopac AcqKnowledge 3.8.1 computer software.

After a 1-h equilibration period, a baseline sample of the cardiac effluent (2.5 ml) was collected, and then 60 ml of Krebs buffer with 10 nM angiotensin II (ANG II, Bachem, Torrance, CA) were recirculated through the heart for 60 min. All effluent samples were acid-matched 1:1 (vol:vol) with 1% heptafluorobutyric acid (HFBA) to abolish metabolism of the peptides at the following times: 1) 5 min of recirculation, 2) 15 min, 3) 30 min, 4) 60 min. Half of the hearts received 1 μM of the ACE2 inhibitor MLN-4760 (Millennium Phar-

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A pharmaceuticals, Cambridge, MA) immediately after the collection of the 15-min sample. Previous studies demonstrated that MLN-4760 specifically inhibits ACE2 at the doses employed here (7). Furthermore, we directly tested the inhibitory action of MLN-4760 by assaying the conversion of angiotensin II into ANG-(1–7) in the cardiac effluent by high-performance liquid chromatography (HPLC) (see Biochemical procedures). Hearts were weighed at the end of the experiment to calculate the heart weight-to-body weight ratio.

Biochemical procedures. Angiotensin peptides were extracted from the acid-matched samples using C18 SepPak columns (Waters, Milford, MA). Each SepPak was activated with 5 ml of 80% methanol (MeOH)-0.1% HFBA, followed by 5 ml of 0.1% HFBA. The 5-ml samples were then applied to the columns, followed by 10 ml of 0.1% HFBA. The columns were rinsed with 5 ml of MilliQ water, and the peptides were eluted in 3 ml of 80% MeOH-0.1% HFBA. The eluate was then analyzed by radioimmunoassay (RIA) for both ANG II and ANG-(1–7) as described previously (12, 13), which was coupled with HPLC analysis of the eluate to verify the presence of ANG-(1–7) as described previously (4). HPLC fraction numbers 10–25 were analyzed for immunoreactive ANG-(1–7). The minimum detectable limits of the ANG II and ANG-(1–7) RIA assays were 0.8 and 2.5 pg/ml, respectively.

ACE2 activity by HPLC. Effluent collected from the hearts of three Tg(+/H11002) and three Tg(+/H11001) rats (without the ACE2 inhibitor) at the end of the 60-min recirculation period were concentrated using an Amicon Ultra 10,000 molecular weight cut-off centrifugal filters (Millipore, Billerica, MA) and washed twice with 15 ml of HEPES buffer containing 120 mM NaCl and 10 μM ZnCl2, pH 7.4. ACE2 activity was determined in the concentrate by quantifying the conversion of 125I-labeled ANG II to ANG-(1–7) for 180 min at 37°C by HPLC as described elsewhere (12).

Immunoblot. ACE2 protein was determined in the Tg(+/H11002) and Tg(+/H11001) cardiac effluent by immunoblot using an NH2-terminally directed antibody (AN1212) developed by us that recognizes ACE2 but not ACE or collectrin (15, 26). We previously confirmed that this antibody can be blocked by the peptide with which it was raised (unpublished observations). We applied 25 μl of the concentrated perfusate to 10% SDS polyacrylamide gels (Bio-Rad, Hercules, CA) for 1 h at 120 volts in Tris-glycine SDS, transferred onto a polyvinylidifluoride membrane and subsequently blocked for 1 h with 5% Bio-Rad dry milk and Tris-buffered saline with Tween before incubation with the ACE2 antibody (1:2,000). Immunoblots were then resolved with Pierce Super Signal West Pico Chemiluminescent substrate (Chicago, IL), as described by the manufacturer, and exposed to Amersham Hyperfilm ECL (Piscataway, NJ).

Fig. 1. Angiotensin II (ANG II) disappearance was not different between normotensive Sprague-Dawley [Tg (+/H11002)] hearts (n = 10, A) and hypertensive [mRen2]27 [Tg (+/H11001)] hearts (n = 7, B) over the course of the recirculation (P > 0.05), and ACE2 inhibition had no effect on the decay of recirculating ANG II in either strain (P > 0.05).

Fig. 2. ANG-(1–7) formation from ANG II measured by radioimmunoassay (RIA) in Tg(+/H11002) (n = 10, A) and Tg(+/H11001) (n = 7, B) hearts. ANG-(1–7) production from ANG II was significant from baseline values (represented at time = 0) in both the Tg(+/H11002) and Tg(+/H11001) rat strains; however, there was no difference in ANG-(1–7) generation between the two strains. The Tg(+/H11002) hearts that received the ACE2 inhibitor at 15 min exhibited no significant reduction of ANG-(1–7) generation at 30 min, but the Tg(+/H11001) ANG-(1–7) production at 30 min decreased by 54.7%. ACE2 inhibition reduced ANG-(1–7) levels by 83.1% (57.3 ± 18.6 pM, P < 0.01) in the Tg(+/H11001) hearts at 60 min but had no significant effect in the Tg(+/H11002) hearts. *P < 0.05 vs. baseline; **P < 0.001 vs. baseline; †P < 0.05 vs. respective control time; ††P < 0.01 vs. respective control time.
Statistical analyses. All values are reported as means ± SE. Student’s t-test and one-way ANOVA were used to determine significant differences at a probability <0.05. Tukey’s post hoc test for multiple comparisons was used following the one-way ANOVA. For the RIA, values at or below the minimal detectable limits of the assays were assigned that value for statistical purposes.

RESULTS

The heart weight-to-body weight ratio (mg/g) for the Tg(−) rats (5.13 ± 0.17) was 26% higher than those ratios determined in the Tg(+) hearts (4.08 ± 0.12; P < 0.001), verifying that the hypertensive animals exhibited marked cardiac hypertrophy. Basal levels of perfusion pressure and heart rate were similar between Tg(−) and Tg(+) hearts, although the heart rate was slightly lower in the Tg(+) hearts (perfusion pressure: Tg(−), 63.8 ± 2.7 mmHg vs. Tg(+), 60.7 ± 3.2 mmHg, P > 0.05; heart rate: Tg(−), 254 ± 6.8 beats/min vs. Tg(+), 219 ± 9.5 beats/min, P < 0.05).

The overall rate of ANG II disappearance in perfused hearts was not different between the Tg(−) and Tg(+) rats. The calculated half-life (t1/2) of the peptide was 42 ± 7 min for the Tg(−) rats and 46 ± 5 min (P > 0.05) for the Tg(+) rats (Fig. 1, A and B). Moreover, ACE2 inhibition did not change the ANG II levels in either strain. ANG-(1–7) production from exogenous ANG II as determined in the effluent of hearts perfused with ANG II peaked at 30 min to an average of 510 ± 55 pM (P < 0.001 vs. baseline) and 497 ± 63 pM (P < 0.001 vs. baseline) in Tg(−) and Tg(+) rats, respectively (Fig. 2, A and B). Addition of the ACE2 inhibitor MLN-4760 in the Tg(−) hearts caused no significant changes in ANG-(1–7) production at either the 30- or 60-min time points. In contrast, ACE2 inhibition in the Tg(+) rats resulted in a sustained reduction in ANG-(1–7) averaging 54.7% (P < 0.05) and 83.1% (P < 0.01) at 30 and 60 min, respectively.

![Fig. 3. HPLC-RIA analysis of immunoreactive ANG-(1–7) in the heart perfusate. A: immunoreactive peaks corresponding to ANG-(1–7) and ANG II following perfusion of the heart with exogenous ANG II. B: immunoreactive peak of ANG II in the perfusate buffer containing ANG II. C: absence of ANG II or ANG-(1–7) immunoreactive peaks in the perfusion buffer alone.](http://ajpheart.physiology.org/)

![Fig. 4. Representative chromatographs of cardiac perfusate demonstrates that 125I-labeled ANG-(1–7) formation from 125I-labeled ANG II was abolished by the ACE2 inhibitor MLN-4760 in both the Tg(−) (A) and Tg(+) (B) cardiac effluent. Soluble ACE2 (sACE2) activity was significantly higher in the Tg(+) perfusate compared with the normal Tg(−) perfusate [Tg(−): 1.97 ± 0.26 fmol·ml⁻¹·min⁻¹ vs. Tg(−): 0.82 ± 0.19 fmol·ml⁻¹·min⁻¹, P < 0.05].](http://ajpheart.physiology.org/)
HPLC analysis of the heart perfusate confirmed the immunoreactive identity of ANG-(1–7) that eluted essentially as a single fraction at 18 min (Fig. 3A). The immunoreactive peak at 24 min corresponds to ANG II and most likely represents the cross reactivity of exogenous ANG II with the ANG-(1–7) RIA. Indeed, HPLC analysis of the buffer containing ANG II revealed a similar peak of ANG II (Fig. 3B). Finally, the analysis of the Krebs buffer without ANG II demonstrated no immunoreactive peaks (Fig. 3C).

Although ACE2 is a membrane-bound metallopeptidase, we examined whether ACE2 activity was present in the cardiac effluent. After the 60-min recirculation period, the perfusate was concentrated 40-fold and incubated with 125I-labeled ANG II. As illustrated in Fig. 4, the chromatographs revealed significant conversion to ANG-(1–7) in the effluent collected from perfused Tg(−/) hearts (Fig. 4A) and Tg(+/+) (Fig. 4B) hearts that was completely abolished by addition of the ACE2 inhibitor MLN-4760. The ACE2 activity was significantly higher in the Tg(+/+) perfusate (1.97 ± 0.26 fmol·ml⁻¹·min⁻¹, n = 3) compared with the normal Tg(−/) perfusate (0.82 ± 0.19 fmol·ml⁻¹·min⁻¹, n = 3, P < 0.05). Consistent with the presence of ACE2 activity in the concentrate, we detected an 80-kDa band in the immunoblot of the concentrated effluent from both Tg(−/) hearts (lanes 3–5, Fig. 5A) and Tg(+/+) hearts (lanes 3–5, Fig. 5B) using an NH₂ terminally directed antibody to rat ACE2. Both the human ACE2 standard and the 80-kDa band were blocked in the Tg(−/) and Tg(+/+) heart effluent by preincubation of the ACE2 immunogenic peptide with the antibody (Fig. 5, C and D, respectively), confirming the presence of endogenous soluble ACE2 (sACE2). Moreover, the densities of the sACE2 band in pooled samples of the Tg(−/) and Tg(+/+) heart perfusate (n = 6) were highly associated with ACE2 activity (r = 0.78). Immunoreactive bands at 220 and 60 kDa were also evident, but their intensity was not diminished by blockade with the ACE2 peptide.

Fig. 5. ACE2 immunoblots reveals an 80-kDa protein in the cardiac effluent collected at the end of the 60-min recirculation period. A and B are the Tg(−/) and Tg(+/+) concentrated perfusates, respectively. C and D are immunoblots blocked with the immunogenic ACE2 peptide. Lane 1, magic markers; lane 2, ACE2 standard (20 ng); lanes 3, 4, and 5, cardiac perfusate (25 µl). Arrow indicates the 120-kDa ACE2 standard.

**DISCUSSION**

Although others have investigated the metabolism of ANG I in the heart (9, 21), the present study in the isolated perfused heart preparation is the first demonstration, to our knowledge, of a direct contribution of cardiac ACE2 to the metabolism of ANG II into ANG-(1–7) in the hearts of hypertensive rats. In agreement with previous studies in the human heart tissue (32), we now report that cardiac tissue has a capacity to generate ANG-(1–7) from ANG II in normal and hypertensive rat hearts, albeit the amounts of peptide production are not different in both normal and hypertrophied hearts. In contrast, our study now reveals a very significant dependence on ACE2 in the hypertensive heart for ANG-(1–7) formation, whereas ACE2 had no major role in ANG-(1–7) formation in the normal heart. Likewise, studies by Zisman et al. (31) also demonstrated an increase in ACE2-mediated ANG-(1–7)-forming activity in human failing heart tissue. Together, these studies imply that ACE2 may serve as a compensatory mechanism to preserve ANG-(1–7) levels in response to hypertension-induced cardiac hypertrophy in this strain, although it may not be sufficient to counteract the deleterious effects of ANG II.

We find that a potent and specific ACE2 inhibitor (7) decreased ANG-(1–7) formation (>80%) from exogenous ANG II in isolated Tg(+/+) rat hearts. The failure of this ACE2 inhibitor to reduce cardiac ANG-(1–7) formation in Tg(−/) rats suggests that ACE2 does not contribute to ANG II metabolism in the perfused normal hearts and that other peptidases may be responsible for the production of ANG-(1–7) from ANG II in Tg(−/) heart activity. Whereas we did not ascertain the identity of other ANG-(1–7)-forming enzymes in the heart for normal rats, previous studies suggest that prolyl oligopeptidase (POP) may prove to be a likely candidate as the enzyme cleaves ANG II to ANG-(1–7) and both soluble and particulate fractions of the Tg(−/) heart exhibit activity (5, 29). POP may prove to be...
important in the regulation of angiotensin peptides in the normal heart.

In recently published studies from both our laboratory (19) and others (30), it was demonstrated that cardiac ACE2 mRNA and activity in heart homogenates was reduced in rat models of hypertension and cardiac hypertrophy. Studies by Jessup et al. (19) showed that ACE2 mRNA and activity were blunted in response to lisinopril or losartan treatment in heart homogenates of mRen2.Lewis rats compared with their normotensive controls. Likewise, Zhong et al. (30) reported a reduction in both cardiac ACE2 mRNA and protein in SHR rats compared with the normal Wistar-Kyoto rat. Collectively, these studies indicated an impairment in cardiac homogenate ACE2 activity in the hypertensive heart. The current study investigated the contribution of coronary artery ACE2 to the metabolism of ANG II into ANG-(1–7) ex vivo utilizing the isolated heart preparation, which is not comparable to ACE2 activity in heart homogenate. It is indeed likely that even though cardiac ACE2 activity is impaired in the hypertensive heart, this activity has assumed the role of preserving the ANG-(1–7) levels, as shown by the current study. Since Zisman et al. (31) showed increased ACE2 activity in failing human heart tissue, it may be that ACE2 increases as the heart progresses from cardiac hypertrophy into heart failure in an ill-fated attempt to protect the heart from demise.

The presence of ACE2 activity in the effluent of the heart suggests that an active secreted form of the enzyme may act either locally or be released into the circulation. Immunoblots revealed an 80-kDa band corresponding to sACE2, as well as bands of larger and smaller molecular mass (220 and 60 kDa, respectively). Newton et al. (22) showed the presence of a 72-kDa form of ACE2 in normal rat cerebrospinal fluid. In addition, Warner et al. (28) showed that ACE2 can be secreted from polarized canine kidney epithelial cells, which was blocked by a metallopeptidase inhibitor, suggesting the secretion was mediated by a metallosheddase. In a similar study by Lambert et al. (20), the authors identified tumor necrosis factor-α convertase (ADAM17) that cleaves ACE2 in human embryonic kidney cells. The truncated form of ACE2 in this study corresponded to immunoblot bands of 105 or 95 kDa, depending on the glycosylation state of the enzyme. The ADAM17 protein is present in the human heart, and its expression is significantly increased in the hearts of patients with cardiomyopathy compared with nonfailing hearts (11). Importantly, increased ADAM17 activity was associated with human heart failure (25), suggesting that a loss of ACE2 [and potential loss of ANG-(1–7) production] from the myocardium may facilitate the progression of heart failure. However, ACE2 gene expression was increased in both human and rodent heart failure (3, 16), and the peptidase activity was elevated in the viable and border/infarct zones of the heart. Additional studies are required to address the exact role of ADAM17 in the processing of ACE2 within the heart. It is possible that the catalytic activity of this secreted form of ACE2 may prove to supplement the activity of the membrane-bound form, which has been detected in cardiac myocytes, as well as the vascular endothelium, and the vascular smooth muscle cells of intra-coronary vessels (3). Moreover, the finding that sACE2 activity was higher in the Tg(+/+) perfusate suggests that either the rate of ACE2 secretion is higher or the rate of ACE2 metabolism is lesser in the pathological state of hypertension. From these findings, it may be that the 80-kDa bands found in our studies (Fig. 5) correspond to secreted active metabolites of ACE2.

In summary, that ACE2 ANG-(1–7)-forming activity was increased in the Tg(+/+) hypertensive strain suggests that this enzyme may constitute an important compensatory mechanism to pressure and/or RAS-dependent cardiac hypertrophy and remodeling in this strain, which supports our notion that ACE2 may be a critical feed-forward step in the RAS pathway to limit the cardiac effects of ANG II, as well as facilitating the vasodilatory and antiproliferative actions of ANG-(1–7) (14). Albeit ACE2 was responsible for most of the ANG-(1–7)-forming activity in [mRen2]27 Tg(+/+) rats in this study, it may be insufficient to counteract the deleterious actions of ANG II on the heart. Without the sustained elevated levels of ANG-(1–7) to mitigate the cardiac hypertrophy and fibrosis associated with elevated ANG II, the renin-dependent hypertension and cardiac hypertrophy will eventually lead the heart to failure, which may be more exacerbated in the absence of ACE2. In agreement with this notion, Crackower et al. (6) showed that genetic deletion of ACE2 resulted in severe cardiac contractility defects and cardiac dilatation, which was reversed when ACE and ACE2 were knocked out concomitantly, suggesting that the balanced expression of these two enzymes is critical in maintaining normal heart function.

The discovery of ACE2 and the continual unveiling of its role in the RAS have elucidated a need for balance between ACE and ACE2 because they work to regulate the net levels of the known biologically active peptides ANG II and ANG-(1–7). These peptide levels, both tissue and circulating, may be disrupted in many disease states, such as hypertension, cardiac fibrosis, and myocardial infarction. Understanding this balance will be key to unravel the ways in which these disease states can be best treated.

ACKNOWLEDGMENTS

The authors thank Drs. Che-Ping Cheng, R. Mark Payne, and James Jordan for help and counsel in setting up the Langendorff apparatus. The ACE2 inhibitor MLN-4760 was obtained from Millennium Pharmaceuticals (Cambridge, MA).

GRANTS

Funding from National Heart, Lung, and Blood Institute Grants HL-51952, HL-56973 provided support for this project. Additionally, the authors gratefully acknowledge grant support in part provided by Unifi, Greensboro, NC, and Farley-Hudson Foundation, Jacksonville, NC.

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