Cardiac angiotensin-(1–12) expression and systemic hypertension in rats expressing the human angiotensinogen gene

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1Department of Surgery, Wake Forest School of Medicine, Winston Salem, North Carolina; 2Hypertension and Vascular Research Center, Wake Forest School of Medicine, Winston Salem, North Carolina; 3Max-Delbrück-Center for Molecular Medicine (MDC), Berlin, Germany; 4Division of Cardiovascular Disease, University of Alabama at Birmingham and Department of Veterans Affairs, Birmingham Veterans Affairs Medical Center, Birmingham, Alabama; 5Department of Anesthesiology, Wake Forest School of Medicine, Winston Salem, North Carolina; and 6Departments of Medicine-Nephrology and Physiology-Pharmacology, Wake Forest School of Medicine, Winston Salem, North Carolina

Submitted 2 November 2015; accepted in final form 8 February 2016

Ferrario CM, VonCannon J, Jiao Y, Ahmad S, Bader M, Dell’Italia LJ, Groban L, Varagic J. Cardiac angiotensin-(1–12) expression and systemic hypertension in rats expressing the human angiotensinogen gene. Am J Physiol Heart Circ Physiol 310: H995–H1002, 2016. First published February 12, 2016; doi:10.1152/ajpheart.00833.2015.—Angiotensin-(1–12) [ANG-(1–12)] is processed into ANG II by chymase in rodent and human heart tissue. Differences in the amino acid sequence of rat and human ANG-(1–12) render the human angiotensinogen (hAGT) protein refractory to cleavage by renin. We used transgenic rats harboring the hAGT gene [TGR(hAGT)L1623] to assess the non-renin-dependent effects of increased hAGT expression on heart function and arterial pressure. Compared with Sprague-Dawley (SD) control rats (n = 11), male homozygous TGR(hAGT)L1623 (n = 9) demonstrated sustained daytime and nighttime hypertension associated with no changes in heart rate but increased heart rate lability. Increased heart weight/tibial length ratio and echocardiographic indexes of cardiac hypertrophy and fibrosis in TGR(hAGT)L1623 rats were associated with a fourfold increase in cardiac ANG II content. Chymase enzymatic activity, using the rat or human ANG-(1–12) as a substrate, was not different in the cardiac tissue of SD and hAGT rats. Since both cardiac angiotensin-converting enzyme (ACE) and ACE2 activities were not different among the two strains, the changes in cardiac structure and function, blood pressure, and left ventricular ANG II content might be due to incomplete suppression of ACE activity and to increased paracrine of ANG-(1–12). Chymase enzymatic activity was modestly increased in heart tissue of TGR(hAGT)L1623 rats. The COOH-terminal sequence of ANG-(1–12) in rats (Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8-His9-Leu10-Leu11-Tyr12) (26). Thus we explored the availability of an experimental construct in which the impact of the hANG-(1–12)/chymase axis could be fully characterized. The transgenic rat [TGR(hAGT)L1623] expressing the human angiotensinogen (hAGT) gene (19) became

ANG II-forming enzymes that, expressed in the rat heart, possess hydrolitic activity on hAGT.

AGENTS THAT BLOCK THE renin-angiotensin system (RAS), angiotensin-converting enzyme (ACE) inhibitors and angiotensin II (ANG II) type 1 receptor blockers (ARB), are established therapies for patients with hypertension and cardiac remodeling based on their ability to counteract the vasoconstrictor and growth-promoting effects of ANG II. Retrospective analysis of large randomized studies document a high residual risk of cardiovascular events in patients treated with RAS inhibitors that has been explained as due to incomplete suppression of ANG II synthesis (32) or ineffective blockade of intracellular sites at which the peptide acts to induce pathology (12, 15, 16).

Current research has established the existence of alternate biochemical pathways for the synthesis of bioactive angiotensins, including the presence of a counteracting axis constituted by the heptapeptide angiotensin-(1–7) [ANG-(1–7)], the monokarboxypeptidase ACE2, and the mas receptor (17, 37). The existence and function of non-renin-dependent pathways for ANG II formation have received little attention although past studies underscored a pivotal role for chymase (EC 3.4.21.39) as an ANG II-forming enzyme from ANG I in humans (14, 21, 34). A resurgence of interest in evaluating non-canonical pathways of ANG II synthesis is related to the identification of an extended form of ANG I, the dodecapeptide angiotensin-(1–12) [ANG-(1–12)] (26), which functions as a tissue precursor substrate for direct non-renin-dependent ANG II formation (33) in the rodent heart (3, 29) and in human left atrial and left ventricular (LV) tissues (1, 2, 4, 27). While further characterization of ANG-(1–12) metabolism in both the circulation (25) and Wistar-Kyoto and spontaneously hypertensive rats neonatal myocytes in culture (3) showed a role of ACE in ANG II formation from ANG-(1–12), chymase rather than ACE was the enzyme generating ANG II directly from ANG-(1–12) (1, 4, 27).

The COOH-terminal sequence of ANG-(1–12) in rats (Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8-His9-Leu10-Leu11-Tyr12), heretofore defined as native rANG-(1–12), is not the same as that in the human (h)ANG-(1–12) form (Asp1-Arg2-Val3-Tyr4-Ile5-His6-Pro7-Phe8-His9-Leu10-Val11-Ile12) (26). Thus we explored the availability of an experimental construct in which the impact of the hANG-(1–12)/chymase axis could be fully characterized. The transgenic rat [TGR(hAGT)L1623] expressing the human angiotensinogen (hAGT) gene (19) became

NEW & NOTEWORTHY

Characterization of a transgenic rat harboring the human angiotensinogen (AGT) gene [TGR(hAGT)L1623] as a novel model to investigate the direct contribution of human relevant renin-independent ANG II formation via chymase or other

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http://www.ajpheart.org H995

an attractive possibility since the species specificity of renin catalytic activity prevents rat renin from cleaving the Leu10-
Val11 bond of hAGT (10, 19). A pilot study suggesting the presence of hANG-(1–12) immunoreactivity in the heart of TGR(hAGT)L1623 rats led us to fully characterize this experimental model in terms of the long-term effects of the hAGT transgene on: 1) blood pressure and echocardiographic indexes of cardiac structure and function; 2) angiotensin peptides expression in their blood and cardiac tissue; and 3) the activity of ACE, ACE2, and chymase on both the human and rat forms of ANG-(1–12).

METHODS

Experimental protocol. Male hAGT (n = 9) and Sprague-Dawley (SD; n = 11) rats were housed from the established colonies at the Wake Forest University Hypertension and Vascular Research Center. Rats were housed in an American Association of Laboratory Animal Care-approved facility in a temperature-controlled room (22 ± 2°C) with a 12:12-h light-dark cycle (lights on 6:00 AM to 6:00 PM) and allowed free access to food and water. The rats were handled in accordance with National Institutes of Health guidelines; our Institutional Animal Care and Use Committee approved the study in advance. At age 10–11 wk and under aseptic conditions, radiotelemetry probes (PA-C40; Data Science International, St. Paul, MN) were chronically implanted while the rats were under anesthesia for continuous monitoring of arterial pressure and heart rate as described elsewhere (36). 24 h measures of blood pressure and heart rate commenced 2 wk postimplantation of the probes and continued for 18 days thereafter. Transthoracic echocardiography was performed once the conscious telemetric blood pressure monitoring period was concluded. At the age of 16–17 wk, animals were decapitated and trunk blood was collected for measurements of RAS components. Hearts were removed and weighed; tibia lengths were measured with a micrometer; and cardiac tissue samples were quickly frozen on dry ice for later measurement of angiotensin peptides, ACE, chymase, and ACE2 activities.

Echocardiography. Transthoracic echocardiography was performed in rats anesthetized lightly with isoflurane (1.5%) using the VevoLAZR Imaging system (VisualSonics, Toronto, Canada). LV M-mode images were obtained in the two-dimensional short axis view close to the papillary muscles and posterior (PWT) and interventricular septal wall thicknesses (IVS) at end diastole and LV end-diastolic and end-systolic dimensions (EDD and EDD) were measured as we described previously (35). Relative wall thickness (RWT) was calculated according to the formula: RWT = (2 × PWT)/EDD. In addition, ejection time (ET) was measured as the time from the beginning to the end of the pulmonary flow wave and indexes of global systolic function including fractional shortening [FS (%); FS = (EDD − EDD)/EDD] and velocity of circumferential shortening [Vcf (circ/s); Vcf = FS/ET] were also calculated. Mitral inflow measurements of early filling velocities (E) were obtained using pulsed Doppler, with the sample volume placed at the tips of mitral leaflets from an apical four-chamber orientation. Doppler tissue imaging to assess early mitral valve septal annular velocities (e’) was also obtained from the four-chamber view. E/e’ was calculated as a measurement of LV filling pressure. All systolic and diastolic indexes are the average of at least three consecutive cardiac cycles.

Immunohistochemistry for tissue hANG-(1–12) in LV myocardium. Immunohistochemistry was performed in sections from the formalin fixed paraffin-embedded LV. Five-micrometer sections were mounted on slides, deparaffinized in xylene, and rehydrated in a graded series of ethanol. After being blocked with 1× PBS/1% casein and incubated overnight at 4°C with the human (1:1,000; affinity purified custom-made antibody; AnaSpec) antibody, slides were incubated for 1 h with the desmin antibody (1:200; Abcam, Cambridge, MA) at room temperature. Alexa Fluor 594- and Alexa Fluor 488-conjugated secondary antibodies (1:500 each; Molecular Probes, Eugene, OR) were applied to visualize the ANG-(1–12) (red) and desmin (green) fluorescence in the tissue. Nuclei were stained (blue) with DAPI (1.5 μg/ml; Vector Laboratories, Burlingame, CA). Image acquisition (×100 objective, ×4,000 video-screen magnification) was performed on a Leica DM6000 epifluorescence microscope with SimplePCI software (Compix, Cranberry Township, PA). Images were adjusted appropriately to remove background fluorescence. Previous studies documented the lack of cross reactivity between the hANG-(1–12) antibody and rANG-(1–12) peptide (15).

Plasma and tissue hormone assays. Plasma rat renin concentration (PRC) and plasma ANG II and serum aldosterone levels were measured by radioimmunoassays (RIA) as previously described (36). Total serum hAGT was detected by ELISA (Immuno-Biological Laboratories). In addition, concentrations of ANG II, rANG-(1–12), and ANG (1–7) were also measured in heart tissue (36). hANG-(1–12) assays were not done because no sensitive RIA procedure is currently available.

Enzyme assays. Tissue enzyme activities were determined by HPLC as previously described (1, 36). The rat and human radiola
de
ten amino acid sequences of ANG-(1–12) were used as substrates for the measurements of chymase enzymatic activities. Human chymase affinity for ANG-(1–12) and ANG I were employed as substrates for ACE activity. 125I-ANG II was used as a substrate for the measurement of ACE2 activity.

Western blot analysis of hANG and rANG in LVs. Hearts from SD and TGR(hAGT)L1623 transgenic rats were removed and LV tissue samples were frozen on dry ice and stored at −80°C for analysis of AGT expression by Western blot (3). Frozen tissue was homogenized in a buffer containing 10 mM HEPES (pH 7.4), 125 mM NaCl, 1 mM EDTA, 1 mM NaF, 10 μg/ml leupeptin, 10 μg/ml pepstatin A, and 1 mM PMSF final concentrations. Homogenates were centrifuged at 2,000 g and then at 100,000 g for 60 min at 4°C. Protein concentration in supernatant fractions was determined by the Bradford method using a Bio-Rad kit (Bio-Rad, Hercules, CA). Samples of supernatant were separated by gel electrophoresis, and then proteins were eluted from the gels by Hybond PVDF membranes (Bio-Rad) for 1 h at 100 V. Nonspecific binding was blocked in 5% nonfat dried milk in 0.1% Tween 20 in TBS for 60 min at room temperature. The blots were incubated with an anti-hANG (1:5,000; R&D Systems, Minneapolis, MN) or anti-rAGT antibody (1:1,000; gift from Dr. M. C. Chappell) raised against hAGT (residues 34–485) and RAGT (residues 44–56), respectively. For loading control, blots were probed with anti-β-tubulin antibody (1:5,000; Millipore). The immunoblots were then resolved with Pierce Super Signal West Pico Chemiluminescent substrates as described by the manufacturer and exposed to Amershams Hyperfilm enhanced chemiluminescence (Amersham Biosciences, Piscataway, NJ). Signal quantification was performed using an image analysis program (MCID Image Analysis Solutions) and optical densities were expressed as the ratio between corresponding protein and β-tubulin. The data are reported as the percentage of the controls.

Statistics. All values are expressed as the means ± SE. Data were analyzed with the Student t-test except for analysis of blood pressure, which was analyzed by repeated ANOVA followed by Newman-Keuls’ posttest. P < 0.05 was considered to be of statistical significance.

RESULTS

Blood pressure and heart weight. Twenty-four-hour average systolic and diastolic blood pressures were 136 ± 1/97 ± 1 mmHg in TGR(hAGT)L1623 transgenic rats and 127 ± 1/88 ± 1 in SD controls (P < 0.001). Figure 1 shows that both day- and nighttime blood pressures were significantly higher in hAGT rats compared with SD controls during 18 days of
continuous measurement. Although average 24-h heart rates were comparable in rats with the hAGT transgene (358 ± 5 beats/min) and their SD controls (354 ± 4 beats/min, P > 0.05), the daytime standard deviation of the heart rate was significantly higher in transgenic rats (12.94 ± 0.36 beats/min) compared with control rats (10.67 ± 0.38 beats/min, P < 0.001). In addition, transgenic rats harboring the hAGT gene were heavier (452 ± 7 vs. 399 ± 5 g, P < 0.05); their cardiac weight (1.510 ± 33 vs. 1.155 ± 14 mg, P < 0.0001) and tibia lengths (44 ± 0.12 vs. 41 ± 0.13 mm; P < 0.0001) were also higher compared with SD rats. Cardiac weight-to-body weight ratio (3.32 ± 0.03 vs. 2.89 ± 0.02 mg/g) and cardiac weight-to-tibia length ratio (34.59 ± 0.68 vs. 28.61 ± 0.33 mm/mm, P < 0.0001) were also higher than those in SD controls.

Cardiac function and structure. Echocardiographic differences in cardiac structure and function between strains include significant increases in LV PWT and EDD (Table 1) in the hAGT vs. SD rats, although RWT was similar between strains. There was no difference in FS; however, the Vcf was decreased in hAGT rats revealing an early sign of systolic dysfunction. Indexes of diastolic function, e’, isovolumic relaxation time corrected for the heart rate, and filling pressure (E/e’) were similar between the two strains (Table 1).

Circulating and cardiac ANG. PRC, plasma ANG II, and rAGT concentrations were not different in hAGT transgenic and SD control rats (Fig. 2). hAGT in transgenic rats averaged 1.6 ± 0.1 mg/ml (n = 9). There was no difference in serum aldosterone levels between SD (10.54 ± 1.92 ng/dl; n = 9) and hAGT rats (13.20 ± 1.54 ng/dl; n = 9). On the other hand, comparative cardiac tissue rANG-(1–12) levels in positive and transgene negative rats were associated with a fourfold increase in cardiac tissue ANG II in TGR(hAGT) L1623 rats (Fig. 2). Cardiac concentrations of ANG-(1–7) averaged 13.37 ± 1.66 fmol/mg in SD rats and 18.02 ± 1.53 fmol/mg in rats containing the hAGT gene (P > 0.05). The marked increases in heart ANG II content accounted for a 57% increase in the cardiac tissue ANG II/ANG-(1–7) ratio (2.10 ± 0.26 in hAGT rats vs. 0.90 ± 0.18 in SD rats, P < 0.001).

Further characterization of the expression of ANG-(1–12) in heart tissue and the catalytic activities of enzymes involved in the processing of angiotensin peptides are illustrated in Figs. 3–5. As documented elsewhere (1, 2, 4), differences in the epitopes for rAGT and hAGT and ANG-(1–12) facilitated the development of specific antibodies for ANG-(1–12) showing no cross-reactivity between species. Strong immunofluorescence for the human sequence of ANG-(1–12) was detected in the atria and LV chambers of hAGT rats (Fig. 3). Immunoreactive (ir)-hANG-(1–12) fluorescence was visualized both

Table 1. Echocardiographic measurements in transgenic TGR(hAGT)L1623 rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>SD (n = 11)</th>
<th>hAGT (n = 9)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heart rate, beat/min</td>
<td>400 ± 8</td>
<td>368 ± 11</td>
<td>&lt;0.05</td>
</tr>
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<td>IVSd, mm</td>
<td>2.02 ± 0.04</td>
<td>2.14 ± 0.10</td>
<td>NS</td>
</tr>
<tr>
<td>PWTd, mm</td>
<td>2.03 ± 0.06</td>
<td>2.29 ± 0.06</td>
<td>NS</td>
</tr>
<tr>
<td>ESD, mm</td>
<td>4.05 ± 0.14</td>
<td>4.23 ± 0.30</td>
<td>NS</td>
</tr>
<tr>
<td>EDD, mm</td>
<td>7.46 ± 0.16</td>
<td>8.14 ± 0.21</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>RWT</td>
<td>0.55 ± 0.02</td>
<td>0.57 ± 0.03</td>
<td>NS</td>
</tr>
<tr>
<td>FS, %</td>
<td>46 ± 1</td>
<td>47 ± 2</td>
<td>NS</td>
</tr>
<tr>
<td>ET, ms</td>
<td>58 ± 2</td>
<td>70 ± 2</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Vcf, mm/s</td>
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<td>0.67 ± 0.03</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>E, mm/s</td>
<td>1.128 ± 38</td>
<td>944 ± 36</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>IVRT, ms</td>
<td>22 ± 0.7</td>
<td>23 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>IVRT/RR</td>
<td>1.40 ± 5</td>
<td>1.37 ± 5</td>
<td>NS</td>
</tr>
<tr>
<td>E/e’</td>
<td>58 ± 2</td>
<td>70 ± 2</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SE. hAGT, human angiotensigen; TGR(hAGT)L1623, hAGT gene; SD, Sprague Dawley; IVSd, intraventricular septum at end diastole; PWTd, posterior wall thickness at end diastole; RWT, relative wall thickness; ESD, end-systolic diameter; EDD, end-diastolic diameter; FS, fractional shortening; ET, ejection time; Vcf, velocity of circumferential shortening; E, peak early filling velocity; IVRT, isovolumic relaxation time; RR, cycle length; e’, early mitral annular velocity; NS, not significant.

Fig. 1. Time course of the changes in systolic and diastolic arterial blood pressures (SBP and DBP) in Sprague-Dawley controls (SD; square markers with dash-lines) and rats expressing the human angiotensigen transgene (hAGT; filled circles and solid lines) during 18 days of 24-h monitoring. Values are means ± SE of daily averages, P < 0.001, compared with SD control rats.
within cardiac LV and left and right atrial myocytes. Additional ir-hANG-(1–12) fluorescence was also found in association with the membranes of myocytes. As illustrated in Fig. 3, no ir-hANG-(1–12) fluorescence was visualized in hearts collected from SD controls. In addition, there was dramatically less cardiac ANG-(1–12) immunofluorescence for the rat sequence of the peptide and there was no difference in expression of this sequence in heart tissue from SD and hAGT rats (data not shown).

Cardiac ACE and ACE2 activities were not different in SD and hAGT rats (Fig. 4). The use of the human sequence of ANG-(1–12) as the substrate for the measurement of ACE enzymatic activity yielded no detectable peaks of either ANG I or ANG II in the collected HPLC fractions (Fig. 5). Chymase

Fig. 2. Renin angiotensin system components in plasma and heart tissue of rats expressing the human angiotensinogen gene and SD controls. Values are means ± SE; *P < 0.001, compared with SD control rats. Other abbreviations as in Fig. 1.

Fig. 3. Illustrative example of the expression of human immunoreactive angiotensin-(1–12) [ANG-(1–12)] in the cardiac chambers of a rat expressing the human angiotensinogen gene (hAGT). The human sequence of ANG-(1–12) is absent in SD rats. Red, human ANG-(1–12); green, desmin; blue, nuclei.

Fig. 4. Cardiac Angiotensin II levels in SD and hAGT rats.
activity using either rat or hANG-(1–12) as substrates was not different in SD and hAGT rats (Fig. 4). In agreement with past studies (2), chymase activity in the heart of SD and hAGT rats is almost ninefold higher compared with ACE activity (Fig. 4). Representative chromatographs in Fig. 5 extended our previous findings in the human heart (1, 4) by showing that chymase cleaved hANG-(1–12) in native membranes isolated from the LVs of hAGT rats.

The protein expression of hAGT and rAGT in the LVs from SD and transgenic rats were determined by Western blot analysis of 60-kDa immunoreactive bands using appropriate antibodies. As shown in Fig. 6, the hAGT protein was present in all LV samples from hAGT transgenic rats but totally absent in SD rats. The expression of the rat sequence of AGT was not different in hAGT and SD rats.

DISCUSSION

Transgenic rats harboring the hAGT transgene are hypertensive and display an increased daytime heart rate lability in association with morphometric and echocardiographic indexes indicative of cardiac hypertrophy and early systolic dysfunction as reflected by the decreased Vcf. The hypertension in rats expressing the hAGT gene is not accompanied by changes in

Fig. 4. Comparison of enzymatic activities of ANG II-forming [angiotensin-converting enzyme (ACE) and chymase] and -degrading (ACE2) enzymes in the heart tissue of TGR(hAGT)L1623 transgenic and SD rats.

Fig. 5. Representative chromatographs of human ANG-(1–12) metabolism by ACE and chymase in hAGT rat heart. Left: regardless of the presence of the ACE inhibitor lisinopril, no detectable peaks of either ANG I or ANG II were shown in the collected HPLC fractions when the human sequence of ANG-(1–12) was used as the substrate for the measurement of ACE enzymatic activity in soluble membrane fraction isolated from hAGT hearts. Right: in contrast, more than 50% of $^{125}$I-hANG-(1–12) was metabolized by native membranes isolated from hAGT hearts in the absence of the chymase inhibitor chymostatin.
The hypertension in TGR(hAGT)L1623 rats did not alter the alterations in the circadian rhythm of these parameters (24) documenting whether the hypertension was associated with transgenic positive and negative rats, as well as allowing for determination of differences in the blood pressure phenotype between cellular form of ANG-(1–12).

ANG II formation afforded by the availability of the intracellular form of ANG-(1–12) as evidenced by the strong generalized increase in the LV tissue ANG II content. Although differences in detecting the presence of hypertension in TGR(hAGT)L1623 rats, it was reported that the high concentrations of human circulating AGT were undetected in transgene-negative rats while blood pressure as well as plasma prorenin, renin, rAGT, and ANG II were not different in transgenic positive and negative rats. While the liver showed the highest hAGT expression, both the kidney and the heart had similar levels of gene expression (19). In agreement with our current studies, negative and positive transgene rats had comparable levels of PRC and serum rAGT (19). Concentrations of circulating hAGT were in milligrams per milliliter in agreement with the previous report (19). On the other hand, in their original characterization of the TGR(hAGT)L1623 rats and subsequent studies (8, 10, 11) the German investigators did not report whether the insertion of the hAGT transgene led to significant changes in tissue ANG II content. Although differences in detecting the presence of hypertension in TGR(hAGT)L1623 rats between their and our studies may be accounted for by the fact that we used homozygous rather than the heterozygous TGR(hAGT)L1623 rats, hypertension was also not reported in their study in homozygous TGR(hAGT)L1623 rats (8). Their inability to detect the presence of elevated blood pressure may be related to the fact that blood pressure was measured in ether-anesthetized rats by the tail-cuff method and a control transgene negative group was not included (9).

Following the initial discovery of ANG-(1–12) in organ extracts from Wistar rats (26), we demonstrated that renin is not an ANG-(1–12)-forming or -converting enzyme (18, 33) and that ACE degraded ANG-(1–12) with high affinity in the rat circulation (25) and rat neonatal cardiac myocytes (3), while chymase, not ACE, generated ANG II directly from ANG-(1–12) as evidenced by the strong generalized increase in the LV tissue ANG II content. Although differences in detecting the presence of hypertension in TGR(hAGT)L1623 rats between their and our studies may be accounted for by the fact that we used homozygous rather than the heterozygous TGR(hAGT)L1623 rats, hypertension was also not reported in their study in homozygous TGR(hAGT)L1623 rats (8). Their inability to detect the presence of elevated blood pressure may be related to the fact that blood pressure was measured in ether-anesthetized rats by the tail-cuff method and a control transgene negative group was not included (9).

PRC, plasma ANG II, serum aldosterone levels or circulating levels of rAGT when compared with the values found in SD normotensive controls. Circulating hAGT in serum was present in milligrams per milliliter and the protein was also found in LV tissue of TGR(hAGT)L1623 rats but no SD controls. Lack of activation of circulating RAS components native to the rat was associated with the detection in the heart of the hAGT protein and a robust fourfold increase in LV tissue ANG II content. The source for the increased heart ANG II in hAGT rats is most likely the result of the high content of hANG-(1–12) as evidenced by the strong generalized increase in the hANG-(1–12) immunofluorescence of the LV found only in the TGR(hAGT)L1623 transgenic rats. Since the hAGT protein is not cleaved by rat renin (19), the expression of human cardiac ANG-(1–12) and the fourfold increase in cardiac ANG II content demonstrate the existence of a non-renin-dependent enzymatic mechanism cleaving hANG-(1–12) into ANG II. Since ANG-(1–12) chymase catalytic activities, assessed by the action of the enzyme on the rat or human sequences of ANG-(1–12), were the same in transgenic positive and negative rats, these data suggest that the higher cardiac ANG II content reflects the increased substrate availability for direct ANG II formation afforded by the availability of the intracellular form of ANG-(1–12).

Chronic instrumentation of rats for continuous blood pressure and heart rate monitoring allowed a detailed characterization of differences in the blood pressure phenotype between transgenic positive and negative rats, as well as allowing for documenting whether the hypertension was associated with alterations in the circadian rhythm of these parameters (24). The hypertension in TGR(hAGT)L1623 rats did not alter the daily rhythm of the blood pressure as the higher blood pressure values during the night hours in transgenic positive and negative rats reflects their most active period of activity (24). The hypertensive phenotype in the hAGT rats translated into structural changes in the heart as indicated by increases in heart weight-to-body weight or heart weight-to-tibia length ratios and LV PWT and EDD. While FS was not different between the two strains, decreased Vcf revealed an early sign of systolic dysfunction under basal conditions in the transgenic rats. The increase in EDD might be due, in part, to the lower heart rate under anesthesia associated with increase in diastolic filling time and increase in cardiac preload. Although circulating levels of aldosterone and ANG II were not different between the two strains, a parallel activation of the renal RAS leading to sodium retention and blood volume expansion may contribute to an increased preload.

Rats expressing the hAGT gene were developed on the background genome of SD rats by Ganten et al. (19) to facilitate in vivo research on the interaction between renin and AGT of human origin in experimental animals (11, 19). In the original description of Ganten et al. (19) of the TGR(hAGT)L1623 rats, it was reported that the high concentrations of human circulating AGT were undetected in transgene-negative rats while blood pressure as well as plasma prorenin, renin, rAGT, and ANG II were not different in transgenic positive and negative rats. While the liver showed the highest hAGT expression, both the kidney and the heart had similar levels of gene expression (19). In agreement with our current studies, negative and positive transgene rats had comparable levels of PRC and serum rAGT (19). Concentrations of circulating hAGT were in milligrams per milliliter and in agreement with the previous report (19). On the other hand, in their original characterization of the TGR(hAGT)L1623 rats and subsequent studies (8, 10, 11) the German investigators did not report whether the insertion of the hAGT transgene led to significant changes in tissue ANG II content. Although differences in detecting the presence of hypertension in TGR(hAGT)L1623 rats between their and our studies may be accounted for by the fact that we used homozygous rather than the heterozygous TGR(hAGT)L1623 rats, hypertension was also not reported in their study in homozygous TGR(hAGT)L1623 rats (8). Their inability to detect the presence of elevated blood pressure may be related to the fact that blood pressure was measured in ether-anesthetized rats by the tail-cuff method and a control transgene negative group was not included (9).
reconsidering chymase in the biochemical processing of ANG peptides in both human and rodent hearts (15, 39, 40).

Using human-specific ANG-(1–12) antibodies we show the presence of ANG-(1–12) immunoreactivity in the heart of TGR(hAGT)L1623 rats and its total absence in transgene negative SD rats. The predominance of chymase over ACE in the heart suggests that this enzyme, in the presence of a high ANG-(1–12) content, accounts for the elevations in cardiac ANG II and the presence of cardiac hypertrophy in hAGT rats. The absence of any differences in ACE2 activity excluded the possibility that the high cardiac ANG II content is related to a decrease in ACE2 catalytic activity. On the other hand, our study was not designed to identify the enzyme or enzymes that may generate hANG-(1–12) or ANG II directly from the hAGT. While preliminary studies suggest that a member of the kallikrein family may cleave ANG-(1–12) from AGT (15), tonin, and cathepsin G may be involved as well.

Prior studies from this laboratory (1–4, 15) and the data documented here regarding the expression of hANG-(1–12) in the heart of transgenic positive rats favor the existence of a system in which ANG-(1–12), either synthesized intracellularly or taken up from extracellular spaces, is acted on by chymase to form ANG II directly. We showed chymase mRNA and protein to be localized in the heart of transgenic rats expressing the Ren-2 gene (38) and the right and left atria in humans, respectively (27). Therefore, the data reported here are in keeping with the previous suggestion that ANG-(1–12) may serve as an intermediate, rapid response, substrate for the intracellular generation of ANG II (15).

The existence of intracellular RAS in the heart and other cardiovascular and reproductive organs is no longer a controversial issue. Several laboratories have provided strong documentation for the existence of a cardiac intracrine RAS, the activity of which may not be abrogated through the use of ACE inhibitors or ARBs (5, 6, 12, 13, 22, 23, 30, 31). In agreement with this interpretation, cardiac ANG II content in rats was not affected by chronic administration of an ACE inhibitor, an ARB, or even both drugs combined (16, 36). Pharmacological approaches geared to prevent ANG II synthesis or activity via ACE inhibition or the binding of the peptide to AT1 receptors epitomize the foundation of current approaches to the treatment and prevention of heart disease and heart failure. However, these therapies have not been reported to inhibit ANG II intracellular formation or activity (6, 12). As pointed out by Ferrario et al. (15), the residual risk for cardiovascular events in patients medicated with ACE inhibitors or ARBs is substantial and often higher than the corresponding benefit achieved by the treatments. The most recently published meta-analysis of 10 prospective randomized placebo-controlled ACEI or ARB trials studying patients with a combination of risk factors confirmed a relatively small reduction of cardiovascular mortality and nonfatal myocardial infarction in the trials using ACE inhibitors [cycle length (RR) = 0.89] and no benefit with ARB therapy (RR = 1.00) (28). In other words, ACE inhibition benefited only 11% of the 77,633 patients recruited in these trials and none of the subjects given ARB (28). Similar conclusions were derived from earlier meta-analysis of RAS blockers in heart failure progression (20). In this meta-analysis, the overall reduction achieved for the composite end point of mortality or hospitalization for congestive heart failure averaged 22% in those treated with an ACE inhibitor compared with 33% for those given placebo (20). While the ACE inhibitors were clearly effective in this population, these data imply that 78% of the patients assigned to the active treatment arm had no benefit from the intervention (20). The gap in understanding between the outcomes of clinical trials using RAS blockers and the advanced knowledge of the central role played by the circulatory and tissue RAS in cardiovascular and renal pathophysiology argues strongly for the existence of independent mechanisms for cardiac ANG II generation that, by its intracrine nature, is not affected by the use of agents that act in the surface of cellular membranes or the immediate extracellular milieu.

In summary, detection of hANG-(1–12) in the hearts of rats harboring the hAGT gene demonstrates the existence of a non-renin-dependent enzymatic mechanism able to cleave ANG-(1–12) from the human substrate. Compared with SD control rats, transgenic rats exhibited hypertension associated with cardiac hypertrophy and systolic dysfunction, higher cardiac tissue ANG II, and no differences in cardiac chymase, ACE, and ACE2 activities. We conclude that hAGT rats represent a novel tool to investigate the direct contribution of human relevant renin-independent ANG II formation via chymase or other ANG II-forming enzymes that, expressed in the rat heart, possess hydrolytic activity on hAGT. The increased realization that intracrine ANG II actions are not blocked by ACE inhibitors or even ARBs (7, 12, 13, 16, 22) requires closer examination since the precise characterization of primary and alternate ANG II-generating pathways in humans is of critical importance in the further development of therapies that will effectively suppress RAS contribution to the progression of human cardiovascular disease.

GRANTS
This research was supported by National Heart, Lung, and Blood Institute Grant PO1-HL-051952 (to C. M. Ferrario) and Wake Forest University TSC-Reynolda Grant U01079 (to J. Varagic).

DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

REFERENCES


