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Localization of the novel angiotensin peptide, angiotensin-(1-12), in heart and kidney of hypertensive and normotensive rats

Jewell A. Jessup¹, Aaron J. Trask¹, Mark C. Chappell¹, Sayaka Nagata², Johji Kato², Kazuo Kitamura², and Carlos M. Ferrario¹

¹ Hypertension and Vascular Research Center and Department of Physiology and Pharmacology, Wake Forest University School of Medicine, Winston-Salem, North Carolina

² Circulatory and Body Fluid Regulation, Faculty of Medicine, University of Miyazaki, Kiyotake, Miyazaki, Japan

Abstract

A low expression of angiotensinogen in the heart has been construed as indicating a circulating uptake mechanism to explain the local effects of angiotensin II on tissues. The recent identification of angiotensin-(1–12) in an array of rat organs suggests this propeptide may be an alternate substrate for local angiotensin production. To test this hypothesis, tissues from 11-wk-old spontaneously hypertensive rats (SHR) and Wistar-Kyoto (WKY) rats ($n = 14$) were stained with purified antibodies directed to the COOH terminus of angiotensin-(1–12). Robust angiotensin-(1–12) staining was predominantly found in ventricular myocytes with less staining found in the medial layer of intracoronary arteries and vascular endothelium. In addition, angiotensin-(1–12) immunoreactivity was present in the proximal, distal, and collecting renal tubules within the deep cortical and outer medullary zones in both strains. PreadSORption of the antibody with angiotensin-(1–12) abolished staining in both tissues. Corresponding tissue measurements by radioimmunoassay showed 47% higher levels of angiotensin-(1–12) in the heart of SHR compared with WKY rats ($P < 0.05$). In contrast, renal angiotensin-(1–12) levels were 16.5% lower in SHR compared with the WKY rats ($P < 0.05$). This study shows for first time the localization of angiotensin-(1–12) in both cardiac myocytes and renal tubular components of WKY and SHR. In addition, we show that increased cardiac angiotensin-(1–12) concentrations in SHR is associated with a small, but statistically significant, reduction in renal angiotensin-(1–12) levels.

Keywords

angiotensinogen; angiotensin I; angiotensin II; hypertension; renin

Although numerous studies have documented the presence of a local renin-angiotensin system (RAS), independent of the circulating system (2,9), it has been questioned whether the reported concentrations of renin and angiotensinogen (Aogen) available for angiotensin production within tissues are sufficient to account for the higher concentrations of angiotensin II (ANG II) (16,17). These data have been interpreted as suggesting that the endogenous production of tissue ANG II may result from Aogen uptake from the circulation (8). With the discovery of angiotensin-(1–12) [ANG-(1–12)], first identified by Nagata et al. (20), we hypothesized that this novel propeptide, cleaved from the Aogen protein, may be an alternate precursor for the local production of angiotensins facilitating intra- or paracrine RAS functions (25). With this

in mind, this study characterized for the first time the expression and localization of ANG-(1–12) within cardiac and renal tissue of both normotensive and hypertensive rats using the combined procedures of immunohistochemistry and tissue peptide measures by radioimmunoassay (RIA).

METHODS

Animals

Experiments, approved by the Wake Forest University School of Medicine Institutional Animal Care and Use Committee, were performed on 11-wk-old male SHR ($n = 14$) and WKY rats ($n = 14$) from Charles River Laboratories (Wilmington, MA), which were fed standard rodent chow ad libitum and housed for 1 wk in an American Association for Accreditation of Laboratory Animal Care-approved facility maintained on a 12-h:12-h light-dark cycle at a constant temperature and humidity.

Experimental protocol

Baseline systolic blood pressure was measured for 3 days by tail-cuff plethysmography (Narco Bio-Systems; Houston, TX) following acclimatization to the housing facility. Following euthanasia by decapitation, the heart and kidneys were quickly excised and divided. One half of the tissue was frozen on dry ice for peptide measurements, whereas the remaining tissue was submerged in 4% paraformaldehyde, fixed for 24 h at 4°C, postfixed in 70% ethanol, processed and embedded into paraffin blocks, and sectioned at 4 μ m for histological examination.

Histology and immunohistochemistry

Immunohistochemistry was performed using two separate polyclonal antibodies directed to the COOH-terminus of the rat ANG-(1–12) sequence, Asp¹-Arg²-Val³-Tyr⁴-Ile⁵-His⁶-Pro⁷-Phe⁸-His⁹-Leu¹⁰-Leu¹¹-Tyr¹². One provided by Dr. Kato (University of Miyazaki, Japan) was affinity purified and previously characterized as having no cross-reactivity with smaller angiotensin fragments (20). The second antibody, prepared for us by AnaSpec (San Jose, CA), was IgG purified using protein A. Western blot analyses were performed on both antibodies to ensure that they did not recognize the larger parent protein, Aogen. This analysis showed that neither antibody cross-reacted with any of the cellular proteins ranging in size from 20–120 kDa. Additionally, we evaluated the ability of ANG I, ANG II, or ANG-(1–7) to bind both ANG-(1–12) antibodies in competition studies using ¹²⁵I-ANG-(1–12) peptide. These binding assays showed no cross-reactivity with ANG I (0.032% cross-reactivity), ANG II (<0.001% cross-reactivity), or ANG-(1–7). Both antibodies were independently used to detect immunoreactive ANG-(1–12) using the avidin-biotin horseradish peroxidase technique as previously reported by our laboratory (1). Endogenous peroxidase activity was blocked with hydrogen peroxide. Sections independently treated with normal goat serum in the absence of the primary antibody served as negative controls. Additional controls included sections treated with the primary antibody preincubated with 10 μ mol/l of the ANG-(1–12) peptide to which the antibodies were directed. To ensure there was no cross-reactivity with smaller angiotensin peptides, more controls were conducted by preincubating the antibody with 10 μ mol/l ANG I, ANG II, and ANG-(1–7).

Staining with each antibody was further validated using an alkaline phosphatase method (27), which used a biotinylated anti-rabbit secondary antibody as the linking reagent and alkaline phosphatase-conjugated streptavidin (BioGenex, San Ramon, CA) for labeling. The Vector red chromogen, obtained as Vector red substrate kit no. 1 (Vector, Burlingame, CA), was diluted in Tris (pH 8.2 to 8.5) and applied to slides for 5 to 10 min at 30° to 35°C. The Tris buffer contained 0.5% casein to block nonspecific protein binding. Negative controls included

sections incubated with nonimmune serum (Bio-Genex) rather than the primary antibody. In preliminary experiments, adjacent sections were immunohistochemically stained using the alkaline phosphatase method with antibodies specific to the NH₂ and COOH terminus of Aogen, respectively, to determine whether there was colocalization of ANG-(1–12) and Aogen. The NH₂-terminus antibody was directed against residues 1–14 (Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-Leu-Tyr-Tyr-Ser) of Aogen, whereas the COOH-terminus antibody targeted residues 428–441 (Glu-Glu-Gln-Pro-Thr-Glu-Ser-Ala-Gln-Gln-Pro-Gly-Ser-Pro) (5). These antibodies for Aogen, raised in rabbit, were generated for us by AnaSpec. Because the COOH-terminus angiotensinogen antibody has no common recognition site for ANG-(1–12), we report the findings using that antibody here.

Photomicrographs of the resultant immunoreactive staining were acquired using a bright-field Nikon microscope system (Melville, NY), including a Diagnostic Instruments Digital SPOT RT, three-pass capture, thermoelectrically cooled charge-coupled camera (Sterling Heights, MI), and processed using the SPOT Advanced software.

RIA

In studies independent of the immunohistochemistry analyses and carried out in the laboratory of Nagata et al. (20) in Japan, cardiac and renal tissue concentrations of ANG-(1–12) were assessed in 100 mg of each tissue by RIA designed to specifically detect the COOH terminus of ANG-(1–12) using a 1:6,300 dilution of purified ANG-(1–12) antibody as previously described. Additionally, ANG I and ANG II in cardiac and renal tissues were determined using anti-COOH-terminus ANG I and anti-COOH-terminus ANG II RIAs as detailed by Nagata et al. (20). In these studies (21), HPLC analyses in rat kidney revealed that the peaks of immunoreactivity corresponded with ANG I, ANG II, and ANG-(1–12). The minimum detectable levels of the assays were 0.5 fmol/tube for ANG I, 1.0 fmol/tube for ANG II, and 2.0 fmol/tube for ANG-(1–12). The intra-assay coefficients of variation averaged 5.46% for ANG-(1–12), 8.02% for ANG I, and 9.68% for ANG II. Previous studies by Nagata et al. (20) documented the efficiency of the extraction procedure at levels >90%, which included boiling the specimens immediately after resection so as to denature the proteins and inactivate the proteinases (13).

Statistical analysis

Values are expressed as means $\pm$ SE. Comparisons between the strains were performed with a two-tailed, unpaired Student's *t*-test (GraphPad Prism 5 software, San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Systolic blood pressure averaged 193 ± 2 mmHg in SHR, whereas the average blood pressure in normotensive WKY rats was 122 ± 3 mmHg ($P < 0.001$). Hypertension was associated with cardiac hypertrophy measured as the ratio of heart weight to body weight (3.48 ± 0.09 mg/g SHR vs. 2.99 ± 0.03 mg/g WKY; $P < 0.001$). Both ANG-(1–12)-directed antibodies demonstrated identical patterns of tissue staining as illustrated in Fig. 1.

ANG-(1–12) in cardiac tissue

ANG-(1–12) staining was visualized within the cardiac tissue of both normotensive WKY and SHR. Although staining was predominantly localized to ventricular myocytes, ANG-(1–12) immunostaining was also found in the medial layer and surrounding adventitia of some intracoronary arteries. As shown in Fig. 2, *top*, the immunoreactive staining was primarily concentrated in the cytoplasm of cardiac myocytes.

There were striking differences between the staining patterns within the heart of WKY versus the SHR (Fig. 2, *top*). In the WKY rats, clusters of ANG-(1–12)-positive myocytes existed along the inner portion of the left and right ventricle with occasional appearances in the interventricular septum. In contrast, immunoreactive ANG-(1–12) staining in the SHR was evenly distributed throughout the entire sections encompassing all of the right and left ventricular walls as well as the septal regions (Fig. 2). When viewed at higher magnification, almost all myocytes showed some degree of positive staining in the SHR, whereas in the WKY rats, large regions were devoid of any staining (Fig. 2).

Expression of the propeptide in the kidney

In both strains, immunoreactive staining for ANG-(1–12) was widely distributed throughout the proximal, distal, and collecting renal tubules, particularly within the deep cortical and outer medullary zones (Fig. 2, *bottom*). Glomeruli and adjacent arterioles were mostly devoid of staining (Fig. 2).

Specificity of the staining

ANG-(1–12) staining was abolished by the preadsorption of the antibody with ANG-(1–12) in both heart and kidney sections. In contrast, the preadsorption of the antibody with ANG I, ANG II, and ANG-(1–7) did not block the staining in both heart and kidney sections. Further confirmation of the ANG-(1–12) antibody specificity was demonstrated using the antibody directed to the COOH-terminus of Aogen. As illustrated in Fig. 3, Aogen within the heart was barely detectable using the COOH-terminus antibody at a 1:25 dilution. In particular, the antibody directed against the COOH-terminal region of Aogen clearly revealed immunoreactive staining patterns different from those detected with the ANG-(1–12) antibodies in heart sections. In the kidney, Aogen staining was readily detectable only in the apical zone of the cortical proximal tubules, including the brush border and luminal margin (Fig. 3).

Tissue content of ANG-(1–12)

Sections of the left ventricle of WKY and SHR were processed for ANG-(1–12) peptide content using the specific ANG-(1–12) antibody generated by Nagata et al. (20). In agreement with the patterns of immunoreactive ANG-(1–12) staining, the content of ANG-(1–12) in the left ventricle of WKY rats averaged 218 ± 13 fmol/g ($n = 6$), a value significantly less than the concentration of the propeptide in the cardiac tissue of SHR (319 ± 32 fmol/g, $P < 0.05$, $n = 6$) (Table 1). Likewise, cardiac tissue concentrations of both ANG I and ANG II were higher ($P < 0.05$) in SHR compared with WKY rats (Table 1). In contrast, renal concentrations of ANG-(1–12) were lower in SHR (753 ± 28 fmol/g) compared with normotensive WKY rats (902 ± 34 fmol/g, $P < 0.01$, Table 1). Although ANG I content in the kidney was not different between the two strains, the renal content of ANG II was higher in the SHR compared with the WKY rats ($P < 0.05$, Table 1).

DISCUSSION

The identification of the novel angiotensin peptide, ANG-(1–12), led us to hypothesize that ANG-(1–12), acting as an angiotensin-forming propeptide, may be an alternate pathway for the generation of tissue angiotensin peptides, in contrast with the generally accepted principle that Aogen is the sole upstream precursor for ANG I formation (19). In support of this hypothesis, we now report the location of immunoreactive ANG-(1–12) in cardiac myocytes and renal tubular cells in addition to concentration measurements of ANG-(1–12) in the heart and kidney of normotensive WKY and hypertensive SHR. The low abundance of positive Aogen staining in cardiac tissue of both strains, as well as the dissimilar distribution of the immunoreactive staining of ANG-(1–12) and Aogen in both cardiac and renal tissues, suggests

that ANG-(1–12) exists as a separate precursor from Aogen. These data agree with our demonstration that ANG-(1–12) can act as a precursor of angiotensin peptide formation in the isolated heart obtained from either normotensive or hypertensive rat strains (29).

The immunohistochemical findings are further validated by the separate and direct demonstration of left ventricular ANG-(1–12) concentrations higher than ANG I and ANG II in both WKY and SHR by RIA. In keeping with immunohistochemical observations, cardiac tissue levels of ANG-(1–12) are higher in SHR compared with WKY rats. Increased ANG-(1–12) content in SHR was associated with higher tissue levels of ANG I and ANG II. That the increases in cardiac concentrations of ANG-(1–12) in SHR may reflect an important contribution of an alternate pathway for cardiac ANG I formation agrees with previous reports showing increases in cardiac Aogen protein concentration and transcripts as well as ANG II in SHR (12,26).

In the kidney, ANG-(1–12) was preferentially expressed in proximal and distal convoluted tubules, whereas Aogen was preferentially restricted to the luminal surface of the proximal tubular cells as reported previously by Navar et al. (21,22) and Lodwick et al. (18). Moreover, the distribution of renal Aogen found in our studies is consistent with the immunohistochemical distribution of the substrate in the rat kidney reported by Kobori et al. (14) and Thomas et al. (28). These data suggest a differential compartmentalization of Aogen and ANG-(1–12) in the kidney. Similar to cardiac tissue, ANG-(1–12) was the predominant peptide since renal concentrations of the propeptide were 17% and 14% higher, on average, than ANG I in both WKY and SHR, respectively. The less apparent differences in the immunoreactive staining of ANG-(1–12) between WKY and SHR agree with reduced renal content of the propeptide in SHR compared with WKY rats and parallel the reported decreases in renal Aogen mRNA expression in both adult (23) and older (>25 wk) (18) SHR.

Noteworthy is the observation that tissue levels of ANG-(1–12) in the heart of both WKY and SHR agree well with the corresponding concentrations of ANG-(1–12) in Wistar rats previously reported by Nagata et al. (20). On the other hand, we now show that renal concentrations of ANG-(1–12) in both adult WKY and SHR are approximately twice as high as those measured in their Wistar rats (20). Part of the difference between the previous and current studies may be explained by the use of younger Wistar rats (6 wk of age) in their original report (21). Strain differences between Wistar and WKY rats in terms of tissue peptide concentrations, particularly related to the kidney, have been documented previously (30). The differences in tissue concentration within these strains imply differential regulation of locally generated biologically active peptides (15). Since we demonstrated that neprilysin is responsible for the conversion of ANG-(1–12) to smaller ANG fragments [i.e., ANG-(1–7)] within the kidney (4), it is plausible that less ANG-(1–12) will accumulate in the SHR kidneys since this strain exhibits heightened renal neprilysin activity compared with normotensive rats (11). This interpretation is in keeping with the demonstration of higher renal concentrations of ANG II in both our present study and that of Kobori et al. (15) also in SHR. On the other hand, we cannot eliminate the possibility that the higher levels of ANG-(1–12) might reflect reduced peptide metabolism, although this is not likely since renal ANG II levels were increased in SHR.

The differential pattern of Aogen expression in the tissues of the WKY and SHR argues further for a potential role of ANG-(1–12) as a substrate for ANG I production since 1) Aogen immunoreactivity was minimally expressed in the ventricle of both strains and 2) both in the heart and kidney, the sites of immunoreactive Aogen did not coincide with the broader distribution of ANG-(1–12). Danser et al. (8) reported that the low levels of cardiac Aogen found in pigs are consistent with its diffusion from plasma into the cardiac interstitium. Although these data do not negate that Aogen transcripts are found in heart tissue, in general

mRNA levels are lower than those found in other tissues (24). Although the demonstration of reduced ANG-(1–12) levels in the kidney of SHR requires further investigation, the data suggest that different factors may regulate the expression of ANG-(1–12) in the heart and kidney. This interpretation is in keeping with the observation of a differential regulation of tissue Aogen content in the liver and kidney of rodents exposed to changes in salt intake (10).

Functional roles for ANG-(1–12) have been described by Nagata et al. (20) and by our laboratory (29) whereby the administration of ANG-(1–12) is associated with hypertensive responses. ANG-(1–12) stimulates constriction of isolated aortic rings, whereas bolus injections of the propeptide elicited an immediate augmentation of blood pressure that was abolished by angiotensin-converting enzyme inhibition or blockade of the ANG II type 1 receptor (20).

Although it is acknowledged that renin plays a critical role in the cleavage of angiotensinogen in the circulation, as recently reported by Yanai et al. (31), data in renin knockout mice emphasize a nonessential role for renin in the processing of Aogen in the brain. Many studies have documented the independence of RASs in the tissues and the circulation. The data reported here expand our knowledge of the cellular mechanisms associated with the biochemical pathways of angiotensin peptides formation, and in keeping with Chai and Danser's (3) analysis of the problem, our data underscore the existence of additional mechanisms for cellular processing of Aogen in the heart and the kidneys.

It can be concluded from these studies that hypertension is associated with significant changes in the expression of ANG-(1–12) in the heart and kidney. As documented in RESULTS, renal content of ANG-(1–12) surpasses by fourfold the cardiac concentrations of ANG-(1–12) in normotensive rats, whereas the hypertensive state minimizes this variation to a twofold difference between the tissues, an implication that hypertension as a disease process regulates the expression of the propeptide similarly to what has been described for other components of tissue RAS (6,7,9,15,24). A further investigation of the mechanisms related to its release from Aogen, cellular uptake or synthesis, and formation of angiotensin peptides within or outside the cell should provide new and important information as to the mechanisms that regulate the cellular actions of the angiotensins.

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Fig. 1. Comparative adjacent sections of ANG-(1–12) immunoreactivity obtained from the heart of a Wistar-Kyoto (WKY) rat with either the affinity purified polyclonal antibody generated by Nagata et al. (Ref. 20) (*top*) or the purified protein A polyclonal antibody produced by AnaSpec (*bottom*). Magnification, $\times 400$.

Fig. 2.

Representative photomicrographs of ANG-(1–12) immunoreactive staining in the WKY and spontaneously hypertensive rats (SHR) with the purified protein A antibody. *Top*: alkaline phosphatase-stained cardiac myocytes. *Bottom*: ANG-(1–12) staining in the kidney of WKY and SHR. *Top, right*, and *bottom, right*: negative controls for each respective tissue. Scale bar indicates 100 µm.

Fig. 3. Immunohistochemical localization of angiotensinogen in cardiac (*top*) and renal (*bottom*) sections from WKY rats determined using a COOH terminally directed antibody. Scale bar indicates 100 μm.

Table 1

Tissue concentrations of angiotensin peptides

	Heart		Kidney	
	WKY	SHR	WKY	SHR
ANG-(1–12), fmol/g	218 ± 313	319 ± 32 [*]	902 ± 34	753 ± 28 [*]
ANG I, fmol/g	123 ± 38	207 ± 15 [*]	748 ± 50	645 ± 73
ANG II, fmol/g	25 ± 1	162 ± 68 [*]	81 ± 4	99 ± 2 [*]

Data are means ± SE. SHR, spontaneously hypertensive rats.

^{*} $P < 0.05$ vs. Wistar-Kyoto rats (WKY).