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Differential regulation of angiotensin-(1-12) in plasma and cardiac tissue in response to bilateral nephrectomy

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Ferrario CM, Varagic J, Habibi J, Nagata S, Kato J, Chappell MC, Trask AJ, Kitamura K, Whaley-Connell A, Sowers JR. Differential regulation of angiotensin-(1-12) in plasma and cardiac tissue in response to bilateral nephrectomy. *Am J Physiol Heart Circ Physiol* 296: H1184–H1192, 2009. First published February 13, 2009; doi:10.1152/ajpheart.01114.2008.—We examined the effects of 48 h bilateral nephrectomy on plasma and cardiac tissue expression of angiotensin-(1-12) [ANG-(1-12)], ANG I, and ANG II in adult Wistar-Kyoto rats to evaluate functional changes induced by removing renal renin. The goal was to expand the evidence of ANG-(1-12) being an alternate renin-independent, angiotensin-forming substrate. Nephrectomy yielded divergent effects on circulating and cardiac angiotensins. Significant decreases in plasma ANG-(1-12), ANG I, and ANG II levels postnephrectomy accompanied increases in cardiac ANG-(1-12), ANG I, and ANG II concentrations compared with controls. Plasma ANG-(1-12) decreased 34% following nephrectomy, which accompanied 78 and 66% decreases in plasma ANG I and ANG II, respectively ($P < 0.05$ vs. controls). Contrastingly, cardiac ANG-(1-12) in anephric rats averaged 276 ± 24 fmol/mg compared with 144 ± 20 fmol/mg in controls ($P < 0.005$). Cardiac ANG I and ANG II values were 300 ± 15 and 62 ± 7 fmol/mg, respectively, in anephric rats compared with 172 ± 8 fmol/mg for ANG I and 42 ± 4 fmol/mg for ANG II in controls ($P < 0.001$). Quantitative immunofluorescence revealed significant increases in average grayscale density for cardiac tissue angiotensinogen, ANG I, ANG II, and AT_1 receptors of WKY rats postnephrectomy. Faint staining of cardiac renin, unchanged by nephrectomy, was associated with an 80% decrease in cardiac renin mRNA. These changes were accompanied by significant increases in $p47^{phox}$, Rac1, and Nox4 isoform expression. In conclusion, ANG-(1-12) may be a functional precursor for angiotensin peptide formation in the absence of circulating renin.

renin; angiotensinogen; angiotensin II; blood pressure

RESEARCH ON THE RENIN-ANGIOTENSIN system's mechanisms of action provides a wealth of information about its role in physiology and cardiovascular pathology. Characterization of newer biochemical pathways by which angiotensin peptides act on diverse cellular receptors to exert paracrine and intracrine mechanisms include the identification of angiotensin-converting enzyme 2 (ACE2), the vasodilator and antiproliferative peptide angiotensin-(1-7) [ANG-(1-7)], and the mas receptor (16, 36). Upstream within the biochemical cascade leading to the formation of angiotensin peptides, angiotensinogen (Aogen) remains the undisputed large molecular protein from which angiotensin I (ANG I) is generated. A newly described propeptide cleaved from Aogen may be an alternate substrate for the formation of biologically active angiotensins. Angiotensin-(1-12) [ANG-(1-12)] was isolated by Nagata et al. (31) in rat's tissue. Administration of the peptide induced an increase in blood pressure that was blocked by either the administration of an ACE inhibitor or a type 1 angiotensin II (AT_1) receptor blocker (31). The possibility that ANG-(1-12) may serve as an alternate substrate for the generation of bioactive angiotensins led us to document increased expression of ANG-(1-12) in cardiac myocytes of adult spontaneously hypertensive rats (SHR) compared with Wistar-Kyoto (WKY) controls (24). Additional studies showed the generation of ANG I, ANG II, and ANG-(1-7) from exogenous ANG-(1-12) in the effluent of isolated hearts from Sprague-Dawley, normotensive Lewis, and congenic mRen2 hypertensive Lewis rats, as well as WKY and SHR (39). Of importance, production of angiotensin peptides from exogenous ANG-(1-12) was not abated in the isolated heart by the preadministration of a selective inhibitor of rat renin (39).

Further characterization of the conditions regulating the expression of ANG-(1-12) in both the circulation and the tissues may add newer insights as to how local renin-angiotensin systems contribute to the regulation of tissue functions and shed light as to whether Aogen is the only active substrate form capable to generate angiotensin peptides. Characterization of alternate pathways for angiotensin peptide formation upstream of ANG I may further impact the understanding of the efficacy of orally active renin inhibitors (43) and the development of newer agents to block the enzyme(s) processing ANG-(1-12) into angiotensin peptides. With this in mind, we evaluated whether renal renin contributes to the circulating and tissue production of this novel peptide by studying the effects of 48 h bilateral nephrectomy on plasma and cardiac tissue expression of ANG-(1-12) and angiotensin peptides in WKY rats.

METHODS

Forty-six male WKY rats (15–16 wk) obtained from Charles River Laboratories (Wilmington, MA) were either nephrectomized bilaterally ($n = 27$, body weight 292 ± 4 g) or sham operated ($n = 19$, body weight 300 ± 4 g, $P > 0.05$) under isoflurane anesthesia via an abdominal incision using aseptic procedures. Forty-eight hours later,

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rats were killed by decapitation and their trunk blood collected in chilled tubes (~4°C) containing a cocktail of protease inhibitors to prevent peptide metabolism, as described by us elsewhere (14). The chest cavity was opened through a midsternal incision, and the heart was excised and placed on a chilled plate. The left ventricle was excised free from the rest of the cardiac chambers and divided in transverse sections for processing. Sections of the ventricle destined for radioimmunoassay (RIA) procedures were snap-frozen on dry ice while alternate sections were immersed in 4% paraformaldehyde for histological processing or liquid nitrogen for gene expression analysis. Experiments were approved by the Institutional Animal Care and Use Committee of the Wake Forest University School of Medicine. Prior to experimentation, rats were housed in an American Association for Accreditation of Laboratory Animal Care-approved facility, provided free access to a standard chow diet and tap water, and kept on a 12:12 h light-dark cycle at a constant temperature and humidity.

Immunohistochemical quantification of cardiac angiotensin system components. The polyclonal ANG-(1-12) antibody, directed to the COOH-terminal sequence of the rat peptide, was IgG affinity purified against protein A as described by us elsewhere (24). This ANG-(1-12) antibody shows no cross-reactivity with Aogen, ANG I, ANG II, and ANG-(1-7) or any cellular protein ranging in sizes between 20 and 120 kDa (24). Characterization of expression of renin-angiotensin system components in the heart of WKY rats by immunofluorescent confocal microscopy was performed at the University of Missouri as described elsewhere (19, 20, 24, 37). Briefly, the harvested left ventricles were immersed and fixed in 4% paraformaldehyde. Heart sections (4 μm) were dewaxed in CitriSolv, rehydrated in ethanol series, and rinse in HEPES wash buffer. The epitopes were retrieved in citrate buffer, pH = 6.0, at 95°C for 25 min. Nonspecific binding sites were blocked by donkey blocker (5% BSA, 5% donkey serum, 0.01% sodium azide) for 4 h. Sections were incubated with 1:50 mouse Aogen (Fitzgerald Industries Int., Concord, MA), rabbit polyclonal ANG (1-12) (1:50) (AnaSpec, San Jose, CA), or mouse anti renin 1:50 (Fitzgerald Industries Int.) overnight. Sections were washed with HEPES and incubated with Alexa fluor 647 donkey anti-rabbit [ANG-(1-12)] and donkey anti-mouse (Aogen and renin) (Invitrogen, Eugene, OR) for 4 h. Sections were then incubated with 1:2,000 4′-6-diamidino-2-phenylindole (DAPI) for 15 min, washed, and mounted with Mowiol. The slides were checked under a biphoton confocal microscope (Zeiss LSM; 510 MLO, Thornwood, NY), and the images were captured with a laser scanning microscopy imaging system (19, 20). Signal intensities were analyzed with MetaView. Immunohistochemical measures of ANG II and AT_1 receptors were obtained from five 4 μm sections of heart tissues. On each image equal areas (×200) selected at random were analyzed and quantified with MetaView. The average density values obtained from each of five sections were average in each animal, and final statistics were derived from group values of five animals. Nonspecific binding sites were blocked as described above. The sections were incubated overnight with either goat ANG II or rabbit AT_1 receptors at a 1:100 dilution (Santa Cruz Biotechnology). Then the sections were incubated with the secondary antibodies and DAPI, and the images were captured and analyzed as described above.

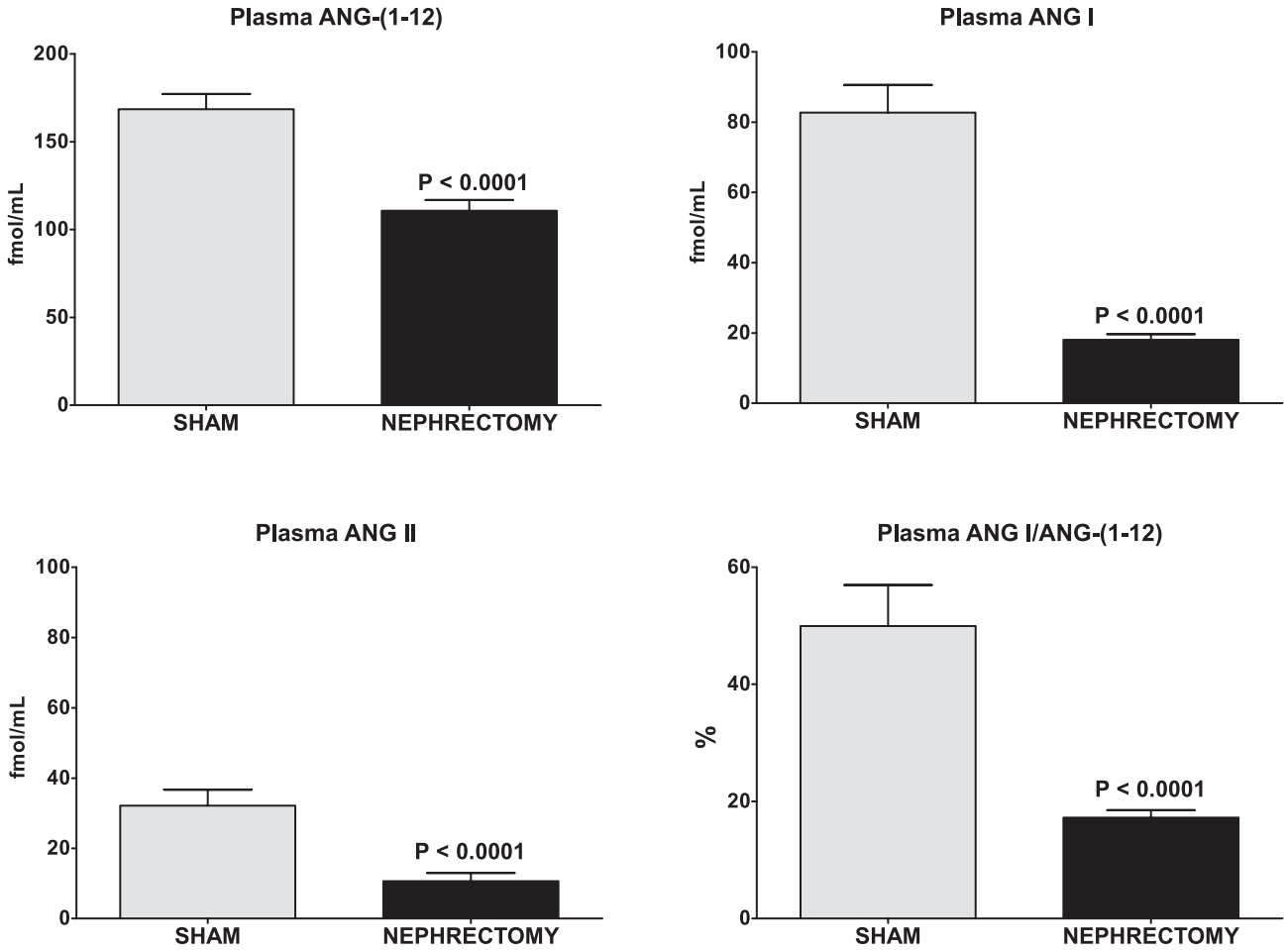

Fig. 1. Plasma concentrations of angiotensin-(1-12) [ANG-(1-12)], angiotensin I (ANG I), angiotensin II (ANG II) and the ratio of ANG I/ANG II in sham-operated and 48 h bilaterally nephrectomized Wistar-Kyoto (WKY) rats. Values are means ± SE.

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Immunostaining of NADPH oxidase subunits. The tissues were harvested and prepared, and the experiments were conducted as previously described (19, 20). Heart tissues were sectioned, deparaffinized in CitriSolv, and rehydrated in ethanol and HEPES wash buffer. Nonspecific binding sites were blocked with donkey blocker and then incubated with primary antibodies, 1:100 goat $p47^{phox}$, 1:100 Nox2, 1:100 Nox4 (Santa Cruz Biotechnology), and mouse 1:200 Rac1 (Upstate, Lake Placid, NY) overnight. After being washed thoroughly with HEPES, the sections were incubated with Alexa fluor 647 donkey anti-goat for $p47^{phox}$, Nox2, Nox4, and donkey anti-mouse for Rac1 (Invitrogen) for 4 h. Sections were incubated with 1:2,000 DAPI for 15 min, washed, and mounted with Mowiol. The slides were checked under a biphoton confocal microscope (Zeiss LSM), and the images were captured with LSM imaging system. Signal intensities were analyzed with MetaView.

RIAs. Plasma and tissue concentrations of angiotensin peptides were assessed by separate and selective RIAs after extraction with Sep-Pak C18 cartridges at the University of Miyazaki (Kiyotake, Miyazaki, Japan) as reported by us elsewhere (24, 31). The RIA for ANG-(1-12) specifically detects the COOH terminus of ANG-(1-12) using a 1:6,300 dilution of purified ANG-(1-12) antibody. As reported elsewhere, the ANG-(1-12) antibody showed no cross-reactivity with Aogen, ANG I, ANG II, ANG III, ANG IV, ANG-(1-7), ANG(1-14), and ANG(1-17) (31). Additionally, left ventricular ANG I and ANG II concentrations were determined using anti-COOH-terminal ANG I and anti-COOH-terminal ANG II RIAs as detailed by Nagata et al. (31). In these studies (31), HPLC analyses 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. (31) documented the efficiency at levels >90% of the extraction procedure, which included boiling the specimens immediately after extraction so as to denature the proteins and inactivate the proteinases (25).

Aogen and renin mRNAs. The methodology employed for the isolation of RNA and reverse transcriptase, real-time PCR is described (15). RNA was isolated from cardiac tissue, using the TRIZOL reagent (GIBCO Invitrogen, Carlsbad, CA), as directed by the manufacturer. The RNA concentration and integrity were assessed using an Agilent 2100 Bioanalyzer with an RNA 6000 Nano LabChip (Agilent Technologies, Palo Alto, CA). Approximately 1 μg of total RNA was reverse transcribed using AMV reverse transcriptase in a 20 μl reaction mixture containing deoxyribonucleotides, random hexamers, and RNase inhibitor in reverse transcriptase buffer. Heating the reverse transcriptase reaction product at 95°C terminated the reaction. For real-time PCR, 2 μl of the resultant cDNA was added to TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA) with the appropriate gene-specific primer/probe set (Applied Biosystems), and amplification was performed on an ABI 7000 Sequence Detection System. The mixtures were heated at 50°C for 2 min, at 95°C for 10 min followed by 40 cycles at 95°C for 15 s and 60°C for 1 min. All reactions were performed in triplicate, and 18S ribosomal RNA, amplified using the TaqMan Ribosomal RNA Control Kit (Applied Biosystems), served as an internal control. The results were quantified as Ct values, where Ct was defined as the threshold cycle of PCR at which amplified product is first detected, and expressed as relative gene expression (the ratio of target/18S rRNA control).

Statistics. Values are expressed as means ± SE. Comparisons between the strains were performed with either one- or two-tailed, unpaired Student's *t*-test (GraphPad Prizm 5 Software, San Diego, CA). $P < 0.05$ was considered statistically significant.

RESULTS

Plasma concentrations of ANG-(1-12), ANG I, and ANG II were significantly reduced in anephric WKY rats (Fig. 1). The decrease in circulating ANG-(1-12) following bilateral nephrectomy is less than that of ANG I, as the ratio of ANG I/ANG-(1-12) averages 50 ± 7% in sham-operated controls and 17 ± 1% in bilaterally nephrectomized WKY rats (Fig. 1). This indicates that in anephric rats circulating ANG I comprises 17% of the ANG-(1-12) levels. In addition, the finding of a significant correlation between plasma ANG I and ANG-(1-12) levels in nephrectomized WKY but not sham-operated controls implies, but obviously does not prove, that the residual concentrations of ANG I in plasma may be derived from circulating ANG-(1-12) (Fig. 2).

The concentrations of ANG-(1-12), ANG I, and ANG II were also measured in the left ventricle of both sham-operated and bilaterally nephrectomized WKY rats. Confirming our previous study in WKY rats (24), left ventricular content of ANG-(1-12) averaged 181 ± 31 fmol/mg compared with 218 ± 13 fmol/mg ($P > 0.05$) in our previous publication (24). Corresponding values for left ventricular content of ANG I and

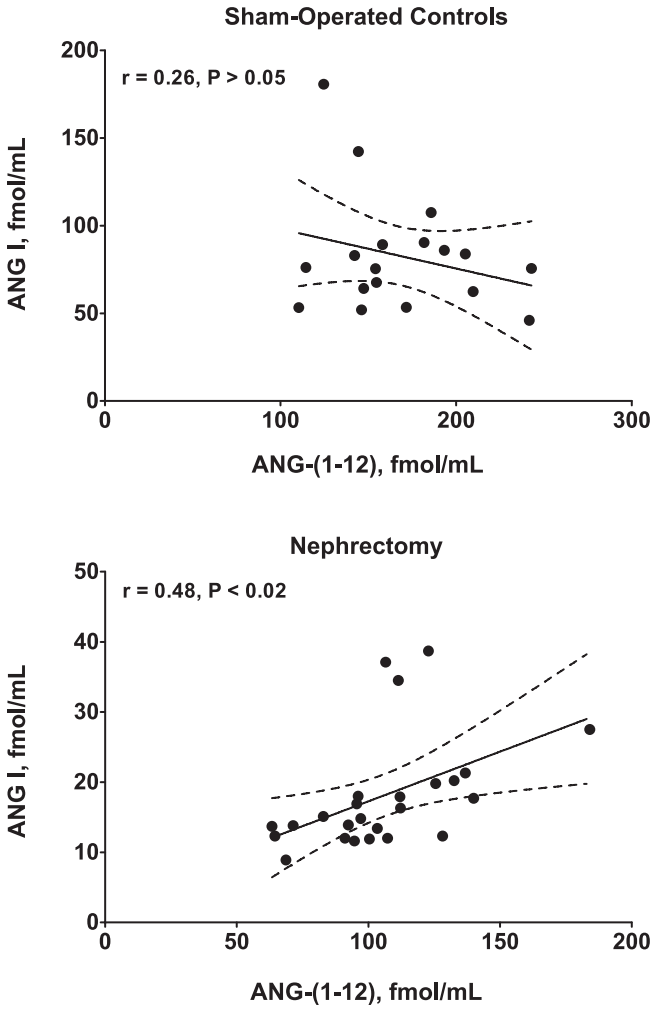

Fig. 2. Scatter gram of plasma concentrations of ANG-(1-12) as a function of plasma ANG I in sham-operated (*top*) and 48 h bilaterally nephrectomized (NX, *bottom*) WKY. Regression line and ± 95% confident limits are denoted in each graph.

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ANG II did not differ significantly from those reported in the same rat strain by us elsewhere (24, 31). As illustrated in Fig. 3, removal of the kidneys augmented the tissue content of ANG-(1-12), ANG I, and ANG II. This was accompanied by a nonstatistically significant reduction in the ANG I/ANG-(1-12) ratio (Fig. 3), further evidencing a dependence of ANG I formation from ANG-(1-12).

We then examined whether nephrectomy altered the expression of renin and Aogen transcripts in samples obtained from the left ventricle of sham and anephric rats. Figure 4 (*top*) shows the presence of a weak immunofluorescence for cardiac renin with equivalent average grayscale intensities in both sham-operated and bilaterally nephrectomized rats. Comparative analysis of cardiac samples assayed for renin mRNA from both WKY groups of rats revealed the absence of this gene transcript. In contrast, cardiac Aogen mRNA detected in sham-operated animals was markedly reduced in anephric rats (Fig. 4, *bottom*).

Figure 5 provides further evidence that removal of the kidneys does not result in the loss of angiotensins in cardiac myocytes. Positive immunostaining for Aogen, ANG-(1-12), ANG II, and AT_1 receptors are expressed in cardiac myocytes from both sham-operated and anephric WKY rats. Quantitative fluorescence intensities for Aogen, ANG II, and AT_1 receptors are augmented in the myocardium of bilateral nephrectomized rats. Additional evidence for increased expression of renin-angiotensin system components in the heart of anephric rats is documented in Fig. 6 through the increased myocardial immunostaining of $p47^{phox}$, Rac1, and Nox4.

DISCUSSION

Aogen is considered the only known functional in vivo precursor of the angiotensins. The current experiments both confirm (24, 31, 39) and expand knowledge of the existence of an intermediate peptide that, upstream from ANG I and derived from Aogen, functions as a substrate for the formation of ANG I and ANG II in both the circulation and the cardiac tissue. Bilateral nephrectomy, a technique long established as a maneuver to determine the contribution of renal renin to angiotensin peptide generation, differentially influenced the expression of ANG-(1-12) in the blood and the heart from WKY rats. In anephric rats, removal of the renal source of circulating renin was associated with a large decline in circulating levels of ANG I and ANG II accompanied by a much smaller fall in plasma ANG-(1-12) levels. The changes in circulating levels of these angiotensins contrasted markedly with the corresponding changes of these peptides in the left ventricle of anephric rats. In this tissue, direct RIA measures of the content of ANG-(1-12), ANG I, and ANG II showed significant increases in the

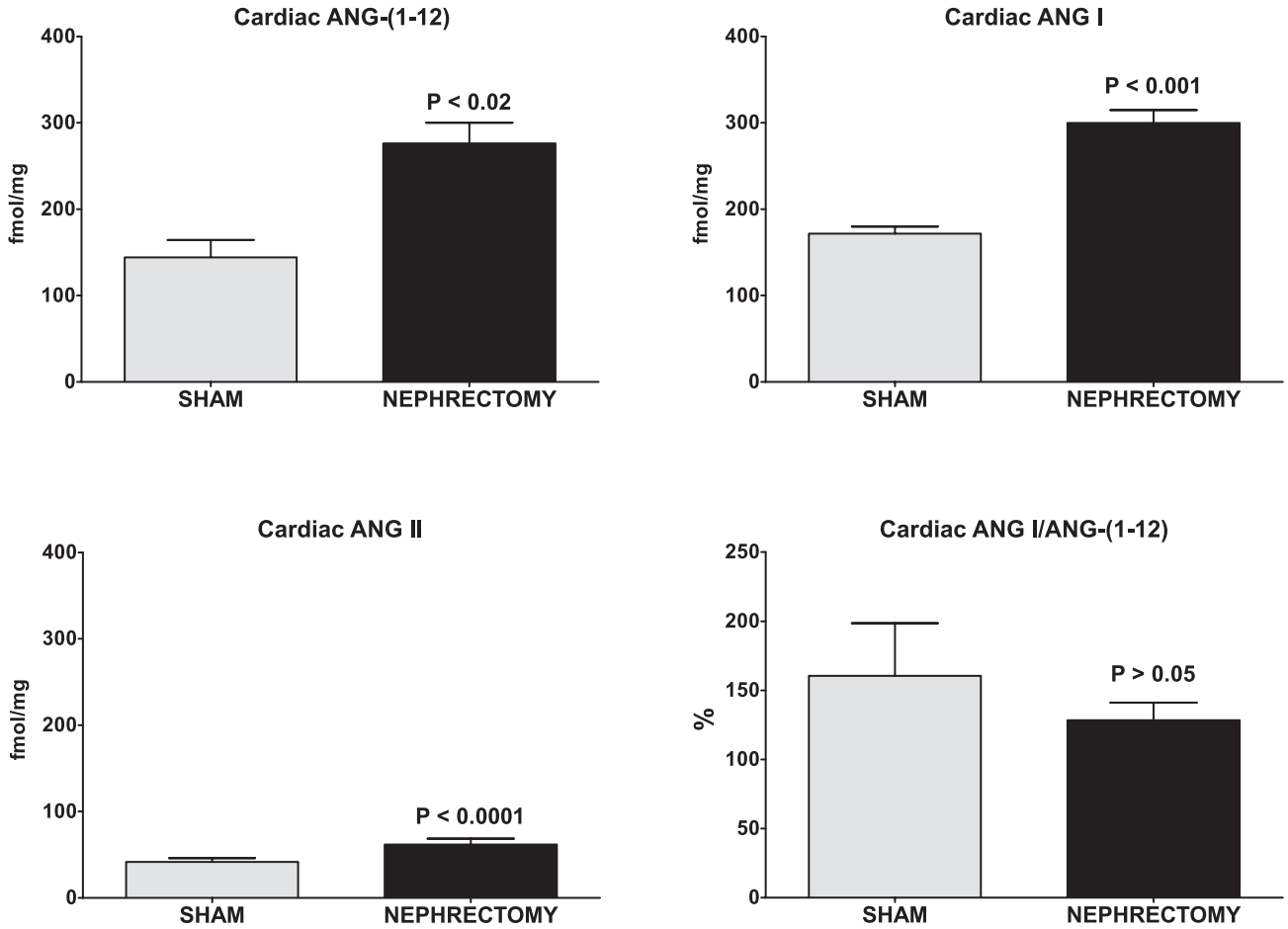

Fig. 3. Left ventricular concentrations of ANG-(1-12), ANG I, ANG II, and the ratio of ANG I/ANG II in sham-operated and 48 h bilaterally nephrectomized WKY. Values are means ± SE. Abbreviations as in Fig. 1.

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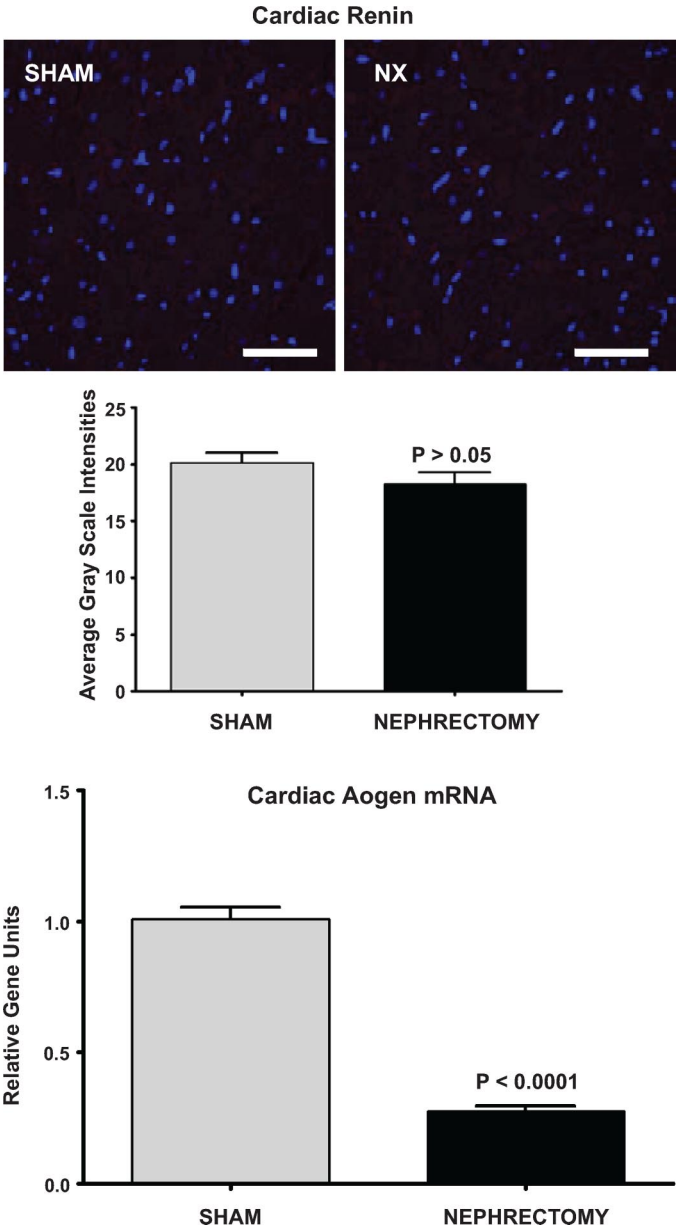

Fig. 4. *Top*: representative immunofluorescent image of cardiac renin revealing a faint expression of the enzyme and no changes after bilateral nephrectomy (also see *middle*). *Bottom*: angiotensinogen (Aogen) gene expression in the left ventricle of sham-operated and bilaterally nephrectomized rats. Values are means ± SE. Bar = 50 μm.

concentration of angiotensin peptides that were also reflected by separate quantitative measures of the immunostaining for cardiac Aogen, ANG II, and AT_1 receptors. A comparative increased immunostaining of cardiac $p47^{phox}$, Rac1, and Nox4 suggest that the increased ANG II activity in the cardiac myocytes following removal of the kidneys may reflect increased generation of radical oxygen species (38). Data on the expression of renin-angiotensin system components in the heart following bilateral nephrectomy are sparse. Our experiments now show that removal of circulating renin is associated with a marked upregulation of both the myocardial content of angiotensins and markers of oxidative stress, a novel finding that is in keeping with the hypothesis of an independent regulation of renin-angiotensin system components in both the circulation and the heart (12, 35).

The experiments were performed using procedures and assays extensively documented in the literature. Nephrectomy is an established method to assess the contribution of renal renin to formation and actions of ANG II in the circulation (2, 6–9, 17) as well as a tool to unravel whether tissues contain renin-angiotensin systems that are independent from the circulation (4, 9–11, 18, 26). In agreement with previous studies (2, 8, 17, 29), the anephric state at 48 h has not yet exerted lethal azotemia as rats maintained weight, remained alert, and continued to drink at will. Antibodies we used to visualize expression and quantitate cardiac ANG-(1-12) levels were validated in previous studies from this (24, 39, 42) and another laboratory (31). In addition, several other publications from our laboratory have documented the sensitivity and specificity of RIA measures of ANG I and ANG II employed here (14).

As expected, blood ANG I and ANG II levels were substantially reduced in the renoprival state, reflecting a loss of the hydrolytic activity of circulating renin after removal of the kidneys. Although ANG-(1-12) levels in blood decreased concurrently, the lower ratio of ANG I/ANG-(1-12) in blood suggests that the effect of nephrectomy on plasma ANG-(1-12) was not as pronounced as that for either ANG I or ANG II. Although plasma ANG I and ANG-(1-12) was positively correlated in nephrectomized but not sham-operated controls, these data cannot be taken as evidencing that the residual levels of ANG I were generated from ANG-(1-12). On the other hand, our findings do exclude a significant participation of renal renin in the conversion of Aogen into ANG-(1-12), in agreement with our previous demonstration that administration of a selective rat renin inhibitor did not block the formation of ANG I, ANG II, and ANG-(1-7) in the isolated heart preparation from both WKY and SHR (39).

Few studies have directly inquired on the effect of depletion of renal renin on the cardiac renin-angiotensin system. A study of the effect of 48 h bilateral nephrectomy from Sprague-Dawley rats showed that the reduction in cardiac ANG I and ANG II was less than those in plasma and not affected by the concurrent administration of ramipril (5). Campbell and colleagues (5) proposed that ANG II formation in anephric rats may be the result of a direct formation from Aogen via a nonrenin-dependent mechanism. Their earlier studies are in keeping with the characterization of ANG-(1-12) as an alternate mechanism for the tissue production of angiotensin peptides in view of the contrasting effect of nephrectomy on ANG-(1-12) and ANG II in the heart and the circulation. In the anephric state, we now show increased cardiac immunostaining of Aogen, ANG II, and AT_1 receptors. The weak immunofluorescence of renin found in bilateral nephrectomized rats, in the absence of any detectable level of renin mRNA, confirms that renin in cardiac myocytes is derived from the circulation (1, 3, 8–10, 21, 27).

Additional lines of evidence suggest that cardiac renin is mostly a result of uptake from the circulation as its levels decline markedly and progressively after removal of the kidneys (8–12, 18). The antihypertensive effect of systemic delivery of an anti-renin antibody was eliminated in bilateral nephrectomized SHR rats (23), while Leenen et al. (28) and Nagano et al. (30) showed that the increase in cardiac ANG II induced by coronary artery ligation or isoproterenol infusion

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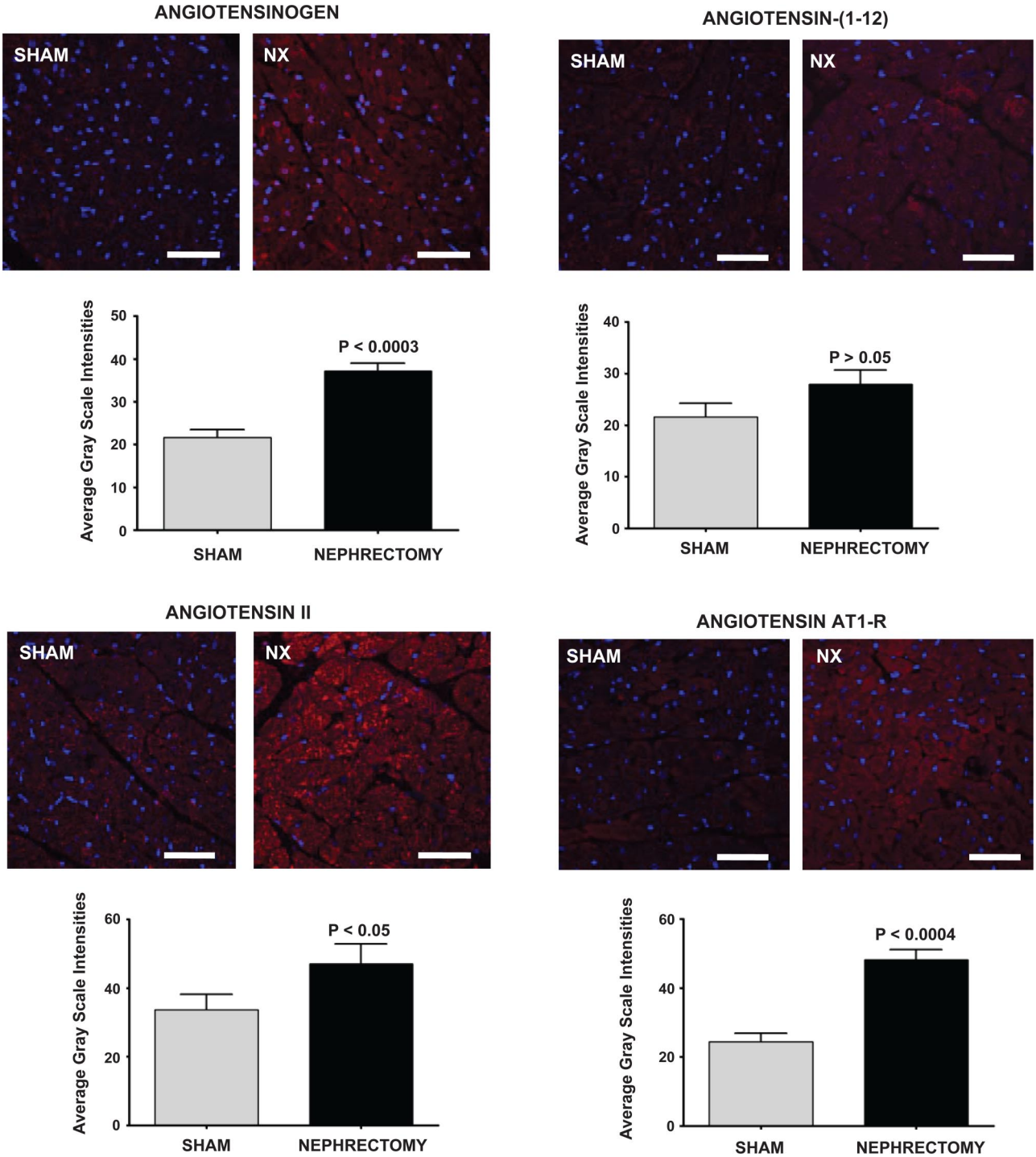

Fig. 5. Representative fluorescent images obtained from the left ventricle of sham-operated and bilaterally nephrectomized rats for Aogen, ANG-(1-12), ANG II, and the type 1 angiotensin II receptor (AT_1R). Bar graphs are means ± SE of average grayscale intensity values of the fluorescent signals for each of the peptides and the receptor. Bars = 50 μm.

was not reduced in rats by prior removal of their kidneys. In the isolated heart preparation from WKY and SHR, the active renin protein present in the effluent did not eliminate production of ANG I, ANG II, and ANG-(1-7) in the presence of a potent and specific rat renin inhibitor (39). Therefore, increased content of ANG-(1-12) in WKY anephric rats may be due to the action of a nonrenin enzyme. Since renin specifically cleaves the Leu^{10}-Leu^{11} bond of rat Aogen to form ANG I, the cleavage between the two aromatic residues Tyr^{12}-Tyr^{13} to liberate ANG-(1-12) requires the participation of another enzyme.

While the paralleling effect of nephrectomy on left ventricular concentrations and expression of Aogen, ANG I, ANG II, and AT_1 receptors provides further evidence for a local cardiac renin-angiotensin system, increased expression of these components in the heart may be considered as evidence for the

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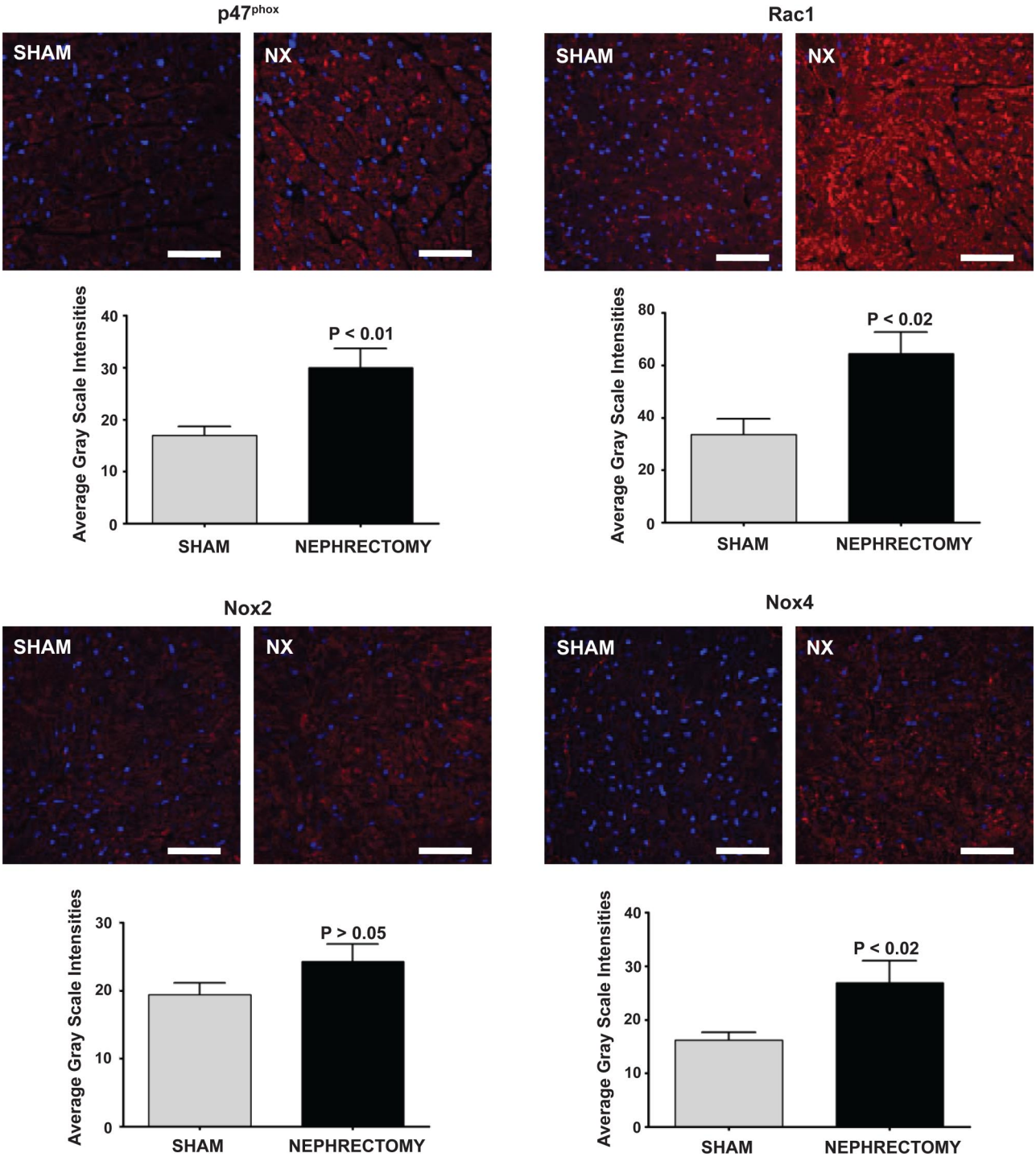

Fig. 6. Representative fluorescent images obtained from the left ventricle of sham-operated and bilaterally nephrectomized rats for the p47^{phox} and Rac1 regulatory subunits of NADPH oxidase and Nox2 and Nox4 isoforms. Graph below each photomicrograph set is means ± SE of quantification of converted signals. Bars = 50 μm.

utilization of ANG-(1-12) as a substrate contributing to the production of the peptides in cardiac tissue. Our experiments do not answer the question of whether the increase in cardiac ANG-(1-12) postnephrectomy is the result of intracellular formation from Aogen, uptake of ANG-(1-12) from the circulation, or a combination of both. The reduced expression of Aogen transcripts following nephrectomy may be construed as indirect evidence of an extracellular mechanism for ANG-(1-12) or even uptake of blood Aogen. On the other hand, a decrease in Aogen mRNA may be a result of nuclear inhibition of gene expression in response to a high intracellular ANG II content as shown by Dostal et al. (13) in neonatal cardiac fibroblasts.

Since the original submission of our study, a new report by Prosser et al. (34) has both confirmed and extended our original studies on ANG-(1-12). These investigators showed that infu-

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sion of ANG-(1-12) in isolated hearts from Sprague-Dawley rats induced a dose-dependent coronary artery vasoconstriction and worsened ischemia-reperfusion injury. As previously demonstrated by Nagata et al. (31), Prosser et al. (34) report that both coronary artery vasoconstriction and worsened ischemia-reperfusion were blocked in the presence of the AT_1-receptor blocker candesartan. However, in their experiments the ANG II-mediated effects following administration of ANG-(1-12) were partially inhibited by pretreatment of chymostatin, an inhibitor of the chymase, a serine protease, shown by Urata and Ganten (40) to be the predominant ANG II-forming enzyme from ANG I in human and rat hearts. These findings and those reported by us previously in the isolated heart of several rat strains (39) underscore the existence of alternate enzymatic mechanisms for the tissue production of angiotensin peptides. In addition, these results contribute, collectively, to explain the relative limited ability of ACE inhibitors (22, 32, 41) and now direct renin inhibitors (33) to induce sustained blockade of ANG II production.

In summary, our experiments provide evidence for dynamic and inverse changes in the content of ANG-(1-12) in the blood and cardiac tissue of anephric WKY rats. The divergent effect of removal of the kidneys on the circulating and cardiac tissue levels of the angiotensins adds weight to the suggestion of a differential regulation of local vs. systemic mechanisms for the expression of the renin-angiotensin system while adding new data about the role of ANG-(1-12) as an alternate intermediate substrate for the generation of cardiac tissue ANG II.

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REFERENCES

1. **Admiraal PJ, Derkx FH, Danser AH, Schalekamp MA.** In vivo production of plasma angiotensin I: is plasma renin sufficient? *J Hypertens Suppl* 7: S222–S223, 1989.
2. **Basso N, De la Riva IJ.** Increased cardiovascular reactivity after bilateral nephrectomy in the rat. *Acta Physiol Latinoam* 23: 1–8, 1973.
3. **Basso N, Taquini AC.** Changes in the renin-angiotensin system after bilateral nephrectomy. *Acta Physiol Latinoam* 20: 316–321, 1971.
4. **Campbell DJ.** Tissue renin-angiotensin system: sites of angiotensin formation. *J Cardiovasc Pharmacol* 10, *Suppl* 7: S1–S8, 1987.
5. **Campbell DJ, Kladis A, Duncan AM.** Nephrectomy, converting enzyme inhibition, and angiotensin peptides. *Hypertension* 22: 513–522, 1993.
6. **Campbell DJ, Kladis A, Skinner SL, Whitworth JA.** Characterization of angiotensin peptides in plasma of anephric man. *J Hypertens* 9: 265–274, 1991.
7. **Coleman TG, Bower JD, Langford HG, Guyton AC.** Regulation of arterial pressure in the anephric state. *Circulation* 42: 509–514, 1970.
8. **Danser AH, Admiraal PJ, Derkx FH, de Bruyn JH, Schalekamp MA.** Changes in plasma renin and angiotensin run in parallel after nephrectomy. *J Hypertens Suppl* 11: S238–S239, 1993.
9. **Danser AH, Admiraal PJ, Derkx FH, Lamers JM, Verdouw PD, Saxena PR, Schalekamp MA.** Cardiac renin is kidney-derived. *J Hypertens Suppl* 11: S224–S225, 1993.
10. **Danser AH, van Kats JP, Admiraal PJ, Derkx FH, Lamers JM, Verdouw PD, Saxena PR, Schalekamp MA.** Cardiac renin and angiotensins. Uptake from plasma versus in situ synthesis. *Hypertension* 24: 37–48, 1994.
11. **De Lannoy LM, Danser AH, van Kats JP, Schoemaker RG, Saxena PR, Schalekamp MA.** Renin-angiotensin system components in the interstitial fluid of the isolated perfused rat heart. Local production of angiotensin I. *Hypertension* 29: 1240–1251, 1997.
12. **De Mello WC, Danser AH.** Angiotensin II and the heart : on the intracrine renin-angiotensin system. *Hypertension* 35: 1183–1188, 2000.
13. **Dostal DE, Booz GW, Baker KM.** Regulation of angiotensinogen gene expression and protein in neonatal rat cardiac fibroblasts by glucocorticoid and beta-adrenergic stimulation. *Basic Res Cardiol* 95: 485–490, 2000.
14. **Ferrario CM, Brosnihan KB, Chappell M.** Measurements of angiotensin peptides. *Hypertension* 26: 843–845, 1995.
15. **Ferrario CM, Jessup J, Chappell MC, Averill DB, Brosnihan KB, Tallant EA, Diz DI, Gallagher PE.** Effect of angiotensin-converting enzyme inhibition and angiotensin II receptor blockers on cardiac angiotensin-converting enzyme 2. *Circulation* 111: 2605–2610, 2005.
16. **Ferrario CM, Trask AJ, Jessup JA.** Advances in biochemical and functional roles of angiotensin-converting enzyme 2 and angiotensin-(1-7) in regulation of cardiovascular function. *Am J Physiol Heart Circ Physiol* 289: H2281–H2290, 2005.
17. **Gabelman EH, Rondell PA.** Protracted pressor response to angiotensin after bilateral nephrectomy in rats. *Circ Res* 18: 705–713, 1966.
18. **Gondo M, Maruta H, Arakawa K.** Direct formation of angiotensin II without renin or converting enzyme in the ischemic dog heart. *Jpn Heart J* 30: 219–229, 1989.
19. **Habibi J, Whaley-Connell A, Hayden MR, Demarco VG, Schneider R, Sowers SD, Karuparthi P, Ferrario CM, Sowers JR.** Renin inhibition attenuates insulin resistance, oxidative stress, and pancreatic remodeling in the transgenic Ren2 rat. *Endocrinology* 149: 5643–5653, 2008.
20. **Habibi J, Whaley-Connell A, Qazi MA, Hayden MR, Cooper SA, Tramontano A, Thyfault J, Stump C, Ferrario C, Muniyappa R, Sowers JR.** Rosuvastatin, a 3-hydroxy-3-methylglutaryl coenzyme a reductase inhibitor, decreases cardiac oxidative stress and remodeling in Ren2 transgenic rats. *Endocrinology* 148: 2181–2188, 2007.
21. **Hilgers KF, Veelken R, Muller DN, Kohler H, Hartner A, Botkin SR, Stumpf C, Schmieder RE, Gomez RA.** Renin uptake by the endothelium mediates vascular angiotensin formation. *Hypertension* 38: 243–248, 2001.
22. **Hollenberg NK.** Pharmacologic interruption of the renin-angiotensin system and the kidney: differential responses to angiotensin-converting enzyme and renin inhibition. *J Am Soc Nephrol* 10, *Suppl* 11: S239–S242, 1999.
23. **Inagami T, Murakami T, Higuchi K, Nakajo S.** Roles of renal and vascular renin in spontaneous hypertension and switching of the mechanism upon nephrectomy. Lack of hypotensive effects of inhibition of renin, converting enzyme, and angiotensin II receptor blocker after bilateral nephrectomy. *Am J Hypertens* 4: 15S–22S, 1991.
24. **Jessup JA, Trask AJ, Chappell MC, Nagata S, Kato J, Kitamura K, Ferrario CM.** Localization of the novel angiotensin peptide, angiotensin-(1-12), in heart and kidney of hypertensive and normotensive rats. *Am J Physiol Heart Circ Physiol* 294: H2614–H2618, 2008.
25. **Kitamura K, Ichiki Y, Tanaka M, Kawamoto M, Emura J, Sakakibara S, Kangawa K, Matsuo H, Eto T.** Immunoreactive adrenomedullin in human plasma. *FEBS Lett* 341: 288–290, 1994.
26. **Krop M, Danser AH.** Circulating versus tissue renin-angiotensin system: on the origin of (pro)renin. *Curr Hypertens Rep* 10: 112–118, 2008.
27. **Krop M, de Bruyn JH, Derkx FH, Danser AH.** Renin and prorenin disappearance in humans post-nephrectomy: evidence for binding? *Front Biosci* 13: 3931–3939, 2008.
28. **Leenen FH, Skarda V, Yuan B, White R.** Changes in cardiac ANG II postmyocardial infarction in rats: effects of nephrectomy and ACE inhibitors. *Am J Physiol Heart Circ Physiol* 276: H317–H325, 1999.
29. **Miyamori I, Matsubara T, Takeda Y, Koshida H, Soma R, Takeda R.** Angiotensin II generation in mesenteric arteries in rats: effects of nephrectomy, deoxycorticosterone and dexamethasone. *Endocrinol Jpn* 37: 9–17, 1990.
30. **Nagano M, Higaki J, Nakamura F, Higashimori K, Nagano N, Mikami H, Ogihara T.** Role of cardiac angiotensin II in isoproterenol-induced left ventricular hypertrophy. *Hypertension* 19: 708–712, 1992.
31. **Nagata S, Kato J, Sasaki K, Minamino N, Eto T, Kitamura K.** Isolation and identification of proangiotensin-12, a possible component of the renin-angiotensin system. *Biochem Biophys Res Commun* 350: 1026–1031, 2006.
32. **Nussberger J, Waeber B, Brunner HR.** Clinical pharmacology of ACE inhibition. *Cardiology* 76, *Suppl* 2: 11–22, 1989.
33. **Oparil S, Yarows SA, Patel S, Fang H, Zhang J, Satlin A.** Efficacy and safety of combined use of aliskiren and valsartan in patients with hypertension: a randomised, double-blind trial. *Lancet* 370: 221–229, 2007.
34. **Prosser HC, Forster ME, Richards AM, Pemberton CJ.** Cardiac chymase converts rat proAngiotensin-12 (PA12) to angiotensin II: effects of PA12 upon cardiac haemodynamics. *Cardiovasc Res*. In press.

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35. **Ruzicka M, Leenen FH.** Update on local cardiac renin-angiotensin system. *Curr Opin Cardiol* 12: 347–353, 1997.
36. **Santos RA, Simoes e Silva AC, Maric C, Silva DM, Machado RP, de Buhr I, Heringer-Walther S, Pinheiro SV, Lopes MT, Bader M, Mendes EP, Lemos VS, Campagnole-Santos MJ, Schultheiss HP, Speth R, Walther T.** Angiotensin-(1-7) is an endogenous ligand for the G protein-coupled receptor Mas. *Proc Natl Acad Sci USA* 100: 8258–8263, 2003.
37. **Stas S, Whaley-Connell A, Habibi J, Appesh L, Hayden MR, Karuparthi PR, Qazi M, Morris EM, Cooper SA, Link CD, Stump C, Hay M, Ferrario C, Sowers JR.** Mineralocorticoid receptor blockade attenuates chronic overexpression of the renin-angiotensin-aldosterone system stimulation of reduced nicotinamide adenine dinucleotide phosphate oxidase and cardiac remodeling. *Endocrinology* 148: 3773–3780, 2007.
38. **Toda N, Ayajiki K, Okamura T.** Interaction of endothelial nitric oxide and angiotensin in the circulation. *Pharmacol Rev* 59: 54–87, 2007.
39. **Trask AJ, Jessup JA, Chappell MC, Ferrario CM.** Angiotensin-(1-12) is an alternate substrate for angiotensin peptide production in the heart. *Am J Physiol Heart Circ Physiol* 294: H2242–H2247, 2008.
40. **Urata H, Ganten D.** Cardiac angiotensin II formation: the angiotensin-I converting enzyme and human chymase. *Eur Heart J* 14, *Suppl* I: 177–182, 1993.
41. **Van de Wal RM, Plokker HW, Lok DJ, Boomsma F, van der Horst FA, van Veldhuisen DJ, van Gilst WH, Voors AA.** Determinants of increased angiotensin II levels in severe chronic heart failure patients despite ACE inhibition. *Int J Cardiol* 106: 367–372, 2006.
42. **Varagic J, Trask AJ, Jessup JA, Chappell MC, Ferrario CM.** New angiotensins. *J Mol Med* 86: 663–671, 2008.
43. **Whaley-Connell A, Habibi J, Cooper SA, Demarco VG, Hayden MR, Stump CS, Link D, Ferrario C, Sowers JR.** Effect of renin inhibition and AT_1R blockade on myocardial remodeling in the transgenic Ren2 rat. *Am J Physiol Endocrinol Metab* 295: E103–E109, 2008.

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