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Local Renin-Angiotensin System in Human Adrenals and Aldosteronomas

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The local renin-angiotensin system may regulate adrenal cell growth and function. Angiotensinogen, renin, and angiotensin converting enzyme gene expression were studied in four normal adrenal glands (removed from patients with renal carcinomas) and five aldosterone-secreting adenomas. Northern blot analysis showed expression of angiotensinogen messenger RNA (mRNA) in normal adrenals at levels approximately 35-fold lower than liver and sixfold lower than kidney. Similar angiotensinogen mRNA levels were present in two aldosteronomas, whereas a third had levels approximately 50% of those found in kidney. Renin mRNA was detectable in most normal adrenals and in three adenomas, one of which had relatively high renin mRNA levels. Angiotensin converting enzyme gene was expressed in adrenal tissue and in three adenomas. Portions from these normal adrenals and two of these aldosteronomas, as well as samples from two other adrenals and three aldosteronomas, were also studied in an in vitro superfusion system coupled with active renin radioimmunometric assay, angiotensin II/III, and aldosterone radioimmunoassay. Total amounts of active renin and angiotensin II/III released from normal adrenals during 270 minutes of superfusion were higher than the amounts released from aldosteronomas (312±35 versus 187±43 and 823±100 versus 436±55 pg/100 mg tissue, respectively; mean±SEM, $p<0.05$), whereas aldosterone release from the adenomatous tissue was approximately threefold higher (320±21 versus 115±18 ng/100 mg tissue; mean±SEM, $p<0.01$). Total amounts of active renin and angiotensin II/III released by normal or adenomatous adrenal samples exceeded threefold to fourfold the amounts extracted from similar samples of the same surgical specimen. These findings provide evidence for a local renin-angiotensin system in human adrenals and in at least some aldosteronomas. (*Hypertension* 1992;19:702–707)

KEY WORDS • **angiotensinogen** • **renin** • **angiotensin II** • **adrenal glands** • **aldosterone** • **human studies** • **angiotensin converting enzyme**

The renin-angiotensin system (RAS) has been considered as an endocrine system whose components are synthesized by different organs and interact in the circulation to generate the active peptide angiotensin II (Ang II), which then reaches target cells. In the past decade, several studies conducted on animals found evidence for a complete RAS within various tissues, suggesting that locally generated Ang II may act as an autocrine or paracrine mediator that might be independently regulated from circulating RAS.[1,2] In the adrenals, local production of Ang II and angiotensin III (Ang III) may stimulate mineralocorticoid synthesis and secretion[3] and growth of adrenal cells[4] and may have an important role in the pathogenesis of high blood pressure, as suggested by studies on transgenic rats with high adrenal renin expression.[5] Many studies reported the presence of some RAS components in the adrenals of various animal species. Renin messenger RNA (mRNA), as well as renin immunoreactivity and activity, has been demonstrated in mouse and rat adrenals[6] and localized in the zona glomerulosa cells.[7,8] Angiotensinogen mRNA has been detected in mouse and rat adrenals,[6] even though it appears to be mainly expressed in periadrenal fibroblast-like cells and brown adipocytes.[9] The presence of angiotensin converting enzyme (ACE) in rat adrenals has been suggested by binding studies with labeled ACE inhibitors.[10,11] Moreover, angiotensin I (Ang I), Ang II, and Ang III have been found in rat adrenals,[12,13] and the production of renin and Ang II by adrenal explant cultures has been demonstrated.[14]

In humans, the RAS is the major physiological modulator of aldosterone secretion, but only a few observations suggest that at least some components of the RAS are present in human adrenal tissue. Reninlike activity has been found in human adrenals and aldosteronomas,[15,16] and renin has been localized by immunohistochemistry in the zona glomerulosa.[17] ACE binding sites have been found in the zona glomerulosa and the medulla of human adrenals.[11] Recently, we found that human adrenal glands, as well as aldosteronomas, release in vitro both renin and Ang II/III in a pulsatile fashion.[18]

The present study was undertaken to look for evidence of local RAS synthesis in human adrenals. Gene

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expression for angiotensinogen, renin, and ACE was analyzed in nonadenomatous human adrenals and in aldosteronomas. Active renin and Ang II/III immunoreactivity were determined in tissue extracts, and their secretion from human adrenal tissue was studied in an in vitro superfusion system.

Methods

Patients and Tissue Samples

A total of 14 patients was studied. Six adrenal glands were obtained from patients who underwent unilateral expanded nephrectomy for kidney cancer. All patients (four men and two women, 46–68 years old) were normotensive, and none had clinical symptoms of adrenal dysfunction. Histological examination revealed normal adrenal morphology and absence of cancer cells. Aldosterone-producing adenomas (aldosteronomas) were removed from eight patients (five men and three women, 34–62 years old) with primary aldosteronism. The patients had hypertension, hypokalemia, low and unresponsive (to upright posture and furosemide) plasma renin activity and Ang II, and elevated urinary aldosterone levels. The differential diagnosis between hyperplasia and aldosterone-producing adenoma was based on at least three of the following tests: 1) postural or captopril response of plasma aldosterone, 2) adrenal scintiscan with ^{75}Se-labeled cholesterol after dexamethasone suppression, 3) computerized axial tomography, and 4) adrenal venography and aldosterone measurement in adrenal venous blood. Both groups of patients were on a diet containing 120–150 mmol sodium and approximately 60 mmol potassium daily and were off any drug for at least 2 weeks before surgery. In all patients, the diagnosis of adenoma was confirmed by pathological study of the surgical specimens. After surgery, blood pressure and serum potassium level promptly returned to normal in all patients. Nonadenomatous adrenals and aldosteronomas were carefully freed from surrounding tissues before portions were taken for analysis. Samples of human liver (obtained from a patient that underwent partial hepatectomy for echinococciasis), lung (taken from lobectomies for lung cancer), and kidney (obtained after uninephrectomies for renal cancer) were used as control tissues for angiotensinogen, renin, and ACE gene expression, respectively. All patients gave informed consent, and the use of human samples followed the guidelines of the Universities of Ancona and Padova.

RNA Extraction and Analysis

Portions of four normal adrenals and five aldosteronomas were quickly frozen in liquid nitrogen and stored at −80°C until processed as previously described with minor modifications of the guanidinium thiocyanate/cesium chloride centrifugation method.[19,20] In brief, frozen tissue samples were homogenized and transferred to Quick Seal tubes (Beckman Instruments, Inc., Palo Alto, Calif.) containing 6 ml of 5.7 M CsCl. Tubes were spun at 36,000 rpm in a 70.1 Ti rotor (Beckman) for 18 hours. Ten micrograms of total RNA in each lane was used for ACE and angiotensinogen mRNA detection, and 30 μg of total RNA was used for renin mRNA detection. RNA samples were separated by electrophoresis through a 0.9% agarose/1.4 M formaldehyde gel containing 0.5 μg/ml ethidium bromide. The gels were photographed routinely to verify quality and quantity of RNA samples, and subsequent gels were adjusted accordingly. Northern blotting and hybridizations were performed as described,[21] except that the hybridization buffer was 5× SSPE (1× SSPE is 0.15 M NaCl, 0.01 M sodium phosphate, and 0.001 M Na2EDTA, pH 7.4), 4% sodium dodecyl sulfate, 10% dextran sulfate, 500 μg/ml heparin, and 100 μg/ml sonicated and denatured salmon sperm DNA. Most of the hybridizations were performed in a hybridization oven (HB-1 Hybridiser, Techne Ltd., Cambridge, UK) at 68°C. After high stringency washings, blots were exposed to X-Omat AR films (Eastman Kodak Co., Rochester, N.Y.) between two intensifying screens (Cronex lightning plus, Du Pont de Nemours, Firenze, Italy) for 16 hours to 10 days at −80°C. Developed films were scanned with an EC910 densitometer coupled with EC934 software/hardware (E-C Apparatus Corp., North St. Petersburg, Fla.) to quantify relative signal intensities of the bands. Renin 1.6 kb mRNA, angiotensinogen 2.0 kb mRNA, and ACE 4.7 kb mRNA were selected for densitometric quantitation. The size in kilobase of the detected mRNAs was calculated on the basis of the 18S and 28S ribosomal RNA migration from the gel wells. Blots were always rehybridized with at least two different probes to compare relative signal intensities on the same blotted RNA.

Complementary DNA Probes

Human angiotensinogen complementary DNA (cDNA) (clone pHag 3) was kindly provided by Dr. S. Nakanishi.[22] A human renin cDNA was obtained from Dr. K. Murakami.[23] A human ACE partial cDNA (525 bp) was cloned after polymerase chain reaction amplification of human lung cDNA, using two oligonucleotide primers complementary to bases No. 10–33 and 495–534 of the published human ACE cDNA sequence.[24] Rat β-actin cDNA[25] was used to validate the differences in hybridization signal intensities. DNAs were labeled with [α-^{32}P]dCTP (>3,000 Ci/mmol) using random primer extension (Multiprime kit, Amersham). The specific activities obtained were estimated to be $>1\times10^9$ dpm/μg DNA.

Tissue Extraction for Renin and Angiotensin II/III Assays

Portions of six normal adrenals and five aldosteronomas were weighed, rapidly frozen in liquid nitrogen, and stored at −80°C until processed as previously described.[18] In brief, for active renin assay, samples were homogenized in 9 vol (vol/wt) ice-cold 0.05 M Tris-HCl, pH 7.4, 10 mM EDTA, 2 mM phenylmethylsulphonyl fluoride, and 0.1 mM captopril (kindly provided by Squibb, Princeton, N.J.). Homogenates were centrifuged for 30 minutes at 14,000g, and supernatants were used for renin assay. The extraction procedure allowed the recovery of 86.4±5.8% (mean±SEM, $n=4$) of 50 pg and 88.2±6.1% of 100 pg ($n=4$) of Medical Research Council human renin standard (1 pg=1.6×10^{-6} Goldblatt Units, World Health Organization International Reference Preparation 68/356) added to adrenal samples.

For Ang II/III assay, samples were first homogenized in 100 μl of 8 M urea and then rehomogenized in 9 vol

(vol/wt) 80% methanol, 10 mM sodium acetate, and 0.5% trifluoracetic acid, pH 5.6. Homogenates were centrifuged for 30 minutes at 14,000*g*, and supernatants were transferred to tubes containing 50 μl of 50% glycerol. The recovery of exogenous Ang II (Peninsula Laboratories, Inc., Belmont, Calif.) added to similar samples was 92.2±3.8% (n=4) for 20 pg and 93.4±4.1% for 80 pg, respectively.

Superfusion Experiments

As previously reported,[14,18] portions (0.1–0.2 g wet wt) of adrenal tissue were quickly placed in ice-cold superfusion Medium 199 (GIBCO, Grand Island, N.Y.), pH 7.4, with 0.1% bovine serum albumin (radioimmunoassay grade, Sigma Chemical Co., St. Louis, Mo.). Tissue samples were finely minced, rinsed several times with ice-cold medium, and placed in 1-ml superfusion chambers together with Bio-Gel P-2 (Bio-Rad, Richmond, Calif.) used as a support matrix. The superfusion chambers, kept at 37°C, were perfused at a flow rate of 0.5 ml/min and gassed with 95% O_2–5% CO_2. After an initial 30-minute period of superfusion, the superfusate was collected in chilled tubes with 5 mM EDTA and 0.1 mM captopril for a total period of 270 minutes. Fractions corresponding to 15 minutes superfusion were lyophylized and stored frozen until the assays for active renin, Ang II/III, and aldosterone. Fractions were also assayed for lactic dehydrogenase activity with a commercial kit (Sigma Chimica, Milano, Italy), as an index for cell damage. Lactic dehydrogenase activity in the superfusate fractions did not show significant variations over the time of superfusion, ranging from 24±6 to 28±4 milliunits/100 mg tissue/15 min (p=NS, n=16).

Radioimmunoassays

Dried samples from either extracts or superfusate were resuspended in a buffer consisting of 0.05 M K_2PO_4, 0.03 M EDTA, 0.02% NaN_3, 0.01% Triton X-100, pH 7.4, and 2.5 g/l bovine serum albumin. The concentration of active renin was measured by an immunoradiometric assay kit (Diagnostics Pasteur, Marnes La Coquette, France) that uses two monoclonal antibodies against human renin (3E8 and 4G1), as previously described.[26,27] Briefly, the first antibody, 3E8, covalently linked to magnetic particles, binds both active and inactive renin, whereas the second antibody, 4G1, labeled with iodine-125, specifically recognizes active renin immobilized by 3E8. Antibody 4G1 did not bind 1) prorenin purified from human kidney or chorionic fluid, 2) prorenin produced by chorionic cells in culture, 3) six distinct synthesized renin fragments, or 4) related proteins such as pepsin or cathepsin D.[27] Immunoradiometric assay results were derived from a standard curve obtained by using monkey serum renin calibrated in Medical Research Council units. The limit of detection was 5 pg/ml, the intra-assay coefficient of variation (CV) was 6% (n=10), and the interassay CV was 10% (n=20). Ang II was assayed using a radioimmunoassay with a first antibody (Arnel Products Co., Inc., New York) that has 100% cross-reactivity with Ang III and all other C-terminal fragments and <0.1% with Ang I.[18] The lowest concentration of Ang II/III detected was 1 picogram per tube. Intra-assay CV was 7% (n=12), and interassay CV was 10% (n=18). Aldosterone was measured by radioimmunoassay with a commercial kit (ALDOK-^{3}H, Sorin, Italy). Detectability was 0.01 ng/ml, intra-assay CV was 5% (n=10), and interassay CV was 8% (n=20).

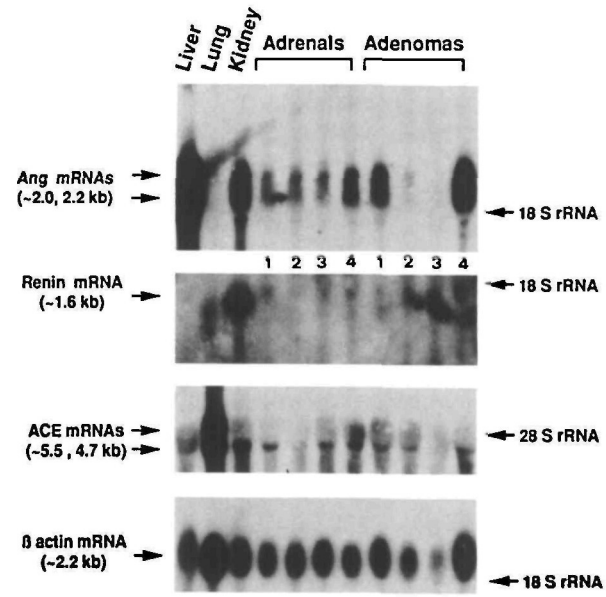

FIGURE 1. *Northern analysis of angiotensinogen (Ang), renin, and angiotensin converting enzyme (ACE) messenger RNA (mRNA) in human liver, lung, kidney, adrenals, and aldosteronomas. Each lane of the same blot contains equal amounts of total RNA; 28S and 18S indicate respective positions of ribosomal RNA transferred to nylon membranes. Estimated mRNA sizes are noted. Exposure times were 7, 10, and 5 days, respectively. The same blots were rehybridized with other renin-angiotensin system complementary DNAs (cDNAs) and with β-actin cDNA. The results of a fifth aldosteronoma that did not express renin-angiotensin system mRNAs are not shown in the figure.*

Statistics

Data are reported as mean±SEM. Comparisons between groups were evaluated by Student's t test for unpaired data. Values of $p<0.05$ were taken to indicate statistical significance.

Results

Angiotensinogen mRNAs of approximately 2.0 and 2.2 kb were very abundant in liver and were also expressed in kidney at levels approximately sixfold lower than liver, whereas human lung tissue did not have detectable angiotensinogen mRNAs (Figure 1). Two distinct angiotensinogen mRNAs were also present in all four nonadenomatous adrenal glands studied and in three aldosteronomas, whereas in two other adenomas, the messages were undetectable. The mRNA sizes, as estimated after electrophoretic migration, were indistinguishable from hepatic and renal angiotensinogen mRNAs and were expressed at levels approximately sixfold lower than kidney in all four nonadenomatous adrenals studied. In the aldosteronomas expressing the angiotensinogen gene, the levels of mRNA were very variable, ranging from levels of approximately 50% of

TABLE 1. Individual Extraction and Superfusion Results Compared With Renin and Angiotensinogen Messenger RNA Levels of Six Fully Studied Surgical Specimens

Patient No.	Tissue	Aldosterone in superfusate (ng/100 mg/ 270 min)	Extracted tissue active renin (pg/100 mg)	Active renin in superfusate (pg/100 mg/ 270 min)	Extracted tissue Ang II/III (pg/100 mg)	Ang II/III in superfusate (pg/100 mg/ 270 min)	Renin mRNA	Angiotensinogen mRNA
1	Adrenal	94	146	308	212	1,174	++	+++
2	Adrenal	48	88	344	182	856	+	+++
3	Adrenal	10	122	414	216	490	+	+++
4	Adrenal	120	72	164	240	1,008	++	++++
2	Aldosteronoma	114	62	322	140	310	+	++
3	Aldosteronoma	346	82	102	82	586	++	0/+

Each patient's number corresponds to the number used in Figure 1 for the same patient. Relative messenger RNA (mRNA) abundance was obtained from comparison of densitometric readings after hybridization with renin or angiotensinogen probes. Relative renin mRNA abundances cannot be compared with relative angiotensinogen mRNA abundances because of lengths, melting temperatures, and specific activities of the two probes were different, and the renin and angiotensinogen probes were not used at the same time in the same hybridization buffer. Ang, angiotensin.

those detected in kidney down to barely detectable levels.

Renin mRNA of approximately 1.6 kb was detected in total RNA extracted from samples of human kidney but not in liver or lung (Figure 1). A mRNA of similar size to renal renin mRNA was also present at low levels in three normal adrenals and in three adenomas (Figure 1). Among these adrenal samples, the highest renin mRNA levels (approximately eightfold lower than kidney) were found in an aldosteronoma (Figure 1, last lane).

The gene encoding for ACE was expressed at the highest levels in lung, followed by kidney (Figure 1). In the lung, two messages of approximately 4.7 kb and 5.5 kb were expressed, whereas only the approximately 4.7 kb mRNA was expressed at lower levels in most adrenal samples (Figure 1).

During superfusion, each of the six normal adrenal samples and each of the five aldosteronoma samples analyzed spontaneously released active renin, Ang II/III, and aldosterone. Cumulative amounts of active renin released by normal adrenals during 270 minutes of superfusion were significantly higher than the amounts released by aldosteronomas (312±35 versus 187±43 pg/100 mg tissue, respectively, $p<0.05$). Similarly, the total amounts of released Ang II/III were higher in the superfusate from normal adrenals in comparison with aldosteronomas (823±100 versus 436±55 pg/100 mg tissue, respectively, $p<0.05$). On the contrary, total released aldosterone was higher in aldosteronomas than in normal adrenal tissues (320±21 versus 115±18 ng/100 mg tissue/270 min, respectively, $p<0.01$). Total amounts of renin and Ang II/III extracted by either normal glands (106.3±10.7 and 222.6±14.4 pg/100 mg tissue, respectively) or aldosteronomas (68.8±5.1 and 122±17.4 pg/100 mg tissue, respectively) were threefold to fourfold lower than total amounts released during superfusion of similar portions of the same surgical specimens. Overall, four normal adrenals and two aldosteronomas were studied both by Northern analysis and by extraction and superfusion for renin and Ang II/III (Table 1). We found a concordance between the expression of the RAS genes and the presence of immunoreactive renin and Ang II/III.

Discussion

Many studies have indicated that a local RAS is present in various extrarenal tissues of experimental animals. Renin and angiotensinogen mRNAs have been found in mouse and rat adrenals, suggesting local synthesis of preprorenin and preangiotensinogen.[6–8]

In the present study, we have found that the angiotensinogen gene is coexpressed with renin and ACE genes in human adrenal tissue (Figure 1). Adrenal angiotensinogen expression in humans has not been reported yet. In the rat, using in situ hybridization, periadrenal brown adipocytes and pericapsular fibroblast-like cells apparently contained most of angiotensinogen mRNA.[9] Contamination of our adrenal samples with pericapsular cells is unlikely but cannot be ruled out. However, we detected angiotensinogen mRNAs also in three aldosteronomas that were nonencapsulated and composed of cells that are similar to normal glomerulosa and fasciculata cells, as also reported by others[28]; thus, it is likely that human adrenocortical cells also can express the angiotensinogen gene.

We have also found the presence of low levels of renin mRNA in three nonadenomatous human adrenals and in three aldosteronomas. In accordance with our results, a recent preliminary report refers to the presence of renin mRNA in human adrenals identified by a mRNA protection assay.[29]

Active renin, measured by a sensitive and specific immunoradiometric assay,[26,27] was found in the extracts of normal and pathological adrenal tissue, as it was found in similar studies done by others.[16] We also found that the amount of active renin extracted from whole normal adrenal gland and the amount released during the superfusion were significantly higher than active renin amounts extracted or released from aldosteronomas. This may be due to reduced synthesis of renin in the adenomas or to the presence of other renin-rich cell types in nonadenomatous adrenal samples (e.g., in the medulla). Although we did not measure either prorenin or preprorenin, the primary translation product of the mature transcripts of the human renin gene, prohormone-to-hormone conversion appears to occur into the adrenal tissue, even though we cannot exclude the possibility that the conversion to active renin occurred during the processing of the tissue samples or during the

superfusion. Continued synthesis and release of renin, rather than a leak from intracellular storage, is also suggested by our finding that total amounts of active renin released during the superfusion exceeded the total amounts present in similar samples frozen after surgery. In accordance with our findings, angiotensinogen and renin gene expression and high concentrations of active renin and Ang I were recently found in a variety of human adrenal samples by others (P. Corvol, personal communication).

We have also found that the ACE gene is expressed in normal adrenals and in aldosteronomas. Very limited observations have been published about ACE gene expression in human tissues,[24] but in mouse tissues, ACE expression in lung and kidney was characterized by two mRNAs of similar size to those we have detected in human lung and kidney.[30] Preliminary indirect evidence for ACE mRNA in human adrenals has already been reported,[31] and binding sites for ACE have been found in normal human adrenal gland.[11] Moreover, we have shown an adrenal Ang II/III production that was at least in part suppressed by the ACE inhibitor quinaprilat,[18] supporting the concept that ACE is synthesized and active in human adrenal tissue.

Immunoreactive Ang II/III was present in the tissue extracts, and, similarly to renin, the total amount of Ang II/III released by adrenal tissue was threefold to fourfold higher than the Ang II/III extracted from similar tissue samples. Thus, our data indicate that human adrenal tissue can synthesize Ang II/III in vitro from local precursors, suggesting the presence of angiotensinogen, active renin, and ACE. Similar findings have been reported by others using rat adrenal explant cultures,[14] and Ang II has been detected in human adrenals.[32] As for renin, normal adrenals contained and released significantly higher amounts of Ang II/III than the aldosteronomas studied. Therefore, it is tempting to speculate that the lower Ang II/III release may be related to the lower renin content of the adenomas. It is also possible that lower active renin and Ang II/III production in the aldosteronomas might be the result of downregulation of the local RAS by aldosterone, like systemic RAS, but we cannot exclude that medullary catecholamines present in normal adrenal samples might stimulate cortical renin and Ang II/III production.

In summary, we found coexpression of renin, angiotensinogen, and ACE genes in at least some human adrenals and aldosteronomas. The presence of adrenal active renin and Ang II/III immunoreactivity and the evidence for their synthesis and release in vitro have also been shown. Our data indicate that a local RAS does exist in human adrenals. Locally formed Ang II/III might play an important role as an autocrine or paracrine regulator of adrenal function, whereas an abnormal activity of the local RAS might be involved in the pathogenesis of abnormal adrenal growth, abnormal mineralocorticoid production, and hypertension.

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