Local Renin-Angiotensin System Is Involved in K⁺-Induced Aldosterone Secretion from Human Adrenocortical NCI-H295 Cells

Urte Hilbers, Jörg Peters, Stefan R. Bornstein, Fernando M.A. Correa, Olaf Jöhren, Juan M. Saavedra, Monika Ehrhart-Bornstein

Abstract—NCI-H295, a human adrenocarcinoma cell line, has been proposed as a model system to define the role of the renin-angiotensin system in the regulation of aldosterone production in humans. Because the precise cellular localization of the components of the renin-angiotensin system in human adrenal cortical cells remains unclear, we investigated their localization in this defined cell system. NCI-H295 cells expressed both angiotensinogen and renin as shown by reverse transcriptase polymerase chain reaction and immunohistochemistry. Human angiotensin-converting enzyme (ACE) was not detectable by immunocytochemistry, ACE binding, or reverse transcriptase polymerase chain reaction. However, 3.5 mmol/L K⁺ stimulated the formation of both angiotensin I and angiotensin II 1.9- and 2.5-fold, respectively, and increased aldosterone release 3.0-fold. The K⁺-induced stimulation of aldosterone release was decreased by captopril and enalaprilat (24% and 26%, respectively) and by the angiotensin type 1 (AT1-) receptor antagonist losartan (28%). Angiotensin II–induced stimulation of aldosterone release was abolished by losartan treatment. Specific [125I]Sar1-angiotensin II binding was detected by receptor autoradiography. The binding of [125I]Sar1-angiotensin II was completely displaced by the AT₁ antagonist losartan but not by the AT₂ receptor ligand PD 123319, confirming the expression of angiotensin II AT₁ receptors in NCI-H295 cells. Our results demonstrate that NCI-H295 cells express most of the components of the renin-angiotensin system. Our failure to detect ACE, however, suggests that the production of angiotensin II in NCI-H295 cells may be ACE independent. NCI-H295 cells are able to produce angiotensin II, and K⁺ increases aldosterone secretion in part through an angiotensin-mediated pathway. The production of angiotensin II in NCI-H295 cells demonstrates that this human cell line can be useful to characterize the role of locally produced angiotensin II in the regulation of aldosterone release. (Hypertension. 1999;33:1025-1030.)

Key Words: NCI-H295 cell line ■ renin-angiotensin system ■ angiotensin II ■ aldosterone ■ potassium

The renin-angiotensin-system (RAS) plays an important role in the regulation of blood pressure as well as sodium and volume homeostasis. In addition to the circulating RAS, local RAS have been documented in a number of animal and human tissues including the adrenal gland (for review, see Reference 2). In the adrenal cortex, the role of the local RAS has not been fully characterized. The human adrenal zona glomerulosa expresses all the components of the RAS, including angiotensinogen (AOG), renin, and angiotensin-converting enzyme (ACE) as well as the prohormone convertase PC5 mRNA. Locally produced angiotensin II (Ang II) has been proposed to exercise an autocrine/paracrine control of aldosterone secretion. In many tissues, the various components of the RAS are found in different cells; and it is not clear whether the components of the adrenocortical RAS are produced by different cells or if one cell contains all components. Precise data on the localization and regulation of the RAS components are a requirement for the investigation and interpretation of this system. The examination of an isolated adrenal RAS is especially important to differentiate between the effects caused by circulating and local adrenal systems.

The human adrenocortical carcinoma cell line NCI-H295 is a widely accepted model for human adrenocortical studies. This cell line, originally cultured from a human adrenocortical tumor in 1980, represents the first cell line to maintain the ability to produce all adrenocortical steroids, expressing the 3 major pathways of adrenal steroidogenesis including the main steroidogenic enzymes. As in normal human adrenocortical cells, synthesis of aldosterone is regulated by Ang II through AT₁ receptors and potassium. These cells therefore may be a suitable system for the characterization of a local RAS.
We asked the questions whether the complete RAS system was coexpressed in NCI-H295 cells, whether these cells could produce Ang II after stimulation, whether the increase in aldosterone release after Ang II could be modified by inhibition of Ang II synthesis or blockade of AT₁ receptors, and whether K⁺-stimulated aldosterone release was dependent on Ang II.

**Methods**

**Cell Culture**

NCI-H295 cells were grown at 37°C in a 5% CO₂ humidified atmosphere in RPMI 1640 (Gibco BRL) containing hydrocortisone (3.625 µg/mL), insulin (5 µg/mL), transferrin (100 µg/mL), estradiol (2.724 µg/mL), selenite (5 µg/L), 2% fetal calf serum, and antibiotics. For immunohistochemistry, adherent cells were selected and grown on culture slides for 48 hours in the above medium and for 24 hours in serum-free medium containing ascorbic acid (10⁻² mol/L), transferrin (100 µg/mL), BSA (0.01% wt/vol), and bacitracin (0.01% wt/vol). Viability of cells was 90% by trypan blue exclusion test.

**RNA Extraction and Reverse Transcriptase Polymerase Chain Reaction**

Total RNA was isolated from 5×10⁶ NCI-H295 cells (RNAeasy Kit, treated with DNase I (Boehringer Mannheim GmbH) and screened for contamination with genomic DNA in a control polymerase chain reaction (PCR) for GAPDH. cDNA was synthesized (First Strand cDNA synthesis kit, Boehringer Mannheim) and amplified with the use of specific primers for GAPDH (as a control for reverse transcriptase [RT]), AOG, and renin (Table). No genomic DNA was amplified when using nontranscribed mRNA. In all reactions, GAPDH, AOG, and renin (rabbit anti-AOG, 1:200; Diagnostic International), renin (rabbit antirenin, 1:100; Diagnostic International), and ACE (monoclonal antibody MAB 4057, 1:50 to 1:500; Biozol). As controls, antigen-preincubated antibodies (10-fold excess) were applied. Bound antibodies were detected by the streptavidin-biotin-peroxidase method (Dianova) with AEC (3-amino-9-ethylcarbazole) as described previously. No staining was observed in control sections in which the antibodies were replaced by rabbit IgG or mouse IgG, respectively.

**Incubation Procedure and Hormone Measurements**

NCI-H295 suspension cells were incubated with secretagogues and/or inhibitors after 24 hours of preincubation in serum-free medium. In a typical set of experiments, 2.5×10⁶ cells/750 µL were incubated with 3.5 mmol/L or 9 mmol/L K⁺ with or without the simultaneous incubation with the ACE inhibitors (ACEIs) captopril or enalaprilat (Merck Sharp & Dohme) or the Ang II type 1 (AT₁) receptor antagonist losartan (Merck Sharp & Dohme). In another set, cells were incubated with Ang II (10⁻⁸ to 10⁻⁷ mol/L) and losartan (10 µmol/L).

Aldosterone concentrations in the medium were measured by radioimmunoassay (Immunotech). For determination of Ang I and Ang II, refer to Reference. Additions of 3.5 mmol/L or 9 mmol/L K⁺ to the medium did not influence either assay.

**ACE Activity**

ACE activity was determined in 10⁶ cells/assay from different cultivation days. Cells were washed twice with PBS, centrifuged, and the pellet resuspended in 0.1 mol/L TES, pH 7.2, containing 0.3% Triton X100. Fifty microliters of the sample (or buffer as reference) was incubated at 37°C for 1 hour with 400 µL phosphate buffer, pH 8.0, and 50 µL substrate (10 mmol/L Z-Phe-His-Leu; Bachem) with or without 10 µmol/L captopril or 10 µmol/L enalaprilat. The reaction was terminated by removing 100 µL and addition to 1 mL 0.1N NaOH at ambient temperature. Subsequently, complex formation between L-His-L-Leu and the reagent was measured, using 100 µL L-His-L-Leu (Bachem) as standard. Twenty-five microliters of orthophthalaldehyde was added, and the mixture was incubated for 30 minutes in the dark. Reaction was terminated by addition of 0.8N HCL (1 mL), and after centrifugation (3000 rpm, 5 minutes), fluorescence was monitored for 1 hour (fluorescence spectrometer RF-1502, Shimadzu).

**ACE Binding**

NCI-H295 cells were washed twice with PBS and 10⁶ cells were pelleted by centrifugation (573g, 4°C for 10 minutes). Cryostat sections (16 µm) of these pellets and human adrenals were cut at −20°C. Binding of [¹²⁵I] 351A to ACE was performed as described.

### Design RT-PCR of GAPDH, AOG, Renin, and ACE Specific Primer Pairs for RT-PCR

<table>
<thead>
<tr>
<th>Primer</th>
<th>Accession No. (EMBL)</th>
<th>Primer sequence (F: forward; R: reverse)</th>
<th>Product, bp</th>
<th>Cycles, n</th>
<th>Ta, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>F M33197 (mRNA)</td>
<td>5'-GCAGGGGGGAGCCAAAGGG-3'</td>
<td>353</td>
<td>35</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>R M33197 (mRNA)</td>
<td>5'-TCAGGGGGGAGCCAAAGGG-3'</td>
<td>356</td>
<td>26</td>
<td>64</td>
</tr>
<tr>
<td>AOG</td>
<td>F AC 24668, exon 2</td>
<td>5'-CACACTGAGAAGGGGCTTTG-3'</td>
<td>320</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>R AC 24667, exon 3</td>
<td>5'-TGTCATCCAGTGGGAAGG-3'</td>
<td>306</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td>Renin</td>
<td>F AC X 0 1696, exon 3</td>
<td>5'-CAATGGAGGACCGCTCTCTAACA-3'</td>
<td>320</td>
<td>35</td>
<td>65</td>
</tr>
<tr>
<td></td>
<td>R AC X 0 1696, exon 5</td>
<td>5'-ACCCCTTGGAGAGATGATGTT-3'</td>
<td>353</td>
<td>35</td>
<td>70</td>
</tr>
</tbody>
</table>

bp indicates base pairs; Ta, annealing temperature.
earlier. Compound 351A is the p-hydroxybenzamidine derivative of MK 521 (lisinopril), which in turn is the Lys-Pro analogue of MK 422, the active diacid from of the specific inhibitor enalapril (MK421). It was iodinated by DuPont New England Nuclear to a specific activity of 2000 Ci/mmol.

Optical densities in the autoradiograms were measured by computerized microdensitometry with the use of the NIH Image 1.61 analysis system. [125 I] 351A concentrations were quantified by comparison with [125 I]-labeled microscales and transformed to corresponding values of femtomoles per milligram of protein. Data were analyzed with the use of GraphPad Prism Software.

**Ang II Receptor Binding**

Sar1-Ang II was iodinated by New England Nuclear to a specific activity of 2200 Ci/mmol. Competition studies were conducted by incubating consecutive sections of NCI-H295 cell pellets with [125 I]Sar1-Ang II and increasing concentrations of Ang II (10^{-11} to 10^{-5} mol/L; Peninsula) or selective ligands for AT1 (losartan, 10^{-11} to 10^{-5} mol/L; Parke-Davis) or AT2 (PD 123319, 10^{-11} to 10^{-5} mol/L; Parke-Davis) as described earlier. Quantitative autoradiography was performed as described for ACE binding.

**3 H-Thymidine Incorporation Assay**

Cells were plated on 96-well dishes (10^5 cells/well) and incubated in serum-free medium for 24 hours in the presence of either 3.5 mmol/L or 9 mmol/L K^+ with or without the addition of captopril or enalaprilat, the AT1 receptor antagonist losartan, or with the inhibitors alone. To assess proliferation, cells were incubated with 2.5 mCi/mL 3 H-thymidine (Peninsula) for 24 hours, removed from the culture plate by repeated pipetting, and 3 H-thymidine incorporation was assayed.

**Statistical Analysis**

Results are expressed as mean±SEM, and statistical significance was determined by ANOVA with the software package SPSS for Windows, version 6. Differences were considered significant at P<0.05. All experiments were repeated for a minimum of 3 different cell passages with 4 wells per experiment.

**Results**

The present study provides proof for a complete functional RAS in the human adrenocortical cell line NCI-H295. RT-PCR for AOG mRNA and renin mRNA yielded the expected cDNA fragments of 320 bp for AOG (Figure 1A) and 306 bp for renin (Figure 1B). Enzymatic digestion of the PCR product for AOG with HaeIII/TaiI led to 2 fragments with the correct sizes of 230 bp/90 bp (HaeIII) and 190/130 bp (TaiI), and digestion of the PCR product for renin with the use of GsuI resulted in the expected pattern of 262 bp/44 bp. Identity of PCR products was also confirmed by subsequent sequencing (data not shown). No expression of ACE mRNA was observed in NCI-H295 cells (Figure 1C).

Immunohistochemical staining for AOG (Figure 2A) and renin (Figure 2C) revealed ~98% positive NCI-H295 cells. No staining (AOG, B) and nearly complete suppression (renin, D) were observed when using an antibody preincubated with specific antigen (10-fold excess).

In response to 12 hours of incubation with 3.5 mmol/L and 9 mmol/L K^+, the aldosterone secretion in NCI-H295 cells increased 3-fold (P<0.001) and 4.4-fold (P<0.001), respectively (Figure 3). This K^+-induced stimulation was reduced by addition of both ACE inhibitors. Enalaprilat (10 μmol/L) reduced aldosterone secretion after 3.5 mmol/L K^+ by 25.5% (P<0.001) and after 9 mmol/L K^+ by 12.8% (P<0.05). Captopril (10 μmol/L) reduced the effects of K^+ (3.5 mmol/L
and 9 mmol/L) by 23.5% ($P<0.05$) and by 12.1% ($P<0.05$), respectively (Figure 3).

The AT$_1$ receptor antagonist losartan (10 $\mu$mol/L) significantly inhibited K$^+$-stimulated aldosterone secretion. Coincubation with 3.5 mmol/L K$^+$ reduced aldosterone secretion by 27.6% ($P<0.001$) and aldosterone secretion induced by 9 mmol/L K$^+$ by 24.8% ($P<0.001$) (Figure 3).

Incubation with 3.5 mmol/L K$^+$ and 9 mmol/L K$^+$ stimulated the formation of Ang I and Ang II by NCI H295 cells. Addition of 3.5 mmol/L K$^+$ for 12 hours resulted in a 2.0-fold increase in Ang I ($P<0.05$) and a 4.9-fold increase in Ang II ($P<0.001$) (Figure 3).

ACE-like activity was measured in membrane preparations of NCI-H295 cells (14.40$\pm$2.51 ng L-His-L-Leu/min per milligram of membranes). This activity could be suppressed completely by addition of 10 $\mu$mol/L captopril and reduced by 60% ($P<0.01$) by addition of 10 $\mu$mol/L enalaprilat to the incubation mixture (Figure 6). Binding of $^{[125]}$I$^{351}$A to ACE was not found in the NCI-H295 cells, whereas human adrenal glands were found to bind specifically $^{[125]}$I$^{351}$A.

Specific $^{[125]}$I$^{351}$A binding was detected by receptor autoradiography in pellet sections of NCI-H295 cells. The binding of $^{[125]}$I$^{351}$A-Ang II was completely displaced by the AT$_1$ antagonist losartan (Figure 7A) but not by the AT$_2$ receptor ligand PD 123319 (Figure 7A), confirming the
expression of Ang II AT$_1$ receptors in NCI-H295 cells. Competition experiments revealed that $[125]$I Sar$^1$-Ang II binding was displaced by Ang II and losartan with an $IC_{50}$ of $7.2 \times 10^{-7}$ mol/L and $4.9 \times 10^{-7}$ mol/L, respectively (Figure 7B).

**Discussion**

The present study demonstrates that renin and AOG coexist in the human adrenocortical carcinoma cell line NCI-H295 and that these cells, on $K^+$ stimulation, produce physiologically active Ang II, resulting in a modulation of aldosterone secretion. The presence of AOG and renin mRNA in both normal and pathological human adrenal tissue has been demonstrated in normal and abnormal human glomerulosa, fasciculata, and medullary cells. We have demonstrated that both AOG and renin mRNA are expressed in NCI-H295 cells. Both AOG and renin were produced by the cells themselves rather than taken up from the medium, since cultivation in serum-free medium did not decrease immunostaining.

The last step in Ang II formation is the conversion of Ang I to Ang II, which, in the classic RAS, requires ACE. Indeed, NCI-H295 cells were able to increase the formation of Ang II on $K^+$ stimulation (see below) and expressed ACE activity that could be inhibited by the ACEIs captopril and enalaprilat. However, neither ACE mRNA, immunoreactive ACE, or $[125]$I$351$A binding to ACE could be detected in NCI-H295 cells. ACE binding has been previously demonstrated in human zona glomerulosa cells, and we detected $[125]$I$351$A binding to sections from human adrenals in our experiments. We therefore can postulate that in NCI-H295, a peptidase other than ACE is active in the conversion of Ang I to Ang II. Such peptidase is recognized by captopril and enalaprilat but not by the anti-ACE antibody or the $[125]$I$351$A ligand and has a nucleotide sequence with a low homology to that of ACE. The existence of non-ACE pathways catalyzing Ang I to Ang II has been reported in different tissues. Non-ACE kinases that could be inhibited by ACE inhibitors include enzymes that are related to, or isomers of, ACE or aminopeptidase P, or those that are related to endopeptidase 24.15.24 Therefore, the absence of ACE binding in the NCI-H295 cells with a concurrent decrease of aldosterone release by ACEIs suggests a non-ACE activity. The inhibition of ACE activity by enalaprilat or captopril despite absence of $[125]$I$351$A binding to the cells probably is due to higher selectivity of lisinopril to ACE. It remains to be elucidated if this ACE-like activity is expressed by normal human adrenocortical cells.

Our results indicate that the NCI-H295 RAS could be activated by $K^+$, resulting in increased formation of Ang II and its precursor Ang I, an effect that was maximum at 3.5 mmol/L $K^+$, in accordance with data from rat adrenal. At this concentration, Ang II concentrations were enhanced 5 times over basal levels. We also show that Ang II–induced aldosterone release could be totally blocked by losartan, indicating that Ang II is physiologically active in NCI-H295 cells by stimulation of an AT$_1$ receptor, as it has been previously demonstrated in this cell line and in human and rat adrenocortical cells. The expression of AT$_1$ receptors and not AT$_2$ receptors could be confirmed by binding studies.

Potassium stimulated the secretion of aldosterone in NCI-H295 cells, an effect partially inhibited (by 20% to 28%) by captopril, enalaprilat, and losartan. This indicated an involvement of the local RAS, and of locally formed Ang II, as one of the mechanisms activated by $K^+$ in the modulation of aldosterone release. It is well known that potassium is a potent regulator of aldosterone secretion by opening plasma membrane Ca$^{2+}$ channels in adrenal zona glomerulosa cells and thus increasing intracellular Ca$^{2+}$. This Ca$^{2+}$ signaling pathway also is involved in $K^+$–induced stimulation of steriodogenesis in NCI-H295 cells. Therefore, $K^+$ stimulates aldosterone directly by an increase in intracellular Ca$^{2+}$ and, to a lesser extent, indirectly by the formation of Ang II. A contribution of the adrenal RAS in $K^+$–induced aldosterone release has been suggested from animal models such as rat adrenocortical preparations and bovine adrenocortical cells in primary culture.

Our observation that Ang II is produced by the steroid-producing cells themselves supports the hypothesis that Ang II regulates the secretion of aldosterone in an autocrine/paracrine manner. This is in agreement with data from other experimental systems, for example, superfused rat glomerulosa tissue and dispersed cells, in which the secretion of aldosterone and Ang II were highly significantly correlated. In addition to these acute effects, the local RAS may be required to maintain adrenocortical cells in an appropriate functional state. In the hypertensive transgenic (TGR mRen-2)277 rats that are transfected with the Ren-2 mouse renin gene, high concentrations of adrenal renin exist, not only in the glomerulosa but also in inner adrenocortical zones. In these animals, aldosterone production was also found in the inner adrenocortical zones, indicating that one role of the
adrenal RAS may be to support aldosterone synthase expression.34,35

In conclusion, our experiments provide evidence that a local RAS exists in the human adrenocortical NCI-H295 cell line. These cells express AOG, renin, Ang I and II, and AT1 receptors. The conversion of Ang I to Ang II in NCI-H295 cells is catalyzed by a peptidase different from human ACE. The identity of this enzyme remains to be elucidated. Both Ang II and aldosterone production can be triggered by a potassium challenge, and blockade of Ang II formation or Ang II AT1 receptors significantly decreases potassium-stimulated aldosterone release. Therefore this RAS fulfills all criteria of a functional autocrine system under the control of physiological stimuli. These cells are the first human model used to study the function of adrenal RAS and its involvement in the regulation of adrenocortical steroidogenesis. Although these cells are tumor cells that might differ from normal adrenal cells in some respects, the NCI-H295 cell line appears to be a highly suitable and attractive model system toward a better understanding of the regulation of local RAS in human cells and ultimately its implication on hypertension.

Acknowledgments
This study was supported by the Deutsche Forschungsgemeinschaft (grants Bo1141/2-3 to S.R.B and PE 366/3-2 to J.P.) and the Wilhelm Sander Stiftung (grant 95.033.1 to M.E.B.). We thank Merck Sharp & Dohme for providing losartan. In addition, we thank Silke Brauer, Sandy Laue, and Jutta Zimmer for their excellent technical assistance and the National Institutes of Health Scientific Computer Resource Center for image processing.

References
Local Renin-Angiotensin System Is Involved in K⁺-Induced Aldosterone Secretion from Human Adrenocortical NCI-H295 Cells
Urte Hilbers, Jörg Peters, Stefan R. Bornstein, Fernando M. A. Correa, Olaf Jöhren, Juan M. Saavedra and Monika Ehrhart-Bornstein

Hypertension. 1999;33:1025-1030
doi: 10.1161/01.HYP.33.4.1025

Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
Copyright © 1999 American Heart Association, Inc. All rights reserved.
Print ISSN: 0194-911X. Online ISSN: 1524-4563

The online version of this article, along with updated information and services, is located on the World Wide Web at:
http://hyper.ahajournals.org/content/33/4/1025