Our previous studies indicated that the amount of renin present in cultured adrenal zona glomerulosa cells increased after stimulation with adrenocorticotropic hormone or potassium. In the present study, we investigated the effects of adrenocorticotropic hormone or potassium on renin gene expression in cultured rat adrenal zona glomerulosa cells. The amount of rat renin messenger RNA (mRNA) was measured by complementary DNA synthesis and the competitive polymerase chain reaction method. The effects of adrenocorticotropic hormone or potassium on adrenal zona glomerulosa cell renin activity and renin mRNA content were compared with the activity and content of control cells. After 1 and 4 hours of stimulation by adrenocorticotropic hormone or potassium, total renin in the medium increased slightly; at the same time, the percent change in the amount of renin mRNA was 281% and 291%, respectively, in the adrenocorticotropic hormone-stimulated group and 218% and 348%, respectively, in the potassium-stimulated group. Twenty-four hours after adrenocorticotropic hormone or potassium stimulation, total renin in the medium increased significantly, by 689% and 220%, respectively; percent change in the renin mRNA content was 754% and 278%, respectively. These results demonstrate that adrenocorticotropic hormone and potassium increased the activity of adrenal renin through an increase in the level of renin mRNA.

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KEY WORDS • adrenal glands • polymerase chain reaction, competitive • adrenocorticotropic hormone • potassium • zona glomerulosa • renin • RNA, messenger

The renin-angiotensin system plays an important role in the control of blood pressure and volume/ electrolyte balance. The main source of renin is the juxtaglomerular cells of the kidney. Renal renin is synthesized as preprorenin, which is converted to prorenin and then to active renin by proteolytic cleavage.1 After release into the circulation, renin catalyzes the first step in the formation of angiotensin II. Studies using renin assay of tissue extracts have detected reninlike activity in many extrarenal tissues, including the submaxillary gland,5 uterus, placenta,3 brain,4 pituitary,5 testis,7 and adrenal gland.8 In the adrenal gland, renin has been found in various species, including rabbits, mice, rats, dogs, cattle, and humans.9–13 The physiological function of adrenal renin is not completely understood. Previous studies indicated that the renin activity increases in rat adrenal zona glomerulosa (ZG) tissue after a low sodium or high potassium diet or after nephrectomy, and there is a positive correlation between the adrenal renin and adrenal aldosterone concentrations,14,15 suggesting that the local renin-angiotensin system may play a role in the control of aldosterone production.

Although most circulating renin is of renal origin,16 some extrarenal tissues also express the renin gene.17–19 Renin messenger RNA (mRNA) has been detected in the adrenal glands of the rat18–21 and mouse.22 In the rat, renin mRNA is identified mainly in adrenal ZG tissue and little is found in the zona fasciculata/medullary tissue, as measured by in situ hybridization and RNA dot-blot hybridization.20,21 More recently Ekker and coworkers23 studied transcription of the renin genes in multiple extrarenal tissues by using the polymerase chain reaction (PCR) method and found renin transcripts in the adrenal glands, testes, and ovaries as well as in the liver, brain, hypothalamus, spleen, thymus, lung, and prostate. A previous study in our laboratory indicated that renin mRNA is located mainly in the adrenal ZG cells,16 and the amount of renin mRNA increased in the adrenal ZG tissue from sodium-depleted rats.21 These studies present important evidence indicating that renin is synthesized in the adrenal gland, rather than taken up from the circulation.

In adrenal ZG cell monolayer cultures, adrenocorticotropic hormone (ACTH) and potassium stimulate production of renin as well as aldosterone.24 The renin could be released from storage granules, synthesized de novo as a preprorenin molecule, or both, processed, and then secreted into the culture medium. To address this question, in the present study the amount of renin mRNA in ACTH- or potassium-stimulated cultured adrenal ZG cells was determined at different times by using complementary DNA (cDNA) synthesis and the competitive PCR method.

Methods

Cell Culture

For each experiment, 10 female Sprague-Dawley rats (Harlan Sprague Dawley, Inc., Indianapolis, Ind.)
Rat renin map with position of PCR primers.

RNA Preparation

Total RNA was isolated from the ZG cells by the RNAzol method (CINNA/BIOTECX Laboratories International Inc., Friendswood, Tex.). Briefly, the cell pellet was lysed in 0.8 ml RNAzol and total RNA was extracted with 80 µl chloroform. The RNA was precipitated with 0.4 ml isopropanol for 3 hours at -20°C and then washed once with 1 ml 75% ethanol. The RNA pellet was dried briefly under vacuum for 5 minutes and dissolved in a diethylpyrocarbonate-treated ribonuclease (RNase)-free solution containing 10 mM Tris HCl/1 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.5).

First Strand cDNA Synthesis

Before PCR amplification, RNA was converted to cDNA by the reverse-transcription reaction.25,26 The reverse-transcription reaction was carried out in a final volume of 20 µl 1× PCR buffer (50 mM KCl, 20 mM Tris HCl, 2.5 mM MgCl₂, and 100 µg/ml nuclelease-free bovine serum albumin) containing 1 mM of each of the four deoxyribonucleotide triphosphates (dNTPs), 1 unit/µl of the RNase inhibitor RNasin (Promega Corp., Madison, Wis.), 100 pmol random hexamer (Pharmacia LKB Biotechnology Inc., Piscataway, N.J.), 100 units MuLV reverse transcriptase (GIBCO-BRL, Gaithersburg, Md.), and the total RNA isolated from 6–8×10⁵ cells. The reaction mixture was incubated for 60 minutes at 42°C. The reaction was stopped by heating the tube in a 95°C water bath for 10 minutes.

Polymerase Chain Reaction Primers and Competitive Template

The PCR primers were designed to span a small intron (intron 7, the smallest intron) of the rat renin gene (Figure 1). The upstream primer (R7E) is located in exon 7, and its sequence is 5′ TGT ATG GCA GTG GTG GAC ACT 3′. The downstream primer (R8E) is located in exon 8, and its sequence is 5′ GTT GCT GAG AGT GTA GGT CCT 3′. The primers were synthesized by a DNA synthesizer (model 391, Applied Biosystems Inc., Foster City, Calif.).

The competitive template (genomic DNA [gDNA]), a fragment of DNA used as an internal standard in the competitive PCR method, was created by PCR amplification of normal rat liver gDNA with primers R7E and R8E. A single 450-bp-long fragment was amplified (data not shown). The amplified DNA was then purified by agarose gel electrophoresis followed by electroelution. The concentration of gDNA was measured by spectrophotometry at wavelengths of 260 nm and 280 nm. Stock solutions of gDNA ranging from 8 fg/µl to 1 fg/µl were prepared by serial dilutions. This makes it possible for us to distinguish amplified cDNA from amplified gDNA by their different sizes after the competitive PCR when using the same pair of primers. Using this pair of primers, PCR amplification will generate a 450-bp fragment from rat renin genomic DNA and a 186-bp fragment from rat renin complementary DNA (cDNA).
amplified from renin cDNA and a 450-bp fragment will be amplified from gDNA.

**Competitive Polymerase Chain Reaction**

Renin cDNA was measured according to the competitive PCR method of Gilliland et al. In this method, a competitive template with known concentrations is co-amplified with the target cDNA by using the same pair of primers, and the competitive template can be distinguished from the target cDNA after amplification by the difference of their sizes. A master mix was prepared in a 1.5-ml Eppendorf tube containing 20 μl of reverse transcriptase reaction mixture as a source of cDNA, 200 μM dNTPs, 0.2 μM each of the R7E and R8E primers, 3.5 units *Thermus aquaticus* (Taq) DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 1× PCR buffer (pH 8.3, Perkin-Elmer Cetus) as a final concentration. The concentration of cDNA was then determined by adding 90 μl master mix into each of seven tubes that contained 80, 40, 20, 10, 5, 1, or 0.5 fg gDNA in 10 μl. Samples were amplified by 35 cycles of PCR in a MicroCycler (Programmable Heating/Cooling System, Eppendorf Inc., Fremont, Calif.): denaturation at 94°C for 1 minute, annealing of primers at 61°C for 1 minute, and extension of primers at 72°C for 1 minute. After amplification, an aliquot of each reaction mixture was subjected to electrophoresis on 1.5% agarose gels. A photograph of the ethidium bromide-stained gel was taken with Polaroid film (type 55, Cambridge, Mass.), and the negative film was used for measurement of the density of the fragments on a Shimadzu Dual-Wave- length Thin-Layer Chromatoscanner (model CS-930, Shimadzu Corp., Kyoto, Japan). The ratio of the density of gDNA versus that of cDNA was plotted against the gDNA concentration after logarithmic transformation. The point at which log(gDNA×0.41)/cDNA equals 0 is where the gDNA concentration equals the cDNA concentration and indicates the concentration of renin mRNA in the cDNA sample. The results are calculated as femtograms renin mRNA per 10⁶ cells and are normalized to percentage increase over the control concentration.

**Radioimmunoassay of Renin Activity**

Because renin in the medium is mainly prorenin, total renin activity in the medium was measured by the trypsin activation method as previously described. Briefly, 200 μl medium (pH 7.4) was incubated with trypsin (0.1 mg/ml sample, type III, Sigma) for 1 hour at 4°C. After the reaction was stopped by lima bean trypsin inhibitor (0.3 mg/ml sample), the medium was incubated for 30 minutes at 37°C, a 50-μl aliquot of the reaction mixture was assayed for generated angiotensin I by using a commercially available kit (New England Nuclear, Boston, Mass.). The results are calculated as picograms angiotensin I per microgram protein per 8 hours and are normalized to percentage change.

**Statistical Analysis**

The concentration of renin mRNA and the renin activity from each control group were considered as 100%, and the concentrations of renin mRNA from the ACTH- and potassium-stimulated groups were calculated as the percentage changes as (concentration in stimulated sample - concentration in control sample x 100%). Then an unpaired, one-tailed Student's *t*-test was used to compare the data from the stimulated group with data from the control group. The results are shown as mean±SEM; *p*<0.05 was considered significant.

**Results**

Figure 2 shows a Polaroid photograph of an ethidium bromide-stained agarose gel. A single concentration of cDNA was coamplified with a series of dilutions of a known concentration of gDNA. As predicted, fragments of size 450 bp from gDNA and of size 186 bp from renin cDNA were coamplified in the same tube (Figure 2). After agarose gel electrophoresis, the amounts of renin cDNA and gDNA were quantified by measuring the density of the two fragments with the chromatoscanner. On a log/log scale, a plot of the ratio of gDNA to cDNA
versus the known concentration of gDNA is linear (Figure 3). The linearity of the log/log plots indicates that the amplification of gDNA is not affected by its small intron; the cDNA and gDNA copies of these genes should be amplified with the same efficiency. The point at which the amounts of gDNA and cDNA are equivalent (i.e., log([gDNA×0.41]/cDNA)=0) is where the concentration of renin cDNA before the PCR is equal to the known starting concentration of gDNA. The amount of gDNA is multiplied by the ratio of the size of cDNA to that of gDNA (that is, 186 bp+450 bp=0.41) to correct for the increased ethidium bromide staining per mole of the larger fragment.

To test the reproducibility of the competitive PCR method, we isolated the total RNA from a rat kidney instead of from the rat adrenal ZG (which has a limited number of cells); the RNA was divided into aliquots, and the entire protocol was repeated in two separate experiments, each containing three groups. The data are shown in Table 1. The results were reproducible within experiments, but not between experiments. For this reason, we always measured renin mRNA in ACTH- or potassium-stimulated cells and control cells in the same experiment. Because of interexperiment variation, the concentration of renin mRNA is expressed as the percentage change.

Figure 3 shows examples of log/log plots of the ratio of gDNA to cDNA versus the known concentrations of gDNA before and after 4 hours of treatment. Figure 3A shows plots of the competitive PCR from control (upper plot) and ACTH-stimulated (lower plot) cells. The concentration of renin cDNA was 15.55 fg in 88,571 control cells (175.59 fg/10^6 cells) and 58.50 fg in 90,000 ACTH-treated cells (649.95 fg/10^6 cells). Figure 3B shows data from chromatoscanner as log([gDNA×0.41]/cDNA) after polymerase chain reaction (PCR) amplification versus log gDNA added to original PCR mixture. To correct for differences in molecular weight, chromatoscanner readings from gDNA fragments were multiplied by 186×450=0.41. Panel A: Plots from control and adrenocorticotropic hormone (ACTH)-stimulated groups at 4 hours. Panel B: Plots from control and potassium-stimulated groups at 4 hours. gDNA, genomic DNA; cDNA, complementary DNA.

**TABLE 1. Measurement of Renin Messenger RNA From Rat Kidney Total RNA by Complementary DNA Synthesis and by Competitive Polymerase Chain Reaction Method**

<table>
<thead>
<tr>
<th>Group</th>
<th>Experiment 1 (fg mRNA per 2 μg total RNA)</th>
<th>R</th>
<th>Experiment 2 (fg mRNA per 2 μg total RNA)</th>
<th>R</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(gDNA×0.41)/cDNA=0)</td>
<td></td>
<td>(gDNA×0.41)/cDNA=0)</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.026</td>
<td>0.96</td>
<td>2.024</td>
<td>0.97</td>
</tr>
<tr>
<td>2</td>
<td>1.161</td>
<td>0.97</td>
<td>2.878</td>
<td>0.88</td>
</tr>
<tr>
<td>3</td>
<td>0.806</td>
<td>0.99</td>
<td>1.571</td>
<td>0.96</td>
</tr>
<tr>
<td>Mean</td>
<td>0.998</td>
<td></td>
<td>2.158</td>
<td></td>
</tr>
<tr>
<td>SD</td>
<td>0.179</td>
<td></td>
<td>0.664</td>
<td></td>
</tr>
<tr>
<td>CV</td>
<td>17.961</td>
<td></td>
<td>30.759</td>
<td></td>
</tr>
</tbody>
</table>

mRNA, messenger RNA; R, correlation factor of log (genomic DNA×0.41)/complementary DNA versus log(gDNA); SD, standard deviation; CV, coefficient of variation.
TABLE 2. Measurement of Renin Messenger RNA From Rat Adrenal Zona Glomerulosa Cell Total RNA by Complementary DNA Synthesis and Competitive Polymerase Chain Reaction Method

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Renin mRNA/10^6 ZG cells (fg)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1 hour</td>
</tr>
<tr>
<td>Control</td>
<td>308.53</td>
</tr>
<tr>
<td>Adrenocorticotropic hormone</td>
<td>868.05</td>
</tr>
<tr>
<td>n</td>
<td>2</td>
</tr>
<tr>
<td>Control</td>
<td>43.27</td>
</tr>
<tr>
<td>Potassium</td>
<td>94.69</td>
</tr>
<tr>
<td>n</td>
<td>2</td>
</tr>
</tbody>
</table>

mRNA, messenger RNA; ZG, zona glomerulosa. Values are mean of n experiments.

shows plots from control and potassium-stimulated cells. The concentration of renin cDNA was 6.04 fg in 104,286 control cells (57.92 fg/10^6 cells) and 16.03 fg in 101,429 potassium-treated cells (158.04 fg/10^6 cells). Table 2 shows the mean concentrations of renin mRNA.

We also measured the renin mRNA and total renin levels at different times (1, 4, and 24 hours) after ACTH or potassium stimulation (Figure 4). Figure 4A shows the results for renin mRNA. After ACTH stimulation, the renin mRNA level increased by 281%, 291%, and 754±367% at 1, 4, and 24 hours, respectively. In the potassium-stimulated group, the renin mRNA level increased by 218%, 348%, and 278±185% at 1, 4, and 24 hours, respectively. The pattern of increase in renin mRNA in the ACTH-stimulated group appeared to differ from that in the potassium-stimulated group. The renin mRNA level started to increase 1 hour after ACTH stimulation and reached the highest level at 24 hours. In the potassium-stimulated group, the renin mRNA level started to increase at 1 hour, reached the highest level at 4 hours, and returned to near control levels at 24 hours. Total renin activity also increased after stimulation with ACTH or potassium (Figure 4B). Although total renin activity was detectable 1 and 4 hours after stimulation, the enzyme activity was significantly increased only after 24 hours of stimulation (689±374% in the ACTH-stimulated group and 220±53% in the potassium-stimulated group).

Discussion

Our previous studies indicated that the activity of renin present in adrenal ZG cells increases after stimulation with ACTH or potassium. However, the presence of renin in a tissue does not demonstrate synthesis of the protein in the tissue. Dzau et al studied sequential changes in renin secretion-synthesis coupling in response to acute β-adrenergic receptor stimulation and found that an immediate response to acute β-adrenergic receptor stimulation was the release of renin from storage. In our study, a better indication that renin production is actually stimulated by ACTH and potassium is the demonstration of an increase in the amount of renin mRNA in ZG cells after treatment with these agents. The increase in renin mRNA could be the result of increased transcription or increased stability of the messenger. Although it is possible to study regulation of the adrenal renin gene at the transcriptional level by detecting mRNA by DNA/RNA hybridization, these methods require large amounts of adrenal total RNA and are not sensitive enough to detect mRNA from a small cell population, particularly when there are both a few cells and only a few mRNA copies in each cell. Other studies indicate that rat adrenal renin mRNA is expressed at a relatively lower level than rat kidney renin mRNA. The recently developed competitive PCR method is very powerful and sensitive and enabled us to measure the renin mRNA content in cultured rat ZG cells (10^6 cells per reaction). To find out the mechanism by which ACTH and potassium increased the adrenal renin activity, we measured the amount of renin mRNA by using cDNA synthesis and the competitive PCR method and the renin activity level by using an enzyme assay. The renin mRNA content from the ACTH-stimulated group started to increase 1 and 4 hours after stimulation and reached the highest level at 24 hours. In the potassium-stimulated group, the mRNA level started to increase 1 hour after stimula-
tion, reached the highest level at 4 hours, and decreased (almost to baseline) at 24 hours. This implies that ACTH has a more prolonged effect on renin mRNA expression than potassium. The renin activity increased nonsignificantly at 1 and 4 hours after stimulation, whereas at 24 hours the total renin activity increased significantly. These studies suggest a proportional change between ACTH-stimulated (not potassium-stimulated) ZG cell renin protein and its mRNA. Potassium may have a very brief effect on renin gene expression in rat ZG cells. The finding of mRNA in a tissue indicates expression of the gene in that tissue, and the presence of increased renin activity in the same cells indicates that the renin mRNA is being translated as well. We found that the concentration of renin mRNA and the activity of its product increased in the same cell culture after ACTH and potassium stimulation.

The PCR method is novel and very sensitive for detecting small amounts of mRNA in tissues. By using this method, Ekker et al. were the first to study the transcription of renin genes in extrarenal tissues. These researchers found renin transcripts in the adrenal glands, testes, and ovaries and lower levels of transcripts in liver, brain, hypothalamus, spleen, thymus, lung, and prostate. In the present study, we measured renin mRNA in cultured monolayer adrenal ZG cells by using the competitive PCR method, which enabled us to detect as well as measure renin mRNA. In the competitive PCR method, the internal control is gDNA, and it is assumed that all renin mRNA has been reverse-transcribed. The efficiency of reverse transcription is not 100% and may vary from experiment to experiment. This may lead to underestimation of the actual amount of renin mRNA present in ZG cells. Also, this may explain the interexperiment and intraexperiment variability.

In summary, we found an early increase in the renin mRNA content in rat adrenal ZG cells after stimulation with ACTH or potassium. This increase in renin mRNA correlates with an increase in the medium renin activity. The data, therefore, suggest that ACTH and potassium increase adrenal renin production by regulation of renin gene expression in rat adrenal ZG cells. Further studies need to be done to investigate whether the increase in the amount of renin mRNA is due to an increase in the transcriptional rate of the renin gene or an increase in the stability of the mRNA.

Acknowledgments

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