Aldosterone Induces Angiotensin-Converting-Enzyme Gene Expression in Cultured Neonatal Rat Cardiocytes

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Background—The cardiac renin-angiotensin-aldosterone system is activated in failing hearts in proportion to the severity of the disease. We hypothesized that a positive feedback mechanism might exist within this system and contribute to the progression of the heart failure.

Methods and Results—To test this hypothesis, we examined whether angiotensin II or aldosterone induces the expression of angiotensin-converting-enzyme (ACE) mRNA in cultured neonatal rat ventricular cardiocytes. Expression of ACE mRNA was detected and quantified using real-time reverse transcription–polymerase chain reaction. Exposure to angiotensin II (10^{-5} mol/L) for 24 hours had no significant effect on the expression of ACE mRNA (0.7 ± 0.5-fold versus control, P = NS), but similar treatment with aldosterone (10^{-5} mol/L) induced a 23.3 ± 7.9-fold increase (P < 0.01) in ACE mRNA expression. The effect of aldosterone was both time- (maximal effect, 24 hours) and dose-dependent (EC_{50}, 4 × 10^{-7} mol/L), and it was significantly (P < 0.01) inhibited by spironolactone, a specific mineralocorticoid receptor antagonist.

Conclusions—Aldosterone upregulates ACE mRNA expression, which is blocked by spironolactone in neonatal rat cardiocytes. Thus, spironolactone may suppress the progression of heart failure by blocking the effects of aldosterone and angiotensin II. (Circulation. 2001;104:137-139.)

Key Words: angiotensin ■ aldosterone ■ polymerase chain reaction ■ cells

Angiotensin II is formed in the circulation, but recent evidence indicates that angiotensin II is also synthesized locally in various tissues, including the heart and blood vessels, where it takes part in an autocrine/paracrine system.\(^1\,^2\) We and others have demonstrated that cardiac angiotensin-converting enzyme (ACE) activity and gene expression are increased in the failing hearts of humans and animals.\(^3\,^\ldots\,^5\)

Aldosterone, which is a component of the renin-angiotensin-aldosterone system (RAAS), promotes the retention of sodium and the loss of potassium, activates the sympathetic nervous system, stimulates myocardial and vascular fibrosis, and causes baroreceptor dysfunction.\(^6\,^\ldots\,^8\) Although previously thought to be synthesized solely in the adrenal cortex, recent animal studies have shown that aldosterone is also produced in such extra-adrenal tissues as the heart and blood vessels,\(^9\,^\ldots\,^11\) and we have reported that aldosterone is produced in the failing human heart.\(^12\)

The mechanisms by which the cardiac RAAS is activated in the hypertrophied or failing heart are not completely understood. It is known that in heart failure, RAAS activity is upregulated in proportion to the severity of the condition. One explanation for this would be the existence of a positive feedback mechanism within the cardiac RAAS that contributes to the progression of heart failure. The present study was designed to examine whether aldosterone or angiotensin II affects the expression of ACE mRNA in cultured cardiocytes. Cardiocyte culture is a well-established model for studying cardiac hypertrophy,\(^13\) and cardiocyte cultures including non-myocytes provide a more physiological condition for testing.\(^14\,^\ldots\,^15\) The effects of angiotensin II and aldosterone on ACE mRNA expression in cultured cardiocytes, including non-myocytes, were analyzed using real-time reverse transcription–polymerase chain reaction (RT-PCR), a rapid and highly sensitive method of detecting gene expression.\(^16\)

Methods

Cell Cultures
The investigation conforms to the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of
Health. We prepared neonatal rat cardiocyte cultures, consisting of myocytes and nonmyocytes in their native proportion, as previously described.\textsuperscript{15} Prepared cardiocytes were plated at a density of 1.0×10\textsuperscript{4} cells/cm\textsuperscript{2} in gelatin-coated 6-well plates (Becton Dickinson).

The cardiocytes were cultured for 30 hours in 10% FCS (Hazleton Biologies)–containing medium and then transferred to serum-free medium containing 0.1% BSA (Sigma) for an additional 10 hours. After this preconditioning period, which was used to examine the effects of aldosterone or angiotensin II, the cultures were provided with fresh medium containing 0.1% BSA, to which angiotensin II (Peptide Institute) or aldosterone (Sigma) was added for selected periods of time. To block aldosterone receptors, spironolactone (10\textsuperscript{–6} to 10\textsuperscript{–11} mol/L; Sigma) was added 1 hour before the addition of the agonists.

**Real-Time RT-PCR for ACE and GAPDH**

**Design of Primers and Probes**

Oligonucleotide primers and TaqMan probes for rat ACE were designed from the GenBank databases (U03734) using Primer Express version 1.0 (Perkin-Elmer Applied Biosystems, Inc), as previously described.\textsuperscript{16,17} The forward primer was 5′-GGAAGAGCTTACAGTGTAGCC-3′, the reverse primer was 5′-CACACCCAAAGCAATTCTTC-3′, and the TaqMan probe was 5′-AATGGCCACGTCCCGGAAAT-3′. In addition, primers and the TaqMan probe for rat GAPDH were purchased from Perkin-Elmer Applied Biosystems.

**Isolation of Total RNA**

Total RNA was extracted from cardiocytes cultured in 6-well plates using a Qiagen RNeasy kit, as previously described.\textsuperscript{18} The RNA was then treated by DNase I (Qiagen) to minimize genomic DNA contamination.

**RT-PCR**

RT-PCR was performed with the extracted RNA in an ABI PRISM 7700 Sequence Detector (Perkin-Elmer Applied Biosystems) using 96 samples per assay (50 µL per tube). RT was carried out for 1 cycle at 50°C for 2 minutes, 60°C for 30 minutes, and 95°C for 5 minutes; the PCR protocol consisted of 40 cycles of 94°C for 15 seconds and 60°C for 30 seconds, and 95°C for 5 minutes. To verify that the amplified products were the target genes, the products from one sample tube were sequenced.

**Statistical Analysis**

Data were expressed as mean±SD. Statistical analysis was performed using one-way ANOVA followed by multiple comparisons using Fisher’s protected least-significant difference and unpaired Student’s t tests, as appropriate. P<0.05 was considered significant.

**Results**

**Quantification by Real-Time RT-PCR**

The linear range of the standard curve for target gene mRNAs (ACE and GAPDH) was determined using the total RNA isolated from the lung tissue of neonatal rats (Figure 1). The threshold cycle was plotted as a function of initial template concentration to generate the standard curve. With this system, the intra-assay and interassay coefficients of variation were 19.5% and 16.9% (n=8 to 10), respectively.

**Effect of Angiotensin II and Aldosterone on Expression of ACE mRNA in Cardiocyte Cultures**

Figure 2 shows the level of ACE mRNA expression by cardiocytes after 24 hours of treatment with either angiotensin II (10\textsuperscript{–6} mol/L) or aldosterone (10\textsuperscript{–5} mol/L). Although angiotensin II had no significant effect on ACE expression (0.7±0.5-fold versus control, P=NS), aldosterone treatment increased ACE mRNA expression 23.3±7.9-fold (P<0.01).
aldosterone to cardiac ACE, which would create a reinforcing, circular cascade within the cardiac RAAS.

Aldosterone was originally thought to be important in the pathophysiology of heart failure only because of its ability to increase sodium retention and potassium loss. Recently, however, Pitt et al.5 showed that inhibiting aldosterone using a low dose of spironolactone substantially reduced the risk of morbidity and mortality among patients with severe heart failure (Randomized Aldactone Evaluation Study) and that the efficacy and mortality among patients with severe heart failure.19 Moreover, given the positive feedback from aldosterone to cardiac ACE, which would create a reinforcing, circular cascade within the cardiac RAAS.

In summary, our results directly demonstrate that aldosterone increases the expression of ACE mRNA and that this effect is blocked by spironolactone, an inhibitor of the mineralocorticoid receptor, in neonatal rat cardiocytes. Thus, a positive feedback pathway from aldosterone to ACE exists within the local cardiac RAAS.

References

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