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 (0.76±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.3–5

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.6–8 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,9–11 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.14,15 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
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 1.0×10^6 cells/cm^2 in gelatin-coated 6-well plates (Becton Dickinson).

The cardiocytes were cultured for 30 hours in 10% FCS (Hazleton Biologics)—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^-6 to 10^-4 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'-CGCGAGACCGACTTACAGTGTAGCC-3', the reverse primer was 5'-2360 AATGGCCACGTCCCGGAAAT-2379-3', and the TaqMan probe was 5'-2282 GGAGACGACTTACAGTGTAGCC-2303-3'. 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, 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^-5 mol/L) or aldosterone (10^-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).

**Characteristics of Aldosterone Effects on Expression of ACE mRNA**

Examination of the time-dependency of effects revealed that aldosterone (10^-5 mol/L) induced significant increases in ACE mRNA expression within 6 hours (versus 0 hours); expression levels then peaked after 24 hours (Figure 3a).

The concentration-dependency of the upregulation of ACE mRNA expression by aldosterone was assessed by examining the effects of exposing cardiocytes to 10^-8 mol/L to 5×10^-5 mol/L aldosterone for 24 hours. As shown in Figure 3b, the effect of aldosterone was concentration-dependent. The EC_{50} was ~4×10^-7 mol/L.

As shown in Figure 3c, spironolactone significantly and dose-dependently inhibited the upregulation of ACE mRNA expression induced by treating cardiocytes with aldosterone for 24 hours.

**Discussion**

In the present study using real-time RT-PCR, we found that aldosterone induces the expression of ACE mRNA in cultured neonatal rat cardiocytes. Although earlier studies have described the upregulation and progression of cardiac RAAS activity in heart failure, the mechanism responsible remains unclear.\textsuperscript{1,2} This study indicates that a positive feedback pathway exists from...
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.43 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 of the aldosterone blockade was likely due entirely to the prevention of sodium retention and potassium loss. We recently reported that aldosterone production is activated in the failing ventricles of humans.12 Moreover, given the positive feedback from aldosterone to ACE within the cardiac RAAS demonstrated in the present study, spironolactone might inhibit the positive feedback within the cardiac RAAS by suppressing the effects of angiotensin II and aldosterone.

Angiotensin II did not increase the expression of ACE mRNA at the same molar level as aldosterone. However, increased production of angiotensin II may indirectly increase ACE mRNA expression by activating aldosterone production.

In experimental model, the concentration of myocardial aldosterone is reported from $10^{-8}$ mol/L to $10^{-7}$ mol/L.20 These levels of spironolactone are approximately one order of magnitude greater than those of cardiac aldosterone. Therefore, a clinical dose of spironolactone is thought to be effective by inhibiting positive feedback from aldosterone to ACE in patients with heart failure.

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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