Glucocorticoid regulation of angiotensin-converting enzyme in primary culture of adult cardiac fibroblasts

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1Laboratory of Genetics and Molecular Cardiology, Heart Institute-InCor and Department of Medicine/Laboratório de Investigação Médica 13, University of São Paulo Medical School, São Paulo 05403-000; and 2Department of Anatomy, Instituto de Ciências Biomédicas, University of São Paulo, São Paulo 05508-900, Brazil

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Barreto-Chaves, M. L. M., I. Anéas, and J. E. Krieger. Glucocorticoid regulation of angiotensin-converting enzyme in primary culture of adult cardiac fibroblasts. Am J Physiol Regulatory Integrative Comp Physiol 280: R25–R32, 2001.—The effect of dexamethasone on angiotensin-converting enzyme (ACE) in primary culture of adult cardiac fibroblasts was analyzed in this study. ACE is central to cardiac remodeling in conditions such as myocardial infarction (MI). Some studies indicate that glucocorticoids are often increased post-MI, whereas other studies suggest that glucocorticoids stimulate ACE activity in various cell types. Most cardiac cells are fibroblasts, which have an important function in cardiac remodeling. Therefore, we studied the effects of glucocorticoids on ACE activity and mRNA levels in primary cultures of adult rat cardiac fibroblasts. Steady-state ACE activity was very low, but it increased sixfold with dexamethasone (1 μM for 48 h) treatment. ACE activation occurred within 12 h and peaked at 96 h, after treatment. RNase-protection assays revealed an associated threefold increase (P < 0.05) in ACE mRNA. Dexamethasone’s stimulatory effect was abolished by an RNA synthesis inhibitor (actinomycin D, 5 μg/ml) but was potentiated by a protein synthesis inhibitor (cyclheximide, 5 μg/ml). The glucocorticoid-mediated response appears to be specific, because mineralocorticoid treatment did not alter ACE activity. These findings indicate that both transcriptional and posttranscriptional mechanisms are involved in glucocorticoid regulation of ACE expression in rat cardiac fibroblasts.

dexamethasone; renin-angiotensin system

The relative contribution of circulating versus local RAS remains to be determined in physiological and pathological conditions. However, increasing evidence supports the notion that ANG II exerts a long-term effect on the structural changes that take place in several cardiovascular conditions (e.g., cardiac hypertrophy, interstitial fibrosis) (2) as well as remodeling in various tissues, including the heart, under normal and pathological conditions (16). Several earlier studies in pressure-overloaded left ventricular hypertrophy and experimental heart failure demonstrated induction of ACE activity and ACE mRNA synthesis (13, 27). Some studies have revealed that the functions of cardiac ACE go beyond blood pressure control and that they may be associated with the degree of hypertrophy, fibrosis, and the inflammatory response (9, 23). Putative cells that may contribute to local activation of ACE are endothelial cells, myocytes, macrophages, vascular smooth muscle cells, and fibroblasts. However, the cells responsible for ACE induction associated with these diseases have not yet been clearly identified.

The heart is composed of cardiac myocytes surrounded by cardiac interstitium and diverse nonmyocyte cells, most of which are fibroblasts, that comprise two-thirds of the myocardial cell population, being under separate and specific control (30) and have also been noted to stimulate cardiac myocyte hypertrophy through paracrine mechanisms (8, 16).

The influence of various hormones and second messengers on ACE production and release by different types of cells in culture has been reported (32). Previous studies have shown that glucocorticosteroids stimulate ACE activity in rabbit alveolar macrophages (11), bovine aorta endothelial cells (17, 20), and vascular smooth muscle (10). By this induction, glucocorticoids increase in both ANG II formation and bradykinin degradation in vascular smooth muscle (10). Furthermore, Sato and colleagues (26) have shown that glucocorticoids promote an increase in the ANG II type 1 receptor in vascular smooth muscle cells.
Then, considering ANG II’s growth-promoting effects on the myocardium and glucocorticoids’ effects on ACE activity, we investigated ACE regulation in well-defined primary cultures of adult rat cardiac fibroblasts after dexamethasone treatment.

**METHODS**

**Preparation and culture of adult rat cardiac fibroblasts.** Cardiac fibroblasts were prepared by selective attachment as described by Crabos and colleagues (3). Briefly, adult male Wistar rats (200–250 g) were decapitated and cleaned with ethanol before the thorax was incised. Hearts were excised, minced, and washed in PBS (GIBCO) supplemented with antibiotics (penicillin 100 U/ml and streptomycin 100 mg/ml; GIBCO). The tissue was then incubated at 37°C for 20 min with 0.1% trypsin (Sigma Chemical, St. Louis, MO), 0.1% collagenase (Type CLS, Worthington Biochemicals), and 0.1% BSA (Sigma Chemical). Dissociated cells were centrifuged (for 5 min at 1,000 rpm) at the end of each of several incubation periods, and the cell pellet was resuspended in DMEM (GIBCO) supplemented with 10% fetal bovine serum (FBS; GIBCO) and antibiotics (penicillin and streptomycin; GIBCO). Cells were then seeded into a 25-cm² flask (Nucon; GIBCO) and incubated at 37°C in a humidified atmosphere of 5% CO₂-95% air. After 2 h, unattached cells and debris were discarded, and attached cells (nonmuscle cells, mostly fibroblasts) were cultured further in DMEM containing 10% FBS. This procedure yielded cultures of cells that were almost exclusively fibroblasts by first passage, as determined by immunocytochemical techniques. On reaching confluence, cells were rinsed in PBS, harvested by enzymatic dissociation (in trypsin solution), centrifuged, and resuspended in DMEM containing 10% FBS. Cells were then seeded into 12-well plastic cell-culture dishes (Corning) or into 150-cm² flasks (Nucon; GIBCO), and medium changes were made every 2 days. Cell viability was determined by trypan blue test, and the cells from passages 1 to 4 were used for all experiments.

**Immunofluorescence cell staining.** Cultures were phenotypically characterized by indirect fluorescent immunocytochemistry. Aortic smooth muscle cells, rabbit aortic endothelial cells, and neonatal rat cardiac myocytes (1) were used as positive controls for the primary antibodies. The following primary antibodies were tested: a monoclonal antibody against von Willebrand factor (Boehringer) for the detection of endothelial cells, a monoclonal antibody against sarcomeric tropomyosin (Sigma Immunochemicals) for cardiomyocytes, a monoclonal antibody against smooth muscle myosin heavy chain (Sigma Immunochemicals) for vascular smooth muscle cells, and a monoclonal antibody against vimentin (Sigma Immunochemicals) for fibroblasts. The staining procedures were performed on cells seeded on glass coverslips and maintained in serum-free media for at least 24 h. After aspiration of the culture media, cells were washed with PBS and fixed in methanol for 15 min. Cells were permeabilized with 0.3% (wt/vol) Triton X-100 for 10 min. To block nonspecific protein binding, cells were incubated in PBS containing 0.5% (wt/vol) BSA for 20 min. Next, cells were incubated for 30 min with the first antibody, which was diluted (1:1,000), and then for a further 30 min with fluorescein thiocyanate-conjugated anti-mouse IgG secondary antibody (Sigma Chemical). After being washed, preparations were mounted and observed via confocal microscopy.

**Treatment of cardiac fibroblasts.** Dexamethasone (Sigma Chemical) was prepared as a stock solution of 10 mg/ml in absolute ethanol. Before treatment with dexamethasone, all cells were growth arrested for 48 h in serum-free media. In some experiments, cells were incubated, as described, with their respective media containing actinomycin D (5 µg/ml; Sigma Chemical) to block new transcription. In other cases, cells were incubated with cycloheximide (CHX; 5 µg/ml; Sigma Chemical) to block protein synthesis. These drugs were added to the media 30 min before the dexamethasone treatment.

**ACE activity assay.** ACE activity was determined by a fluorimetric assay on the basis of the rate of generation of His-Leu by hydrolysis of Hippuryl-His-Leu substrate (Sigma Chemical), according to Oliveira and colleagues (22). Cells were harvested into assay buffer containing 8 mM Hippuryl-His-Leu in 0.4 M sodium borate buffer (pH 8.3) with 0.1% Triton X-100. The samples were incubated for 3 h at 37°C in a water bath. Substrate hydrolysis was found to be linear during this period of time. ACE specificity was confirmed by inhibition of enzymatic activity with the use of the specific inhibitor, MK-422 (Merck). o-Phthaldialdehyde (20 mg/ml; Sigma Chemical) was then added to the aliquots, and the fluorescence of the His-Leu product was measured at 495 nm with an excitation wavelength of 365 nm (Luminescence Spectrometer; SLM-AMINCO, Rochester, NY). All assays were performed in triplicate. Protein was measured via the method of Bradford, with the use of BSA as a standard. Activity calculations were based on the basis of Michaelis-Menten first-order kinetics.

**Isolation of total cellular RNA.** The cardiac fibroblasts were washed three times with PBS. Total cellular RNA was isolated with the use of triZOL (GIBCO). The integrity of RNA was confirmed by agarose gel electrophoresis and subsequent ethidium bromide staining of total cellular RNA samples.

**Probes.** An antisense cRNA probe labeled with [α-32P]uridine 5'-triphosphate (800 Ci/mmol; Amersham) was synthesized with the use of the protocol described in the Maxiscript kit (Ambion). The ACE probe was prepared by subcloning into a plasmid vector (pBluescript; Stratagene) and was confirmed by sequencing; the probe was 300 nucleotides in length. Template DNA (1 µg) was linearized by restriction digestion and then transcribed with T3 RNA polymerase, according to the manufacturer’s recommendations. A β-actin cDNA probe (125 nucleotides in length; Ambion) was used as an internal standard.

**RNase-protection assay.** Briefly, total cellular RNA (20 µg) and the ACE-radiolabeled antisense probe (1 × 10⁵ cpm/sample) were cohybridized in 80% formamide, 100 mM sodium citrate, 300 mM sodium acetate, and 1 mM EDTA (pH 6.4) overnight at 45°C. RNase digestion (RNase cocktail; Ambion) was performed at 30°C for 30 min. After precipitation, protected fragments were separated by 8 M urea-5% polyacrylamide gel electrophoresis. Gel was vacuum-dried before exposure to Kodak Hyperfilm (Amersham). RNA expression was quantified from the autoradiogram by densitometric scanning (Eagle Eye; Stratagene).

To demonstrate the absence of nonspecific binding, probes were hybridized with 10 µg of yeast tRNA in the presence and absence of RNase cocktail after the Ambion RNase-protection assay (RPA) II protocol. Undigested probes were 70 nucleotides larger than the protected fragment because of sequences transcribed from the plasmid. Expression of ACE mRNA was compared in both experimental conditions (cells with or without dexamethasone treatment) with the use of the β-actin mRNA signal as an internal standard to account for loading differences onto the gel.

**Transfection experiments.** Primary cardiac fibroblasts were grown in 12-well plates on DMEM supplemented with 10%
FBS and antibiotics until the cells were 80% confluent. Before transfection experiments, cells were serum starved for 24 h, receiving DMEM alone. Cells were transfected with the use of a liposome formulation (Lipofectamine Reagent; Gibco) with a 1,273-bp rat ACE promoter driving a luciferase reporter gene (pGL2 vector; Promega) or constructs containing progressive deletions of this promoter controlling the expression of the same reporter gene (0.65 μg of DNA·3.0 μl of Lipofectamine-1·well-1). As an internal reference for transfection efficiency, pLTR-β-galactosidase (Promega) was cotransfected in each experiment (0.5 μg of DNA·3.0 μl of Lipofectamine·1·well-1). A 2.2-kb III fragment from pMMTV-CAT containing the mouse mammary tumor virus promoter cloned into the Hind III site of pGLO driving a luciferase reporter gene was used as a positive control for glucocorticoid stimulation. Cell lysis buffers and the luciferase assay system were from Promega. The β-galactosidase assay system was from Tropix.

Data presentation. All data from multiple experiments are expressed as means ± SD. All n values indicate the number of separate experiments performed with the use of different cell preparations. The results were analyzed by two-tailed t-test or ANOVA followed by the Bonferroni t-test as a post hoc test. Statistical significance was set at P < 0.05.

In transfection assays, luciferase activity was normalized for β-galactosidase activity driven by a cotransfected LTR-β-galactosidase reporter gene to correct for differences in transfection efficiency.

RESULTS

Characterization of fibroblasts. Cardiac fibroblasts were isolated from other cardiac cell types with the use of a differential plastic attachment procedure. Under the confocal microscope, the fibroblasts appeared as mononucleated, bipolar, or multipolar cells spread out on the dish and did not form the cell islands characteristic of endothelial cells. The cytosol was translucent, and the cell perimeter was not sharply defined. The cells (at first passage) stained positive for vimentin, one of five major groups of intermediate filaments characteristic of fibroblasts, but they were negative for smooth muscle myosin heavy chain (vs. positive vascular smooth muscle cells), von Willebrand factor (vs. positive rabbit aortic endothelial cells), and sarcomeric tropomyosin (vs. positive neonatal rat cardiomyocytes).

Effects of dexamethasone on ACE activity. Figure 1 shows that ACE activity was virtually undetectable in cardiac fibroblasts under basal conditions. The addition of different concentrations of dexamethasone to the culture medium resulted in a dose-dependent increase in ACE activity (Fig. 1A). Enzymatic activity after 48 h of treatment increased with added dexamethasone concentrations between 10⁻⁹ and 10⁻⁵ M. Next, cells were incubated with 1 μM dexamethasone for various periods of time before harvesting. ACE activity was found to be increased after 10 h of exposure to dexamethasone, with a progressive increase to 0.10 nmol His-Leu·min⁻¹·mg cell protein⁻¹ at 96 h (Fig. 1B). Dexamethasone had no effect on the protein concentration. Additional experiments showed no increase in ACE activity in the culture supernatants (data not shown).

In addition, the specificity of glucocorticoid response in regard to mineralocorticoid activation was investigated. Aldosterone (10⁻⁶ and 10⁻⁸ M) treatment for 48 h resulted in no changes in fibroblast ACE activity (Table 1).

Effect of dexamethasone on ACE mRNA levels. Because of the low amount of ACE mRNA, a sensitive

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<th>Table 1. Effect of aldosterone and dexamethasone on ACE activity of cardiac fibroblasts</th>
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<td>ACE activity, nmol His-Leu·min⁻¹·mg cell protein⁻¹</td>
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Values represent the means ± SD for 3 determinations. ACE (angiotensin-converting enzyme) activity was measured after 48 h of culture in the presence or absence (control) of steroid. *Statistically significant differences from the control value.
RPA developed in our laboratory was used to determine the steady-state expression of ACE mRNA in cardiac fibroblasts, as shown in Fig. 2A. Dexamethasone (1 μM) treatment increased ACE expression approximately threefold after 48 h of treatment (n = 3, P < 0.001). Results from the quantitative analysis of ACE mRNA levels in the cells are illustrated in Fig. 2B.

Time-course response of dexamethasone on ACE mRNA levels. Figure 3A shows that treatment of adult cardiac fibroblasts with dexamethasone (1 μM) led to a significant increase in steady-state mRNA levels from 12 to 72 h. Results from the quantitative analysis of ACE mRNA levels in the cells are illustrated in Fig. 3B.

Effects of transcription and protein synthesis inhibition on ACE expression. The following studies were aimed at clarifying the molecular mechanisms underlying the regulation of ACE gene expression by dexamethasone. To further analyze whether dexamethasone stimulates ACE at the level of transcription, cardiac fibroblasts were treated with the transcription inhibitor actinomycin D (Fig. 4A). In the presence of actinomycin D, the effect of dexamethasone on increasing ACE expression was virtually abolished (3.81 ± 1.27 to 1.16 ± 0.20, P < 0.05). Results from the quantitative analysis of ACE mRNA levels in these cells are illustrated in Fig. 4B. Actinomycin D, at the dose used, had no significant effect on control cells.

The protein synthesis inhibitor cycloheximide (CHX) was used to examine the involvement of posttranscriptional mechanisms in the dexamethasone-induced ACE gene activation. The steady-state levels of ACE mRNA were not influenced by protein synthesis inhibition. In contrast, the increase in steady-state levels of ACE mRNA induced by dexamethasone was further potentiated by pretreatment of the cultures with CHX (3.62 ± 0.15 vs. 6.84 ± 1.76, P < 0.05) (Fig. 5A).
together, these data indicate that the modulation of ACE expression by dexamethasone does not require de novo protein synthesis, suggesting that modification of factors necessary for activation of ACE expression is already present before glucocorticoid treatment.

**Transfection assays.** To define potential regulatory elements required for ACE promotor activity, a series of constructs containing progressive deletions was investigated. Similar to what has been observed in humans, rabbits, and mice, the ACE promotor in rats contains a glucocorticoid response element (GRE). This sequence, TGTTCT, is located −703 bp from the transcription initiation site. Therefore, we used a reporter gene approach to test whether this GRE participates in dexamethasone-mediated ACE gene induction. Figure 6 shows the results of transient transfections with a series of ACE promotor constructs driving a luciferase reporter gene. Treatment with dexamethasone failed to alter luciferase activity in any of the ACE constructs. Dexamethasone, however, did induce a significant increase in luciferase activity on a positive control heterologous promotor containing a glucocorticoid responsive element (MMTV) (2.50 ± 0.6, P < 0.001). These results indicate that cultured adult cardiac fibroblasts possess the signaling pathway required to express glucocorticoid gene activation.

**DISCUSSION**

Cultures of adult rat cardiac fibroblasts were prepared, and their phenotypic characterization was carried out by various criteria, including morphology and immunologic staining.

The major finding of this study is that ACE activity and mRNA in cardiac fibroblasts are practically unde-
The physiological role of ACE in cardiac fibroblasts is currently unknown. New knowledge about the role of the RAS in the regulation of cardiac remodeling supports the hypothesis that ACE regulation of ANG II production is important in local responses. In an autocrine and/or paracrine system, ANG II regulates collagen turnover by fibroblasts and thereby modulates connective tissue formation at sites of repair (7, 31). In our experiments, we did not observe significant levels of ACE activity or mRNA under steady-state conditions. These findings are in accordance with those of Danilov and colleagues (5), who detected ACE only in endothelial cells by immunohistochemical analysis of myocardial tissue. The elevation of ACE gene expression in response to glucocorticoid stimulation is the result of transcriptional and/or posttranscriptional mechanisms. For most steroid-regulated genes, the major effect occurs at the level of transcription (24). However, glucocorticoids have also been reported to both stabilize (25) and destabilize (15) mRNA transcripts. To investigate molecular mechanisms underlying the regulation of the ACE gene by glucocorticoids, we analyzed 1.3 kb of 5′-flanking region of the rat gene with the use of a reporter gene approach. Promoter activity of this region was tested by constructing various recombinant plasmids containing 5′-flanking fragments of ACE gene fused to luciferase reporter gene. The GRE (TGTTCT) present in this promoter failed to respond to glucocorticoid stimulation in cardiac fibroblasts. These results are in agreement with early observations that a human ACE promoter containing a GRE transfected in endothelial cells failed to show evidence of activation in response to glucocorticoid treatment (29). On the basis of these results, one could postulate that regulatory sequences present up- or downstream from the fragment investigated are required for proper expression of this response. For instance, in the milk protein, whey acidic protein (WAP), important hormonal regulatory elements are located between −6,300 and −3,000 bp upstream from the WAP transcription-start site (6). Alternatively, the 6-phosphofructose-2-kinase-fructose-2,6-biphosphatase contains regulatory elements located in the first intron of the gene (18). Future studies of the ACE promotor region and its interactions with nuclear transcription factors will be required to resolve this issue, considering the existence of any distal elements that could mediate the activation of ACE with dexamethasone treatment.

ACE gene transcription rate was not measured directly because transcription run-off experiments in isolated nuclei could not be employed since significant steady-state ACE mRNA levels were not detected. However, when transcription was blocked by the application of actinomycin D, the stimulatory effects of dexamethasone were completely abolished, suggesting that the glucocorticoid was directly stimulating ACE gene expression.

Superinduction of the dexamethasone-induced response by the protein synthesis inhibitor CHX indicates that there may be a posttranscriptional component in this response (21). However, the quantitative significance of the posttranscriptional compo-
ment could not be reliably estimated, because the ACE mRNA half-life could not be accurately measured in control cells. Thus the superinduction of ACE mRNAs appears to be a consequence of inhibition of protein synthesis per se rather than a reflection of a specific CHX effect and/or toxicity. This finding suggests that the glucocorticoid-induced rapid augmentation of ACE mRNA levels is independent of de novo protein synthesis (33).

Taken together, these results suggest that the induction of ACE mRNA in cardiac fibroblasts by dexamethasone reflects enhanced transcription, independent of protein synthesis. On the basis of our results, it remains unclear whether dexamethasone increases the stability of ACE mRNA. These findings shed light on the molecular mechanisms associated with glucocorticoid induction of the ACE gene, which may accompany pathological states such as MI.

Perspectives

Cardiac fibroblasts represent a major cell constituent of cardiac tissue and possess several components of RAS. Characterization of the pathways underlying the activation of the RAS during physiological and special pathophysiological situations will be important not only to assess the relative role of each cell type involved in this process but also to create new opportunities for therapeutic intervention through the development of intelligent molecules and/or gene therapy approaches.

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