Stimulation of bovine pulmonary artery endothelial cell ACE by dexamethasone: involvement of steroid receptors

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Dasarathy, Y., J. J. Lanzillo, and B. L. Fanburg. Stimulation of bovine pulmonary artery endothelial cell ACE by dexamethasone: involvement of steroid receptors. Am. J. Physiol. 263 (Lung Cell. Mol. Physiol. 7): L645-L649. 1992. After exposure of bovine pulmonary artery endothelial cells in culture to 1 μM dexamethasone for 24-48 h, angiotensin converting enzyme (ACE) activity of these cells was elevated severalfold. The increase in ACE activity was preceded by an increase in ACE mRNA, which could be detected after treatment of cells with dexamethasone for 4 h. When the increase in ACE mRNA produced by dexamethasone at 4 h was blocked by α-amanitin, an RNA polymerase II inhibitor, the increase in ACE activity detected at 48 h was inhibited. RU 38486, a steroid receptor antagonist, inhibited the elevation of both ACE activity and mRNA produced by dexamethasone. Among other steroids tested, only hydrocortisone, aldosterone and corticosterone-21-acetate had a stimulatory effect on ACE activity. RU 38486 effectively blocked the elevation in ACE activity produced by both aldosterone and dexamethasone, but had no effect on the elevation of ACE activity produced by other agents (3-isobutyl-1-methylxanthine, A23187, and dibutyryl adenosine 3',5'-cyclic monophosphate). From these data we conclude that dexamethasone and certain other steroids with an hydroxyl group in the 11th carbon position regulate ACE gene expression of bovine endothelial cells at the transcriptional level via a steroid receptor-mediated mechanism.

EXPERIMENTAL PROCEDURES

Materials. RNAzol was obtained from Biotex Labs, Houston, TX. Fast-Track mRNA isolation kit was purchased from Invitrogen, San Diego, CA. Anti-digoxigenin-AP Fab fragments were from Boehringer Mannheim Biochemicals, Indianapolis, IN. The chemiluminescent substrate AMPPD was from Tropix, Bedford, MA. Uncharged Hybond-N nylon was from Amer sham, Arlington Heights, IL. Molecular biology grade buffers, hippuryl-histidyl-leucine and all steroids were from Sigma Chemical, St. Louis, MO. RU 38486 was obtained as a gift from Roussel-UCLAF, France. The 0.24-to 9.5-kb RNA ladder was obtained from Bethesda Research Labs, Gaithersburg, MD. RPMI 1640 was from Gibco Labs, Grand Island, NY, and medium 199 was from Whittaker Bioproducts, Walkersville, MD. All other chemicals used were of the highest purity commercially available.

METHODS

Cell culture. Endothelial cells were isolated from pulmonary arteries of freshly slaughtered calves as described previously (15), and their identification was confirmed by the presence of factor VIII and ACE as surface markers. Frozen cells from second to third passage were thawed and suspended in RPMI 1640 containing antibiotics and 10% fetal bovine serum and plated in 35 or 100 mm tissue culture dishes. The cells were maintained in culture and grown to confluence at 37°C in a humidified chamber containing 5% CO2. For ACE assays, appropriate agents (specified in the figure legends) were added to the culture medium, and after various periods of incubation, media were aspirated. Cells were washed and scraped into 1.0 ml of phosphate-buffered saline (PBS) per dish and frozen at -20°C until used for the assays of ACE and total protein.

Viability. Viability of cells before and after treatment with agents was tested by trypan blue dye exclusion and was >95% under all reported experimental conditions. Cells exposed to α-amanitin for periods up to 4 h were fully viable after 24 h in culture.

ACE assay. ACE in the cellular sonicates was assayed by the fluorimetric method of Friedland and Silverstein using hippuryl-histidyl-leucine (HHL) as substrate (8). One unit of ACE activity was defined as the amount of enzyme required to hydrolyze 1 μmol of substrate per min at 37°C under standard conditions. The specificity of ACE was tested by using the specific ACE inhibitor, lisinopril, in the assay reaction mixture.

Assay of protein. Total protein in the cell sonicates was measured by the method of Lowry et al. (13).

Probes. For the ACE probe, a 480-bp fragment of human ACE cDNA corresponding to nt 3380-3859 (18) was prepared from whole cell RNA and labeled with digoxigenin (Dg) by the polymerase chain reaction (PCR) as described previously (12). For β actin, an 0.8 kb fragment of chicken cDNA (Oncor, Gaithersburg, MD) served as target for PCR amplification with concomitant Dg incorporation (12). Primers 5'-CGCACATACAGGGACAGCAC (forward) and 5'-TCCCATCTA-GGCATACAGGGGCGACGCAC (reverse) were used to prepare a 390-bp Dg-labeled probe.

Northern analysis. After the cells reached confluence, appropriate agents were added to the endothelial cells, and after specified periods of incubation, media were aspirated and cells were
washed with PBS. The ACE message was enriched by isolating the messenger RNA according to the manufacturer's (Invitrogen) instructions using the Fast Track mRNA isolation kit. Denatured mRNAs were electrophoresed in 1.1% formaldehyde agarose gels and transferred to nylon membrane (Hybond-N) using a Posiblot pressure transfer apparatus (Stratagene) for 60 min. Nucleic acids were ultraviolet (UV) cross-linked at 254 nm for 30 s in a Stratalinker unit (Stratagene) and baked for 2 h at 65°C after which the blots were stored at room temperature until they were ready to be probed. Prehybridization was done for 2 h at 65°C in 25 mM potassium phosphate, pH 7.4, 5× standard saline citrate (SSC) (1× SSC = 15 mM sodium citrate, 150 mM NaCl, pH 7.0), 5× Denhardt's reagent, 50 g/ml sheared salmon sperm DNA and 0.5% sodium dodecyl sulfate (SDS). Hybridizations were done overnight at 65°C in a Robbins hybridization oven with the denatured probe (12 ng/ml) added to the above prehybridization solution in the presence of 5% dextran sulfate. After hybridization, blots were rinsed briefly and washed twice at 65°C for 20 min each time in 20 ml of 1× SSC and 0.5% SDS (high stringency conditions) and then washed four times at ambient temperature. All wash and blocking steps were carried out as described previously (12). The blot was incubated for 30 min with 1/10,000 diluted anti-digoxigenin Fab fragment conjugated to alkaline phosphatase followed by hydrolysis of a phosphorylated dioxetane substrate AMPPD (0.26 mM). The membrane was exposed to Kodak X-OMAT film at ambient temperature and the message was detected after 3–4 h exposure.

**Image analysis.** The images on the X-ray film were subjected to digital image processing and analysis using Image, a public domain program for the Macintosh II. Equivalent areas in both ACE and the corresponding β-actin images were measured and results are represented as ACE/β-actin.

**Statistical analysis.** All experiments were done with different batches of cells and at least three times. Results from representative experiments are shown as means ± SD.

**RESULTS**

Dexamethasone at 1 μM concentration caused a striking increase in the specific activity of ACE on incubation with bovine endothelial cells for 48 h with 75–80% of the total induction of ACE activity being achieved at 24 h (Fig. 1). Cells needed to be in contact with dexamethasone for only 30 min to achieve a full induction of ACE activity at 48 h (Table 1).

![Fig. 1. Time course of effect of dexamethasone on bovine endothelial cellular angiotensin-converting enzyme (ACE) activity.](http://ajplung.physiology.org/)

**Table 1. Duration of incubation with dexamethasone required to elevate cellular ACE**

<table>
<thead>
<tr>
<th>Duration of Incubation with Dexam, h</th>
<th>ACE at 48 h, μU/dish</th>
<th>Protein at 48 h, mg/dish</th>
<th>Sp Activity of ACE, μU/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.10±0.02</td>
<td>0.68±0.02</td>
<td>0.15±0.03</td>
</tr>
<tr>
<td>0.5</td>
<td>1.54±0.15</td>
<td>0.64±0.03</td>
<td>2.38±0.19</td>
</tr>
<tr>
<td>1.0</td>
<td>1.69±0.01</td>
<td>0.66±0.01</td>
<td>2.56±0.04</td>
</tr>
<tr>
<td>48</td>
<td>1.64±0.01</td>
<td>0.68±0.01</td>
<td>2.41±0.02</td>
</tr>
</tbody>
</table>

Values are means ± SD of triplicate determinations from a representative experiment. Confluent endothelial cells were treated with 1 μM dexamethasone (Dex). After various incubation periods, the media were aspirated; cells were washed and incubated further in fresh medium without dexamethasone. After 48 h, cells were washed and scraped into 1.0 ml phosphate-buffered saline/dish and cell sonicates were used for the determination of angiotensin-converting enzyme (ACE) and total protein.

Appropriate inhibitors were used to assess whether the initial events occurring after treatment with dexamethasone involved protein synthesis or a transcriptional process. Treatment of cells with dexamethasone in the presence of 20 μg/ml cycloheximide for only 4 h did not have any effect on the elevation of ACE activity at 48 h produced by dexamethasone (data not shown). However, longer incubations (48 h) with cycloheximide resulted in inhibition of elevation of ACE levels (3–5, 7, 10). On the other hand, treatment of cells with α-amanitin, a RNA polymerase II inhibitor (transcription inhibitor), for the initial 4 h incubation with dexamethasone resulted in almost total inhibition of the dexamethasone-induced ACE activity at 24 h (Fig. 2). Also, pretreatment of cells with actinomycin D for 2 h blocked the elevation of ACE produced by dexamethasone at 24 h (data not shown).

![Fig. 2. Treatment of cells with dexamethasone in presence of α-amanitin.](http://ajplung.physiology.org/)
Northern analysis of bovine ACE mRNA isolated from cells incubated with 1 μM dexamethasone for different periods of time showed that dexamethasone stimulated the transcription of the ACE gene as early as 4 h. The ACE mRNA level peaked between 8 and 24 h of incubation with dexamethasone and returned toward control levels at 48 h (Fig. 3, A and B).

The glucocorticoid receptor antagonist, RU 38486, was tested to determine whether dexamethasone stimulated the ACE gene through a steroid receptor(s). RU 38486 (2 μg/ml) completely inhibited the elevation in ACE activity produced by dexamethasone (Fig. 4). The dexamethasone-induced increase in ACE mRNA level was similarly blocked by RU 38486 (Fig. 5). RU 38486 alone had no effect on ACE activity or ACE mRNA levels.

Since a steroid receptor(s) appears to be involved in the induction of ACE produced by dexamethasone, several other steroids were assessed to determine their effects on ACE. Other than dexamethasone, only aldosterone, hydrocortisone, and corticosterone-21-acetate had a stimulatory effect on ACE activity (Table 2).

To determine whether a corticosteroid receptor(s) is generally involved in the stimulation of endothelial cell ACE, cells were incubated in the presence of RU 38486 with a variety of agents known to elevate cellular ACE. ACE elevation produced by dexamethasone and aldosterone was totally inhibited by RU 38486, but that produced by other nonsteroidal agents was unaffected under the same conditions (Table 3).

**DISCUSSION**

Although it has been previously reported that dexamethasone elevates ACE of endothelial cells in culture, no information has been available as to whether this action represents a transcriptional or translational event. Our current studies provide evidence that ACE gene transcription is increased in these cells after dexamethasone treatment and that augmentation of transcription occurs through a glucocorticoid receptor(s).

Neither bovine ACE cDNA nor the related gene has been sequenced to date. Although the human cDNA probe we used for these studies readily recognizes the human ACE message from total RNA extracts of human
endothelial cells, it did not recognize the ACE message assessed by Northern analysis of total cell RNA isolated from bovine endothelial cells (12). However, when the bovine endothelial cell ACE message was enriched by isolation of mRNA from these cells, the bovine ACE message was easily detected by the human ACE cDNA probe on Northern analysis and its mobility was comparable to bovine endothelial cell ACE message. After 48 h, the mRNA level at this time decreases, suggesting a degradation in ACE message.

The early transcripational stimulatory events after dexamethasone treatment are independent of protein synthesis. Prolonged treatment with cycloheximide probably inhibits ACE synthesis through interference with later translational actions (3-5, 7, 10). Northern analysis shows that the ACE mRNA level starts to increase at 4 h and peaks after 8 h incubation with dexamethasone. The precise time lag between ACE mRNA synthesis and later expression of catalytically active ACE protein in endothelial cells is uncertain. Although ACE activity peaks at 48 h, the mRNA level at this time decreases, suggesting a degradation in ACE message.

ACE gene induction by dexamethasone was blocked by the glucocorticoid receptor antagonist RU 38486, indicating that the increase in ACE mRNA/activity requires interaction of dexamethasone with an intracellular glucocorticoid receptor(s). The ACE activity induced by aldosterone was also inhibited by RU 38486. RU 38486 had no effect on ACE activity induced by other agents such as methylxanthine, A23187, and cAMP elevating agents, supporting the specific involvement of steroid receptor(s) in the ACE stimulatory action of the steroids.

A specific corticosteroid structure appears to be important in the mechanism of ACE induction. Steroids that have been found to induce bovine ACE have an hydroxyl group on carbon atom 11; the corresponding steroids with a keto group on the 11th carbon atom do not induce ACE. It is not known whether the 11 β-OH group of the steroid determines the specificity or affinity for the receptors.

The role that stimulation of ACE by corticosteroid as noted in vitro might play in vivo systems is unclear. Corticosteroids actually reduce the elevated serum levels of ACE associated with sarcoidosis (22), but this ACE is thought to be derived from granulomatous tissue and the granulomas are likely to be simultaneously resolving with treatment. In reports of reduction of serum ACE with corticosteroid treatment of healthy subjects or patients with diseases other than sarcoidosis (9, 20), effects on turnover of ACE are not taken into account. The specific synthesis of ACE by the endothelium following corticosteroid treatment in vivo has never been tested.

We thank Deborah La Perche for assistance in preparing this manuscript.

Table 2. Effect of steroid hormones on endothelial cell ACE

<table>
<thead>
<tr>
<th>Agents</th>
<th>ACE, mU/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.094±0.006</td>
</tr>
<tr>
<td>Estrone</td>
<td>0.106±0.003</td>
</tr>
<tr>
<td>β-Estradiol</td>
<td>0.105±0.003</td>
</tr>
<tr>
<td>Testosterone</td>
<td>0.094±0.027</td>
</tr>
<tr>
<td>Progestosterone</td>
<td>0.104±0.008</td>
</tr>
<tr>
<td>Corticosterone-21-acetate</td>
<td>0.340±0.050*</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.600±0.015*</td>
</tr>
<tr>
<td>Hydrocortisone</td>
<td>1.080±0.040*</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.480±0.050*</td>
</tr>
<tr>
<td>Cortisone</td>
<td>0.103±0.006</td>
</tr>
<tr>
<td>Dehydrocortisone</td>
<td>0.108±0.018</td>
</tr>
</tbody>
</table>

Values are means ± SD of triplicate determinations from a representative experiment. Confluent bovine endothelial cells were treated with 1 μM of the above agents. After 48 h, cells were washed and scraped into 1.0 ml phosphate-buffered saline/dish. Cell sonicates were used for the determination of angiotensin-converting enzyme (ACE) activity and total protein content. * Statistically significant from control P < 0.001.

Table 3. Effect of RU 38486 on the elevation of ACE produced by various agents

<table>
<thead>
<tr>
<th>Agents</th>
<th>Concentration, mM</th>
<th>ACE, mU/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.001</td>
<td>0.480±0.004</td>
</tr>
<tr>
<td>Dexamethasone</td>
<td>0.001</td>
<td>1.703±0.060</td>
</tr>
<tr>
<td>Aldosterone</td>
<td>0.001</td>
<td>0.916±0.006</td>
</tr>
<tr>
<td>IBMX</td>
<td>0.2</td>
<td>0.891±0.044</td>
</tr>
<tr>
<td>A23187</td>
<td>0.00033</td>
<td>0.643±0.019</td>
</tr>
<tr>
<td>DBcAMP</td>
<td>1.0</td>
<td>1.089±0.033</td>
</tr>
</tbody>
</table>

Values are means ± SD of triplicate determinations from a representative experiment. Confluent bovine endothelial cells were treated with the above agents in the presence and absence of 2 μg/ml RU 38486. After 48 h, cells were washed and scraped into 1.0 ml phosphate-buffered saline/dish. Cell sonicates were used for the determination of angiotensin-converting enzyme activity and total protein content. IBMX, 3-isobutyl-1-methylxanthine.
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REFERENCES


