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Dexamethasone stimulates endothelin-1 gene expression in renal collecting duct cells

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ABSTRACT

Aldosterone stimulates the endothelin-1 gene (*Edn1*) in renal collecting duct (CD) cells by a mechanism involving the mineralocorticoid receptor (MR) and the glucocorticoid receptor (GR). The goal of the present study was to determine if the synthetic glucocorticoid dexamethasone affected *Edn1* gene expression and to characterize GR binding patterns to an element in the *Edn1* promoter. Dexamethasone (1 μM) induced a 4-fold increase in *Edn1* mRNA in mIMCD-3 inner medullary CD cells. Similar results were obtained from cortical collecting duct-derived mpkCCD$_{c14}$ cells. RU486 inhibition of GR completely blocked dexamethasone action on *Edn1*. Similarly, 24 h transfection of siRNA against GR reduced *Edn1* expression by approximately 50%. However, blockade of MR with either spironolactone or siRNA had little effect on dexamethasone induction of *Edn1*. Cotransfection of MR and GR siRNAs together had no additive effect compared to GR-siRNA alone. The results indicate that dexamethasone acts on *Edn1* exclusively through GR and not MR. DNA affinity purification studies revealed that either dexamethasone or aldosterone resulted in GR binding to the same hormone response element in the *Edn1 Edn1* promoter. The *Edn1* hormone response element contains three important sequence segments. Mutational analysis revealed that one of these segments is particularly important for modulating MR and GR binding to the *Edn1* hormone response element.

1. Introduction

Endothelin-1 (ET-1) is a 21 amino acid signaling peptide involved in a wide variety of biological processes including development, homeostasis, cell-cycle control and inflammation [1]. The spatial and temporal production of ET-1 is highly regulated in order to maintain proper control over these diverse functions. The primary mechanism controlling ET-1 concentrations occurs at the level of gene transcription [1]. Previously, we and others have reported that the ET-1 gene (*Edn1*) was under direct transcriptional control by the mineralocorticoid aldosterone in the kidney [2,3]. Interestingly, the action of aldosterone on *Edn1* was mediated not only by the classical mineralocorticoid receptor (MR), but also by the glucocorticoid receptor (GR).

MR and GR are both ligand-dependent transcription factors that share extensive structural homology and identical consensus sequences [4]. However, the expression and function of MR is far more restricted than GR. Most notably, MR is expressed in polarized epithelial cells involved in sodium transport including the aldosterone-sensitive cells of the distal nephron and collecting duct in the kidney [5]. In these cells, MR plays a vital role in the maintenance of sodium homeostasis and blood pressure control through the transcriptional regulation of genes involved in transepithelial sodium transport [6–8]. In contrast, GR is ubiquitously expressed in the body and is estimated to modulate 10% of the genes within the human genome [9–11]. Glucocorticoids are involved in a wide variety of physiological processes including the stress response, immune function, reproduction, behavior, and metabolism. The importance of GR is underscored by the fact that exogenous and synthetic glucocorticoids represent one of the most widely used classes of therapeutic compounds due to their efficacy in the treatment of inflammatory, autoimmune and proliferative disorders.

Renal collecting duct cells express both MR and GR *in vivo* [12]. These cells also express 11β-hydroxysteroid dehydrogenase type II (11βHSD-2). Aldosterone is not a substrate for this enzyme, so

Abbreviations: 11βHSD-2, 11β-hydroxysteroid dehydrogenase type II; DAPA, DNA affinity purification assay; ET-1, Endothelin-1 peptide; GR, Glucocorticoid receptor; HRE, Hormone response element; MR, Mineralocorticoid receptor.

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11βHSD-2 acts only on endogenous glucocorticoids, such as cortisol, producing 11-keto metabolites that do not activate MR or GR [13,14]. Therefore, the functional role of GR in renal collecting duct cells is not well defined [8]. However, the absence of 11βHSD-2 in renal collecting duct cells can have important detrimental effects. For example, glucocorticoid hormones can bind to MR with similar affinity to aldosterone [4,15] resulting in inappropriate salt retention and hypertension in human patients [16,17].

Aldosterone can also bind to GR [18,19]. Therefore, it is possible that aldosterone mediates its action through both MR and GR in 11-βHSD2 expressing cells of the collecting duct. Support for this hypothesis is found in transgenic mice that overexpress GR. These animals exhibited an increase in *Scnn1a* (αENaC) levels in the collecting duct and a decrease in urinary aldosterone levels, demonstrating a transient GR-dependent change in sodium balance *in vivo* [20]. In our own studies, aldosterone stimulated both MR and GR binding to a single high affinity hormone response element (termed HRE2) in the *Edn1* promoter [2]. Similar receptor binding patterns have been observed for other aldosterone target genes involved in sodium balance, such as *Atp1a*, *Scnn1a*, *Sgk1* and *Per1* [21–25]. Therefore, it is not surprising that both MR and GR stimulate sodium transport in collecting duct cells [19,26].

While there is mounting evidence suggesting that GR participates in aldosterone action in the kidney, it is not known whether GR acts in concert with MR or if GR functions independently. GR could conceivably function by binding to an alternative response element or by a non-genomic action. The goal of the present study was to determine if GR stimulates *Edn1* expression in the mIMCD-3 collecting duct cell line. Since mIMCD-3 collecting duct cells express 11βHSD-2, selective GR action on *Edn1* was evaluated using dexamethasone. Dexamethasone is a synthetic glucocorticoid that is not subject to inactivation by 11βHSD-2. Dexamethasone has an additional advantage for study of selective GR activation because it exhibits a very high affinity for GR [27]. In this report we show that dexamethasone activates *Edn1* expression via GR binding to HRE2, and that sequence changes in HRE2 alter GR binding to the element.

2. Experimental

2.1. Cell culture and hormone treatment

The mpkCCD$_{c14}$ cells are a mouse cortical collecting duct cell line and were a kind gift of Dr. Alain Vandewalle [28]. The mIMCD-3 cells are a mouse inner medullary collecting duct cell line and were purchased from American Type Culture Collection. All cells were maintained in DMEM/F12 plus 10% FBS and 50 μg/ml gentamicin. For all hormone experiments, cells were plated on 6-well Costar Transwell plates (Corning Inc.). Cells were grown 24 h past confluency and changed to DMEM/F12 plus 10% charcoal-dextran stripped FBS (Invitrogen) for another 24 h prior to hormone treatments. Aldosterone, dexamethasone, spironolactone and RU486 were purchased from Sigma-Aldrich, prepared in 100% ethanol and stored at −20 °C until use. Cells were treated with vehicle (ethanol), 1 μM dexamethasone or 1 μM aldosterone for 1 h. For inhibitor studies, cells were treated with agonist plus RU486 (10 μM) or spironolactone (10 μM). The final concentration of ethanol in all experiments was 0.1%.

2.2. Steady-state mRNA determination

Hormone studies were conducted as described above on growth-arrested confluent cell monolayers grown in 6-well Costar Transwell plates. Total RNA (2 μg) was isolated from cells using TRIzol® Reagent (Invitrogen), treated with DNase I (Ambion) to eliminate genomic DNA, and reverse transcribed using oligo dT, random hexamers and Superscript™ III (Invitrogen). No reverse transcriptase served as a negative control in the cDNA reaction. Resulting cDNAs (32 ng) were used as templates in duplicate quantitative real-time PCR (QPCR) reactions run on an Applied Biosystems QPCR machine. No template cDNA was used a negative control in QPCR experiments. Cycle threshold (C_T) values were normalized against β-actin (*actb*) and relative quantification was performed using the $\Delta\Delta C_T$ method [29]. TaqMan® (Applied Biosystems) primer/probe sets were used and are listed in Supplemental Table 1. QPCR studies were run with standardized conditions using the $\Delta\Delta C_T$ method.

2.3. Hormone receptor siRNA knockdown

MR-siRNA (J-061269-09 NR3C2), GR-siRNA (J-045970-10 NR3C1) and control non-targeting siRNA against luciferase (#2 D-001210-02-05) were purchased from Dharmacon (Lafayette, CO, USA). Cells were seeded at a density of 75,000 cells/cm^2 on 6-well Transwell plates (Corning Incorporated) and transfected for 24 h with 2 μM siRNA in 6 μl of DharmaFect 4. At the time of transfection cells were switched to phenol-red free DMEM/F12 plus 10% charcoal dextran stripped FBS. After 24 h the cells were treated with 1 μM dexamethasone or vehicle for 1 h. RNA was extracted and processed as described above for QPCR.

2.4. DNA affinity purification assay and Western analysis

Hormone experiments were conducted in mIMCD-3 cells as described above except that cells were grown in 200 mm dishes (Corning). Cells were treated with vehicle (0.15% ethanol), aldosterone, or dexamethasone. Cytoplasmic and nuclear extracts were obtained using the NE-PER® kit (Pierce Biotechnology) and DNA affinity purification assays (DAPAs) were performed as described previously [2]. In brief, double stranded DNA probes homologous to the wild-type or mutated *Edn1* HRE2 were biotinylated on 5′ ends (sequences detailed in Fig. 5B). Probes were immobilized on 50 μl of streptavidin coated agarose beads and incubated with 175 μg of nuclear extract in the presence of freshly prepared protease inhibitors (Roche) for 1 h at room temperature with end-over-end rotation. Beads were pelleted and supernatants were removed and assayed for input controls by Western blotting for actin. Pelleted beads were washed four times with ice-cold PBS plus protease inhibitors. After the final wash, all liquid was aspirated from the beads with flat-headed gel loading tips (USA Scientific) and 50 μl of 2× LDS (BioRad) plus βME. Samples were boiled for 5 min, chilled on ice, and loaded onto a 7.5% Tris-HCl SDS-PAGE Ready Gel (Biorad) for electrophoresis. Proteins were transferred to PVDF overnight and blocked with 2% Rodeo blocker plus 0.05% saddle soap (USB) in TBS. The monoclonal MR antibody was a kind gift of Drs. Elise and Celso Gomez-Sanchez and was used at a 1:100 dilution [30]. The GR and actin antibodies were purchased from Santa Cruz and used at 1:5000 and 1:200 dilutions, respectively. Blots were washed with blocking solution and developed with Rodeo Western Detection Reagents (USB). Equal loading was controlled for by Bradford assay and input control Westerns against actin.

2.5. Statistics

Unless otherwise stated, all experiments were performed in duplicate in at least three independent studies. Statistical significance was determined using a two-tailed Student's t test and $p < 0.05$ was considered significant.