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Gene array analysis of the effects of chronic adrenocorticotropic hormone in vivo on immature rat adrenal glands

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Abstract

Development of a mature adrenocortical phenotype is a critical event in the transition of mammals from fetal to postnatal life. We previously reported that the functional maturation of the adrenal glands of newborn rats is accelerated by adrenocorticotropic hormone (ACTH). We report here that chronic exposure of neonatal/juvenile rat pups to ACTH in vivo results in significant changes in expression of over 200 genes in the adrenal glands. ACTH significantly upregulated genes associated with cell signaling, gene transcription, cell migration and tissue remodeling. In addition, ACTH significantly downregulated several genes associated with de novo cholesterol biosynthesis and cholesterol trafficking. Finally, ACTH upregulated genes associated with intracellular metabolism and inactivation of glucocorticoids. The results demonstrate that the developmental effects of ACTH alter expression of a broad range of genes involved not solely in steroid synthesis, but in cellular functions related to growth and differentiation of the glands. In addition, the negative effects of ACTH on genes required for cholesterol synthesis and production of active glucocorticoids, suggests a mechanism whereby excessive production of glucocorticoids, which may have deleterious actions on developing structures like the central nervous system, is prevented.

Keywords: Differential gene expression; ACTH; Cholesterol biosynthesis; Rat

1. Introduction

Depending on the species, the mammalian adrenal gland undergoes a complex series of developmental processes during fetal and/or neonatal life that results in a morphologically and functionally mature gland. Functional maturation can be defined in various ways, but of particular importance is the ability of the glands to synthesize and secrete glucocorticoids. These steroids are required for ex utero survival, notably but not solely because they stimulate pulmonary differentiation [1]. Because of the incidence of premature births in the human population, it is of great interest to determine the mechanisms that govern adrenocortical maturation. Several factors appear to be key determinants of this process, including the expression of orphan nuclear receptors [2–6], specific cytochrome P450 enzymes and dehydrogenases involved in steroid hormone production [7,8], and intracellular mediators of cholesterol entry into mitochondria, such as the peripheral-type benzodiazepine receptor, or PBR [9,10].

We have identified the anterior pituitary hormone ACTH as an important developmental signal that accelerates the appearance of a more mature phenotype in the adrenal glands of young rats [10–12]. In those studies, neonatal rats injected daily with ACTH developed increased sensitivity to the hormone, as evidenced by greater capacity of corticosterone production per unit number of cortical cells. The effect of ACTH was reversible and appeared to be mediated partly by increased PBR expression [9,10]. Of note, however, was the observation that despite hypertrophy and possibly hyperplasia of the gland [10], and the increased steroidogenic capacity [10–12], plasma corticosterone levels following ACTH injection in immature rats failed to reach levels comparable to those we typically observe in fully mature, adult rats [9,11,13]. Thus, although ACTH upregulates the functional capacity of neonatal and juvenile rat adrenal glands, it is unable to completely induce adult-like activity.

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In the present study, we sought to expand what is known about the intracellular effects of chronic ACTH stimulation on rat adrenal gland development, by subjecting glands from ACTH- or saline-treated immature rats to gene microarray analysis. Development of the mammalian adrenal gland entails much more than upregulation of steroidogenic capacity, involving for example hyperplasia, cell migration, and tissue remodeling leading to morphologically and functionally distinct zones within the adrenal cortex. Therefore, we focused particular attention on the effects of ACTH on genes involved in steroidogenesis, signaling pathways, gene transcription, cholesterol biosynthesis, tissue remodeling, and cell proliferation. We also tested the hypothesis that ACTH may induce a subset of genes that act to suppress maximal steroidogenesis. If so, this would explain the repeated observations that regardless of treatment, we have not been able to induce neonatal/juvenile rat adrenal glands to attain an adult-like steroidogenic phenotype [10–12]. We report here the first differential gene expression study performed on developing adrenal glands after chronic ACTH stimulation. The results suggest that chronic ACTH induces or suppresses genes involved in numerous aspects of cell function, including cholesterol biosynthesis and corticosterone metabolism.

2. Material and methods

2.1. Animals

Pregnant Holtzman Sprague–Dawley rats were obtained from Harlan (Indianapolis, IN) on gestation day 18. The animals were housed in individual cages on a 12-h light, 12-h dark cycle (08:00–20:00 h) in the animal care facility with food and water ad libitum. Litters were culled to 12–15 pups per dam. All pups remained with their dam until the last day of injections and were only briefly separated during the administration of injections.

2.2. Effect of ACTH on gene expression

The day of birth was designated postnatal day (pd) 1. Control and experimental animals were chosen randomly and each treatment group was represented by individuals within each litter. Pups were marked with a permanent marker in order to distinguish among the different treatment groups within each litter. Pups of both sexes were injected intraperitoneally twice daily (08:00 and 16:00 h) as previously described [10], from pd 2 to pd 14 with either saline or 20 μg/kg body weight of porcine $ACTH_{1–39}$ (Sigma–Aldrich Corp.) in 100 μl.

On pd 14, pups from each group were randomly divided into two additional groups receiving a final injection of either saline or ACTH. Pups were sacrificed by decapitation at 30 or 120 min after the final saline or ACTH injection.

Treatment groups were as follows: (1) chronic ACTH followed by acute saline, sacrificed at 30 min; (2) chronic ACTH followed by acute ACTH, sacrificed at 30 min; (3) chronic saline followed by acute saline, sacrificed at 30 min; (4) chronic saline followed by acute ACTH, sacrificed at 30 min; (5) chronic ACTH or (6) saline followed by acute ACTH, sacrificed at 120 min. Each of the six groups were represented by pools of 12–15 adrenals from two separate, independent injection studies. These groups were derived from an earlier study [10] in which the steroidogenic response to chronic ACTH was determined. The final injections (ACTH or saline) and times of sacrifice (30 or 120 min post-injection) were performed to confirm that the steroidogenic phenotype was altered by exposure of the animals to chronic ACTH injections. This regimen resulted in stress-like plasma ACTH levels, enlarged adrenal glands, and increased corticosterone production in response to ACTH compared to control animals chronically treated with saline; these data have been reported elsewhere [10] and confirmed our earlier results [11]. In the present study, we were interested only in changes in gene expression which resulted from *chronic* ACTH, not those genes (if any) expression of which changed after the acute treatments. Thus, for statistical analysis of gene expression data, we combined the groups into two: animals receiving ACTH or saline chronically ($n = 6$ each), regardless of acute treatment.

At the end of an experiment, animals were killed by decapitation. Adrenal glands were removed, decapsulated to remove the outer connective tissue and most of the zona glomerulosa cell layer that lies adjacent to the capsule, and frozen immediately on dry ice. Absence of significant amounts of glomerulosa cells was confirmed by RT-PCR analysis of aldosterone synthase (CYP11B2) (not shown).

2.3. Total RNA isolation and purification

Total RNA was isolated from neonatal adrenal tissue with TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA) as recommended by Affymetrix (Santa Clara, CA), followed by column purification (Qiagen; Valencia, CA). RNA quality was determined on the basis of the ratio of absorbance at 260 and 280 nm in 10 mM Tris–HCl solution (pH 7). All RNA samples were checked for integrity of 18 and 28 S RNA by gel electrophoresis.

2.4. Microarray analysis

Reverse transcription, second strand synthesis, labeled cRNA preparation, and hybridization to the rat U34A GeneChip were performed at the Boston University Microarray Resource (Boston University School of Medicine, Boston, MA). Briefly, using a poly-dT primer incorporating a T7 promoter, double-stranded cDNA was synthesized from 10 μg total RNA using a Superscript cDNA Synthesis Kit (Invitrogen). Biotin-labeled cRNA was generated from the double-stranded cDNA template by in vitro transcription with T7 polymerase using a BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY), and purified on RNeasy affinity columns (Qiagen). Biotinylated cRNA (20 μg) was fragmented in 40 mM Tris–acetate,