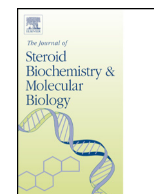
Journal of Steroid Biochemistry & Molecular Biology 143 (2014) 451–459

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry & Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb



Dexamethasone acutely down-regulates genes involved in steroidogenesis in stallion testes

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ARTICLE INFO

Article history:
Received 15 March 2014
Received in revised form 27 June 2014
Accepted 3 July 2014
Available online 7 July 2014

Keywords:
Glucocorticoid
Regulation of gene expression
Steroidogenesis
Testosterone
Estrogen

ABSTRACT

In rodents, livestock and primate species, a single dose of the synthetic glucocorticoid dexamethasone acutely lowers testosterone biosynthesis. To determine the mechanism of decreased testosterone biosynthesis, stallions were treated with 0.1 mg/kg dexamethasone 12 h prior to castration. Dexamethasone decreased serum concentrations of testosterone by 60% compared to saline-treated control stallions. Transcriptome analyses (microarrays, northern blots and quantitative PCR) of testes discovered that dexamethasone treatment decreased concentrations of glucocorticoid receptor alpha (*NR3C1*), alpha actinin 4 (*ACTN4*), luteinizing hormone receptor (*LHCGR*), squalene epoxidase (*SQLE*), 24-dehydrocholesterol reductase (*DHCR24*), glutathione S-transferase A3 (*GSTA3*) and aromatase (*CYP19A1*) mRNAs. Dexamethasone increased concentrations of *NFkB* inhibitor A (*NFKBIA*) mRNA in testes. *SQLE, DHCR24* and *GSTA3* mRNAs were predominantly expressed by Leydig cells. In man and livestock, the *GSTA3* protein provides a major 3-ketosteroid isomerase activity: conversion of Δ^5-androstenedione to Δ^4-androstenedione, the immediate precursor of testosterone. Consistent with the decrease in *GSTA3* mRNA, dexamethasone decreased the 3-ketosteroid isomerase activity in testicular extracts. In conclusion, dexamethasone acutely decreased the expression of genes involved in hormone signaling (*NR3C1, ACTN4* and *LHCGR*), cholesterol synthesis (*SQLE* and *DHCR24*) and steroidogenesis (*GSTA3* and *CYP19A1*) along with testosterone production. This is the first report of dexamethasone down-regulating expression of the *GSTA3* gene and a very late step in testosterone biosynthesis. Elucidation of the molecular mechanisms involved may lead to new approaches to modulate androgen regulation of the physiology of humans and livestock in health and disease.

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1. Introduction

Factors that negatively impact male fertility reduce reproductive success. The testes play a central role in male fertility with their dual functions of steroidogenesis and spermatogenesis. Importantly, the two functions are linked: spermatogenesis requires a local environment with a high concentration of testosterone, provided by the steroidogenic Leydig cells [1,2]. While the enzymes involved in cholesterol biosynthesis and steroidogenesis have been identified, the regulation of steroidogenesis is still being elucidated [3–6]. Identification of the molecular mechanisms involved may reveal new targets for therapeutic augmentation or interruption of steroidogenesis.

Stress is an increasingly relevant health problem of humans and animals. It is well known that stress (which induces high concentrations of circulating glucocorticoids) and treatment with glucocorticoid drugs negatively impact sperm production and fertility in male mammals [7–10]. Dexamethasone is a glucocorticoid drug used clinically for its effective and long lasting anti-inflammatory action [11]. Treatment with dexamethasone acutely reduced levels of circulating testosterone by 60% or more in rams, boars, bulls, stallions and baboons [7,12–15]. In the subsequent weeks, semen quality was impaired: there were increased numbers of sperm with abnormal morphology and biochemical parameters [14,16]. Both effects of dexamethasone were transient, with full recovery of serum testosterone concentrations in days and semen quality as early as 4–6 weeks after treatment. The molecular mechanisms of glucocorticoid impairment of

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http://dx.doi.org/10.1016/j.jsbmb.2014.07.003
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steroidogenesis have been studied primarily in rodents [8,10,17,18]. Glucocorticoid effects on cultured Leydig cells from rodent testes indicate that some dexamethasone actions are direct [19–24]. Previous studies using that model found that dexamethasone down-regulated products of the luteinizing hormone receptor (*LHCGR*), *CYP*11A1, *CYP*17A1 or *HSD3B2* genes [17,20–22]. It is noteworthy that rodents utilize the Δ^4 -pathway of steroidogenesis, in contrast to the Δ^5-pathway used by human and livestock species [25]. The current study used dexamethasone treatment as a model system to study impaired testosterone biosynthesis in stallions, which use the Δ^5-pathway of steroidogenesis.

Stallions are a unique livestock species in which, as in men, individuals are selected for breeding based on factors other than fertility. Male subfertility is relatively common in stallions and men, as is interventional therapy for that problem [26]. In both species, medical data for reproductive parameters and rates of establishing pregnancies for individuals can be extensive. Subfertility in stallions and men can be exacerbated by stress or medical treatment with glucocorticoids. The glucocorticoid receptor gene (*NR3C1*) is highly expressed in stallion testes [27]. In the current work, the acute effects of dexamethasone treatment of stallions on expression of genes in testes were analyzed to discern the molecular mechanism of impaired testosterone production. To our knowledge, this is the first report of results from global, non-biased analyses of dexamethasone effects on gene expression in the testes. Five genes (*SQLE, DHCR24, ACTN4, GSTA3* and *NFKBIA*) were newly discovered to be regulated by dexamethasone in testes, including the down-regulation of the *GSTA3* gene. Dexamethasone also depressed the 3-ketosteroid isomerase activity in testes extracts, the activity of the GSTA3 enzyme. Although this activity was discovered 37 years ago [28], it was more recently recognized as the predominant isomerase in the Δ^5-pathway of steroidogenesis employed by livestock and human, but not rodent, species [3,29–32]. The GSTA3 enzyme isomerizes Δ^5-androstene-3,17-dione to Δ^4-androstene-3,17-dione, the immediate precursor of testosterone, as well as Δ^5-pregnene-3,20-dione to produce progesterone.

2. Materials and methods

2.1. Stallions and in vivo treatments

All animal procedures were approved by the Texas A&M University Animal Care and Use Committee. Eight Quarter Horse stallions (4–10 years of age, 452 ± 12 kg) underwent daily semen and blood collection at 8 A.M. for 10 days [33]. The stallions were housed individually in solid walled stalls that did not allow physical or visual contact between them. Serum was frozen for cortisol and testosterone analyses. The first 7 days were necessary to train stallions for semen collection as well as to deplete extra-gonadal sperm reserves so that the 8th to 10th day collections reflected the daily sperm output of each stallion. Semen parameters were normal and included volume of gel-free ejaculate (35 ± 7 ml), total sperm count per ejaculate ($4.3 \pm 0.5 \times 10^9$), total motility ($82 \pm 2\%$) and progressive motility ($53 \pm 4\%$). Values reported are averages across all stallions for the last 3 days of the experiment. The last two parameters were assessed using a Hamilton-Thorn motility analyzer (Beverly, MA). Total testicular volume (average 305 ± 19 cc) was determined via ultrasonography. The actual daily sperm output (DSO) and the predicted DSO from total testes volume were used to calculate spermatogenic efficiency for each stallion (Table 1), as described in [34]. These values were used to separate the stallions into treatment groups with equivalent spermatogenic efficiencies of testis tissues. At 10 P. M. on day 9 of the study, four stallions were injected with 0.10 mg/kg dexamethasone i.v. while the four control stallions received a similar volume of saline i.v. 12 h after injection, the stallions were castrated [35]. The time point was chosen because the nadir of testosterone concentrations in serum appeared then in this model (manuscript submitted). Fresh testis tissue was fixed in paraformaldehyde for in situ hybridization and in situ reverse transcription-PCR (RT-PCR). Fresh testis tissue (100 g) collected from peripheral sites was minced and mixed to make a "mixed peripheral sample". Aliquots were snap frozen in liquid nitrogen and stored at −80 °C for RNA analyses. All tissue analyses were performed with samples from the right testes.

2.2. Radioimmunoassays of testosterone and cortisol in serum

Serum concentrations of cortisol were determined in duplicate 25-μl aliquots of samples by a single-antibody RIA that utilized polypropylene tubes coated with the cortisol antiserum (MP Biomedicals, Solon, OH; [36]). The radiolabeled tracer was ^{125}I-cortisol. The minimum detectable concentration was 1.2 ng/ml and the intra-assay and inter-assay coefficients of variation was 3.4% and 4.2%, respectively. Serum concentrations of testosterone were determined in duplicate 100-μl aliquots of samples by a single-antibody RIA that used testosterone antiserum obtained from Dr. Niswender (GDN; Dr. Gordon D. Niswender, Colorado State University, Ft. Collins, CO; [37]). The radiolabeled tracer was ^{3}H-testosterone (PerkinElmer-New England Nuclear, Boston, MA). The minimum detectable concentration was 15 pg/ml and the intra-assay and inter-assay coefficients of variation was 6.8% and 9.4%, respectively.

2.3. Isolation of RNA from testes and global analyses of mRNAs

Total cellular RNA was isolated with Tripure reagent (Roche; Indianapolis, IN) from frozen tissue from the right testis of each stallion. RNA concentrations were measured using a Nanodrop ND-

Table 1
Calculation of percent of spermatogenic efficiency, which was used to divide the stallions into two groups with testis tissues of equivalent function. Ejaculates collected on the last 3 days of the study were averaged to determine actual daily sperm production (DSO) for each stallion. Ultrasound measurements of height, length and width of each testis were used to calculate total testis volume in cubic centimeters (cc). These volumes were used to calculate predicted DSO, as in Ref. [34]. The actual DSO divided by the Predicted DSO generated the percent spermatogenic efficiency ("% Efficiency") values for each stallion, which were used to randomize the stallions between the two treatment groups. "% Efficiency" means and standard errors for the treatment groups are in the rightmost column.

Stallion	Actual DSO $\times 10^9$	Total testes size (cc)	Predicted DSO $\times 10^9$	% Efficiency	Treatment	% Efficiency mean ± SE
A	3.90	366	7.52	51.9	Control	72.1 ± 7.5
B	5.09	352	7.19	70.8	Control	
C	4.66	299	5.92	78.7	Control	
D	6.10	345	7.02	86.9	Control	
E	2.48	226	4.16	59.6	Dex	75.8 ± 14.2
F	4.36	337	6.83	63.8	Dex	
G	4.70	241	4.52	104	Dex	

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