General and Comparative Endocrinology 176 (2012) 500–506

Contents lists available at SciVerse ScienceDirect

General and Comparative Endocrinology

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

5α-Reductase, an enzyme regulating glucocorticoid action in the testis of *Rhinella arenarum* (Amphibia: Anura)

Amelia J. Tesone [a], Eleonora Regueira [a], Luis Fabián Canosa [b], Nora R. Ceballos [a,*]

[a] Laboratorio de Endocrinología Comparada, Departamento de Biodiversidad y Biología Experimental, Facultad de Ciencias Exactas y Naturales, Universidad de Buenos Aires, Consejo Nacional de Investigaciones Científicas y Técnicas, Buenos Aires, Argentina

[b] Laboratorio de Neuroendocrinología Comparada, Instituto de Investigaciones Biotecnológicas–Instituto Tecnológico Chascomús (IIB-INTECH), Consejo Nacional de Investigaciones Científicas y Técnicas, Universidad Nacional de General San Martin, Chascomús, Buenos Aires, Argentina

ARTICLE INFO

Article history:
Available online 20 January 2012

Keywords:
5α-Reductase
Androgens
Toad testes
Glucocorticoid-receptor

ABSTRACT

The reduction of A-ring of glucocorticoids to produce 5α-dihydro-derivatives by 5α-reductases has been considered as a pathway of irreversible inactivation. However, 5α-reduced metabolites of corticosterone and testosterone have significant biological activity. In this paper, we investigated whether toad testicular 5α-reductase (5α-Red) is able to transform corticosterone into 5α-dihydrocorticosterone. Furthermore, we studied the role of 5α-reduced metabolite of corticosterone as a glucocorticoid receptor (GR) agonist. The activity of 5α-Red was assayed in subcellular fractions with [^{3}H]corticosterone or [^{3}H]testosterone as substrate. The enzyme localizes in microsomes and its optimal pH is between 7 and 8. The activity is not inhibited by finasteride. These results support the conclusion that toad 5α-Red resembles mammalian type 1 isoenzyme. Kinetic studies indicate that neither K_m nor V_{max} for both corticosterone and testosterone were significantly different among reproductive periods. The K_m value for testosterone was significantly higher than that for corticosterone, indicating that the C-21 steroid is the preferred substrate for the enzyme. Studies of the binding capacity of 5α-dihydrocorticosterone (5α-DHB) to the testicular GR show that 5α-DHB is able to displace the binding of [^{3}H]dexamethasone to testicular cytosol with a similar potency than corticosterone. The inhibition constant (Ki) values for corticosterone and 5α-DHB were similar, 31.33 ± 2.9 nM and 35.24 ± 2.3 nM, respectively. *In vitro* experiments suggest that 5α-DHB is an agonist of toad testicular GR, decreasing the activity of the key enzyme for androgen synthesis, the cytochrome P450 17-hydroxylase, C17,20-lyase.

1. Introduction

Glucocorticoids (GCs) are the principal peripheral effectors of the stress system and are regulated by the hypothalamic–pituitary–adrenal axis [19,54]. The amount of GCs released in response to stressful stimuli induces a wide variety of physiological effects on different systems, among them the reproductive system [53,54]. In vertebrates, GCs exert their actions through the interaction with at least two different intracellular receptors belonging to the nuclear hormone receptor superfamily, type I or mineralocorticoid receptor (MR) and type II or glucocorticoid receptors (GR) [27]. Additionally, rapid non-genomic actions have been proposed for mammals, avian, fish and amphibians [8,10,24,25,43,44].

In mammalian Leydig cells, glucocorticoids (GCs) inhibit testosterone synthesis by different mechanisms [36,45,46,61]. However, the access of GCs to their receptors within rat Leydig cells is regulated, at least in part, by the NADPH/NADP$^+$-dependent 11β-hydroxysteroid dehydrogenase or type 1 isoform (11β-HSD1) [29–32,59].

Previous studies from our laboratory have provided compelling evidence that the testis of the toad *Rhinella arenarum*, formerly named *Bufo arenarum* [28,49], expresses a NADH/NAD$^+$-dependent 11β-HSD with predominantly oxidative activity [22]. Additionally, a cytosolic glucocorticoid receptor similar to mammalian GR was described [23].

The enzyme 5α-reductase (5α-Red) catalyzes the reduction of the A-ring of steroids with a 4,5 double bond and a 3-oxo-group [52]. In mammals, this enzyme is expressed in several tissues such as seminal vesicle, epididymis, prostate and testicular Leydig cells [12,40,55]. In mammalian Leydig cells 5α-Red converts testosterone into the more potent androgen 5α-dihydrotestosterone (5α-DHT) [56]. Currently, two subtypes of 5α-Red with specific pH-optima and inhibitor sensitivities, designated type 1 and 2, have been described in both human and rat [3,4,42].

In adult mammals, testosterone is the major secreted androgen due to the progressive decline in 5α-Red activity after approximately 40-days of age [20,21]. In contrast, 5α-DHT has been shown to be the

* Corresponding author. Fax: +54 11 4576 3384.
E-mail address: nceballo@bg.fcen.uba.ar (N.R. Ceballos).

0016-6480/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
doi:10.1016/j.ygcen.2012.01.004

most important testicular metabolite in different amphibian species [17,35,40,58]. Moreover, the high 5α-Red activity found in the testis of *R. arenarum* is involved in the conversion not only of T into 5α-DHT but also of progesterone into 5α-pregnan-3,20 dione [17].

In mammals, 5α-Red participates also in the metabolism of glucocorticoids by the reduction of the A-ring, and 5α-reduction has been recognized as part of the inactivating pathway [18]. However, 5α-reduced metabolites of other steroids are potent agonists at the same receptor as their parent hormone. For instance, reduced derivatives of progesterone may act as neurosteroids, and allopregnanolone binds to the non-nuclear α-amino butyric acid, type A receptor [50]. Previous evidences indicate that 5α-dihydrocorticosterone (5α-DHB) is practically devoid of glucocorticoid activity and competes for dexamethasone binding sites much less efficiently than corticosterone [18]. However, Baxter and Tomkins [7] found in rat hepatoma cells that 5α-dihydrocortisol induces the enzyme tyrosine aminotransferase to a third of the level induced by cortisol. In addition, 5α-reduced glucocorticoids also regulate gluconeogenic enzymes like phosphoenolpyruvate carboxykinase and fructose-1,6-diphosphatase in rat liver [33] and modulate brain excitability also in rats [12]. Moreover, MacInnes et al. [37] showed that 5α-reduced glucocorticoids bind to and activate GR. Taken together these studies suggest that the transcription of glucocorticoid-regulated genes could be influenced by intracellular levels of corticosterone and its 5α-reduced metabolites. Furthermore, the capacity of 5α-reduced glucocorticoids to suppress inflammation has been demonstrated both *in vitro* and *in vitro* [41,62].

Amphibians, as other vertebrates, are characterized for having a seasonal rhythm in GC concentrations with the highest levels during breeding [39,51]. However, of equal importance of GCs delivery to target tissues is the quantity of active hormones reaching the GR. Therefore, a shift in the relative amounts of 11β-HSD that can activate or deactivate GCs in target tissues could alter the physiological effects of GCs. The toad *R. arenarum* is a seasonal breeder, with the breeding season restricted to the period between September and December, southern springtime. During breeding, males of this species show low levels of circulating androgens [13] and high concentrations of plasma GCs [22]. Amphibian testes in general, and *R. arenarum* in particular, have a high activity of 5α-Red, enzyme that could contribute also to the modulation of GC action. However, the role of 5α-Red as a putative contributor to the regulation of GC action in amphibians remains unknown, but are vital to further understand any physiological consequence of annual GC rhythms. Therefore, the main goal of this paper is to study the role of 5α-Red in the regulation of GC action in the testis of the toad *R. arenarum*.

2. Methods

2.1. Materials

Corticosterone (CORT), 5α-dihydrocorticosterone (5α-DHB), dexamethasone, radioinert steroids and cofactors were from Sigma Chemical Co. (St. Louis, MO, USA) and [^{3}H]dexamethasone (40 Ci/mmol), [^{3}H]corticosterone (70.5 Ci/mmol), [7(n)-^{3}H]pregnenolone (18 Ci/mmol) and [1,2,6,7(n)-^{3}H]testosterone (75.5 Ci/mmol) were from Perkin-Elmer (Boston, MA, USA). Silica gel plates 60GF 254 on aluminums were purchased from Merck (Darmstadt, Germany). All other chemicals were of reagent grade.

2.2. Animals

Testicular tissue was obtained from adult male toads of *R. arenarum* (150–180 g) collected all year long in a non-agricultural area near Buenos Aires City (Argentina). Animals were housed at ambient temperature and natural photoperiod with free access to water and fed with crickets, zophobas and liver. Toads were anaesthetized with 1% MS222, in accordance with the Amphibians: Guidelines for the Breeding, Care and Management of Laboratory Animals (National Academy of Sciences, 1974). Testes were quickly removed and processed according to the experiment (see below).

2.3. 5α-Reductase activity

'For the determination of 5α-Red, testes were homogenized in 50 mM Tris–HCl buffer containing 1 mM Cl_2Mg, 20% glycerol, pH 7.0 (T buffer) in a proportion of 250 mg of tissue/ml. Subcellular fractions were separated from the homogenate by differential centrifugation according to Pozzi et al. [48]. Briefly: after two sedimentations of the nuclear fraction at 800*g* for 10 min, mitochondria were sedimented from the supernatant by centrifugation at 15000*g* for 20 min. The pellet was washed twice with half the initial volume of T buffer. For the separation of microsomal fraction, the 15000*g* supernatants were centrifuged at 105000*g* for 90 min. Mitochondria were purified by using 0.88 M sucrose. Suspensions of mitochondria in T buffer were layered on top of 0.88 M sucrose and centrifuged at 11500*g* for 20 min. The supernatant was discarded and the pellet washed twice with T buffer. All steps were carried out at 4 °C. All fractions were used immediately for enzyme assays. In order to determine pH optima of 5α-Red, microsomes were incubated in buffer consisting of 10 mM Tris and citric acid monohydrate, pH 4.0–8.0, with 0.5 mM NADPH, in a final volume of 1 ml. The reaction catalyzed by 5α-Red was started by the addition of 25 μM of [^{3}H]corticosterone or [^{3}H]testosterone, with or without different concentrations of Finasteride (0–100 μM) depending on the experiment, and samples were incubated for 20 min. These conditions were established by preliminary experiments as the linear zones of time and enzyme curves. The reaction was stopped with cold dichloromethane and media were extracted twice with the same solvent. Substrate and product were separated by thin layer chromatography (TLC) with dichloromethane:acetone (75:5, v/v) as solvent system. The amounts of [^{3}H]5α-DHB and [^{3}H]5α-DHT produced were quantified after chromatography. The mass of the products was calculated from the known specific activities of the substrate. Positions of radioactive steroids on TLC were determined by fluorography with EN^{3}HANCE spray (NEN, Boston, MA) as a fluorographic intensifier and Kodak films X-OMAT LS (Sigma Chem. Co., St. Louis, MO). Standard 3-oxo-4-ene steroids were detected by UV absorption. Primuline was used to reveal the position of non-absorbing standard steroids [60].

Kinetic parameters, K_m and V_{max}, of 5α-Red were determined by measuring the microsomal conversion of [^{3}H]corticosterone and [^{3}H]testosterone for 10 min at 28 °C with different concentrations of both substrates (0.01 to 10 μM). Protein concentration was adjusted so that the rate of product formation was linear for up to 10 min. After incubations, steroids were extracted, separated and quantified as described above. K_m and V_{max} were estimated from Lineweaver–Burk linearization.

2.4. Subcellular marker enzymes

Succinate-dehydrogenase (SDH) as mitochondrial marker was determined according to Pennington [47] (1961), and glucose-6-fosfatase (Glu-6P) for microsomes according to Aronson and Touster [6]. Inorganic phosphorous were determined by the methods of Ames [2] (1966).

2.5. Glucocorticoid receptor binding assays

Binding assays were performed as previously described [23]. Briefly: testicular tissue was homogenized in ice-cold GR buffer (10 mM Hepes buffer with 5 mM EDTA, 10% (v/v) glycerol,

20 mM Na_2MoO_4 and 0.1 mM PMSF, pH 7.4). Cytosolic fraction was prepared from the homogenate by differential centrifugation [48]. Protein concentration was estimated by the method of Bradford [9]. Binding was assayed in triplicate employing 400–600 μg cytosolic proteins and 3.5 nM [^{3}H]dexamethasone in GR buffer. Binding parameters were obtained by the displacement of [^{3}H] dexamethasone specific binding with different concentrations of unlabeled dexamethasone, corticosterone or 5α-DHB (2.5–1000 nM). All incubations were carried out in a final volume of 0.5 ml at 4 °C. After equilibrium was reached, unbound [^{3}H]dexamethasone was removed by the incubation with an equal volume of charcoal-dextran (2%:0.2%) in PBS, pH 7.4 during 20 min and subsequent centrifugation. Specific binding was calculated by subtracting non-specific binding obtained in parallel samples after the addition of a 1000-fold excess of unlabeled dexamethasone. Binding parameters - dissociation constants (Kd) and the number of binding sites (B) – were obtained employing the Ligand Programme (Ligand Software David Rodbard, NIH).

2.6. Long-term in vitro incubations

Testes were quickly dissected under sterile conditions, placed on Leibovitz (L15) culture medium, and fat bodies, mesorchia and Bidder's organ were removed. Testes were cut into slices of approximately 2 mm thick (approximately 20 mg). Slices were individually transferred to culture plates containing 2 ml L15–10 mM Hepes-10% fetal bovine serum (FBS) plus antibiotic (penicillin, 100 IU/ml, streptomycin, 0.1 mg/ml) and antimycotic (amphotericin B, 0.25 mg/ml) [14]. Fetal bovine serum was inactivated at 55 °C and depleted of steroids by charcoal:dextran treatment prior to use. Testicular explants were cultured for 24 h with different concentrations of dexamethasone or 5α-DHB. After incubations, slices were individually homogenized for the determination of the cytochrome P450 17-hydroxylase, C17,20-lyase (Cyp450c17) activity, a key enzyme in androgen biosynthesis. The activity of Cyp450c17 was measured in 50 mM potassium phosphate buffer, pH 7.4, containing 0.1 mM EDTA, 0.4 mM β-mercaptoethanol, 3 mM $MgCl_2$ and 20% glycerol (T buffer). Enzyme activity was determined according to Fernández Solari et al. [26] as follows: 200 μg proteins of the homogenate were incubated for 10 min with 5 μM [^{3}H]pregnenolone in T buffer, pH 7.4, containing, 0.5 mM NADPH and 1 IU/ml glucose-6-phosphate dehydrogenase. Pregnenolone, dehydroepiandrosterone and 17-hydroxypregnenolone were separated by TLC using methylene chloride: acetone (75:5, v/v). The specific activities of the enzymes were expressed as pmole of product/min/mg of protein. Positions of radioactive steroids on TLC were performed as described before.

2.7. Western blot analysis

Homogenates from fragments used in long-term *in vitro* incubations were also processed for Western blot to detect changes in the total amount of Cyp450c17 protein. For this purpose, 100 μg of proteins mixed with sample buffer (40 mM Tris–HCl, 2% sodium lauryl sulfate (w/v), 4% glycerol (v/v), 0.001% bromophenol blue (w/v), 2 mM β-mercaptoethanol, pH-6.8) were electrophoresed at 130 V in a 10% sodium dodecyl sulfate–polyacrylamide gel (SDS–PAGE). After electrophoresis, proteins were transferred to PVDF membranes (Hybond LFP, Amersham) at 300 mA for 90 min. After transference, membranes were incubated in phosphate buffered solution containing 0.1% Tween-20 (PBS-T) and 3% peroxide hydrogen for 10 min and blocked for 1 h at room temperature in PBS-T containing 5% low-fat powdered milk. Incubations with primary antibodies were performed overnight at 4 °C in PBS-T with 0.4 ug/ml of rabbit polyclonal anti mouse Cyp450c17 antibody (Santa Cruz Biotechnology, Santa Cruz, CA) and 0.4 μg/ml of rabbit anti actin antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Then, membranes were incubated with the secondary antibody coupled to horse radish peroxidase (0.2 μg/ml goat antirabbit IgG, Chemicon International, USA). Immunopositive bands were visualized by using enhanced chemiluminiscent detection reagent (1.25 mM luminol, 0.198 mM cumaric acid, 0.038 v/v H_2O_2, 100 mM Tris–HCl buffer) in a Fujifilm LAS-1000 chemiluminescent detection system. Densitometric analysis of corresponding bands was performed with ImageGauge software (Fuji photo film CO., LTD, Altura Software, Inc). In order to validate the semi-quantitative method, the relationship between protein concentration and optical density was analyzed. This ratio was linear in the range of the concentration used (30–100 μg of protein).

2.8. Radioactivity determination

Radioactivity was measured by liquid scintillation counter. Scintillation counting was carried out with Wallac 1409 DSA equipment (Wallac Co, Turku, Finland). The scintillation cocktail for all samples was OptiPhase-Hi safe 3 (Wallac Co, Turku, Finland).

2.9. Statistical analysis

Results were expressed as means standard error. In binding assays and Western blot analysis, a randomized block design was used to minimize the variability between experiments. Two-way ANOVAs followed by Tukey's multiple comparisons tests were used to detect significant differences among treatments and experiments. Statistical analyses were performed with STATISTICA 6.0 (StatSoft, Inc., Tulsa, OK). Differences were considered significant with $P < 0.05$. Before statistical analysis data were tested for normality and homoscedasticity by using Lilliefors and Bartlett's test, respectively. Data from the Western blot analysis were log transformed [$ln(X + 1)$] to correct for heteroscedasticity.

3. Results

3.1. Subcellular distribution

The activity of 5α-Red was assayed in subcellular fractions obtained by differential centrifugation using [^{3}H]corticosterone or [^{3}H]testosterone as substrate. Table 1 shows that 5α-Red activity was localized in the microsomal fraction regardless of the substrate employed. After additional purification of mitochondrial fraction, the 5α-Red activity in this fraction decreased along with the Glu-6Pase activity, a microsomal marker, to become non-detectable.

3.2. pH preference

The pH preference of enzyme activity is a criterion for identifying 5α-Red subtypes. Fig. 1 shows the activity of microsomal 5α-

Table 1
Subcellular distribution of 5α-Red activity.

	5α-Red (A)	5α-Red (B)	SDH	Glu-6P
Homogenate	0.48 ± 0.11	0.28 ± 0.10	5.2 ± 1.2	2.3 ± 0.9
Nucleus	0.18 ± 0.10	0.18 ± 0.11	2.1 ± 0.9	1.9 ± 0.9
Mitochondria	0.92 ± 0.13	0.32 ± 0.09	11.3 ± 1.2	2.9 ± 1.0
Mitochondria*	N.D	N.D	31.0 ± 3.4	N.D
Microsomes	2.50 ± 0.38	0.71 ± 0.26	0.6 ± 0.2	29.7 ± 3.8
Citosol	N.D	N.D	N.D	N.D

5α-Red activity was measured with 25 μM of [^{3}H]corticosterone (A) or testosterone (B) as substrates and 25 μg protein in T buffer. NADPH concentration was 0.5 mM. Incubation time was 20 min at 28°. Glu-6P, glucose 6-phosphatase; SDH, succinate dehydrogenase; Mitochondria*, purified mitochondria; ND, non-detectable. Values represent means of five triplicate experiments ± SE.

Red in a pH-range between 4.0 and 8.0 with testosterone or corticosterone as substrates. The activity increases at pH 6.0 with both substrates and keeps constant as far as pH 8.0. However, at more acidic pH values (4.0–5.0), very low activity was detected. Therefore, the optimal pH of toad testicular 5α-Red resembles the mammalian type 1 isoenzyme [1].

3.3. Effect of Finasteride on 5α-Red activity

Finasteride (FIN) is an inhibitor of mammalian 5α-Red type 2 enzyme at nmolar concentrations (Ki = 3–5 nM). In contrast, FIN is a relatively poor inhibitor of mammalian 5α-Red type 1 (Ki ⩾ 300 nM) [1,34]. Fig. 2 shows that FIN was unable to significantly inhibit 5α-Red activity, even at micromolar concentrations. This result additionally supports the conclusion that toad 5α-Red resembles mammalian type 1 isoenzyme.

3.4. Kinetic studies

Kinetic parameters, K_m and V_{max}, of 5α-Red were determined in three different periods: pre-reproductive (Pre-R) from April to August, reproductive (R) from September to December, and post-reproductive (Post-R) from January to March. Pre-R and Post-R periods correspond to that previously defined as non-reproductive period in this species [13]. Neither K_m nor V_{max} for both corticosterone and testosterone were significantly different among reproductive periods (Table 2). Thus, the K_m and V_{max} values from different periods were pooled to get a unique value of each parameter. As shown in Table 3, the K_m value for testosterone was significantly higher than that for corticosterone, indicating that the C-21 steroid is the preferred substrate for the enzyme.

3.5. Competition experiments

To characterize the binding capacity of 5α-DHB to the testicular GR, the specific binding of [^{3}H] dexamethasone to cytosol was displaced with radioinert dexamethasone, corticosterone and 5α-DHB. Competition studies show that 5α-DHB displaced the binding of [^{3}H]dexamethasone with a similar potency than corticosterone (Fig. 3). The inhibition constant (Ki) values for corticosterone and 5α-DHB were similar, 31.33 ± 2.9 nM and 35.24 ± 2.3 nM, respectively, indicating that 5α-DHB could compete efficiently with corticosterone for GR. The Kd value for dexamethasone was 12.4 ± 1.2 nM.

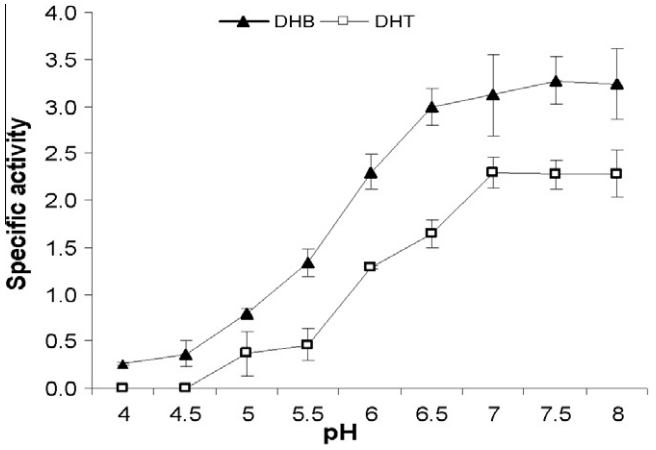

Fig. 1. pH profile of 5α-reductase activity in microsomal fraction in the pH range of 4.0–8.0 with 0.5 mM NADPH as cofactor and 25 μM testosterone or corticosterone (DHB) as substrates. DHT, 5α-dihydrotestosterone; DHB, 5α-dihydrocorticosterone. Incubations were carried out for 20 min at 28 °C in 10 mM Tris and citric acid monohydrate buffer, pH 4.0–8.0 with a concentration of 0.5 mM NADPH. After incubations, substrates and products were separated as described in Section 2. Specific activity is expressed as nmole/min/mg protein. Values are expressed as means of four triplicate experiments ± SE.

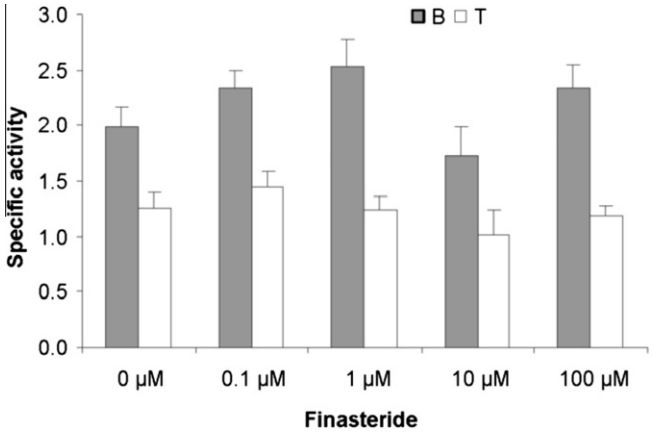

Fig. 2. Effect of different concentrations of Finasteride on 5α-Red activity. 5α-Red activity was measured with 25 μM of [^{3}H]corticosterone or testosterone as substrates and 25 μg protein in Tris–HCl buffer, pH 7.5, and 0.5 mM NADPH. Incubation time was 20 min at 28°. Specific activity is expressed as nmole/min/mg protein. Values represent means of four triplicate experiments ± SE.

Table 2
Kinetic parameters of 5α-Red.

Epoca K_m (nM)	V_{max}	(pmoles DHB/min·mg prot)
Pre-reproductive	509.7 ± 53.6	2.7 ± 0.6 (n = 6)
Reproductive	687.9 ± 160.1	3.0 ± 0.8 (n = 6)
Post-reproductive	519.3 ± 33.3	2.6 ± 0.5 (n = 9)

Kinetic parameters corresponding to different seasons. K_m and V_{max} were obtained after Lineweaver–Burk linearization. Values were measured by using different concentrations of corticosterone (2.5–1 μM) in the presence of 0.25 mM NADPH at pH 7.0. Values are expressed as media ± SE. Number of assays is between brackets.

Table 3
Kinetic constants for 5α-Red activity.

Substrate K_m	(nM)	V_{max} (nmole/min/mg protein)
Corticosterone	569 ± 59**	2.39 ± 0.14**
Testosterone	1190 ± 230**	2.74 ± 0.09**

K_m and V_{max} were measured with various concentrations (0.01 to 10 μM) of either corticosterone or testosterone with 0.5 mM NADPH in T buffer. K_m and V_{max} were calculated by Lineweaver–Burk linearization. Values represent means of four triplicate experiments ± SE. **: differences were significant between substrate s with $p < 0.01$.

3.6. Effects of dexamethasone and 5α-DHB on Cyp450$_{c17}$ activity and protein level

In order to determine whether 5α-DHB is an agonist or an antagonist of GR, the effect of dexamethasone and 5α-DHB on the activity of Cyp450c17 was examined. Fig. 4 shows that 150 and 1500 nM dexamethasone significantly inhibited the activity of the key enzyme for androgen biosynthesis, Cyp450$_{c17}$. At the same concentrations 5α-DHB evoked a similar response. This result is consistent with an agonistic activity of 5α-DHB. The effect of 5α-DHB on Cyp450c17 was also determined by Western blot analysis. As shown in Fig. 5 and 24 h treatment with 150 and 1500 nM 5α-DHB, and 1500 nM of dexamethasone produced a significant decrease in the amount of protein.

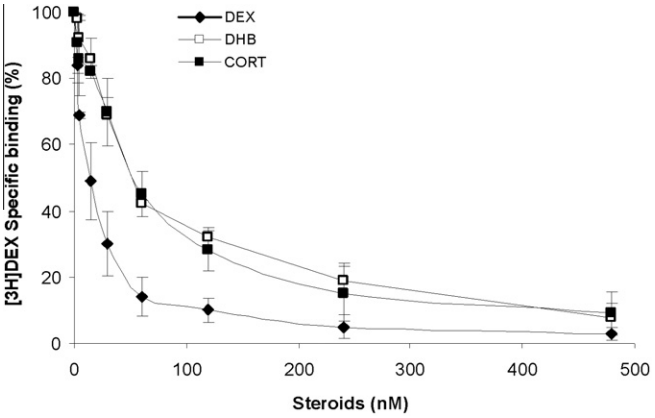

Fig. 3. Displacement curves of specific binding of [^{3}H]dexamethasone by various steroids. Testes of six toads were employed in each experiment. Error bars correspond to standard deviations. Results correspond to 5 independent experiments.

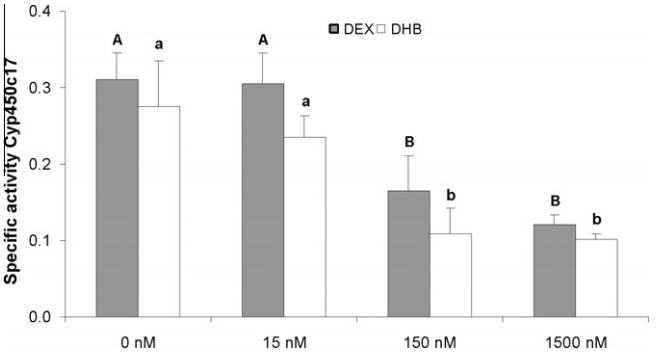

Fig. 4. Effect of dexamethasone and 5α-DHB on Cyp450c17 activity. Testes were cultured with different concentrations of dexamethasone and 5α-DHB. After 24 h incubation, enzymatic activity was determined as describe in Section 2. Activity is expressed as nmole/min x mg protein. Results correspond to 6 independent experiments ± SE. Different letters mean significant differences among concentrations after 24 h with $P<0.05$.

4. Discussion

The action of GC on Leydig cells is determined by several factors. Undoubtedly, the concentration of plasma GCs is an important issue to take into account, particularly in cold-blooded vertebrates having marked changes in this parameter. In amphibians, an increase in GC concentration during the breeding season has been clearly demonstrated in several species [39,51]. Nevertheless, there are other factors determining the sensitivity of one particular cell to GC action. For instance, the expression in target tissues of an 11β-HSD with oxidative activity, could decrease the local concentration of GCs to an insufficient level for the activation of GR. Concurrently, an increase in the number and affinity of GR could increase the sensitivity to GCs. Concomitantly, the amount of plasma binding proteins could be involved in the regulation of the cellular response to GC. For instance, corticosteroid binding globulin (CBG) binds GC with high affinity and therefore could regulate the availability of these hormones. The binding of GCs to CBG may serve as a buffering mechanism regulating the amount of free hormone that enter target tissues, or alter GC clearance rates [11,63]. Nevertheless, there is another factor that has not been taking into account, the expression and activity of 5α-Red in GC target tissues, like testicular Leydig cells.

As described in several species, amphibian testes produce high quantities of 5α reduced-steroids such as 5α-DHT and 5α-pregnanedione [5,17,35,58], making them an excellent model to study the role of 5α-Red in the regulation of GC availability.

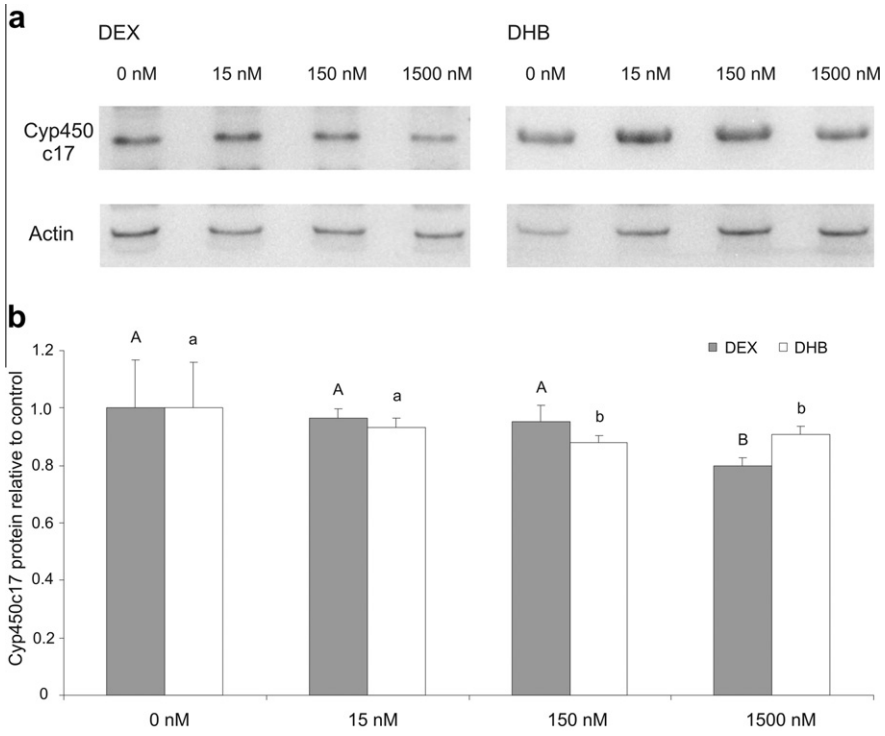

Fig. 5. Effect of dexamethasone and 5α-DHB on Cyp450c17 protein level. After treatments, testes were processed as described in Section 2. Western blot analysis was performed with the cell homogenate. (a) Membrane corresponding to one representative experiment. (b) Densitometric analysis of Cyp450c17-immunoreactive band. Values of Cyp450c17 were normalized to the corresponding actin level and expressed as the ratio treatment/control. Values are expressed as means of the different experiments ($n = 5$ for DEX treatment and $n = 4$ for -DHB treatment) ± SE. Upper case letters and lowercase letters are used to denote significant differences between the treatments with DEX or DHB, respectively. Different letters mean significant differences between treatments with $p < 0.05$.

In *R. arenarum*, testicular 5α-Red is an enzyme associated with the microsomes with no detectable activity in nuclear fraction. Classically, mammalian type 1 and 2 5α-Red activities are characterized by their pH optima. Type 2 5α-Red can indeed be assessed by its narrow pH-profile, with higher activity at pH 5.0 [57]. Furthermore, FIN specifically inhibits type 2 enzyme at nmolar concentrations. Distinctly, the mammalian type 1 5α-Red has a broad pH-optima [57] and FIN is a poor inhibitor of its activity [34]. The pH-profile of 5α-Red activity in toad testis reveals minimal activity at pH 5.0 and high activity at pH values up to 6.0, when testosterone or corticosterone are used as substrates.

As shown in mammals, in toad testes the microsomal enzyme utilizes pregnene and androstene steroids as alternative substrates with NADPH as a cofactor. However, toad testicular enzyme has a greater affinity for corticosterone than for testosterone.

This study demonstrates not only that the testis of *R. arenarum* is able to 5α-reduce glucocorticoids but also that 5α-DHB binds to GR in testicular cells with an affinity similar to corticosterone. To address whether 5α-DHB binds to GR as an antagonist or agonist, we investigated the GR-dependent responses in testes. The results indicate that the 5α-reduced metabolite is able to induce a response similar to dexamethasone by decreasing the activity of $Cyp450_{c17}$. The difference between the decrease of the activity and the diminution in the amount of protein could be due to post-translational modifications and/or GC-dependent changes in the quantity of the cytochrome b5 [38]. Together, these findings imply that, when present at physiological concentrations, 5α-reduced glucocorticoids could contribute to GR activation. Potentially, this could also happen wherever 5α-reductase was expressed, including glucocorticoid target tissues such as liver and adipose tissue. This work also provides further evidence for another intracrine mechanism, over and above the influence of 11β-hydroxysteroid dehydrogenases, by which concentrations of active glucocorticoids could be modulated independently of circulating corticosterone levels [23]. Whether 5α-reductase contributes to GR activation is now the subject for further investigations that should also be aimed to address the physiological result of the competition between the activating action of 5α-Red and the inactivating activity of 11β-HSD. Moreover, the effect of 5α-DHB on $Cyp450_{c17}$ activity and protein level may be part of the mechanism of the switch in steroidogenic capability of toad testes that occurs during the reproductive season [13] along with other factors such as, FSH and mGnRH [14–16]. This fact is of particular interest since it was demonstrated that GC serum levels increase during reproductive season in several amphibian species [39,51].

Taken together, these studies demonstrate for the first time in amphibian testis that 5αRed has the capacity to reduce corticosterone into its agonistic derivative, 5α-DHB. Besides, the conversion of testosterone and corticosterone into 5α-DHT and 5α-DHB, respectively, is catalyzed by one enzyme with higher affinity for corticosterone than testosterone. In addition, 5αRed activity exhibits the same pH dependence for both substrates lacking activity at the more acidic pH and having broad pH-optima for both substrates. Furthermore, FIN has no effect on the transformation of both substrates. These characteristics suggest that toad testis express a 5α-Red possessing features similar to type 1 mammalian enzyme.

Acknowledgments

This work was supported by grants from The University of Buenos Aires (UBACyT X042) and The National Research Council of Argentina (PIP 2433). The experiments comply with de "Principles of animal care", publication No. 86-23, and revised 1985 of the National Institute of Health and also with the argentine laws.

References

[1] S. Aggarwal, S. Thareja, A. Verma, T.R. Bhardwaj, M. Kumar, An overview on 5α-reductase inhibitors, Steroids 75 (2010) 109–153.
[2] B.N. Ames, Assay of inorganic phosphate, total phosphate and phosphatases. Methods Enzymol. VIII (1966) 115–118.
[3] S. Anderson, R.W. Bishop, D.W. Russell, Expression, cloning and regulation of steroid 5α-reductase, an enzyme essential for male sexual differentiation, J. Biol. Chem. 264 (1989) 16249–16255.
[4] S. Anderson, D.W. Russell, Structural and biochemical properties of cloned and expressed humanan and rat steroid 5α-reductases, Proc. Natl. Acad. Sci. USA 87 (1990) 3640–3644.
[5] E. Antila, A. Saure, Seasonal variation of androgen interconversion in testicular tissue of Rana temporaria in vitro, Experientia 35 (1978) 326–328.
[6] N.N. Aronson, O. Touster, Isolation of rat liver plasma membrane fragments in isotonic sucrose. Methods Enzymol. XXXI (1974) 90–94.
[7] J.D. Baxter, G.M. Tomkins, Specific cytoplasmic glucocorticoid hormone receptors in hepatoma tissue culture cells, Proc. Natl. Acad. Sci. 68 (1971) 932–937.
[8] R.J. Borski, Nongenomic membrane actions of glucocorticoids in vertebrates, Trends Endocrinol. Metab. 11 (2000) 427–436.
[9] M.M. Bradford, A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding, Anal. Biochem. 72 (1976) 248–254.
[10] C.W. Breuner, M. Orchinik, Pharmacological characterization of intracellular, membrane, and plasma binding sites for corticosterone in house sparrows, Gen. Comp. Endocrinol. 163 (2009) 214–224.
[11] C.W. Breuner, M. Orchinik, Beyond carrier proteins. Plasma binding proteins as mediators of corticosteroid action in vertebrates, J. Endocrinol. 175 (2002) 99–112.
[12] N. Bruchovsky, J.D. Wilson, The conversion of testosterone to 5α-androstan-17-β-ol-3-one by rat prostate *in vivo* and *In vitro*, J. Biol. Chem. 243 (1968) 2012–2021.
[13] L.F. Canosa, N.R. Ceballos, Seasonal changes in testicular steroidogenesis in the toad *Bufo arenarum* H, Gen. Comp. Endocrinol. 125 (2002) 426–434.
[14] L.F. Canosa, N.R. Ceballos, *In vitro* hCG and human recombinant FSH actions on testicular steroidogenesis in the toad *Bufo arenarum*, Gen. Comp. Endocrinol. 126 (2002) 318–324.
[15] L.F. Canosa, A.G. Pozzi, C. Rosemblit, N.R. Ceballos, N.R., Steroid production in toad. J. Steroid Biochem. Molec. Biol. 85 (2003) 227-233.
[16] L.F. Canosa, A.G. Pozzi, G.M. Somoza, N.R. Ceballos, Effects of mammalian GnRH on testicular steroidogenesis in the toad *Bufo arenarum*, Gen. Comp. Endocrinol. 127 (2002) 174–180.
[17] L.F. Canosa, A.G. Pozzi, N.R. Ceballos, Pregnenolone and progesterone metabolism by the testes of *Bufo arenarum*, J. Comp. Physiol. B 168 (1998) 491–496.
[18] J. Carlstedt-Duke, J.A. Gustafsson, S.A. Gustafsson, O. Wrange, Interactions of corticosterone, 5α-dihydrocorticosterone and dexamethasone with proteins in rat-liver cytosol, Eur. J. Biochem. 73 (1977) 231–238.
[19] G.P. Chrousos, Stress and disorders of the stress system, Nat. Rev. Endocrinol. 5 (2009) 374–381.
[20] J.C. Coffey, F.S. French, S.D. Nayfeh, Metabolism of progesterone by rat testicular homogenate. IV. Further studies of testosterone formation in immature testes *in vitro*, Endocrinology 89 (1971) 865–872.
[21] G.M. Cooke, F. Pothier, B.D. Murphy, The effect of progesterone, 4,16-androstadien-3-one and MK-434 on the kinetics of pig testis microsomal testosterone-4-ene-5α reductase activity, J. Steroid Biochem. Molec. Biol. 60 (1997) 353–359.
[22] D. Denari, N.R. Ceballos, 11-hydroxysteroid dehydrogenase in the testis of *Bufo arenarum*: changes in its seasonal activity, Gen. Comp. Endocrinol. 143 (2005) 113–120.
[23] D. Denari, N.R. Ceballos, Cytosolic glucocorticoid receptor in the testis of *Bufo arenarum*. Seasonal changes in its binding parameters, Gen. Comp. Endocrinol. 147 (2006) 247–254.
[24] S.J. Evans, T.F. Murray, F.L. Moore, Partial purification and biochemical characterization of a membrane glucocorticoid receptor from an amphibian brain, J Steroid Biochem. Molec. Biol. 72 (2000) 209–221.
[25] N.K. Evanson, J.P. Herman, R.R. Sakai, E.G. Krause, Nongenomic actions of adrenal steroids in the central nervous system, J. Neuroendocrinol. 22 (2010) 846–861.
[26] J.J. Fernández Solari, A.G. Pozzi, N.R. Ceballos, Seasonal changes in the activity of the cytochrome $P450_{c17}$ from the testis of *Bufo arenarum*, J. Comp. Physiol. B 172 (2002) 685–690.
[27] J.W. Funder, Glucocorticoid and mineralocorticoid receptors: biology and clinical relevance, Annu. Rev. Med. 48 (1997) 231–240.
[28] D.R. Frost, T. Grant, J. Faivovich, R.H. Bain, A. Haas, C.F.B. Haddad, R.O. de Sá, A. Channing, M. Wilkinson, S.C. Donnellan, C.J. Raxworthy, J.A. Campbell, B.L. Blotto, P. Moler, R.C. Drewes, R.A. Nussbaum, J.D. Lynch, D.M. Green, W.C. Wheeler, The amphibian tree of life, Bull. Am. Mus. Nat. Hist. 297 (2006) 1–370.
[29] H-B. Gao, R-S. Ge, V. Lakshmi, A. Marandici, M.P. Hardy, Hormonal regulation of oxidative and reductive activities of 11β-hydroxysteroid dehydrogenase in rat Leydig cells. Endocrinology 138 (1997) 156–161.
[30] H-B. Gao, L-X. Shan, C. Monder, M.P. Hardy, Suppression of endogenous corticosterone levels in vivo increases the steroidogenesis capacity of purified rat Leydig cells *in vitro*. Endocrinology 137 (1996) 1714–1418.

[31] R.S. Ge, M.P. Hardy, Initial predominance of the oxidative activity of type 1 11β-hydroxysteroid-dehydrogenase in primary rat Leydig cells and transfected cell lines, J. Androl. 21 (2000) 303–310.
[32] R.S. Ge, Q. Dong, E-M. Niu, C.M. Sottas, D.O. Hardy, J.F. Catterall, S.A. Latif, D.J. Morris, M.P. Hardy, 11β-Hydroxysteroid-dehydrogenase 2 in rat Leydig cells: its role in blunting glucocorticoid action at physiological levels of substrate, Endocrinology 146 (2005) 2657–2661.
[33] S.W. Golf, F. Bepperling, V. Graef, Effect of 5α-dihydrocorticoids on enzymes of gluconeogenesis in rat liver, Steroids 43 (1984) 85–91.
[34] K.S. Hirsch, C.D. Jones, J.E. Audia, S. Andersson, L. McQuaid, N.B. Stamm, B.L. Neubauer, P. Pennington, R.E. Toomey, D.W. Russell, LY191704: A selective, nonsteroidal inhibitor of human steroid 5α-reductase type I, Proc. Natl. Acad. Sci. USA 90 (1993) 5277–5281.
[35] D.E. Kime, E.A. Hews, Androgen biosynthesis *in vitro* by testes from Amphibia, Gen. Comp. Endocrinol. 35 (1978) 280–288.
[36] S.A. Latif, M. Shena, R-S. Geb, C.M. Sottas, M.P. Hardy, D.J. Morris, Role of 11-OH-C19 and C21 steroids in the coupling of 11β-HSD1 and 17β-HSD3 in regulation of testosterone biosynthesis in rat Leydig cells, Steroids 76 (2011) 682–689.
[37] K.J. McInnes, C.J. Kenyon, K.E. Chapman, D.E. Livingstone, L.J. Macdonald, B.R. Walker, R. Andrew, 5α-reduced glucocorticoids, novel endogenous activators of the glucocorticoid receptor, J. Biol. Chem. 279 (2004) (2004) 22908–22912.
[38] W. Miller, Regulation of steroidogenesis by electron transfer, Endocrinology 146 (2005) 2544–2550.
[39] I.T. Moore, T.S. Jessop, Stress, reproduction, and adrenocortical modulation in amphibians and reptiles, Horm. Behav. 43 (2003) 39–47.
[40] C.H. Muller, Plasma 5-dihydrotestosterone and testosterone in the bullfrog, Rana catesbeiana: Stimulation by bullfrog LH, Gen. Comp. Endocrinol. 33 (1977) 214–227.
[41] M. Nixon, R. Upreti, R. Andrew, 5-reduced glucocorticoids – A story of natural selection, J. Endocrinol. (2011), doi:10.1530/JOE-11-0212.
[42] K. Normington, D.W. Russel, Tissue distribution and kinetic characteristics of rat 5α-reductase isozymes, J. Biol. Chem. 267 (1992) 19548–19554.
[43] M. Orchinik, L. Matthews, P.J. Gasser, Distinct specificity for corticosteroid binding sites in amphibian cytosol, neuronal membranes, and plasma, Gen. Comp. Endocrinol. 118 (2000) 284–301.
[44] M. Orchinik, T.F. Murray, F.L. Moore, A corticosteroid receptor in neuronal membranes, Science 252 (1991) 1848–1851.
[45] T.E. Orr, D.R. Mann, Role of glucocorticoid in the stress-induced suppression of testicular steroidogenesis in adult male rats, Horm. Behav. 26 (1992) 350–363.
[46] T.E. Orr, M.F. Taylor, A.K. Bhattacharyya, D.C. Collins, D.R. Mann, Acute immobilization stress disrupts testicular steroidogenesis in adult male rats by inhibiting the activities of 17α-hydroxylase and 17,20-liase without affecting the binding of LH/hCG, J. Androl. 15 (1994) 302–308.
[47] R.J. Pennington, Biochemistry of dystrophic muscle: mitochondrial succinate-tetrazolium reductase and adenosine triphosphatase, Biochem. J. 80 (1961) 649–654.
[48] A.G. Pozzi, C.P. Lantos, N.R. Ceballos, Subcellular localization of 3β-hydroxysteroid dehydrogenase isomerase in testis of *Bufo arenarum* H, Gen. Comp. Endocrinol. 106 (1997) 400–406.
[49] J.B. Pramuk, T. Robertson, J.W. Sites Jr., B.P. Noonan, Around the world in 10 million years: biogeography of the nearly cosmopolitan true toads (*Anura: Bufonidae*), Global Ecol. Biogeogr. 17 (2008) 72–83.
[50] A.J. Rapkin, M. Morgan, L. Goldman, D.W. Brann, D. Simone, V.B. Mahesh, Progesterone metabolite allopregnanolone in women with premenstrual syndrome, Obstet. Gynecol. 90 (1997) 709–714.
[51] L.M. Romero, Seasonal changes in plasma glucocorticoid concentrations in free-living vertebrates, Gen. Comp. Endocrinol. 128 (2002) 1–24.
[52] D.W. Russell, J.D. Wilson, Steroid 5α-reductase: two genes/two enzymes, Annu. Rev. Biochem. 63 (1994) 25–61.
[53] R.M. Sapolsky, Stress-induced suppression of testicular function in the wild baboon: role of glucocorticoids, Endocrinology 116 (1985) 2273–2278.
[54] R.M. Sapolsky, M.L. Romero, A.U. Munck, How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions, Endocr. Rev. 21 (2000) 55–89.
[55] H. Schmidt, I. Noack, K.D. Voigt, Metabolism and mode of action of androgens in target tissues of male rats. II. Mode of action of testosterone and 5-dihydrotestosterone at a cellular level on seminal vesicles and prostates of rats. Acta Endocrinol. (Copenh) 69 (1972) 165–173.
[56] P.K. Sitteri, J.D. Wilson, Testosterone formation and metabolism during male sexual differentiation in the human embryo, J. Clin. Endocrinol. Metab. 38 (1974) 113–125.
[57] P.N. Span, Th.J. Benraad, C.G.J. Sweep, A.G.H. Smals, Kinetic analysis of rat steroid 5α-reductase activity in prostate and epididymis homoenates at neutral pH: Evidence for type I activity in epididymis, J. Steroid Biochem. Molec. Biol. 57 (1996) 95–101.
[58] M. Wada, J.C. Wingfield, A. Gorbman, Correlation between blood levels of androgen and sexual behaviour in male leopard frog Rana pipiens, Gen. Comp. Endocrinol. 29 (1976) 72–77.
[59] Q. Wang, P. Zhang, H.B. Gao, Luteinizing hormone induces expression of 11β-hydroxysteroid dehydrogenase type 2 in rat Leydig cells, Reprod. Biol. Endocrinol. 4 (2009) 7–39.
[60] R.S. Wright, A reagent for the non-destructive location of steroids and some other lipophilic materials on silica gel thin layer chromatograms, J. Chromatogr. 59 (1971) 220–221.
[61] Y-C. Xiao, Y-D. Huang, D.O. Hardy, X-K. Li, R-S. Ge, Glucocorticoid Suppresses Steroidogenesis in Rat Progenitor Leydig Cells. J. Androl. 31 (2010) 365–371.
[62] C.A. Yang, M. Nixon, C.J. Kenyon, D.E.W. Livingstone, R. Duffin, A.G. Rossi, B.R. Walker, R. Andrew, 5-reduced glucocorticoids exhibit dissociated anti-inflammatory and metabolic effects, British J. Pharmacol. (2011). doi 4 10.1111/j.1476-5381.2011.01465.x.
[63] C. Yding Andersen, Possible new mechanism of cortisol action in female reproductive organs: physiological implications of the free hormone hypothesis, J. Endocrinol. 173 (2002) 211–217.