11β-Hydroxysteroid dehydrogenase is a predominant reductase in intact rat Leydig cells

C M Leckie, L A M Welberg and **J R Seckl**

Molecular Endocrinology Laboratory, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, UK

(Requests for offprints should be addressed to J R Seckl, Molecular Endocrinology Unit, Molecular Medicine Centre, University of Edinburgh, Western General Hospital, Edinburgh EH4 2XU, UK)

Abstract

11β-Hydroxysteroid dehydrogenases (11β-HSDs) interconvert active corticosterone and inert 11-dehydrocorticosterone. In tissue homogenates, 11β-HSD type 1 (11β-HSD-1) exhibits both 11β-dehydrogenase (corticosterone inactivating) and 11β-reductase (corticosterone regenerating) activities, whereas 11β-HSD type 2 (11β-HSD-2) is an exclusive dehydrogenase. In the rat testis, 11β-HSD has been proposed to reduce glucocorticoid inhibition of testosterone production, promoting puberty and fertility. This hypothesis presupposes dehydrogenation predominates. 11β-HSD-1 immunoreactivity has been localised to Leydig cells. However, recent studies suggest that 11β-HSD-1 is predominantly an 11β-reductase in many intact cells. We therefore examined the expression and reaction direction of 11β-HSD isozymes in cultures of intact rat Leydig cells.

Reverse transcriptase PCR demonstrated expression of 11β-HSD-1, but not 11β-HSD-2 mRNA in rat testis. Primary cultures of intact rat Leydig cells showed predominant 11β-reductase activity, activating 50–70% of 11-dehydrocorticosterone to corticosterone over 3 h, whereas 11β-dehydrogenation was <5%. Although both dexamethasone (10 nM) and corticosterone (1 μM) modestly inhibited LH-stimulated testosterone production by Leydig cells, inert 11-dehydrocorticosterone (1 μM) had similar effects, suggesting 11β-reductase is functionally important. Carbenoxolone (10^{-5} M) inhibited 11β-reduction in intact Leydig cells. However, although carbenoxolone reduced Leydig cell testosterone production, this also occurred in the absence of glucocorticoids, suggesting effects distinct from modulation of corticosteroid access to Leydig cells.

In conclusion, rat Leydig cell 11β-HSD-1 is unlikely to reduce glucocorticoid access to testicular receptors. More likely, 11β-reductase amplifies glucocorticoid action, perhaps to maintain Leydig cell metabolic and endocrine functions.

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Introduction

11β-Hydroxysteroid dehydrogenase (11β-HSD) catalyses the interconversion of active glucocorticoids (cortisol, corticosterone) and inert 11-keto forms (cortisone, 11-dehydrocorticosterone), thus determining glucocorticoid access to intracellular receptors (Monder & White 1993). Two isozymes have been identified, the products of distinct genes (Seckl 1993, White *et al.* 1997). 11β-HSD-type 2 (11β-HSD-2) is a high affinity, NAD-dependent, exclusive dehydrogenase, largely confined to aldosterone target tissues and the placenta (Albiston *et al.* 1994, Brown *et al.* 1996). 11β-HSD-2 excludes glucocorticoids from intrinsically non-selective mineralocorticoid receptors in the distal nephron and null mutations of the 11β-HSD-2 gene are responsible for the clinical features of the syndrome of apparent mineralocorticoid excess (Mune *et al.* 1995, Stewart *et al.* 1996, White *et al.* 1997).

In contrast, considerable debate persists over the possible role of 11β-HSD type 1 (11β-HSD-1). This lower affinity isozyme was first isolated and cloned from rat liver (Lakshmi & Monder 1988, Agarwal *et al.* 1989) and is widely expressed, with highest activity in liver, kidney, lung and testis, at least in the rat (Monder & White 1993). So prominent was expression of 11β-HSD-1 mRNA in rat testis that a testicular library was used to isolate the human cDNA (Tannin *et al.* 1991). Recent studies have suggested that rat testicular 11β-HSD-1 is induced around puberty in the Leydig cell (Neumann *et al.* 1992), spawning the notion that it acts as an 11β-dehydrogenase, reducing inhibition of testosterone production by glucocorticoids (Phillips *et al.* 1989). This view was supported by the effects of enzyme inhibitors, which potentiate the otherwise modest inhibition of testosterone production by corticosterone (Monder *et al.* 1994*a*). In rat models of social and sexual dominance–subordinacy, the correlations of testicular 11β-HSD activity, inversely with corticosterone and directly with testosterone, further supported the hypothesised role of testicular 11β-HSD-1 as a 'gating-mechanism' to reduce glucocorticoid

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inhibition of testosterone production (Monder *et al.* 1994*b*).

However, we and others have recently shown that, whereas 11β-HSD-1 is bidirectional in homogenates and organelle preparations, 11β-reduction (regeneration of active corticosterone from inert 11-dehydrocorticosterone) often predominates in intact cells. 11β-Reductase predominance is seen in most transfected cells (Duperrex *et al.* 1993, Low *et al.* 1994) and in primary cultures of rat hepatocytes (Jamieson *et al.* 1995), lung cells (Hundertmark *et al.* 1995), neurons (Rajan *et al.* 1996), vascular smooth muscle cells (Brem *et al.* 1995) and human adipose cells (Bujalska *et al.* 1997). Moreover, 11β-HSD-1 reductase appears functionally important, since it amplifies glucocorticoid action via glucocorticoid receptors (GR) in transfected cells (Low *et al.* 1994) and primary cultures (Hundertmark *et al.* 1995, Rajan *et al.* 1996). This amplification of glucocorticoid action would not conform with the proposed testicular 'barrier' role for 11β-HSD. The present study therefore re-examined rat testicular 11β-HSD, to determine the isozymes present and the reaction direction in intact cells.

Materials and Methods

All sterile cell culture disposable plastic ware was obtained from Costar UK Ltd (High Wycombe, Bucks, UK), and liquid cell culture products from Gibco BRL (Paisley, UK). [1,2,6,7-^{3}H]Corticosterone ([^{3}H]corticosterone) was obtained from Amersham International (Aylesbury, Bucks, UK). The tritiated metabolite of [^{3}H]corticosterone, [1,2,6,7-^{3}H]11-dehydrocorticosterone ([^{3}H]11-dehydrocorticosterone) was prepared using human placental extract as described previously (Leckie *et al.* 1995).

PCR

Total RNA was isolated from rat kidney, liver and testes (RNeasy Total RNA Kit, Qiagen, Surrey, UK) and 1 μg was reverse transcribed (Reverse Transcription System, Promega, Southampton, Hants, UK). The cDNA was denatured at 96 °C for 15 min and subjected to 30 cycles of PCR (96 °C for 30 s, 55 °C for 45 s and 72 °C for 90 s, plus a final elongation step at 72 °C for 10 min) with primers designed to the rat 11β-HSD-1 sequence (forward 5′-AAAGCTTGTCAC(AT)GGGGCCAGCAAA, reverse 5′-AGGATCCA(AG)AGCAAACTTGCTTGCA) and the rat 11β-HSD-2 sequence (forward 5′-TGCTGCA GATGGACCTGACCAA, reverse 5′-TAGTAGTGG ATGAAGTACATGAGC).

Leydig cell isolation

The testes were removed from four adult male Han–Wistar rats (250 g) and placed in warmed Medium 199 containing Hanks' salts (Gibco) supplemented with 0·5 mg/ml BSA. Testes were decapsulated, trimmed of blood vessels and placed in pairs in 7 ml Medium 199 containing Hanks' salts supplemented with 20 mM Hepes buffer, 0·5 mg/ml collagenase (Worthington Lorne Laboratories, Twyford, UK), 0·1% soybean trypsin inhibitor (Sigma Chemical Co., Poole, Dorset, UK) and 1·5 mg/ml BSA, and placed in a shaking waterbath at 37 °C for 40–50 min. The dissociated cells were diluted in Medium 199 containing Hanks' salts and 0·5 mg/ml BSA and the seminiferous tubules were allowed to settle before filtering the supernatant through 60 μm nylon gauze. The resulting cell suspension was centrifuged at 160 ***g*** for 10 min at room temperature and resuspended in 10 ml Medium 199 containing Hanks' salts and 0·5 mg/ml BSA. The crude interstitial preparation was applied to a Percoll gradient consisting of five 10 ml layers of Percoll with densities of 1·09, 1·07, 1·05, 1·03 and 1·00 g/ml (formed by mixing isotonic Percoll (Percoll diluted 9:1 with 10 × Medium 199 containing Hanks' salts) with Medium 199 containing Hanks' salts). Five millilitres of cells were added to each of two Percoll gradients and centrifuged at 500 ***g*** for 24 min at room temperature. Cells were collected from the 1·05/1·07 g/ml interface, diluted in Medium 199 containing Hanks' salts and 0·5 mg/ml BSA and centrifuged at 200 ***g*** for 10 min at room temperature. The cells were counted, diluted to 250 000 cells/ml and plated on 24-well plates in a volume of 1 ml in Medium 199 containing Earle's salts and supplemented with 0·5 mg/ml BSA, 2 mM L-glutamine, 100 IU/ml penicillin and 100 μg/ml streptomycin.

The proportion of Leydig cells present in the culture was determined by staining for 3β-hydroxysteroid dehydrogenase (Payne *et al.* 1980). Cells were incubated overnight at 34 °C and the medium removed and replaced with a solution prepared by mixing 1 mg nitro-blue tetrazolium dissolved in 0·6 ml 1 mg/ml 5β-androston-3β-ol-17-one in dimethylsulphoxide with 10 mg β-NAD in 9·5 ml Dulbecco's PBS. The cells were returned to the incubator for several hours, the solution was removed and the cells were fixed in 10% formalin in Dulbecco's PBS. The proportion of stained cells was typically >90%.

11β-HSD assay

To assay 11β-HSD in intact Leydig cells, the cells were incubated for 1, 2, 5 or 6 days at 34 °C and the medium removed and replaced with medium containing 25 nM corticosterone or 11-dehydrocorticosterone with 2 nM [^{3}H]corticosterone or [^{3}H]11-dehydrocorticosterone respectively as tracer. Two hundred microlitres medium were removed at 2, 3 or 6 h, tritiated steroids extracted in 1 ml ethyl acetate, the upper organic phase removed, evaporated under air and the steroids resuspended in 100 μl ethanol containing 2·5 mg/ml each of corticosterone and 11-dehydrocorticosterone. Steroids were

separated on TLC plates (Merck, Hoddesdon, Herts, UK) in chloroform:95% ethanol (92:8); bands were visualised under UV light and scraped into scintillation vials containing 1 ml liquid scintillant (Cocktail T, BDH, Poole, Dorset, UK), as previously described (Rajan *et al.* 1996). Steroid conversion was calculated from the radioactivity in each fraction expressed as [product]/[substrate+product]. Recovery of radioactivity was >98% and no significant bands of radioactivity were found on the TLC plates outside the recovery areas of corticosterone and 11-dehydrocorticosterone.

Testosterone assay

Cells were cultured in medium containing 10 nM dexamethasone, 1 μM corticosterone or 11-dehydrocorticosterone in the presence and absence of 10 μM carbenoxolone overnight. The medium was removed after 18 h and replaced with medium containing the appropriate steroids, with or without carbenoxolone, supplemented with 100 ng/ml ovine luteinising hormone (LH). The medium was removed after 6 h and frozen for analysis of testosterone by RIA (Webb *et al.* 1985).

Results

Reverse transcription PCR (RT-PCR) amplified 11β-HSD-1 transcripts of the anticipated size from RNA derived from rat liver, kidney and testis. In contrast, 11β-HSD-2 transcripts were not detected in testis, although a strong band of the predicted size was amplified from kidney (Fig. 1) and a weaker band from liver.

Medium containing 25 nM corticosterone or 11-dehydrocorticosterone was added to the cultured Leydig cells after 1, 2, 5 and 6 days of culture. After 3 h, 200 μl medium were removed for measurement of steroid conversion on each day of measurement. Over this time in culture, 11β-dehydrogenase activity (corticosterone to 11-dehydrocorticosterone conversion) remained below 5% (Fig. 2). In contrast, 11β-reductase was clearly detected with 50–70% of 11-dehydrocorticosterone metabolised to corticosterone over the 3 h incubation period on all days of assessment. These data suggest that in intact Leydig cells 11β-HSD activity is primarily in the 11β-reductase direction and that this activity is maintained over at least a week in culture. Carbenoxolone pretreatment of intact Leydig cells in culture inhibited 11β-reductase activity with an ED_{50} of $\sim 5 \times 10^{-6}$ M (Fig. 3). The small amount of dehydrogenase activity present was also inhibited by carbenoxolone, and this occurred at a lower concentration of carbenoxolone (ED_{50} of $\sim 5 \times 10^{-7}$ M). An alternative 11β-HSD inhibitor, glycyrrhetinic acid, also inhibited 11β-HSD activity in intact Leydig cells with an ED_{50} of $\sim 10^{-6}$ M.

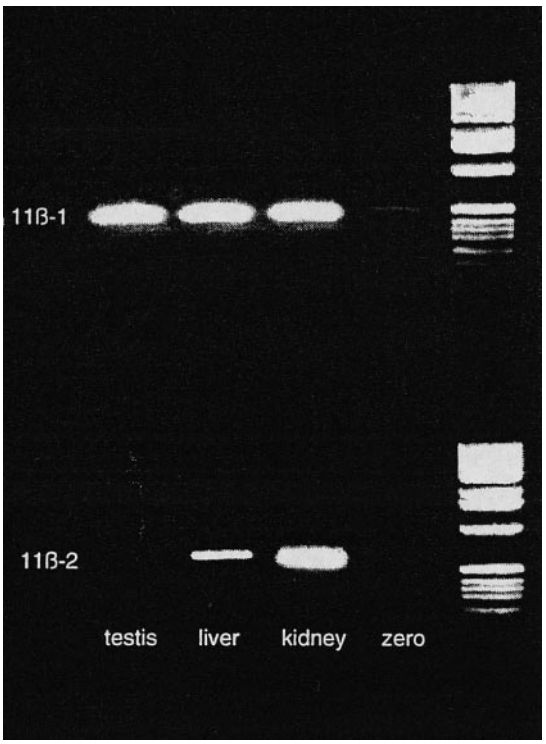

Figure 1 Expression of 11β-HSD-1, but not 11β-HSD-2, transcripts in rat testis following RT-PCR. Note the presence of 11β-HSD-1 mRNA in testis, liver and kidney, whereas 11β-HSD-2 mRNA is detected in the kidney (positive control), but not in the testis (weak expression of 11β-HSD-2 transcripts in liver may reflect the documented expression in biliary ducts).

LH (100 ng/ml) stimulated testosterone production from cultured Leydig cells. This stimulation was inhibited by pretreatment of the Leydig cells with 10 nM

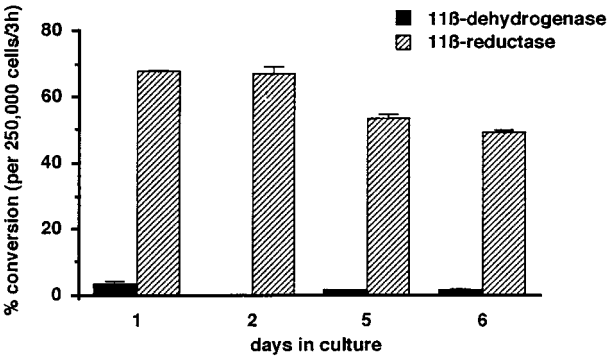

Figure 2 11β-HSD activity in both 11β-dehydrogenase and 11β-reductase directions in intact rat Leydig cells in primary culture for periods of 1, 2, 5 and 6 days. Enzyme activity was assessed with addition of [^{3}H]corticosterone and [^{3}H]11-dehydrocorticosterone respectively, and estimation of the production of steroid product per 250 000 cells over 3 h. Note the marked predominance of 11β-reductase throughout the period of culture.

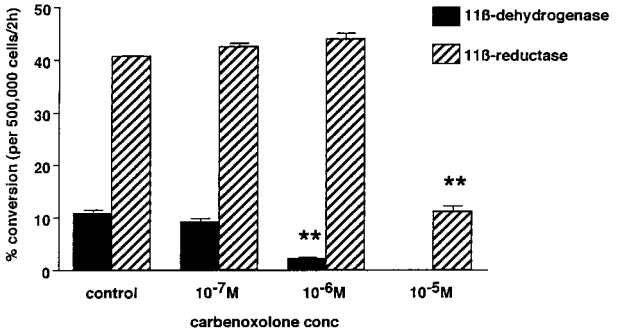

Figure 3 Effects of carbenoxolone upon 11β-reductase and 11β-dehydrogenase in rat Leydig cells in primary culture for 6 days. Enzyme activity was assessed with addition of [^{3}H]corticosterone and [^{3}H]11-dehydrocorticosterone respectively, with estimation of the production of steroid product per 500 000 cells over 2 h. $^{**}P<0{\cdot}01$ compared with control.

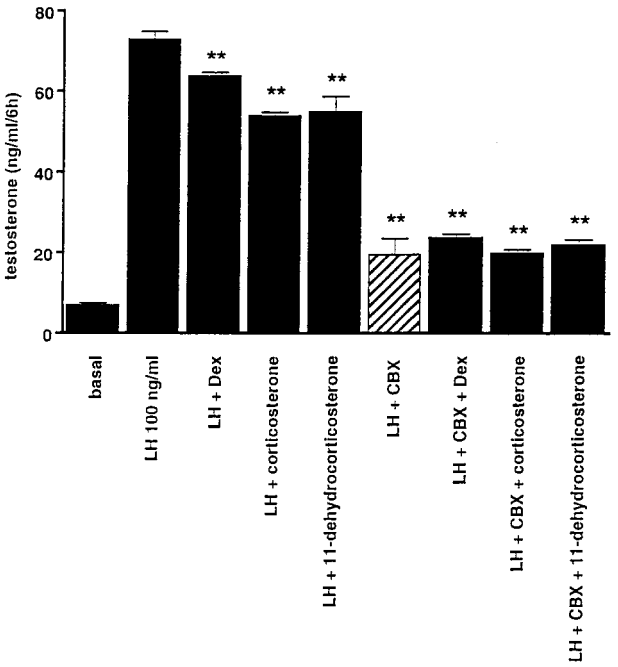

Figure 4 Effects of glucocorticoids (dexamethasone (Dex), corticosterone and 11-dehydrocorticosterone) and carbenoxolone (CBX) upon LH-stimulated testosterone production by rat Leydig cells in primary culture. $^{**}P<0{\cdot}01$ compared with LH alone. Note the modest inhibition of LH-stimulated testosterone production by dexamethasone (10 nM) and corticosterone (1 μM). Otherwise inert 11-dehydrocorticosterone (1 μM) has similar effects to corticosterone, suggesting 11β-reductase activity may be functionally important. Also note the direct and more potent inhibitory effects of carbenoxolone (10 μM) alone in the absence of corticosteroids (hatched bar) than glucocorticoids themselves and the lack of additional effects of corticosteroids with carbenoxolone.

dexamethasone, 1 μM corticosterone and 1 μM 11-dehydrocorticosterone (Fig. 4). Ten micromolar carbenoxolone, a concentration required to inhibit appreciably 11β-reductase activity, itself inhibited testosterone production from Leydig cells in culture in the absence of any added steroid (Fig. 4); addition of glucocorticoids to carbenoxolone had no additional effect upon LH-stimulated testosterone production. It was therefore clearly impossible to determine the effect of carbenoxolone on the inhibition of testosterone production by glucocorticoids.

Discussion

Sensitive RT-PCR showed 11β-HSD-1, but no 11β-HSD-2, mRNA expression in the rat testis. The data confirm previous reports of 11β-HSD-1 mRNA and immunoreactivity in the rat testis (Agarwal *et al.* 1989, Monder & Lakshmi 1990) and the absence of 11β-HSD-2 transcripts in rat testicular extracts (Zhou *et al.* 1995). Immunocytochemical studies have suggested that 11β-HSD-1 is localised to the Leydig cell (Phillips *et al.* 1989), although the presence on Western blots of testicular extracts of immunoreactive species smaller than the presumed authentic 34 kDa 11β-HSD-1 may reflect some polyspecificity of the antisera employed (Agarwal *et al.* 1989, Monder & Lakshmi 1990). Our unpublished *in situ* hybridisation data show high 11β-HSD-1 mRNA expression in the interstitium, compatible with the immunolocalisation.

11β-HSD in intact rat Leydig cells in culture was a predominant 11β-reductase. These results conform with most previous studies of 11β-HSD-1 in intact cells (Duperrex *et al.* 1993, Low *et al.* 1994, Hundertmark *et al.* 1995, Jamieson *et al.* 1995, Rajan *et al.* 1996), and more recently, with the predominant reaction direction in whole organs (Jamieson *et al.* 1997) and *in vivo* (Kotelevtsev *et al.* 1997). However, Monder *et al.* (1994*a*) found significant 11β-dehydrogenation in rat Leydig cells, although reductase activity was not determined. More recently, Gao *et al.* (1997) reported bidirectional 11β-HSD activity, with predominant 11β-dehydrogenation, in rat Leydig cells. The reason for the discrepancies between these studies and our own is unclear. In the studies of Gao *et al.*, Leydig cells were harvested from culture dishes and taken into suspension before assay of reaction direction. 11β-HSD-1 shows predominant 11β-reduction in intact cells, but is bidirectional in homogenates or even when damaged cells are present (Low *et al.* 1994, Jamieson *et al.* 1995, Rajan *et al.* 1996). Moreover, in homogenates 11β-dehydrogenation is apparently more stable than 11β-reduction, so even limited cellular disruption will favour dehydrogenation, a contention supported by the detection by these authors of dehydrogenation in similarly treated hepatocytes, whereas activity in undisturbed hepatocyte cultures and in intact liver is predominantly reductive (Jamieson *et al.* 1995, 1997). Alternative explanations, of

possible strain differences in Leydig cell reaction direction or the existence of novel 11β-HSD isozymes in Leydig cells, lack experimental support. Moreover, two further results suggest that 11β-HSD-1 functions as a reductase. First, the effects of dexamethasone and corticosterone to inhibit testosterone production by Leydig cells were similar, which does not support the notion that physiological glucocorticoid effects are modulated by 11β-dehydrogenase. Secondly, otherwise inert 11-dehydrocorticosterone was as potent as corticosterone in inhibiting the output of testosterone from Leydig cell cultures. Thus, it is probable that Leydig cell 11β-HSD-1 is activating 11-dehydrocorticosterone to corticosterone, which itself reduces testosterone production.

Our data also confirm previous work (Monder *et al.* 1994*a*) and show that glucocorticoids modestly inhibit LH-stimulated testosterone production by Leydig cells (Welsh *et al.* 1982). If 11β-dehydrogenation reduces this action, then 11β-HSD inhibitors should amplify the effects of corticosterone. However, carbenoxolone alone, at the minimum concentration to inhibit 11β-HSD-1 in rat Leydig cells, itself markedly reduced the production of testosterone in response to LH. This effect occurred in the absence of glucocorticoids and was of considerably greater magnitude than the action of even the potent synthetic glucocorticoid dexamethasone, which is not a substrate for 11β-HSD-1. Although the mechanism of this effect is obscure, it renders impossible the determination of whether pharmacological inhibition of Leydig cell 11β-HSD-1 has any effect upon the glucocorticoid control of testosterone production.

The function of an 11β-reductase in rat Leydig cells is unknown. GR binding sites and immunoreactivity have been reported in Leydig cells (Neumann *et al.* 1992, Schultz *et al.* 1993). Whilst glucocorticoids suppress gonadotrophin-induced testosterone production by Leydig cells, by inhibiting transcription of steroidogenic enzymes (Welsh *et al.* 1982, Hales & Payne 1989, Payne & Sha 1991), higher doses of corticosterone may stimulate basal testosterone production (Orr & Mann 1992). Indeed, glucocorticoids are necessary for expression of gonadotrophin receptors in Leydig cells (Engel & Frowein 1974). Thus 11β-reductase might amplify gonadotrophin responsiveness. In addition, glucocorticoids are necessary for many constitutive metabolic processes (Miller & Tyrrell 1995). The prominent diurnal rhythm of corticosterone may not provide sufficient ligand during the nadir, when 'free' corticosterone levels (allowing for 90–95% binding by corticosteroid-binding globulin) are very low (Akana *et al.* 1992). In contrast, 11-dehydrocorticosterone levels are around 50 nM in rat plasma (R Best and J R Seckl, unpublished data) and in humans cortisone circulates, largely unbound, at around 100 nM (Walker *et al.* 1992), providing plentiful substrate for an 11β-reductase. Alternatively, glucocorticoids may not be the major substrate for testicular 11β-HSD-1. 11-Hydroxy-androgens or 11-hydroxy-progesterones may affect rat Leydig cell testosterone production (Monder & White 1993), though the presence of 11-hydroxy-progesterone has not been demonstrated in mammalian tissues or human urine (Morita *et al.* 1996). Finally, 11β-HSD activity and 11β-HSD-1 mRNA are absent from mouse (Rajan *et al.* 1995) and squirrel monkey (Moore *et al.* 1993) testis. These data do not suggest that 11β-HSD-1 provides any generic mammalian system to 'gate' glucocorticoid effects in the post-pubertal testes. Indeed male mice with targeted disruption of the 11β-HSD-1 gene are fertile (Kotelevtsev *et al.* 1997).

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