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

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Abstract

11β-Hydroxysteroid dehydrogenases (11β-HSDs) interconvert active corticosterone and inert 11-dehydrocortico-sterone. In tissue homogenates, 11β-HSD type 1 (11β-HSD-1) exhibits both 11β-dehydrogenase (cortico-sterone inactivating) and 11β-reductase (cortico-sterone 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-dehydrocortico-sterone 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-dehydrocortico-sterone (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.


Introduction

11β-Hydroxysteroid dehydrogenase (11β-HSD) catalyses the interconversion of active glucocorticoids (cortisol, corticosterone) and inert 11-keto forms (cortisone, 11-dehydrocortico-sterone), 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. 1994a). 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
inhibition of testosterone production (Monder et al. 1994b).

However, we and others have recently shown that, whereas 11β-HSD-1 is bidirectional in homogenates and organellar 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). [1,2,6,7-3H]Corticosterone ([3H]corticosterone) was obtained from Amersham International (Aylesbury, Bucks, UK). The tritiated metabolite of [3H]corticosterone, [1,2,6,7-3H]11-dehydrocorticosterone ([3H]11-dehydrocorticosterone) was prepared using human placental liquid cell culture products from Gibco BRL (Paisley, UK). [1,2,6,7-3H]Corticosterone ([3H]corticosterone) was obtained from Amersham International (Aylesbury, Bucks, UK). The tritiated metabolite of [3H]corticosterone, [1,2,6,7-3H]11-dehydrocorticosterone ([3H]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 (RNasy 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′-AAAGCTTGTCACATGTTGCCAGCA, reverse 5′-AGGATCCAGGAGCAACCTTGAGCA and the rat 11β-HSD-2 sequence (forward 5′-TGCTGTCAATGGACCTGCAC, reverse 5′-TAGATGTGGGATGAAATCGTC). 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-androstene-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 [3H]corticosterone or [3H]11-dehydrocorticosterone respectively as tracer. Two hundred microliters 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 ED50 of \(2.5 \times 10^{-6}\) M (Fig. 3). The small amount of dehydrogenase activity present was also inhibited by carbenoxolone, and this occurred at a lower concentration of carbenoxolone (ED50 of \(5 \times 10^{-7}\) M). An alternative 11β-HSD inhibitor, glycyrrhetinic acid, also inhibited 11β-HSD activity in intact Leydig cells with an ED50 of \(10^{-6}\) M.

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

**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).

**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 \([3H]\)corticosterone and \([3H]\)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.
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. (1994a) 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. 1994a) 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 liquid 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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References


Hundertmark S, Buhler H, Ragoch V, Dinkelborg L, Arabin B & Weitzel HK 1995 Correlation of surfactant phosphatidylcholine


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