

11 β -hydroxysteroid dehydrogenase type 2 expression in the newly formed Leydig cells after ethane dimethanesulphonate treatment of adult rats

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Abstract: The enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD) catalyzes the reversible conversion of physiologically active corticosterone to the biologically inert 11 β -dehydrocorticosterone in rat testis and protect the Leydig cells (LCs) against the suppressive effect of glucocorticoids. The developmental pathway of the adult LCs population is accompanied with an increase in the 11 β -HSD activity. Thus, 11 β -HSD together with its role in controlling the toxicological effect of glucocorticoids on LCs can be used as a marker for their functional maturity. Ethane 1,2-dimethanesulphonate (EDS) treatment of adult rats become unique appropriate model, which enable to answer many questions related to the differentiation of adult LCs in the prepubertal rat testis. The aim of the present study was to investigate the specific changes in the 11 β -HSD type 2 immunoreactivity in tandem with the expression of androgen receptor (AR) during renewal of LCs population after EDS treatment. In the present study, we observed the first appearance of immunostaining for 11 β -HSD2 in new LCs population on day 14 after EDS administration when the progenitor LCs were detected. Our immunohistochemical analysis revealed progressive increases in the 11 β -HSD2 reaction intensity on 21 days after EDS treatment and reached a maximum on day 35. AR immunexpression was found in new LCs on day 14 and 21 after EDS injection with an increasing curve of intensity. The most prominent AR immunostaining in new population LCs was evident by 35 days after EDS and that coincided with the increased number of LCs and restoration of adult LCs population. Our results demonstrated similar pattern of immunoreactivity for 11 β -HSD2 and AR in new LCs population after EDS treatment and suggested that the changes in 11 β -HSD2 expression can be used for evaluation of adult LCs differentiation in rat testis.

Key words: 11 β -HSD2 - Leydig cells - Testis - EDS

Introduction

The hydroxysteroid dehydrogenases (HSD) are essential for the biosynthesis and metabolism of steroids in Leydig cells (LCs). The enzyme 11 β -hydroxysteroid dehydrogenase (11 β -HSD) catalyzes the reversible conversion of physiologically active corticosterone to the biologically inert 11 β -dehydrocorticosterone in rat

testis. This enzyme is hypothesized to modulate LCs steroidogenesis by controlling the intracellular concentration of glucocorticoids. By doing so, 11 β -HSD can protect the LCs against the suppressive effect of glucocorticoids [1-4].

Two isoforms of 11 β -HSD have been characterized: type 1 and 2. Type 1 enzyme (11 β -HSD1) is NADP(H)-dependent bidirectional enzyme that has wide tissue distribution and functions as either dehydrogenase or an oxidoreductase, converting 11 β -dehydrocorticosterone to corticosterone in several tissues. In contrast, 11 β -HSD type 2 (11 β -HSD2) is unidirectional dehydrogenase that inactivates glucocorticoids and use NAD as cofactor [4-7]. Various reports

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showed that rat LCs contain 11 β -HSD type 1 mRNA and the corresponding protein whereas type 2 was undetectable [1,2,4,5,8]. Recently, it has been established that 11 β -HSD2 is present in rat LCs and the amount of 11 β -HSD2 mRNA was about thousand-fold lower compared with 11 β -HSD type 1 [9,10,11]. Systematic developmental data about the immunohistochemical expression of 11 β -HSD2 in regenerating rat LCs are still lacking.

The postnatal development of the rat LCs includes three distinct functional stages of differentiation-progenitors (PLCs), immature type (ILCs) and adult LCs (ALCs) [12,13]. The developmental pathway of the ALCs population is accompanied with an increase in the 11 β -HSD activity and thus the enzyme can be used as a marker for functional maturation of LCs [4, 14,15,16]. An examination of 11 β -HSD in the LCs revealed that both oxidative and reductive activities were barely detectable in PLCs, intermediate in ILCs, and highest in ALCs. The ratio of the two activities favored reduction in PLCs and ILCs and oxidation in ALCs [1]. ALCs population expresses high levels of 11 β -HSD1 oxidative activity [1,17]. However, the enzymatic behavior of 11 β -HSD in LCs is not consistent with the presence of type 1 alone [16,18]. It is still obscure whether the switch of 11 β -HSD activity from reduction to oxidation during the transition from PLCs to ALCs [1] can be associated with the presence of 11 β -HSD2.

There is strong evidence that androgen action, mediated through the androgen receptor (AR) [19] is required for the onset of ALCs differentiation [20,21]. The pattern of AR expression reflects the importance of androgen action on LCs differentiation and the levels of AR mRNA and protein in the LCs peak in the immature animal and decline with sexual maturity [22]. A possible correlation between the developmental pattern of AR expression and ontogenetic course of 11 β -HSD2 in rat LCs is still not examined.

Ethane 1,2-dimethanesulphonate (EDS) selectively and temporarily destroys ALCs resulting in rapid loss of testosterone (T) production [23,24]. Hence, EDS-treatment of adult rats is unique appropriate model that enables to answer many questions related to the differentiation of ALCs in developing rat testis. The presence of 11 β -HSD in adult testis after EDS administration is still not examined. In this respect the present study aimed to investigate the specific changes in expression of 11 β -HSD2 in tandem with AR during renewal of LCs population after EDS treatment.

Materials and methods

EDS treatment. Mature male Wistar rats were housed under normal light (12L: 12D) and food and water ad libitum and were divided into two groups. One group of the rats received a single intraperitoneal (*i.p.*) injection of EDS (75 mg/kg body weight) dis-

solved in dimethyl sulphoxide (DMSO): water (1:3 v/v). A second group of rats received a single *i.p.* injection only of DMSO: water. EDS was synthesized according the instructions of Jackson and Jackson [25] because it was not available commercially. The animals were killed 24 hours, 7, 14, 21 and 35 days after treatment. Testicular fragments approximately 4-5 mm thick were fixed by immersion in Bouin's solution for 24 hours at room temperature (RT), embedded in paraffin and prepared for routine histological examination (staining with hematoxylin-eosin) and immunohistochemistry.

Immunohistochemistry for 11 β -HSD type 2. Paraffin sections (6 μ m thick) were incubated in 3% (v/v) H₂O₂ in methanol to inhibit endogenous peroxidase activity and treated with 2.0% normal rabbit serum to block non-specific binding. After that the sections were incubated with polyclonal sheep anti 11 β -HSD type 2 antibody (Chemicon, USA, dilution 1:1000) for 24 hours at 4°C in a humid chamber. For the detection of the 11 β -HSD2 immunoreactivity an amplification combination of the peroxidase anti-peroxidase (PAP) and the avidin-biotin-peroxidase complex (ABC) methods was applied [26]. In the next steps biotinylated anti-goat IgG in final dilution 1:250 (provided by Dakopatts, Denmark) and goat PAP (Dakopatts, Denmark, diluted 1:100) were used. In the last step ABC (Vector, USA) at final dilution 1:250 was applied. The peroxidase activity was then developed by 3,3'-diaminobenzidine tetra-hydrochloride (DAB) chromogene substrate kit, (Vector, USA). Sections were dehydrated and coverslipped using Vector-mount (Vector, USA) medium.

As controls, sections were used in which the primary, secondary or tertiary antibodies were replaced by phosphate-buffered saline (PBS) and only the peroxidase activity was visualized.

Immunohistochemistry for AR. Visualization of AR was performed on dewaxed sections (5 μ m) with antigen retrieval (5 min in 0.01 M citrate buffer, pH 6.0). This was followed by endogenous peroxidase blocking in 3% (v/v) H₂O₂ in methanol for 30 min at RT. Tissue sections were blocked in TBS containing normal swine serum (NSS; 1:4) and 5% BSA before an overnight incubation at 4°C with primary antibody diluted in NSS. For detection of AR a rabbit polyclonal antibody clone N20, Santa Cruz Biotechnology, USA, diluted 1:200 was applied. A swine anti-rabbit biotinylated secondary antibody (DAKO, Denmark), diluted 1:500 in NSS was applied for 30 min at RT. Bound antibodies were visualized by incubating the sections with ABC complex/HRP reagent (DAKO, Denmark) for 30 min followed by color development with liquid DAB+kit (DAKO, Denmark), monitored microscopically. Sections were counterstained with hematoxylin, dehydrated and coverslipped using Pertex mounting medium (Cell Path plc, Hemel Hempstead, UK)

The specificity of immunostaining for AR was checked using previously established procedures - this involved preabsorption of the primary antibody with peptide immunogen (Santa Cruz N-20 peptide).

Quantification of immunohistochemistry. The intensity of the reactions for 11 β -HSD2 and AR in LCs were assessed through the special software DP-Soft (Olympus, Japan, version 3, 2 for Windows) on Microphot-SA (Nikon, Japan) microscope, equipped with a Camedia-5050Z (Olympus, Japan) digital camera. The values of intensity were in the interval 0-256, where 0 represents -white and 256 -black. The immune reaction intensity in positive LCs was evaluated in different microscopic fields of each testicular section (at least 100 cells). Mean intensity of antigen expression for each animal of group was calculated.

Statistical analysis. The results were analyzed using one-way ANOVA and depending on homogeneity of variances Games-Howell or Tukey *post hoc* test for intergroup differences in 11 β -

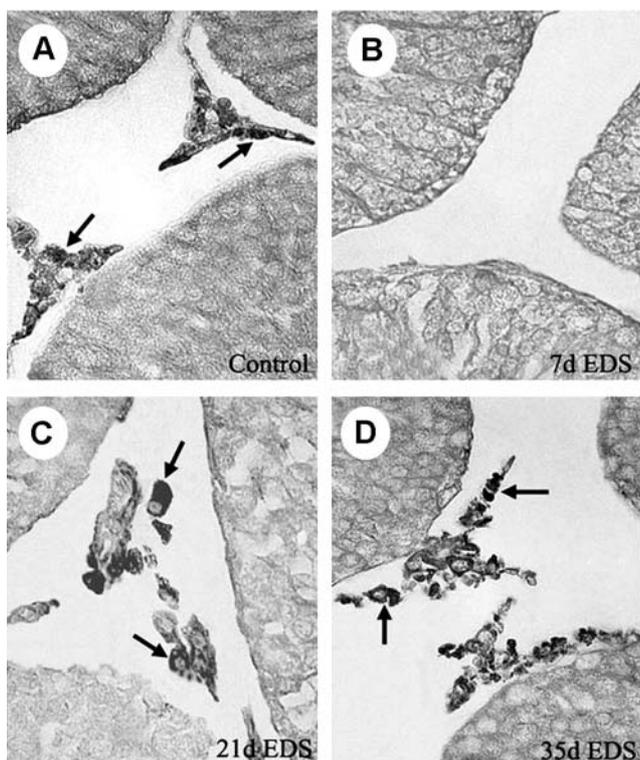


Fig. 1. Rat testis. Immunorexpression of 11 β -HSD2 in the Leydig cells (arrows) after EDS administration $\times 200$. **A.** Control testis. 11 β -HSD type 2 immunoreactivity with strong intensity in the cytoplasm of LCs. **B.** 7 days after EDS administration. Lack of 11 β -HSD2 expression in testis. **C.** 21 days after EDS. Immunoreactivity for 11 β -HSD2 in the newly formed LCs. **D.** 35 days post EDS. 11 β -HSD2 expression with increased staining intensity in the new population of LCs.

HSD2 and AR expression respectively were applied. Statistical significance was set at $P < 0.05$. Data obtained at day 7 after EDS treatment was not included in the statistical analysis because there was a lack of LCs and respectively no immune reaction. Data is presented as mean \pm SEM.

Results

As previously reported EDS induced total loss of LCs within the first week after treatment and they reappeared by 14 day increasing markedly in number following day 21 [27].

In control animals strong intensity of 11 β -HSD type 2 immunoreactivity was detected in the cytoplasm of LCs (Fig. 1A). Seven days after EDS administration the lack of 11 β -HSD2 immunostaining coincided with total loss of LCs in the testis (Fig. 1B). The first single 11 β -HSD2 immunoreactive LCs were detected in the testicular interstitium of experimental rats by 14 days after EDS and on day 21 intense immunolabelling was found in the cytoplasm of newly-formed LCs (Fig. 1C). Maximal intensity of 11 β -HSD2 immunorexpression was observed in the new population of LCs by 35 day after EDS (Fig. 1D).

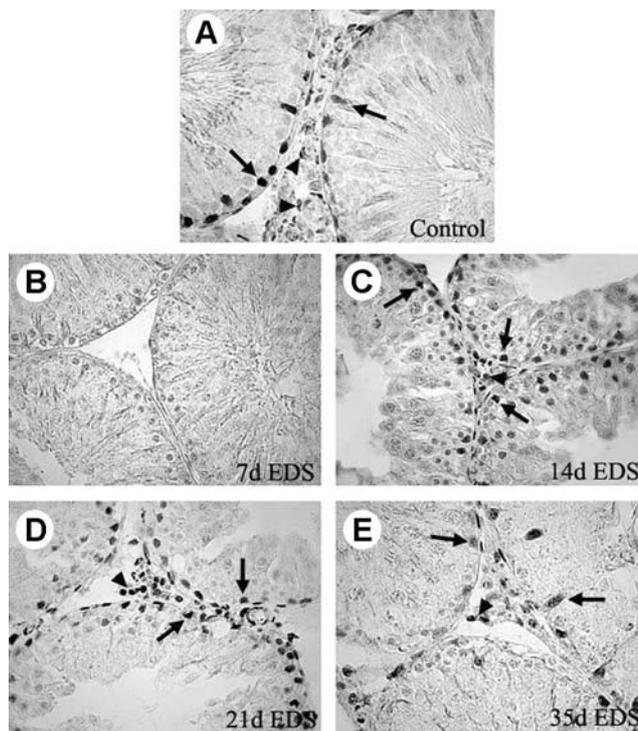


Fig. 2. Rat testis. Immunorexpression of AR in the Leydig cells after EDS treatment. $\times 400$. **A.** Control testis. AR immunoreactivity with nuclear localization in the LCs (arrowheads), Sertoli cells (arrows) and peritubular myoid cells. **B.** 7 days after EDS. Total loss of AR immunoreactivity in testis. **C.** 14 day post EDS. AR immunorexpression in the new LCs. **D.** 21 days after EDS. AR immunoreactivity with an increased staining intensity in repopulation of LCs. **E.** 35 days post EDS. Strong AR immunostaining in new population LCs.

No immunoreactivity was observed in peritubular and Sertoli cells of the testes from control and treated rats.

In the testes of control rats nuclear AR immunoreactivity was found in the LCs, Sertoli cells and peritubular myoid cells (Fig. 2A) whereas stage-specific expression for AR was evident in the Sertoli cells, as reported previously [28,29]. Seven days after EDS administration lack of AR immunoreactivity in the testis was accompanied by complete elimination of LCs (Fig. 2B). AR immunorexpression was found in new LCs on day 14 and 21 after EDS injection (Fig. 2C, D). The most prominent AR immunostaining in new population LCs was evident by 35 days after EDS and that coincided with the increased number of LCs (Fig. 2E) and restoration of adult LCs population.

Quantitative measurements of immunohistochemical reactions using DP-Soft software revealed parallel changes in immunoreactivity for both, 11 β -HSD2 and AR (Fig. 3 and Fig. 4). Increasing curves of intensity of immune reactions are evident following their total lack on day 7th after EDS treatment.

Histological analysis of spermatid differentiation on day 7 after EDS administration revealed loss of

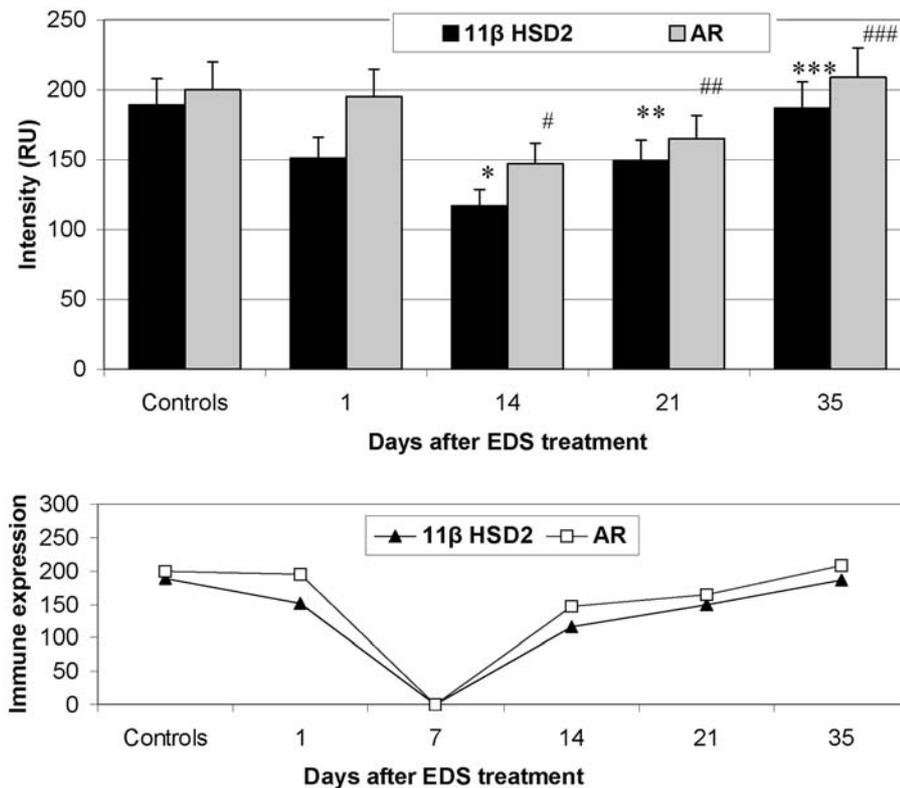


Fig. 3. Intensity of immune reactions for 11 β -HSD2 and AR in the Leydig cells after EDS treatment. *** $P < 0.05$, $P < 0.001$, $P < 0.01$ (in comparison with days 1, 14 and 21 after EDS); ** $P < 0.01$, $P < 0.001$, $P < 0.01$ (in comparison with controls, and days 14 and 35 after EDS); * $P < 0.001$, $P < 0.05$ (in comparison with controls and day 1 after EDS). ### $P < 0.01$, $P < 0.001$, $P < 0.001$ (in comparison with controls, and days 1, 14 and 21 after EDS); # $P < 0.001$ (in comparison with controls, and days 1, 14 after EDS); # $P < 0.001$ (in comparison with controls and day 1 after EDS).

Fig. 4. Quantitative changes in immune expression of 11 β -HSD2 and AR in the Leydig cells after EDS treatment.

elongated spermatids from late stages of the cycle of the seminiferous epithelium. Massive loss of elongated spermatids in all the stages was evident on day 14 after treatment (see Fig. 2B); then they reappeared on day 21 in late stages and on day 35 in all the stages of the cycle (see Fig. 2D).

As we previously have show, a severe drop in T concentration on day 7 after EDS treatment was followed by gradual increase in T levels following day 14 that correlate with loss and recovery of LCs population [28].

Discussion

The enzyme 11 β -HSD has been suggested as a marker for the functional maturity of ALCs in rats [3,4,14,17]. The appearance of 11 β -HSD correlates with the post-natal increase in LCs number, testicular weight, total surface area of the intracellular membranes, and T production by LCs [4]. Neumann *et al.* [15] reported a temporal coincidence of the first appearance of elongated spermatids in the seminiferous epithelium and the first histochemical demonstration of 11 β -HSD in the rat LCs on 35 pnd. By using EDS experimental model the present study provides new data about expression pattern of 11 β -HSD during renewal of LCs population. Our results bring additional elucidation on the role of this enzyme in the process of LCs differentiation. Moreover, together with our previous studies [27] the current data point out the relationship between

11 β -HSD and kinetics of spermatid differentiation and restoration of T production by new LCs population after EDS.

Several authors have demonstrated that 11 β -HSD in LCs is predominantly an oxidase [1,2,3]. Developmental analysis of 11 β -HSD in rat LCs during their pubertal differentiation revealed a reduction/oxidation switch in enzyme activity during the transition from PLCs to ALCs [1]. According to Schafers *et al.* [16], clear recognizable oxidative activity of 11 β -HSD is present from 31 pnd onward, first in single ALCs and later in majority of these cells. It seems that the increase in 11 β -HSD oxidative activity in ALCs is not consistent with the presence of type 1 alone [5,18,30]. Recently it was demonstrated that LCs expressed not only 11 β -HSD type 1, an oxidoreductase but also type 2, an unidirectional oxidase [9,10]. The inhibition of 11 β -HSD1 predominantly lowered reductase activity whereas by inhibition of 11 β -HSD2 alone, the oxidase activity was more prominent suppressed [10].

In the present study, we observed the first appearance of immunostaining for 11 β -HSD2 in new LCs population on day 14 after EDS administration when the PLCs were detected [24,27,31]. Our quantitative immunohistochemical analysis of 11 β -HSD2 pattern revealed progressive increases in the reaction intensity on 21 days after EDS treatment and reached a maximum on day 35 that is a turning point in the development of immature and mature LCs [14]. These changes

in 11 β -HSD2 expression are consistent with previous data related to the process of structural and functional maturation of the new population LCs after EDS [27,31]. Therefore, 11 β -HSD2 can be a useful marker for ALCs differentiation and the course of its reaction intensity might be associated with increased 11 β -HSD oxidative activity that occurred during the transition from PLCs to ALCs in postnatal rat testis [1,16]. Moreover, gene profiling of rat PLCs, immature LCs and ALCs showed increased expression level of 11 β -HSD2 gene and parallel increases in 11 β -HSD2 enzyme activity during postnatal development [11]. Our data for elevated 11 β -HSD2 expression in differentiating LCs after EDS treatment are consistent with above mentioned findings and the view that restoration of new LCs population after EDS recovers the normal dynamics of LCs postnatal development.

Androgen action in the testis, as in other tissues, requires the AR to mediate transcriptional activation [19]. In the testis, AR was expressed in LCs, Sertoli cells, and peritubular myoid cells [29]. The AR level in the nuclei of adult Sertoli cells depends mainly on the level of androgens whereas in the case of LCs and peritubular cells this dependency is more limited [32]. AR expression in the LCs did not vary with the stage of the cycle of the seminiferous epithelium [29]. Several lines of evidence demonstrated the importance of autocrine regulation by androgens in the differentiation of PLCs into immature LCs [20,21] indicated by increased AR level in ILCs [22]. In the present study, the increasing curve of AR immunoreactivity in new LCs following day 14 after EDS is consistent with the appearance of PLCs and their transformation into immature LCs and ALCs [27,31]. Our results demonstrated similar pattern of immunoreactivity for 11 β -HSD2 and AR in new LCs population after EDS treatment and suggested that the changes in 11 β -HSD2 expression can be used for evaluation of ALCs differentiation in rat testis.

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