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# Efficiency of the sulfate pathway in comparison to the Δ4- and Δ5-pathway of steroidogenesis in the porcine testis

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ABSTRACT

Sulfonated steroids are increasingly recognized as a circulating reservoir of precursors for the local production of active steroids in certain target tissues. As an alternative to sulfonation of unconjugated steroids by cytosolic sulfotransferases, their direct formation from sulfonated precursors has been described. However, productivity and physiological relevance of this sulfate pathway of steroidogenesis are still widely unclear. Applying the porcine testis as a model, conversion of pregnenolone sulfate (P5S, sulfate pathway) by CYP17A1 was assessed in comparison to the parallel conversions of pregnenolone (P5, Δ5-pathway) and progesterone (P4, Δ4-pathway). To characterize conversions in the virtual absence of competing enzyme activities, in a first series of experiments porcine recombinant CYP17A1 was incubated with the respective substrate in the presence of bovine recombinant cytochrome P450 oxidoreductase (CPR) and cytochrome b5 (b5). Moreover, porcine testicular microsomal fractions were used as a source of homologous CYP17A1, CPR and b5. Invariably 17α-hydroxylation of P5S was, if at all, only minimal and no formation of dehydroepiandrosterone sulfate from P5S was detectable. Consistent with earlier studies porcine CYP17A1 efficiently metabolized P4 and P5 in both assay systems. Metabolism of P4 and P5 by testicular microsomal protein varied substantially between the five animals tested. In conclusion, a physiologically relevant sulfate pathway for the production of C19-steroids from P5S *via* CYP17A1 is very unlikely in the porcine testis.

## 1. Introduction

Among the males of mammalian domestic animal species the boar is rather exceptional in that it exhibits high concentrations of numerous sulfonated steroids in the systemic circulation [1–6]. Therefore we use the boar as a model for the study of metabolism, transport and function of sulfonated steroids. Besides estrogens [2–4,6], in boars sulfonated steroids can be mainly related to intermediates or metabolites arising from the synthesis of androgens [1,3,4,7,8] and steroidal pheromones [5,9,10] encompassing numerous hydroxylated C19- and C21-steroids. Data from comparative measurements in testicular artery and vein [1,5–7,9,11,12], in lymph fluid of the spermatic cord [11] and from determinations in testicular tissue point to the testis as their primary site of production [5,13], and results from *in vitro* experiments using purified Leydig cells indicate that they are the main producers of sulfonated estrogens and several sulfonated C19-steroids [10,14–16]. However, the metabolic steps bringing about the broad spectrum of sulfonated steroids in boars are still poorly characterized.

Sulfonated steroids are commonly considered to arise from the sulfonation of unconjugated steroids by several members of the large superfamily of cytosolic sulfotransferases [17,18]. As an alternative, the broad spectrum of sulfonated steroids produced in the porcine testis could partly arise from direct conversions of sulfonated steroids, possibly starting from cholesterol sulfate (sulfate pathway of

*Abbreviations:* A4, androstenedione; Aβ, andien β-synthase activity of CYP17A1; ACN, acetonitrile; ANβ, androsta-5,16-dien-3β-ol; b5, cytochrome b5; CPR, cytochrome P450 oxidoreductase; CYB5A, CYB5 B isoforms of cytochrome b5; CYB5R, cytochrome b5 reductase; DHEA, dehydroepiandrosterone; DHEAS, dehydroepiandrosterone sulfate; DTT, dithiothreitol; DLPC, dilauroyl phosphatidylcholine; E, estrogens; GC–MS, gas chromatography–mass spectrometry; LC–MS/MS, liquid chromatography–tandem mass spectrometry; MeOH, methanol; OHP4, 17α-hydroxyprogesterone; OHP5, 17α-hydroxypregnenolone; OHP5S, 17α-hydroxypregnenolone sulfate; P4, progesterone; P5, pregnenolone; P5S, pregnenolone sulfate; PMSF, phenylmethanesulfonyl fluoride; T, testosterone; UM6, -7, -13, -22, -32, unidentified metabolites, named after the fraction in which they elute during HPLC analysis)

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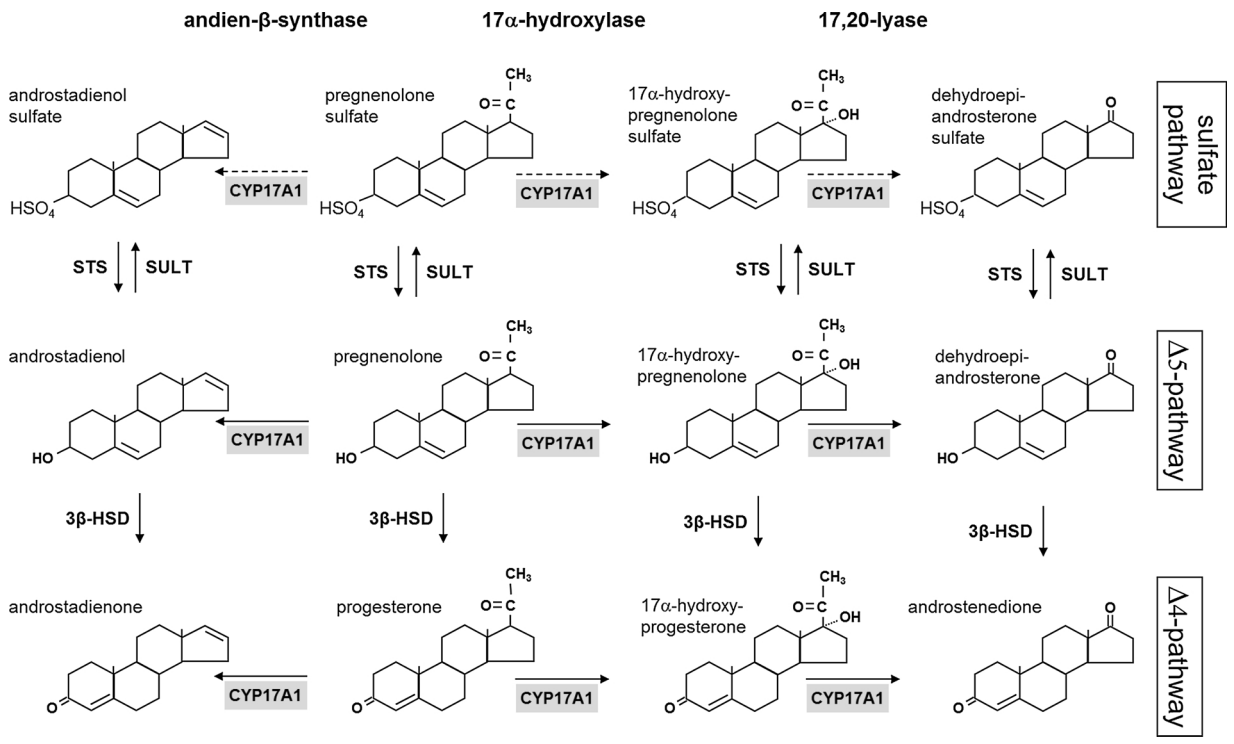


**Fig. 1.** Reactions catalyzed by porcine CYP17A1 including the hypothetical sulfate pathway investigated in this study.
SULT: members of the cytosolic sulfotransferases family; 3β-HSD: 3β-hydroxysteroid dehydrogenase-Δ5,4-isomerase; STS: steroid sulfatase.

steroidogenesis; see Fig. 1). Accordingly, conversions of sulfonated steroids were described mainly in the sixties until the eighties in various steroidogenic organs from different species including the boar [19–27]. However, these studies did not provide definite information on the efficiencies of these conversions in comparison to the metabolism of their unconjugated counterparts and their physiological relevance. The existence of an efficient sulfate pathway in the porcine testis would not only explain the broad spectrum of numerous sulfonated steroids but would also provide a possible explanation for the high steroid sulfatase expression of porcine Leydig cells [28], which could control the steps of the steroidogenic cascade where sulfonated precursors may enter the pool of unconjugated steroids (see Fig. 1).

A crucial enzyme at the key branch point of steroidogenesis is CYP17A1, a multifunctional enzyme exhibiting several activities and accepting several substrates, which catalyzes both 17α-hydroxylase and 17,20-lyase reactions on the Δ4- and Δ5-pathway, respectively (Fig. 1). Both activities of CYP17A1 involve electron transfers from reduced nicotinamide adenine dinucleotide phosphate (NADPH) *via* cytochrome P450 oxidoreductase (CPR) to the heme group of the enzyme. The decision concerning whether the enzyme stops after 17α-hydroxylation or proceeds to 17,20-lyase activity depends on several factors such as a high molar abundance of CPR [29], serine/threonine phosphorylation of CYP17A1 [30] or the presence of cytochrome b5 (b5). Cytochrome b5 occurs in two isoforms with CYB5A (type 1) localized in microsomal membranes and CYB5B (type 2) occurring in the outer mitochondrial membrane. Although it is a hemoprotein that in cooperation with other enzymes may also undergo redox chemistry, it is widely accepted that it stimulates the 17,20-lyase activity of CYP17A1 rather as an allosteric facilitator of the electron transfer from CPR to the enzyme than as an alternative electron donor (for reviews on CYP17A1 function see [31–34]). Recently, it was shown in electrochemical studies that the allosteric effect of b5 leads to altered electron transfer kinetics of CYP17A1, thereby promoting the enzyme's lyase activity [35]. Nevertheless, a role of b5 as a redox donor in CYP17A1 mediated androgen synthesis is still a matter of debate [36]. Generally, CYP17A1 efficiently 17α-hydroxylates progesterone (P4) and pregnenolone (P5). Concerning the efficiency of the subsequent 17,20-lyase reaction, there are significant differences between species [31,33,37,38], with a clear preference for 17α-hydroxypregnenolone (OHP5) in primates [39,40], cat [31] and ruminants [31,38,41–44], whereas in guinea pig [31,37] 17α-hydroxyprogesterone (OHP4) clearly is the preferred substrate. As in horse and hamster, in pigs CYP17A1 has been reported to exhibit considerable 17,20-lyase activity for the metabolism of both OHP4 and OHP5 [31,38,45]. In addition to 17α-hydroxylation and 17,20-lyase activity, depending on the species CYP17A1 has been demonstrated to possess further enzymatic activities [38]. With respect to the boar the andien-β-synthase (Aβ) activity of CYP17A1 is of particular importance as it catalyzes the first step leading to the production of steroidal pheromones (Δ16-steroids), providing androsta-4,16-dien-3-one from P4 and androsta-5,16-dien-3β-ol (ANβ) from P5 (see Fig. 1) [46–48]. Different from its role for the 17,20-lyase activity of CYP17A1, during stimulation of Aβ activity b5 may act as an electron donor itself mediating the electron transfer from NADH cytochrome b5 reductase (CYB5R) to CYP17A1 [34]. Accordingly, CYB5R3 significantly enhanced the Aβ activity of the porcine CYP17A1/CPR/b5 system [48,49] whereas increasing amounts of CPR only marginally augmented ANβ production [50]. In pigs, CYB5A has been shown to efficiently stimulate 17,20-lyase and Aβ activities, whereas CYB5B stimulated 17,20-lyase activity only [48].

Due to the importance of CYP17A1 for the production of androgens, estrogens and steroidal pheromones from C21 steroids, the aim of this study was to compare the activity of this enzyme for P5S conversion (sulfate pathway) with the parallel utilization of P5 (Δ5-pathway) and P4 (Δ4-pathway). To characterize the enzymatic reactions in the virtual absence of other competing enzyme activities, in a first series of experiments recombinant porcine CYP17A1 was incubated with the respective substrate in the presence of bovine recombinant CPR and b5, which were available from a previous study on the bovine enzyme [51]. Moreover, to compare the conversion of P5S, P5 and P4 in a pure porcine system microsomal fractions prepared from porcine testis were used as a source of CYP17A1, CPR and b5.