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Steroid-Metabolizing Cytochrome P450 (CYP) Enzymes in the Maintenance of Cholesterol and Sex Hormone Levels

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Abstract
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The enzymes CYP27A1 and CYP7B1 are widely expressed in various human tissues and perform catalytic reactions in cholesterol homeostasis and endocrine signaling.

We have investigated the metabolism of a synthetic oxysterol. In this study, we show that CYP27A1 is the enzyme responsible for a 28-hydroxylation of this oxysterol and that the rate of CYP27A1-mediated metabolism is relatively slow. This may give an explanation for the prolonged inhibitory effects on cholesterol biosynthesis that have been shown for this oxysterol. The current study contributes to the knowledge of synthetically produced oxysterols and their potential use as cholesterol lowering drugs.

In two studies we investigated CYP7B1-mediated metabolism of different sex hormones. Our data indicate that CYP7B1 may carry out a previously unknown catalytic reaction involving an androgen. Taken together the data suggest that varying steroid concentrations in cells and tissues may be important for CYP7B1-dependent metabolism of sex hormones and sex hormone precursors. CYP7B1-mediated hydroxylation of sex hormones may influence the cellular levels of these steroids and may be a potential pathway for elimination of the steroids from the cell.

Some known CYP7B1 substrates are agonists for ERα and ERβ but the reported role(s) of CYP7B1 for ER action are not fully understood. In the last study we investigated the role(s) of CYP7B1-mediated metabolism for ER-mediated action. Our data indicate that CYP7B1-mediated conversion of steroids that affect ER-mediated response into their 7α-hydroxymetabolites will result in loss of action. This indicates that CYP7B1 may have an important role for regulation of ER-mediated processes in the body.

In summary, results from this thesis contribute to the knowledge on the metabolism of synthetic oxysterols of potential use as cholesterol lowering drugs and the role(s) of CYP7B1-mediated metabolism for processes related to the functions of sex hormones.

Keywords: CYP27A1, CYP7B1, cytochrome P450, estrogen receptor, androgen receptor, cholesterol, sex hormone, hydroxylation, steroid metabolism, regulation of steroid levels

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To you

List of Papers

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

I Pettersson, H., Norlin, M., Andersson, U., Pikuleva, I., Björkhem, I., Misharin, A.Y., and Wikvall, K. Metabolism of a novel side chain modified Δ8(14)-15-ketosterol, a potential cholesterol lowering drug: 28-hydroxylation by CYP27A1. *Biochim. Biophys. Acta.* 1781, 383-390 (2008).

II Pettersson, H., Holmberg, L., Axelson, M., and Norlin, M. CYP7B1-mediated metabolism of dehydroepiandrosterone and 5α-androstane-3β,17β-diol - potential role(s) for estrogen signaling. *FEBS J.* 275, 1778-1789 (2008).

III Pettersson, H., Oliw, E., and Norlin, M. CYP7B1-mediated metabolism of 5α-androstane-3α,17β-diol (3α-Adiol): A novel pathway for potential regulation of the cellular levels of androgens and neurosteroids. Submitted

IV Pettersson, H., Lundqvist, J., and Norlin, M. Studies on the effects of CYP7B1-mediated catalysis on estrogen receptor activation. Manuscript

Contents

Abbreviations

3α-Adiol	Androstane-3α,17β-diol
3α-Atriol	5α-Androstane-3α,7α,17β-triol
3β-Adiol	5α-Androstane-3β,17β-diol
3β-Atriol	5α-Androstane-3β,7α,17β-triol
Aene-diol	5-Androstene-3β,17β-diol
Aene-triol	5-Androstene-3β,7α,17β-triol
ANOVA	Analysis of variance
AR	Androgen receptor
ARE	Androgen response element
CYP	Cytochrome P450
DHEA	Dehydroepiandrosterone
DHT	Dihydrotestosterone
DMSO	Dimethyl sulfoxide
E2	17β-estradiol
ER	Estrogen receptor
ERE	Estrogen response element
FBS	Fetal bovine serum
GABA	Gamma-amino butyric acid
GC-MS	Gas chromatography-mass spectrometry
HEK	Human embryonic kidney
HMG-CoA	3-Hydroxy-3-methylglutaryl-coenzyme A
HPLC	High-pressure liquid chromatography
LXR	Liver X Receptor
NADPH	Nicotinamide adenine dinucleotide phospate, reduced form
ONPG	Ortho-Nitrophenyl-β-D-galacto pyranoside
RLU	Relative light units
RP-HPLC	Reverse phase-HPLC
SREBP	Sterol regulatory element binding protein
SP-HPLC	Straight phase-HPLC

Introduction

The steroid-metabolizing cytochrome P450 (CYP) enzyme system

Enzymes are biological catalysts. They increase the rate of chemical reactions within cells without endogenous overall change. All enzymes are proteins and many of the enzymes need a non-protein component, a co-factor, for catalytic activity.

In 1961 there were reports of an unusual membrane-bound pigment in mammalian liver tissue. This pigment was able to form a CO complex that had an absorbance peak at 450 nM. The protein(s) were isolated, characterized and named cytochrome P450 [1].

Cytochrome P450 (CYP) enzymes are involved in a wide variety of oxidative metabolic processes as for example steroid synthesis and metabolism, cholesterol homeostasis and metabolism of vitamin D_3, drugs and environmental contaminants [1, 2]. In drug metabolism the CYP-enzymes are classified as phase I enzymes. The phase I metabolism is an introduction of an oxygen group and this oxygen is usually used as a site for the phase II enzymes [1]. A typical phase I reaction for a CYP enzyme is described in Figure 1.

Today this enzyme family has grown into a superfamily consisting of 57 *CYP* genes and 58 pseudogenes arranged into 18 families and 43 subfamilies for humans [3]. When Nebert and Gonzalez [1] compared sequences of cytochrome P450 originating from human and bacteria there were astonishing similarity between the sequences. They suggested that this superfamily originated from a common ancestor.

There are given rules on how a CYP enzyme shall be named. CYPs are classified into families and subfamilies based on their similarity of amino acid sequence. The enzyme is denoted CYP followed by a designating number (family), a letter (subfamily) and an individual number (gene). If the amino acid sequences share more than 40% identity the enzymes belong to the same family (*e.g.* CYP27) and to be members of the same subfamily more than 55% of the sequence shall be identical (CYP27A). Each individual enzyme is represented by an individual number (CYP27A1). An updated list of CYP genes, sequences and activities can be accessed on a P450-homepage on the internet http://drnelson.utmem.edu/CytochromeP450.html

All known structures of CYP enzymes show a conserved general overall structural fold that resembles a shape of a triangle turned upside-down [4, 5]. Human CYPs are usually membrane-bound proteins located to the mitochon-

dria or the endoplasmic reticulum of the cell. For a catalytic reaction to occur the CYP enzyme is dependent of electron transfer from NADPH (mammalian cells) or NADH (bacterial cells) as well as a co-system, NADPH-cytochrome P450 reductase. The mitochondrial CYP enzymes need a ferredoxin and ferredoxin reductase system [6, 7].

$$\mathbf{RH + O_2 + 2e^- \rightarrow ROH + H_2O}$$

Figure 1. A typical CYP enzyme reaction requires delivery of two electrons to the CYP heme group. The metabolism results in an oxygenated metabolite which is more water soluble.

Cholesterol

Cholesterol is the most abundant sterol in vertebrate tissues. This molecule participates in a number of essential processes in the body. For example cholesterol is a precursor for bile acids, vitamin D_3, several hormones, and a crucial molecule for structure and viscosity in the cell membranes [8]. Cholesterol has a distinguished nucleus base feature of four composite carbon rings that cannot be metabolized. This nucleus will be intact throughout the cholesterol metabolic process into other compounds. The metabolic synthesis pathways is shown in Figure 2.

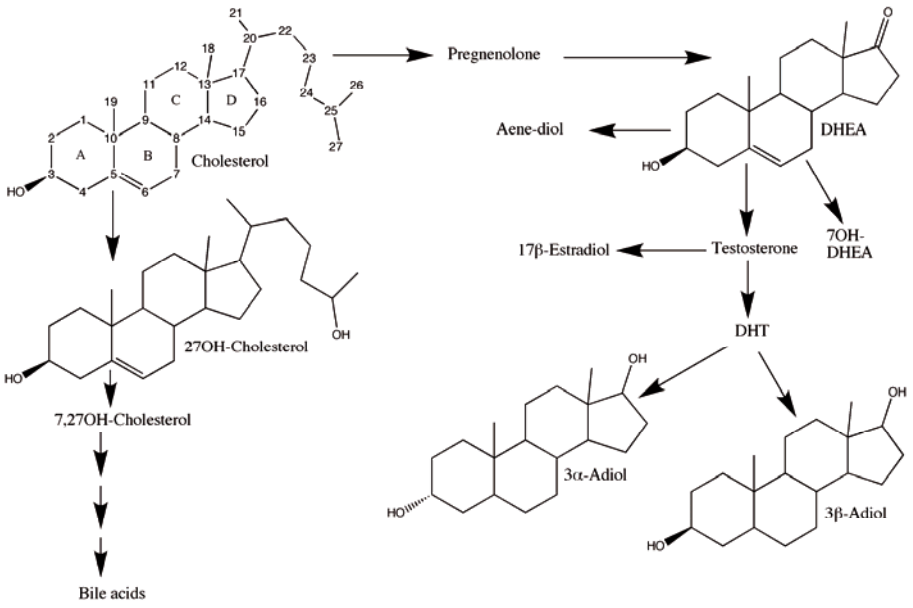


Figure 2. Metabolic pathways for conversion of cholesterol into bile acids and sex hormones. The cholesterol molecule is shown with carbon numbering.

Cholesterol homeostasis and cholesterol metabolism

Cholesterol is synthesized in many different tissues within the body *e.g.* liver, adrenal cortex and reproductive tissues. The rate-limiting step in this process is catalyzed by the enzyme HMG-CoA reductase which regulates the conversion of HMG-CoA to mevalonic acid. Mevalonic acid will by additional metabolic steps be converted to cholesterol.

The metabolism of cholesterol into bile acids is mainly through two pathways; the neutral (classical pathway) or acidic (alternative) pathway. The neutral pathway is an important pathway for bile acid biosynthesis and the acidic pathway may maintain the synthesis of bile acid under conditions when the neutral pathway is disturbed [9]. It has been estimated that approximately 400-600 mg of cholesterol will be metabolized and eliminated through the neutral pathway per day, whereas the acidic pathway will convert 18-20 mg of cholesterol on a daily basis under normal conditions [10].

The mechanisms of cholesterol homeostasis are complex and involve a number of different interactions. The regulation of cholesterol homeostasis consists of a network of enzymes, which in themselves are tightly controlled by several hormones, oxysterols and cholesterol [8, 11]. These pathways are regulated by negative feed-back to maintain a balance between cholesterol synthesis and cholesterol metabolism and excretion. Elevated cholesterol concentration will inhibit the enzyme HMG-CoA reductase and by this the cholesterol plasma level is lowered. Elimination of cholesterol occurs by the conversion of cholesterol to bile acids and bile salts which are transported to the intestine for elimination. If these mechanisms fail this may lead to serious consequences. Too much cholesterol in the circulation may result in atherosclerosis [12]. Accumulation of cholesterol in the bile may result in an increased risk for gallstones [13, 14]. If the cholesterol homeostasis is deficient in the brain this may lead to neurodegeneration *e.g.* Alzheimer's disease [15-18].

Bile acid biosynthesis

Bile acids are synthesized in the liver, stored in the gallbladder and secreted into the intestine through the bile duct. Bile acids enhance fat absorption and promote excretion of unmetabolized cholesterol. Regulation of bile acid biosynthesis is crucial and failure in the regulation may result in different diseases. The rate-limiting step in bile acid synthesis is the introduction of a 7α-hydroxyl group by the liver-specific microsomal enzyme CYP7A1. This enzyme can be regulated by bile acids and cholesterol [19, 20].

Oxysterols

Oxysterols are cholesterol derivatives and have been reported to be gene regulatory molecules in cholesterol biosynthesis, efflux, transport, excretion and absorption [21, 22]. The synthesis of oxysterols from cholesterol is considered to have impact because cholesterol and cholesterol esters do not have

the same ability to activate the genes themselves [23]. In the oxysterol synthesis an oxygen group, often a hydroxyl group, is incorporated into the cholesterol nucleus or into the side chain. This hydroxyl introduction will in most cases result in a lowering of the compound's half-life [22].

Oxysterols have been ascribed a number of different effects *e.g.* down-regulation of cholesterol synthesis, increase of bile acid synthesis, induction of CYP7A1 and mediation of sterol transport, cell growth and cholesterol elimination [9, 22, 24-26]. Some well known oxysterols are 27-, 25-, 24- and 7α-hydroxycholesterol [19, 22, 25, 27]. Many effects of endogenous oxysterols are mediated by the key transcriptional factor LXR (nuclear liver X receptor) [22, 26, 27]. The LXR's are regulatory factors for the cholesterol homeostasis and an activation of the receptor will result in an up-regulation of cholesterol transport proteins. These proteins are important for the elimination of cholesterol from the cell. Studies on $LXR^{-/-}$ mice have shown that these mice are unable to convert cholesterol into bile acids which results in an accumulation of cholesterol in different tissues, *e.g.* spleen, liver, lung and artery [8, 19, 28]. CYP enzymes of importance for the oxysterol synthesis are CYP3A4, CYP27A1, CYP7A1 and CYP46A1. Many of the oxysterols are very similar in structure but they have a significant difference in the biological responses [27].

Steroid hormones

Steroid hormones are complex fat-soluble molecules. All endogenous steroid hormones derive from cholesterol via the initial conversion of cholesterol to pregnenolone. Steroid hormones are divided into glucocorticoids, mineralocorticoids and sex hormones (androgens and estrogens). Steroid hormone synthesis and secretion occur in many tissues including adrenal cortex, ovaries, placenta, testes, brain and prostate. The biosynthesis of sex hormones is shown in Figure 2. Because of their hydrophobic feature the sex hormones must form a complex with a carrier protein (sex hormone binding protein, SHBP) when they are secreted into the circulation for further transport to the target tissue (endocrine response). Hormones can also exert paracrine, autocrine or intracrine responses.

Steroid hormones diffuse directly across the plasma membrane of the target cell and bind to a receptor. Ligand binding will activate the receptor and result in stimulation or in an inhibitory response [29]. Steroid hormones have high affinity for their receptor(s). Because of this high affinity generally low concentrations (nanomolar) are needed for responses in the target tissue. Steroid hormones have a broad variation of functions within the body as for example effects on sodium and potassium uptake and excretion, inflammatory response, bone development, metabolism, behaviour and sexual development. A number of different diseases are caused by deficiencies of specific metabolizing enzymatic steps throughout the steroid hormone biosynthesis. These defects may result in female-like genitalia in males, masculinization of external genitals in females and early virilization in males, elevated cell growth, and neurological problems among others [30-32].

The sex hormone precursor dehydroepiandrosterone

A steroid precursor is a molecule that other steroids derive from through different metabolic steps. Dehydroepiandrosterone (DHEA) is an important steroid precursor for many of the androgenic and estrogenic hormones. DHEA is synthesized from cholesterol via pregnenolone and 17α-hydroxypregnenolone in the adrenal cortex and is the most abundant steroid in circulation together with its sulfate DHEA-S. DHEA has been shown to have two separate concentration peaks through human life. One is just before birth and the DHEA level will decrease after birth. At adolescence the concentration will increase and reach its peak in humans at the age of 20-30 years. After this second peak, the concentration will decrease again. At age 65-75 the concentration will be about 25% of the peak level [33]. DHEA has been suggested to affect a number of different processes in various tissues, including CNS function, immune system, lipid profiles and cellular growth [34-36].

The sex hormones estrogens and androgens

Estrogens are considered to be 'female' hormones and androgens are considered to be 'male' hormones. However, both hormones are represented in both sexes, but in different concentration levels. In males, estrogen levels are low in plasma but the concentration in semen can be even higher than found in female serum [37].

Estrogens are known to influence a number of processes in the body *e.g.* both female and male reproduction systems, bone development, cell proliferation, hormone-dependent carcinogenesis, immune response and CNS functions [27, 38-40]. Most effects exerted by estrogens are mediated via the estrogen receptors ERα and ERβ [41]. The role of estrogenic action may differ for different types of estrogens and/or receptors and/or cellular environments that require different co-regulators [27, 42, 43]. 17β-Estradiol (E2) is the most potent and also the most abundant endogenous estrogenic ligand with equal binding affinity to both ER sub-types [27, 44].

Androgens are steroids that play a crucial role in several steps in the male development. The most abundant androgens are testosterone and dihydrotestosterone (DHT) that is formed by 5α-reduction of testosterone. The 5α-reduction of testosterone to DHT is a key step in androgenic action especially for sexual development and testicular functions [45-49]. These androgenic steroids exert their effects via interactions with the androgen receptor (AR) [45]. Both testosterone and DHT have affinity to AR although DHT is a more potent androgen [49-52]. If the androgenic action is disturbed this may result in deformed genitals, or even absence of outer male genitals [53]. If there is an overexpression of DHT this may contribute to development of diseases like benign prostate hyperplasia or prostate cancer or virilization of urogenitals in females [30, 38, 54].

In the brain androgens can be metabolized either to estrogens or to DHT [48]. Circulating levels of androgens can influence the behavior of humans and other vertebrates. This is because there are neurons that are sensitive to

stimulation by androgenic hormones. Androgens have been suggested to have an impact on human aggression [55].

5α-Androstane-3α,17β-diol and 5α-androstane-3β,17β-diol

The steroids 5α-androstane-**3α**,17β-diol (3α-Adiol) and 5α-androstane-**3β**,17β-diol (3β-Adiol) (Figure 3) are both formed from the potent androgen DHT in different tissues [47, 48, 56-58]. In the conversion of DHT into 3α-Adiol and 3β-Adiol the formation of 3β-Adiol is favored compared to formation of 3α-Adiol [59-61]. Although these two DHT metabolites are very similar in structure they have been reported to have distinguished roles of action in the cell.

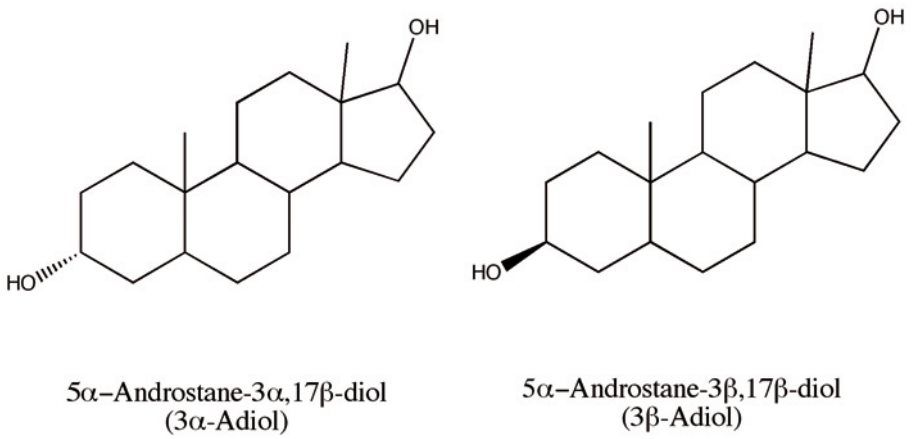


Figure 3. Structural similarity of the two DHT metabolites 3α-Adiol and 3β-Adiol.

3α-Adiol is suggested to be a less active form of DHT and the formation may thereby lower the androgenic effects on for example prostatic cell growth. However, the conversion of DHT to 3α-Adiol is reversible which may give 3α-Adiol the role of a source for DHT [57, 59, 62]. There are reports that 3α-Adiol may be a crucial component involved in different processes related to urogenital functions [62-65]. In a study by Dozmorov *et al*. [66] 3α-Adiol is suggested to be a crucial component in the mechanism that control cell viability and cell survival. In addition to 3α-Adiol's role in inactivation of DHT or as a source for this steroid and/or preventing apoptosis there are studies indicating that 3α-Adiol has a physiological role as modulator for the $GABA_A$ receptor in the brain. 3α-Adiol is synthesized *de novo* by glial cells in the brain [48, 67]. The modulation of the $GABA_A$ receptor may result in an anti-convulsant and/or analgesic effect [58, 68, 69]. In contrast to the reported androgenic effects of this steroid, Edinger and Frye [70] proposed that 3α-Adiol enhances memory and learning by ERβ activation.

3β-Adiol has been reported to be an active hormone [43, 71-73]. There are studies showing that 3β-Adiol is an agonist for the estrogen receptor β (ERβ). This steroid is considered to be an important intraprostatic estrogen [74, 75].

Guerini *et al.* [42] suggested that 3β-Adiol inhibits prostate cancer cell migration acting through ERβ activation. Gustafsson and collaborators proposed a pathway for hormonal control of prostate growth [43, 76]. According to this pathway the androgenic stimulation of growth via the androgenic receptor (AR) is counteracted via 3β-Adiol-mediated stimulation of ERβ. Other theories of the means of action by this steroid are that 3β-Adiol may act as a paracrine growth factor stimulating replicative DNA synthesis of developing male germ cells and may act as a modulator of ERβ-mediated gene transcription in neuronal and Sertoli cells [72, 77, 78]. Baum *et al.* [79] presented results indicating that interaction of 3β-Adiol with ER may result in activation of mounting behavior in male rats.

Receptors

Receptors are proteins on the cell surface or located intracellularly that are able to bind molecules (ligands) and thereby constitute a complex. This complex can trigger different responses depending on sort of ligand and receptor. The formed complex will result in some form of signal within the cell. The signal pathways can be of different types and often involve a production of a second messenger (for example cAMP). Receptors may act as transport channels through the cell membrane for organic and inorganic molecules. Steroid receptors belong to the large family of nuclear receptors. In the absence of hormones the steroid receptor is mainly found in the cytoplasm [40, 80]. Steroid receptors include; one androgen receptor (AR), two estrogen receptors (ER) named ERα and ERβ, the progesterone receptor, the glucocorticoid receptor and the mineralocorticoid receptor.

The estrogen receptors (ER)

In 1986 Green *et al.* [81] first cloned and sequenced an estrogen receptor. Ten years later a second estrogen receptor was cloned by Kuiper *et al*. [82]. The first estrogen receptor was named ERα and the later described one was named ERβ. These subtypes are encoded by different genes and located on different chromosomes. ERα and ERβ share similar DNA-binding domains (96% homology) and similar ligand-binding domains (56% homology) and they also bind to the same hormone receptor element (ERE) on DNA [83, 84]. Analyses of the ligand-binding sequences between human ERα and ERβ revealed that only 2 out of the total 22 amino acids that interact with the ligand differ between the two ERs [85].

Even though ERα and ERβ possess a high homology of the ligand-binding sequence, the relative binding affinity differs for several hormones between the two receptor sub-types [74]. The receptors have been shown to have overlapping expression patterns with only a few exceptions. Epididymis, kidney, prostate stroma and adrenal are examples of tissues where only ERα is expressed and prostate epithelium, lung and brain stem where only ERβ is expressed [84, 86].

Most nuclear receptors are ligand-activated and they regulate gene transcription. In the classic model of ER activation, the activated ER forms dimers and regulate gene transcription by binding to ERE in the promoter of target genes where it interacts with transcription factors and co-regulators. The activation of ERα and ERβ may result in similar or opposite biological effects [44, 84, 87]. The opposite responses may be due to different factors like intracellular environment, type of ligand, receptor conformation and recruitment of different co-regulators [40, 88].

The androgen receptor (AR)

In 1988 Trapman *et al.* [89] cloned and sequenced the androgen receptor (AR). AR is expressed in many tissues and interacts in a broad variety of physiological processes such as effects on bone density, skeletal muscle growth, behavioral moods and prostate tissue growth [90]. The AR is very similar in its DNA- and ligand-binding sequences compared to other members of the steroid hormone receptors [46, 81, 89]. The ligand-binding sequence consists of 20 amino acids that interact more or less directly with the binding ligand. Only two residues differ between the human AR and the human progesterone receptor [91]. Defective or mutated AR is directly involved in disorders including benign prostatic hyperplasia (BPH), androgen insensitivity syndrome (AIS), hirsutism, spinal bulbar muscular atrophy (SBMA), also named Kennedy's disease, and prostate cancer [90, 92, 93].

The $GABA_A$ receptor

Gamma-amino-butyric acid A ($GABA_A$) receptors are located in the ganglia synapses in the brain. The $GABA_A$ receptor has been suggested to have an important role in mediation of behavioral moods such as aggression, stress and anxiety [94, 95]. This receptor may have an inhibitory effect on the synaptic signaling of nervous transmission [96]. Anti-analgesic or anti-convulsant effects as well as effects on memory are connected to the activation of $GABA_A$ [58, 68, 70]. Local steroid metabolites may act as modulators for this synaptic receptor [97]. 3α-Adiol is believed to have stimulatory effects on the $GABA_A$ receptor whereas 3β-Adiol has been reported to have an inhibitory effect [98].

Selective estrogen receptor modulators (SERM)

Selective estrogen receptor modulators (SERM) are a class of compounds that will act on estrogen receptors. Characteristic for SERMs are that they can act as agonists in one tissue and antagonists in other tissues. When ER binds to a SERM this permits the receptor to undergo a conformational change that is an intermediate between the active and inactive state. This complex can interact with different co-regulators (activators or suppressors)

that would not interact with ER under normal conditions [99]. Tamoxifen and raloxifene are two SERMs that are used for treatment of breast cancer and prevention of osteoporosis [40].

Sterol 27-hydroxylase (CYP27A1)

The enzyme CYP27A1 (sterol 27-hydroxylase, P450c27, mitochondrial vitamin D_3 25-hydroxylase are other names fore the same enzyme) was first isolated from rabbit liver mitochondria in 1984 [100], from rat liver in 1988 [101] and also from pig kidney in 1991 [102]. This is a multi-functional enzyme that is expressed in many tissues such as the brain, prostate, liver, lung, kidney, small intestines, arterial endothelium, fibroblasts and macrophages [9, 103-106]. CYP27A1 catalyzes several metabolic steps in cholesterol homeostasis and vitamin D_3 metabolism. This enzyme catalyzes the 27-hydroxylation of intermediates in the neutral pathway of bile acid biosynthesis and 27-hydroxylation of cholesterol in the acidic pathway [9, 19, 20]. CYP27A1 generates 27-hydroxycholesterol which, via the LXR, activates genes coding for transport proteins that shuttle intracellular cholesterol to the outer membrane for further transport and elimination [107]. CYP27A1 has broad substrate specificity and is expressed in most tissues in the body. The CYP27A1-formed 27-hydroxycholesterol will suppress HMG-CoA reductase, the rate limiting enzyme in cholesterol homeostasis [24, 108].

CYP7B1 and related enzymes

7α-hydroxylases

Several hydroxylating enzymes are involved in metabolism that leads to bile acids or hormones. Hydroxylation may be a key reaction in the biosynthesis process. There are at least three different enzymes that are able to catalyze 7α-hydroxylation of the steroid structure.

CYP7A1 and CYP39A1

Cholesterol 7α-hydroxylase (CYP7A1) is a key enzyme involved in the maintenance of cellular cholesterol balance [19, 107]. Studies on CYP7A1 $^{-/-}$ mice showed that these mice were able to perform 7α-hydroxylation in an alternative pathway via the related enzyme CYP7B1 [109, 110]. The expression of CYP7A1 is restricted to the liver [27, 111]. CYP39A1 is a liver-specific oxysterol 7α-hydroxylase enzyme that, together with CYP7A1, metabolizes 24S-hydroxycholesterol which originates in the brain [27]. Ikeda *et al.* [112] have reported findings of this enzyme also in bovine eye cells.

CYP7B1

In 1995 Stapleton *et al.* [113] found a transcript in the rat hippocampus which was 39% similar to the sequence of the already known cholesterol 7α-hydroxylase (CYP7A1). This novel cytochrome P450 was named oxysterol 7α-hydroxylase (CYP7B1). CYP7B1 has metabolic activity against steroids including 27-hydroxycholesterol, DHEA, pregnenolone, 5α-androstane-3β,17β-diol (3β-Adiol) and 5-androstene-3β,17β-diol (Aene-diol) [36, 76, 114, 115]. The enzyme is widely distributed in different tissues including liver, brain, lung and prostate among others [113, 116]. CYP7B1 may exert its hydroxylation not only at carbon number 7 but also at carbon number 6. There are reported findings of 6α/6β- and 7α/7β-hydroxylation [117-119]. There are some reports indicating that metabolites formed by CYP7B1 may have physiological effects of their own [120, 121]. In recent years it has been proposed that CYP7B1-mediated metabolism might play an important role in cellular growth, for example in the prostate tissue. CYP7B1 metabolizes some of the ERβ ligands. As a result of this hydroxylation the ligand(s) may be extruded from the cell [43, 72, 120, 122, 123].

Diseases connected to deficiencies of the enzymes CYP7B1 or CYP27A1

Disorders in cholesterol biosynthesis and metabolism may result in severe health problems *e.g.* neurological disease, cholestasis and malabsorbtion of fat-soluble vitamins [124]. Deficiency of CYP7B1 has been described by Setchell *et al.* [124]. This study reports a severe biliary cholestasis in a newborn boy that had a mutation in the *cyp7B1* gene but an intact *cyp7A1* gene. This mutation was lethal and the boy died at the age of about 5 months after extensive medical and surgical efforts. The patient had liver failure which may be due to the elevated levels of oxysterols which are known to result in hepatotoxicity.

Cerebrotendinous xanthomatosis (CTX) is a rare genetic sterol storage disease caused by point mutations in the *cyp27A1* gene. Characteristic features are neurological dysfunction, dementia and cataracts among others. Biochemical characteristics are reduced primary bile acid synthesis, elevated biliary, urinary and fecal excretion of bile salts, low plasma concentration of cholesterol and elevated tissue cholesterol levels, including in the brain [125, 126]. CTX may result in accelerated atherosclerosis and neurological deterioration. CTX can be treated by oral bile acid therapy [9].

Aims of the present investigation

The overall aim of the present investigation was to investigate enzymatic reactions of relevance for sex hormone signaling and cholesterol homeostasis

The specific aims were:

- to study and compare metabolism of synthetic sterols which inhibit cholesterol biosynthesis
- to study the properties of CYP7B1-mediated metabolism of steroids that may influence estrogen signaling
- to study the potential CYP7B1-mediated metabolism of androgens
- to study the role of CYP7B1-mediated catalysis for estrogen receptor-mediated response

Experimental Procedures

Materials

The pCMV6 vector containing cDNA encoding for human CYP7B1 was kindly provided by D.W. Russell (University of Texas, Dallas, TX, USA). Human liver microsomes were obtained from Genetest. The human ERα/β and AR expression vectors were generous gifts from P. Chambon (Institut de génétique et de biologie moléculaire et cellulaire, Strasbourg, France) and A. Brinkmann (Erasmus Medical Centre, Rotterdam, the Netherlands), respectively. The ERE and ARE_2-TATA luciferase reporter vectors were generously provided by K. Arcaro (University of Massachusetts, MA, USA) and J. Trapman (Erasmus Medical Centre, Rotterdam, the Netherlands), respectively. 3β-Hydroxy-24S-methyl-5α-cholesta-8(14),22-dien-15-one was synthesized as described by Misharin and co-workers [127, 128]. All remaining materials were purchased from commercial sources.

Preparation of ^{3}H-labeled 5α-androstane-3β,17β-diol (3β-Adiol)

^{3}H-Labeled 3β-Adiol was prepared from ^{3}H-labeled DHT by bioconversion using hydroxysteroid dehydrogenase from *Pseudomonas testosteroni* [129]. A mixture of labeled and unlabeled DHT was incubated with hydroxysteroid dehydrogenase and NADH in potassium phosphate buffer for 10 min at 37°C in a water bath. The reaction was quenched and extracted with 5 ml ethyl acetate. The organic phase was evaporated under N_2-gas, dissolved in a small amount of acetone and applied on a silica gel TLC-plate. The TLC-plate was developed in toluene/methanol 90:10 (v/v). TLC-plates with unlabeled DHT, 3β-Adiol and 3α-Adiol were used as references and developed together with the sample plate. The sample TLC-plate was scanned for localisation of the radioactive products. The reference TLC-plates were exposed to iodine vapours (o/n) to visualize the steroids and the retention times of reference compounds were compared with those of the sample plate. Under these conditions the main product formed was 3β-Adiol. The formed radioactive 3β-Adiol was extracted from the silica gel with ethyl acetate. The obtained solution of ^{3}H-labeled 3β-Adiol was evaporated under N_2-gas and dissolved in ethyl acetate. The radioactivity, expressed in cpm/µl, was determined by injection of an aliquot on a reverse phase HPLC (RP-HPLC) as described in paper II. The solution of ^{3}H-labeled 3β-Adiol was diluted to a working concentration of 50,000 cpm/µl.

Preparation of ^{3}H-labeled 5-androstene-3β,17β-diol (Aene-diol)

^{3}H-Labeled Aene-diol was prepared from ^{3}H-labeled DHEA by using a similar methodology as described above (Preparation of ^{3}H-labeled 5α-androstane-3β,17β-diol). The solution of ^{3}H-labeled Aene-diol was diluted to a working concentration of 50,000 cpm/μl.

Animals and tissue sample collection

Tissues from adult pigs were obtained from the Funbo-Lövsta Research Centre, Department of Animal Breeding and Genetics, Swedish University of Agricultural Sciences (SLU), Ultuna. Tissues from male uncastrated piglets were a generous gift from Professor Per Wallgren, Department of Ruminant and Porcine Diseases, National Veterinary Institute (SVA). All the animals were healthy and untreated at the time of euthanasia. All tissue samples were stored at -80°C until microsomal preparation was performed.

Microsomal preparation from pig tissues

The pig tissues were weighed, minced and homogenized in sucrose buffer containing 0.25 M sucrose, 10 mM Tris-Cl, pH 7.4, and 1 mM EDTA to a 20% suspension. Microsomes were prepared as described [130] and stored at -80°C until incubation. Protein contents of the microsomes were assayed by the method of Lowry *et al.* [131].

Incubation and analysis procedures

Incubations with microsomes were carried out at 37°C for 20 or 30 min. The amount of microsomes used per incubation sample was 0.5-1 mg/ml. 3β-Hydroxy-5α-cholest-8(14)-en-15-one, 3β-hydroxy-24S-methyl-5α-cholesta-8(14),22-dien-15-one, DHEA, 3β-Adiol, 3α-Adiol or Aene-diol, dissolved in acetone, were incubated with microsomes and NADPH in a total volume of 1 ml of Tris-acetate buffer. The incubations were quenched and extracted with 5 ml ethyl acetate. The organic phase was collected and stored at -20°C until analysis. Incubations without NADPH were performed at the same time and used as negative controls.

For incubations where steroids were added as potential inhibitors, incubations with labeled substrates were carried out as described above except that various amounts of unlabeled steroids were added to the incubation mixtures as inhibitors.

For assay of CYP7B1-mediated activity the organic phase of the incubations was evaporated under N_2-gas, dissolved in mobile phase methanol/water and subjected to the RP-HPLC. The RP-HPLC mobile phase system

consisted of methanol/water 50:50 (v/v) for 10 minutes, a linear gradient from 50 to 100% methanol for the next 10 minutes and then 100% methanol for the remaining 10 minutes [114]. Incubation and analysis procedures were as described in Paper II and III.

Incubations with recombinant human CYP27A1 (0.05 nmol) were carried out at 37ºC for 10 or 15 minutes and the activity towards 7α-hydroxy-4-cholestene-3-one, 3β-hydroxy-5α-cholest-8(14)-en-15-one or 3β-hydroxy-24S-methyl-5α-cholesta-8(14),22-dien-15-one was assayed by SP-HPLC using a mobile phase of hexane/isopropanol 92:8 or 94:6 (v/v). Incubation and analysis procedures were as described in Paper I.

Mammalian cell culture

Cells were seeded on 60-mm tissue culture dishes approximately one day prior to experiments and grown to 60-70% confluence. Enzymatic activity towards steroids of interest was examined by addition of 15 μg substrate dissolved in dimethyl sulfoxide (DMSO) to the medium and incubation for about 24 hours at 37ºC with 5% CO_2. Following incubation with substrate, the medium was collected and extracted and the organic phase was analyzed for hydroxylated metabolites as described below. Incubations terminated immediately after addition of substrate (corresponding to an incubation time of 0 hours) were used as negative controls. Protein contents of the cells were assayed by the method of Lowry *et al*. [131].

Incubations where steroids were added as potential inhibitors were carried out as described above except that 1.5-150 μg of unlabeled steroid were added to the cell media together with the labeled substrates.

Analysis of cell incubations

Cell incubations were analyzed as described in paper I-III. The organic phase was evaporated under N_2-gas, dissolved in mobile phase methanol/water and subjected to radio RP-HPLC. The RP-HPLC mobile phase system consisted of methanol/water 50:50 (v/v) for 10 minutes, a linear gradient from 50 to 100% methanol for the next 10 minutes and then 100% methanol for the remaining 10 minutes [114].

Transient transfection and overexpression of recombinant human CYP7B1 in mammalian cells

The cells were cultured as described above. In experiments to study the effects on ER- and AR-mediated response, vectors containing human ERα, ERβ or AR were transiently transfected in HEK293 or LNCaP cells using calcium co-precipitation or Lipofectamine 2000 (Invitrogen). Control experiments were performed with empty vector and vehicle (EtOH).

For experiments with overexpression of CYP7B1, HEK293 cells were transfected with the vector containing cDNA encoding for human CYP7B1. Transfection was carried out by electroporation (Gene Pulser II, Bio-Rad) or by Lipofectamine 2000. Following transfection, the cells were cultured for about 24 h on 60-mm plates in medium containing substrate dissolved in DMSO. Following incubations with substrate, the medium was collected and extracted and the organic phase was analyzed for hydroxylated metabolites as described in Paper II and III.

Preparation of 5α-androstane-3β,7α,17β-triol (3β-Atriol)

3β-Atriol was obtained by bioconversion, using microsomal incubation with 3β-Adiol and separation by HPLC as described in Paper IV.

Analysis of ER- and AR-mediated response by luciferase reporter assay

HEK293 or LNCaP cells were transiently transfected with an ER- or AR-responsive luciferase reporter vector together with expression vectors containing either human ERβ, ERα or AR and a β-galactosidase plasmid (in order to control for transfection efficiency), using co-precipitation or Lipofectamine 2000 as described in Paper II-IV.

The ER-responsive vector contains a strong estrogen response element (ERE) coupled to luciferase. Similarly the AR-responsive vector contains an androgen response element (ARE) coupled to luciferase. Transfected cells were treated with steroids in varying concentrations, dissolved in ethanol, and the levels of estrogen- or androgen-dependent luciferase activity in steroid-treated cells were compared with the luciferase levels in cells treated with the same volume of vehicle. Luciferase and β-galactosidase activities were assayed as described in Paper II-IV. Luciferase activity is expressed as relative light units (RLU) divided by β-galactosidase activity (expressed as absorbance at 420 nm).

Statistical analysis

Analysis of statistical significance was performed using analysis of variance (ANOVA) or Student's t-test. P values ≤ 0.05 were considered statistically significant. The software used was Minitab®, release 14 or MS Excel.

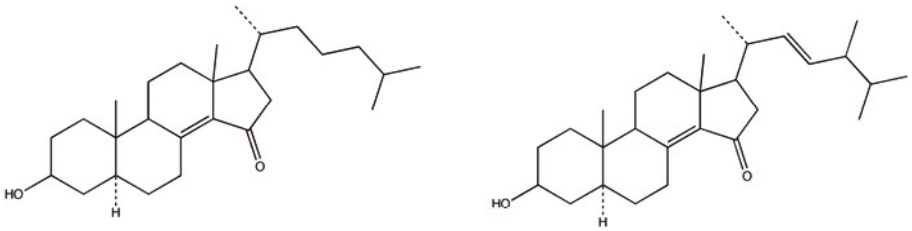
Results and Discussion

CYP27A1-mediated metabolism of synthetic 15-ketosterols (Paper I)

Biological oxysterols are formed by metabolic transformation of cholesterol. These molecules have diverse functions, including effects on cholesterol homeostasis by regulation of crucial genes. Oxysterols usually undergo rapid metabolism and thereby lose their regulatory effect. CYP27A1 is an enzyme active in formation and metabolism of biological oxysterols. It is of interest to produce synthetic oxysterols with inhibitory effect on the cholesterol synthesis that may be potential cholesterol lowering drugs. How synthetic oxysterols may act and be metabolized is not well known today. It has been suggested that these compounds may be rapidly metabolized by liver cells resulting in loss of action.

Schroepfer and coworkers described a synthetic 15-ketosterol (3β-hydroxy-5α-cholest-8(14)-en-15-one), hereinafter referred to as the originally described 15-ketosterol [132]. The structure of the original 15-ketosterol is shown in Figure 4. This originally described 15-ketosterol is known to have an inhibitory effect, although short-lived, on cholesterol biosynthesis. A problem with this compound is that it will undergo a rapid metabolism and thereby the regulatory effect will be abolished.

There are naturally occurring sterols in plants, *e.g.* stigmasterol and campesterol, which undergoes a slow metabolism due to the structure of the side chain. Misharin and coworkers synthesized a 15-ketosterol (3β-hydroxy-24S-methyl-5α-cholesta-8(14),22-dien-15-one), hereinafter referred to as the side chain modified 15-ketosterol, originating from the originally described 15-ketosterol (Figure 4) [127, 128]. This side-chain modified 15-ketosterol has a methyl group inserted as carbon 28 and there is also an unsaturated bond between carbon 22 and 23. In this study we investigated the metabolism of this side chain modified 15-ketosterol.



3β-hydroxy-5α-cholest-8(14)-en-15-one
(originaly described 15-ketosterol)

3β-hydroxy-24S-methyl-5α-cholesta-8(14),22-dien-15-one
(side chain modified 15-ketosterol)

*Figure 4.*Structures of the two 15-ketosterols. (A) shows the originally described sterol, 3β-hydroxy-5α-cholest-8(14)-en-15-one, and (B) shows the side-chain modified sterol, 3β-hydroxy-24S-methyl-5α- cholesta-8(14),22-dien-15-one.

We hypothesized that, because of the side-chain structure, the modified 15-ketosterol might be metabolized slowly and that this might explain its prolonged regulatory effect compared to the originally described 15-ketosterol. The second hypothesis was that, because of the structural similarity to known CYP27A1 substrates, the side-chain modified 15-ketosterol might be a substrate for CYP27A1.

In the initial experiments we examined polar metabolites endogenously formed from the two 15-ketosterol variants in two different cell-lines, HEK293 and HepG2, both expressing CYP27A1. After incubation with the substrates the cell medium was analyzed by HPLC. The results showed that polar metabolites are formed from both 15-ketosterols.

In experiments conducted with purified recombinantly expressed human CYP27A1, the results support the hypothesis that CYP27A1 is the enzyme responsible for the hydroxylation of both the originally described 15-ketosterol and the side-chain modified 15-ketosterol. HPLC chromatograms are shown in Figure 5. These incubations resulted in formation of two CYP27A1-mediated metabolites (product 1 and product 2) from each of the sterols as analyzed by HPLC. The retention time for peak 1 from both 15-ketosterols was very similar whereas the retention times differed for peak 2. Also the amount of metabolite formed differed between the 15-ketosterol substrates.

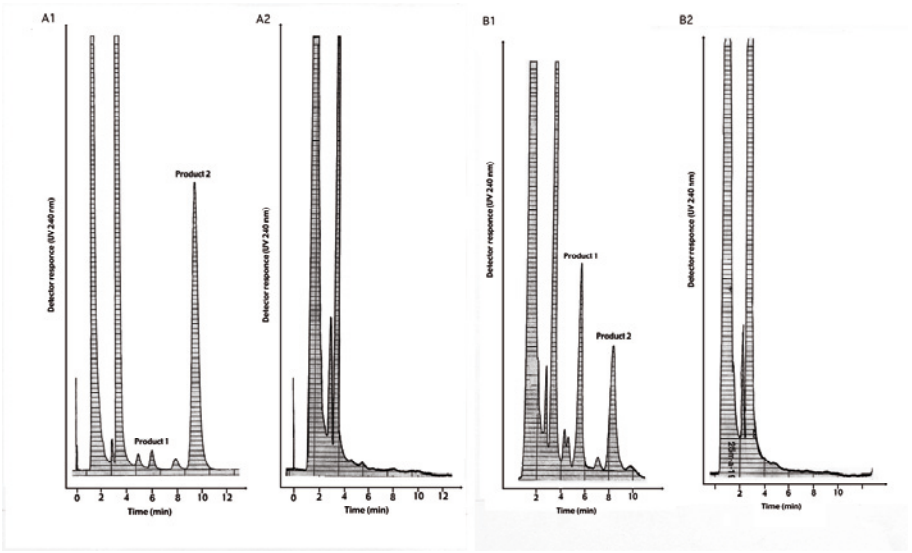


Figure 5. HPLC chromatograms of CYP27A1-mediated metabolism of the originally described (A) and the side-chain modified (B) 15-ketosterols. A1 and B1 show the chromatogram of incubations when the purified enzyme was saturated with substrate. A2 and B2 show chromatogram of incubations without electron donating system (NADPH) used as negative controls.

For determination of the metabolite structure we collected fractions from the HPLC, corresponding to the retention time of the eluted hydroxy metabolites, and analyzed the metabolites in these fractions with GC/MS. The GC/MS pattern indicated that the originally described 15-ketosterol was metabolized mainly to a 27-hydroxylated product. The minor product formed was consistent with a 25-hydroxylated product. For the side-chain modified 15-ketosterol the GC/MS results showed that the main product formed is a hydroxylation at the C-28 position and that the minor product formed is a 27-hydroxy metabolite.

We determined the enzyme kinetics for CYP27A1-mediated hydroxylation of both 15-ketosterols. As shown in Table 1 the K_{cat}/K_m is dramatically lower for the side-chain modified 15-ketosterol compared to the originally described 15-ketosterol indicating that the rate of metabolism is slower for the side-chain modified 15-ketosterol. These kinetic data indicate that the side-chain modified 15-ketosterol, due to its slower metabolism, may exhibit its inhibitory effect on cholesterol biosynthesis for a longer time period compared to the originally described 15-ketosterol.

Table 1. Kinetic parameters for the CYP27A1-mediated metabolism of the two 15-ketosterols of interest for this study.

	K_{cat} (min^{-1})	K_m (μM)	K_{cat}/K_m (μM^{-1} min^{-1})
3β-Hydroxy-5α-cholest-8(14)-en-15-one	4.4±1.2	4.7±2.3	0.94±0.16
3β-Hydroxy-24S-methyl-5α-cholesta-8(14),22-dien-15-one	1.1±0.2	17.6±5.9	0.06±0.01

As shown by the results in this study, human CYP27A1 is the enzyme responsible for the hydroxylation of both 15-ketosterols and a previously not known CYP27A1-mediated metabolism was discovered. The CYP27A1-mediated metabolism of the side-chain modified 15-ketosterol lead mainly to a 28-hydroxylated product. This is the first reported finding that CYP27A1 is able to carry out a hydroxylation at carbon nr 28. Both 15-ketosterols were metabolized to 27-hydroxylated products and the originally described 15-ketosterol was also metabolized to a 25-hydroxylated product. This study revealed that the side-chain modified sterol is metabolized nearly 15 times slower compared to the originally described sterol. These results may be important findings in the future design of cholesterol lowering drugs.

The results in the current and other studies indicate that CYP27A1 is an enzyme of potential importance for development of cholesterol lowering drugs. This enzyme promotes cholesterol elimination and might be a potential target for treatment of hypercholesterolemia.

CYP7B1-mediated metabolism of hormones with potential effects on estrogen signaling (Paper II)

CYP7B1 is a widely expressed enzyme within different tissues and different species. The enzyme is reported to be involved in numerous key events in cellular processes including metabolism of dehydroepiandrosterone (DHEA), a hormone precursor, and 5α-androstane-3β,17β-diol (3β-Adiol) a previously reported estrogen receptor β agonist. Within the body, diverse processes may be regulated through activation or inactivation of hormonal nuclear signaling receptors. CYP7B1-mediated metabolism may result in 6-and/or 7-hydroxymetabolites that can be eliminated from the cellular environment. CYP7B1 may therefore have an important role in regulation of hormonal levels in the cells and/or tissues.

In this study we examined the CYP7B1-mediated conversion of the steroids DHEA and 3β-Adiol. We used microsomes prepared from pig organs and a CYP7B1 expressing human kidney cell line (HEK293) as source for CYP7B1 enzyme. Many results on CYP7B1 are reported from experiments conducted with rodents. The pig has a high content of CYP7B1 and the CYP enzymatic system is more similar between human and pig than between hu-

man and rodents [133]. Pig organs for microsomal preparation can be obtained from an abattoir. To our knowledge, there are no previous reports on CYP7B1-mediated metabolism of 3β-Adiol in pig tissues.

We investigated if there might be a gender difference in metabolic rate and/or if there may be a difference in metabolism between castrated and uncastrated pigs. Sex differences have been reported for CYP enzymes in other species. Also there may be difficulties to determine at the abattoir the sex of the animal from whom the organs were collected. Most male pigs that are bred for human consumption are castrated at a young age, before they reach full sexual development. Experiments were conducted to analyze if the CYP7B1-mediated metabolism of DHEA and 3β-Adiol is dependent on tissue/organ, age and/or sex in pigs.

Results from these incubations indicate that there is no difference in metabolism between the genders or castrated/not castrated male pigs. On the other hand, tissue specificity and age were found to have an impact on the hydroxylation of DHEA and 3β-Adiol. Results from kinetic experiments indicate that the CYP7B1-dependent metabolic rate is in the same order of magnitude for both hormones. It may be concluded from these results that the synthesis and metabolism of these two steroids, in pigs, probably will vary throughout the individual's age and between different tissues.

The hormonal concentration levels may vary between tissues and species. Because of this we examined the potential effects of 3β-Adiol on the CYP7B1-mediated hydroxylation of DHEA and vice versa. Results are shown in Figure 6. Results from pig liver microsome incubations imply that 3β-Adiol is able to inhibit the metabolism of DHEA with about 60% when both steroids are present in equimolar concentrations. Results from experiments with DHEA as inhibitor of the hydroxylation of 3β-Adiol indicate that DHEA has to be present at about 10 times higher concentration compared to 3β-Adiol before a significant inhibition is achieved in incubations with pig liver microsomes. In corresponding experiments conducted with HEK293 cells, a human kidney-derived cell line, the results showed that 3β-Adiol and DHEA are able to inhibit each others hydroxylation at low concentrations.

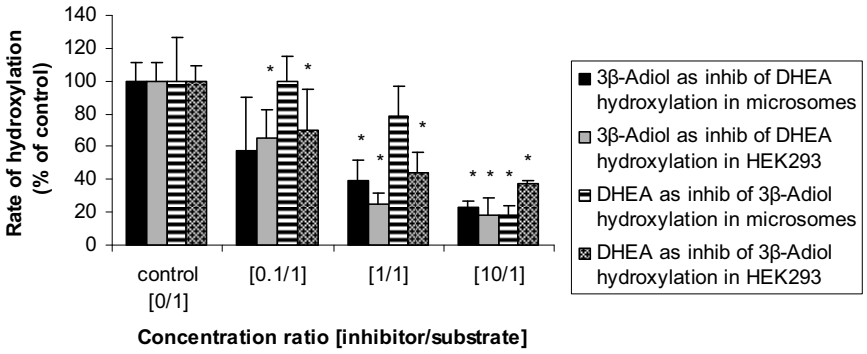


Figure 6. Effects of 3β-Adiol on the CYP7B1-mediated hydroxylation of DHEA and vice versa. Experiments were carried out in pig liver microsomes and human HEK293 cells. Concentrations are shown as ratio between added inhibitor and substrate. Controls consisted of incubation without added inhibitor [0/1].

To study the inhibitory effects by these two steroids we conducted experiments and the results were analyzed by construction of Dixon plots. The patterns of the Dixon plots were not consistent with a pure competitive or non-competitive inhibition, for either of the steroids. Instead these plots suggest that 3β-Adiol and DHEA may have a mixed inhibitory effect on each others CYP7B1-mediated metabolism.

For further information on the CYP7B1-mediated metabolism of 3β-Adiol in pig we determined the structures of the metabolites by GC/MS. Eluted fractions corresponding to retention times for hydroxymetabolites were collected from the HPLC and the structures were determined. The results from GC/MS showed that a majority of 5α-androstane-3β,7α,17β-triol was formed and minor formation of trace amounts of 6-hydroxy and 7β-hydroxy metabolites was also observed.

There are discrepancies in previously reported results concerning the role of CYP7B1 for ERβ signaling. Weihua *et al.* [76] have reported that 3β-Adiol can activate the receptor and that CYP7B1 in this case will eliminate this ligand by metabolism. On the other hand Martin *et al.* [120] conclude that CYP7B1 will generate an ERβ ligand by mediating the hydroxylation of DHEA to 7α-hydroxy-DHEA (7OH-DHEA). We investigated the two contradictory hypotheses by comparing the effects of 7OH-DHEA and 3β-Adiol in the same experiment and with the same methodology. Receptor activation was studied by reporter assay and the result indicated that 3β-Adiol is a more efficient activator of ERβ compared to 7OH-DHEA at least at steroid concentrations of 100 nM. Martin *et al.* reported activation at a much higher concentration, 5 μM.

As shown in this study pig organs can be used as CYP7B1 source for studying metabolism of 3β-Adiol and DHEA. Rodents are a good model for experiments that require *in vivo* results. Pigs are more alike humans in the CYP enzymatic system compared to rodents. It should be born in mind, how-

ever, that there may be species-specific differences that must be considered when any animal species is used as model for human metabolic studies.

There were no significant gender differences in CYP7B1-mediated metabolism of DHEA and 3β-Adiol but age-dependent as well as tissue-dependent differences were shown. The results on metabolite structure obtained in this study by GC/MS are in agreement with result from previously reported findings by other researchers conducted with other species.

The fact that CYP7B1 mediates formation of both 6- and 7-hydroxy metabolites which stereochemically may be in both α and β orientation indicates that CYP7B1 might implement its hydroxylation differently, perhaps depending on species, tissue and/or cellular environment. The kinetic analysis indicates that the rates of metabolism of DHEA and 3β-Adiol are similar. The results from the inhibition studies that we conducted may be an indication that cellular steroid concentration is of importance. Voigt and Bartsch have reported that the ratio of these two intracellular steroids is [DHEA] > [3β-Adiol] [134] and Mohler *et al.* [50] have reported that the DHEA level exceed the 3β-Adiol level by at least 10-fold in prostate tissue. The relatively high DHEA/3β-Adiol ratio together with the results from our study may lead to the conclusion that elevated DHEA levels can decrease the metabolism of 3β-Adiol and thereby increases ERβ-mediated signaling.

In summary, this study indicates that tissue-specific steroid concentrations may have an impact on the CYP7B1-dependent metabolism and thereby also on the levels of some CYP7B1 steroid substrates that may act as ERβ activators.

A novel CYP7B1-mediated hydroxylation and potential regulation of androgen levels in the cell (Paper III)

Maintenance of cellular levels of the potent androgen dihydrotestosterone (DHT) is of importance for different processes, including sexual functions and development of male urogenitals and normal tissue growth [45, 53, 59]. The steroid 5α-androstane-3α,17β-diol (3α-Adiol) is a metabolite formed from DHT. This conversion is a reversible process. 3α-Adiol has been proposed to serve as a less active or inactive source for DHT, thereby regulating the androgen-dependent effects. Another suggestion for 3α-Adiol action is that this steroid has physiological effects of its own on the $GABA_A$ receptor. 3α-Adiol is reported to exert sedative and anti-epileptic activities the brain [58, 135].

The structure of 3α-Adiol is very similar to the structure of the known CYP7B1 substrate 3β-Adiol (Figure 3). Although 3α-Adiol and 3β-Adiol are similar in structure these steroids have been reported to have different effects on *e.g.* cellular growth. 3β-Adiol is an ERβ agonist and 3α-Adiol has been reported to possess androgenic properties. However, other data suggest that 3α-Adiol may exhibit ERβ-mediated effects on learning and memory [57, 136].

Because of the structural resemblance between 3α-Adiol and 3β-Adiol we investigated the possibility that 3α-Adiol may be metabolized by CYP7B1. Hydroxylation of steroids is a possible way of regulating the cellular steroid concentrations. All previously known CYP7B1 substrates have a 3β-hydroxy structure. The results from this study indicate a previously unknown metabolic pathway for a 3α-hydroxy steroid, involving CYP7B1.

Results from initial experiments on the potential inhibitory effect of 3α-Adiol on the CYP7B1-mediated hydroxylation of 3β-Adiol showed that at equimolar concentrations, 3α-Adiol inhibits 3β-Adiol metabolism by about 50% in pig liver microsomes (Figure 7). Inhibition of 3β-Adiol hydroxylation may result in elevated concentration of this ERβ agonist and thereby a stronger intracellular estrogenic effect. Previous studies reported an androgenic suppression on the CYP7B1 promoter in LNCaP cells [137]. Other studies suggest that CYP7B1 might decrease estrogenic actions [76] or that CYP7B1 instead may contribute to formation of estrogenic ligands [120]. The present and previous results may lead to the conclusion that CYP7B1 actions may control androgenic and estrogenic intracellular balance.

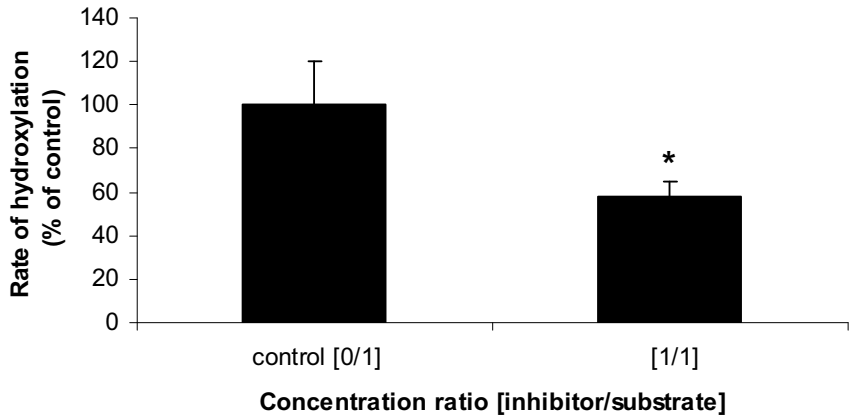


Figure 7. Effects of 3α-Adiol on the CYP7B1-mediated hydroxylation of 3β-Adiol in pig liver microsomes. The right bar shows the rate of hydroxylation of 3β-Adiol when an equimolar concentration of unlabeled 3α-Adiol is present. The left bar represents control incubations with no inhibitor added.

To evaluate if 3α-Adiol may be metabolized by a CYP-enzyme we examined if 3α-Adiol will undergo NADPH-dependent metabolism in microsome incubations. Because of the structural similarity between 3α-Adiol and 3β-Adiol we hypothesized that the retention times of the potential hydroxy-products from 3α-Adiol might correspond to the retention times for 3β-Adiol metabolites in the HPLC gradient system. One major metabolite was formed from 3α-Adiol and this had a retention time close to 3β-Adiol's major trihydroxy-metabolite. In addition, two or three minor products were formed.

Incubations without NADPH resulted in no product formation, indicating that the metabolism of 3α-Adiol in pig liver microsomes is CYP-dependent.

As described above the retention time for the major metabolite formed from 3α-Adiol was similar to the retention time of a known trihydroxy-metabolite from CYP7B1-mediated metabolism of 3β-Adiol. To determine the structure of the major 3α-Adiol metabolite we collected fractions from the HPLC and analyzed these on GC/MS. The results support the conclusion that the major product formed in pig liver microsomes from 3α-Adiol is a trihydroxy-metabolite with the structure 5α-androstane-3α,7α,17β-triol. The GC/MS spectrum is consistent with previous findings on 3α-Adiol metabolites formed in rat liver [138]. Also, the GC/MS pattern resembles the spectrum of the very similar steroid 5α-androstane-3β,7α,17β-triol, known to be formed by CYP7B1, as reported by other groups [139] and in paper II. The structural resemblance of these two metabolites indicates that the enzyme responsible for the metabolism of 3α-Adiol may be CYP7B1. There are earlier reports of 2- and 18-hydroxymetabolites formed from 3α-Adiol in rat liver. We did not observe any of these metabolites in this study. Our results do not exclude the possibility of an additional enzyme/ enzymes that may be involved in the hydroxylation of 3α-Adiol.

To obtain more evidence for the hypothesis that CYP7B1 is the enzyme responsible for the 7αhydroxylation of 3α-Adiol we conducted experiments with overexpression of human CYP7B1. HEK293 cells were transfected with an expression vector containing cDNA for CYP7B1 and analyzed for enzymatic activity. The enzymatic activity was compared to endogenous activity in cells without overexpression of the enzyme. The results showed a significant increase of metabolic activity when cells were transfected with CYP7B1 cDNA compared to non-transfected cells. These results further support the theory that CYP7B1 is able to conduct a 7αhydroxylation of this 3α-hydroxy steroid.

It has been proposed that the hormone receptors AR and ERβ may have counteracting actions, thereby balancing androgenic and estrogenic processes [59, 76]. As a part of this study we wanted to compare the effects of 3α-Adiol's action on these two nuclear receptors. HEK293 cells were transfected with expression vectors containing human AR or ERβ cDNA, together with ARE- or ERE-luciferase reporter vectors, and treated with hormones. Cells treated with DHT or 17β-estradiol were used as positive controls. These results indicate that 3α-Adiol may be more potent as an activator for AR compared to ERβ at a concentration of 0.1 μM. 3α-Adiol may have an important role as regulator of hormonal signaling by controlling the levels of more potent agonists such as 3β-Adiol and DHT. A suggested role for CYP7B1-mediated metabolism in the balance of androgenic and estrogenic activity is shown in Figure 8.

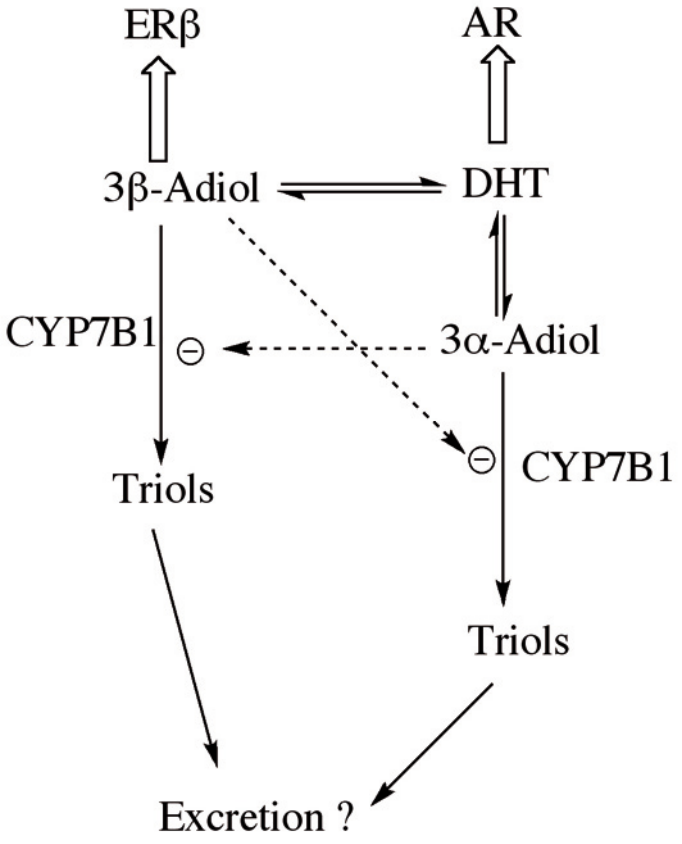


Figure 8. A suggested role for CYP7B1-mediated metabolism of 3α-Adiol and 3β-Adiol in balancing cellular androgenic and estrogenic hormone levels.

It may be speculated from the results obtained in the present study that CYP7B1-mediated metabolism of 3α-Adiol might enhance the elimination of 3α-Adiol from the cell, thereby preventing conversion back to DHT. This may be a potential regulatory mechanism for controlling the androgenic stimulation of cell growth. 3α-Adiol has been proposed to be a powerful $GABA_A$ modulator in the brain where CYP7B1 is also highly expressed. CYP7B1 also might be important for the maintenance of 3α-Adiol levels in the brain.

Effects of CYP7B1-mediated catalysis on ER activation (Paper IV)

Many biological effects in the body are mediated through the estrogen receptors ERα and ERβ. These effects include sexual functions, bone development and cell/tissue growth. It has been proposed that different types of estrogens may alert different responses. There are also suggestions that ERα and ERβ may counteract each others effects on cellular growth.

In this study we focused on the effects by CYP7B1-mediated catalysis on the estrogen-mediated response. There is a discrepancy in reported data on how CYP7B1 may affect activation of the ER. Suggestions have been made that CYP7B1-mediated metabolism of DHEA will form an ERβ agonist, 7OH-DHEA [120]. On the other hand there are suggestions that CYP7B1-mediated metabolism of another ERβ ligand, 3β-Adiol, will result in elimination of the agonist [76].

Since there are contradictory reports, either suggesting that CYP7B1 will form an ERβ ligand or that the enzyme is responsible for an elimination process, we investigated this by comparing the effects of CYP7B1 substrates and the CYP7B1-formed 7α-hydroxylated products. Experiments were carried out with an ERE (estrogen response element)-luciferase reporter system. HEK293 cells were transfected with human ERβ vector and then treated with 3β-Adiol, 5-androstene-3β,17β-diol (Aene-diol), DHEA and pregnenolone or their CYP7B1-formed 7α-hydroxy products (3β-Atriol, Aene-triol, 7OH-DHEA and 7OH-pregnenolone).

The results showed that only 3β-Adiol and Aene-diol are able to trigger a significant ERβ response in this system. Neither DHEA, pregnenolone nor the CYP7B1-formed 7α-hydroxy products were able to activate this nuclear receptor in our experiments. Results shown in Figure 9 imply that the action of CYP7B1 will abolish the estrogenic activities of 3β-Adiol and Aene-diol.

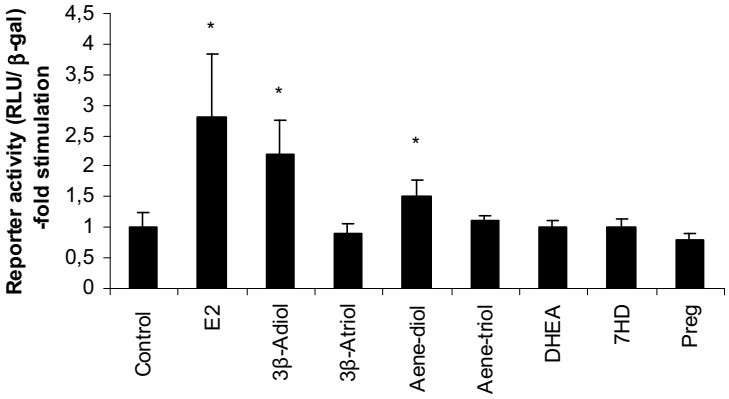


Figure 9. Effects of CYP7B1 steroid substrates and CYP7B1-formed products on ERβ-mediated response. The result indicates that only 3β-Adiol and Aene-diol are able to trigger a significant response on the ERβ receptor in this system. Ethanol and 17β-estradiol are used as negative and positive controls, respectively. 7OH-DHEA, 7HD; 17β-estradiol, E2.

The results from treatment with 7OH-DHEA are in contrast with results reported by Martin *et al* [120] which suggest that 7OH-DHEA is active as an ERβ ligand. We conducted experiments with different DHEA and 7OH-DHEA concentrations to obtain more information in this matter. We used one high concentration (10 μM) and one low concentration (10 nM), more similar to a physiological level [50]. Our results did not give any indication that either DHEA or 7OH-DHEA is able to trigger an ERβ-response. Factors that could contribute to the conflicting results are different cell types and available co-regulators that might influence ER-signaling.

Our results do not support the suggestion that CYP7B1 might form an ERβ agonist. Instead the results from this study imply that CYP7B1-mediated metabolism of 3β-Adiol and Aene-diol will abolish the estrogenic effect.

An oxysterol metabolized by CYP7B1, 27-hydroxycholesterol, has recently been shown to have pro-estrogenic activity in some cells and has also been connected to ER-mediated effects in vascular cells [140]. We investigated the ability of different oxysterols to activate ERα. The oxysterols of interest were 27-hydroxycholesterol, 7α-hydroxycholesterol, 7α-hydroxy-4-cholesten-3-one (two 7α-hydroxy structures) and a 15-ketosterol, 3β-hydroxy-5α-cholest-8(14)-en-15-one, with a similar structure as 27-hydroxycholesterol. Experiments were carried out with an ERE-(estrogen response element) luciferase reporter system. The results indicate that despite the structural similarity compared to CYP7B1 steroid substrates none of the oxysterols were able to activate ERα. The results from present and previous studies suggest that oxysterol-mediated effects on ER may be different in different cells and may be dependent on cell type and intracellular components [140, 141].

It has been suggested that the estrogenic response may differ between ERα and ERβ. To obtain more information on the role of CYP7B1 and Aene-diol for ER-mediated responses we conducted experiments with both receptor subtypes and treatment with Aene-diol and Aene-triol. To study how these responses may appear at a more physiological concentration, we added the steroids in a concentration of 10 nM. The results shown in Figure 10 indicate that Aene-diol may trigger a response equally well for both estrogen receptors and that CYP7B1-mediated metabolism abolishes the ER-response for both receptor subtypes. It can not be excluded from this study that the ER-mediated response may differ when the steroid concentrations are even lower. Kuiper *et al.* [86] have reported that Aene-diol has higher affinity for ERβ than for ERα although both appear to bind at nanomolar concentrations.

There are reports that Aene-diol is present at higher concentrations in some tissues (*e.g.* prostate) compared to 3β-Adiol [134] and in a review by Lardy *et al.*[142] there are reports of high yields of Aene-diol formed from DHEA in several different species.

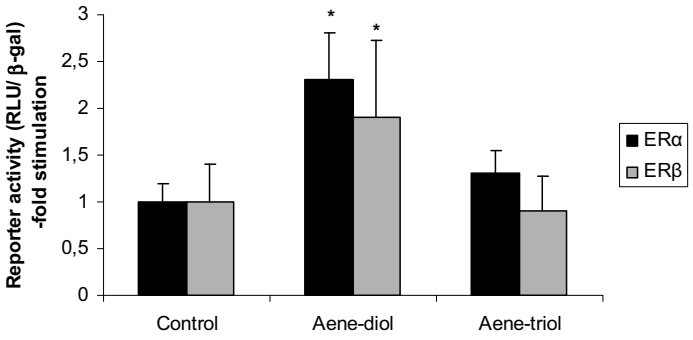


Figure 10. Effects of Aene-diol and Aene-triol, at a concentration of 10 nM, on ERα and ERβ-mediated response. The results indicate that Aene-diol may activate both ER subtypes and that CYP7B1-mediated hydroxylation of Aene-diol abolishes the ER-response.

Considering previous reports on binding affinity to ER and cellular steroid concentrations, together with the results from this study, it may be concluded that CYP7B1 hydroxylation may have an important role in regulating the intracellular concentrations of steroids with estrogenic effects. Other conclusions from these and previous results may be that the cellular environment, with regard for example to intracellular co-factors, is important for the ability of oxysterols to act as ER modulators.

The results from the current study add to the knowledge on the role of CYP7B1 in control of ER activation. This metabolism may be of importance for cellular growth and other ER-mediated processes.

Summary and conclusions

The enzyme CYP27A1 is active in both formation and metabolism of various steroid compounds including oxysterols. Oxysterols are molecules that have been suggested to be of importance for cholesterol homeostasis. When oxysterols are metabolized, by for example CYP27A1, they will most probably lose their regulatory effects.

Paper I describes the metabolism of a synthetic oxysterol (3β-hydroxy-24S-methyl-5α-cholesta-8(14),22-dien-15-one). In this study, we show that CYP27A1 is the enzyme responsible for a 28-hydroxylation of this oxysterol and that the rate of CYP27A1-mediated metabolism is relatively slow. This may give an explanation for the prolonged inhibitory effects on cholesterol biosynthesis that have been shown for this oxysterol. The current study contributes to the knowledge of synthetically produced oxysterols of potential use as cholesterol lowering drugs.

CYP7B1 is a widely expressed enzyme in different species and tissues. This enzyme has broad substrate specificity and is responsible for metabolism of several sex hormones. CYP7B1 may have an important role in regulation of hormonal levels in tissues and cells.

Paper II investigates the CYP7B1-mediated metabolism of DHEA (dehydroepiandrosterone), a sex hormone precursor, and 3β-Adiol (5α-androstane-3β,17β-diol), a previously reported ERβ agonist, in pig tissues and in a human cell line. Results from our experiments show that 3β-Adiol inhibits the hydroxylation of DHEA into 7αOH-DHEA by about 50-60% when the steroids are present in equimolar concentrations. DHEA has an inhibitory effect on 3β-Adiol's hydroxylation into 3β-Atriol, particularly in high concentrations. Kinetic studies indicated a mixed inhibition type. The rate of CYP7B1-mediated hydroxylation of DHEA and 3β-Adiol was similar. Taken together the data suggest that varying steroid concentrations may be important for CYP7B1-dependent catalysis. CYP7B1-mediated hydroxylation of these two steroids may influence the cellular levels of ERβ ligands.

The results of paper III describe a potential regulation of androgen levels and a novel CYP7B1-mediated hydroxylation of an androgen metabolite (5α-androstane-3α,17β-diol / 3α-Adiol). All previously known CYP7B1 substrates have a 3β-hydroxy structure. In this study we examined the possibility that CYP7B1 also might have metabolic activity towards a steroid with a 3α-hydroxy structure (3α-Adiol). Experiments with overexpression of the human CYP7B1 enzyme in HEK293 cells revealed that CYP7B1 is capable of metabolism of 3α-Adiol. The results also show that 3α-Adiol is able to inhibit the hydroxylation of 3β-Adiol and thereby might increase the level of this ERβ agonist. CYP7B1-mediated hydroxylation of 3α-Adiol might be a

potential pathway for elimination of this steroid thereby preventing back conversion into the potent androgen receptor agonist DHT (dihydrotestosterone).

Nuclear receptor-mediated processes include activation of the receptors ERα and ERβ. Some known CYP7B1 substrates are agonists for these receptors but the reported roles of CYP7B1 for ER action are contradictory in different studies. Paper IV investigates the role of CYP7B1-mediated metabolism for ER-mediated action. The results from this study indicate that CYP7B1 activity decreases the ER-mediated response. We examined the possibility that oxysterols with a structural similarity to CYP7B1 substrates may activate ER response. Although there are reports that some of these oxysterols may affect ER action, we did not observe any effects in our experimental system. Our data indicate that low concentrations of Aene-diol (5-androstene-3β,17β-diol) is able to trigger ER-mediated response equally well for both ERα and ERβ and that CYP7B1-mediated conversion of Aene-diol into a 7α-hydroxymetabolite will result in loss of action. The results contribute to the knowledge on the role of CYP7B1 for ER-mediated response. Hydroxylation by CYP7B1 might be of importance for regulation of ER-mediated processes like bone development and cellular growth.

Svensk sammanfattning /Summary in Swedish

Denna avhandling berör området metabola omvandlingar av steroider med betydelse för kolesterolbalansen och könshormonernas funktion. Tonvikten ligger på enzymerna CYP27A1 och CYP7B1. Dessa två enzymer, som tillhör enzymfamiljen cytokrom P450 (CYP), anses inneha viktiga men i viss mån outredda funktioner vad gäller kolesterolutsöndring och signalering som kan påverka hormonberoende celltillväxt. I avhandlingen studeras enzymatiska reaktioner som medieras av CYP27A1 eller CYP7B1. Avhandlingen rapporterar även tidigare okända metabola reaktioner utförda av ovanstående två enzymer.

Enzymet CYP27A1 är aktivt inom uppbyggnad samt nedbrytning av olika steroider, bland annat oxysteroler. En oxysterol är en kolesterolmolekyl med ett extra syre. Oxysteroler har föreslagits vara av vikt för kolesterolbalansen. När oxysteroler omvandlas (metaboliseras) av t ex CYP27A1 medför detta ofta att molekylen förlorar sin regulatoriska effekt.

CYP7B1 återfinns i flertalet olika vävnader. Enzymet metaboliserar flera olika könshormoner och tros vara viktig vad gäller reglering av hormonkoncentrationerna i olika organ.

I Arbete I genomfördes experiment för att utreda metabolismen av en ny syntetiskt framställd oxysterol (3β-hydroxy-24S-metyl-5α-cholesta-8(14),22-dien-15-one) som i djurstudier har visats ha kolesterolsänkande egenskaper. Denna kolesterolsänkande effekt kan vara av intresse för framställning av nya läkemedel mot höga kolesterolnivåer. Det var dock inte känt tidigare hur denna oxysterol metaboliseras. Det framkom att det humana CYP27A1 ger upphov till dels en 27- och dels en 28-hydroxylerad metabolit från denna syntetiska oxysterol. 28-Hydroxylering är en tidigare okänd reaktion för CYP27A1. Vi visar också att hastigheten för CYP27A1:s metabolism av 3β-hydroxy-24S-metyl-5α-cholesta-8(14),22-dien-15-one är mycket lägre jämfört med metabolismhastigheten för en sedan tidigare känd syntetisk kolesterolsänkande oxysterol. Att metabolismen är långsammare kan vara en bidragande orsak till den mer långvarigt hämmande effekt på kolesterolsyntesen som 3β-hydroxy-24S-metyl-5α-cholesta-8(14),22-dien-15-one visats ha.

I Arbete II studeras CYP7B1-medierad metabolism av steroiderna 5α-androstan-3β,17β-diol (3β-Adiol) och dehydroepiandrosteron (DHEA). DHEA är ett viktigt utgångsämne för bildning av könshormoner. 3β-Adiol har rapporterats vara en östrogenreceptor-agonist. Bägge steroiderna är således involverade i hormonell signalering. Resultat från denna studie visar att 3β-Adiol kan hämma hydroxylering av DHEA till 7α-hydroxy-DHEA med ca 50-60% om steroiderna återfinns i samma koncentrationer. Höga koncentrationer av DHEA har en hämmande effekt på 3β-Adiol's metabolism till hydrox-

ylerade metaboliter. Kinetiska analyser visar att CYP7B1:s metabola aktivitet mot 3β-Adiol och DHEA är i stort sett lika men att en hög DHEA/3β-Adiol kvot, liknande den kvot som föreligger i flera olika humana vävnader, kraftigt hämmar CYP7B1:s aktivitet. Detta tyder på att varierande steroidnivåer kan ha en viktig betydelse för CYP7B1-medierad metabolism av steroidhormonerna 3β-Adiol och DHEA.

I Arbete III studeras metabolismen av 5α-androstan-3α,17β-diol (3α-Adiol), vilket är en steroid som bildas från dihydrotestosteron (DHT) i bl a prostatavävnad. DHT är en mycket potent androgen som bland annat har en stimulerande effekt på vävnadstillväxt genom att aktivera androgenreceptorn (AR). Bildningen av 3α-Adiol från DHT anses minska de androgena effekterna då DHT är en starkare ligand för AR jämfört med 3α-Adiol. Bildningen av 3α-Adiol från DHT är dock en reversibel reaktion vilket innebär att 3α-Adiol kan ge upphov till DHT. 3α-Adiol har föreslagits ha egna fysiologiska effekter på bl a smärta genom att påverka $GABA_A$ receptorn i hjärnan. Resultaten från denna studie beskriver en möjlig reglering av cellens androgennivå genom en tidigare okänd CYP7B1-medierad metabolism av 3α-Adiol till hydroxylerade metaboliter. Alla tidigare kända CYP7B1 substrat har en distinkt 3β-hydroxy-struktur. I denna studie visar vi att CYP7B1 är katalytiskt aktiv mot en struktur som innehar en 3α-hydroxylgrupp. Denna enzymatiska reaktion kan vara en möjlig metabol väg för att eliminera 3α-Adiol och förhindra återbildning till DHT. Detta skulle kunna reglera celltillväxten i vävnaden.

I Artikel IV undersöker vi vilken roll CYP7B1-medierad metabolism har för östrogenreceptor-signalering. Processer som startar genom aktivering av cellkärnereceptorer inkluderar aktivering av östrogenreceptor α och β (ERα och ERβ). Det finns vissa kända CYP7B1 substrat som är agonister för dessa två nukleära receptorer. Dock är dessa rapporterade fynd i viss mån motstridiga. Resultaten i arbete IV tyder på att CYP7B1:s aktivitet kan leda till en minskad ER-aktivering för både ERα och ERβ. I ett annat experiment undersökte vi om oxysteroler eventuellt kan aktivera ER-signalering. Det finns vissa studier som rapporterar att några oxysteroler kan ha en effekt på ER. I denna studie kunde vi dock inte observera någon effekt av oxysteroler på ER-signalering. Resultaten från denna studie bidrar till ökade kunskaper om CYP7B1:s roll för ER-medierad signalering. De hydroxylerings-reaktioner som CYP7B1 utför kan vara viktiga för reglering av ER-medierade processer såsom skelettutveckling och celltillväxt.

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