Enzymatic Regulation of Steroidogenesis and Nuclear Receptor Activation

Special Focus on Vitamin D and Sex Hormones

JOHAN LUNDQVIST
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Abstract

Enzyme-catalyzed reactions are important to regulate steroidogenesis and nuclear receptor activation. The present investigation examines the role of steroid metabolism catalyzed by CYP7B1 for regulation of hormone receptor activation and the effects of vitamin D on enzymatic regulation of steroidogenesis.

The study reports data indicating that CYP7B1 can regulate estrogenic signaling by converting estrogens into inactive or less active metabolites. Similar results were obtained for CYP7B1-mediated metabolism of some androgen receptor ligands, indicating that CYP7B1 can be involved also in the regulation of androgenic signaling. CYP7B1 substrates and metabolites were found to exert androgenic effects in a cell line-specific manner. Furthermore, cell line differences were observed in the expression pattern for androgen receptor coactivators.

This thesis reports that 1α,25-dihydroxyvitamin D₃ alters the gene expression and enzyme activity of CYP21A2 and CYP17A1 leading to suppressed production of aldosterone, dehydroepiandrosterone and androstenedione in adrenocortical cells. These are novel findings on vitamin D action.

A mechanism is reported for the vitamin D-mediated regulation of the CYP21A2 gene. Data indicate that vitamin D receptor interacting repressor (VDIR) and Williams syndrome transcription factor (WSFT) are key coactivators in this novel vitamin D receptor (VDR)-mediated mechanism. Furthermore, the results indicate that altered expression levels of VDIR and WSFT can shift the suppressing effect of vitamin D to a stimulatory effect. Also, epigenetic components were found to be involved in the effects of vitamin D on CYP21A2 transcriptional rate. In addition, a functional vitamin D response element was identified in the CYP21A2 promoter.

This study also reports that 1α,25-dihydroxyvitamin D, affects sex hormone production in a tissue-specific way. Gene expression and enzyme activity of aromatase were found to be downregulated in cells derived from breast, but not in cells derived from prostate and adrenal cortex. The production of estradiol and dihydrotestosterone was altered in a tissue-selective manner following vitamin D treatment. These findings are of importance for the discussion on vitamin D as a potential anti-breast cancer agent.

Keywords: adrenal steroidogenesis, CYP7B1, vitamin D, calcitriol, enzymatic regulation, transcriptional regulation, CYP21A2, aromatase, sex hormone, estrogen, androgen, nuclear receptor

Johan Lundqvist, Department of Pharmaceutical Biosciences, Box 591, Uppsala University, SE-75124 Uppsala, Sweden.

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“These experiments clearly demonstrate the existence of a fourth vitamin whose specific property, as far as we can tell at present, is to regulate the metabolism of the bones.”

McCollum et al., Journal of Biological Chemistry, 1922
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., Lundqvist, J. and Norlin, M.
   Effects of CYP7B1-mediated catalysis on estrogen receptor activation.

II  Lundqvist, J. and Norlin, M.
   Effects of CYP7B1-related steroids on hormone receptor activation in different cell lines.
   *Manuscript*

III  Lundqvist, J., Norlin, M. and Wikvall, K.
    1α,25-Dihydroxyvitamin D₃ affects hormone production and expression of steroidogenic enzymes in human adrenocortical NCI-H295R cells.

IV  Lundqvist, J., Norlin, M. and Wikvall, K.
    1α,25-Dihydroxyvitamin D₃ exerts tissue-specific effects on estrogen and androgen metabolism.

V  Lundqvist, J., Norlin, M. and Wikvall, K.
    Vitamin D-mediated regulation of CYP21A2 transcription – a novel mechanism for vitamin D action.
    *Submitted*

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<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>3β-Adiol</td>
<td>5α-androstane-3β,17β-diol</td>
</tr>
<tr>
<td>3β-Atriol</td>
<td>5α-androstane-3β,7α,17β-triol</td>
</tr>
<tr>
<td>3β-HSD</td>
<td>3β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>5-Aza</td>
<td>5-aza-2′-deoxycytidine</td>
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<tr>
<td>7α-hydroxy-DHEA</td>
<td>7α-hydroxy-dehydroepiandrosterone</td>
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<td>17β-HSD</td>
<td>17β-hydroxysteroid dehydrogenase</td>
</tr>
<tr>
<td>Aene-diol</td>
<td>5-androstene-3β,17β-diol</td>
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<tr>
<td>Aene-triol</td>
<td>5-androstene-3β,7α,17β-triol</td>
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<tr>
<td>AR</td>
<td>androgen receptor</td>
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<tr>
<td>ARE</td>
<td>androgen response element</td>
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<tr>
<td>bp</td>
<td>base pair</td>
</tr>
<tr>
<td>CAH</td>
<td>congenital adrenal hyperplasia</td>
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<tr>
<td>Calcidiol</td>
<td>25-hydroxyvitamin D₃</td>
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<tr>
<td>Calcitriol</td>
<td>1α,25-dihydroxyvitamin D₃</td>
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<tr>
<td>CAR</td>
<td>constitutive androstane receptor</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<tr>
<td>DHEA</td>
<td>dehydroepiandrosterone</td>
</tr>
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<td>DHEA-S</td>
<td>dehydroepiandrosterone sulfate</td>
</tr>
<tr>
<td>DHT</td>
<td>dihydrotestosterone</td>
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<tr>
<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<tr>
<td>DNA</td>
<td>deoxyribonucleic acid</td>
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<tr>
<td>ELISA</td>
<td>enzyme-linked immunosorbent assay</td>
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<tr>
<td>ERα</td>
<td>estrogen receptor α</td>
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<td>ERβ</td>
<td>estrogen receptor β</td>
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<tr>
<td>ERE</td>
<td>estrogen response element</td>
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<td>estradiol</td>
<td>17β-estradiol</td>
</tr>
<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
</tr>
<tr>
<td>HPLC</td>
<td>high-performance liquid chromatography</td>
</tr>
<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
</tr>
<tr>
<td>NCoR-1</td>
<td>nuclear receptor co-repressor 1</td>
</tr>
<tr>
<td>nVDRE</td>
<td>negative vitamin D responsive element</td>
</tr>
<tr>
<td>POR</td>
<td>P450 oxidoreductase</td>
</tr>
<tr>
<td>pVDRE</td>
<td>positive vitamin D responsive element</td>
</tr>
<tr>
<td>RLU</td>
<td>relative light units</td>
</tr>
<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
</tr>
<tr>
<td>RXR</td>
<td>retinoid X receptor</td>
</tr>
</tbody>
</table>
SF-1  steroidogenic factor-1
SMRT  nuclear receptor co-repressor 2
SRA   homo sapiens steroid receptor RNA activator
SRC-1 human steroid receptor coactivator
StAR  steroidogenic acute regulatory protein
SULT2A1 sulfotransferase 2A1
TBP   TATA box binding protein
TLC   thin layer chromatography
TSA   trichostatin A
UV    ultraviolet
VDIR  VDR interacting repressor
VDR   vitamin D receptor
VDRE  vitamin D responsive element
WSTF  Williams syndrome transcription factor
Introduction

Production and action of steroid hormones

All steroid hormones are synthesized from the common precursor cholesterol, which can be obtained from the diet or de novo synthesized from acetyl CoA. The production of steroid hormones is regulated via a number of enzymes of which a majority belongs to the cytochrome P450 (CYP) superfamily. An overview of steroids and enzyme-catalyzed reactions of importance for this thesis is shown in figures 1, 2 and 3.

Steroid hormones exert a wide range of physiological responses, including functions in the immune system, protein and carbohydrate metabolism, water and salt balance, reproductive system and development of sexual characteristics. Steroid hormones are synthesized in steroidogenic tissues such as the adrenal cortex, breast, ovaries, prostate and testis, either from cholesterol or from steroidogenic precursors secreted from other steroidogenic tissues. In the work presented in this thesis, enzymatic regulation of the production and action of steroid hormones has been investigated.

Adrenal steroidogenesis

The adrenal cortex produces steroid hormones such as aldosterone, corticosterone, cortisol, dehydroepiandrosterone (DHEA) and androstenedione. The adrenal steroidogenesis is quantitatively regulated by the transcription of CYP11A1 (cholesterol side-chain cleavage enzyme) and the activity of steroidogenic acute regulatory protein (StAR).

There are three adrenocortical zones, each with a distinct role in the production of steroid hormones; zona glomerulosa produces mineralocorticoids (e.g. aldosterone), zona fasciculata produces glucocorticoids (e.g. cortisol) and zona reticularis is the point of synthesis for adrenal androgens (e.g. DHEA). The qualitative regulation of adrenal steroidogenesis, determining which type of steroid that will be produced, is performed by the transcription and activity of CYP17A1. CYP17A1 catalyzes two different reactions, namely the 17α-hydroxylation and the 17,20-lyase reaction. The expression and activity of CYP17A1 differs between the three adrenal zones [1-3]. CYP17A1 is not expressed in zona glomerulosa leading to the produc-
Figure 1. Enzyme-catalyzed reactions in the adrenal steroidogenesis that are of importance for the work presented in this thesis.

Figure 1. Enzyme-catalyzed reactions in the adrenal steroidogenesis that are of importance for the work presented in this thesis.

tion of mineralocorticoids. In zona fasciculata, CYP17A1 is expressed and catalyzing 17α-hydroxylation, but not the 17,20-lyase activity, leading to glucocorticoid production. In zona reticularis, CYP17A1 catalyzes both 17α-hydroxylation and 17,20-lyase activities and adrenal androgens are therefore the main product of adrenal steroidogenesis in this adrenal zone [1]. Hence, in order to control the production of steroid hormones it is necessary to control the two activities of CYP17A1 separately. It is well-known that the manners of regulation for the two activities of CYP17A1 are principally different. The 17α-hydroxylase activity is regulated via the expression
of the CYP17A1 gene, while the 17,20-lyase activity is regulated by post-
transcriptional mechanisms [1-7]. The adrenal zone-specific activities of
CYP17A1 are summarized in figure 2.

Three posttranscriptional mechanisms for regulation of 17,20-lyase activ-
ity have been described; the abundance of P450 oxidoreductace (POR) [8,
9], allosteric action of cytochrome b5 [10] and serine phosphorylation of
CYP17A1 [11-14]. Isolated 17,20-lyase deficiency is a rare condition first
described by Zachmann et al. 1972 [15]. It has recently been reported that
the 17,20-lyase deficiency could be a result of a mutated cytochrome b5
[16]. CYP17A1 deficiency, or impaired CYP17A1 activity due to altered
posttranscriptional mechanisms, may lead to hypertension, hypokalemia and
impaired development of sexual characteristics due to decreased production
of adrenal androgens. Patients that are genetically male often present com-
plete male pseudohermaphroditism while female patients may be infertile
[17-19].

It has been reported that CYP17A1 strongly prefers 17α-
hydroxypregnenolone over 17α-hydroxyprogesterone as a substrate for
17,20-lyase activity in humans [1, 20]. 17α-Hydroxyprogesterone may,
however, be a substrate for CYP17A1 in other species [20].

CYP21A2 is a steroid 21-hydroxylase catalyzing the production of 11-
deoxycorticosterone and 11-deoxycortisol, which are precursors for the pro-
duction of corticosterone, aldosterone and cortisol. 21-Hydroxylase defi-
ciency may lead to severe conditions such as congenital adrenal hyperplasia
(CAH) and Addison’s disease [21]. CYP21A2 is exclusively expressed in
the adrenal cortex [1]. Therefore, glucocorticoids and mineralocorticoids can
not be synthesized in other tissues than the adrenal cortex.
Figure 2. Adrenal zone-specific activities of CYP17A1.

Sex hormone production

Sex hormones are mainly produced in the gonads, breast and prostate. The sex hormones in these tissues are produced either by in situ synthesis from cholesterol or by enzyme catalyzed conversion of DHEA or androstenedione excreted to the circulation from the adrenal cortex.

The sex hormones are divided into two groups; androgens and estrogens. Androgens such as testosterone and 5α-dihydrotestosterone (DHT) are produced via reactions catalyzed by 17β-hydroxysteroid dehydrogenase (17β-HSD) and 5α-reductase. Estrogens, such as 17β-estradiol (estradiol), are produced by aromatization of androgenic precursors, a reaction catalyzed by CYP19A1 (aromatase). Hence, the tissue-selective expression and activity of 5α-reductase and aromatase regulates the production of androgens and estrogens [22-24]. Enzyme-catalyzed reactions in the sex hormone production of importance for this thesis are summarized in figure 3. For some of these steroids, it remains unclear if they act as estrogens or as androgens or if they are inactive metabolites [25]. To fully understand the estrogenic and/or androgenic signaling exerted by these steroids is of utmost importance in research on e.g. breast and prostate carcinogenesis.

It is crucial to regulate the levels of sex hormones in order to achieve normal gonadal development. Abnormally high levels of estrogens and androgens are associated with increased risk for breast cancer and prostate cancer, respectively [23, 26-28].
The role of steroid 7α-hydroxylation

The widely expressed steroid 7α-hydroxylase CYP7B1 is involved in the metabolism of a number of steroids reported to influence estrogenic and androgenic signaling. For instance, CYP7B1 converts DHEA, 5-androstene-3β,17β-diol (Aene-diol) and 5α-androstane-3β,17β-diol (3β-Adiol), to the corresponding 7α-hydroxy steroids [29-31]. Furthermore, it has recently been reported that 5α-androstane-3α,17β-diol is a substrate for CYP7B1 [32]. The effect of this CYP7B1-catalyzed conversion of steroids on their ability to exert estrogenic and androgenic effects has been disputed. Some researchers have suggested that CYP7B1 substrates, but not products, may stimulate estrogen receptor β (ERβ), indicating that CYP7B1 activity could be a mechanism to decrease ERβ activity [33]. Other researchers have proposed that CYP7B1 products, but not substrates, can activate ERβ, suggesting that CYP7B1 activity would increase estrogenic signaling [34].

Conflicting data have been published regarding the effect of DHEA and 7α-hydroxy-DHEA on the androgen receptor. Mo et al. [35] have reported that DHEA, but not 7α-hydroxy-DHEA, exerts androgenic effects. Chen et al. [36] suggests that DHEA rather acts as an antagonist to the androgen receptor and Evalul et al. [37] have reported that DHEA has to be converted to androstenedione, a reaction catalyzed by 3β-hydroxysteroid dehydrogenase (3β-HSD), to gain androgenic effect. Aene-diol has been reported to
have androgenic effects by some researchers [38-40], while other researchers state that Aene-diol has to be converted to testosterone to gain androgenic properties [37]. In conclusion, the role of these CYP7B1 substrates and metabolites, and thereby the role of CYP7B1 activity, in estrogenic and androgenic signaling is still not fully understood.

Estrogen and androgen receptors

Estrogens and androgens exert most of their effects via nuclear receptors. Estrogenic effects are mediated by estrogen receptor α (ERα) and estrogen receptor β (ERβ) while androgenic response is mediated by the androgen receptor (AR). These receptors are nuclear transcription factors that, when ligand activated, are able to regulate gene expression.

The two estrogen receptors are encoded by different genes and have separate expression patterns. ERα is expressed in breast, liver, bone, brain, urogenital tract and in the cardiovascular system while ERβ is expressed in breast, bone, urogenital tract, gastrointestinal tract, lung, cardiovascular system and in the brain [41]. Estrogens can either activate both ERα and ERβ or be a specific ligand for one of the receptors. When ligand activated, the estrogen receptors alter gene expression of estrogen sensitive genes by dimer formation (ERα:ERα, ERα:ERβ or ERβ:ERβ). The ligand activated receptor dimer binds to an estrogen receptor response element in the gene promoter and alters the transcriptional rate via recruitment of coactivators. Estrogens have also been found to exert rapid responses, which have been suggested to be mediated by membrane-bound receptors [42].

The androgen receptor is expressed in neuroendocrine and musculoskeletal tissues and in the male urogenital system [43]. Ligand-activated androgen receptor alters gene expression of androgen-sensitive genes by a mechanism resembling the one for estrogen receptors. Ligand-activated androgen receptor forms a homodimer, binds to an androgen response element in the gene promoter and alters the transcriptional rate by recruitment of coactivators.

Sex hormone-dependent cancers

Sex hormone signaling plays important roles in both breast and prostate carcinogenesis.

A large majority of all breast cancers are classified as estrogen receptor positive, and most of these tumors rely on estrogens to proliferate [26]. For estrogen-dependent carcinomas arising in postmenopausal women, when the ovarian estrogen production is decreased, the local estrogen production in breast is crucial for the tumor development [44]. Therefore, regulation of estrogenic signaling and estrogen production has become key strategies in breast cancer treatment [23, 26]. The enzyme aromatase catalyzes the conversion of androgenic precursors to estrogens. The gene expression of aro-
matase is regulated by different promoters in a tissue-specific manner [24, 45]. The aromatase expression is higher in breast cancer tissue than in normal breast tissue and the local estrogen levels in breast cancer tissue are higher than the circulating levels [46, 47]. Treatment with the antiestrogen tamoxifen has been first line endocrine therapy for more than thirty years, but today treatment with a third generation aromatase inhibitor like letrozole, anastrozole or exemestane is the recommended first line endocrine therapy for most postmenopausal women. However, treatment with both aromatase inhibitors and anti-estrogens are associated with risk of adverse effects (eg. osteoporosis) due to effects on peripheral estrogen metabolism [48, 49].

Androgenic signaling is important in a broad range of physiological processes, including normal prostate development and prostate carcinogenesis. A majority of all prostate cancers are androgen dependent when diagnosed (ie. they rely on androgens to proliferate) [50]. Furthermore, cells in benign prostate hyperplasia rely on androgens to proliferate. Therefore, decreased androgen production and/or inhibited androgenic signaling have become key strategies in the treatment of prostate cancer and benign prostate hyperplasia [51] (eg. antiandrogens and 5α-reductase inhibitors). As an alternative strategy to decrease androgen production, a CYP17A1 inhibitor has undergone a phase III clinical trial as a treatment against castration-resistant prostate cancer [52]. Furthermore, ERβ is expressed in the prostate and has been proposed to be involved in prostate growth inhibition [53, 54], although the role of estrogenic signaling in the regulation of prostate proliferation remains unclear.

From vitamin D towards hormone D

Vitamin D was discovered in the early 20th century when it was described that rickets could be cured by sunlight or cod liver oil. This fat-soluble vitamin was the fourth vitamin described and therefore designated as vitamin D. Vitamin D was found to regulate intestinal absorption and renal reabsorption of calcium and bone metabolism. Later research has demonstrated that vitamin D can be obtained from the diet or be de novo synthesized from 7-dehydrocholesterol. Further, the active form of vitamin D, 1α,25-dihydroxyvitamin D₃, interacts with a receptor in the target cells, showing that vitamin D should be biochemically characterized as a hormone rather than as a vitamin [55].

During the last decades, the outlook on vitamin D has widened, from being a vitamin solely involved in bone metabolism and calcium homeostasis, to being a multifunctional hormone known to affect a broad range of physiological processes. This includes effects on the immune system, brain and fetal development, insulin secretion, cancer, apoptosis, cell proliferation and differentiation as well as the cardiovascular system via the vitamin D recep-
tor (VDR) [55-58]. The vitamin D receptor is widely expressed and it has been suggested that 1α,25-dihydroxyvitamin D₃ may have other roles yet undiscovered [57, 59, 60]. It has recently been suggested that 1α,25-dihydroxyvitamin D₃ may affect the expression of up to 200 genes in humans [61].

Production, bioactivation and metabolism

There are two forms of vitamin D, vitamin D₂ (ergocalciferol) which is synthesized in plants, yeast and fungi and vitamin D₃ (cholecalciferol) which is synthesized in animals. Vitamin D₃ is synthesized in the skin from 7-dehydrocholesterol upon exposure to UV-B radiation. Vitamin D₃ is then bioactivated in two subsequent steps to gain the biologically active form of vitamin D (figure 4). In the first step, vitamin D₃ is 25-hydroxylated to 25-hydroxyvitamin D₃ (calcidiol). 25-Hydroxylation of vitamin D is a reaction that can be catalyzed by the mitochondrial CYP27A1 and the microsomal CYP3A4, CYP2R1 and CYP2J2 in humans. 25-Hydroxylation of vitamin D is mainly performed in the liver and calcidiol is then excreted into the circulation. Calcidiol is converted to 1α,25-dihydroxyvitamin D₃ (calcitriol) by 1α-hydroxylation, mainly performed in the kidneys. The principal human 1α-hydroxylase for 25-hydroxyvitamin D₃ is CYP27B1. The 1α,25-dihydroxyvitamin D₃ produced is excreted into the circulation and acts as a hormone [55, 57, 58, 62-64].

Extrarenal 1α-hydroxylation of 25-hydroxyvitamin D₃ has been reported for a wide range of tissues, including colon, brain, mammary tissue, breast, pancreatic islets, parathyroid glands, placenta, prostate and keratinocytes [57, 62]. These findings indicate that 1α,25-dihydroxyvitamin D₃ may be produced locally and act in an intracrine or paracrine fashion. It may be speculated that the local concentration of 1α,25-dihydroxyvitamin D₃ in these tissues could be higher than the circulating levels.

The normal serum level of calcidiol is 50-100 nM and for calcitriol 50-125 pM [57]. Calcitriol is the most potent form of vitamin D even though calcidiol can exert some biological effects as well. The circulating levels of 1α,25-dihydroxyvitamin D₃ is tightly regulated via a feed-back mechanism where 1α,25-dihydroxyvitamin D₃ downregulates the expression of CYP27B1 and upregulates the expression of CYP24A1 [63].

Both calcidiol and calcitriol is metabolized by CYP24A1 to the less active compounds 24,25-dihydroxyvitamin D₃ and 1α,24,25-trihydroxyvitamin D₃ respectively [65]. It has recently been reported that CYP11A1 can catalyze the production of 20-hydroxyvitamin D₃ from vitamin D₂ and the production of 1α,20-dihydroxyvitamin D₃ from 1α-hydroxyvitamin D₃ [66-70]. Both these metabolites have been reported to exert biological effects on cell differentiation and gene expression in a way resembling the one of 1α,25-
dihydroxyvitamin D$_3$ [66, 67]. The physiological role, if any, of this CY11A1-mediated metabolism of vitamin D remains to be clarified.

![Figure 4. Bioactivation of vitamin D$_3$ to its hormonally active form, 1α, 25-dihydroxyvitamin D$_3$.](image)

**Mode of action**

The bioactivated form of vitamin D alters the gene expression of a large number of genes. 1α,25-Dihydroxyvitamin D$_3$ may increase the expression of certain genes while it decreases the expression of other genes. Both these effects of 1α,25-dihydroxyvitamin D$_3$ are mediated by activation of the vitamin D receptor (VDR) which forms a heterodimer with the retinoid X receptor (RXR). Extensive research has been conducted to understand the mechanism for 1α,25-dihydroxyvitamin D$_3$-mediated induction of gene expression. The mechanism is now well known and based on a direct interaction between the ligand-activated VDR-RXR complex and a vitamin D responsive element (VDRE) in the gene promoter. These positive VDRE (pVDRE) consist of a hexameric direct repeat of the consensus sequence 5′-RGKTCA (R=A or G, K=G or T) [71]. The two half sites are separated by a three nucleotide spacer. The ligand-activated VDR-RXR complex interacts with the pVDRE and acts as a transcription factor to increase the transcriptional rate by recruiting coactivators [57].

The mechanism for vitamin D-mediated downregulation of gene expression, on the other hand, has in large part remained unclear [72]. Studies on the mechanism for 1α,25-dihydroxyvitamin D$_3$-mediated downregulation of gene expression have been performed for only a few genes. The proposed mechanism for those genes includes recruitment of corepressors such as VDR interacting repressor (VDIR) and Williams syndrome transcription factor (WSTF) as well as epigenetic mechanisms such as histone deacetylation and DNA methylation [73-77]. The negative vitamin D responsive elements (nVDRE) that have been described only vaguely resembles the pVDRE described [77]. It has been suggested that the mechanism for vitamin D-mediated downregulation of gene expression does not include a direct
interaction between the ligand-activated VDR-RXR complex and the nVDRE, but rather an interaction between nVDRE and VDIR [73].

Vitamin D has also been reported to exert rapid effects only seconds or minutes after treatment. Due to the quick response, it has been suggested that these effects are non-genomic and mediated by membrane-bound receptors [78].

Vitamin D and adrenal steroidogenesis

There are a few early reports in the literature suggesting a connection between vitamin D and the adrenal steroidogenesis [79, 80]. De Toni et al. [79] reported in 1959 several clinical cases describing children with rickets having changes in urinary 17-ketosteroid levels. The authors suggested that the disturbance in steroid metabolism may have some bearing on the action of vitamin D and that it exists some relationship between such metabolic disturbance and the sensitivity or resistance of organisms to antirachitic vitamins. In a study with puppies, Guseinova and collaborators found a decrease in the secretion of 17-ketosteroids after rachitogenic treatment [80]. In a more recent publication, a case has been reported where a woman with osteomalacia had elevated serum levels of aldosterone and low levels of 25-hydroxyvitamin D [81]. The condition was normalized after 24 months of treatment with vitamin D. Chatterjee and collaborators [82, 83] have reported that ligand-activated VDR upregulates the expression of sulfotransferase 2A1 (SULT2A1), an enzyme that catalyzes the conversion of dehydroepiandrosterone (DHEA) to dehydroepiandrosterone-sulfate (DHEA-S).

Vitamin D and sex hormones

The production of sex hormones is regulated by multiple enzymes (figure 3). Vitamin D has been reported to affect the expression and activity of several of these enzymes. Wang and Tuohimaa [84] have reported that 1α,25-dihydroxyvitamin D₃ upregulates the mRNA level for 17β-hydroxysteroid dehydrogenases (17β-HSD) type 2, 4 and 5 in cell lines derived from human prostate. In keratinocytes, Hughes et al. [85] have reported that 1α,25-dihydroxyvitamin D₃ stimulates the expression of 17β-HSD type 1 and 2. It has been shown that 1α,25-dihydroxyvitamin D₃ alters the aromatase activity in placental cells [86, 87], prostate cells [88] and osteoblasts [89, 90]. Kinuta et al. [91] have reported that vitamin D receptor null mutant mice have a decreased aromatase activity in the ovary, testis and epididymis. Recently, it was reported that 1α,25-dihydroxyvitamin D₃ alters the gene expression of aromatase in a tissue-selective manner [92]. In breast cancer cell lines, vitamin D treatment resulted in decreased aromatase gene expression, while the same treatment increased the aromatase gene expression in osteosarcoma cell lines. 1α,25-Dihydroxyvitamin D₃ has therefore been
proposed to be a tissue-selective aromatase modulator [92]. Furthermore, $1\alpha,25$-dihydroxyvitamin D$_3$ and analogs have been reported to alter sex hormone signaling in breast cancer cells by suppressing the expression of estrogen receptor $\alpha$ [93-96]. It has also been reported that vitamin D deficiency alters reproductive functions in both male and female rats, indicating that vitamin D may affect sex hormone signaling [97, 98].

Vitamin D and breast cancer

It is well-known that $1\alpha,25$-dihydroxyvitamin D$_3$ exerts anti-proliferative and pro-differentiating effects and has therefore been proposed to be of potential use as an anti-cancer agent [99-102]. Epidemiological studies have revealed relationships between solar UV-B exposure, which is needed for vitamin D$_3$ production, and decreased incidence and mortality in breast cancer. Low 25-hydroxyvitamin D$_3$ serum level has been found to be correlated to breast cancer risk [100, 103]. $1\alpha,25$-Dihydroxyvitamin D$_3$ has also been reported to affect apoptosis and expression of tumor suppressor genes [100, 103]. As discussed above, it has been reported that $1\alpha,25$-dihydroxyvitamin D$_3$ may alter the estrogenic signaling by suppression of gene expression for aromatase [92] and estrogen receptor $\alpha$ [93-96]. These effects are of importance for the discussion of vitamin D as an anti-cancer agent against breast cancer.
Aims of the present investigation

The overall aim of the present investigation was to study the enzymatic regulation of steroidogenesis and nuclear receptor activation with a special focus on the effects of vitamin D on steroidogenesis and the effects of metabolism of steroids for their ability to activate estrogen and androgen receptors.

The specific aims were:

- To study the role of CYP7B1-mediated catalysis for nuclear receptor activation
- To study the effects of 1α,25-dihydroxyvitamin D3 on adrenal steroid hormone production and expression and activity of key adrenocortical enzymes
- To study the effects of 1α,25-dihydroxyvitamin D3 on enzymes of importance for estrogen and androgen metabolism
- To study the mechanism for 1α,25-dihydroxyvitamin D3-mediated effects on CYP21A2
Experimental procedures

Materials

1α,25-Dihydroxyvitamin D₃ was obtained from Solvay, Duphar, The Netherlands. CYP21A2 promoter-luciferase reporter constructs were a kind gift from professor Walter L. Miller, University of California, San Francisco. Expression vectors for VDIR and WSTF were kind gifts from professor Shigeaki Kato, University of Tokyo. The full-length human vitamin D receptor (VDR) expression vector was a gift from Dr. Leonard Freedman, the Merck Research Laboratories, West Point. The expression vector for the human retinoid X receptor (RXR) was a kind gift from Dr. Ronald M. Evans, Howard Hughes Medical Institute, The Salk Institute for Biological Studies, San Diego. The human ERβ and ERα expression vectors and the ERE (estrogen response element) luciferase reporter vector were generous gifts from Dr. P. Chambon, Institut de génétique et de biologie moléculaire et cellulaire, Strasbourg, and Dr. K. Arcaro, University of Massachusetts, respectively. The human AR expression vector and the ARE (androgen response element) luciferase reporter vector were generous gifts from Dr. A. Brinkmann and Dr. J. Trapman, Erasmus Medical Centre, Rotterdam. The vitamin D receptor antagonist TEI-9647 was a generous gift from Teijin Pharma, Tokyo.

NCI-H295R cells and Y-1 cells were a kind gift from professor Ingvar Brandt and Caco-2 cells from professor Per Artursson, both at Uppsala university. All other chemicals were of analytical grade and purchased from various commercial sources.

Cell culture and treatment

Human adrenocortical carcinoma NCI-H295R cells (ATCC CRL-2128) were cultured as a monolayer in Dulbecco’s modified Eagle’s medium/Nutrient Mixture F-12 Ham (Sigma) supplemented with 1% ITS Plus premix (BD Biosciences), 2.5% NuSerum (VWR), 1% L-glutamine (Gibco) and 1% antibiotic/antimycotic (Gibco). The cells were subcultured once a week and the medium was changed two to three times a week.

Human breast adenocarcinoma MCF-7 cells (ATCC HTB-22), human embryonic kidney HEK-293 cells (ATCC CRL-1573) and human epithelial colorectal adenocarcinoma Caco-2 cells (ATCC HTB-37) were cultured as a
monolayer in Dulbecco’s modified Eagle’s medium (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotics/antimycotics (Gibco). MCF-7 cells were subcultured once a week and the medium was changed once a week. Caco-2 cells were subcultured twice a week. HEK-293 cells were subcultured twice a week.

Mouse adrenocortical Y-1 cells (ATCC CCL-79) were cultured as a monolayer in F-12K medium (ATCC) supplemented with 15% horse serum (ATCC) and 2.5% fetal bovine serum (Gibco) and 1% antibiotic/antimycotic (Gibco). Cells were subcultured once a week and the medium was changed every 48 hour.

Human prostate adenocarcinoma LNCaP cells (ATCC CRL-1740) and human prostate carcinoma DU-145 cells (ATCC HTB-81) were cultured in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco) and 1% antibiotic/antimycotic (Gibco). The cells were subcultured twice a week.

All cells were cultured in a humidified environment at 37°C with 5% CO₂. Cells were treated with different hormones or other substances dissolved in ethanol or dimethyl sulfoxide (DMSO). The control group was treated with the same amount of vehicle (ethanol and/or DMSO).

Analysis of steroid hormone secretion

In order to analyze the steroid hormone secretion, the cell culture medium from treated cells was collected and the hormone levels were analyzed using enzyme-linked immunosorbent assay (ELISA). Secretion of aldosterone, corticosterone, cortisol, androstenedione, DHEA, DHEA-S, estradiol, testosterone and dihydrotestosterone was measured. The ELISA kits were purchased from Demeditech Diagnostics GmbH, Germany. The absorbance was measured at 450 nm, using a Polarstar Optima (BMG Labtech) plate reader. The analyses were performed in accordance with the manufacturer’s recommendations.

Reverse transcriptase-polymerase chain reaction (RT-PCR)

Expression of CYP11A1, CYP17A1, CYP21A2, 3β-HSD, CYP11B1, CYP11B2, VDR, VDR interacting repressor (VDIR), Williams syndrome transcription factor (WSTF), 5α-reductase, aromatase, Hem45, AR, nuclear receptor co-repressor 1 (NCoR-1), nuclear receptor co-repressor 2 (SMRT), human steroid receptor coactivator (SRC-1) and homo sapiens steroid receptor RNA activator (SRA) mRNA in different cell lines were measured by RT-PCR. RNA was isolated using RNeasy Mini kit (Qiagen) and reversed tran-
scribed to cDNA by Reverse Transcription System (Promega). The PCR reaction was performed with the AmpliTaq Gold system (Applied Biosystems).

Quantitative real time RT-PCR
Relative expression level of CYP11A1, CYP11B1, CYP11B2, CYP17A1, CYP21A2, 3β-HSD, VDIR, 5α-reductase, aromatase, Hem45 and NCoR-1 mRNA in different cell lines were measured by real time RT-PCR. RNA was isolated using RNeasy Mini kit (Qiagen) and reversed transcribed to cDNA by Reverse Transcription System (Promega).

The real time RT-PCR analysis was performed with Power SYBR Green Master Mix (Applied Biosystems) or iQ SYBR Green Supermix (Bio-Rad) using an iQ5 Real-Time PCR Detection System (Bio-Rad). All experiments were conducted in accordance with the manufacturer’s recommendations. Human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) or TATA binding protein (TBP) was used as endogenous control. All real time RT-PCR data were normalized to the endogenous control. The relative mRNA expression was calculated with the standard curve method and expressed as fold change compared to the control group. Non-template wells were used as negative control. Melt curve analysis was performed routinely.

Assays of enzyme activity
The enzyme activity of CYP21A2 and CYP17A1 was measured in NCI-H295R cells. The CYP21A2 activity was measured by addition of [3H]-17α-hydroxyprogesterone which was 21-hydroxylated to 11-deoxycortisol. The substrate and metabolite was separated using thin layer chromatography with trichloroethane:acetone 70:30 as mobile phase. The silica gel plates were then analyzed on a LB2723 Berthold scanner.

In order to measure the 17α-hydroxylation activity of CYP17A1, cells were pretreated with the CYP11B1 inhibitor metyrapone (Sigma). 11-deoxycorticosterone was then added and metabolized to 11-deoxycortisol. The substrate and metabolite was separated in a reversed phase high performance liquid chromatography system on a 125 × 4 mm LiChrosphere RP 18 column (5 μm; Merck) with acetonitril:water 40:60 as mobile phase and monitored with an UV detector.

The 17,20-lyase activity of CYP17A1 was measured by analyzing the conversion of 17α-hydroxyprogrenolone into DHEA in cell culture, while inhibiting 3β-HSD activity with trilostane. The amount of DHEA secreted was determined using ELISA, as described above.

The aromatase enzyme activity was determined in MCF-7 cells, LNCaP cells and NCI-H295R cells, by measuring the conversion of testosterone to
estradiol while preventing the 5α-reductase-catalyzed conversion of testosterone to dihydrotestosterone with finasteride. Testosterone was added to the cell culture and the production of estradiol was measured using ELISA, as described above.

Analysis of nuclear receptor activation

Cells were transiently transfected with a nuclear receptor-responsive luciferase reporter vector (AR-responsive or ER-responsive) together with expression vectors containing cDNA for either the androgen receptor (AR), the estrogen receptor α (ERα) or the estrogen receptor β (ERβ). All cells were cotransfected with a pCMVβ-galactosidase plasmid (in order to standardize for transfection efficiency). The transfection was carried out using Lipofectamine (Invitrogen) (NCI-H295R cells, HEK-293 cells, DU-145 cells and LNCaP cells) in accordance with the manufacturer’s recommendations or calcium co-precipitation (HEK-293 cells) as previously described [104]. The AR-responsive vector contains an androgen response element (ARE) coupled to luciferase. The ER-responsive vector contains an estrogen response element (ERE) coupled to luciferase. Transfected cells were treated for 24-40 hours with different steroids dissolved in ethanol and the levels of androgen-dependent or estrogen-dependent luciferase activity were measured as previously described [104, 105] and compared with the luciferase levels in cells treated with the same volume of ethanol. Luciferase activity is expressed as relative light units (RLU) divided by β-galactosidase activity. Data are expressed as fold change compared to control ± standard deviation.

Analysis of CYP21A2 promoter activity

Cells were transiently transfected with luciferase reporter constructs containing different lengths of the CYP21A2 promoter, spanning from -9027 to +13, coupled to the luciferase gene [106]. In all experiments, pCMVβ-galactosidase plasmid was cotransfected, in order to standardize for transfection efficiency. Cells were transfected using Lipofectamine 2000 (Invitrogen) in accordance with the manufacturer’s recommendations. In some experiments, the cells were cotransfected with expression vectors for human vitamin D receptor (VDR), human retinoid X receptor (RXR), human VDIR and/or human WSTF.

Following transfection, the cells were treated for 24 hours with different substances dissolved in ethanol or dimethyl sulfoxide. Control samples were treated with the same amount of ethanol and/or dimethyl sulfoxide.

After the treatment, cells were lysed and luciferase and β-galactosidase activities were measured as previously described [104, 105]. Luciferase re-
porter activity is expressed as relative light units (RLU) divided by β-galactosidase activity. Reporter activity is presented as fold change compared to the control group ± standard deviation.

Gene silencing by small interfering RNA

Transfection of siRNA was used to silence the expression of VDIR in NCI-H295R cells. Transfection was carried out with DharmaFECT transfection reagent 1 (Thermo Scientific) in accordance with the manufacturer’s recommendations. As siRNA, the siGENOME SMARTpool M-009384-00-0005 (Thermo Scientific) was used. Following gene silencing, the effect of 1α,25-dihydroxyvitamin D₃ on the mRNA level of CYP21A2 was determined, using real-time RT-PCR. The effectiveness of siRNA silencing was controlled by measuring VDIR mRNA levels using real time RT-PCR. The VDIR mRNA was decreased by 70% following siRNA transfection.

In silico analysis of gene promoters

The CYP21A2 promoter (9 kb upstream) and different aromatase promoters were in silico-analyzed for putative VDREs. The promoter sequences were manually searched for consensus sequences for VDRE described by Matilainen et al. [71] and for previously described negative VDREs [77].

Mutagenesis

Mutagenesis was used to produce deletion mutants where putative nVDREs in the CYP21A2-promoter luciferase constructs had been deleted. Quick-Change II XL Site-Directed Mutagenesis Kit (Agilent Technologies) was used in accordance with the manufacturer’s recommendations. Primers were purchased from Thermo Fisher Scientific GmbH, Germany.

Statistical analysis

Analysis of statistical significance was performed using either ANOVA or Student’s t-test in Microsoft Excel. P-values <0.05 were considered statistically significant.
Results and discussion

Effects of CYP7B1-related steroids on estrogen receptor activation

In paper I, we have examined the role of CYP7B1-mediated catalysis in estrogenic signaling. This research has been conducted by investigating the estrogenic effects exerted by some CYP7B1 substrates and metabolites.

The enzyme CYP7B1 catalyzes 7α-hydroxylation of a number of steroids. The chemical structures of some CYP7B1 substrates and metabolites of importance for this study are shown in figure 5. The aim of paper I was to examine the effect of this 7α-hydroxylation on the steroids’ ability to stimulate estrogen receptor α (ERα) and estrogen receptor β (ERβ). Previously, conflicting data have been published regarding the effect of CYP7B1-mediated metabolism on estrogenic signaling where some researchers have reported that 7α-hydroxylation abolishes the estrogenic effect of steroids [33] while other have reported that 7α-hydroxylated products are more potent estrogens than the corresponding CYP7B1 substrates [34].

In the present study, we examined the estrogenic effects of several CYP7B1-related steroids using an estrogen response element (ERE) luciferase reporter system transiently transfected into human embryonic kidney HEK-293 cells. Our results showed a significant stimulation of both estrogen receptor α and estrogen receptor β by the CYP7B1 substrates Aene-diol and 3β-Adiol while DHEA did not stimulate any of the estrogen receptors. To study the effects of CYP7B1-mediated steroid metabolism on a steroid’s ability to activate the estrogen receptor β, we treated transfected cells with the corresponding 7α-hydroxylated products (Aene-triol, 3β-Atriol, 7α-hydroxy-DHEA). Neither of these steroids were able to activate estrogen receptor β. Furthermore, we found that Aene-diol, but not Aene-triol, could activate estrogen receptor α. These results indicate that CYP7B1-mediated 7α-hydroxylation of Aene-diol and 3β-Adiol may alter the estrogenic signaling by metabolizing ERα and ERβ ligands to inactive metabolites.

The mRNA level of HEM45, a gene known to be stimulated by estrogens, was markedly upregulated by Aene-diol while the CYP7B1-formed metabolite exerted significantly less effect on HEM45 gene expression, lending further support to the conclusion that 7α-hydroxylation abolishes the estrogenic effect of Aene-diol. CYP7B1-mediated metabolism of 3β-Adiol has been proposed to influence ERβ-mediated growth suppression [33].
results indicate that Aene-diol, a steroid reported to be present in higher intraprostatic concentrations than 3β-Adiol [107], also might be important for ER-related pathways. Our data indicate that low concentrations of Aene-diol can 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. These findings do not support the suggestion by Martin et al. [34] that CYP7B1-catalyzed 7α-hydroxylation increases estrogenic signaling. In conclusion, data presented in this paper show that CYP7B1 may act as a regulator of estrogenic signaling by metabolizing estrogenic steroids to inactive metabolites. This paper also reports findings indicating that Aene-diol may play a role in estrogenic signaling in the prostate.

To understand the estrogenic and/or androgenic effects of these steroids, and the role of CYP7B1-mediated catalysis, is of importance to understand normal prostate development as well as prostate carcinogenesis. Furthermore, information regarding the regulation of estrogenic and androgenic signaling can be used in the development of new treatment strategies against prostate cancer.
Figure 5. Some CYP7B1 substrates and metabolites.

Effects of CYP7B1-related steroids on androgen receptor activation

In paper II, we have investigated the role of CYP7B1 in androgenic signaling. Androgenic signaling is important in normal prostate development as well as in prostate carcinogenesis. Since a majority of all prostate cancers rely on androgens to proliferate, it is of importance to investigate the mechanisms that regulate the levels of androgens in the prostate. CYP7B1 is a widely expressed steroid hydroxylase involved in the metabolism of a number of steroids reported to influence estrogen and androgen signaling. The effect of CYP7B1-mediated metabolism on a steroid’s ability to exert effect on sex hormone receptors has been disputed.

In this study, we have examined whether the CYP7B1 substrates DHEA and Aene-diol and their corresponding 7α-hydroxy products, formed in a reaction catalyzed by CYP7B1, could trigger an androgenic response in an
androgen-dependent luciferase reporter system. These experiments have been conducted in different cell lines derived from human embryonic kidney, human adrenal cortex and human prostate. The results indicate significantly lower androgen receptor activation by CYP7B1-formed metabolites than by the corresponding substrate. Thus, CYP7B1-mediated metabolism may be of importance in the regulation of androgenic signaling, by converting AR ligands into less active metabolites. Furthermore, we found cell line-specific differences in the steroids’ ability to trigger the androgen-dependent response (figure 6). These differences between cell lines could be a result of cell line differences in expression of comodulators of importance for the androgen receptor or by cell line-specific metabolism of the steroids during the assay.

Discussion on the cell line differences

The studied steroids were able to trigger an AR-dependent response in a cell-line specific manner. In human embryonic kidney HEK-293 cells, dihydrotestosterone stimulated the AR-dependent reporter system but neither treatment with CYP7B1 substrates DHEA or Aene-diol nor CYP7B1 metabolites 7α-hydroxy-DHEA or Aene-triol resulted in an AR-dependent response. In human adrenocortical carcinoma NCI-H295R cells, dihydrotestosterone and the CYP7B1 substrates DHEA and Aene-diol acted as androgens while the corresponding 7α-hydroxylated steroids 7α-hydroxy-DHEA and Aene-triol did not. Furthermore, we studied the androgenic effects of these steroids in two cell lines derived from human prostate; LNCaP and DU-145. In DU-145 cells, both CYP7B1 substrates and metabolites stimulated the AR-dependent reporter system, but the effect of 7α-hydroxy-DHEA and Aene-triol was significantly lower than the effect of the corresponding CYP7B1 substrates. In LNCaP cells, on the other hand, Aene-diol acted as an androgen, while DHEA, 7α-hydroxy-DHEA and Aene-triol were unable to stimulate the reporter system. This indicates that the cellular milieu may vary between LNCaP cells and DU-145 cells in a manner that is of importance for androgenic signaling.

To investigate the observed cell line differences in androgenic signaling, we studied cell line differences in AR comodulator expression and putative cell-specific metabolism of DHEA and Aene-diol. Over 170 potential AR comodulators have been reported [43, 108] and it has been proposed that the expression levels of these coactivators and corepressors could alter the androgenic signaling. Overexpression of multiple AR coactivators has been reported to be associated with prostate carcinogenesis and prostate cancer progression [43]. Tissue-selective expression of AR comodulators has also been described, indicating that the expression level of comodulators could be important in the regulation of tissue-specific androgenic signaling [108]. Furthermore, animal knockout models, as well as studies where different
comodulators have been overexpressed, demonstrate that the expression level of comodulators could be of importance for AR’s ability to trigger androgenic response [108]. Therefore, we studied whether the expression of four AR comodulators differed between the four cell lines, using RT-PCR. Such a cell line specificity in comodulator expression could be a possible mechanism for the observed differences in androgenic signaling. We found that all four cell lines expressed the comodulators NCoR-1, SRC-1, SRA and SMRT. However, the result from the RT-PCR experiments indicated that the expression level of NCoR-1 differed between the cell lines. In order to study this in detail, the expression level of NCoR-1 mRNA was measured using real-time RT-PCR. We found that the expression level of the comodulator NCoR-1 differed markedly between the studied cell lines, where NCoR-1 mRNA level in LNCaP >> HEK-293 > NCI-H295R ≈ DU-145. It has been reported by Miyamoto et al. [38] that overexpression of the AR comodulator ARA70 altered the capacity of Aene-diol to trigger an AR dependent response. The observed differences in NCoR-1 expression between the cell lines studied in our paper could play a role for the observed differences in androgenic signaling. Further research is, however, needed to fully understand the role of comodulator expression in cell line-specific regulation of androgenic signaling.

Another possible mechanism for the observed cell line differences in androgenic signaling is cell-specific metabolism of the added steroid, during the assay. To investigate this, we studied different aspects of expression and activity of the enzyme 3β-HSD. Evaul et al. [37] have suggested that both DHEA and Aene-diol have to be converted to androstenedione and testosterone, respectively, to gain androgenic effect, reactions catalyzed by 3β-HSD. That suggestion indicates that cell line-specific expression of 3β-HSD could be an explanation for the cell line differences that we have observed. However, we found that all four studied cell lines did express 3β-HSD. Furthermore, we found that both DHEA and Aene-diol exert androgenic effects in DU-145 cells even when 3β-HSD is inhibited by trilostane. In summary, these findings do not support the suggestion that cell line differences in 3β-HSD expression could explain the observed differences in androgenic signaling between cell lines nor that 3β-HSD-catalyzed conversion is required to obtain androgenic effect of DHEA and Aene-diol.

In conclusion, data presented in paper II indicate that CYP7B1 may be a regulator of androgenic response, at least in some tissues, by converting AR ligands into less active metabolites. Further research has to be conducted to examine the mechanism and role for the observed tissue selectivity in androgen receptor activation.
Effects of CYP7B1 substrate Aene-diol and CYP7B1 metabolite Aene-triol on AR-mediated response in HEK-293 cells, NCI-H295R cells, LNCaP cells and DU-145 cells. Cells were transiently transfected with AR-responsive luciferase reporter system and an expression vector containing cDNA for AR. The cells were then treated with 1 μM of either Aene-diol or Aene-triol. *Statistically significant response compared to negative control (p<0.05).

Figure 6. Effects of CYP7B1 substrate Aene-diol and CYP7B1 metabolite Aene-triol on AR-mediated response in HEK-293 cells, NCI-H295R cells, LNCaP cells and DU-145 cells. Cells were transiently transfected with AR-responsive luciferase reporter system and an expression vector containing cDNA for AR. The cells were then treated with 1 μM of either Aene-diol or Aene-triol. *Statistically significant response compared to negative control (p<0.05).

Effects of 1α,25-dihydroxyvitamin D₃ on adrenal steroidogenesis

Previous clinical studies have reported a connection between vitamin D and steroidogenesis but the characteristics of this relationship have remained uninvestigated. The aim of the study presented in paper III was to investigate the possible effects of vitamin D on steroidogenesis at molecular level. The active form of vitamin D, 1α,25-dihydroxyvitamin D₃, is a multifunctional hormone known to affect a broad range of physiological processes, mainly by altering gene expression. Human adrenocortical carcinoma NCI-H295R cells, a widely used model for human adrenal cortex [109-113], were cultured and treated with 1α,25-dihydroxyvitamin D₃. The amount of hormone produced was then measured using enzyme-linked immunosorbent assay (ELISA), the mRNA levels of steroidogenic enzymes were determined using real time RT-PCR and the enzyme activities of key enzymes were measured using thin layer chromatography, high-performance liquid chromatography or ELISA.

Following 1α,25-dihydroxyvitamin D₃ treatment, hormone production, mRNA levels and enzyme activities were altered for key components in the
adrenal steroidogenesis. In summary, this paper reports previously unknown biological effects of vitamin D on the adrenal steroidogenesis. The effects of 1α,25-dihydroxyvitamin D3 are summarized in figure 7.

Figure 7. Summary of effects of 1α,25-dihydroxyvitamin D3 on adrenal steroidogenesis. + represents increased gene expression and/or enzyme activity while - represents decreased hormone production, gene expression and/or enzyme activity.

Effects of vitamin D on gene expression

The mRNA levels of three key enzymes in the adrenal steroidogenesis, CYP11A1, CYP17A1 and CYP21A2 were found to be altered by 1α,25-dihydroxyvitamin D3 treatment. CYP11A1 and CYP17A1 mRNA levels were upregulated after vitamin D treatment, while CYP21A2 mRNA level was suppressed by the same treatment. No significant changes were observed in the mRNA levels of CYP11B1, CYP11B2 and 3βHSD.

Effects of vitamin D on hormone production and enzyme activity

We found that 1α,25-dihydroxyvitamin D3 treatment decreased the production of corticosterone, androstenedione, dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S), while the production of aldosterone and cortisol was unaltered. Furthermore, we measured the enzyme activity of CYP21A2 and CYP17A1 by adding a known amount of substrate to the cell culture and measuring the turnover of substrate to product. In resemblance with the effects on mRNA level, CYP21A2 enzyme activity was suppressed.
by 1α,25-dihydroxyvitamin D3. CYP17A1 catalyzes two separate reactions, the 17α-hydroxylation and the 17,20-lyase reaction. We found that treatment with vitamin D resulted in increased 17α-hydroxylation activity of CYP17A1, but in decreased 17,20-lyase activity of CYP17A1.

It is well-known that the manners of regulation for the two activities of CYP17A1 are principally different. The 17α-hydroxylase activity is regulated via the expression of the CYP17A1 gene, while the 17,20-lyase activity is regulated by posttranscriptional mechanisms [1, 4-7]. Three posttranscriptional mechanism for regulation of 17,20-lyase activity have been described; the abundance of P450 oxidoreductase (POR) [8, 9], allosteric action of cytochrome b5 [10] and serine phosphorylation of CYP17A1 [11-14]. The discrepancy between the 1α,25-dihydroxyvitamin D3-mediated increase in expression of CYP17A1 mRNA and the suppression of 17,20-lyase activity could be a result of posttranscriptional mechanisms affected by 1α,25-dihydroxyvitamin D3.

Discussion on adrenal bioactivation of vitamin D

Extrarenal production of active vitamin D metabolites has been reported for multiple organs. Slominski and collaborators [66-70] have reported that the adrenal enzyme CYP11A1 catalyzes the conversion of vitamin D3 to 20-hydroxyvitamin D3 and the conversion of 1α-hydroxyvitamin D3 to 1α,20-dihydroxyvitamin D3. 1α-hydroxyvitamin D3 is an exogenous substance used to treat hypovitaminosis D caused by insufficient 1α-hydroxylase capacity. Both 20-hydroxyvitamin D3 and 1α,20-dihydroxyvitamin D3 have been reported to exert effects resembling those of 1α,25-dihydroxyvitamin D3 [66, 67]. Due to the high expression of CYP11A1 in the adrenal cortex, it may be speculated that 20-hydroxyvitamin D3 and 1α,20-dihydroxyvitamin D3 are produced in the adrenal cortex. However, it remains to be investigated whether these vitamin D metabolites alters the adrenal steroidogenesis in the same manner as 1α,25-dihydroxyvitamin D3.

Tissue-selective effects of vitamin D on sex hormone production

The aim of paper IV was to examine the effects of 1α,25-dihydroxyvitamin D3 on estrogen and androgen metabolism in different cell lines of human origin derived from adrenal cortex, breast and prostate. The effects of 1α,25-dihydroxyvitamin D3 on endogenous production of estradiol, testosterone and dihydrotestosterone as well as the aromatase enzyme activity and the mRNA expression of aromatase and 5α-reductase were determined. The effects are summarized in table 1.
Interestingly, we found that 1α,25-dihydroxyvitamin D3 exerted cell linespecific effects on estrogen and androgen metabolism. In breast cancer MCF-7 cells, aromatase gene expression and estradiol production were decreased, while production of androgens was markedly increased. In adrenocortical NCI-H295R cells, 1α,25-dihydroxyvitamin D3 stimulated aromatase expression and decreased dihydrotestosterone production. In prostate cancer LNCaP cells, aromatase expression increased after the same treatment, as did production of testosterone and dihydrotestosterone. Furthermore, we studied the effects of 1α,25-dihydroxyvitamin D3 on three different aromatase promoters, and found that the transcriptional rate of these promoters were affected by 1α,25-dihydroxyvitamin D3 in a cell line-specific manner.

This study reports data important for the discussion about NCI-H295R cells as a model for effects on estrogen and androgen metabolism. Furthermore, this paper reports important knowledge about 1α,25-dihydroxyvitamin D3 as an interesting substance for further research in the field of breast cancer prevention and treatment.

Table 1. Effects of 1α,25-dihydroxyvitamin D3 on estrogen and androgen metabolism, summary of results reported in paper IV. ↑ represents increased; ↓ represents decreased; - represents unaltered.

<table>
<thead>
<tr>
<th>Cell line Effect of treatment with 1α,25-dihydroxyvitamin D3</th>
<th>Aromatase mRNA level</th>
<th>Aromatase enzyme activity</th>
<th>Estradiol production</th>
<th>Dihydrotestosterone production</th>
<th>Expression of promoter specific aromatase transcripts</th>
</tr>
</thead>
<tbody>
<tr>
<td>MCF-7 ↓</td>
<td>MCF-7 ↓</td>
<td>MCF-7 ↓</td>
<td>MCF-7 ↓</td>
<td>MCF-7 ↑</td>
<td>MCF-7 ↓ (p I.3, p I.4 and p II)</td>
</tr>
<tr>
<td>LNCaP ↑</td>
<td>LNCaP -</td>
<td>LNCaP -</td>
<td>LNCaP -</td>
<td>LNCaP ↑</td>
<td>LNCaP No statistically significant alterations</td>
</tr>
</tbody>
</table>
Vitamin D as a potential anti-breast cancer agent

1α,25-Dihydroxyvitamin D3 and vitamin D analogs exert anti-proliferative and pro-differentiating effects on cells in vitro and these compounds have therefore been proposed to be of potential use as anti-cancer agents. Epidemiological studies have revealed relationships between solar UV-B exposure, which is needed for vitamin D3 production, and decreased incidence and mortality in breast cancer as well as between 25-hydroxyvitamin D3 serum levels and breast cancer risk [103]. 1α,25-Dihydroxyvitamin D3 has also been reported to affect apoptosis and expression of tumor suppressor genes [100, 103].

To regulate estrogen production and signaling are key strategies in breast cancer treatment, since a large part of all breast cancers depends on estrogens to proliferate. To regulate the activity of aromatase, that catalyzes the conversion of testosterone to estradiol, is therefore of utmost importance in breast cancer treatment. It has recently been reported that 1α,25-dihydroxyvitamin D3 alters the gene expression and enzyme activity of aromatase in a tissue-specific manner [92]. The results presented in paper IV add knowledge showing that aromatase promoter activity, expression and enzyme activity are regulated by 1α,25-dihydroxyvitamin D3 in a tissue-selective manner. This lends further support to the hypothesis that the bioactivated form of vitamin D acts as a selective aromatase modulator [92]. Furthermore, the suppressing effect of 1α,25-dihydroxyvitamin D3 on the aromatase enzyme activity is abolished by cotreatment with a VDR inhibitor, showing that the effect is mediated by VDR.

Treatment with both aromatase inhibitors and anti-estrogens are associated with risk of adverse effects due to effects on peripheral estrogen metabolism (eg. osteoporosis) [48, 49]. The vitamin D-mediated tissue-selective regulation of aromatase expression and activity is therefore an interesting strategy to affect estrogen levels in breast without effects on the peripheral estrogen metabolism. Hence, vitamin D or analogs are interesting substances in further research on breast cancer treatment and prevention.

NCI-H295R cells as a model to study sex hormone production

The NCI-H295R cell line has been proposed as a screening tool to study the effects of endocrine disruptors on estrogen and androgen production and has undergone a validation program supported by the Organization for Economic Cooperation and Development (OECD) [114-117]. Therefore, we studied whether this cell line reacted to 1α,25-dihydroxyvitamin D3 treatment in the same way as cell lines derived from important endocrine target tissues. As shown in table 1, adrenocortical NCI-H295R cells respond to 1α,25-dihydroxyvitamin D3 treatment in a different way than breast cancer MCF-7 cells and prostate cancer LNCaP cells. The observed differences between
NCI-H295R cells and cells derived from important endocrine target tissues, and the molecular basis for these differences, need to be addressed and clarified if NCI-H295R cells should be used as a model for estrogen and androgen metabolism.

Discussion on putative mechanisms for tissue-selective regulation of aromatase

The mechanism for the vitamin D-mediated tissue-selective regulation of aromatase remains to be clarified. Aromatase uses different promoters in different tissues and a negative vitamin D response element has been proposed in the aromatase promoter I.3 [92]. Therefore, it has been suggested that the cell line differences in vitamin D-mediated alterations in aromatase expression could be a result of tissue-selective use of aromatase promoter. However, our finding that 1α,25-dihydroxyvitamin D₃ upregulates the promoter I.3-specific transcript in NCI-H295R cells but downregulates the same promoter-specific transcript in MCF-7 cells does not support this suggested mechanism. It may be speculated that the observed tissue-differences could be a result of varying expression of comodulators in the different cell lines. Further research is needed to elucidate the mechanism for this tissue-selective effect of 1α,25-dihydroxyvitamin D₃.

Mechanism for vitamin D-mediated effects on CYP21A2

In Paper III, we reported that 1α,25-dihydroxyvitamin D₃ decreases mRNA level and enzyme activity of the steroid 21-hydroxylase CYP21A2. In paper V, we have studied the mechanism for the 1α,25-dihydroxyvitamin D₃-mediated effects on CYP21A2. In this paper, the effect of 1α,25-dihydroxyvitamin D₃ on the promoter activity of CYP21A2 has been studied in a luciferase reporter assay system in cell lines derived from human adrenal cortex, human colon and mouse adrenal cortex.

1α,25-Dihydroxyvitamin D₃ was found to alter the promoter activity of CYP21A2 via a vitamin D receptor (VDR)-mediated mechanism. We report that the mechanism for 1α,25-dihydroxyvitamin D₃-mediated suppression of CYP21A2 includes the key comodulators VDR interacting repressor (VDIR) and Williams syndrome transcription factor (WSTF) as well as epigenetic mechanisms such as histone deacetylation and DNA methylation.

In conclusion, this paper reports a mechanism for the effects of 1α,25-dihydroxyvitamin D₃ on CYP21A2. This is a previously unknown regulatory pathway for CYP21A2 transcription. Furthermore, we report data indicating a novel mechanism of vitamin D action, showing that the expression level of
key comodulators may shift a suppressing effect of vitamin D to a stimulatory effect.

Effects of nuclear receptor and comodulator expression level

We found that altered expression levels of nuclear receptors VDR and RXR as well as the expression levels of comodulators VDIR and WSTF influenced the effects of 1α,25-dihydroxyvitamin D₃ on the promoter activity of CYP21A2. When the CYP21A2 promoter-luciferase reporter system was treated with 1α,25-dihydroxyvitamin D₃, the promoter activity was decreased in all cell lines studied. Surprisingly, when VDR and RXR was overexpressed, the effect of vitamin D shifted from a suppressing effect on the promoter activity to a stimulatory effect. Thus, overexpression of VDR and RXR reversed the previously observed effect of vitamin D on CYP21A2 promoter activity from suppression to stimulation. Consequently, the expression level of the nuclear receptors VDR and RXR are crucial for the effects of 1α,25-dihydroxyvitamin D₃ on CYP21A2 promoter activity.

Although the mechanism for vitamin D-mediated suppression of gene expression remains unclear, it has been reported that VDIR and WSTF act as key comodulators in vitamin D-mediated transcriptional repression of CYP27B1 and the parathyroid hormone gene. Both these comodulators were overexpressed in NCI-H295R cells in order to examine if they play a role in the vitamin D-mediated effects on CYP21A2 transcription. When both VDR/RXR and VDIR/WSTF were overexpressed, 1α,25-dihydroxyvitamin D₃ suppressed the transcriptional rate of CYP21A2 by approximately 50%, which is in the same order of magnitude as the suppression of CYP21A2 mRNA level and enzyme activity that were reported in paper III. The suppressing effect of 1α,25-dihydroxyvitamin D₃ on CYP21A2 gene expression was abolished when the VDIR expression was silenced using siRNA. This result lends further support to the suggestion that VDIR is a key comodulator in vitamin D-mediated repression of gene expression.

Our results show that both VDIR and WSTF play important roles in the 1α,25-dihydroxyvitamin D₃-mediated suppression of CYP21A2 and that altered expression levels of these comodulators may shift a suppressing effect of 1α,25-dihydroxyvitamin D₃ to a stimulatory effect.

Epigenetic mechanisms

It has been proposed that epigenetic mechanisms, such as DNA demethylation and histone deacetylation, may affect 1α,25-dihydroxyvitamin D₃-mediated suppression of transcription [75]. Furthermore, epigenetic components in expression control have been reported for multiple cytochrome P450 enzymes [118, 119]. To examine whether these mechanisms are involved in the 1α,25-dihydroxyvitamin D₃-mediated effects on CYP21A2 transcription,
NCI-H295R cells transfected with the CYP21A2 promoter-luciferase construct and expression vectors for VDR and RXR, were treated with 1α,25-dihydroxyvitamin D$_3$ and the methyltransferase inhibitor 5-aza-2'-deoxycytidine and/or the histone deacetylase inhibitor trichostatin A.

Treatment with 1α,25-dihydroxyvitamin D$_3$ alone increased the promoter activity of CYP21A2. However, the 1α,25-dihydroxyvitamin D$_3$-mediated increase in promoter activity was significantly decreased when the cells were co-treated with a methyltransferase inhibitor and/or a histone deacetylase inhibitor. In a separate experiment, we showed that the 1α,25-dihydroxyvitamin D$_3$-mediated effect on CYP21A2 mRNA level was altered when the cells were cotreated with 5-aza-2'-deoxycytidine and trichostatin A.

Our finding that the effect of vitamin D on CYP21A2 transcriptional rate was altered when cells were cotreated with a histone deacetylase inhibitor and/or a DNA methyltransferase inhibitor, indicate that epigenetic modifications are involved in the effects of vitamin D on CYP21A2. This indicates that the complex formed by nuclear receptors and comodulators that mediates the effect of vitamin D on CYP21A2 also may include factors that function as histone deacetylases and DNA methyltransferases.

Identification of a functional VDRE

NCI-H295R cells were transfected with progressive deletion constructs of the CYP21A2 promoter and expression vectors containing cDNA for VDR and RXR. When transfected cells were treated with 1α,25-dihydroxyvitamin D$_3$, a 9 kb long promoter construct was markedly upregulated by the treatment. The other deletion constructs were not affected to the same extent. This result indicates that the promoter sequence most important for 1α,25-dihydroxyvitamin D$_3$-mediated effects on CYP21A2 transcription is located in the region between -5.6 kb and -9 kb.

An in silico analysis of the CYP21A2 promoter revealed putative vitamin D response elements, based on sequence similarity to previously described response elements for vitamin D. We found that when one of the putative response elements was deleted using mutagenesis, the effect of 1α,25-dihydroxyvitamin D$_3$ was abolished. We conclude from this, that the sequence CACCTG located at -8205 to -8210 in the CYP21A2 promoter (figure 8) is important for the effects of 1α,25-dihydroxyvitamin D$_3$ on the promoter activity and that it might function as a VDRE.

\[-8214 \text{gggCACCTGggc} -8202\]

*Figure 8. The functional VDRE identified in the CYP21A2 promoter.*
Conclusions

This thesis reports previously unknown biological effects of vitamin D on steroidogenesis and new data on the role of steroid metabolism for regulation of sex hormone signaling. Based on the results presented in this thesis, it can be concluded that:

- Estrogenic signaling may be altered by CYP7B1-mediated metabolism of ERα and ERβ ligands to less active metabolites.
- Androgenic signaling may be altered by CYP7B1-mediated metabolism of AR ligands to less active metabolites in a cell line-specific manner.
- The hormone production in human adrenocortical NCI-H295R cells is affected by 1α,25-dihydroxyvitamin D₃ via alteration of CYP17A1 and CYP21A2 mRNA levels and enzyme activities.
- The metabolism of androgens and estrogens is altered by 1α,25-dihydroxyvitamin D₃ in a cell line-specific manner.
- The transcriptional rate of CYP21A2 is repressed by 1α,25-dihydroxyvitamin D₃. The effect is mediated by nuclear receptors VDR and RXR and comodulators VDIR and WSTF via a functional VDRE in the CYP21A2 promoter.
Populärvetenskaplig sammanfattning

Enzymer är ämnen som påskyndar kemiska reaktioner. De kemiska reaktionerna i en viss vävnad, till exempel tillverkningen av hormoner, kan styras genom att reglera mängden av ett eller flera enzymer som finns i den vävnaden. I denna avhandling studeras hur dessa enzymsystem kan regleras för att styra tillverkningen av binjurehormoner och könshormoner.

Könshormoner kan vara antingen östrogener eller androgen, vilka har olika effekter i cellerna. Att förstå hur tillverkning, effekt och nedbrytning av könshormoner sker är viktigt ur flera aspekter, till exempel för att få ytterligare kunskap om vad som orsakar bröstcancer och prostatacancer samt hur nya behandlingsstrategier för dessa sjukdomar kan utvecklas. I kroppen tillverkas flera könshormon-liknande ämnen, för vilka det fortfarande är oklart om de fungerar som östrogener eller som androgen. I den här avhandlingen studeras om dessa steroider kan fungera som östrogener, som androgen eller som både östrogener och androgen. Jag har funnit att steroiden 5-androsten-3β,17β-diol kan fungera både som östrogen och som androgen i vart fall i vissa vävnader. Ytterligare forskning behövs för att klarlägga varför 5-androsten-3β,17β-diol fungerar som androgen i vissa vävnadstyper men inte i andra.


Vitamin D är ett fettlösligt vitamin som bildas i huden då den bestrålas av solljus. Vitamin D kan även tas upp från kosten. I kroppen aktiveras vitamin D i två steg till ett ämne, 1α,25-dihydroxyvitamin D₃, som har många olika effekter. Det har länge varit känt att vitamin D är viktigt för skelettet, men under de senaste årtiondena har det rapporterats att vitamin D också påverkar många andra fysiologiska processer. Brist på vitamin D har kunnat kopplas till ökad risk att drabbas av olika cancerformer och autoimmuna sjukdomar. Vitamin D anses också påverka immunförsvaret.

Jag har även studerat hur vitamin D påverkar tillverkningen av könshormoner. Mina resultat visar att vitamin D påverkar tillverkningen av östrogener på olika sätt i olika vävnadstyper. I celler från bröstvävnad minskade tillverkningen av östrogener då cellerna behandlades med vitamin D. I celler från binjurevävnad och prostatevävnad hade vitamin D motsatt effekt och tillverkningen av östrogener ökade eller förblev oförändrad. Att vitamin D påverkar östrogenproduktionen på detta sätt är ett viktigt fynd som kan användas för att i framtiden utveckla vitamin D-liknande läkemedel mot bröstcancer.

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