SHENGJUN QIAO

Fatty Acid Metabolism, Vitamin D\textsubscript{3} and Prostate Cancer

ACADEMIC DISSERTATION
To be presented, with the permission of the Faculty of Medicine of the University of Tampere, for public discussion in the small auditorium of Building B, Medical School of the University of Tampere, Medisiinarinkatu 3, Tampere, on May 20th, 2006, at 12 o’clock.
To My Parents and My Family
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ABSTRACT

1α,25(OH)2D3 is the natural most active metabolite of vitamin D3 and has antiproliferative effects in a variety of normal and cancer cells. The actions of 1α,25(OH)2D3 are mediated by vitamin D receptor (VDR), which is a nuclear receptor and acts as a transcription factor to regulate target gene expression. We used cDNA microarray to screen 1α,25(OH)2D3-responsive genes and found that fatty acid synthase (FAS) and long-chain fatty-acid-CoA ligase 3 (FACL3), which is also known as long-chain fatty acyl-CoA synthetase (ACS3), were regulated by 1α,25(OH)2D3 in human prostate cancer LNCaP cells. FAS expression was indirectly and androgen-dependently downregulated by 1α,25(OH)2D3. Inhibition of FAS activity resulted in a robust suppression of LNCaP cell growth. FACL3, a downstream enzyme of FAS in the fatty acid metabolism pathway, was upregulated by 1α,25(OH)2D3 at mRNA, protein and activity levels. The upregulation of FACL3 expression by 1α,25(OH)2D3 was androgen/AR-dependent. Inhibition of FACL3 activity significantly attenuated 1α,25(OH)2D3-induced growth inhibition of LNCaP cells. Further study suggested that FACL3 mediated the 1α,25(OH)2D3-repression of FAS expression in terms of feedback inhibition by long-chain fatty acyl-CoAs, which were synthesized by FACL3 during its upregulation by 1α,25(OH)2D3. In addition, FACL3 expression was constitutively low in more malignant human prostate cancer cells, PC3 and DU145, compared to less malignant LNCaP cells. Taken together, the data, for the first time, suggest that fatty acid metabolism may play a role in the antiproliferative actions of 1α,25(OH)2D3 in prostate cancer cells by regulating FAS and FACL3 expression. The upregulation of FACL3 expression by 1α,25(OH)2D3 increases the synthesis of long-chain fatty acyl-CoAs, which repress FAS expression by means of feedback inhibition. Decreased FAS expression may lead to a decrease in the de novo fatty acid synthesis resulting in a growth inhibition of prostate cancer cells.
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>1α,25(OH)2D3</td>
<td>1α,25-dihydroxyvitamin D3</td>
</tr>
<tr>
<td>1α-hydroxylase</td>
<td>25-hydroxyvitamin D$_3$-1α-hydroxylase</td>
</tr>
<tr>
<td>15-PGDH</td>
<td>15-hydroxyprostaglandin dehydrogenase</td>
</tr>
<tr>
<td>25(OH)D$_3$</td>
<td>25-hydroxyvitamin D$_3$</td>
</tr>
<tr>
<td>25-hydroxylase</td>
<td>Vitamin D$_3$-25-hydroxylase</td>
</tr>
<tr>
<td>5LO</td>
<td>5-lipoxygenase</td>
</tr>
<tr>
<td>AKT</td>
<td>Protein kinase B (PKB)</td>
</tr>
<tr>
<td>Apaf-1</td>
<td>Apoptotic protease activating factor-1</td>
</tr>
<tr>
<td>ACS</td>
<td>Long-chain fatty acyl-CoA synthetase</td>
</tr>
<tr>
<td>ACS3</td>
<td>Long-chain fatty acyl-CoA synthetase 3</td>
</tr>
<tr>
<td>AF-2</td>
<td>Activation function 2 domain</td>
</tr>
<tr>
<td>AA</td>
<td>Arachidonic acid</td>
</tr>
<tr>
<td>AR</td>
<td>Androgen receptor</td>
</tr>
<tr>
<td>AMACR</td>
<td>Alpha-methylacyl-CoA racemase</td>
</tr>
<tr>
<td>ACC</td>
<td>Acetyl-CoA carboxylase</td>
</tr>
<tr>
<td>Bcl2</td>
<td>B-cell CLL/lymphoma 2</td>
</tr>
<tr>
<td>BRCA1</td>
<td>Breast cancer 1, early onset</td>
</tr>
<tr>
<td>BPH</td>
<td>Benign prostatic hyperplasia</td>
</tr>
<tr>
<td>Cdk2</td>
<td>Cyclin-dependent kinase 2</td>
</tr>
<tr>
<td>CBP</td>
<td>CREB binding protein</td>
</tr>
<tr>
<td>CYP24</td>
<td>25-hydroxyvitamin D$_3$-24-hydroxylase</td>
</tr>
<tr>
<td>CREB</td>
<td>Cyclic AMP (cAMP) response element binding protein</td>
</tr>
<tr>
<td>COX-2</td>
<td>Cyclooxygenase-2</td>
</tr>
<tr>
<td>C/EBPdelta</td>
<td>CCAAT/enhancer-binding protein delta</td>
</tr>
<tr>
<td>DUSP10</td>
<td>Dual specificity phosphatase 10</td>
</tr>
<tr>
<td>DHA</td>
<td>Docosahexaenoic acid</td>
</tr>
<tr>
<td>DHT</td>
<td>Dihydrotestosterone</td>
</tr>
<tr>
<td>DCC-serum</td>
<td>Dextran-coated, charcoal-treated serum</td>
</tr>
<tr>
<td>EPA</td>
<td>Eicosapentaenoic acid</td>
</tr>
<tr>
<td>EGFR</td>
<td>Epidermal growth factor receptor</td>
</tr>
<tr>
<td>FAS</td>
<td>Fatty acid synthase</td>
</tr>
<tr>
<td>FACL</td>
<td>Long-chain fatty-acid-CoA ligase</td>
</tr>
<tr>
<td>FACL1(2)</td>
<td>Long-chain fatty-acid-CoA ligase 1(2)</td>
</tr>
<tr>
<td>FALC3</td>
<td>Long-chain fatty-acid-CoA ligase 3</td>
</tr>
<tr>
<td>FACL4</td>
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</tr>
<tr>
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</tr>
<tr>
<td>FACL6</td>
<td>Long-chain fatty-acid-CoA ligase 6</td>
</tr>
<tr>
<td>FARE</td>
<td>Fatty acyl-CoA response element</td>
</tr>
<tr>
<td>FBS</td>
<td>Fetal bovine serum</td>
</tr>
<tr>
<td>GTA</td>
<td>General transcription apparatus</td>
</tr>
<tr>
<td>GLRE</td>
<td>Glucose response element</td>
</tr>
<tr>
<td>HER-2</td>
<td>v-erb-b2 erythroblastic leukemia viral oncogene homolog 2</td>
</tr>
<tr>
<td>IGFBP-3</td>
<td>Insulin-like growth factor binding protein-3</td>
</tr>
<tr>
<td>IRE</td>
<td>Insulin response element</td>
</tr>
<tr>
<td>KGF</td>
<td>Fibroblast growth factor 7/keratinocyte growth factor</td>
</tr>
<tr>
<td>LH</td>
<td>1α,25(OH)2-16-ene-23-yne-26,27-F6-19-nor-D3</td>
</tr>
<tr>
<td>mVDR</td>
<td>Membrane VDR</td>
</tr>
</tbody>
</table>
nVDRE  negative VDRE
NCoR  nuclear receptor corepressor
NDRG1  N-myc downstream regulated
pRb  retinoblastoma protein
PSAP  prostate-specific acid phosphatase
PSA  prostate-specific antigen
PDF  prostate-derived factor
PAP  prostatic acid phosphatase
PTHrP  parathyroid hormone-related protein
PCNA  proliferating cell nuclear antigen
PUFAs  polyunsaturated fatty acids
PIN  prostatic intraepithelial neoplasia
PI3K  phosphatidylinositol-3 kinase
PTEN  phosphatase and tensin homolog
PLAB  prostate differentiation factor
RXR  retinoid X receptor
SRC-1  steroid receptor coactivator 1
SMRT  silencing mediator of retinoid and thyroid hormone receptor
SDS-PAGE  SDS-polyacrylamide gel electrophoresis
TNF  tumor necrosis factor
VDR  vitamin D receptor
VDRE  vitamin D response element
LIST OF ORIGINAL COMMUNICATIONS


II. **Shengjun Qiao** and Pentti Tuohimaa (2004): The role of long-chain fatty-acid-CoA ligase 3 in vitamin D₃ and androgen control of prostate cancer LNCaP cell growth. Biochemical and Biophysical Research Communications 319:358-368.


IV. **Shengjun Qiao** and Pentti Tuohimaa: Expression and vitamin D₃ regulation of long-chain fatty-acid-CoA ligase 3 in human prostate cancer cells (submitted for publication).

The above original papers are referred to in the text of this dissertation by their Roman numerals.
INTRODUCTION

The protective functions of vitamin D$_3$ against prostate cancer are evident (Corder et al. 1993; Ahonen et al. 2000; Tuohimaa et al. 2004). These functions are thought to be due to the antiproliferative effects of vitamin D$_3$. The antiproliferative effects of vitamin D$_3$ are exerted through vitamin D receptor (VDR)-mediated regulation of target gene expression, resulting in cell cycle arrest, cell apoptosis and cell differentiation. Therefore, the analysis of vitamin D$_3$-responsive genes and their biological functions is critical for an understanding of the mechanisms behind vitamin D$_3$ actions including its antiproliferative effects.

Fatty acid synthase (FAS), a pivotal enzyme for de novo long-chain fatty acid synthesis, has been found to be associated with many cancers. In prostate cancer, FAS is highly overexpressed, selectively activated and closely associated with disease initiation and development (Shurbaji et al. 1996; Pizer et al. 2001; Bull et al. 2001; Welsh et al. 2001; Myers et al. 2001; Swinnen et al. 2002; Verhoeven 2002; Rossi et al. 2003; Ettinger et al. 2004; Moore et al. 2005). Inhibition of FAS activity or knockdown of FAS mRNA results in cell proliferation suppression and apoptosis (Furuya et al. 1997; Pizer et al. 2001; Pflug et al. 2003; De Schrijver et al. 2003; Brusselmans et al. 2003; Kridel et al. 2004; Brusselmans et al. 2005; Alli et al. 2005). Long-chain fatty-acid-CoA ligase 3 (FACL3) is a downstream fatty acid metabolic enzyme of FAS and belongs to a subfamily of long-chain fatty-acid-CoA ligase. It converts long-chain fatty acids, preferentially myristic acid, arachidonic acid (AA) and eicosapentaenoic acid (EPA), into long-chain acyl-CoAs, which play important roles in many ways such as signal transduction, protein acylation and energy production (Kuhajda 2000). FACL3 has moreover been found to be involved in EPA-induced apoptosis of leukemia cells (Finstad et al. 2000; Heimli et al. 2003).

To date, there is no report on a connection between vitamin D$_3$ actions and fatty acid metabolism. One recent study indicates that vitamin D$_3$ inhibits the expression of cyclooxygenase-2 (COX-2) in prostate cancer cells (Moreno et al. 2005a). COX-2 utilizes polyunsaturated fatty acid arachidonic acid (AA) to synthesize prostaglandins, which are involved in the initiation and development of many cancers including prostate cancer. In the present study, cDNA microarray analysis of vitamin D$_3$-regulated genes revealed that FAS and FACL3 were regulated by 1α,25(OH)$_2$D$_3$, a hormonally active form of vitamin D$_3$, in prostate cancer LNCaP cells. This finding led to further studies on FAS and FACL3 in vitamin D$_3$ actions in prostate cancer cells.
1. **Vitamin D₃ and Prostate Cancer**

1.1 **Vitamin D₃ and risk of prostate cancer**

Prostate cancer is one of the leading causes of cancer death in males worldwide (Ekman 1999; Tayeb et al. 2003 and 2004). Its initiation, progression and development to invasiveness and metastasis, a life-threatening form of the disease, are complicated and associated with genetic and epigenetic factors (Waalkes and Rehm 1994; Farkas et al. 2000; Nwosu et al. 2001; Luo and Yu 2003; Visakorpi 2003; Schaid 2004; Karayi and Markham 2004; De Marzo et al. 2004; Cussenot and Cancel-Tassin 2004; Bostwick et al. 2004; Konishi et al. 2005; Wolk 2005; Sonn et al. 2005).

Vitamin D₃ is a lipophilic chemical molecule produced endogenously from precursor 7-dehydrocholesterol in skin by exposure to ultraviolet rays of sunlight or obtained from the diet (Holick et al. 1977; Okano et al. 1977; Dusso et al. 2005). Vitamin D₃ is transported in the circulation by binding to plasma proteins such as vitamin D binding protein (Cooke and Haddad 1989) and metabolized to 25-hydroxyvitamin D₃ (25(OH)D₃) by vitamin D₃-25-hydroxylase (25-hydroxylase) in the liver (Okuda 1994) and subsequently to 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃) by 25-hydroxyvitamin D₃-1α-hydroxylase (1α-hydroxylase) in the kidney (Fraser et al. 1970). 1α,25(OH)₂D₃ is thought to be a steroid hormone and an active form of vitamin D₃. In addition to its traditional biological functions in the maintenance of calcium and bone homeostasis in animal and human bodies (Heaney 1997; Heaney RP et al. 1997), a number of studies suggest that vitamin D₃ levels in blood circulation are associated with the risk of prostate cancer and are thought to play a role in the etiology and the development of prostate cancer. Serum levels of 1α,25(OH)₂D₃ are found to be significantly decreased in cases compared with controls and higher levels of 1α,25(OH)₂D₃ could reduce the risk of prostate cancer (Corder et al. 1993; Tavani et al. 2001). The lower the serum levels of 25(OH)D₃ are, the more aggressive the prostate cancer appears to be and high levels of 25(OH)D₃ can delay prostate cancer development (Ahonen et al. 2000; Tuohimaa et al. 2004). Several other studies suggest that the levels of vitamin D₃ metabolites (25(OH)D₃, 1α,25(OH)₂D₃) seem to be not associated with prostate cancer risk (Braun et al. 1995; Gann et al. 1996; Kristal et al. 2002; Platz et al. 2004).

Studies on exposure to ultraviolet radiation and dietary factors support the idea that vitamin D₃ (25(OH)D₃ and/or 1α,25(OH)₂D₃) has a protective effect against prostate cancer (Corder et al. 1995; Giovannucci 1998; Chan et al. 1998; Giovannucci et al. 1998; Bodiwala et al. 2003). Therefore, vitamin D₃ deficiency might be a risk factor for prostate cancer and supplementation could delay the progression of the disease.

Polymorphism of vitamin D receptor (VDR) has also been found to be related to prostate cancer. The homozygosity for F allele (FF alleles) at the FokI site (single nucleotide polymorphism) of VDR gene is associated with the risk of prostate cancer in African Americans (Oakley-Girvan et al. 2004), whereas VDR ff FokI genotype (homozygosity for f allele at the FokI site) is inversely associated with prostate cancer risk in the population with higher levels of insulin-like growth factor binding protein-
3 (IGFBP-3) (Chokkalingam et al. 2001). A benign prostatic hyperplasia (BPH) patient with B TaqI polymorphism in VDR gene has an increased risk for development of prostate cancer, suggesting that VDR B TaqI polymorphism may be a predisposing factor for prostate cancer (Tayeb et al. 2004). BsmI bb polymorphism in VDR gene is associated with increased risk of prostate cancer, whereas BsmI polymorphism with ‘BB’ or ‘Bb’ genotype decreases the risk of prostate cancer (Cheteri et al. 2004; Huang et al. 2004). Long poly(A) allele (A18 to A20) of VDR gene is correlated with advanced prostate cancer (Ingles et al. 1997). On the other hand, some studies suggest that these polymorphisms (TaqI, FokI, BsmI and poly(A) repeat) of VDR gene are not related to prostate cancer (Ntais et al. 2003).

Interaction between different VDR polymorphisms appears also to play a role in prostate cancer risk. VDR BsmI b allele has been shown to significantly decrease the risk of advanced prostate cancer in men with a long (L) allele of the poly(A) microsatellite in VDR gene (bL haplotype), whereas, BsmI B allele further increases the risk of advanced prostate cancer with VDR long (L) allele of the poly(A) (BL haplotype). This suggests that BsmI/poly(A) BL polymorphism in VDR gene may be associated with more advanced prostate cancer (Ingles et al. 1998).

In addition, serum vitamin D\textsubscript{3} levels seem to have some impact on VDR polymorphism-associated prostate cancer risk. For example, serum levels of 25(OH)D\textsubscript{3} below the median in subjects with the BB genotype show a reduced risk of prostate cancer compared with the subjects with the bb genotype (Ma et al. 1998).

1.2. Antiproliferative effects of vitamin D\textsubscript{3}

The antiproliferative effects of 1α,25(OH)\textsubscript{2}D\textsubscript{3}, an active form of vitamin D\textsubscript{3}, and its analogs are mainly due to their ability to induce cell cycle arrest, apoptosis and differentiation.

1.2.1. Cell cycle

Cell cycle is defined as the sequence of events occurring during the life span of a cell and composed of four major periods termed G1, S, G2 and M phase (King 2004). The antiproliferative actions of 1α,25(OH)\textsubscript{2}D\textsubscript{3} and its analogs on normal and malignant cells can be exerted through inducing cell cycle arrest at G0/G1 (Kobayashi et al. 1998a; Pettersson et al. 2000; Akutsu et al. 2001; Liu et al. 2002; Ryhanen et al. 2003; Wagner et al. 2003; Han et al. 2003; Mehta et al. 2003; Molnar et al. 2003; Furigay and Swamy 2004; Li et al. 2004; Hager et al. 2004), G1/S (Wang et al. 1996; Wang et al. 2000; Jensen et al. 2001; Schwartz et al. 2004) and G2/M (Kobayashi et al. 1993; Kobayashi et al. 1998b; Jiang et al. 2003; Dai et al. 2004) phase.

In prostate cancer cells, 1α,25(OH)\textsubscript{2}D\textsubscript{3} and its analogs induce cell cycle arrest mainly at the G0/G1 phase (Campbell et al. 1997a; Elstner et al. 1999; Johnson et al. 2002; Rao et al. 2002; Stewart et al. 2004). For example, 1α,25(OH)\textsubscript{2}D\textsubscript{3} markedly induces G0/G1 phase arrest and this phase arrest in the cell cycle is accompanied by significant growth inhibition of prostate cancer ALVA-31 and LNCaP cells (Krishnan et al. 2003a; Polek et al. 2003; Guzey et al. 2004). 1α,25(OH)\textsubscript{2}D\textsubscript{3} and its analogs also
arrest cell cycle at the G1/S phase in prostate cancer cells such as PC-3 and DU145 (Campbell et al. 1997a; Koike et al. 1999).

Effects of 1α,25(OH)2D3 and its analogs on cell cycle arrest are mainly due to modulation of cyclin-dependent kinases (Cdks) and its inhibitors as well as retinoblastoma protein (pRb) phosphorylation, which is mediated by cyclin/Cdk complexes (Tamrakar et al. 2000). Cdks are activators of the cell cycle by binding to cyclins, whereas Cdk inhibitors are repressors by inhibiting the activity of Cdks. The phosphorylation state of pRb regulates G1 to S phase transition in the cell cycle (Tamrakar et al. 2000). 1α,25(OH)2D3 and its analogs increase cyclin-dependent kinase inhibitors p21(waf-1) and/or p27(kip1) proteins and decrease Cdk2 activity and phosphorylated pRb in prostate cancer cells (Faiella et al. 1996; Campbell et al. 1997a; Zhuang and Burnstein 1998; Elstner et al. 1999; Moffatt et al. 2001; Krishnan et al. 2003a; Rao et al. 2004;). The increases in the p21 and/or p27 proteins lead to inhibition of the activities of Cdks such as Cdk2 and subsequent dephosphorylation of pRb, resulting in G0/G1 or G1/S phase arrest in the cell cycle (Zhuang and Burnstein 1998; Yang et al. 2002; Yang and Burnstein 2003).

1.2.2. Apoptosis

In contrast to the cell cycle through which cells proliferate, apoptosis (programmed cell death) is a way through which cells die. 1α,25(OH)2D3 and its analogs have an apoptosis-inducing effect (James et al. 1996; Welsh et al. 1998; Lamprecht et al. 2001; Banerjee and Chatterjee 2003; Elias et al. 2003; Molnar et al. 2004; Crescioli et al. 2004 and 2005). In prostate cancer, 1α,25(OH)2D3 induces apoptosis of LNCaP and ALVA-31 cells (Fife et al. 1997; Guzey et al. 2002; Polek et al. 2003) and this apoptotic effect is accompanied by decreased anti-apoptotic protein Bcl-2 and Bcl-X(L) (Blutt et al. 2000a). Further study indicates that the apoptotic effect of 1α,25(OH)2D3 on LNCaP and ALVA-31 cells is mediated by the intrinsic (mitochondrial) pathway but not the extrinsic (death receptor) pathway, since activation of caspase 9 but not caspase 8 is seen in 1α,25(OH)2D3-induced apoptosis (Guzey et al. 2002).

1α,25(OH)2D3 analogs such as EB1089 and LH ([1alpha,25(OH)2-16-ene-23-yne-26,27-F6-19-nor-D3) appear to be more potent than 1α,25(OH)2D3 in inducing prostate cancer cell apoptosis. They are able not only to induce apoptosis in LNCaP cells (Campbell et al. 1999; Blutt et al. 2000b; Hisatake et al. 2001) but also in prostate cancer PC3 and DU145 cells, which are less sensitive to 1α,25(OH)2D3 (Campbell et al. 1997b; Swamy et al. 2003 and 2004). Induction of apoptosis by 1α,25(OH)2D3 analogs has also been shown to be mediated by the intrinsic (mitochondrial) pathway in benign prostatic hyperplasia (BPH) and prostate cancer cells by reducing Bcl-2 (Crescioli et al. 2002a and 2002b). In addition, 1α,25(OH)2D3 and its analogs induce prostatic cell apoptosis in vivo (rat models) (Nickerson and Huynh 1999; Crescioli et al. 2003 and 2004).

1.2.3. Differentiation

Vitamin D3 and its analogs induce differentiation of different cell types including prostate cancer cells (Hisatake et al. 2001; Osborne and Hutchinson 2002; Ulsperger et al. 2003; Chen and Holick 2003; Leibowitz and Kantoff 2003; Molnar et al. 2003).
For instance, 1\(\alpha\),25(OH)\(_2\)D\(_3\) and its analogs are able to induce or promote the differentiation of LNCaP cells (Miller et al. 1992; Esquenet et al. 1996; Bauer et al. 2003), as indicated by stimulating the secretion of prostate-specific acid phosphatase (PSAP) and prostate-specific antigen (PSA), two markers of the differentiated prostatic phenotype (Hedlund et al. 1997). 1\(\alpha\),25(OH)\(_2\)D\(_3\) can also stimulate a marked differentiation in rat prostate (Konety et al. 1996).

In addition to their ability to induce cell cycle arrest, cell apoptosis and cell differentiation, 1\(\alpha\),25(OH)\(_2\)D\(_3\) and its analogs seem to inhibit angiogenesis (Osborne and Hutchinson 2002; Beer and Myrthue 2004), to reduce invasiveness (Lokeshwar et al. 1999; Peehl and Feldman 2004a) and metastasis of prostate cancer cells (Chen and Holick 2003; Young et al. 2004).

### 1.3. Molecular basis of vitamin D3 actions

#### 1.3.1. Nuclear vitamin D receptor

The biological actions of 1\(\alpha\),25(OH)\(_2\)D\(_3\) are mediated by its cognate nuclear vitamin D receptor (VDR). VDR is a ligand-dependent transcription factor and belongs to the subfamily of steroid hormone receptors (Dusso et al. 2005). Human VDR cDNA with a full-length of 4605 base pairs was cloned in 1988 (Baker et al. 1988) and composed of 11 exons (Miyamoto et al. 1997) which encode a single polypeptide chain composed of 427 amino acids (human VDR protein). Based on different functions, human VDR protein is divided approximately into four domains (Malloy et al. 1999): the N-terminal A/B domain (1-24 amino acids), which interacts with other transcriptional factors, the DNA-binding domain (24-90 amino acids) containing two Zn\(_2\)+-fingers, the flexible hinge region (90-242 amino acids), which affects VDR transcriptional activity (Shaffer et al. 2005) and the C-terminal ligand-binding domain containing an activation function 2 (AF-2) domain, which is required for 1\(\alpha\),25(OH)\(_2\)D\(_3\)-induced RXR/VDR heterodimerization and the interaction between VDR and coactivators (Liu et al. 2000). The basic structure of the VDR protein is shown in Figure 1.

![Figure 1. Schematic representation of the VDR protein structure](image)
After binding to $1\alpha,25(OH)_2D_3$, VDR is activated and forms a heterodimer with retinoid X receptor (RXR) (Barsony and Prufer 2002; Bettoun et al. 2003), another transcriptional factor, on vitamin D response element (VDRE), which is a short DNA sequence composed typically of two hexameric half-sites arranged either as direct repeats separated by three nucleotides (DR3) or inverted palindromes spaced by nine nucleotides (IP9) (Quack et al. 1998), in the promoter region of a target gene. The VDR-RXR heterodimer on the VDRE of the target gene promoter then interacts with general transcription apparatus (GTA) (Christakos et al. 2003) by recruiting coactivators (MacDonald et al. 2001) such as steroid receptor coactivator 1 (SRC-1) (Onate et al. 1995; Gill et al. 1998) and CREB binding protein (CBP)/p300 (Kim et al. 2005). The recruited coactivators destabilize the chromatin structure and facilitate the initiation of the transcription by GTA (Bannister and Kouzarides 1996; Chiba et al. 2000), resulting in target gene transcription (Figure 2). During the VDR-mediated target gene transactivation, phosphorylation of VDR is also seen and plays a role in its transcriptional activity (Hsieh et al. 1991; Jones et al. 1991)

![Figure 2. Schematic illustration of VDR-mediated gene transactivation.](image)

In most cases, VDR directly stimulates the transcription of target genes such as those of osteocalcin and 25-hydroxyvitamin D$_3$-24-hydroxylase (CYP24) (Haussler et al. 1995). In some cases, ligand-activated VDR can directly inhibit transcription by binding to a negative VDRE (nVDRE), which contains only one of the two heptameric motifs of the putative VDRE (Demay et al. 1992) in the promoter region of the target gene and subsequent recruitment of corepressors such as nuclear receptor corepressor (NCoR) (Horlein et al. 1995; Yu et al. 2005) and silencing mediator of retinoid and thyroid hormone receptor (SMRT) (Chen and Evans 1995; Li et al. 1997;
Yu et al. 2005). The corepressors recruited by VDR stabilize the chromatin where the target gene is located and thus prevent the transcription (Rastinejad et al. 1995), for example, in the VDR-mediated transcriptional repression of parathyroid hormone (PTH) and parathyroid hormone-related protein (PTHrP) genes (Liu et al. 1996; Nishishita et al. 1998; Tovar Sepulveda and Falzon 2003).

In addition to nuclear VDR, through which 1α,25(OH)2D3 exerts its genomic actions, some studies suggest the presence of membrane VDR (mVDR) (Nemereet et al. 1994; Baran et al. 2000) which mediates nogenomic rapid actions of 1α,25(OH)2D3 such as regulation of phosphoinositide metabolism (Lieberherr et al. 1989; Bourdeau et al. 1990) and cytosolic calcium levels (Lieberherr 1987; Hruska et al. 1988; Sugimoto et al. 1988; Morelli et al. 1993), but this is still controversial and needs to be further studied.

1.3.2. VDR-regulated genes involved in antiproliferative effects

A number of 1α,25(OH)2D3-responsive genes have been found in prostate cancer cells (Krishnan et al. 2003b; Guzey et al. 2004; Krishnan et al. 2004; Matilainen et al. 2005; Ikezoe et al. 2005; Dunlop et al. 2005) and some of them have been shown to be associated with the antiproliferative effects of 1α,25(OH)2D3. The followings are examples.

1) Insulin-like growth factor binding protein-3 (IGFBP-3) seems to be a direct (primary) 1α,25(OH)2D3-responsive gene (Peng et al. 2004). It is markedly upregulated by 1α,25(OH)2D3 and supposed to be one component associated with the antiproliferative effects of 1α,25(OH)2D3 in human prostate cancer LNCaP cells by inducing cell cycle arrest through regulation of cell cycle inhibitor p21, since p21 protein is increased and the increase in p21 appears to be mediated by IGFBP-3 in LNCaP cells treated with 1α,25(OH)2D3 (Boyle et al. 2001; Krishnan et al. 2003b and 2004). Another study suggests that 1α,25(OH)2D3-induced upregulation of IGFBP-3 is not required for the growth inhibitory effects of 1α,25(OH)2D3 on prostate cancer cells including LNCaP cells (Stewart and Weigel 2005a). Therefore, the role of IGFBP-3 in the antiproliferative effects of 1α,25(OH)2D3 is controversial.

2) Bcl-2 and Bcl-X(L), two anti-apoptotic proteins, are downregulated by 1α,25(OH)2D3 and associated with 1α,25(OH)2D3-induced apoptosis of LNCaP cells through the intrinsic apoptotic (mitochondrial) pathway (Blutt et al. 2000a) and contribute to the antiproliferative effects of 1α,25(OH)2D3 on LNCaP cells.

3) Prostate-derived factor (PDF), a proapoptotic protein belonging to the TGF-β superfamily, is upregulated by 1α,25(OH)2D3 in LNCaP cells and suggested to be, at least in part, related to 1α,25(OH)2D3-induced growth inhibition of LNCaP cells. The action of PDF in the antiproliferative effects of 1α,25(OH)2D3 seems to be through a non-classical TGF-β signalling pathway, but the detailed mechanisms are not known (Nazarova et al. 2004).

4) Cyclooxygenase-2 (COX-2) is a key enzyme for the synthesis of prostaglandins (PGs). 15-hydroxyprostaglandin dehydrogenase (15-PGDH), in contrast to COX-2, is an enzyme for catabolizing PGs. COX-2 is downregulated and 15-PGDH is upregulated by 1α,25(OH)2D3 in prostate cancer LNCaP cells. The repression of COX-2 and stimulation of 15-PGDH expression are thought to be one contributor to
the growth inhibitory actions of 1α,25(OH)2D3 in prostate cancer cells by reducing the levels of PGs, which are proliferative stimuli of prostate cancer cell growth (Moreno et al. 2005a). Therefore, the regulation of PG metabolism by 1α,25(OH)2D3 is proposed to be a new mechanism associated with 1α,25(OH)2D3 antiproliferative effects in prostate cancer cells (Moreno et al. 2005b).

5) 25-hydroxyvitamin D3-24-hydroxylase (CYP24), which inactivates 1α,25(OH)2D3, has been found to be one of the most upregulated genes in prostatic cells including prostate cancer cells and suggested to be one component involved in the antiproliferative effects of 1α,25(OH)2D3 in terms of decreasing 1α,25(OH)2D3 by forming 1α,24,25(OH)3D3. This suggests that vitamin D3 metabolism plays a role in the antiproliferative actions of 1α,25(OH)2D3 (Peehl et al. 2004b).

6) CCAAT/enhancer-binding protein delta (C/EBPdelta) is a transcription factor and belongs to the superfamily of CCAAT/enhancer binding proteins (C/EBPs) (Yukawa et al. 1998; Sivko and DeWille 2004). C/EBPdelta is upregulated by 1α,25(OH)2D3 in LNCaP cells and correlated with growth inhibition of LNCaP cells in response to 1α,25(OH)2D3. The upregulation of C/EBPdelta appears to be mediated by androgen receptor, but the detailed mechanism is not yet known (Ikezoe et al. 2005).

7) Prostatic acid phosphatase (PAP) is a prostate-related antigen and serves as a tumor marker for prostate cancer (Wang et al. 2005). PAP is upregulated by 1α,25(OH)2D3 and contributes to the antiproliferative effects of 1α,25(OH)2D3 on LNCaP cells and its derived prostate cancer C81 LN cells. 1α,25(OH)2D3 upregulation of PAP is thought to cause a decrease in tyrosine kinase signalling which is associated with cell cycle progression by tyrosine phosphorylation of cell cycle stimulators such as c-Myc, a proto-oncogene and v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 (HER-2), a member of the epidermal growth factor receptor (EGFR) family (Stewart et al. 2005b).

In addition, parathyroid hormone-related protein (PTHrP), proliferating cell nuclear antigen (PCNA), breast cancer 1, early onset (BRCA1) and dual specificity phosphatase 10 (DUSP10) have also been found to be 1α,25(OH)2D3-regulated and related to the antiproliferative effects of 1α,25(OH)2D3 on prostate cancer cells, but the mechanisms are not clear (Hsieh et al. 1996; Campbell et al. 2000; Tovar Sepulveda and Falzon 2003; Peehl et al. 2004).

Therefore, the antiproliferative effects of 1α,25(OH)2D3 seem to be mediated by many different genes, which are involved in the regulation of various intracellular events in prostate cancer cells.

1.4. Vitamin D3 and androgen interaction

Androgen plays a pivotal role in the development of normal prostate, and the maintenance and the differentiation of adult prostate (Cunha et al. 1987; Wilding 1995). Studies also show that androgen is closely associated with the initiation and progression of prostate cancer (Buchanan et al. 2001). The biological functions of androgen are mediated by androgen receptor (AR) (Gelmann 2002). AR is a nuclear receptor and, like other nuclear receptors, it acts as a transcription factor to control
target gene expression after binding to its cognate ligand. AR is expressed in prostatic cells and is a critical factor involved in prostate cancer development (Buchanan et al. 2001), especially in the progression of androgen-sensitive to androgen-refractory disease (Taplin and Balk 2004). Therefore, androgen ablation is currently one effective way for the treatment of hormone-sensitive prostate cancer (Stewart and Weigel 2004; Roscigno et al. 2005; Beekman et al. 2005; Ryan et al. 2005; Stewart et al. 2005) and the suppression of AR activity is proposed to be potent for treatment of hormone-refractory disease (Ichikawa et al. 2005).

On the other hand, androgen and AR play a crucial role in the antiproliferative actions of 1α,25(OH)2D3 in androgen-dependent prostate cancer cells. For example, in LNCaP cells which are androgen-receptor positive and sensitive to androgen, the growth inhibitory actions of 1α,25(OH)2D3 have been shown to be androgen/AR dependent (Miller et al. 1992; Zhao et al. 1997). In addition, little or no response to the growth inhibitory effects of 1α,25(OH)2D3 in AR-negative prostate cancer cells (Bao et al. 2004) and requirement of androgen for 1α,25(OH)2D3 inhibition of normal prostatic cell growth (Danielpour et al. 1994; Konety et al. 1996; Leman et al. 2003a) also support the notion that androgen/AR plays an important role in the antiproliferative effects of 1α,25(OH)2D3. The mechanisms behind this phenomenon is still not fully understood, but AR appears to act as a mediator for 1α,25(OH)2D3-induced growth inhibition of prostate cancer cells (Bao et al. 2004). 1α,25(OH)2D3 upregulates AR expression at both mRNA and protein levels (Hsieh et al. 1996; Esquenet et al. 1996; Zhao et al. 1999) and increase AR nuclear localization and its ligand binding in LNCaP cells (Hsieh and Wu 1997; Leman and Getzenberg 2003b). The upregulation of AR by 1α,25(OH)2D3 seems to be indirect and mediated by an unknown protein (Zhao et al. 1999). Recent studies have indicated that some 1α,25(OH)2D3 target genes are also androgen-responsive genes such as PSA (Krishnan et al. 2003b and 2004). This suggests that the interaction between 1α,25(OH)2D3 and androgen in the repression of androgen-dependent prostate cancer cell growth may be through the cross talk between their nuclear receptors in terms of co-regulation of target gene expression. Therefore, the identification of target genes regulated by both 1α,25(OH)2D3 and androgen may be critical for a further understanding of the molecular mechanisms by which AR mediates the antiproliferative effects of 1α,25(OH)2D3 on androgen-responsive prostate cancer cells.

2 Fatty Acids and Prostate Cancer

2.1. Fatty acids and prostate cancer risk

Fatty acids are hydrocarbon chains with a carboxyl group at one end and a methyl group at the other end (Hardman 2004). Fatty acids can be endogenously produced by organisms or obtained exogenously from food and have diverse biological functions in cells including lipid biosynthesis, energy storage, protein acylation and signal transduction (Kuhajda 2000).

Epidemiological studies have found a connection between fatty acids and the risk of cancers (Ghadirian et al. 1996; Giovannucci et al. 1997; Willett 1997; Santiago et al. 1997).
In human prostate cancer, high levels of saturated fatty acids phytanic acid, palmitic acid and myristic acid in serum are associated with increased risk of the disease (Harvei et al. 1997; Mannisto et al. 2003; Xu et al. 2005). The risk effect of phytanic acid is proposed to be related to alpha-methylacyl-CoA racemase (AMACR). AMACR is overexpressed in prostate cancer and associated with disease development. Phytanic acid is a substrate of AMACR and stimulates AMACR expression in prostate cancer cells (Mobley et al. 2003). Total saturated fatty acids appear to increase prostate cancer risk but this remains to be further investigated (Slattery et al. 1990; Vlajinac et al. 1997; Yang et al. 1999). Among monounsaturated fatty acids, C18 trans-fatty acids (King et al. 2005), palmitoleic acid (Harvei et al. 1997) and oleic acid (Heshmat et al. 1985; Schuurman et al. 1999) are associated with high risk of prostate cancer. The association between total monounsaturated fatty acids and prostate cancer risk is controversial (Whittemore et al. 1995; Ghadirian et al. 1996; Norrish et al. 2000; Bidoli et al. 2005).

The effects of polyunsaturated fatty acids (PUFAs) on the risk of prostate cancer are mainly from studies on omega-3 (the first double bond in 3 carbons from the n end: n-3) and omega-6 (the first double bond in 6 carbons from the n end: n-6) fatty acids. The outcomes are a mixture. A number of studies suggest that omega-3 PUFAs from fish and marine products are inversely associated with the risk of prostate cancer (Giovannucci et al. 1993; Rose 1997b; Yang et al. 1999; Terry et al. 2001; Sonoda et al. 2004; Narayanan et al. 2005) and slower the progression, invasiveness and metastasis of the disease (Freeman et al. 2000; Augustsson et al. 2003). For instance, eicosapentaenoic acid (EPA; 20:5n-3) and docosahexaenoic acid (DHA; 22:6n-3) both reduce the risk of total and advanced prostate cancer (Norrish et al. 1999; Leitzmann et al. 2004). This is in line with laboratory studies in which they are able to inhibit prostate cancer cell growth (Karmali et al. 1987; Rose et al. 1992; Godley et al. 1996a; Pandalai et al. 1996; Connolly et al. 1997; Motaung et al. 1999; Chung et al. 2001; Terry et al. 2003; Larsson et al. 2004; Aktas and Halperin, 2004; Narayanan et al. 2005). The actions of EPA and DHA against prostate cancer are thought to be via inhibition of arachidonic acid-derived eicosanoid biosynthesis such as prostaglandin E2 (PGE2), which is associated with prostate carcinogenesis (Karmali et al. 1987; Chaudry, et al. 1994; Connolly et al. 1997; Norrish et al. 1999; Vang and Ziboh, 2005). Modulation of gene expression such as upregulation of peroxisome proliferator-activated receptor gamma (PPARγ) and repression of nuclear transcription factor κB (NF-κB) are also possible mechanisms of the antiproliferative effects of EPA and/or DHA (Chung et al. 2001; Larsson et al. 2004; Narayanan et al. 2005). On the other hand, some studies have not found a clear relationship between omega-3 PUFAs and prostate cancer risk (Godley et al. 1996b; Schuurman et al. 1999; Kristal et al. 2002; Mannisto et al. 2003). Alpha-linolenic acid (ALA; 18:3n-3) has been proposed to be associated with the risk of prostate cancer in the majority of studies (Giovannucci et al. 1993; Gann et al. 1994; Harvei et al. 1997; De Stefani et al. 2000; Newcomer et al. 2001; Leitzmann et al. 2004; Brouwer et al. 2004).

Omega-6 PUFAs seem to be positively associated with prostate cancer risk (Rose 1997a and 1997b; Yang et al. 1999; Newcomer et al. 2001) such as arachidonic acid (AA; 20:4n-6), which is associated with increased levels of PSA and the risk of prostate cancer (Ritch et al. 2004), but this is still controversial. The notion that AA is associated with prostate cancer risk is supported by laboratory experiments in which
AA stimulates the growth of prostate cancer cells (Connolly et al. 1997; Rose 1997b; Ghosh and Myers 1997 and 1998; Anderson et al. 1988; Myers 1999; Hughes-Fulford et al. 2005 and 2006). The stimulatory effect of AA on prostate cancer is thought to be due to the activation of eicosanoid biosynthesis, for example, by 5-lipoxygenase (5LO) and/or cyclooxygenase-2 (COX-2) and mediated through the phosphatidylinositol-3 kinase (PI3K)/protein kinase B (AKT) pathway (Chaudry, et al. 1994; Ghosh and Myers 1997 and 1998; Myers and Ghosh 1999; Hughes-Fulford et al. 2005 and 2006).

The ratio of omega-6/omega-3 PUFA is most probably associated with the risk of prostate cancer (Yang et al. 1999; Aronson et al. 2001; Ritch et al. 2004) and suggests that the balance between omega-3 and omega-6 PUFA may play a role in prostatic carcinogenesis. In addition, the correlation between high fat intake and prostate cancer risk suggests a role for fatty acids in the risk of prostate cancer (Heshmat et al. 1985; Giovannucci et al. 1993; Hietanen et al. 1994; Rose 1997a and 1997b; Zhou and Blackburn 1997; Willett 1997; Gupta et al. 2001).

2.2. Fatty acid synthesis

Biosynthesis of fatty acids is a universal event in cells from low organisms like bacteria to high organisms such as plants and animals (Kuhajda 2000). Fatty acid synthesis occurs in the cytoplasm and initiates with two-carbon-containing molecule, acetyl-CoA. Acetyl-CoA is mainly produced by degradation of glucose in mitochondria. By sequential combination of two-carbon units derived from acetyl-CoA, malonyl-CoA, a 14-carbon-containing molecule, is first formed. After the formation of malonyl-CoA, a unique intermediate for fatty acid synthesis, acetyl-CoA interacts with malonyl-CoA to produce a 16-carbon fatty acid, palmitate as a terminal product of fatty acid synthesis (Figure 3) (Smith 1994; Kuhajda 2000). Therefore, the de novo fatty acid synthesis is the process of condensing eight two-carbon units (acetyl groups from acetyl-CoA) one by one to form a 16-carbon saturated long-chain fatty acid. Palmitate can then be modified by chain elongation and/or desaturation to give rise to the other fatty acids such as longer chain fatty acids and unsaturated fatty acids (Cinti et al. 1992; Bezard et al. 1994; Grammatikos et al. 1994; Sprecher et al. 1995; Moon et al. 2003; Kniazeva et al. 2003; Bulotta et al. 2003; Pereira et al. 2003; Wong et al. 2004). Many enzymes are involved in fatty acid synthesis, of these acetyl-CoA carboxylase (ACC) and fatty acid synthase (FAS) are two pivotal enzymes (Kuhajda 2000). ACC catalytically synthesizes malonyl-CoA from acetyl-CoAs and is the first rate-limiting enzyme. FAS utilizes malonyl-CoA and acetyl-CoA as substrates to synthesize palmitate and thus acts as the main synthetic enzyme (Figure 3) (Kuhajda 2000).
2.3. Fatty acid activation and long-chain fatty-acid-CoA ligase 3

Before fatty acid degradation for energy production occurs in mitochondria through β-oxidation, the fatty acids must be activated to fatty acyl-CoAs (Black et al. 2000; Schnurr et al. 2002; Bembenek et al. 2004; Wang et al. 2004). Short-chain fatty acids (C4-C8) and medium-chain fatty acids (C8-C12) can diffuse across the mitochondrial membrane (Sim et al. 2002) and are activated in the mitochondria and degraded there (Kunau et al. 1995; Yang and He 1999). Long-chain fatty acids (C >12) are mainly activated in the endoplasmic reticulum and then transported into the mitochondria with the aid of a carrier protein called carnitine, which is located on the membrane of mitochondria (Eaton 2002; Sim et al. 2002; Reda et al. 2003; Czeczot and Scibior 2005), for β-oxidation (Kondrup and Lazarow 1985; Kunau et al. 1995; Yang and He 1999).

In human cells, long-chain fatty acids are activated catalytically by long-chain fatty-acid-CoA ligase (FACL), also known as long-chain fatty acyl-CoA synthetase (ACS). There are five isoforms of human FACL present, termed long-chain fatty-acid-CoA ligase 1(2) (Abe et al. 1992; Fujino et al. 1992; Cantu et al. 1995), 3 (Minekura et al. 1997 and 2001), 4 (Cao et al. 1998), 5 (Oikawa et al. 1998) and 6 (Malhotra et al. 1999; Mashek et al. 2004) respectively. The different isoforms of human FACL have different substrate specificities and tissue distributions. Human long-chain fatty-acid-CoA ligase 3 (FACL3) preferentially utilizes myristic acid, arachidonic acid (AA) and eicosapentaenoic acid (EPA) as substrates to form corresponding long-chain fatty-acid-CoAs (Fujino et al. 1997). Human FACL3 gene is localized on chromosome 2 (q34-q35) (Minekura et al. 1997) and expressed in a variety of human tissues such as brain, heart, placenta, prostate, skeletal muscle, testis and thymus (Minekura et al. 2001; Fujimoto et al. 2004). In addition to synthesis of long-chain fatty-acid-CoA, little is known about the other biological functions of FACL3. One study indicates that EPA-induced apoptosis of leukemia cells is accompanied by an increased expression of FACL3 (ACS3) at mRNA and protein levels (Finstad et al. 2000). Another study shows that inhibition of FACL (ACS) activity blocks EPA-induced leukemia cell apoptosis (Heimli et al. 2003). This suggests that FACL3 may be associated with EPA-induced leukemia cell apoptosis.
2.4. Regulation of de novo fatty acid synthesis

The regulation of fatty acid synthesis occurs basically by two ways, short-term regulation and long-term regulation. The short-term regulation of fatty acid synthesis is achieved by control of acetyl-CoA carboxylase (ACC) activity via allosteric regulation and reversible phosphorylation (Munday 2002). Citrate is an allosteric activator of ACC, and increases the activity of ACC by polymerization of ACC (Halestrap and Denton 1974; Munday et al. 1988) and thus stimulates de novo fatty acid synthesis. On the other hand, ACC can be phosphorylated by AMP-activated protein kinase (AMPK) or cAMP-dependent protein kinase (PKA), resulting in inactivation (Munday et al. 1988) and subsequent fatty acid synthesis inhibition. Therefore, modulation of ACC activity is an effective way to control the de novo fatty acid synthesis. In addition, allosteric activation and inactivation by phosphorylation of ACC appear not to be independent of each other, since the highly polymerized ACC (activity increased) appears to be relatively dephosphorylated and the phosphorylated form of ACC (activity decreased) is less sensitive to the allosteric activator citrate (Munday et al. 1988; Thampy and Wakil 1988), suggesting that the interaction between allosteric activation and inactivation by phosphorylation of ACC also plays a role in the regulation of fatty acid synthesis.

In comparison with the short-term regulation, which occurs at epigenic levels, long-term regulation of fatty acid synthesis occurs at genic level through regulation of ACC gene transcription by nutrients and hormones. These nutrients and hormones include glucose, fatty acyl-CoA and insulin. Glucose, insulin and fatty acyl-CoA response elements (GLRE, IRE and FARE) have been identified to be present in the promoter region of the ACC gene. GLRE and IRE are positive responsive elements and FARE is a negative responsive element (Kim 1997). Therefore, glucose and insulin stimulate ACC gene expression (Kim et al. 2005), which leads to increases in ACC protein and activity, resulting in the stimulation of de novo fatty acid synthesis, whereas fatty acyl-CoA represses ACC gene transcription resulting in inhibition of de novo fatty acid synthesis (Kamiryo et al. 1976; Ogiwara et al. 1978; Nikawa et al. 1979). This indicates that fatty acyl-CoA is a feedback inhibitor of ACC gene transcription and plays an important role in the feedback inhibition of de novo fatty acid synthesis. In addition, insulin regulation of FAS gene expression and long-chain fatty acyl-CoA inhibition of FAS in yeast may also play a crucial role in the control of de novo fatty acid synthesis (Sumper and Trauble 1973; Griffin and Sul 2004).

2.5. Fatty acid synthase and prostate cancer

Mammalian fatty acid synthase (FAS) is a cytosolic and multifunctional enzyme. It is composed of different monofunctional enzymes (domains) such as β-ketoacyl synthase and enoyl reductase and functions as a homodimer (Smith et al. 2003; Asturias et al. 2005). In normal human tissues, because the diet usually provides most fatty acids for the requirement such as energy production and survival, only little endogenous fatty acid synthesis occurs (Weiss et al. 1986; Kuhajda 2000; Baron et al. 2004). Also, FAS expression is quite low or even undetectable in most normal human tissues and under strict regulation (Kuhajda 2000; Baron et al. 2004). In contrast, de novo synthesis of fatty acids in tumor tissues occurs at a high rate despite adequate ambient fatty acids and has been proposed to be a hallmark of cancer cells (Rashid et al. 1997; Gansler et al. 1997; Milgraum et al. 1997; Swinnen et al. 2000; Kuhajda
FAS is also highly expressed and its expression appears to be out of control in a large number of human cancers including prostate cancer (Kuhajda 2000; Baron et al. 2004). Therefore, the overexpression of FAS is a common event in human cancer and is associated with the disease.

In human prostate cancer, FAS overexpression is one of the earliest and most frequent molecular alterations in prostate carcinogenesis (Moore et al. 2005). High levels of FAS expression are widely seen in prostate cancer cells, prostatic intraepithelial neoplasia (PIN), and primary and advanced prostate cancer tissues (Swinnen et al. 1997; Bull et al. 2001; Welsh et al. 2001; Pizer et al. 2001; Myers et al. 2001; Ettinger et al. 2004). Furthermore, the expression of FAS is increased from low grade to high grade prostate cancer and reaches the highest level in invasive and metastatic prostate cancer (Shurbaji et al. 1996; Pizer et al. 2001; Swinnen et al. 2002; Verhoeven 2002; Rossi et al. 2003; Ettinger et al. 2004). This suggests that overexpression of FAS is not only associated with the initiation but also strongly associated with the progression of prostate cancer to the more malignant and invasive/metastatic form, which is the main cause of death from this disease.

The finding of FAS overexpression in cancer has led to studies on the effects of FAS activity inhibition or downregulation on prostate cancer growth. Inhibition of FAS activity by inhibitors such as C75 or knockdown of FAS mRNA results in growth inhibition of human prostate cancer cells and/or their xenografts (Furuya et al. 1997; Pizer et al. 2001; Pflug et al. 2003; De Schrijver et al. 2003; Brusselmans et al. 2003; Kridel et al. 2004; Brusselmans et al. 2005; Alli et al. 2005). This suggests that inhibition of FAS expression or activity is a potent way to treat prostate cancer. Growth inhibition mediated by suppression of FAS in prostate cancer is mainly due to apoptosis and cell cycle arrest, which is accompanied by increased expression of cyclin-dependent kinase inhibitors p21 and p27 and decreased expression of cyclin D1, suggesting that the growth stimulatory action of FAS in prostate cancer cells occurs through promotion of the cell cycle and reduction of apoptosis (Furuya et al. 1997; Myers et al. 2001).

Overexpression of FAS in prostate cancer appears to be correlated with activation of phosphatidylinositol-3 (PI3) kinase/Akt kinase pathway, since inhibition of PI3k/Akt kinase pathway or increase in the expression of its inactivator phosphatase and tensin homolog (PTEN), a tumor suppressor gene, leads to decreased FAS expression in prostate cancer (Van de Sande et al. 2002 and 2005; Bandyopadhayay et al. 2005), but the detailed mechanisms have still not been fully elucidated.
AIMS OF THE STUDY

The aim of this study was to analyse the mechanisms by which the growth inhibitory effects of vitamin D$_3$ are mediated in human prostate cancer cells. The specific aims were:

1. To search for new vitamin D$_3$-regulated genes using cDNA microarrays

2. To study vitamin D$_3$ regulation of FAS and FACL3 that were two genes recognised in cDNA microarrays

3. To study the roles of FAS and FACL3 in the antiproliferative actions of vitamin D$_3$ in prostate cancer cells

4. To study possible mechanism by which vitamin D$_3$ regulates FAS and FACL3 expression
MATERIALS AND METHODS

1. Materials

1α,25(OH)2D3, EB1089 and CB1093 were kindly supplied by Leo Pharmaceuticals (Ballerup, Denmark). RPMI-1640 medium was purchased from Sigma-Aldrich (Saint Louis, Missouri, USA). Fetal bovine serum (FBS) was obtained from Gibco BRL (Life Technology, Paisley, Scotland). TRIzoL Reagent was purchased from Invitrogen (Carlsbad, USA). Casodex was obtained from AstraZeneca (London, UK). Dihydrotestosterone (DHT), cycloheximide, polyadenylic acid (poly dA), leupeptin, myristic acid, cerulenin, CoA, ATP and triacsin C were purchased from Sigma (Missouri, USA). [1-14C]-labelled myristic acid was purchased from BIOTREND (Cologne, Germany). Cy3-dUTP (25 nM), Cy5-dUTP (25 nM), dNTP (5 mM dATP, 5 mM dCTP, 5 mM dGTP, 2 mM dTTP) and Oligo (dT) primers (1 mg/ml) were purchased from Amersham Pharmacia Biotech (Piscataway, USA). Human COT-1 DNA (1 mg/ml), SuperScript II RT (200 U/µl) and yeast tRNA (10 mg/ml) were purchased from Gibco BRL (Grand Island, USA). rRNasin® RNase inhibitor (40 U/µl) was purchased from Promega (Madison, USA). 10x Dig blocking buffer was from Roche Diagnostics (Mannheim, Germany). High Capacity DNA Archive kit and SYBR Green PCR Master Mix Kit were purchased from Applied Biosystems (Foster City, USA). M-PER™ Mammalian Protein Extraction Reagent and BCA Protein Assay Reagent Kit were obtained from PIERCE (Rockford, USA). ECL™ Western Blotting Detection Reagents was from Amersham Biosciences UK limited (UK). Rabbit antiserum to human FACL3/ACS3 was supplied by Dr. Yasuyuki Fujimoto (Teikyo University, Japan). Human prostate cancer cell lines LNCaP, PC-3, DU145 were obtained from American Type Culture Collection (Rockville, MD). Human 2-1 chip containing 3,000 gene probes was purchased from Turku Centre for Biotechnology (Turku, Finland). Human BBC_13K_3 Chip containing more than 12,000 gene probes was obtained from the Biomedicum Biochip Center (Helsinki, Finland)

2. Methods

2.1. Cell culture and treatments

Human prostate cancer cell lines LNCaP, PC-3 and DU145 were maintained in RPMI-1640 medium supplemented with 10% FBS, 3 mM L-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin at 37°C in a humidified atmosphere of 5% CO2. For the treatments, LNCaP, PC-3 or DU145 cells were grown to approximate 50% - 70% confluence and treated with 1α,25(OH)2D3, analogs and/or other reagents for times indicated. Vehicle ethanol treatment was used as the controls for 1α, 25(OH)2D3, EB1089, CB1093, DHT, cycloheximide, casodex or myristic acid treatment and vehicle DMSO treatment as the controls for cerulenin or triacsin C treatment.

2.2. RNA isolation

RNA was isolated using TRIzoL Reagent (Invitrogen, USA) according to the instructions of the manufacturer. In Brief, 1 ml of TRIzoL Reagent was added to 5
cm² of the culture area, cells were homogenized by passing through a pipette several times in TRIzol Reagent and incubating for 5 min at room temperature. After the homogenization, chloroform was added to the cell lysate at a volume of one fifth of TRIzol Reagent and mixed vigorously by hand for 15 s. The mixture was centrifuged at 12,000g for 15 min at 4°C after 3 min incubation at room temperature. The aqueous phase was transferred to a sterile tube and an equal volume of isopropyl alcohol was added to precipitate RNA for 15 min at room temperature. RNA pellet was obtained by centrifuging as mentioned above and washed twice in 75% ethanol. The RNA pellet was redissolved in RNase-free water and the concentration was measured using GeneQuant II (Pharmacia Biotech, USA).

2.3. cDNA microarray and data analysis

2.3.1. Human 2-1 chip

cDNA microarray was performed according to the manufacturer’s instructions. In brief, 20 µg of RNA sample from 1α,25(OH)2D₃-treated cells was labelled with Cy5-dUTP (25 nM) by reverse transcription under Oligo(dT)₁₂₋₁₈ primer direction. In parallel, an equal amount of RNA sample from untreated cells was labelled with Cy3-dUTP (25 nM) as control. RNA labelling was done at 42°C for 80 min. When the labelling was completed, RNA was removed from synthesized cDNA by the addition of a small amount of NaOH solution (1 M) followed by neutralization with Tris-HCl (1 M, pH 7.5). Cy3-labelled cDNA and Cy5-labelled cDNA were combined in one Microcon Column (Millipore Corporation. Bedford, USA) and washed 4 times in TE buffer (pH 7.4) by centrifugation. In the final washing step, COT-1 DNA, PolyA and yeast tRNA were added to the washing buffer, and centrifuged to make the final volume of labelled cDNA mixture less than 10 µl. For hybridization, Human 2-1 Glass chip containing 3,000 genes (Turku Centre for Biotechnology. Turku, Finland) was pretreated with 0.1% SDS, sterile H₂O and 95% ethanol respectively and air-dried. The labelled cDNA mixture was hybridized with the chip in a humid chamber at 65°C overnight. After the hybridization, the chip was washed 4 times with slight shaking, and spun dry by centrifugation.

2.3.2. Human BBC_13K_3 chip

cDNA microarray was performed according to the instructions of the manufacturer. Briefly, 50 µg of RNA sample from 1α,25(OH)2D₃-treated cells was labelled with Cy5-dUTP (25 nM) and 50 µg of RNA sample from control cells was labelled with Cy3-dUTP (25 nM) by reverse transcription using Oligo(dT)₁₂₋₁₈ as primers. The labelling reaction was done at 42°C for 60 min. Thereafter, RNA was removed from the labelled cDNA by the addition of a certain amount of NaOH solution (1 M) at 65°C for 30 min and the solution was neutralized with Tris-HCl (1 M, pH 7.5) at room temperature. Cy3-labelled cDNA was mixed with a small amount of human COT-1 DNA in one Microcon column (Millipore Corporation. Bedford, USA) and Cy5-labelled cDNA was mixed with an equal amount of COT-1 DNA in another Microcon column followed by washing in TE buffer (pH 7.4) by centrifugation until the volume was less than 50 µl for each labelled cDNA. The Cy3-labelled cDNA and Cy5-labelled cDNA were combined in a new Microcon column and washed once in TE buffer (pH 7.4) by centrifugation to a final volume of 8-25 µl. The labelled cDNA (8-25 µl) was mixed with a certain amount of poly dA and yeast tRNA, and then
hybridized with the chip containing over 12,000 genes in a humid chamber at 65°C overnight. After the hybridization, the chip was washed 3 times with slight shaking, and spun dry by centrifugation.

### 2.3.3. Data analysis

The hybridized chips were scanned in ScanArray 4000 Series (Packard BioScience) and the images were analysed using QuantArray Microarray Analysis Software (Packard BioScience). Difference in gene expression between 1α,25(OH)2D3-treated and untreated samples was expressed as fold. The fold equal to or more than 1.8 was considered to be upregulation. A fold equal to or less than 0.55 for Human BBC_13K_3 chip and 0.58 for Human 2-1 chip was considered to be downregulation. The data were finally normalized to Median using Excel Date Normalization Macro.

### 2.4. cDNA synthesis

cDNA was synthesized using High Capacity Archive Kit (Applied Biosystems, USA) following the instructions of the manufacturer. Briefly, 10 µg of total RNA dissolved in 50 µl of RNase-free H2O was combined with 50 µl of 2x RT Master Mix (10 µl of 10x Reverse Transcription Buffer, 4 µl of 25x dNTPs, 10 µl of 10x random primers, 5 µl of MultiScribe Reverse Transcriptase (50 U/µl) and 21 µl Nuclease-free H2O ). Reverse transcription was done at 25ºC for 10 min and then at 37ºC for 120 min.

### 2.5. Real-time quantitative PCR

Real-time quantitative PCR (QPCR) was performed with SYBR Green PCR Master Mix kit in ABI PRISM 7000 Detection System (Applied Biosystems, USA) following the instructions of the manufacturer. Briefly, 20 µg of cDNA was combined with primers of target gene and 2xSYBR Green PCR Master Mix to the final volume of 30 µl or 50 µl per reaction. Real-time quantitative PCR was done at 95ºC for 10 min followed by 40 cycles at 95ºC, 15 sec and 60ºC, 1 min in ABI PRISM 7000 Detection System. The PCR product was examined by agarose gel electrophoresis and dissociation curve to ensure that the product was specific for the target gene. The data were analysed using ABI Prism 7000 SDS Software and normalized to the housekeeping gene to verify uniform RNA loading. The final result was expressed as N-fold difference in gene expression between treatment and control \{N(fold)=[ target gene (treatment) / housekeeping gene (treatment)]/[ target gene (control) / housekeeping gene (control)]\}. The values used for calculation in above formula were obtained from the corresponding standard curve. All primers used for analysis of target gene expression and housekeeping genes were designed using compatible software Primer Express for ABI PRISM 7000 Detection System. The primer sequences used in the study are listed in Table 1.

### 2.6. Protein extraction

#### 2.6.1. Cytosolic and nuclear protein preparation

The cellular proteins were prepared according to a modified version of the method of Hurst (Hurst et al. 1990). In brief, cells were treated or untreated with compounds indicated in 10% DCC-FBS medium for 24 h and lysed with buffer I (20 nM Hepes
Ph 8.0, 20 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitor cocktail (Boehringer-Mannheim, Mannheim, Germany) for 5 min on ice. Supernatant was taken as the cytosolic protein after centrifugation at 13,200 rpm for 1 min at 4°C. The pellet at the bottom of the tube was the nuclear fraction and extracted with buffer II (20 nM Hepes pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 µM MgCl₂, 2 µM EDTA pH 8.0, 1 mM dithiothreitol, and protease inhibitor cocktail) for 30 min on ice. The nuclear proteins were obtained by centrifuging as above. The protein concentration was determined using BCA Protein Assay Reagent Kit (PIERCE, USA).

2.6.2. Total protein preparation

The total proteins were extracted from the cells using M-PER Mammalian Protein Extraction Reagent following the instructions of the manufacturer. Briefly, 400 µl of M-PER Reagent was added to cells in a 6 cm culture dish after carefully removing the culture medium. The cells were incubated with M-PER Reagent for 5 min by shaking gently at room temperature. Cell lysate was collected and centrifuged at 27,000 g for 5 min to pellet the cell debris. After the centrifugation, supernatant was transferred to a fresh tube for protein analysis. The concentration of total proteins was measured using BCA Protein Assay Reagent Kit according to the instructions of the manufacturer.

2.7. Western blotting analysis

Equal amounts of protein extracts from different samples were heated in SDS sample buffer at 95°C for 5 min and subjected to SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (7.5% SDS-PAGE for androgen receptor (AR) protein and 9.5% SDS-PAGE for long-chain fatty-acid-CoA ligase 3 (FACL3) protein analysis). After SDS-PAGE, proteins were transferred to nitrocellulose membrane and the membrane was blocked in TBS buffer (50 mM Tris, 0.9% NaCl, 0.05%, pH 8.0) containing 5% nonfat milk and 0.1% Tween 20 for 1 h at 37°C. Thereafter, membranes were incubated with primary antibodies (anti-human AR antibody AR 70, 1 mg/ml, 1:200; antiserum to human FACL3/ACS3, 1:2000 ) in TBS buffer containing 1% nonfat milk and 0.1% Tween 20 at 4°C overnight. Next day, the membrane was washed 3 x 15 min in TBS buffer containing 0.1% Tween 20 and incubated with peroxidase-conjugated goat IgG fraction to rabbit IgG for 1 hour at room temperature by shaking gently. After rewashing as mentioned above, the membranes were incubated with ECL™ Western Blotting Detection Reagents according to the instructions of the manufacturer. Blot was developed with enhanced chemiluminescence system (Konica SRX-101A, USA).

2.8. Enzyme activity assay

The activity of long-chain fatty-acid-CoA ligase (FACL/ACS) was measured as described by Malhotra (Malhotra et al. 1999) with minimal modification. Briefly, the FACL/ACS activity was determined by measuring the formation of [1-14C] myristoyl-CoA from [1-14C]-labelled myristic acid, a preferential substrate for FACL3/ACS3. The cell lysate equivalent of 10⁷ cells was incubated for 20 min in 0.15 ml of a standard reaction mixture containing 15 µmol of TRIS/HCl, pH 8.0, 1 µmol of ATP, 100 nmol of CoA, 750 nmol of dithiothreitol, 3 µmol of MgCl₂ and 40 µl of a solution containing 50 mM NaHCO₃, 7.5 mM Triton X-100, 10 nmol of myristic acid and 2 x
10^5 d.p.m. of [1-\textsuperscript{14}C]-labelled myristic acid. The reaction was stopped by the addition of 2.25 ml of a mixture of propan-2-ol:heptane:2 M sulphuric acid (40:10:1), followed by 1.5 ml of heptane and 1 ml of water and vigorous shaking. After centrifugation at 2,000 g for 5 min, the upper layer was removed and the lower aqueous phase was washed three times with 2 ml of heptane. The radioactivity in the lower (myristoyl-CoA) and upper (myristic acid) phases was measured by scintillation counting (WALLAC, VICTOR 1420 MULTILABEL COUNTER). The activity of FAACL/ACS was expressed as [1-\textsuperscript{14}C] myristoyl-CoA formation.

2.9. Cell growth assay

Cells were grown in 96-well plates and treated with 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} and other reagents for the times indicated. After the treatments, cell growth was assayed using crystal violent staining. In brief, the cells were fixed by 11\% of glutaraldehyde solution in a 96-well plate for 15 min by shaking at 500 rpm and air-dried after washing. Crystal violent solution (0.1\%) was added to stain the fixed cells for 20 min by shaking, excess dye was removed by extensive washing with deionized water, the plates were air-dried before the addition of 10\% of acetic acid to extract cell-bound dye. The optical density of dye extracts was measured directly in the plates at the wavelength of 590 nm using the Microplate Reader (WALLAC, VICTOR 1420 MULTILABEL COUNTER, Turku, Finland).

2.10. Cell viability assay

Cells were detached by trypsinization and suspended in culture medium at a concentration of 10^6 cells/ml. One drop of cell suspension was mixed with one drop of trypan blue. The mixture (20 \mu l) was immediately transferred to the counting chamber and left for 1-2 min before performing cell number counting under low-power microscopy. The total number of cells and the number of stained cells (dead cells) were counted to determine the viable cell numbers.

2.11. Statistical methods

Data were statistically analysed using a paired two-tailed t-test and expressed as mean ± SD. A difference of p < 0.05 was considered significant, and p < 0.001 highly significant.
<table>
<thead>
<tr>
<th>Gene names</th>
<th>primer sequences</th>
<th>positions</th>
</tr>
</thead>
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<tr>
<td>human FAS</td>
<td>Forward primer: 5’-AACTCCAAGGACACAGTCACCAT-3’</td>
<td>(exon 12)</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-CAGCTGCTCCACGAACCTCAA-3’</td>
<td>(exon 13)</td>
</tr>
<tr>
<td>human FACL1 (FACL2)</td>
<td>Forward primer: 5’-TCTTTCCCCGTGGTTCCAA-3’</td>
<td>(exon 12)</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-TGGTGGTTGCTGGTCCGAAA-3’</td>
<td>(exon 13)</td>
</tr>
<tr>
<td>human FACL3/ACS3</td>
<td>Forward primer: 5’-ACTCCACTGTGCAAGCTTT-3’</td>
<td>(exon 9)</td>
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<tr>
<td></td>
<td>Reverse primer: 5’-CACCACACAAAGGACAGCA-3’</td>
<td>(exon 10)</td>
</tr>
<tr>
<td>human FACL4</td>
<td>Forward primer: 5’-CAGGCCAGTGTGAAAAGAATACCT-3’</td>
<td>(exon 11)</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-AGTCTAGCAGATGCAAAGG-3’</td>
<td>(exon 12)</td>
</tr>
<tr>
<td>human FACL5</td>
<td>Forward primer: 5’-TGGCTTCACACAGGAGACATTTG-3’</td>
<td>(exon 18)</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-GTCTCTTTTTACGGTGATGAGATCTTTC-3’</td>
<td>(exon 19)</td>
</tr>
<tr>
<td>human FACL6</td>
<td>Forward primer: 5’-CACATTTTCTATTTGCTTTGCA-3’</td>
<td>(exon 13)</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5’-GCCCTCCGTGGCAATAGAC-3’</td>
<td>(exon 14)</td>
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<tr>
<td>human PBGD</td>
<td>Forward primer 5’-CCACACACAGCTACTTTCCA-3’</td>
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<td></td>
<td>Reverse primer 5’-TTTCTTCCGCGCTGCA-3’</td>
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<td>human GAPDH</td>
<td>Forward primer 5’-CCACATCGTCAGACACC-3’</td>
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<tr>
<td></td>
<td>Reverse primer 5’-ACCAGGCGCCCAATAC-3’</td>
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</table>
RESULTS

1. Vitamin D₃-responsive Genes in LNCaP Cells (I, II)

LNCaP cells were treated or left untreated with 10 nM 1α,25(OH)₂D₃ for 24 h. After the treatments, RNA was isolated and applied for cDNA microarray. The cDNA microarrays were done at least in duplicate and the results indicated that in human 2-1 Chip, 24 genes were regulated and in human BBC_13K_3 Chip, up to 400 genes were regulated by 10 nM 1α,25(OH)₂D₃ 24 h after the treatments. Two fatty acid metabolism genes, fatty acid synthase (FAS) and long-chain fatty-acid-CoA ligase 3 (FACL3) were revealed, for the first time, to be regulated by 1α,25(OH)₂D₃. FAS was one of the most downregulated genes (0.41±0.08-fold) and FACL3 was the most upregulated gene (4.12±1.07-fold) by 10nM 1α,25(OH)₂D₃ in LNCaP cells.

2. Fatty Acid Synthase (FAS) (I)

2.1. Vitamin D₃ downregulates FAS expression

Based on the cDNA microarray results in which FAS expression was shown to be downregulated by 1α,25(OH)₂D₃, the regulation of FAS expression was further studied using real-time quantitative PCR. LNCaP cells were treated with 10 nM 1α,25(OH)₂D₃ for 6, 24 and 48 h respectively. Real-time quantitative PCR confirmed the downregulation of FAS expression by 1α,25(OH)₂D₃. Repression of FAS expression was stronger 6 h after 1α,25(OH)₂D₃ treatment (0.43±0.05-fold, p<0.001). At 24 and 48 h the inhibitory effects of 1α,25(OH)₂D₃ on FAS expression were slightly decreased but still significant (24 h, 0.60±0.02-fold, p<0.05; 48 h, 0.63±0.023-fold, p<0.05).

To see whether the regulatory effect of 1α,25(OH)₂D₃ on FAS expression is direct, cycloheximide, a protein synthesis inhibitor, was used to treat LNCaP cells in the presence of 10 nM 1α,25(OH)₂D₃ for 6 h. Cycloheximide abolished the downregulation of FAS expression by 1α,25(OH)₂D₃. Since the actions of 1α,25(OH)₂D₃ in LNCaP cells have been shown to be androgen-dependent (Zhao et al. 1997), we sought to see whether androgen had any effect on 1α,25(OH)₂D₃ regulation of FAS expression. LNCaP cells were treated with 10 nM 1α,25(OH)₂D₃ in the absence of androgen (DCC-serum medium) for different times (6, 24 and 48 h) or in the presence of antiandrogen, Casodex. Real-time quantitative PCR analysis showed that 1α,25(OH)₂D₃ failed to regulate FAS expression in the absence of androgen or in the presence of Casodex (Figure 4, unpublished data).
2.2. Inhibition of FAS activity represses LNCaP cell growth

To see whether FAS plays a role in cell growth, cerulenin, a specific inhibitor of FAS activity, was applied to treat LNCaP cells for different times (0, 2, 4 and 6 days). Cell growth assay indicated that inhibition of FAS activity by cerulenin resulted in highly significant suppression of LNCaP cell growth on days 2, 4 and 6 respectively (p<0.001). This growth suppressive effect was shown to be time-dependent, with the maximal growth inhibition (over 90%) seen on day 6. Since Casodex, an androgen antagonist, was shown to block 1\(\alpha\),25(OH)\(_2\)D\(_3\) downregulation of FAS expression, the effects of Casodex on 1\(\alpha\),25(OH)\(_2\)D\(_3\)-induced LNCaP cell growth inhibition were studied. Casodex attenuated the growth inhibitory actions of 1\(\alpha\),25(OH)\(_2\)D\(_3\) in LNCaP cells.

3. Long-Chain Fatty-Acid-CoA Ligase 3 (FACL3) (II, IV)

3.1. Vitamin D\(_3\) upregulates FACL3 expression

Long-chain fatty-acid-CoA ligase 3 (FACL3) is a downstream enzyme of FAS in fatty acid metabolism pathway. Our cDNA microarrays revealed that FACL3 expression is upregulated by 10 nM 1\(\alpha\),25(OH)\(_2\)D\(_3\) in LNCaP cells 24 h after treatment. The upregulation of FACL3 expression by 1\(\alpha\),25(OH)\(_2\)D\(_3\) was further confirmed by real-time quantitative PCR analysis and shown to be time-dependent. At 24 and 48 h FACL3 expression was upregulated more than two-fold respectively (2.15±0.06-fold, p<0.001; 2.68±0.12-fold, p<0.001). The upregulation reached a peak of 3.64±0.06-fold (p<0.001) at 96 h and then gradually decreased at 144 (2.92±0.04-fold, p<0.001) and 192 h (2.24±0.03-fold, p<0.001) in LNCaP cells. Western blotting analysis showed that FACL3 protein (75 kDa) was also increased by 10 nM 1\(\alpha\),25(OH)\(_2\)D\(_3\) in LNCaP cells. 1\(\alpha\),25(OH)\(_2\)D\(_3\)-stimulated FACL3 protein increase is slight 24 h, but more than two-fold 48 h after the treatments.
Since both mRNA and protein expression of FACL3 were upregulated by 10 nM 1α,25(OH)₂D₃ in LNCaP cells, we investigated whether 1α,25(OH)₂D₃ affects the activity of FACL3. LNCaP cells were treated with 10 nM 1α,25(OH)₂D₃ for 24, 48, 96 and 144 h respectively. FACL3 activity was analysed using whole cell lysates and determined by measuring the formation of [1-¹⁴C]myristoyl-CoA from [1-¹⁴C]-labelled myristic acid, a preferential substrate for FACL3, and finally expressed as relative activity to the control. The results indicated that the activity of FACL3 was elevated by 10 nM 1α,25(OH)₂D₃ in LNCaP cells and shown to be time-dependent. At 24 h FACL3 activity was slightly increased (1.22±0.01-fold, p>0.05). The increase in the FACL3 activity by 1α,25(OH)₂D₃ was significant at 48 h (1.48±0.07-fold, p<0.05), reached a peak at 96 h (4.04±0.33-fold, p<0.05) and then declined to 1.53±0.04-fold at 144 h (p<0.05).

Androgen/AR is required for the growth inhibitory action of 1α,25(OH)₂D₃ in LNCaP cells (Zhao et al. 1997). To investigate the effects of androgen and AR on the regulation of FACL3 expression by 1α,25(OH)₂D₃, LNCaP cells were treated with 10 nM 1α,25(OH)₂D₃ in the absence of androgen (DCC-serum medium) or in the presence of androgen (normal serum medium) and antiandrogen Casodex. 1α,25(OH)₂D₃ had no significant effect on FACL3 expression in androgen-depleted DCC-serum medium. Antiandrogen Casodex completely blocked 1α,25(OH)₂D₃ upregulation of FACL3 expression in androgen-containing normal serum medium (1α,25(OH)₂D₃ treatment (24 h): 2.16±0.13-fold, 1α,25(OH)₂D₃ plus Casodex treatments (24 h): 0.72±0.05-fold, p<0.001). To further confirm the effects of androgen and AR on 1α,25(OH)₂D₃ regulation of FACL3 expression in LNCaP cells, dihydrotestosterone (DHT) was used to treat the cells in the presence of 1α,25(OH)₂D₃ or the combination of 1α,25(OH)₂D₃ and Casodex in androgen-deprived DCC-serum medium. The stimulatory effect of 1α,25(OH)₂D₃ on FACL3 expression was totally restored by DHT and this effect of DHT was abolished by Casodex. At 6 h FACL3 expression was upregulated approximately 2-fold by 1α,25(OH)₂D₃ in the presence of 10 nM DHT. The upregulation of FACL3 by 1α,25(OH)₂D₃ was more than 5.5-fold in the presence of 10 nM DHT and approximately 1.5-fold in the presence of 1 nM DHT 24 h after the treatments. At 48 h the upregulation was around 2-fold in the presence of 1 nM DHT and over 3 fold in the presence of 10 nM DHT (Table 2). Antiandrogen Casodex thoroughly blocked the effect of DHT on the upregulation of FACL3 expression by 1α,25(OH)₂D₃ [1α,25(OH)₂D₃+DHT (24 h): 4.97±0.38-fold, 1α,25(OH)₂D₃+DHT+Casodex (24 h): 0.99±0.08-fold, p<0.001]. Western blotting analysis showed that 1α,25(OH)₂D₃ had no significant effect on AR protein expression in androgen-depleted DCC-serum medium but increased AR protein expression in the presence of androgen (DHT) compared with DHT alone at 24 h. In AR-negative prostate cancer PC-3 and DU145 cells, 1α,25(OH)₂D₃ had no effects on FACL3 expression. In addition, cycloheximide, a protein synthesis inhibitor, appeared to block 1α,25(OH)₂D₃ upregulation of FACL3 expression in LNCaP cells [1α,25(OH)₂D₃ treatment (24 h): 2.55±0.07-fold, 1α,25(OH)₂D₃ plus cycloheximide treatments (24 h): 1.64±0.05-fold, p<0.05].
Table 2. DHT effects on 1α,25(OH)2D3 regulation of FACL3 expression in LNCaP cells in DCC-serum medium (* p<0.05, ** p<0.001)

<table>
<thead>
<tr>
<th>Treatments</th>
<th>6 h (fold)</th>
<th>24 h (fold)</th>
<th>48 h (fold)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control (0.1% ethanol)</td>
<td>1±0.01</td>
<td>1±0.01</td>
<td>1±0.00</td>
</tr>
<tr>
<td>1α,25(OH)2D3 (10 nM)</td>
<td>1.13±0.03</td>
<td>1.02±0.02</td>
<td>1.01±0.04</td>
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<tr>
<td>DHT (1 nM)</td>
<td>0.94±0.02</td>
<td>1.08±0.04</td>
<td>1.24±0.02</td>
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<tr>
<td>DHT(1 nM)+1α,25(OH)2D3(10 nM)</td>
<td>1.19±0.05</td>
<td>1.43±0.05**</td>
<td>1.99±0.13**</td>
</tr>
<tr>
<td>DHT (10 nM)</td>
<td>1.33±0.03</td>
<td>2.55±0.05**</td>
<td>2.12±0.15**</td>
</tr>
<tr>
<td>DHT(10 nM)+1α,25(OH)2D3(10 nM)</td>
<td>1.85±0.05**</td>
<td>5.62±0.29**</td>
<td>3.02±0.23**</td>
</tr>
</tbody>
</table>

Some vitamin D3 analogs are more potent than 1α,25(OH)2D3 in cell growth inhibition. We examined the effects of EB1089 and CB1093, two synthetic analogs of 1α,25(OH)2D3, on FACL3 expression. LNCaP cells were treated with 10 nM EB1089 or 10 nM CB1093 for 24 and 48 h respectively. FACL3 mRNA expression was analysed using real-time quantitative PCR and the protein expression was measured using Western blotting. FACL3 mRNA was significantly increased by both EB1089 (24 h: 3.35±0.07-fold, p<0.001. 48 h: 4.01±0.68-fold, p<0.001) and CB1093 (24 h: 4.65±0.21-fold, p<0.001. 48 h: 6.80±0.79-fold, p<0.001) in LNCaP cells. In parallel with their upregulation of FACL3 mRNA, EB1089 and CB1093 increased the protein expression of FACL3 in LNCaP cells. Increase in FACL3 protein expression by either of them was greater 48 h (over 2-fold) than 24 h (less than 1.5-fold) after the treatments.

3.2. Inhibition of FACL3 activity attenuates the growth inhibitory action of vitamin D3

To investigate the possible role of FACL3 in 1α,25(OH)2D3-induced LNCaP cell growth inhibition, triacsin C, an inhibitor of FACL3/ACS3 activity, was used to treat LNCaP cells in the presence of 1α,25(OH)2D3 for 0, 2, 4 and 6 days respectively. Cell growth was analysed using crystal violet staining. Cell growth inhibition was expressed as % of controls. Inhibition of FACL3 activity partially blocked the growth suppressive effects of 1α,25(OH)2D3 on LNCaP cells. 10 nM 1α,25(OH)2D3 induced significant growth inhibition of LNCaP cells on days 4 (45.8%±0.036, p<0.001) and 6 (57.5%±0.041, p<0.001). In the presence of triacsin C, the growth inhibitory effects of 1α,25(OH)2D3 were significantly reduced in LNCaP cells on both days 4 (26.1%±0.02, p<0.05) and 6 (42.6%±0.02, p<0.05) compared with the 1α,25(OH)2D3 treatment alone. The viability of the LNCaP cells was not affected by 1α,25(OH)2D3, triacsin C or the combination of both during the treatments as measured by trypan blue exclusion assay.

3.3. FACL3 is a dominant isoform in LNCaP cells

FACL3 is a member of the human FACL family which includes four other members termed respectively FACL1(2), FACL4, FACL5 and FACL6. We analysed the expression profile of all five members of the FACL family in LNCaP cells. The cells were grown in normal serum medium for 24, 48 and 96 h respectively. Expression
levels of each isozyme were analysed using real-time quantitative PCR and expressed as % of total expression of all isoforms. FACL3 was a major isoform expressed in LNCaP cells. The expression of FACL3 was stable through times tested and accounted for 68.46% on average (24 h: 67.37%, 48 h: 71.5%, 96 h: 66.5%) in the total expression of all isozymes in LNCaP cells.

3.4. Effects of vitamin D₃ on other isoforms of FACL

To see whether 1α,25(OH)₂D₃ has any effects on the expression of other isozymes of FACL, LNCaP cells were treated with 10 nM 1α,25(OH)₂D₃ for different times (6, 24, 48, 96, 144 and 192 h respectively) and the expression of each isozyme was measured using real-time quantitative PCR. 1α,25(OH)₂D₃ showed no effects on the expression of FACL1(2), FACL4 and FACL6 respectively. FACL5 expression was downregulated approximately 2-fold by 1α,25(OH)₂D₃ at 24 (0.59±0.03, p<0.05) and 48 h (0.62±0.02, p<0.05). No regulation effect on FACL5 expression was seen at later time points (96, 144 and 192 h).

3.5. Low FACL3 expression in PC-3 and DU145 cells

1α,25(OH)₂D₃ and its analogs were shown to upregulate FACL3 expression and inhibition of FACL3 activity significantly attenuated the growth inhibitory effects of 1α,25(OH)₂D₃ on prostate cancer LNCaP cells. We further examined the constitutive expression of FACL3 in LNCaP and more malignant prostate cancer PC-3 and DU145 cells (Campbell et al. 1998 and 1999) using real-time quantitative PCR and Western blotting. FACL3 mRNA expression was expressed as fold relative to the housekeeping gene and protein expression was expressed as fold of the FACL3 protein in LNCaP cells. The expression of FACL3 was constitutively low at mRNA and protein levels in both PC-3 and DU145 cells compared to that in LNCaP cells. In normal serum medium, FACL3 mRNA expression dramatically decreased in PC-3 (2.23±0.12-fold, p<0.001) and DU145 (1.37±0.06-fold, p<0.001) cells compared to LNCaP cells (8.67±0.76-fold). In DCC-serum medium, FACL3 mRNA expression decreased in LNCaP cells (4.7±0.17-fold) compared to that (8.67±0.76-fold) in normal serum, but the mRNA expression (4.7±0.17-fold) in LNCaP cells was still significantly high compared to that in PC-3 (2.37±0.12-fold, p<0.001) and DU145 (1.33±0.06-fold, p<0.001) cells. In both normal serum and DCC-serum media, FACL3 protein expression was high in LNCaP cells (over 2-fold) compared to that in PC-3 and DU145 cells. For each cell line, no evident difference in FACL3 protein expression was seen between normal serum and DCC-serum media.

4. FACL3 Mediates Vitamin D₃ Downregulation of FAS Expression (III)

FAS and FACL3 are two fatty acid metabolic enzymes involved respectively in de novo long-chain fatty acid synthesis and activation. 1α,25(OH)₂D₃ was shown to downregulate FAS expression and upregulate FACL3 expression in human prostate cancer LNCaP cells. To explore the possible mechanisms through which 1α,25(OH)₂D₃ regulates both FAS and FACL3 expression, LNCaP cells were treated in the presence of 1α,25(OH)₂D₃ with cerulenin, an enzyme activity inhibitor of FAS, or triacsin C, an enzyme activity inhibitor of FACL3. The expression of FAS and FACL3 was analysed using real-time quantitative PCR. Triacsin C was shown to
completely abolish 1α,25(OH)_2D_3 downregulation of FAS expression [1α,25(OH)_2D_3 (24 h): 0.6±0.06-fold, 1α,25(OH)_2D_3 + triacsin C (24 h): 1.09±0.15-fold, p< 0.05], whereas cerulenin showed no significant effect on the upregulation of FACL3 expression [1α,25(OH)_2D_3 (24 h): 2.17±0.11-fold, 1α,25(OH)_2D_3 + cerulenin (24 h): 1.87±0.10-fold, p>0.05]. Triacsin C alone had no evident effect on FAS expression (1.02±0.11, p>0.05). Cerulenin alone had no significant effect on FACL3 expression (1.01±0.1, p>0.05). Since FACL3 was not regulated by 1α,25(OH)_2D_3 in AR-negative prostate cancer PC-3 cells, we examined FAS expression in this cell line and found that no regulation of FAS expression was seen by 1α,25(OH)_2D_3 in PC-3 cells.

To further investigate the mechanism of FACL3-mediated downregulation of FAS expression by 1α,25(OH)_2D_3, myristic acid, one of the preferential substrates for FACL3, was applied to treat LNCaP cells in the presence of 1α,25(OH)_2D_3 and FACL3 activity inhibitor, triacsin C. FAS expression was measured using real-time quantitative PCR. Myristic acid had no significant effect on 1α,25(OH)_2D_3 downregulation of FAS at 24 h (1α,25(OH)_2D_3: 0.54±0.16-fold, 1α,25(OH)_2D_3 + myristic acid: 0.7±0.03-fold, p>0.05), but enhanced the downregulation of FAS expression by 1α,25(OH)_2D_3 at 48 h in LNCaP cells (1α,25(OH)_2D_3: 0.59±0.09-fold, 1α,25(OH)_2D_3 + myristic acid: 0.37±0.04-fold, p<0.05). Triacsin C blocked this effect of myristic acid (1α,25(OH)_2D_3 + myristic acid: 0.37±0.04-fold, 1α,25(OH)_2D_3 + myristic acid + triacsin C: 1.53±0.31-fold, p<0.05). Myristic acid alone showed no significant effect on FAS expression (24 h: 0.88±0.05-fold, p>0.05. 48 h: 0.85±0.06-fold, p>0.05).
DISCUSSION

1. cDNA Microarray Analysis of Vitamin D₃-regulated Genes

Antiproliferative actions of 1α,25(OH)₂D₃ are exerted through VDR-mediated expression of target genes. In the present study we used cDNA microarray to explore 1α,25(OH)₂D₃-responsive genes in human prostate cancer LNCaP cells, which have been shown to be sensitive to 1α,25(OH)₂D₃. At the beginning of the study, a chip containing 3,000 genes was used to screen 1α,25(OH)₂D₃-regulated genes and 24 genes were found to be regulated by 10 nM 1α,25(OH)₂D₃ 24 h after the treatments (≥1.8-fold difference in gene expression between treatment and no treatment considered to be upregulation, ≤0.58-fold difference to be downregulation). The results were obtained from three independent microarrays and represented the first expression profile of vitamin D₃-regulated genes in prostate cancer cells revealed by cDNA microarray. To further investigate 1α,25(OH)₂D₃-regulated genes, a different chip containing more than 12,000 genes was used. In this chip, two independent microarray results showed that up to four hundred genes were regulated by 10 nM 1α,25(OH)₂D₃ in LNCaP cells 24 h after the treatments (≥1.8-fold considered to be upregulation, ≤0.55-fold considered to be downregulation) including the genes revealed from the previous chip containing 3,000 genes and the genes already known to be 1α,25(OH)₂D₃-regulated such as prostate specific antigen (PSA) (Hsieh et al. 1996), androgen receptor (AR) (Zhao et al. 1999), insulin-like growth factor binding protein 3 (IGFBP-3) (Boyle et al. 2001), fibroblast growth factor 7 (keratinocyte growth factor) (KGF) (Aksenov et al. 2001), N-myc downstream regulated (NDRG1), hydroxyprostaglandin dehydrogenase 15 (15-PGDH), CD24 antigen (small cell lung carcinoma cluster 4 antigen), prostate differentiation factor (PLAB) and claudin 4 (Krishnan et al. 2004). Cyclin-dependent kinase inhibitor 1A (p21/Cip1), a 1α,25(OH)₂D₃ target gene associated with 1α,25(OH)₂D₃-induced cell cycle arrest (Moffatt et al. 2001), was slightly upregulated (1.67-fold on average, below the threshold of 1.8-fold used). 25-Hydroxyvitamin D(3)-24-hydroxylase, a more sensitive 1α,25(OH)₂D₃ target gene (Peehl et al. 2003), and cyclin-dependent kinase inhibitor p27, which is 1α,25(OH)₂D₃-regulated and associated with cell cycle arrest (Huang et al. 2004), were not in the chip. Among the new 1α,25(OH)₂D₃-regulated genes revealed in the current microarray, long-chain fatty-acid-CoA ligase 3 (FACL3) was shown to be the most upregulated gene in LNCaP cells.

2. Vitamin D₃ Regulation of FACL3 Expression

Human long-chain fatty-acid-CoA ligase 3 (FACL3) is a fatty acid metabolic enzyme. It catalytically converts long-chain fatty acids, preferentially myristic acid, arachidonic acid (AA) and eicosapentaenoic acid (EPA) to the corresponding acyl-CoA (Fujino et al. 1997). Our cDNA microarrays revealed that FACL3 expression was mostly upregulated by 10 nM 1α,25(OH)₂D₃ in prostate cancer LNCaP cells 24 h after the treatments. The upregulation of FACL3 expression was further confirmed by real-time quantitative PCR analysis and shown to be time dependent, the maximal upregulation being more than 3.5-fold 96 h after the response to 10 nM 1α,25(OH)₂D₃. This is the first study to indicate that FACL3 is a new vitamin D₃ target gene.
Cycloheximide, a protein synthesis inhibitor, was able to block 1α,25(OH)₂D₃ upregulation of FACL3 expression, suggesting that FACL3 is not a primary target gene of 1α,25(OH)₂D₃. This is consistent with no vitamin D responsive element (VDRE) found in the promoter region of FACL3 gene (Minekura et al. 2001). In the absence of androgen (DCC-serum), 1α,25(OH)₂D₃ failed to regulate FACL3 expression. DHT completely restored the upregulatory effect of 1α,25(OH)₂D₃ on FACL3 expression in LNCaP cells grown in DCC-serum medium. This indicates that 1α,25(OH)₂D₃ upregulation of FACL3 expression is androgen-dependent. Casodex, an antagonist of androgen which blocks androgen actions by binding to the androgen receptor (AR), abolished 1α,25(OH)₂D₃ upregulation of FACL3 expression in the presence of androgen, suggesting that AR is essential for the upregulation of FACL3 expression by 1α,25(OH)₂D₃. In addition, no regulatory effects of 1α,25(OH)₂D₃ on FACL3 expression found in AR-negative human prostate cancer PC-3 and DU145 cells also support this finding. Therefore, the upregulation of FACL3 expression by 1α,25(OH)₂D₃ is mediated by both androgen and AR in human prostate cancer LNCaP cells.

FACL3 expression has been also shown to be upregulated by androgen DHT in LNCaP cells, consistent with a previous report (DePrimo et al. 2002). The regulatory effect of DHT on FACL3 expression was blocked by Casodex, indicating that the upregulation of FACL3 expression by DHT is mediated by AR. This is in line with the fact that the actions of androgen are mediated by AR. Abrogation of DHT effect on FACL3 expression by cycloheximide suggests that the regulatory effect of DHT on FACL3 expression is not direct. This is consistent with the finding that androgen response element (ARE) is not present in the promoter region of FACL3 gene (Minekura et al. 2001).

AR expression is indirectly upregulated by 1α,25(OH)₂D₃ in LNCaP cells (Zhao et al. 1999). We analysed AR protein expression by Western blotting using both cytosolic protein and nuclear protein extracts from LNCaP cells grown in androgen-depleted DCC-serum medium. A similar pattern of AR expression was seen in both protein extracts. 1α,25(OH)₂D₃ (10 nM) had no evident effect on the expression of AR protein in the absence of androgen in LNCaP cells 24 h after the treatments. This is consistent with an earlier report (Zhao et al. 1999). In the presence of androgen, DHT (10 nM), 1α,25(OH)₂D₃ increased AR protein expression compared with DHT alone. This indicates that the effect of 1α,25(OH)₂D₃ on FACL3 expression is parallel to its effect on AR protein expression in the presence of 10 nM DHT and suggests that increased AR protein expression is associated with the upregulation of FACL3 expression by 1α,25(OH)₂D₃ in LNCaP cells. Casodex had no effect on 1α,25(OH)₂D₃ upregulation of AR protein expression, suggesting that the blocking effect of Casodex on the upregulation of FACL3 expression may be due to interference with the interaction between androgen and AR.

The activity of FACL3 was increased by 1α,25(OH)₂D₃ in LNCaP cells. Consistent with 1α,25(OH)₂D₃ upregulation of FACL3 mRNA expression, the increase in the FACL3 activity by 1α,25(OH)₂D₃ reached a peak at 96 h (around 4-fold) and then decreased at 144 h. Since myristic acid, a preferential substrate for FACL3, was used to measure the activity of FACL3 in the presence of 1α,25(OH)₂D₃, it is possible that myristic acid is utilized by other isoforms of FACL3 and may have an impact on the
1α,25(OH)₂D₃ induction of FACL3 activity. 1α,25(OH)₂D₃ had no evident effect on the expression of isoforms FACL1(FACL2), FACL4 and FACL6 respectively. FACL5 expression was shown to be downregulated about 2-fold by 1α,25(OH)₂D₃ at 24 and 48 h, and no regulation was seen at other times. In addition, FACL3 is the major isoform of FACL expressed in LNCaP cells (over 68% in total FACL expression). The expression of FACL3 was over 2-fold more than that of FACL1(FACL2) and over a hundred thousand-fold more than that of FACL4, FACL5 or FACL6. Therefore, the isoforms FACL1(FACL2), FACL4, FACL5 and FACL6 make no significant contribution to the increased activity of FACL3 by 1α,25(OH)₂D₃ in LNCaP cells.

FACL3 protein expression was also upregulated by 1α,25(OH)₂D₃ in LNCaP cells, consistent with 1α,25(OH)₂D₃ upregulation of FACL3 mRNA expression. Furthermore, the data suggest that the increase in the activity of FACL3 is due to the increase in FACL3 protein expression by 1α,25(OH)₂D₃. In addition, the FACL3 protein was detected as a single band with a molecular weight of 75 kDa by Western blotting analysis in three human prostate cancer LNCaP, PC-3 and DU145 cell lines. This is in line with a report indicating that FACL3 is expressed as a 75 kDa protein in human hepatocyte cells (Fujimoto et al. 2004). This suggests that human FACL3 protein is expressed as a single isoform, not like the mouse FACL3 protein, which is expressed as two different isoforms (79 and 80 kDa) due to alternative translation initiation of mRNA (Fujino et al. 1997).

Vitamin D₃ is potential as a therapeutic intervention for cancer including prostate cancer, but its application in clinical trials has been limited by its induction of hypercalcemia at the pharmacological doses. Vitamin D₃ analogs seem to be more promising in clinical application with reduced calcemic activity. We studied the regulation of FACL3 expression by EB1089 and CB1093, two synthetic analogs of 1α,25(OH)₂D₃, in human prostate cancer cells. Both EB1089 and CB1093 stimulated FACL3 mRNA expression significantly in AR-positive LNCaP cells, consistent with 1α,25(OH)₂D₃ upregulation of FACL3 mRNA expression. EB 1089 showed a capacity similar to that of 1α,25(OH)₂D₃ in the upregulation of FACL3 mRNA expression. CB1093 was more effective than 1α,25(OH)₂D₃ in the stimulation of FACL3 mRNA expression. In parallel with the upregulation of FACL3 mRNA expression, EB1089 and CB1093 both increased the expression of FACL3 protein in LNCaP cells. EB1089 and CB1093 had effects similar to that of 1α,25(OH)₂D₃ on FACL3 protein expression. No regulation of FACL3 expression by EB1089 or CB1093 found in AR-negative human prostate cancer PC-3 and DU145 cells suggests that the regulatory effects of EB1089 and CB1093 on FACL3 expression may also be dependent on androgen/AR.

Triacsin C, an inhibitor of FACL3 activity, is able to block fatty acid-induced cell apoptosis (Shimabukuro et al. 1998; Mu et al. 2001). In the present study, triacsin C was shown to significantly reverse 1α,25(OH)₂D₃-induced inhibition of LNCaP cell growth, indicating that FACL3 is involved in the growth inhibitory effects of 1α,25(OH)₂D₃ on LNCaP cells. The strongest effect of triacsin C in blocking 1α,25(OH)₂D₃ inhibition of LNCaP cell growth was seen on day 4 (96 h), on which the highest increase in the enzyme activity of FACL3 by 1α,25(OH)₂D₃ was seen. This suggests that increased enzyme activity of FACL3 is directly associated with the growth inhibitory actions of 1α,25(OH)₂D₃ in LNCaP cells.
Some studies show that triacsin C is not specific for the inhibition of FACL3 activity. This implies that the activity of other isozymes may be competitively inhibited by triacsin C, and this inhibition may also have some effects on $1\alpha,25(\text{OH})_2\text{D}_3$ actions, especially if the expression of these isozymes is affected by $1\alpha,25(\text{OH})_2\text{D}_3$. FACL3 is a dominant isozyme expressed in LNCaP cells, its expression being over two-fold higher than that of FACL1(FACL2) and over a hundred thousand-fold higher than that of FACL4, FAACL5 or FACL6. In addition, $1\alpha,25(\text{OH})_2\text{D}_3$ had no effect on the expression of isozymes FACL1(FACL2), FAACL4 and FACL6 in LNCaP cells. This suggests that inhibition of the activity of these isoforms by triacsin C could have no significant effect on $1\alpha,25(\text{OH})_2\text{D}_3$ inhibition of cell growth. FACL5 was shown to be downregulated about 2-fold by $1\alpha,25(\text{OH})_2\text{D}_3$ at 24 and 48 h, but no regulation of FACL5 was seen at later times such as 96 and 144 h, at which the attenuation of $1\alpha,25(\text{OH})_2\text{D}_3$-induced cell growth inhibition by triacsin C was seen. On the other hand, one study indicates that the activity of FACL5 is not inhibited by triacsin C (Kim et al. 2001). Therefore, the blocking effect of triacsin C on the inhibition of LNCaP cell growth by $1\alpha,25(\text{OH})_2\text{D}_3$ is a consequence of the triacsin C inhibition of the increased FACL3 activity by $1\alpha,25(\text{OH})_2\text{D}_3$ in LNCaP cells.

Human prostate cancer LNCaP cells are more differentiated and less aggressive of the cell lines used. PC-3 and DU145 are less differentiated and more aggressive human prostate cancer cells (Campbell et al. 1998 and 1999). FACL3 expression has been found to be constitutively low in PC-3 and DU145 cells compared to LNCaP cells. This suggests that decreased FACL3 expression may be one event occurring during the development of prostate cancer into a more malignant phenotype.

3. Vitamin D$_3$ Regulation of FAS Expression

Fatty acid synthase (FAS) is overexpressed in many cancers, including prostate cancer. In prostate cancer, FAS is strongly associated with the initiation, progression and metastatic development of the disease (Epstein et al. 1995; Shurbaji et al. 1996; Swinnen et al. 2000 and 2002). Inhibition of FAS activity or breakdown of FAS mRNA results in significant growth inhibition of prostate cancer cells (Furuya et al. 1997; De Schrijver et al. 2003). Our cDNA microarray analysis of $1\alpha,25(\text{OH})_2\text{D}_3$-responsive genes revealed that FAS expression was downregulated by $1\alpha,25(\text{OH})_2\text{D}_3$ in prostate cancer LNCaP cells and the regulation was further confirmed by real-time quantitative PCR analysis. Cycloheximide, a protein synthesis inhibitor, blocked $1\alpha,25(\text{OH})_2\text{D}_3$-induced repression of FAS expression, suggesting that the downregulation of FAS expression by $1\alpha,25(\text{OH})_2\text{D}_3$ is not direct and may be mediated by an intermediate protein. Androgen withdrawal or antiandrogen, Casodex, abolished the $1\alpha,25(\text{OH})_2\text{D}_3$ downregulation of FAS expression. This suggests that the suppression of FAS expression by $1\alpha,25(\text{OH})_2\text{D}_3$ is androgen-dependent in LNCaP cells. Inhibition of FAS activity by cerulenin, an enzyme activity inhibitor of FAS, results in a strong growth inhibition of LNCaP cells, suggesting that repression of FAS expression is one event associated with $1\alpha,25(\text{OH})_2\text{D}_3$-induced growth inhibition of LNCaP cells.

Since androgen has been shown to upregulate FAS expression in LNCaP cells (Swinnen et al. 1997), this may explain the less inhibitory effect of $1\alpha,25(\text{OH})_2\text{D}_3$ on FAS expression seen at later time points (24 and 48 h). $1\alpha,25(\text{OH})_2\text{D}_3$ increases AR
expression in LNCaP cells (Zhao et al. 1999), and the increased AR expression may enhance the upregulatory effect of androgen present in the serum of culture medium on FAS expression, resulting in attenuation of 1α,25(OH)₂D₃ repression of FAS expression in LNCaP cells.

4. FACL3-mediated Downregulation of FAS Expression

FAS catalytically de novo synthesizes long-chain fatty acids and FACL3, a downstream enzyme of FAS, converts long chain fatty acids into long chain fatty acyl-CoAs. Some studies indicate that fatty acid synthesis is inhibited by long-chain fatty acyl-CoAs (McGee and Spector 1975). This led us to investigate the mechanisms by which 1α,25(OH)₂D₃ regulates the expression of FAS and FACL3 in LNCaP cells.

Inhibition of FACL3 activity by triacsin C, an activity inhibitor of FACL3, resulted in the complete abolishment of 1α,25(OH)₂D₃ repression of FAS mRNA expression, whereas inhibition of FAS activity by cerulenin, an inhibitor of FAS activity, had no significant effect on 1α,25(OH)₂D₃ upregulation of FACL3 mRNA expression, indicating that downregulation of FAS expression by 1α,25(OH)₂D₃ is mediated by FACL3 in LNCaP cells. This is consistent with the findings that 1α,25(OH)₂D₃ downregulation of FAS expression is indirect. In addition, in AR-negative PC-3 cells, 1α,25(OH)₂D₃ had no effect on FAS expression, consistent with the lack of regulation of FACL3 expression by 1α,25(OH)₂D₃ in this cell line.

1α,25(OH)₂D₃ increased the activity of FACL3. An increase in FACL3 activity could lead to an increase in the synthesis of long chain fatty acyl-CoAs. Myristic acid, one of the substrates preferential for FACL3, enhanced 1α,25(OH)₂D₃ repression of FAS expression and this effect was abolished by inhibition of FACL3 activity, indicating that the repressive effect of myristic acid on FAS expression in the presence of 1α,25(OH)₂D₃ is due to the formation of myristic acid-CoA (myristoyl-CoA) in LNCaP cells. The repression of FAS expression by myristic acid was only seen in the presence of 1α,25(OH)₂D₃, suggesting that the myristic acid-CoA, which is responsible for the repression of FAS expression, is synthesized by FACL3 during its upregulation by 1α,25(OH)₂D₃. Therefore, FACL3-mediated 1α,25(OH)₂D₃ suppression of FAS expression is through the mechanism of feedback inhibition by long-chain fatty acyl-CoAs in LNCaP cells. Myristic acid had no significant effect on the 1α,25(OH)₂D₃ repression of FAS expression at 24 h, consistent with the finding that the formation of myristoyl-CoA is not significant at 24 h in the presence of 1α,25(OH)₂D₃ as indicated by the measurement of FACL3 activity using 14C-labelled myristic acid. The data suggest that the downregulation of FAS expression by 1α,25(OH)₂D₃ is a consequence of feedback inhibition of FAS expression by long-chain fatty acyl-CoAs formed during 1α,25(OH)₂D₃ upregulation of FACL3 expression in LNCaP cells.

Inhibition of fatty acid synthesis by long chain fatty acyl-CoAs is evident. Some studies suggest that long chain fatty acyl-CoAs inhibit fatty acid synthesis by feedback inhibition of ACC (Bortz and Lynen 1963; Kim 1997; Faergeman and Knudsen 1997; Munday 2002), the first and rate-limit enzyme in fatty acid synthesis pathway. Based on the present study, we suggest that long chain fatty acyl-CoAs formed by FACL3 during its upregulation by 1α,25(OH)₂D₃ repress FAS mRNA
expression, the second and pivotal enzyme in the fatty acid synthesis pathway. Therefore, inhibition of FAS expression by long chain fatty acyl-CoAs may represent a different mechanism of long-term regulation of de novo fatty acid synthesis. Studies also indicate that inhibition of ACC by long chain fatty acyl-CoA is due to a decrease in the amount of enzyme. In addition, the changes in the amount of ACC caused by alterations in dietary fat are accompanied by similar changes in the amount of FAS (Majerus and Kilburn 1969). These findings imply that feedback inhibition of ACC by long chain fatty acyl-CoAs may be due to a repression of ACC mRNA expression and a link between inhibition of FAS and ACC by long chain fatty acyl-CoAs. Fatty acyl-CoA response elements (FARE) has been identified in the promoter region of ACC gene (Kim 1997) and a direct inhibition of ACC by long chain fatty acyl-CoAs has been reported (Ogiwara et al. 1978). It may be possible that the inhibition of ACC expression by long chain fatty acyl-CoAs could affect FAS expression and result in the decrease in FAS mRNA. The relationship between inhibition of ACC and FAS expression by long chain fatty acyl-CoAs remains to be investigated.

In summary, two fatty acid metabolic enzymes FAS and FACL3 are 1α,25(OH)2D3-regulated in human prostate cancer LNCaP cells. 1α,25(OH)2D3 upregulates FACL3 expression and this regulation appears to be mediated by androgen/AR signalling pathway. Increased FACL3 expression by 1α,25(OH)2D3 results in increased synthesis of long-chain fatty acyl-CoAs, which repress FAS expression by feedback inhibition. The repression of FAS expression, probably due to attenuation of de novo fatty acid synthesis, results in cancer cell growth inhibition. The data suggest that fatty acid metabolism plays a role in the antiproliferative effects of 1α,25(OH)2D3 in prostate cancer cells. The modulation of fatty acid metabolism by 1α,25(OH)2D3 through regulation of FACL3 and FAS expression revealed in the present study may be one mechanism by which 1α,25(OH)2D3 exerts its growth inhibitory actions in an androgen-dependent manner.
CONCLUSIONS

The present study identified two 1α,25(OH)_{2}D_{3}-regulated genes, FAS and FACL3. FAS is a pivotal de novo fatty acid synthesis enzyme and has been found to be overexpressed in and associated with many cancers, including prostate cancer. FACL3 is a downstream enzyme of FAS in the fatty acid metabolism pathway. It converts long-chain fatty acids, the products of FAS, into long-chain fatty acyl-CoAs for further utilization such as energy production in mitochondria.

FAS expression is downregulated by 1α,25(OH)_{2}D_{3} in human prostate cancer LNCaP cells. The regulation is indirect and androgen-dependent. The inhibition of FAS enzyme activity by its inhibitor cerulenin results in the strong suppression of LNCaP cell growth, suggesting that 1α,25(OH)_{2}D_{3}-induced repression of FAS expression is involved in its growth inhibitory effects on LNCaP cells.

FACL3 expression and activity are increased by 1α,25(OH)_{2}D_{3} in LNCaP cells. Upregulation of FACL3 expression by 1α,25(OH)_{2}D_{3} may be mediated by androgen/AR signalling. The inhibition of FACL3 enzyme activity by inhibitor triacsin C significantly attenuates 1α,25(OH)_{2}D_{3}-induced growth inhibition of LNCaP cells, indicating that upregulation of FACL3 expression by 1α,25(OH)_{2}D_{3} is one component associated with 1α,25(OH)_{2}D_{3}-induced repression of LNCaP cell growth. In addition, FACL3 expression is constitutively low in the more malignant human prostate cancer PC-3 and DU145 cells compared to the less malignant LNCaP cells.

Downregulation of FAS expression by 1α,25(OH)_{2}D_{3} is mediated by FACL3 through feedback inhibition by long-chain fatty acyl-CoAs, which are synthesized by FACL3 during its upregulation by 1α,25(OH)_{2}D_{3} in LNCaP cells. The FACL3-mediated 1α,25(OH)_{2}D_{3} repression of FAS expression contributes to the growth inhibition of LNCaP cells by 1α,25(OH)_{2}D_{3} (Figure 4).

![Figure 4](image_url). Schematic illustration of FACL3-mediated 1α,25(OH)_{2}D_{3} suppression of FAS expression and cell growth inhibition in human prostate cancer LNCaP cells.
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Shengjun Qiao
REFERENCES


cancer and a PI 3-kinase inhibitor synergizes with FAS siRNA to induce apoptosis. Oncogene 24(34):5389-5395.


ORIGINAL COMMUNICATIONS
Inhibition of fatty acid synthase expression by 1α,25-dihydroxyvitamin D₃ in prostate cancer cells

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Abstract

1α,25-Dihydroxyvitamin D₃ (1α,25(OH)₂D₃) and its derivatives are a potential treatment of human prostate cancer. The antiproliferative action of 1α,25(OH)₂D₃ is mainly exerted through nuclear vitamin D receptor (VDR)-mediated control of target gene transcription. To explore the target genes which are regulated by 1α,25(OH)₂D₃ in human prostate cancer LNCaP cells, cDNA microarray was performed by using a chip that contains 3000 gene probes. The results showed that 24 genes were regulated by 1α,25(OH)₂D₃. Five of them encode proteins which belong to metabolic enzymes and fatty acid biosynthesis. Fatty acid synthase (FAS) was found to be down-regulated by 1α,25(OH)₂D₃, and the regulation was confirmed by real-time quantitative RT-PCR analysis. Inhibition of FAS expression by 1α,25(OH)₂D₃ in LNCaP cells was more than 50% at 6 h. Inhibitory effect of 1α,25(OH)₂D₃ on FAS expression was completely blocked in the presence of protein synthesis inhibitor cycloheximide, indicating that the down-regulation of FAS gene expression by 1α,25(OH)₂D₃ was indirect in LNCaP cells. An inhibition of FAS activity by cerulenin resulted in a strong inhibition of LNCaP cell proliferation. The inhibition of FAS expression and cell proliferation by 1α,25(OH)₂D₃ seemed to be androgen-dependent, since antiandrogen, casodex and DCC-treatment of serum blocked the vitamin D action. The findings suggest that FAS is involved in the antiproliferative effect of 1α,25(OH)₂D₃ in presence of androgens on prostate cancer LNCaP cells.

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Keywords: Fatty acid synthase; Gene regulation; 1α,25-Dihydroxyvitamin D₃; Antiandrogen; Cell proliferation

1. Introduction

The protective function of vitamin D against prostate cancer is evident [1–3]. This function is thought to be exerted through vitamin D receptor (VDR)-mediated pathway controlling the target gene expression, resulting in G1/S-phase arrest in cell cycle, apoptosis and cell differentiation [2]. The knowledge of the vitamin D-regulated genes is necessary for understanding of prostate cancer development. LNCaP, a well established human prostate cancer cell line, is commonly employed to study the effects of vitamin D and its analogs on prostate cancer, since this cell line is sensitive to vitamin D.

Fatty acid synthase (FAS/FASN), a key metabolic enzyme involved in the de novo biosynthesis of fatty acids, has been found to be a potential anticancer target [4–6]. FAS expression in human normal tissues is under a strict hormonal control [7–9]. In contrast, FAS expression appears to be independent of hormonal regulation in many cancers, because its expression is commonly up-regulated in these cancers despite high levels of ambient fatty acids [10]. Epithelial cells in human endometrium, one of the fastest growing human tissues, express abundant FAS during their proliferation [9,11], and the regions of endometrial carcinomas with the highest FAS expression are those regions with highest cell proliferation [12] suggesting a correlation between FAS expression and cell proliferation. FAS was found to be overexpressed in breast, prostate, ovary, endometrium and colon cancers [13–18]. In several of these cancers, elevated FAS expression occurs early in cancer progression and is associated with poor prognosis [19,20]. In prostate cancer, fatty acid synthesis pathway was found to be selectively activated in a subset of prostate cancers, and FAS expression elevated markedly not only at mRNA level [21] but also at protein level [14,16]. Immunohistochemical studies showed that the overexpression of FAS tended to increase from low grade to high grade prostatic epithelial neoplasia (PIN), and to invasive carcinoma, whereas FAS expression was negative in normal or benign hyperplastic glandular structures. These
findings indicate that overexpression of FAS is an early and common event in the development of prostate cancer, and suggest that antineoplastic therapy based on FAS inhibition may be a chemoprevention or a curative treatment for prostate cancer [22].

FAS has a specific tissue distribution, a low expression in normal tissues and a high expression in cancers, and it has a unique β-ketoacyl synthase activity. These characteristics make it an attractive target for cancer therapy as FAS inhibitors [10], since the inhibitor-induced cytotoxicity could be limited to cancer cells without significant effect on normal cells. Inhibition of FAS expression by its inhibitor cerulenin resulted in both breast and prostate cancer cell death [23,24]. In this study, we found for the first time that FAS expression was down-regulated by 1α,25(OH)2D3 in prostate cancer LNCaP cells.

2. Materials and methods

2.1. Reagents and buffers

Cy3-dUTP (25 nM), Cy5-dUTP (25 nM), dNTP (dATP, dCTP, dGTP, dTTP) and Oligo(dT)18 (25 nM) primer (1 mg/ml). Superscript II (200 U/µl) and Yeast tRNA (10 mg/ml) were purchased from Gibco BRL (Grand Island, USA). Ten times Dg blocking buffer was from Roche Diagnostics (Mannheim, Germany). Polyadenylic acid (PolyA) and cerulenin were from Sigma (Missouri, USA). rNase inhibitor (40 U/µl) was purchased from Promega (Madison, USA). TRizol Reagent was from Invitrogen (Carlsbad, USA). High Capacity DNA Archive kit and SYBR Green PCR Master Mix kit were purchased from Applied Biosystems (Foster City, USA). 1α,25(OH)2D3 was obtained from Leo Pharmaceuticals (Ballerup, Denmark). FBS was from Gibco BRL (Life Technology, Paisley, Scotland). RPMI-1640 medium was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Hydroyxyflutamide was from Schering-Plough Co. (Helsinki, Finland). Casodex was obtained from AstraZeneca (London, UK).

2.2. Cell culture, treatment and RNA isolation

Human prostate cancer LNCaP cells (ATCC, USA) were maintained in RPMI-1640 medium supplemented with 10% FBS, 3 mM l-glutamine, 100 µg/ml streptomycin and 100 U/ml penicillin at 37 °C in humidified atmosphere of 5% CO2. LNCaP cells grown to 70% confluence were treated with 1α,25(OH)2D3 and other reagents at indicated concentrations. For vitamin D3, cholecalciferol, casodex and hydroxyflutamide treatments, 0.1% of ethanol was used as control. DMSO (0.1%) was applied as control for cerulenin treatment. RNA was isolated with TRIzoL Reagent following the instruction of manufacturer. RNA concentrations were measured at 260 nm with GeneQuant II (Pharmacia Biotech).

2.3. cDNA microarray and data analysis

cDNA microarray was performed according to manufacturer’s instruction. In brief, 20 µg of RNA sample from 1α,25(OH)2D3-treated cells was labelled with Cy3-dUTP (25 nM) by reverse transcription under Oligo(dT)18 primer direction, in parallel, the equal amount of RNA sample from untreated cells was labelled with Cy5-dUTP (25 nM) as control. The RNA labelling reactions were done at 42 °C for 80 min. After the labelling reaction, RNA was removed from synthesized cDNA by addition of small amount of NaOH solution (1 M) followed by neutralisation with Tris-HCl (1 M, pH 7.5). Cy3-cDNA and Cy5-cDNA were combined together in one Microcon Column (Millipore Corporation, Bedford, USA) and washed four times in TE buffer (pH 7.4) by centrifugation. In the final washing step, Cot-1 DNA, PolyA and Yeast tRNA were added to washing buffer and centrifuged to make the final volume of labelled cDNA mixture less than 10 µl. For hybridization, Human2-1 Glass chip containing 3000 cDNA probes (Turku Centre for Biotechnology, Turku, Finland) was pretreated with 0.1% SDS, sterile H2O and 95% ethanol, respectively, and air-dried. The labelled cDNA mixture was hybridized with the chip in a humid chamber at 65 °C over night. After hybridization, the chip was washed four times with slight shaking, and spaced dry by centrifugation. The hybridized chip was scanned in ScanArray 4000 Series (Packard BioScience), the hybridization images were analyzed using QuantArray Microarray Analysis Software (Packard BioScience), and the data were finally normalized to median by using Excel Date Normalization Macro.

2.4. Real-time quantitative RT-PCR analysis

Real-time quantitative RT-PCR was performed in ABI PRISM 7000 Detection System (Applied Biosystems, USA). Reverse transcription (RT) and real-time PCR were done separately with High Capacity Archive kit (Applied Biosystems, USA) and SYBR Green PCR Master Mix kit (Applied Biosystems, USA) following the instruction of manufacturer. Briefly, for reverse transcription, 10 µg of RNA dissolved in 50 µl PCR-compatible buffer was combined with 50 µl of 2× RT Master Mix (10 µl of 10× reverse transcription buffer, 4 µl of 25× dNTPs, 10 µl of 10× random primers, 5 µl of multiscribe reverse transcriptase (50 U/µl) and 21 µl of nuclease-free H2O). RT was performed at 25 °C for 10 min followed by 37 °C for 120 min. For real-time PCR, 20 ng of cDNA was combined with primers and 2× SYBR Green PCR Master Mix to the final volume of 50 µl per reaction. Real-time PCR was done at 95 °C for 10 min followed by 40 cycles at 95 °C, 15 s and 60 °C, 1 min. The PCR product was examined by dissociation
curve and agarose gel electrophoresis to ensure that band was visible only at the expected molecular weight. The data was analyzed by ABI Prism 7000 SDS Software and normalized to housekeeping gene porphobilinogen deaminase (PBGD) to verify uniform RNA loading in experiments. The final result was expressed as N-fold difference in gene expression between treated sample and control sample: \[ \text{N-fold} = \frac{\text{gene (treatment)/PBGD (treatment)}}{\text{gene (control)/PBGD (control)}} \]. The values used for calculation in the formula were obtained from corresponding standard curve. The standard curves were made by series of dilution of untreated samples. All primers used were designed by using compatible software Primer Express for ABI PRISM 7000 Detection System (Applied Biosystems). Primers for human FAS cDNA were as follows: forward primer 5′-AACTCCAAGGACACAGTCACA T-3′ (in exon 12), reverse primer 5′-CAGCTGCTCCAGCAACTCAA-3′ (in exon 13), PCR product was 65 bp in length. Primers for human PBGD cDNA were as follows: forward primer 5′-CCACACACAGCCTACTTTCCA-3′ (in exon 4), reverse primer 5′-TTTCTTCCGCCGTTGCA-3′ (in exon 5), PCR product was 69 bp.

2.5. Cell growth assay

LNCaP cells were grown in 96-well plates and treated with hormones and other reagents at indicated time. After the treatment, cell growth was assayed by using crystal violet staining. In brief, cells were fixed by 11% glutaraldehyde solution in plates for 15 min with shaking at 500 rpm, and air-dried after washing. Crystal violet solution (0.1%) was added to stain fixed cells for 20 min by shaking, excess dye was removed by extensive washing with deionized water, the plates were air-dried before addition of 10% acetic acid to withdraw cell-bound dye. The optical density of dye extracts was measured directly in plates at the wavelength of 590 nm by using the Microplate Reader (WALLAC, VICTOR 1420 MULTILABEL COUNTER, Turku, Finland).

2.6. Statistical method

Data collected in the study was analyzed by a paired two-tailed t-test. Each result was obtained from at least three independent experiments. \( P < 0.05 \) was considered as significant, and \( P < 0.001 \) as highly significant.

3. Results

3.1. 1α,25(OH)2D3-regulated genes revealed by cDNA microarray in LNCaP cells

To investigate the expression profile of the genes regulated by 1α,25(OH)2D3 in prostate cancer LNCaP cells, cDNA microarray was performed by using the chip containing 2500 known genes and 500 ESTs. Three independent cDNA microarrays were done with RNA samples from LNCaP cells treated and untreated by 10nM 1α,25(OH)2D3 for 24h. The microarray results showed that 24 genes expressed differentially between 1α,25(OH)2D3 treated and untreated cells. The genes which were up- or down-regulated more than 1.8-fold (including 1.8-fold) or less than 0.58-fold (including 0.58-fold) were considered. Of the 24 genes, 19 were down-regulated by 1α,25(OH)2D3 (Table 1), and 5 were up-regulated by 1α,25(OH)2D3 (Table 2).

<table>
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<tr>
<th>Accession no.</th>
<th>Description</th>
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<tr>
<td>N29056</td>
<td>ATPase, H+ transporting lysosomal (vacuolar proton pump), α-polypeptide</td>
<td>0.49 ± 0.05</td>
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<tr>
<td>N69204</td>
<td>Chromosome segregation 1 (yeast homology)-like</td>
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<tr>
<td>T65788</td>
<td>EST</td>
<td>0.40 ± 0.11</td>
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<tr>
<td>T50788</td>
<td>Estrogen-regulated translation initiation factor 4E binding protein 2</td>
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<tr>
<td>H53032</td>
<td>Fatty acid synthase</td>
<td>0.41 ± 0.06</td>
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<tr>
<td>N53172</td>
<td>G protein-coupled receptor</td>
<td>0.56 ± 0.23</td>
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<td>H69198</td>
<td>Human clone 23933 mRNA sequence</td>
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<td>K-o-1</td>
<td>0.41 ± 0.04</td>
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<td>KIAA0217 protein</td>
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<td>R93829</td>
<td>Nucleosome assembly protein 1-like-1</td>
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<td>AA5996859</td>
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<td>AA5998487</td>
<td>Phosphoribosylpyrimidine formyltransferase</td>
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<td>Quinone oxidoreductase homology</td>
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<td>H75708</td>
<td>RNA binding protein 2</td>
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<td>AA281635</td>
<td>Suppression of tumorigenicity 16 (melanoma differentiation)</td>
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<td>B00047</td>
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<td>SET translocation (myeloid leukaemia-associated)</td>
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<td>AA599116</td>
<td>Small nuclear ribonucleoprotein polypeptides B and B1</td>
<td>0.46 ± 0.12</td>
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<td>AA490213</td>
<td>Transducer of ERBB2</td>
<td>0.51 ± 0.09</td>
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Table 2

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<td>H75862</td>
<td>KIAA0670 protein/acinus</td>
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<tr>
<td>H65281</td>
<td>ESTs moderately similar to p76 (H. sapiens)</td>
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<td>W86776</td>
<td>Histidine ammonia-lyase</td>
<td>2.23 ± 0.15</td>
</tr>
<tr>
<td>AA478553</td>
<td>Dopachrome tautomerase</td>
<td>1.93 ± 0.35</td>
</tr>
<tr>
<td>AA479093</td>
<td>Inositol 1,4,5 triphosphate receptor</td>
<td>1.84 ± 0.65</td>
</tr>
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3.2. Inhibition of FAS expression by 1α,25(OH)2D3 in LNCaP cells

On the basis of cDNA microarray result, the FAS expression was studied further by using real-time quantitative RT-PCR. RT-PCR results showed that FAS expression was significantly inhibited by 10 nM 1α,25(OH)2D3 at 24 h after the treatment (0.60 ± 0.02-fold in mRNA expression). To study the time course of the FAS expression, LNCaP cells were treated for 6, 24 and 48 h. The results showed...
that the strongest inhibition of FAS expression by 10 nM 1α,25(OH)₂D₃ was seen at 6 h (P = 0.00076). A statistically significant inhibition of FAS expression was also found at 24 h (P = 0.0049) and 48 h (P = 0.0044) (Fig. 1).

To study whether the down-regulation of FAS gene expression by 1α,25(OH)₂D₃ is direct, LNCaP cells were treated with 1α,25(OH)₂D₃ in the presence of protein synthesis inhibitor cycloheximide for 6 h. The result showed that inhibition of FAS expression by 1α,25(OH)₂D₃ was completely blocked by cycloheximide in LNCaP cells (Fig. 2), suggesting that inhibition of FAS expression might be indirect. To study the effect of FAS on cell proliferation, FAS protein inhibitor cerulenin was added in the culture medium. The growth of LNCaP cells was inhibited by cerulenin, and the inhibitory effect was time-dependent (Fig. 3).

Casodex, an androgen antagonist, partially reversed growth inhibition of LNCaP cells by 1α,25(OH)₂D₃ (Fig. 4). In contrast, antiandrogen, hydroxyflutamide seemed to enhance the growth inhibition of LNCaP cells by 1α,25(OH)₂D₃ (Fig. 5). 1α,25(OH)₂D₃ failed to inhibit the FAS expression when the cells were grown in the serum treated with dextran coated charcoal (DCC) (data not shown).

4. Discussion

The result here is the first expression profile of vitamin D-regulated genes in prostate cancer cells revealed by cDNA
Fig. 5. The effect of antiandrogen hydroxyflutamide (HF) on 1α,25(OH)₂D₃-repression of prostate cancer LNCaP cell proliferation. LNCaP cells were seeded in 96-well plates and treated with 10 nM 1α,25(OH)₂D₃ in the presence or absence of 1 μM hydroxyflutamide at indicated time points. Cell proliferation was assayed by crystal violet staining. Hydroxyflutamide was not able to block the antiproliferative effect of 1α,25(OH)₂D₃ on LNCaP cells but enhanced vitamin D inhibitory effect (days 4 and 6) (*P < 0.05, **P < 0.001).

Microarray. The microarray results showed that 24 genes were regulated by 10 nM 1α,25(OH)₂D₃ in prostate cancer LNCaP cells. Nineteen of these genes were down-regulated and five were up-regulated. Of the 24 vitamin D-regulated genes, 5 encode enzymes which belong to metabolic enzymes involved in fatty acid biosynthesis, purine nucleotide biosynthesis and histidine catabolism. FAS, phosphoribosyl-glycinamide formyltransferase (GART) and stearoyl-CoA desaturase (SCD) are down-regulated by 1α,25(OH)₂D₃. Histidine ammonial-lyase (HAL) and dopachrome tautomerase are up-regulated by vitamin D. This suggests that cellular metabolic pathways may play important roles in the action of 1α,25(OH)₂D₃. FAS and GART were found to be associated with cancer development [19,20,22,25]. HAL inhibits the growth of some cancers [26]. The regulation of these metabolic enzymes by vitamin D might be associated with its anticancer ability. Other genes such as G protein-coupled receptor and nuclear mitotic apparatus protein were also found to be regulated by 1α,25(OH)₂D₃ in LNCaP cells.

FAS is highly expressed in prostate cancer and correlates with cancer development [14,16,21,22]. In our study, FAS expression was found to be down-regulated by 1α,25(OH)₂D₃ in LNCaP cells. Inhibitory effect of vitamin D on FAS expression was strongest at 6 h. Inhibition of FAS expression by 1α,25(OH)₂D₃ was completely blocked in the presence of protein synthesis inhibitor, cycloheximide, suggesting that down-regulation of FAS expression by vitamin D is not direct but might be mediated by a new protein synthesized during treatment. FAS protein inhibitor, cerulenin, was able to inhibit the proliferation of LNCaP cells suggesting that FAS is involved in the antiproliferative effect of 1α,25(OH)₂D₃ on LNCaP cells. An earlier study showed that inhibition of LNCaP cell growth by 1α,25(OH)₂D₃ was blocked by an antiandrogen casodex, and suggested that inhibition of LNCaP cell proliferation by vitamin D was dependent upon androgen action [27]. It has been demonstrated that another antiandrogen, hydroxyflutamide, acts as androgen in LNCaP cells due to a mutation in androgen receptor [28]. Similarly in our study, casodex antagonized vitamin D inhibition of LNCaP cell growth. This suggests that inhibition of FAS expression by 1α,25(OH)₂D₃ might be androgen-dependent. We also show that hydroxyflutamide is androgenic in LNCaP cells and it cannot antagonize the inhibition of LNCaP cell proliferation by 1α,25(OH)₂D₃.

Since androgen was found to up-regulate FAS expression in LNCaP cells [29], the androgens present in the serum added to the cell culture medium could affect vitamin D inhibition of FAS expression. Therefore, we treated the serum with DCC to remove steroids, and the vitamin D effect on FAS expression was abolished. This suggests that the inhibition of FAS expression by 1α,25(OH)₂D₃ is androgen-dependent. This is in line with our epidemiological result suggesting that the protective action of vitamin D against prostate cancer is androgen-dependent [3]. Androgen receptor is up-regulated by 1α,25(OH)₂D₃ in LNCaP cells [30]. This might explain why the inhibition of FAS expression by vitamin D was slightly less pronounced at 24
and 48 h than at 6 h. Increased AR may enhance the effect of the small amount of androgens in the serum on stimulation of FAS expression, resulting in less inhibition of FAS expression by 1a,25(OH)2D3 at the later time point.

Inhibition of FAS expression might be a potential treatment for cancer. Human promyelocytic leukemia HL60 cells were arrested in G1-phase of cell cycle by inhibitors of FAS expression [31]. A synthetic inhibitor of FAS, abolished growth of human mesotheliomas xenografts in mice at the level without a significant systemic toxicity except for a reversible weight loss [5]. The inhibition of FAS expression by 1α,25(OH)2D3 provides a possibility of developing vitamin D-based FAS inhibitory drugs for prostate cancer therapy. These drugs may selectively inhibit the growth of prostate cancer cells with high level of FAS expression. The synthetic derivatives of vitamin D are most promising, since they do not possess the side effect of 1α,25(OH)2D3 such as hypercalcemia, which severely limits its clinical use as a drug for prostate cancer therapy.

In conclusion, FAS was for the first time found to be down-regulated by 1α,25(OH)2D3 in prostate cancer LNCaP cells. The regulation was shown to be indirect and androgen-dependent. Inhibition of FAS activity by its inhibitor resulted in a clear-cut suppression of LNCaP cell proliferation. These findings suggest that FAS is involved in the antiproliferative effect of 1α,25(OH)2D3 on prostate cancer LNCaP cells.

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References


The role of long-chain fatty-acid-CoA ligase 3 in vitamin D₃ and androgen control of prostate cancer LNCaP cell growth

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Abstract

The antiproliferative effect of 1α,25(OH)₂D₃ on human prostate cancer cells is well known, but the mechanism is still not fully understood, especially its androgen-dependent action. Based on cDNA microarray results, we found that long-chain fatty-acid-CoA ligase 3 (FACL3/ACS3) might play an important role in vitamin D₃ and androgen regulation of LNCaP cell growth. The expression of FACL3/ACS3 was found to be significantly upregulated by 1α,25(OH)₂D₃ and the regulation was shown to be time-dependent, with the maximal regulation over 3.5-fold at 96 h. FACL3/ACS3 was a dominant isoform of FACL/ACS expressed in LNCaP cells as indicated by measuring the relative expression of each isoform. 1α,25(OH)₂D₃ had no significant effect on the expression of FACL1(FACL2), FACL4 and FACL6 except for its downregulation of FACL5 at 24 and 48 h by around twofold. The upregulation of FACL3/ACS3 expression by 1α,25(OH)₂D₃ was accompanied with increased activity of FACL/ACS as demonstrated by enzyme activity assay using a 14C-labeled substrate preferential for FACL3/ACS3. The growth inhibitory effect of 1α,25(OH)₂D₃ on LNCaP cells was significantly attenuated by FACL3/ACS3 activity inhibitor. Androgen withdrawal (DCC-serum), in the presence of antiandrogen Casodex or in AR-negative prostate cancer cells (PC3 and DU145), vitamin D₃ failed to regulate FACL3/ACS3 expression. The upregulation of FACL3/ACS3 expression by vitamin D₃ was recovered by the addition of DHT in DCC-serum medium. Western blot analysis showed that the expression of androgen receptor (AR) protein was consistent with vitamin D₃ regulation of FACL3/ACS3 expression. Taken together, the data suggest that the upregulation of FACL3/ACS3 expression by vitamin D₃ is through an androgen/AR-mediated pathway and might be one of the contributions of the vitamin D₃ antiproliferative effect in prostate cancer LNCaP cells.

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Keywords: 1α,25(OH)₂D₃; FACL3/ACS3; Expression regulation; Cell growth inhibition

In animal cells, fatty acids synthesized by fatty acid synthase (FAS) must be activated before being oxidized in the mitochondria. Acyl-CoA synthetase (ACS), a fatty acid metabolic enzyme, catalytically activates fatty acid to acyl-CoA, an active form of fatty acid. Acyl-CoA is utilized for cellular lipid synthesis and energy production. In addition, acyl-CoA has also been found to act as an acyl donor in protein acylation [1–4]; a stimulator in intracellular protein transport [5,6]; and a regulator in cells by inhibiting fatty acid synthesis, activating protein kinase C [7], inhibiting nuclear thyroid hormone receptor [8], and modulating the activation of superoxide anion (O₂⁻) generation, and degranulation of neutrophils [9]. Therefore, ACS is an important metabolic enzyme and may play a pivotal role in cell regulation mediated by acyl-CoA.

ACS is associated with fatty acid-induced cell apoptosis, since the apoptosis induced in these cells is shown to be mediated or caused by acyl-CoA and can be blocked by inhibition of ACS activity [10,11]. ACS is also shown to play a role in the suppression of a certain kind of tumor growth. Inhibition of Walker 256 tumor growth by linolenic acid (GLA) in rats is accompanied by an increase in both acyl-CoA content and the mitochondria ACS activity, suggesting that the increased mitochondria ACS...
activity may be involved in GLA-induced tumor growth inhibition [12]. Intragastric administration of several peroxisomal proliferators, which are more potent as hepatocarcinogens, caused inhibition of ACS activity in animals and it was proposed that inhibition of ACS by peroxisomal proliferators elevated free fatty acids which stimulated protein kinase C activity and ultimately promoted tumor formation [13]. The activity of ACS was found to be markedly reduced (30 to 50-fold) in Morris hepatoma 7288C and hepatoma tissue culture (HTC) cells when compared with normal liver [14]. This implies that the decrease in ACS activity in hepatoma may be related to hepatocarcinogenesis.

ACS has several isozymes based on the differences in fatty acid substrate, tissue distribution, and other characteristics [15–20]. Human long-chain fatty-acid-CoA ligase 3 (FACL3), also named human acyl-CoA synthetase 3 (ACS3), is an ACS isozyme [17]. FACL3/ACS3 was shown to be expressed in a wide range of human tissues, the highest level of FACL3/ACS3 expression was seen in prostate, heart, skeletal muscle, and testis [18]. FACL3/ACS3 preferentially utilizes myristate, arachidonate, and eicosapentaenoate (EPA) as substrates to form acyl-CoA [21]. Eicosapentaenoate has been found to have a broader anticancer activity [22–31]. In leukemia Romas cells, EPA-induced cell apoptosis was accompanied by increased EPA-CoA formation and paralleled with a high level of FACL3/ACS3 mRNA expression when compared with EPA-resistant leukemia cells [32]. This suggests that the increased FACL3/ACS3 expression may be associated with EPA-induced Romas cell apoptosis.

1α,25(OH)2D3, an active metabolite of vitamin D3, is a well-known cell antiproliferator. The antiproliferative effect of 1α,25(OH)2D3 is exerted through vitamin D3 receptor (VDR)-mediated pathway to control the target gene expression, resulting in cell cycle arrest in G1/S phase, cell apoptosis, and differentiation [33]. In human prostate cancer LNCaP cells, 1,25(OH)2D3 induces G1 accumulation [34,35] and apoptosis [36–38]. Our epidemiological study also suggests that vitamin D3 is protective against prostate cancer [39]. By using cDNA microarray, we found that the FACL3/ACS3 was one of the most stimulated genes by 1α,25(OH)2D3 in LNCaP cells. Further study indicated that the regulation of FACL3/ACS3 expression by vitamin D3 was mediated by both androgen and AR, and suggested that increased FACL3/ACS3 expression by vitamin D3 was one of the components associated with the antiproliferative effect of 1α,25(OH)2D3 in AR-positive prostate cancer LNCaP cells.

Materials and methods

Reagent. 1α,25(OH)2D3 was obtained from Leo Pharmaceuticals (Ballerup, Denmark). RPMI-1640 medium was purchased from Sigma–Aldrich (Saint Louis, Missouri, USA). FBS was from Gibco-BRL (Life Technology, Paisley, Scotland). TRizol reagent was purchased from Invitrogen (Carlsbad, USA). Casodex was obtained from AstraZeneca (London, UK). Dihydrotestosterone (DHT), cycloheximide, polyadenylic acid (poly(dA)), leupeptin, myristic acid, CoA, ATP, and trisian C were from Sigma (Missouri, USA). 1α,25(OH)2D3 was purchased from BIOTREND (Köln, Germany). Cy3-dUTP (25 mM), Cy5-dUTP (25 mM), dNTP (5 mM dATP, 5 mM dCTP, 5 mM dGTP, and 2 mM dTTP), and oligo(dT)12–18 (1 mg/ml) were purchased from Amersham–Pharmacia Biotech (Piscataway, USA). Human COT-1 DNA (1 mg/ml), SuperScript II RT (200 U/μl), and yeast tRNA (20 ng/ml) were purchased from Gibco-BRL (Grand Island, USA). rRNAsin RNase inhibitor (40 U/μl) was purchased from Promega (Madison, USA). High Capacity DNA Archive Kit and SYBR Green PCR Master Mix Kit were purchased from Applied Biosystems (Forster City, USA).

Cell treatment and RNA isolation. Human prostate cancer LNCaP cells (ATCC, USA) were grown in RPMI-1640 medium supplemented with 10% FBS or 10% FBS treated with dextran coated charcoal (DCC-FBS), 3 mM l-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin at 37°C in a humid atmosphere of 5% CO2, and treated with 1α,25(OH)2D3, DHT and other reagents at indicated concentrations. For 1α,25(OH)2D3, DHT, cycloheximide, and Casodex treatments, 0.1% ethanol treatment was used as a control. DMSO (0.1%) treatment alone was a control for triacsin C application. Cellular RNA was isolated with TRizol reagent following the instructions of the manufacturer. RNA concentrations were measured at 260 nm with GeneQuant II (Pharmacia Biotech, USA).

cDNA microarray. A chip (Biomedical Biochip Center, Helsinki, Finland) arrayed with about 12000 gene probes was used for cDNA microarray according to the manufacturer’s instructions. Briefly, 50 μg of RNA sample from 1α,25(OH)2D3-treated cells was labeled with Cy5-dUTP (25 mM) and 50 μg of RNA sample from control cells was labeled with Cy3-dUTP (25 mM) by reverse transcription using Oligo(dT)12–18 as a primer. The labeling reaction was done at 42°C for 60 min. After that, RNA was removed from the labeled cDNA by addition of a certain amount of NaOH solution (1 M) at 65°C for 30 min and the solution was neutralized with Tris–HCl (1 M, pH 7.5), at room temperature. Cy3-labeled cDNA was mixed with 1 μM poly(dA), 50 μg/ml polyadenylic acid (poly(dA)), leupeptin, myristic acid, CoA, ATP, and triacsin C were from Sigma (Missouri, USA). 1α,25(OH)2D3, DHT, cycloheximide, and Casodex treatments, 0.1% ethanol treatment was used as a control. DMSO (0.1%) treatment alone was a control for triacsin C application. Cellular RNA was isolated with TRizol reagent following the instructions of the manufacturer. RNA concentrations were measured at 260 nm with GeneQuant II (Pharmacia Biotech, USA).

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Real-time quantitative PCR. Reverse transcription (RT) from RNA was done with High Capacity Archive Kit (Applied Biosystems, USA) following the instructions of the manufacturer. In brief, 10 μg of RNA dissolved in 50 μl RNase-free H2O was combined with 50 μl of 2× RT Master Mix (10 μl of 10× reverse transcription buffer, 4 μl of 25× dNTPs, 10 μl of 10× random primers, 5 μl of MultiScribe reverse transcriptase (50 U/μl), and 21 μl nuclease-free H2O) and the RT reaction was performed at 25°C for 10 min followed by 37°C for 120 min. The real-time quantitative PCR (QPCR) was done with SYBR Green PCR Master Mix Kit (Applied Biosystems, USA) following the instructions of the manufacturer in ABI PRISM 7000 Detection System (Applied Biosystems, USA). Briefly, 20 ng of cDNA
the cells were treated with 25(OH)2D3 and triacsin C at indicated concentrations and times. The growth of cells was assayed using crystal violet staining. Briefly, the cells were directly fixed in 96-well plates with 11% glutaraldehyde by shaking at 500 rpm for 15 min, then washed, and air-dried. 0.1% crystal violet solution was added to stain the fixed cells by shaking for 20 min. Excess dyes were washed away with deionized water and the plates were air-dried before the addition of 10% acetic acid to extract out cell-bound dyes. The optical density loading was determined by measuring the formation of [1- 14C]myristoyl-CoA from [1- 14C]myristic acid, a preferential substrate for FACL3/ACS. The cell lysate equivalent of 105 cells was incubated for 20 min in 0.15 ml of a standard reaction mixture containing 15 μmol Tris–HCl, pH 8.0, 1 μmol ATP, 100 nmol CoA, 750 nmol diithiothreitol, 3 μmol MgCl2, and 40 μl of a solution containing 50 mM NaHCO3, 7.5 mM Triton X-100, 10 nmol of myristic acid, and 2 × 10−4 d.p.m. of [1-14C]myristic acid. The reaction was stopped by the addition of 2.25 ml of a mixture of propan-2-ol:heptane:2M sulfuric acid (40:10:1), followed by 1.5 ml heptane and 1 ml water and vigorous shaking. After centrifugation at 2000 g for 5 min, the upper layer was removed and the lower aqueous phase was washed three times with 2 ml heptane. The radioactivity in the lower (myristoyl-CoA) and upper (myristic acid) phase was measured by scintillation counting (WALLAC, VICTOR 1420 MULTILABEL COUNTER). The activity of FACL/ACS was expressed as [1-14C]myristoyl-CoA formation.

### Cytosolic and nuclear protein extraction

The cellular proteins were prepared according to a modified version of the method of Hurst et al. [41]. In brief, LNCaP cells were treated or untreated with compounds indicated in 10% DCC-serum medium for 24 h and lysed with buffer I (20 mM Hepes, pH 8.0, 20 mM NaCl, 0.5% Nonidet P-40, 1 mM dithiothreitol, and protease inhibitor cocktail) (Boehringer–Mannheim, Mannheim, Germany) for 5 min on ice. Supernatant was taken as the cytosolic protein after centrifugation at 13,200 rpm for 1 min at 4°C. The pellet at the bottom of the tube was the nuclear fraction and extracted with buffer II (20 mM Hepes, pH 7.9, 25% glycerol, 0.42 M NaCl, 1.5 μM MgCl2, 2 μM EDTA, pH 8.0, 1 mM dithiothreitol, and protease inhibitor cocktail) for 30 min on ice. The nuclear proteins were obtained by centrifuging as above. The protein concentration was determined using BCA Protein Assay Kit (PIERCE, USA).

### Western blot analysis

Aliquots of 40 μg protein extract prepared as described were heated in SDS sample buffer at 95°C for 5 min and subjected to 7.5% SDS–polyacrylamide gel. After electrophoresis, the proteins were transferred to nitrocellulose membranes, and the membranes were incubated with primary antibody (AR 70, 1 mg/ml, 1:200) against androgen receptor (AR) in TBS containing 1% non-fat milk and 0.05% Tween 20 at 4°C overnight after incubation with blocking buffer (50 mM Tris, 0.9% NaCl, 0.05% Tween 20, and 5% non-fat milk, pH 8.0), for 1 h at 37°C. After washing, the membranes were incubated with goat anti-rabbit IgG conjugated with peroxidase diluted 1:10,000 for 1 h at room temperature. The membranes were rewashed and blots were developed with enhanced chemiluminescence system (Konica SRL-101A, USA).

### Statistics

All data were evaluated for statistical significance by t test and expressed as means ± SD. A difference of p < 0.05 was considered significant and p < 0.001 highly significant.

### Table 1

<table>
<thead>
<tr>
<th>Gene names</th>
<th>Primer sequences</th>
<th>Primer positions</th>
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<tbody>
<tr>
<td>FACL1(FACL2)</td>
<td>Forward primer: 5'-TCTTCCCCGTGGTCTTCAA-3'</td>
<td>Exon 12</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-TGGGTGTTCGGTGCTGGAAAA-3'</td>
<td>Exon 13</td>
</tr>
<tr>
<td>FACL3/ACS3</td>
<td>Forward primer: 5'-ACTCCACTGTCGACGACCTT-3'</td>
<td>Exon 9</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-CACCACACACAGGACGCAA-3'</td>
<td>Exon 10</td>
</tr>
<tr>
<td>FACL4</td>
<td>Forward primer: 5'-CAGGCCAGTGTAAGAAATACCT-3'</td>
<td>Exon 11</td>
</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-ATTTCAGCATACAGCAGCAAAG-3'</td>
<td>Exon 12</td>
</tr>
<tr>
<td>FACL5</td>
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</tr>
<tr>
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<td>Exon 13</td>
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<td></td>
<td>Reverse primer: 5'-GCCTTCAGGCGCAATAGAC-3'</td>
<td>Exon 14</td>
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<td>GAPDH</td>
<td>Forward primer: 5'-CCACATCGTCGACAGACCAT-3'</td>
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</tr>
<tr>
<td></td>
<td>Reverse primer: 5'-ACCAGGCCGCCCCATACG-3'</td>
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</table>
Results

1α,25(OH)₂D₃-regulated genes revealed by cDNA microarray in LNCaP cells

In our previous study, 24 genes were revealed to be regulated by 10 nM 1α,25(OH)₂D₃ in LNCaP cells by cDNA microarray with a chip containing 3000 gene probes [42]. Here a different chip containing more than 12000 genes was applied to further investigate vitamin D₃-regulated genes. The microarray was done in duplicate and the results showed that up to 400 genes were regulated by 10 nM 1α,25(OH)₂D₃ in LNCaP cells after 24 h of treatment in 10% of FBS serum medium (≥1.8-fold regarded as upregulated and ≤0.55-fold as downregulated). Prostate specific antigen (PSA) [43], E-cadherin [44], androgen receptor (AR) [45], insulin-like growth factor binding protein 3 (IGFBP-3) [46], and fibroblast growth factor 7 (keratinocyte growth factor) (KGF) [47], which have already been found to be vitamin D₃-regulated, were also shown to be consistently regulated by 10 nM 1α,25(OH)₂D₃ in LNCaP cells (1.96 ± 0.13-fold for PSA, 2.0 ± 0.26-fold for E-cadherin, 2.87 ± 0.78-fold for AR, 1.91 ± 0.04-fold for IGFBP-3, and 2.16 ± 0.38-fold for KGF). One of the most regulated genes revealed was long-chain fatty-acid-CoA ligase 3, also known as acyl-CoA synthetase 3, which was upregulated by 10 nM 1α,25(OH)₂D₃ treatment. FACL3/ACS3 is a fatty acid metabolic enzyme just downstream of fatty acid synthase (FAS), which was involved in the antiproliferative effect of 1α,25(OH)₂D₃ on LNCaP cells in our previous study.

Expression of FACL3/ACS3 is upregulated by 1α,25(OH)₂D₃ in a time-dependent manner

On the basis of cDNA microarray results, the regulation of FACL3/ACS3 expression by 1α,25(OH)₂D₃ was further analyzed using real-time quantitative RCP (QPCR). The cells were treated or untreated with 10 nM 1α,25(OH)₂D₃ for 24 h. The experiment was repeated three times and the data were normalized to housekeeping gene GAPDH. The results confirmed that the expression of FACL3/ACS3 was significantly upregulated by 1α,25(OH)₂D₃ in LNCaP cells at all indicated time points and the regulation was shown to be time-dependent. The strongest upregulation of FACL3/ACS3 expression by 1α,25(OH)₂D₃ (over 3.5-fold) was reached at 96 h after treatment, thereafter the upregulation gradually decreased (Fig. 1).

FACL3/ACS3 is a dominant isoform of FACL/ACS expressed in LNCaP cells

Human FACL3/ACS3 belongs to the FACL/ACS family that consists of several isozymes named FACL1(FACL2), FACL3, FACL4, FACL5, and FACL6, respectively. To determine the distribution of the expression of these isozymes in LNCaP cells, the cells were grown for 24, 48, and 96 h in normal culture medium, respectively, and the expression level of each isozyme was measured by QPCR. The expression level of each isoform was normalized to the housekeeping gene GAPDH and finally expressed as percentage of total isoforms expression. FACL3/ACS3 was a major isoform of FACL/ACS expressed in LNCaP cells. The expression of FACL3/ACS3 was shown to be stable through the different time points indicated and its average expression accounted for 68.46% (from 67.37% at 24 h, 71.5% at 48 h, and 66.5% at 96 h) of the total expression of all isozymes in LNCaP cells (Fig. 2).

1α,25(OH)₂D₃ has no significant effect on the expression of other isozymes of FACL/ACS except for its downregulation of FACL5 at 24 and 48 h

To see whether 1α,25(OH)₂D₃ affects the expression of other FACL/ACS isoforms, LNCaP cells were treated with 10 nM 1α,25(OH)₂D₃ at the indicated times and the expression of each of these isoforms was measured by QPCR. The expression of FACL5 was shown to be downregulated by up to twofold at 24 and 48 h after vitamin D₃ treatment, no regulation was seen at other indicated times (Fig. 3). 1α,25(OH)₂D₃ had no significant effect on the expression of FACL1(FACL2), FACL4, and FACL6 in LNCaP cells, respectively (data not shown).
Increase in the activity of FACL/ACS by 1α,25(OH)2D3 accompanied and paralleled with the increase in the expression of FACL3/ACS3

The activity of FACL/ACS was determined by measuring the formation of [1-14C]myristoyl-CoA from [1-14C]myristic acid, a preferential substrate for FACL3 as described in Materials and methods section. The experiment was done in duplicate. The activity of FACL/ACS was expressed as the fold relative to the control. The FACL/ACS activity reached a peak of more than fourfold at 96 h after 1α,25(OH)2D3 treatment. At 144 h the activity of FACL/ACS decreased but was still significantly high when compared with no treatment.

The growth inhibitory effect of 1α,25(OH)2D3 is significantly blocked by FACL3/ACS3 activity inhibitor

To investigate the possible role of FACL3/ACS3 in 1α,25(OH)2D3-induced LNCaP cell growth arrest, triacsin C, an inhibitor of FACL3/ACS3 activity, was added to the cells treated with 1α,25(OH)2D3 in normal serum medium for the times indicated. Cell growth was measured by crystal violet staining. Each experiment was repeated at least three times. Triacsin C partially but significantly blocked 1α,25(OH)2D3-repression of LNCaP cell growth at day 4 and day 6 (Fig. 5A). Cell viability was not significantly affected by 1α,25(OH)2D3, triacsin C or the combination of both during the treatments (Fig. 5B).

Regulation of FACL3/ACS3 expression by 1α,25(OH)2D3 is through androgen/AR-mediated pathway

Since the action of 1α,25(OH)2D3 in LNCaP cells has been shown to be androgen-dependent [42,48], dextran
coated charcoal (DCC)-treated serum, DHT, and androgen antagonist Casodex were applied to study the effect of androgen on vitamin D3-regulation of FACL3/ACS3 expression. In DCC-FBS medium, where endogenous steroids are substantially depleted, 1α,25(OH)2D3 failed to regulate FACL3/ACS3 expression (data not shown). In 10% FBS medium the upregulation of FACL3/ACS3 by vitamin D3 was completely blocked by antiandrogen Casodex (Fig. 6). To further examine the effect of androgen on vitamin D3-regulation of FACL3/ACS3 expression, dihydrotestosterone (DHT), a natural androgen, was added to DCC-FBS medium and the cells were treated with 1α,25(OH)2D3. The results showed that the upregulation of FACL3/ACS3 by vitamin D3 was recovered by the addition of DHT to the DCC-serum medium (Fig. 7). At 6 h, FACL3/ACS3 expression was upregulated approximately twofold by 1α,25(OH)2D3 in the presence of 10 nM DHT. The strongest upregulation of FACL3/ACS3 expression (more than 5.5-fold) by 1α,25(OH)2D3 was seen at 24 h.
in the presence of 10 nM DHT and regulation of about 1.5-fold was seen in the presence of 1 nM DHT. The upregulation of FACL3/ACS3 expression was increased to around twofold by vitamin D3 in the presence of 1 nM DHT and over threefold in the presence of 10 nM DHT at 48 h. Antiandrogen Casodex completely blocked the upregulation of FACL3/ACS3 expression by 1α,25(OH)2D3 in the presence of DHT was completely blocked by Casodex. Casodex also blocked DHT-stimulation of FACL3/ACS3 expression. Vitamin D3 alone or Casodex alone had no effect on the expression of FACL3/ACS3 in DCC-serum medium where androgen was depleted. The result was from three independent experiments.

Discussion

In our previous study, we used a chip containing 3000 genes to do cDNA microarray and revealed 24 genes regulated by 1α,25(OH)2D3 in prostate cancer LNCaP cells [42]. In the present study, a different chip containing over 12,000 genes was applied to further investigate vitamin D3 target genes in LNCaP cells. The results showed that up to four hundred genes were regulated by 10 nM 1α,25(OH)2D3 24 h after treatment. The vitamin D3-regulated genes revealed in our previous study were repeatable in the current microarray experiments except for the genes not used in the present chip. Some of genes already identified to be vitamin D3-regulated such as androgen receptor (AR) and insulin-like growth factor binding protein 3 (IGFBP-3) were also revealed to be consistently regulated by 10 nM 1α,25(OH)2D3 in LNCaP cells. Cyclin-dependent kinase inhibitor 1A (p21/Cip1) was also found to be upregulated by about 1.67-fold but dropped below the threshold (≥1.8-fold) defined. Cyclin-dependent kinase inhibitor p27 is not in the chip currently used. Long-chain fatty-acid-CoA ligase 3, also known as acyl-CoA synthetase 3, which catalytically converts long-chain fatty acid to its active form acyl-CoA, was the most upregulated gene by 10 nM 1α,25(OH)2D3 found in the current microarray study. The upregulation of FACL3/ACS3 expression by vitamin D3 was further confirmed by real-time quantitative PCR. To our knowledge, this is the first report on the vitamin D3-modulation of FACL3/ACS3 expression.

The failure of 1α,25(OH)2D3 in the regulation of FACL3/ACS3 expression in DCC-serum medium and androgen DHT capable of recovery of the upregulation of FACL3/ACS3 expression by vitamin D3 in DCC-serum medium suggest that androgen is essential for vitamin D3 action in the regulation of FACL3/ACS3 expression in LNCaP cells. AR is also a necessary and critical factor for vitamin D3 regulation of FACL3/
ACS3 expression since antiandrogen Casodex is able to block the vitamin D action and no regulation of FACL3/ACS3 expression is seen in AR-negative prostate cancer cells PC3 and DU145 by 1α,25(OH)2D3. Therefore, the upregulation of FACL3/ACS3 expression by vitamin D3 is androgen-dependent, and both androgen and AR are required. In other words, vitamin D3 is not able to upregulate the expression of FACL3/ACS3 in the absence of either of them in LNCaP cells. The blockage effect of protein synthesis inhibitor, cycloheximide, on vitamin D3 upregulation of FACL3/ACS3 expression suggests that the regulation of FACL3/ACS3 expression by vitamin D3 might not be direct. This is consistent with the fact that no vitamin D3 response element (VDRE) is found in the promoter region of FACL3/ACS3 gene [18], and the vitamin D3 regulation of FACL3/ACS3 expression is mediated by androgen/AR found in the current study. DHT was also found to upregulate the expression of FACL3/ACS3 in the presence of androgen and suggests that increased AR protein expression is involved in vitamin D3 upregulation of FACL3/ACS3 expression in LNCaP cells. No significant effect of antiandrogen Casodex on vitamin D3 regulation of AR protein expression in the presence of androgen DHT also suggests that the blockage effect of antiandrogen Casodex on vitamin D3 regulation of FACL3/ACS3 expression is due to its interfering with the interaction between androgen and AR.

Androgens showed downregulation of AR mRNA in most cell lines and tissues including prostate [50]. One earlier study reported that the cellular content of AR protein was increased by androgen in LNCaP cells while AR mRNA was decreased by androgen [51]. In the present study, we found that AR protein was downregulated by 10 nM DHT at 24 h after the treatment of LNCaP cells in DCC-serum medium. This inconsistency may be due to the different kind of androgen and the different concentrations of androgen used in the different experiments. 10 nM DHT showed to decrease in AR protein at 24 h in LNCaP cells, but it still upregulated the expression of FACL3/ACS3, since in the control DHT was absent, therefore no regulation of FACL3/ACS3 expression was seen even though AR protein level is higher than that in the DHT treatment. 1α,25(OH)2D3 was shown to antagonize 10 nM DHT decrease of AR protein, in other words, AR protein level was significantly high in the presence of both 1α,25(OH)2D3 and 10 nM DHT when compared with DHT alone. Increased AR protein by vitamin D3 in the presence of DHT enhanced the interaction between DHT and AR, resulting in the increased upregulation of FACL3/ACS3 expression. This is consistent with the findings that the upregulation of FACL3/ACS3 expression by the combination of 1α,25(OH)2D3 and 10 nM DHT was significantly high when compared with 10 nM DHT alone in LNCaP cells.
1α,25(OH)₂D₃ was shown to stimulate the activity of FACL3/ACS in LNCaP cells by using a substrate preferential for FACL3/ACS3. The increase in the FACL/ACS activity by vitamin D₃ reached its peak at 96 h and then decreased at 144 h but was still significant. This is consistent and parallels with the upregulation of FACL3/ACS3 expression by 1α,25(OH)₂D₃ (the upregulation reached a peak at 96 h and then decreased at 144 h). 1α,25(OH)₂D₃ had no significant effect on the expression of isoform FACL1(FACL2), FACL4, and FACL6, respectively. FACL5 expression was shown to be downregulated about twofold by vitamin D₃ at 24 and 48 h, and no regulation was seen at other indicated times. In addition, FACL3/ACS3 is the major isoform of FACL/ACS expressed in LNCaP cells (over 68% in total FACL/ACS expression). The expression of FACL3/ACS3 was over twofold higher than that of FACL1(FACL2) and over a hundred thousand-fold higher than that of FACL4, FACL5, and FACL6, respectively. These suggest that the isoforms FACL1 (FACL2), FACL4, FACL5, and FACL6 made no significant contribution to the increased activity of FACL/ACS by vitamin D₃, the increase in the activity of FACL/ACS is mainly due to the increase in the FACL3/ACS3 activity as the consequence of upregulation of FACL3/ACS3 expression by vitamin D₃. In other words, the upregulation of FACL3/ACS3 expression by 1α,25(OH)₂D₃ is accompanied by an increase in the activity of FACL3/ACS3 in LNCaP cells.

Triacsin C, an inhibitor of FACL3/ACS3 activity, is able to block fatty acid-induced cell apoptosis [10,11]. In the present study, triacsin C was shown to significantly reverse inhibition of LNCaP cell growth by 1α,25(OH)₂D₃ at days 4 and 6. A stronger effect of triacsin C on the blocking vitamin D₃ inhibition of LNCaP cell growth was seen at day 4, consistent with the greatest upregulation of FACL3/ACS3 by vitamin D₃ shown at 96 h (day 4), which was accompanied with the highest increase in the activity of FACL3/ACS3. The increase in FACL3/ACS3 activity by vitamin D₃ leads to the increase in the binding of triacsin C to FACL3/ACS3, resulting in enhanced inhibition of FACL3/ACS3 activity which is responsible for attenuation of vitamin D₃-inhibition of LNCaP cell growth. Some studies showed that triacsin C was not specific for inhibition of FACL3/ACS3 activity. This implies that the activity of other isozymes might be inhibited competitively by triacsin C, and this inhibition may also have some effect on vitamin D₃ actions, especially if the expression of these isozymes is affected by vitamin D₃. FACL3/ACS3 is a major isozyme in LNCaP cells, its expression is over twofold higher than that of FACL1(FACL2) and over a hundred thousand-fold higher than that of FACL4, FACL5, and FACL6, respectively. No significant effect of vitamin D₃ on the expression of isozymes FACL1 (FACL2), FACL4, and FACL6 suggests that inhibition of activity of these isoforms by triacsin C may have no significant effect on vitamin D₃-inhibition of cell growth. FACL5 was shown to be downregulated about twofold by vitamin D₃ at 24 and 48 h. No regulation of FACL5 was seen at other indicated times such as 96 and 144 h, at which the inhibition of cell growth by vitamin D₃ was significantly attenuated by triacsin C. The downregulation of FACL5 by vitamin D₃ may make a contribution to the decrease in the activity of FACL/ACS, but this contribution can be ignored when compared with the contribution of FACL3/ACS3 to the increase in the FACL/ACS activity, since the expression of FACL5 is over a hundred thousand-fold lower than that of FACL3/ASC3. In addition, the recombinant study showed that FACL5 was not inhibited by triacsin C [52]. Taken together, the data suggest that the blockage effect of triacsin C on the inhibition of LNCaP cell growth by 1α,25(OH)₂D₃ is the main consequence of the triacsin C inhibition of the FACL3/ACS3 activity increased by vitamin D₃.

In conclusion, FACL3/ACS3 is a vitamin D₃ target gene which is upregulated by 1α,25(OH)₂D₃ through androgen/AR-mediated pathway in prostate cancer LNCaP cells. Increased FACL3/ACS3 expression may be one component associated with 1α,25(OH)₂D₃ repression of LNCaP cell growth. This study may provide valuable information for the further understanding of the mechanism of vitamin D₃ antiproliferative effect on the cells whereby the vitamin D₃ action is dependent on androgen.

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References


Vitamin D3 inhibits fatty acid synthase expression by stimulating the expression of long-chain fatty-acid-CoA ligase 3 in prostate cancer cells

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Abstract FAS and FACL3 are enzymes of fatty acid metabolism. In our previous studies, we found that FAS and FACL3 genes were vitamin D₃-regulated and involved in the antiproliferative effect of 1\textsubscript{α},25(OH)\textsubscript{2}D₃ in the human prostate cancer LNCaP cells. Here, we elucidated the mechanism behind the downregulation of FAS expression by vitamin D₃. Triacsin C, an inhibitor of FACL3 activity, completely abolished the downregulation of FAS expression by vitamin D₃, whereas an inhibitor of FAS activity, cerulenin, had no significant effect on the upregulation of FACL3 expression by vitamin D₃ in LNCaP cells. In human prostate cancer PC3 cells, in which FACL3 expression is not regulated by vitamin D₃, no regulation of FAS expression was seen. This suggests that the downregulation of FAS expression by vitamin D₃ is mediated by vitamin D₃ upregulation of FACL3 expression. Myristic acid, one of the substrates preferable for FACL3, enhanced the repression of FAS expression by vitamin D₃. The action of myristic acid was abrogated by inhibition of FACL3 activity, suggesting that the enhancement in the downregulation of FAS expression by vitamin D₃ is due to the formation of myristoyl-CoA. The data suggest that vitamin D₃-repression of FAS mRNA expression is the consequence of feedback inhibition of FAS expression by long chain fatty acyl-CoAs, which are formed by FACL3 during its upregulation by vitamin D₃ in human prostate cancer LNCaP cells.

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Keywords: FAS; FACL3; Vitamin D₃; Long chain fatty acyl-CoA; Prostate cancer

1. Introduction

Fatty acid synthesis is controlled by a long term and a short term regulation. The long term regulation of fatty acid synthesis occurs through alterations in the rate of synthesis of acetyl-CoA carboxylase (ACC), the first and rate-limiting enzyme of the fatty acid synthesis, and that of fatty acid synthase (FAS), the second and a key enzyme of the fatty acid synthesis [1–3]. The short term regulation involves cellular fatty acyl-CoAs [4], but the mechanisms are not fully understood. Inhibition of the de novo fatty acid synthesis by free fatty acids appears to depend on the formation of long chain fatty acyl-CoAs [5]. Long chain fatty acyl-CoAs have shown to inhibit ACC [6–9] and FAS [10]. It is proposed that long chain fatty acyl-CoAs exert their effects on ACC and FAS by means of feedback inhibition [10,11]. It is further suggested that the inhibitory effect of long chain fatty acyl-CoA on the fatty acid synthesis may be due to its regulation of lipogenic enzymes in a feedback manner through suppression of the gene transcription [12].

Long chain fatty acyl-CoAs are catalytically synthesized by long-chain fatty-acid-CoA ligase in cells. The inhibitory effect of long chain fatty acyl-CoAs on the fatty acid synthesis implies that long-chain fatty-acid-CoA ligase may play an important role in the suppression of fatty acid synthesis. It was reported that long-chain fatty-acyl-CoA ligase was involved in the inhibition of fatty acid synthesis [13]. Recently, we found that FACL3, which utilizes preferentially myristic acid, eicosapentaenoic acid (EPA), and arachidonic acid as substrates to form long chain fatty acyl-CoAs, was upregulated by vitamin D₃ in both expression and activity levels and contributed to the growth inhibitory effect of vitamin D₃ in human prostate cancer LNCaP cells [14]. We also found that FAS, which is overexpressed and associated with prostate cancer development, was downregulated by vitamin D₃ in LNCaP cells [15]. In the current study, we report that the downregulation of FAS mRNA by vitamin D₃ is due to the feedback inhibition of FAS expression by long chain fatty acyl-CoAs, which are synthesized by FACL3, and suggest that long chain fatty acyl-CoA-mediated inhibition of FAS expression may be one mechanism by which FACL3 contributes to the antiproliferative effect of vitamin D₃ in the human prostate cancer LNCaP cells.

2. Materials and methods

2.1. Reagents

1\textsubscript{α},25(OH)\textsubscript{2}D₃ was obtained from Leo Pharmaceuticals (Ballerpup, Denmark). Cerulenin, Myristic acid and triacsin C were purchased from Sigma (Missouri, USA). RPMI-1640 medium was purchased from Sigma–Aldrich (Saint Louis, Missouri, USA). FBS was from Gibco BRL (Life Technology, Paisley, Scotland). TRIzoL reagent was

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purchased from Invitrogen (Carlsbad, USA). High Capacity DNA Archive kit and SYBR Green PCR Master Mix Kit were purchased from Applied Biosystems (Forster City, USA).

2.2. Cell culture and treatments

Human prostate cancer LNCaP cells (from ATCC, USA) were grown in RPMI-1640 medium supplemented with 10% FBS, 3 mM l-glutamine, 100 μg/ml streptomycin and 100 U/ml penicillin at 37 °C in a humid atmosphere of 5% CO2. After grown to semi-confluence, the cells were treated with 1α,25(OH)2D3, triacsin C, cerulien, myristic acid or the combination as indicated. For the treatment of 1α,25(OH)2D3 or myristic acid, vehicle ethanol which was received by the cells was used as a control. Vehicle DMSO was used as a control for triacsin C or cerulien treatment.

2.3. RNA preparation

After the treatments, total RNA was isolated using TRIzol reagent (Invitrogen, USA) according to the instructions of the manufacturer. Briefly, 2 ml of TRIzol reagent was added to a 25 cm2 culture bottle and passed the cell lysate several times through a pipette for homogenization. 400 μl of chloroform was added to each homogenized sample and mixed it vigorously. After incubation of the sample for 3 min at room temperature, it was centrifuged at 12 000 × g for 15 min at 4 °C, transferred the aqueous phase to a fresh tube and added an equal volume of isopropyl alcohol for RNA precipitation. After the precipitation, the sample was centrifuged as indicated above and RNA pellet was washed with 75% ethanol. Finally, RNA was redissolved in RNase-free water and the RNA concentration was measured using GeneQuant II (Pharmacia Biotech, USA).

2.4. cDNA synthesis

cDNA synthesis from RNA was done using High Capacity Archive Kit (Applied Biosystems, USA) following the instructions of the manufacturer. In brief, 10 μg of RNA dissolved in 50 μl of RNase-free H2O was combined with 50 μl of 2× RT Master Mix (10 μl of 10× Reverse Transcription Buffer, 4 μl of 25× dNTPs, 10 μl of 10× random primers, 5 μl of MultiScribe Reverse Transcriptase (50 U/μl) and 21 μl of MultiScribe Reverse Transcriptase (20 U/μl) and water to the final volume of 30 μl. The reverse transcription was performed at 25 °C for 10 min followed by 37 °C for 120 min.

2.5. Real-time quantitative PCR

The real-time quantitative PCR (QPCR) was performed with SYBR Green PCR Master Mix kit (Applied Biosystems, USA) following the instructions of the manufacturer in ABI PRISM 7000 Detection System (Applied Biosystems, USA). Briefly, 20 ng of cDNA synthesized by reverse transcription was combined with primers for the target gene and 2× SYBR Green PCR Master Mix to the final volume of 30 μl per reaction. The PCR was done at 95 °C for 10 min followed by 40 cycles at 95 °C for 15 s, 60 °C for 1 min. The product of PCR was checked by dissociation curve to ensure that the product was specific for the studied gene. The data were analyzed by ABI Prism 7000 SDS Software and normalized to constitutively expressed glyceraldehyde-3-phosphate dehydrogenase (GAPDH) to verify uniform sample loading. The final result was expressed as N-fold difference in gene expression between treated and untreated samples \((N(%f) = \frac{[\text{gene (treated)}]}{[\text{gene (untreated)}]} \frac{[\text{GAPDH (treated)}]}{[\text{GAPDH (untreated)}]} \)). The values used for the calculation in the formula were obtained from the respective standard curve. The standard curve was made by a series of dilutions of untreated sample. All primers were designed using compatible software Primer Express for ABI PRISM 7000 Detection System. The primers used for QPCR are shown in Table 1.

2.6. Statistics

Data were analyzed using Student’s t test. A difference of \(p < 0.05\) was considered as significant.

3. Results

3.1. FACL3 mediates vitamin D₃ inhibition of FAS mRNA expression

FAS synthesizes fatty acids. FACL3 converts long chain fatty acid to acyl-CoA for further utilization. In our previous study, we demonstrated that FAS mRNA was decreased by vitamin D₃ in the human prostate cancer LNCaP cells and might have a contribution to antiproliferative effect of vitamin D₃ [15]. We also found that FACL3 mRNA and activity were increased by vitamin D₃ in LNCaP cells [14]. To investigate whether there is an association between vitamin D₃ downregulation of FAS expression and its upregulation of FACL3 or the regulations are independent on each other, cerulien, an inhibitor of FAS activity, and triacsin C, the inhibitor of FACL3 activity, were applied to treat the cells in the presence or absence of vitamin D₃, and mRNA levels were measured using real-time QPCR. The results showed that triacsin C completely abolished vitamin D₃ decrease of FAS mRNA (Fig. 1), whereas cerulien had no significant effect on the increase in FACL3 mRNA by vitamin D₃ (Fig. 2) in LNCaP cells. Triacsin C had no effect on vitamin D₃ up-regulation of FACL3 mRNA. FAS and FACL3 mRNA expressions were not affected by cerulien or triacsin C alone. In addition, we found in the previous study that FACL3 expression was not regulated by vitamin D₃ in human prostate cancer PC3 cells [14], which are vitamin D₃ responsive and the action of vitamin D₃ is independent of androgen. We measured FAS expression in PC3 cells and found that FAS expression was not regulated by vitamin D₃ (data not shown).

<table>
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<td>The list of primers used for QPCR analysis</td>
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<td><strong>Gene names</strong></td>
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<td>FACL3</td>
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<td>FAS</td>
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<td>GAPDH</td>
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Fig. 1. Inhibition of FACL3 activity by triacsin C abolishes vitamin D₃ repression of FAS mRNA expression in the human prostate cancer LNCaP cells. LNCaP cells were grown in RPMI-1640 medium supplemented with 10% FBS and treated with 10 nM 1α,25(OH)2D₃, 1 μM triacsin C or the combination of them for 24 h. After the treatments, RNA was isolated and the expression of FAS mRNA was measured using real-time quantitative PCR. The results represent three independent experiments and normalized to housekeeping gene GAPDH. Triacsin C abolished completely the downregulation of FAS mRNA by 1α,25(OH)2D₃. No considerable effect of triacsin C alone on FAS mRNA expression was seen.
3.2. Formation of myristoyl-CoA is responsible for myristic acid-induced repression of FAS expression in the presence of vitamin D₃

There are several substrates preferential for FACL3 in the synthesis of long chain fatty acyl-CoAAs [16]. In our previous study, myristic acid, one of the preferential substrates for FACL3, was applied to measure the enzyme activity and indicated that the formation of myristoyl-CoA was significantly increased at indicated time points (48, 96, and 144 h) in the presence of vitamin D₃ in human prostate cancer LNCaP cells [14]. To investigate the effect of long chain fatty acyl-CoAAs formed by FACL3 on vitamin D₃ inhibition of FAS mRNA expression, myristic acid and triacsin C were used to treat LNCaP cells in the presence or absence of vitamin D₃. Myristic acid showed no significant effect on vitamin D₃ repression of FAS mRNA expression at 24 h, but enhanced the decrease in FAS mRNA by vitamin D₃ at 48 h (Fig. 3). Triacsin C, an inhibitor of FACL3 activity, abrogated the effect of myristic acid on vitamin D₃ inhibition of FAS expression (Fig. 3). Neither myristic acid nor triacsin C alone had significant effect on the expression of FAS mRNA. The data suggest that myristoyl-CoA, but not myristic acid, possesses an ability to inhibit FAS expression and the formation of myristoyl-CoA by FACL3 contributes to the downregulation of FAS expression by vitamin D₃ in LNCaP cells. Myristic acid had no considerable effect on vitamin D₃ upregulation of FACL3 expression in LNCaP cells (data not shown).

4. Discussion

Vitamin D₃ is a potential anti-cancer compound due to its antiproliferative function. In our previous studies, we investigated the genes regulated by 1α,25(OH)₂D₃ in prostate cancer LNCaP cells using cDNA microarray and found that two fatty acid metabolic enzymes, FAS and FACL3, were regulated by vitamin D₃. The further studies suggested that FAS and FACL3 were involved in vitamin D₃ inhibition of LNCaP cells growth [14,15]. FAS catalytically synthesizes fatty acid and FACL3, a downstream enzyme of FAS, converts long chain fatty acid to the corresponding acyl-CoA. A number of studies indicated that fatty acid synthesis was inhibited by long chain fatty acyl-CoAAs [5]. This led us to further investigate whether there is a relationship between the regulation of FAS and FACL3 expressions by vitamin D₃.

Inhibition of FACL3 activity by triacsin C resulted in a complete abolishment of the repression of FAS mRNA expression by vitamin D₃. Cerulenin, an inhibitor of FAS activity, had no significant effect on vitamin D₃ upregulation of FACL3 mRNA expression. Neither the effect of triacsin C on vitamin D₃ increase of FACL3 mRNA nor the effect of cerulenin on the vitamin D₃ decrease of FAS mRNA was seen. These suggest that the downregulation of FAS expression by vitamin D₃ is mediated by FACL3 in LNCaP cells. This is consistent with our previous findings that the action of vitamin D₃ on FAS expression is not direct [15]. In vitamin D₃-responsive human prostate cancer PC3 cells, which are androgen receptor-negative in contrast to LNCaP cells, no regulation of FAS expression by vitamin D₃ was found, consistent with no vitamin D₃ regulation of FACL3 expression in PC3 cells. Vitamin D₃ increases both mRNA expression and the activity of FACL3 [14]. The increase in the FACL3 activity by vitamin D₃ results in an increase in the formation of long chain fatty acyl-CoAAs, which may inhibit FAS mRNA expression. Myristic acid, one of the substrates preferential for FACL3, had no significant effect on the decrease of FAS mRNA by vitamin D₃ at 24 h, but enhanced the decrease in FAS mRNA by vitamin D₃ at 48 h. This is consistent with our previous study that the formation of myristoyl-CoA is not significantly elevated at 24 h and significantly elevated at 48 h in the presence of vitamin D₃ [14]. Myristic acid-induced enhancement in the vitamin D₃-repression of FAS mRNA expression was completely abolished by triacsin C, indicating that the formation of myristoyl-CoA contributes to vitamin D₃ downregulation of FAS expression. The data suggest that the downregulation of FAS mRNA by vitamin D₃ is the result of feedback inhibition of FAS expression by long chain fatty acyl-CoAAs formed during vitamin D₃ upregulation of FACL3 in LNCaP cells.
Inhibition of fatty acid synthesis by long chain fatty acyl-CoAs is evident. Some studies [5,11,17] suggest that long chain fatty acyl-CoAs inhibit fatty acid synthesis in terms of feedback inhibition of ACC, the first enzyme in fatty acid synthesis pathway. Based on this study, we suggest that long chain fatty acyl-CoAs formed by FACL3 during its upregulation by vitamin D3 repress mRNA expression of FAS, the second enzyme in fatty acid synthesis pathway. Inhibition of FAS expression by long chain fatty acyl-CoAs may represent one mechanism of inhibition of fatty acid synthesis. Some results suggest that inhibition of ACC by long chain fatty acyl-CoA is due to the decrease in amounts of enzyme, the amounts of changes in ACC caused by the alteration in dietary fat are accompanied with similar amounts of changes in FAS [3]. These imply that inhibition of ACC by long chain fatty acyl-CoAs might be due to a repression of ACC mRNA expression and a link between inhibitions of FAS and ACC by long chain fatty acyl-CoAs. The direct inhibition of ACC by long chain fatty acyl-CoA has been reported [18]. It might be possible that the inhibition of ACC by long chain fatty acyl-CoA could affect FAS expression and result in the decrease in FAS mRNA. The relationship between inhibitions of ACC and FAS by long chain fatty acyl-CoAs remains to be investigated.

FAS is overexpressed in prostate cancer and associated with prostate cancer development [19–22]. Inhibition of FAS activity or knockdown of FAS mRNA results in human prostate cancer LNCaP cell growth inhibition/apoptosis [15,23]. We found previously that the expression of FACL3 was upregulated by vitamin D3 and contributed to the inhibitory effect of vitamin D3 in LNCaP cells [14]. In this study, we unravell that long chain fatty acyl-CoAs which are formed due to the increase in FACL3 expression by vitamin D3 decrease FAS mRNA. This suggests that the contribution of FACL3 to vitamin D3 inhibition of LNCaP cells growth, at least in part, is due to long chain fatty acyl-CoA-mediated decrease in FAS expression, and could be one mechanism of FACL3 in vitamin D3 antiproliferative effect. Taken together, we conclude that the upregulation of FACL3 by vitamin D3 results in the increase in long chain fatty acyl-CoAs, which repress FAS mRNA expression by means of feedback inhibition. The decrease in FAS expression might contribute to the inhibition of human prostate cancer LNCaP cell growth by vitamin D3 (Fig. 4).

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References