Vitamin D_3 inhibits fatty acid synthase expression by stimulating the expression of long-chain fatty-acid-CoA ligase 3 in prostate cancer cells

Shengjun Qiao[a,b,*], Pentti Tuohimaa[a,b,c]

[a]Department of Anatomy, Medical School, University of Tampere, 33014 Tampere, Finland
[b]Tampere Graduate School in Biomedicine and Biotechnology, University of Tampere, 33014 Tampere, Finland
[c]Department of Clinical Chemistry, Tampere University Hospital, University of Tampere, 33014 Tampere, Finland

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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 D_3-regulated and involved in the antiproliferative effect of $1\alpha,25(OH)_2D_3$ in the human prostate cancer LNCaP cells. Here, we elucidated the mechanism behind the downregulation of FAS expression by vitamin D_3. Triacsin C, an inhibitor of FACL3 activity, completely abolished the downregulation of FAS expression by vitamin D_3, whereas an inhibitor of FAS activity, cerulenin, had no significant effect on the upregulation of FACL3 expression by vitamin D_3 in LNCaP cells. In human prostate cancer PC3 cells, in which FACL3 expression is not regulated by vitamin D_3, no regulation of FAS expression was seen. This suggests that the downregulation of FAS expression by vitamin D_3 is mediated by vitamin D_3 upregulation of FACL3 expression. Myristic acid, one of the substrates preferential for FACL3, enhanced the repression of FAS expression by vitamin D_3. The action of myristic acid was abrogated by inhibition of FACL3 activity, suggesting that the enhancement in the downregulation of FAS expression by vitamin D_3 is due to the formation of myristoyl-CoA. The data suggest that vitamin D_3-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 D_3 in human prostate cancer LNCaP cells.

Keywords: FAS; FACL3; Vitamin D_3; 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 D_3 in both expression and activity levels and contributed to the growth inhibitory effect of vitamin D_3 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 D_3 in LNCaP cells [15]. In the current study, we report that the downregulation of FAS mRNA by vitamin D_3 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 D_3 in the human prostate cancer LNCaP cells.

2. Materials and methods

2.1. Reagents

$1\alpha,25(OH)_2D_3$ was obtained from Leo Pharmaceuticals (Ballerup, 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

* Corresponding author. Fax: +358-3-215-6170.
E-mail address: shengjun.qiao@uta.fi (S. Qiao).

Abbreviations: FAS, fatty acid synthase; FACL3, long-chain fatty-acid-CoA ligase 3; ACC, acetyl-CoA carboxylase; acyl-CoA, acyl-coenzyme A; myristoyl-CoA, myristoyl-coenzyme A

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purchased from Invitrogen (Carlshad, 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% CO_2. After grown to semi-confluence, the cells were treated with 1α,25$(OH)_2D_3$, triacsin C, cerulenin, myristic acid or the combination as indicated. For the treatment of 1α,25$(OH)_2D_3$ 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 cerulenin 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 cm^2 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 H_2O 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 H_2O) and 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 $\{N(\text{fold}) = [\text{gene}(\text{treated})/\text{GAPDH}(\text{treated})]/[\text{gene}(\text{untreated})/\text{GAPDH}(\text{untreated})]\}$. 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 D_3 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 D_3 in the human prostate cancer LNCaP cells and might have a contribution to antiproliferative effect of vitamin D_3 [15]. We also found that FACL3 mRNA and activity were increased by vitamin D_3 in LNCaP cells [14]. To investigate whether there is an association between vitamin D_3 downregulation of FAS expression and its upregulation of FACL3 or the regulations are independent on each other, cerulenin, 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 D_3, and mRNA levels were measured using real-time QPCR. The results showed that triacsin C completely abolished vitamin D_3 decrease of FAS mRNA (Fig. 1), whereas cerulenin had no significant effect on the increase in FACL3 mRNA by vitamin D_3 (Fig. 2) in LNCaP cells. Triacsin C had no effect on vitamin D_3 upregulation of FACL3 mRNA. FAS and FACL3 mRNA expressions were not affected by cerulenin or triacsin C alone. In addition, we found in the previous study that FACL3 expression was not regulated by vitamin D_3 in human prostate cancer PC3 cells [14], which are vitamin D_3 responsive and the action of vitamin D_3 is independent of androgen. We measured FAS expression in PC3 cells and found that FAS expression was not regulated by vitamin D_3 (data not shown).

Table 1
The list of primers used for QPCR analysis

Gene names	Primer sequences
FACL3	Forward primer: 5′-ACTCCACTGTGCGACAGCTTT-3′ Reverse primer: 5′-CACCACACAACAGGAGACGAA-3′
FAS	Forward primer: 5′-AACTCCAAGGACACAGTCACCAT-3′ Reverse primer: 5′-CAGCTGCTCCACGAACTCAA-3′
GAPDH	Forward primer: 5′-CCACATCGCTCAGACACCAT-3′ Reverse primer: 5′-ACCAGGCGCCCAATACG-3′

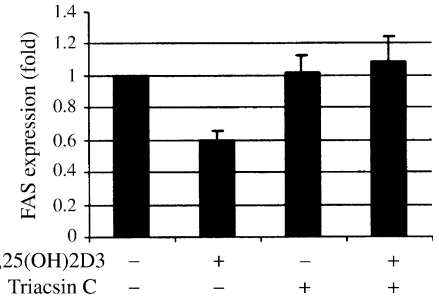

Fig. 1. Inhibition of FACL3 activity by triacsin C abolishes vitamin D_3 repression of FAS mRNA expression in the human prostate cancer LNCaP cells. LNCaP cells were grown in RPMI-1640 medium supplemented with 10% FBS and treated with 10 nM 1α,25$(OH)_2D_3$, 1 μM triacsin C or the combination of them 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$(OH)_2D_3$. No considerable effect of triacsin C alone on FAS mRNA expression was seen.

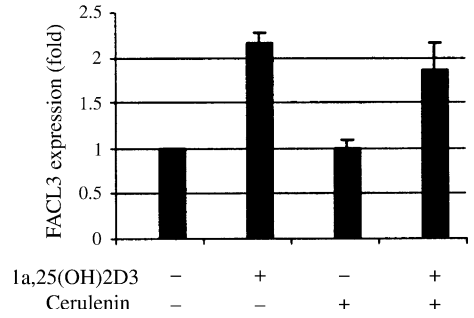

Fig. 2. Inhibition of FAS activity by cerulenin has no significant effect of vitamin D_3 upregulation of FACL3 mRNA expression in prostate cancer LNCaP cells. The cells were grown in RPMI-1640 medium supplemented with 10% FBS and treated with 10 nM $1\alpha,25(OH)_2D_3$, 5 μg/ml cerulenin or the combination of two for 24 h. After the treatments, RNA was isolated and the expression of FACL3 mRNA was measured using real-time quantitative PCR. The results represent three independent experiments and normalized to housekeeping gene GAPDH. Cerulenin showed no significant effect on vitamin D_3 upregulation of FACL3 expression.

3.2. *Formation of myristoyl-CoA is responsible for myristic acid-induced repression of FAS expression in the presence of vitamin D_3*

There are several substrates preferential for FACL3 in the synthesis of long chain fatty acyl-CoAs [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 D_3 in human prostate cancer LNCaP cells [14]. To investigate the effect of long chain fatty acyl-CoAs formed by FACL3 on vitamin D_3 inhibition of FAS mRNA expression, myristic acid and triacsin C were used to treat LNCaP cells in the presence or absence of vitamin D_3. Myristic acid showed no significant effect on vitamin D_3 repression of FAS mRNA expression at 24 h, but enhanced the decrease in FAS mRNA by vitamin D_3 at 48 h (Fig. 3). Triacsin C, an inhibitor of FACL3 activity, abrogated the effect of myristic acid on vitamin D_3 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 D_3 in LNCaP cells. Myristic acid had no considerable effect on vitamin D_3 upregulation of FACL3 expression in LNCaP cells (data not shown).

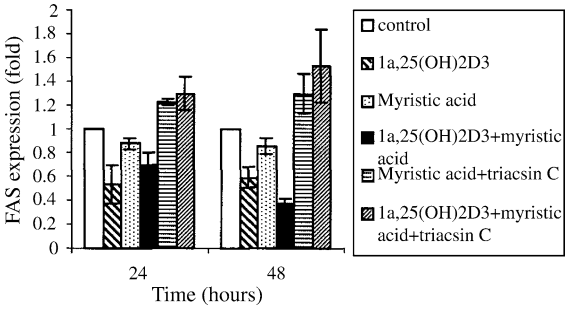

Fig. 3. Enhancement in vitamin D_3 inhibition of FAS expression by myristic acid is attributable to the formation of myristoyl-CoA in prostate cancer LNCaP cells. LNCaP cells were grown in RPMI-1640 medium supplemented with 10% FBS and treated with 10 nM $1\alpha,25(OH)_2D_3$, 10 μM myristic acid, 1 μM triacsin C or the combinations as indicated. FAS mRNA expression was measured using real-time quantitative PCR. The results represent three independent experiments and normalized to housekeeping gene GAPDH. At 24 h, myristic acid showed to attenuate slightly but not significantly the vitamin D_3-repression of FAS expression. The downregulation of FAS expression by vitamin D_3 was significantly enhanced by myristic acid at 48 h and this enhancement was abrogated by triacsin C. Myristic acid alone showed no significant effect on FAS expression.

4. Discussion

Vitamin D_3 is a potential anti-cancer compound due to its antiproliferative function. In our previous studies, we investigated the genes regulated by $1\alpha,25(OH)_2D_3$ in prostate cancer LNCaP cells using cDNA microarray and found that two fatty acid metabolic enzymes, FAS and FACL3, were regulated by vitamin D_3. The further studies suggested that FAS and FACL3 were involved in vitamin D_3 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-CoAs [5]. This led us to further investigate whether there is a relationship between the regulation of FAS and FACL3 expressions by vitamin D_3.

Inhibition of FACL3 activity by triacsin C resulted in a complete abolishment of the repression of FAS mRNA expression by vitamin D_3. Cerulenin, an inhibitor of FAS activity, had no significant effect on vitamin D_3 upregulation of FACL3 mRNA expression. Neither the effect of triacsin C on vitamin D_3 increase of FACL3 mRNA nor the effect of cerulenin on the vitamin D_3 decrease of FAS mRNA was seen. These suggest that the downregulation of FAS expression by vitamin D_3 is mediated by FACL3 in LNCaP cells. This is consistent with our previous findings that the action of vitamin D_3 on FAS expression is not direct [15]. In vitamin D_3-responsive human prostate cancer PC3 cells, which are androgen receptor-negative in contrast to LNCaP cells, no regulation of FAS expression by vitamin D_3 was found, consistent with no vitamin D_3 regulation of FACL3 expression in PC3 cells. Vitamin D_3 increases both mRNA expression and the activity of FACL3 [14]. The increase in the FACL3 activity by vitamin D_3 results in an increase in the formation of long chain fatty acyl-CoAs, 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 D_3 at 24 h, but enhanced the decrease in FAS mRNA by vitamin D_3 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 D_3 [14]. Myristic acid-induced enhancement in the vitamin D_3-repression of FAS mRNA expression was completely abolished by triacsin C, indicating that the formation of myristoyl-CoA contributes to vitamin D_3 downregulation of FAS expression. The data suggest that the downregulation of FAS mRNA by vitamin D_3 is the result of feedback inhibition of FAS expression by long chain fatty acyl-CoAs formed during vitamin D_3-upregulation of FACL3 in LNCaP cells.

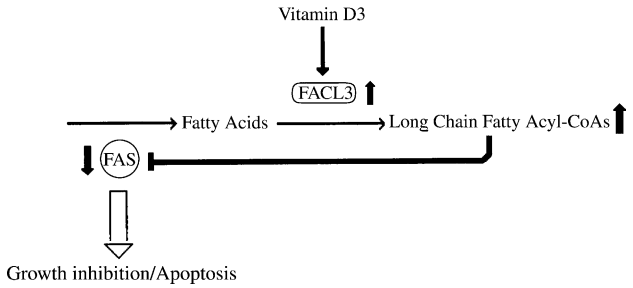

Fig. 4. Schematic illustration of the proposed mechanism of vitamin D_3 inhibition of FAS expression in human prostate cancer LNCaP cells. FACL3 expression is upregulated by vitamin D_3, increased FACL3 expression enhances the formation of long chain fatty acyl-CoAs, which regulate FAS expression by means of feedback inhibition, resulting in the decrease in FAS expression which regulates cell growth and apoptosis.

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 D_3 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-CoAs 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 D_3 and contributed to the inhibitory effect of vitamin D_3 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 D_3 decrease FAS mRNA. This suggests that the contribution of FACL3 to vitamin D_3 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 D_3 antiproliferative effect. Taken together, we conclude that the upregulation of FACL3 by vitamin D_3 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 D_3 (Fig. 4).

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