Curcumin induces human cathelicidin antimicrobial peptide gene expression through a vitamin D receptor-independent pathway

Chunxiao Guo[a,b], Elena Rosoha[a,b], Malcolm B. Lowry[a,c], Niels Borregaard[d], Adrian F. Gombart[a,b*]

[a]Linus Pauling Institute, [b]Department of Biochemistry and Biophysics, [c]Department of Microbiology, Oregon State University, Corvallis, OR 97331

[d]The Granulocyte Research Laboratory, Department of Hematology, National University Hospital, Copenhagen, Denmark

*Corresponding author: Adrian F. Gombart, Linus Pauling Institute, Department of Biochemistry and Biophysics, Oregon State University, 307 Linus Pauling Science Center, Corvallis, OR 97331, tel. 541-737-8018, fax 541-737-5077

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Abstract

The vitamin D receptor (VDR) mediates the pleiotropic biologic effects of 1α,25 dihydroxy-vitamin D_3. Recent *in vitro* studies suggested that curcumin and poly-unsaturated fatty acids (PUFAs) also bind to VDR with low affinity. As potential ligands for the VDR, we hypothesized that curcumin and PUFAs would induce expression of known VDR target genes in cells. In this study, we tested whether these compounds regulated two important VDR target genes - human cathelicidin antimicrobial peptide (*CAMP*) and 1,25-dihydroxyvitamin D_3 24-hydroxylase (*CYP24A1*)- in human monocytic cell line U937, colon cancer cell line HT-29 and keratinocyte cell line HaCaT. We demonstrated that PUFAs failed to induce *CAMP* or *CYP24A1* mRNA expression in all three cell lines, but curcumin up-regulated *CAMP* mRNA and protein levels in U937 cells. Curcumin treatment induced *CAMP* promoter activity from a luciferase reporter construct lacking the VDR binding site and did not increase binding of the VDR to the *CAMP* promoter as determined by chromatin immunoprecipitation assays. These findings indicate that induction of *CAMP* by curcumin occurs through a vitamin D receptor-independent manner. We conclude that PUFAs and curcumin do not function as ligands for the VDR.

1. Introduction

The nuclear receptor superfamily is divided into four groups based on whether the receptor forms a homo- or heterodimer complex and what class of ligand is bound [1]. The endocrine receptors form homodimers and bind steroid hormones produced by endocrine tissues. The xenobiotic receptors function as heterodimers with retinoid-X-receptor (RXR) and bind to xenobiotic compounds, dietary lipids and cholesterol metabolites. The third group forms heterodimers with RXR and binds to thyroid hormone and vitamins A and D while the orphan receptor group lacks known ligands [1]. The vitamin D receptor (*VDR, NR1I1*) is widely expressed in most, if not all, human tissues and possesses characteristics of both the second and third groups [2]. It serves as the receptor for 1α, 25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] which binds with high affinity and for the secondary bile acid lithocholic acid (LCA) that binds with low affinity [3]. Vitamin D is obtained either from food, supplementation or synthesized in the skin by UVB irradiation of 7-dehydrocholesterol [4]. LCA is a secondary bile acid converted from primary bile acids by gut microbiota [5]. Upon engagement of a ligand, VDR forms a heterodimer with RXR and binds to vitamin D response elements (VDREs) present in about 2000 genomic locations and directly regulates approximately 200 genes [6, 7]. Target genes of the VDR contribute to bone mineral homeostasis, detoxification of exogenous and endogenous compounds, cancer prevention, mammalian hair cycling and immune function [8].

The ability of the VDR to bind LCA suggests that it may interact with other novel ligands. To identify additional VDR ligands, Jurutka and colleagues used a mammalian two-hybrid system to

test high concentrations of curcumin (CM) and the polyunsaturated fats (PUFAs) docosahexaenoic acid (DHA), eicosapentaenoic acid (EPA), arachidonic acid (AA), and linolenic acid (LA) [9]. These compounds promoted the dimerization of VDR and RXR suggesting that they may function as novel low-affinity ligands for the VDR [9]. More recently, curcumin was shown to induce expression of the VDR target genes *CYP24A1*, *CYP3A4*, *TRPV6* and *CDKN1A* in the human colon cancer cell line Caco-2 [10].

The human cathelicidin antimicrobial peptide (*CAMP*) gene encodes the hCAP18 pro-protein that is cleaved to release the active peptide LL-37. The *CAMP* gene is directly regulated by binding of the VDR to a VDRE located in its promoter region [11]. Expression of the human *CAMP* mRNA and hCAP18 is strongly induced by both $1,25(OH)_2D_3$ and LCA in keratinocytes and myeloid leukemia cell lines [11, 12]. Induction of the *CAMP* gene by LCA requires a 1000-fold higher concentration of LCA than $1,25(OH)_2D_3$ (1×10^{-5} versus 1×10^{-9} M, respectively) as it is a low-affinity ligand for the VDR . We hypothesized that if CM and PUFAs are low-affinity ligands for the VDR then at high concentrations they may induce the human *CAMP* gene in cells via activation of the VDR. In this study, we showed that PUFAs did not act as VDR ligands and were unable to increase expression of the *CAMP* gene in keratinocyte, colon and myeloid cell lines, but CM acted through a VDR-independent pathway to increase *CAMP* expression.

2. Material and methods

2.1. Compounds

Curcumin (C7727-500MG), cis-4,7,10,13,16,19-docosahexaenoic acid (D2534), cis-5,8,11,14,17-eicosapentaenoic acid (E2011), arachidonic acid (A9673), linolenic acid (L2376), were purchased from Sigma Aldrich (St. Louis, MO, USA).

2.2. Cell culture

Colonic epithelial cell line HT-29 was kindly provided by Dr. Rod Dashwood (Oregon State University, Corvallis, OR). The human monocytic U937 and the keratinocyte HaCaT cell lines were a generous gift from Dr. H. Phillip Koeffler (Cedars-Sinai Medical Center, Los Angeles, CA). U937 cells were maintained in RPMI 1640 medium and HT-29 and HaCaT cells were maintained in DMEM medium (Mediatech Inc., Manassas, VA, USA). All media were supplemented with 10% (v/v) heat-inactivated FBS, 2 mM L-glutamine, and 1% Pen/Strep (Invitrogen Corporation, Carlsbad, CA, USA). Cell cultures were incubated at 37°C in a humidified 5% CO_2 incubator.

2.3. Quantitative real-time PCR (qRT-PCR)

U937, HaCaT and HT-29 cells were treated with compounds as described in the figure legends. Total RNA was isolated using the SV Total RNA Isolation System according to the manufacturer's protocol (Promega Corporation, Madison, WI, USA). RNA (1-2 μg) was converted to cDNA using SuperScript III reverse transcriptase and random hexamer primers (Invitrogen Corporation) according to the manufacturer's recommendations. PCR reactions were set up as described previously [11]. PCR was performed on a Bio-Rad iCycler iQ5 or CFX-96 QPCR system (Bio-Rad Laboratories, Hercules, CA, USA). All the threshold cycle (C_t) numbers

were normalized to 18S rRNA. The probes and primers for the human *CAMP*, *CYP24A1*, *FABP4* and *RN18S1* genes used for qRT-PCR are described in Table 1.

2.4. Intracellular staining, fluorescence activated cell sorting and enzyme-linked immunosorbent assay

U937 cells were treated as indicated in the figure legends. Cells were fixed, permeabilized and blocked using the eBioscience™ Fixation and Permeabilization Kit as described by the manufacturer (eBioscience, Inc., San Diego, CA, USA). Cells were incubated with a rabbit, anti-hCAP18 polyclonal antibody [14] and a Dylight 649 Fab' 2 donkey anti-rabbit antibody (Jackson Immunoresearch, Pike West Grove, PA, USA). Fluorescence activated cell sorting (FACS) was performed on a BD FACSCalibur flow cytometer (BD Biosciences, San Jose, CA, USA) and the results were analyzed by BD CellQuest™ Pro software (BD Biosciences). The enzyme-linked immunosorbent assay (ELISA) was performed as described previously [14].

2.5 CAMP promoter luciferase reporter assay

U937 cells were electroporated using a NEON^{TM} transfection system (Life Technologies, Grand Island, NY, USA) in Tip100 tips at 5×10^7 cells/mL. Electroporation conditions were 1400 mV, 30ms, 1 pulse. A total of 10 µg plasmid was used per electroporation. After transfection, cells were treated with CM or $1,25(OH)_2D_3$ or vehicle as indicated in the figure legends. Cells were lysed and dual luciferase assays were performed as described by the manufacturer (Promega, Madison, WI, USA). The human *CAMP* promoter (nucleotides –693 to 14) containing the VDRE and a 5' deletion of the promoter (ΔHindIII, nucleotides –497 to 14) lacking the VDRE were subcloned into a pXP2 firefly luciferase reporter plasmid previously [11]. A renilla

luciferase reporter (phTKRL, Promega) was co-transfected to normalize firefly luciferase activities in all experiments.

2.6. Chromatin-Immunoprecipitation Assay

Chromatin-immunoprecipitation (ChIP) experiments were performed as described previously [15]. Briefly, U937 cells (10^7 cells/IP) were treated with compounds as specified in the figure legend for 24 hours. Cells then were fixed with 1% (v/v) formaldehyde for 10 minutes at room temperature and quenched by 0.1 M glycine for 5 minutes. Fixed chromatin was sheared to 200-1000 bp fragments by a bath sonicator (Bioruptor™ XL, Diagenode Inc. Denville, NJ) following the manufacturer's recommendations. Anti-VDR antibodies (2 μg C-20 VDR antibody, sc-1008; 2 μg N-20 VDR antibody, sc-1009, Santa Cruz Biotechnology, Santa Cruz, CA) were incubated with sheared chromatin for 16 hours at 4°C. Immunocomplexes were pulled down by Protein A/G Plus Agrose beads (sc-2003, Santa Cruz Biotechnology, Santa Cruz, CA) and DNA was recovered using Chelex® 100 resin (Bio-Rad, Hercules, CA). To evaluate the VDR occupancy at the human *CAMP* gene promoter, quantitative PCR was performed as described in section 2.3. Occupancy by VDR was normalized with respect to chromatin input used for immunoprecipitation. Primers and probe are listed in Table 1.

2.7. Data analysis

All qRT-PCR and ELISA experiments were performed in triplicate or duplicate and results were represented as mean value with SD. Student's t-test was performed using Sigma Plot (Systat Software, San Jose, CA) and Microsoft Excel (Microsoft Corporation, Redmond, WA).

3. Results

3.1. CAMP gene expression is induced by curcumin but not PUFAs

The human *CAMP* and *CYP24A1* are known target genes of the VDR and induced by $1,25(OH)_2D_3$ and LCA [16, 17]. We predicted that compounds that function as low-affinity ligands for the VDR would induce expression of these two genes. To test this, we treated U937 (Fig. 1 A & B), HT-29 (Fig. 1 C & D) and HaCaT (Fig. 1 E & F) cells with CM, DHA, EPA, AA and LA for 24 hours. $1,25(OH)_2D_3$ and LCA were included as positive controls and vehicle (ethanol or DMSO) was used for the untreated control. Because $1,25(OH)_2D_3$ does not induce *CAMP* strongly in HT-29 cells, sodium butyrate (NaB), a known inducer of *CAMP* in colon cancer cell lines, was included for experiments with HT-29 (Fig. 1 C & D)[18]. $1,25(OH)_2D_3$ and LCA strongly induced expression of both the *CAMP* (Fig. 1 A & E) and *CYP24A1* (Fig. 1 B & F) genes in U937 and HaCaT cells. In HT-29 cells *CAMP* was not strongly induced by $1,25(OH)_2D_3$ or LCA, but was induced about four-fold by NaB (Fig. 1 C). *CYP24A1* expression was induced by LCA and $1,25(OH)_2D_3$ in all cells tested (Fig. 1 B, D & F). The PUFAs (DHA, EPA, AA and LA) did not induce human *CAMP* or *CYP24A1* expression in U937, HaCaT or HT-29 cells (Fig. 1 A-F). CM consistently induced expression of human *CAMP* by about 3-fold ($n=3$, $P<0.05$) in U937 and HT-29 cells (Fig. 1 A & C) but not in HaCaT cells (Fig. 1 E). In all three cell lines, CM did not induce *CYP24A1* (Fig. 1 B, D & F). To demonstrate that the PUFAs used in this study were active, we examined expression of *FABP4*, a gene induced by PUFAs binding the PPARγ receptor in monocytes [19]. *FABP4* expression was induced in U937 cells demonstrating that the compounds were functional (Fig. 2). To ensure that induction of CAMP or CYP24A1 did not peak prior to 24 hours, we tested CM and DHA in a time course experiment (0, 3, 6, 12 and 24 hours) and observed maximal induction of *CAMP* by CM at 24 hours and no

induction by DHA (data not shown). Collectively, these data indicate that PUFAs do not act as low-affinity agonists for the VDR and that CM induces *CAMP*, but not *CYP24A1*.

3.2. CM elevates hCAP18 levels

Treatment of U937 cells with 10 nM $1,25(OH)_2D_3$ increases levels of hCAP18 (the protein encoded by the human *CAMP* gene) secreted into the medium [11]. We monitored secreted levels of hCAP18 in the medium by ELISA [11]. As expected, 10 nM $1,25(OH)_2D_3$ increased secretion of hCAP18 into the medium; however, treatment with 100 μM LCA and 1 nM $1,25(OH)_2D_3$, which induce *CAMP* mRNA expression to similar levels, did not enhance hCAP18 secretion and neither did CM nor the PUFAs (Fig. 3). These results suggest that modest increases in *CAMP* mRNA levels may not lead to secretion of hCAP18 proteins in U937 cells.

To determine if induction of *CAMP* mRNA by CM would increase intracellular hCAP18 expression, U937 cells were treated with either 15 μM CM, 100 μM DHA, 100 μM EPA, 100 μM AA, 100 μM LA, 100 μM LCA or 10 nM $1,25(OH)_2D_3$ for 24 hours. The hCAP18 levels were measured by intracellular staining and FACS. The PUFAs did not increase hCAP18 levels (data not shown). CM increased the intracellular hCAP18 levels, however, they were lower than those induced by LCA and $1,25(OH)_2D_3$ (Fig. 4).

3.3. CM does not enhance $1,25(OH)_2D_3$ induction of CAMP expression

It was shown previously that treatment of Caco-2 cells with CM and $1,25(OH)_2D_3$ resulted in a combinatorial activation of a transfected VDRE-Luc reporter construct [9, 10]. To determine if CM plus $1,25(OH)_2D_3$ would activate the *CAMP* gene better than either compound alone, we

treated U937 cells with 15 μM CM and increasing doses of $1,25(OH)_2D_3$. The *CAMP* mRNA levels were evaluated by qRT-PCR (Fig. 5). CM increased *CAMP* mRNA levels by 2.6-fold while 0.1 nM vitamin D induced *CAMP* by 6.5-fold. The combination induced *CAMP* by 5.5-fold indicating no combinatorial activation of the gene. This lack of combinatorial activation was observed with 1 nM and 10 nM $1,25(OH)_2D_3$, as well (Fig. 5).

3.4 Induction of the *CAMP* gene by CM does not require the VDRE in the CAMP promoter.

We predicted that if CM induced *CAMP* through the VDR, then deletion of the VDRE in the *CAMP* promoter should abrogate the induction. We transfected *CAMP* promoter firefly luciferase reporters with or without the presence of the VDRE (pXP2-*CAMP*-luc and pXP2-*CAMP*-ΔHindIII-luc, respectively, Fig. 6 A) into U937 cells. Consistent with our previous report [11], deletion of the VDRE in the *CAMP* promoter almost completely abolished induction of luciferase activity by 10 nM $1,25(OH)_2D_3$ (Fig. 6 B). On the other hand, CM was still capable of increasing *CAMP* promoter activity in the absence of the VDRE in the promoter (Fig. 6 B). 10 μM CM induced the luciferase activities by about two-fold regardless of the presence or absence of the VDRE. From these experiments, we concluded that induction of the *CAMP* gene by CM does require the VDRE.

3.5 CM does not increase VDR binding to the *CAMP* gene promoter

CM does not appear to function as a ligand for the VDR, thus we predicted that it would not increase VDR binding to the human *CAMP* gene promoter. To test this, we performed ChIP for VDR in U937 cells treated with CM, LCA and$1,25(OH)_2D_3$ (Fig. 7). We found that VDR binding to the *CAMP* promoter was increased with $1,25(OH)_2D_3$, and LCA treatment and not by

CM (Fig. 7), strongly suggesting that CM-induced human *CAMP* expression occurs through a VDR- independent mechanism.

4. Discussion

VDR agonists are of great interest because of their potential therapeutic benefits in treating cancer, psoriasis and other diseases [20-24]. Thousands of analogs have been synthesized around the vitamin D backbone to reduce or eliminate its hypercalcemic side effects [25]. Another class of VDR agonists is secondary bile acid LCA and its analogs [26] that activate VDR target genes without inducing hypercalcemia [17]. The identification of new agonists increases the toolbox of backbones upon which additional analogs can be developed. To this end, we tested a group of potential VDR ligands identified by a mammalian two hybrid system [9]. We showed that CM modestly induced CAMP, but not CYP24A1 expression and that PUFAs did not induce the mRNA levels these two VDR target genes in human monocyte (U937), keratinocyte (HaCaT) or colon cancer (HT-29) cell lines. These results suggest these compounds are not functional VDR agonists. On the other hand, the known ligands, LCA and $1,25(OH)_2D_3$ strongly induced both genes. Of the putative ligands tested, only CM increased intracellular levels of hCAP18. This induction was observed in three of four experiments and was less than either LCA or $1,25(OH)_2D_3$. The modest induction of CAMP by CM did not appear to occur through the VDR. ChIP experiments showed that VDR binding to the *CAMP* promoter was not increased by CM as it is by both LCA and $1,25(OH)_2D_3$. Furthermore, we demonstrated by reporter assays that CM activated the *CAMP* promoter in the absence of the VDRE.

CM at the concentration we used can elicit ER stress [27] and a recent study showed ER stress induces human *CAMP* expression in keratinocytes [28]. We tested whether ER stress induced *CAMP* in our cell lines and were unable to demonstrate a role for this mechanism (data not shown); therefore, ER stress elicited by CM is not a likely mechanism for induction of human *CAMP* gene expression in our study. Collectively, these data argue that CM and PUFAs are not low affinity ligands for the VDR and CM activates *CAMP* expression by a currently unknown mechanism(s).

The discrepancy between our work and the previous study [9] could be attributed to several factors. First, recent molecular docking studies proposed that two ligand binding pockets exist in the VDR ligand binding domain: the genomic and alternative pockets. Vitamin D and its metabolites are ligands of the genomic pocket while CM is proposed to mainly bind to the alternative pocket [29]. Therefore, in the mammalian two hybrid system, the possible binding of CM to the alternative pocket may have increased VDR/RXR dimerization; however, since CM was a weak ligand of the genomic pocket, it did not activate transcription of VDR target genes in our cell culture experiments. Second, prior studies demonstrated that CM regulated the VDR target gene CYP24A1 in Caco-2 cells [10]. We did not observe this in U937, HaCaT or HT-29 cells, suggesting that modulation of VDR target genes by CM could be specific to the type of cell used.

Future experiments determining the crystal structure of the VDR/CM complex may further define the role of CM as a VDR alternative pocket ligand. Also, additional studies in other cell

lines may be required to comprehensively understand the possible function of CM and PUFAs as VDR ligands.

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Figure Legends

Figure 1. Alternative VDR ligands fail to activate the human *CAMP* gene. U937 cells (A, B), HT-29 cells (C, D) and HaCaT cells (E, F) were treated with 10 μM curcumin (CM), 100μM docosahexaenoic acid (DHA), 100 μM eicosapentaenoic acid (EPA), 100 μM arachidonic acid (AA) , 100 μM linolenic Acid (LA), 100 μM lithocholic acid (LCA) and 1 nM $1,25(OH)_2D_3$ for 24 hours. For HT-29 cells, 2 mM sodium butyrate (NaB) was used as positive control since $1,25(OH)_2D_3$ is not a potent inducer of CAMP in these cells. qRT-PCR analysis of human *CAMP* (A, C, and E) and *CYP24A1* (B, D and F) mRNA levels were normalized to 18S rRNA. Each panel is from one experiment, but is representative of three independent experiments. *Significant ($P < 0.05$) difference compared with untreated control.

Figure 2. PUFAs and CM induced FABP4 expression in U937 cells. U937 cells were treated with 10 μM CM, 100 μM DHA, 100 μM EPA, 100 μM AA and 100 μM LA for 24 hours. *FABP4* mRNA levels were measured by qRT-PCR using primers and probe as described in Table 1. *Significant ($P < 0.05$) difference compared with untreated control.

Figure 3. Curcumin and PUFAs do not increase levels of secreted hCAP18. U937 cells were treated with 10 μM CM, 100 μM DHA, 100 μM EPA, 100 μM AA, 100 μM LA, 100 μM LCA or $1,25(OH)_2D_3$ (1 nM and 10 nM) for 24 hours. Culture medium was collected and subjected to ELISA to measure extracellular hCAP18 protein levels. *Significant ($P < 0.01$) difference compared with untreated control.

Figure 4. CM increases intracellular levels of hCAP18. U937 cells were treated with 10 μM CM, 100 μM LCA and 1 nM $1,25(OH)_2D_3$ for 24 hours. Intracellular hCAP18 levels were assessed by flow cytometry. This panel is from one experiment, but is representative of four independent experiments.

Figure 5. CM does not cooperatively increase CAMP expression by $1,25(OH)_2D_3$. U937 cells were treated with 15 μM CM in the absence or presence of increasing concentrations of $1,25(OH)_2D_3$ for 24 hours. This data are representative of two independent experiments. Levels of *CAMP* expression were measured by qRT-PCR using primers and probe as described in Table 1.

Figure 6. CM induces CAMP promoter activity in absence of VDRE. A) Schematic diagrams show the structures of the two *CAMP* promoter-luciferase reporter constructs used in this study. Solid filled black box indicates the location of the VDRE in the *CAMP* promoter. B) U937 cells were electroporated with pXP2-CAMP-luc or pXP2-CAMP-ΔHindIII-luc plasmid and then treated with 10 μM CM, 10 nM $1,25(OH)_2D_3$ or vehicle for 20 hours. Data were presented as fold changes over the corresponding untreated control. *Significant ($P < 0.05$, n=3) difference compared with untreated control. This bar chart summarizes three independent experiments.

Figure 7. CM does not enhance VDR binding to the human CAMP promoter. U937 cells were treated with 10 μM CM, 100 μM LCA and 10 nM $1,25(OH)_2D_3$ for 24 hours. Chromatin-IP

was performed as described in section 2.5. The panel represents two independent experiments. *Significant ($P < 0.05$) difference compared with untreated control.

Table 1

Table 1. Primers and probes used for qRT-PCR

Gene	Primer Sequence	Probe Sequence
CAMP	F 5'-GCTAACCTCTACCGCCTCCT -3' R 5'-GGTCACTGTCCCCATACACC -3'	5'-FAM-ACCCCAGGCCCACGATGGAT-BHQ1-3'
CYP24A1	F 5'-GAACGTTGGCTTCAGGAGAA -3' R 5'-TATTTGCGGACAATCCAACA -3'	5'-FAM-TGCGCATCTTCCATTTGGCG-BHQ1-3'
FABP4	F 5'-AGCACCATAACCTTAGATGGGG -3' F 5'- CGTGGAAGTGACGCCTTTCA -3'	5'-FAM-ATTCCACCACCAGTTTATCATCCTCTCGT-BHQ1-3'
CAMP CHIP	F 5'-GGGCAACTTGTCCCTTGCAAGAG-3' F 5'-TGAAAATTAGCCACGCATGA-3'	5'-FAM-CTCTAGGTTGGGGGTGGCTACTGTCTTCAT-BHQ1-3'
RN18S1	F 5'-AAACGGCTACCACATCCAAG -3' R 5'-CCTCCAATGGATCCTCGTTA -3'	5'-FAM-AGCAGGCGCGCAAATTACCC-BHQ1-3'

Figure 1

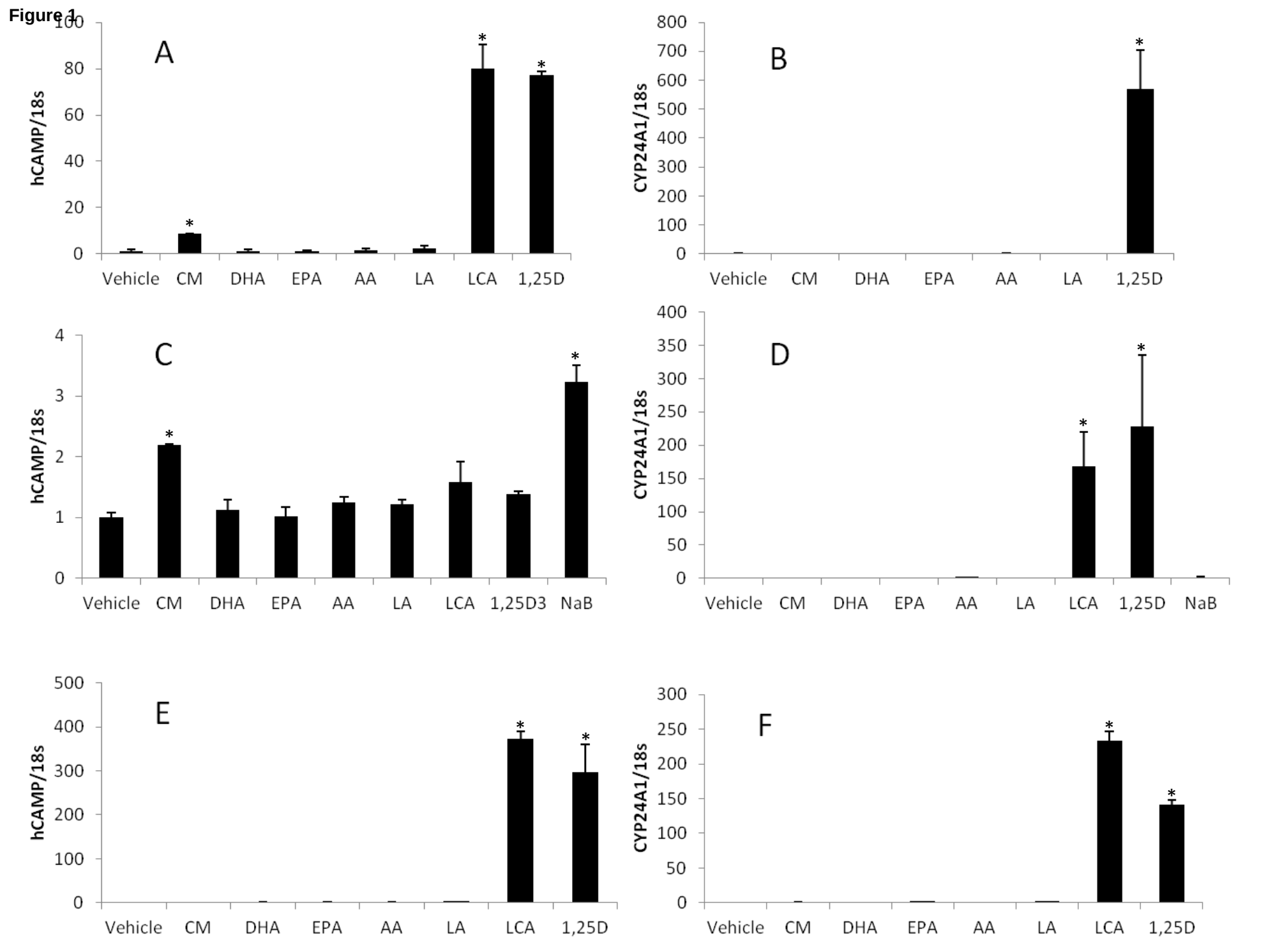

A
hCAMP/18s
100
80
60
40
20
0
Vehicle
CM
DHA
EPA
AA
LA
LCA
1,25D
B
CYP24A1/18s
800
700
600
500
400
300
200
100
0
Vehicle
CM
DHA
EPA
AA
LA
1,25D
C
hCAMP/18s
4
3
2
1
0
Vehicle
CM
DHA
EPA
AA
LA
LCA
1,25D3
NaB
D
CYP24A1/18s
400
350
300
250
200
150
100
50
0
Vehicle
CM
DHA
EPA
AA
LA
LCA
1,25D
NaB
E
hCAMP/18s
500
400
300
200
100
0
Vehicle
CM
DHA
EPA
AA
LA
LCA
1,25D
F
CYP24A1/18s
300
250
200
150
100
50
0
Vehicle
CM
DHA
EPA
AA
LA
LCA
1,25D

Figure 2

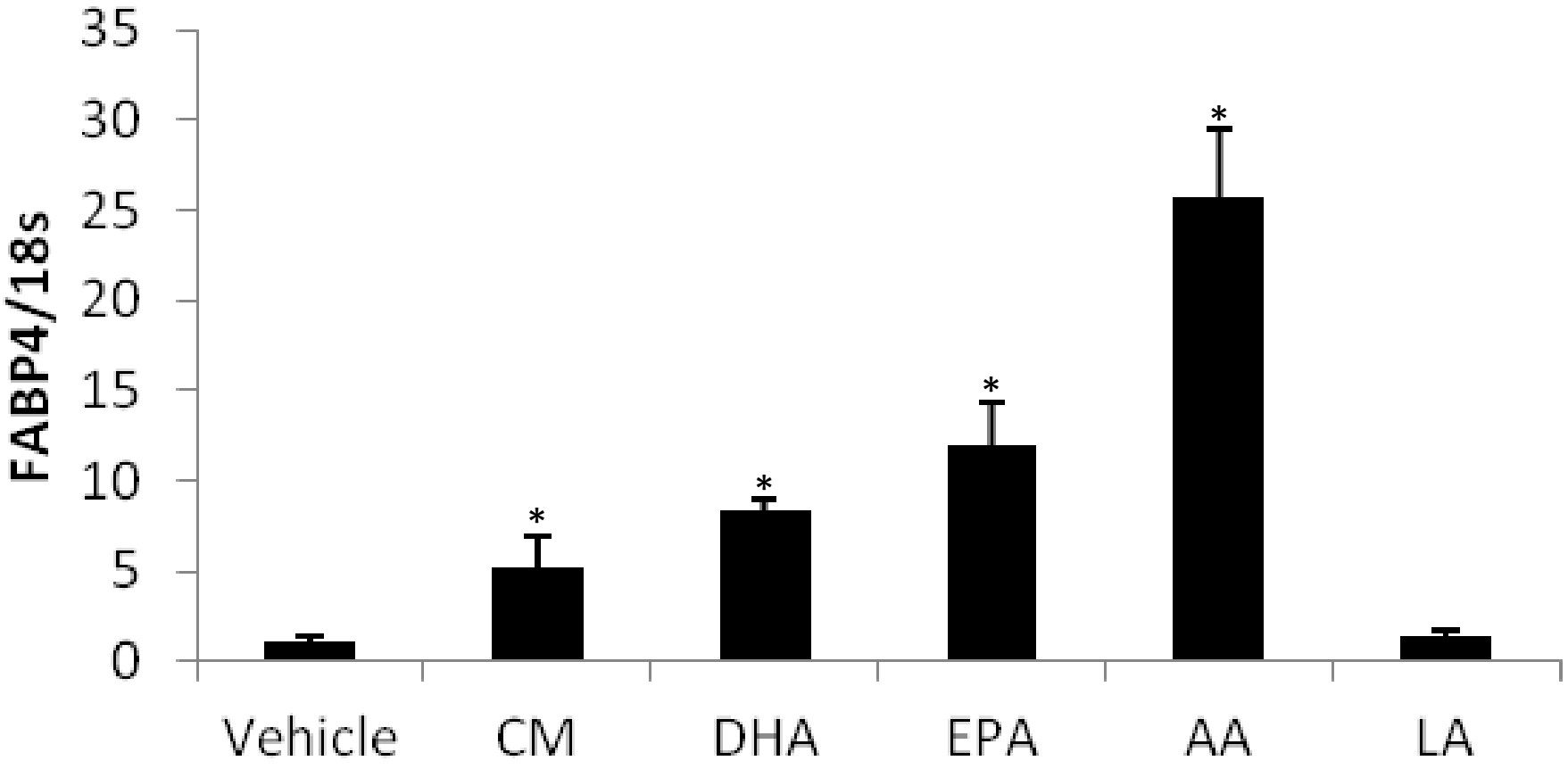


Figure 3

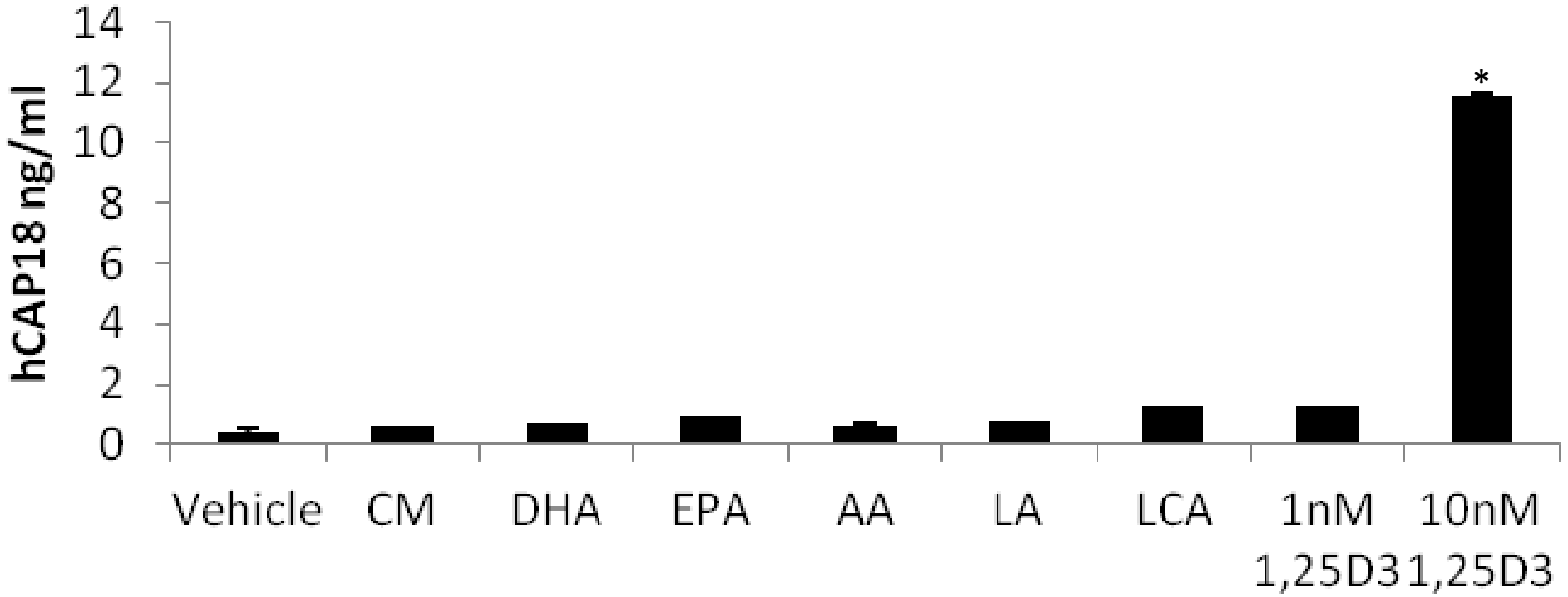

hCAP18 ng/ml
14
12
10
8
6
4
2
0
*
Vehicle
CM
DHA
EPA
AA
LA
LCA
1nM 1,25D3
10nM 1,25D3

Figure 4

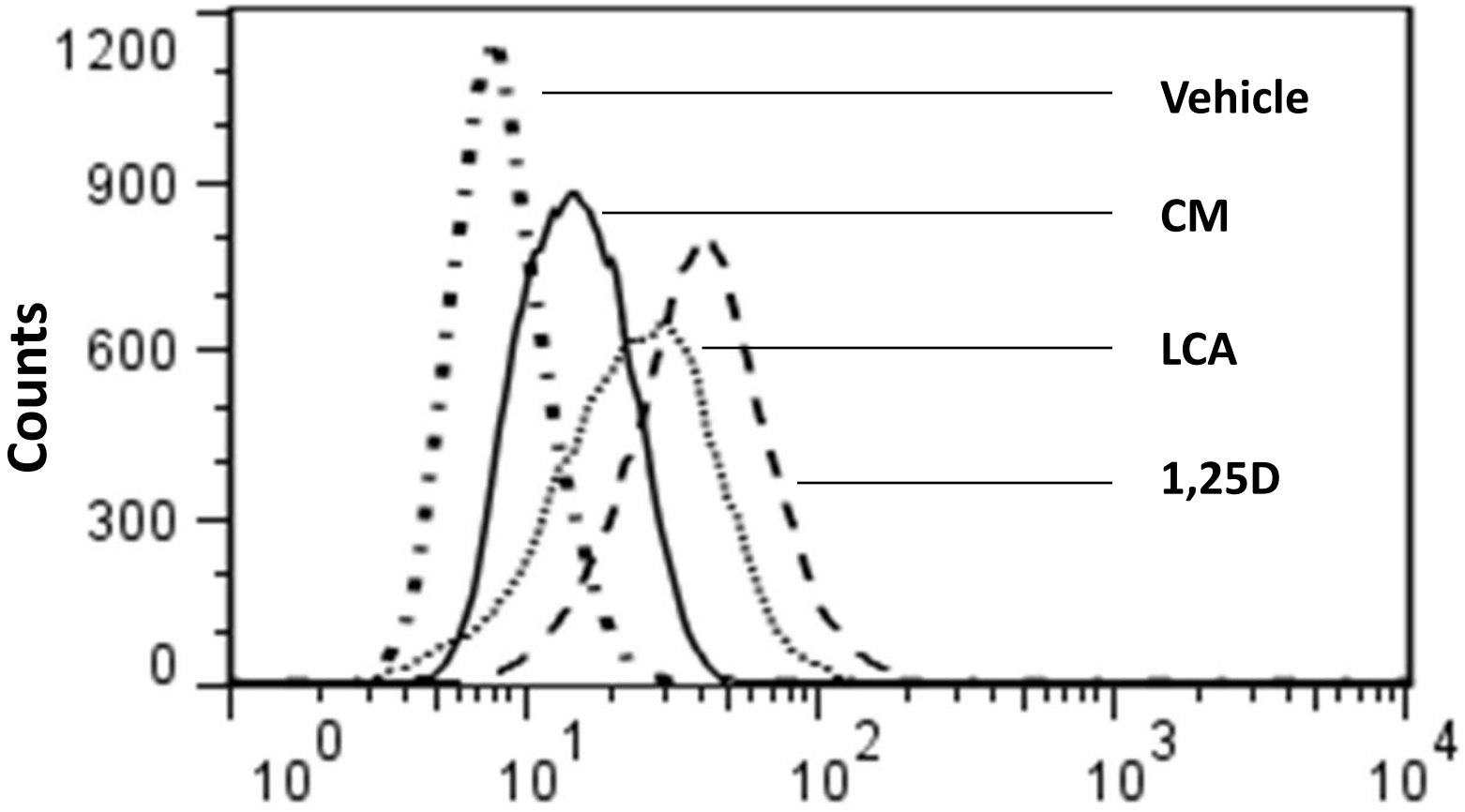

Counts
1200
900
600
300
0
10^0
10^1
10^2
10^3
10^4
Vehicle
CM
LCA
1,25D

Figure 5

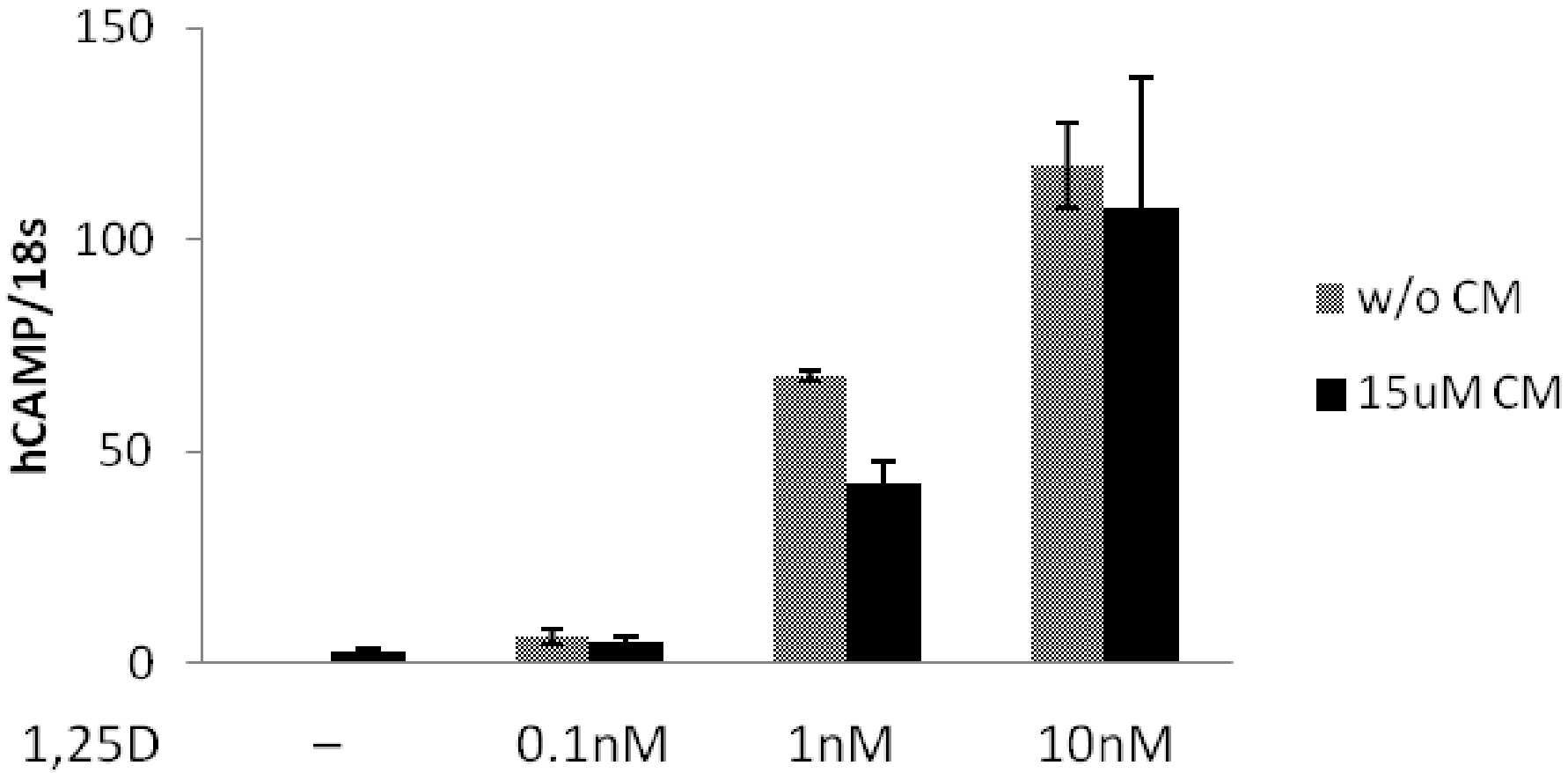


Figure 6

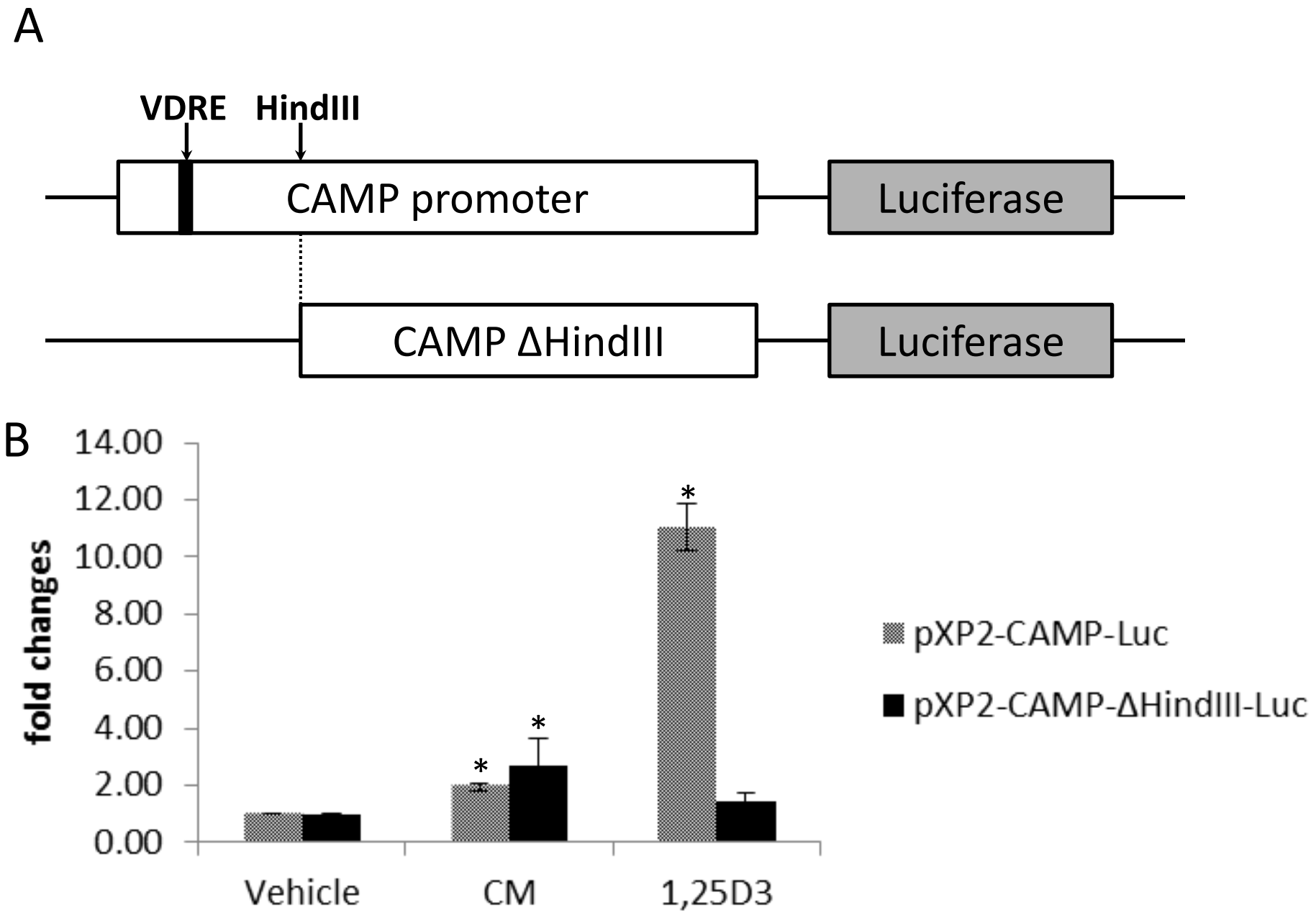
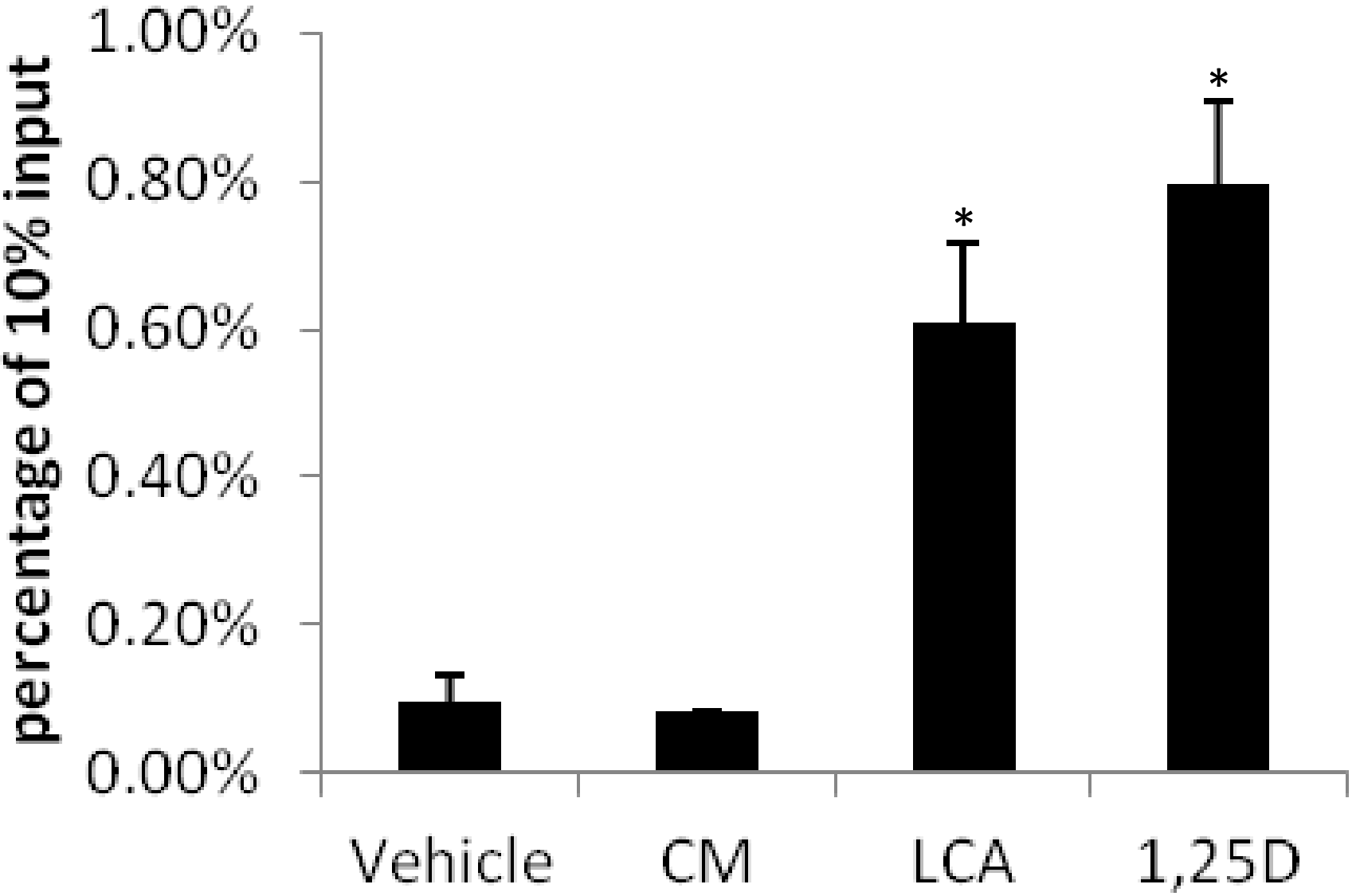

A
VDRE
HindIII
CAMP promoter
Luciferase
CAMP ΔHindIII
Luciferase
B
fold changes
14.00
12.00
10.00
8.00
6.00
4.00
2.00
0.00
Vehicle
CM
1,25D3
pXP2-CAMP-Luc
pXP2-CAMP-ΔHindIII-Luc

Figure 7


percentage of 10% input
1.00%
0.80%
0.60%
0.40%
0.20%
0.00%
*
*
Vehicle
CM
LCA
1,25D