1,25-Dihydroxyvitamin D₃ inhibits growth of the breast cancer cell line MCF-7 and downregulates cytochrome P4501B1 through the COX-2/PGE2 pathway

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Abstract. Cytochrome P4501B1 (CYP1B1) is responsible for tumor progression in estrogen receptor-positive breast cancer due to its key role in estrogen metabolism, which is upregulated by PGE2, the main product of COX-2 that is found to be overexpressed in many breast tumors. Previous studies reported that inhibition of the COX-2/PGE2 pathway, by 1,25-dihydroxyvitamin D₃ in MCF-7 breast cancer cells. The aim of this study was to investigate if the CYP1B1 protein expression shows covariation with the COX-2 and phosphorylated ERα (p-ERα) in human breast cancer. We also investigated whether 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] downregulates CYP1B1 via the COX-2/PGE2 pathway in MCF-7 cells. We analyzed the expression of COX-2, p-ERα and CYP1B1 using an immunohistochemical staining assay. In the present study, COX-2 was correlated to p-ERα ($P<0.001$) and CYP1B1 ($P=0.001$), p-ERα was correlated to CYP1B1 ($P=0.012$). We assessed the effects of 1,25(OH)₂D₃ on MCF-7 cells. 1,25(OH)₂D₃ treatment inhibited MCF-7 cell growth in a time-and dose-dependent manner; the cell cycle was arrested in the G0/G1 phase. Treatment with 100 nmol/l 1,25(OH)₂D₃ for 72 h significantly decreased the expression of COX-2 mRNA in MCF-7 cells ($P<0.05$), decreased the levels of PGE2 in cell culture supernatant ($P<0.01$), and downregulated p-ERK, p-ERα and CYP1B1 protein expression ($P<0.05$). Taken together, these results suggest that the COX-2/PGE2 pathway positively regulates the expression of CYP1B1 in breast cancer. 1,25-Dihydroxyvitamin D₃ inhibits the growth of MCF-7 cells and downregulates CYP1B1 mediated by the COX-2/PGE2 pathway.

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Introduction

Cancer Statistics 2010 indicated that breast cancer incidence is the highest one in female cancer (counts for 23%) and the main cause of cancer death for women (counts for 14%) (1). Large number of epidemiological studies (2) have shown that, breast cancer incidence or mortality was significantly negatively correlated with sun exposure, suggested that lacking of sunlight causes the reduction of vitamin D synthesis which in turns closely related with the disease; additionally, there is evidence that (3) high levels of plasma vitamin D can reduce the risk of breast cancer. Vitamin D is a multifunctional hormone, besides its indispensable role in calcium and phosphorus metabolism, recent research have found that it can inhibit a variety of tumor cells. New research shows that vitamin D has anti-inflammatory and anticancer mechanisms (4).

The role of inflammatory microenvironment in tumor development has been paid increased attention. The interation of the amounts of inflammatory cytokines, growth factors and the oncogene activation engage in fast induction of cyclo-oxygenase-2 (COX-2) expression during carcinogenesis, COX-2 affects tumor progression by participating in the malignant proliferation, invasion and metastasis. COX-2 is overexpressed in a variety of malignancies, including breast cancer, which is estrogen-dependent. It has been reported that (5) once COX-2/PGE2 pathway was activated, it can elevate the activity of estrogen synthesis enzyme, thus in turn increase the level of estrogen in breast cancer, correspondingly promoting breast cancer development. In 2010, a study (6) was reported on the adjustment of cytochrome P4501B1 (CYP1B1) expression by PGE2 in breast cancer cells. CYP1B1 belongs to the cytochrome P450 (CYP) super-gene family and is the only member of CYP1B subtribe, its main function is to catalyze hydroxylation of estrogen, generating the genotoxic carcinogens. These results indicated that, there exists an important relationship between COX-2/PGE2 pathway and the carcinogenic effect of estrogen-metabolizing enzymes (CYP1B1). The anti-inflammatory and anticancer effect of vitamin D is relevant to its negative regulation of CYP1B1 expression followed by interference with the COX-2/PGE2 pathway.

In this study, human breast cancer tissues from different patients and estrogen receptor α (ERα)-positive breast cancer

cell line MCF-7 were used. First we investigated the protein expression of COX-2, p-ERα and CYP1B1 in human breast cancer tissue samples for analyzing the relevance between each of them; besides, 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] which is the active metabolite of vitamin D, was used to treat MCF-7 cells. After treatment, the expression of COX-2, PGE2, p-ERK, p-ERα and CYP1B1 were detected, cell proliferation and cell cycle transformation were observed. We also disscuss the mechanism of 1,25(OH)₂D₃ affecting CYP1B1 through COX-2/PGE2 pathway during human breast cancer tumorigenesis.

Materials and methods

Chemicals and reagents. 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide (MTT) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO, USA). Phenol red-free Dulbecco's modified Eagle's medium (DMEM) was purchased from SAFC Biosciences, Inc. (Lenexa, KS, USA). Newborn calf serum (NBCS) was obtained from Life Technologies, Inc.; rabbit anti-human CYP1B1, phospho-ERα(ser118) and ERK polyclonal antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). Rabbit anti-human COX-2, phospho-ERK1/2 (Thr202/Tyr204) and β-actin polyclonal antibodies; goat anti-rabbit IgG/HRP, goat anti-rabbit IgG/FITC and human PGE2 ELISA kit were from Bioss, Inc. (Beijing, China). Enhanced chemiluminescence (ECL) system and goat anti-rabbit IgG/Cy3 were from Beyotime, Inc. Polyvinylidene difluoride (PVDF) membrane from Millipore, Inc. PCR oligonucleotide primers were synthesized by Sangon Biotech Co. (Shanghai, China). PrimeScript RT reagent kit was from Takara, Inc. (Dalian, China).

Tissue samples. Formalin-fixed, paraffin-embedded sections from 42 surgically removed breast tumors were analyzed, after local ethics committee approval. All samples were obtained from patients that had not undergone any chemotherapy or radiotherapy before surgical resection. All patients were diagnosed and treated in the First Affiliated Hospital of Chongqing Medical University during the period from 2010 to 2011. All patients with breast carcinoma (35 invasive ductal carcinomas, 3 mucinous adenocarcinomas, 3 intraductal carcinomas, 1 malignant myoepithelioma and 1 atypical medullary carcinoma) were woman. The mean patient age was 52 years (range, 36-75). All specimens obtained at surgery or outside histology slides were reviewed by a senior pathologist.

Immunostaining for COX-2, phospho-ERα(ser118) and CYP1B1 protein expression. The streptavidin-biotin peroxidase complex method was used for immunohistochemistry. Briefly, tissue sections (4-μm thick) were dewaxed and antigen retrieval was performed in citrate buffer using a microwave. Sections were incubated for 10 min with a 3% hydrogen peroxide solution to quench endogenous peroxidase activity and then were incubated overnight at 4°C with specific primary antibodies and developed using 3,3-diaminobenzidine for 3 min. The anti-COX-2, anti-phospho-ERα(ser118) and anti-CYP1B1 antibodies were used at 1:100 dilution.

Evaluation of immunohistochemical staining. Evaluation of the COX-2 and CYP1B1 expression was considered as positive when the results of immunohistochemistry in the tumor cells demonstrated an unequivocal granular staining of the cytoplasm. Only nuclear staining was considered as positive for p-ERα(ser118) expression. Each marker immunostain was recorded according to stain intensity (intensity score) and percentage of cancerous cells that stained positively (quantity score). Intensity score was evaluated as negative (0), weak (1), moderate (2) or strong (3), and quantity score was categorized as follows: 0, negative; 1, <10%; 2, 11-50%; 3, 51-75%; and 4, >75%. By multiplying both components, a score (0-12) was obtained. Marker was considered to be positive if it had a score of ≥3 and as negative if it had a score of <3.

Removal of sex hormones by Charcoal/Dextran-treated newborn calf serum (7). Charcoal was washed twice with cold sterile water immediately before use. A 5% charcoal suspension in 0.5% dextran T40 of the same volume as serum was centrifuged at 1000 x g for 10 min. Supernatants were aspirated, and the serum aliquot was mixed with the charcoal pellets. This charcoal-serum mixture was maintained in suspension by mixing 4 times/min at 56°C for 30 min. This suspension was centrifuged at 1000 x g for 20 min. This procedure was repeated twice, and the supernatant was filtered through a 0.22 μm cellulose acetate-filter. The charcoal dextran-treated NBCS (CDNBCS) was stored at -20°C until needed.

Cell culture and treatment. Human breast cancer cell line MCF-7 was obtained from the Department of Pathophysiology, Chongqing Medical University. MCF-7 was grown in phenol red-free medium (DMEM), supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 10% CDNBCS at 37°C in an atmosphere containing 5% CO_2. Stock solutions of 1,25(OH)₂D₃ was prepared in ethanol and added directly to the culture medium for incubation. Control cells were treated only with ethanol. The final ethanol concentration was always <0.5%.

Cell proliferation assay. The cells were made into suspension and added to sterilized 96-well plates at a density of $2x10^4$/ml. There groups were set up on this study: the blank control group containing only 200 μl DMEM medium, the negative control group consisting of only 200 μl cell suspension and the 1,25(OH)₂D₃ treatment group. In the treatment group, 1,25(OH)₂D₃ solution of the same volume but various concentration was added into wells separately so that the final concentration of 1,25(OH)₂D₃ ranged between 1-100 nmol/l. The cells were incubated for 24, 48 and 72 h, respectively. MTT (5 mg/ml) was added to each aspirated well. Cells were incubated for a further 4 h at 37°C. After 4 h, media were aspirated. Then cells were added with 150 μl DMSO and incubated for a further 10 min at 37°C with gentle shaking. Cells viability was asessed by absorbance of the end of the experimental time. The resulting optical density at 570 nm was measured on computer-controlled microplate analyzer. The experiment was repeated for 3 times, and average of 3 results was taken as a final value.

Cell cycle analysis. Cells were collected, washed, suspended in cold PBS, fixed in 75% ethanol at -20°C overnight, washed and resuspended in PBS with RNAase (20 mg/l). Cellular

Table I. Sequences of the primer pairs for COX-2 and human housekeeping genes used for RT-PCR.

Target name	Accession no. (Ref-seq)	Primer sequence
COX-2	NM-000963.2	Forward: 5'-TTCAAATGAGATTGTGGGAAAATTGCT-3'
		Reverse: 5'-AGATCATCTCTGCCTGAGTATCTT-3'
β-actin	NM-001101.3	Forward: 5'-CTGGGACGACATGGAGAAAA-3'
		Reverse: 5'-AAGGAAGGCTGGAAGAGTGC-3'

Table II. Correlation between COX-2, p-ERα(ser118) and CYP1B1 protein expression in breast cancer tissues.

	p-ERα(ser118)				CYP1B1			
	+ n (%)	- n (%)	r	P-value	+ n (%)	- n (%)	r	P-value
COX-2								
+	27 (82)	6 (18)	0.615	0.000	28 (85)	5 (15)	0.481	0.001
-	1 (11)	8 (89)			3 (33)	6 (67)		
p-ERα(ser118)								
+					24 (86)	4 (14)	0.383	0.012
-					7 (50)	7 (50)		

DNA was stained with PI and cell samples were analyzed on Becton-Dickinson Flow Cytometer (BD Biosciences, USA).

Reverse transcription-polymerase chain reaction analysis. Total RNA was extracted from MCF-7 cells using TRIzol (Takara). The first-strand cDNA was synthesized from 1 μg of total RNA using a Prime Script kit (Takara). COX-2 gene expression was quantified by semi-quantitative reverse transcriptase polymerase chain reaction (RT-PCR). β-actin was uesd as an endogenous control. RT-PCRs were performed with primers listed in Table I. The PCR conditions were: 94˚C for 3 min, followed by 35 cycles of 95˚C for 50 sec, 58˚C for 50 sec and 72˚C for 50 sec. Five microliters of the PCR product was separated by electrophoresis in 2% agarose gel and visualized by ethidium bromide staining. Gene expression analysis was performed with the Quantity One Software (Bio-Rad, Hercules, CA, USA).

Measurement of PGE2 by enzyme-linked immunosorbent assay. MCF-7 cells were seeded in 6-well tissue culture plates ($5x10^5$ cells/well). After 24 h the growth media were replaced with phenol red-free DMEM medium containing vehicle or 100 nmol/l 1,25(OH)$_2$D$_3$. After 72 h, PGE 2 concentrations were measured in the conditioned media using an ELISA kit.

Western blot analysis. Cells were treated with either vehicle or 100 nmol/l 1,25(OH)$_2$D$_3$ for 72 h. Then they were harvested, and whole-cell extracts were used. Cell lysates were resolved by 8% SDS-polyacrylamide gel electrophoresis followed by electroblotting onto a PVDF membrane. The membrane was probed with the appropriate primary antibody followed by incubation with secondary antibody. The immunoreactive bands were visualized using an ECL kit, according to the manufacturer's instructions. Proteins expression analysis was performed with the Quantity One Software (Bio-Rad).

Immunofluorescence analysis. MCF-7 Cells, grown on coverslips and incubated with vehicle or 100 nmol/l 1,25(OH)$_2$D$_3$ for 72 h, were fixed with 4% paraformaldehyde in PBS for 30 min and permeabilized with 0.3% Triton X-100 for additional 10 min at 25˚C. Cells were then incubated overnight at 4˚C with the following primary antibodies: anti-COX-2 (1:20 in PBS), anti-phospho-ERα(ser118) (1:20 in PBS) and anti-CYP1B1 (1:20 in PBS). The primary antibodies were visualized, after appropriate washing with PBS, using goat anti-rabbit IgG-FITC (1:50 in PBS) or goat anti-rabbit IgG-Cy3 (1:50 in PBS) for 30 min at 37˚C. Nuclei were stained with DAPI. Coverslips were finally mounted with antifade medium for observation using a fluorescence microscope. Fluorescent density parameter was performed using software Image-Pro Plus 6. 0 (Media Cybernetics, Inc., MD, USA).

Statistical analysis. Results are expressed as means ± SD. Statistical correlation between COX-2 expression, p-ERα(ser118) expression and CYP1B1 expression in cancer tissues were calculated using the χ^2 test. All statistical analyses were performed by SPSS 13.0 using the independent sample t-test for comparing the two sample groups. For all tests, $P<0.05$ was considered statistically significant.

Results

Association of COX-2 staining with p-ERα(ser118) and CYP1B1 staining in breast cancer tissues. In breast cancer tissues, 78.6% of the tumors were positive for COX-2 expression, 66.7% of the tumors were postive for p-ERα(ser118)
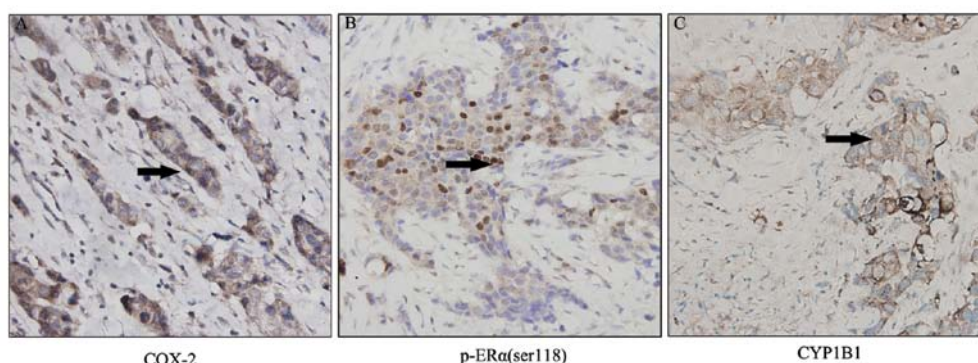
Figure 1. Expression of COX-2, p-ERα(ser118) and CYP1B1 in primary breast carcinomas. Immunohistochemical staining of paraffin-embedded human breast tumor tissues demonstrating positive COX-2, p-ERα(ser118) and CYP1B1 expression. (A) COX-2 positive expression. (B) p-ERα(ser118) positive expression. (C) CYP1B1 positive expression. Original magnification, x400. Arrows indicate positive staining of the cell.
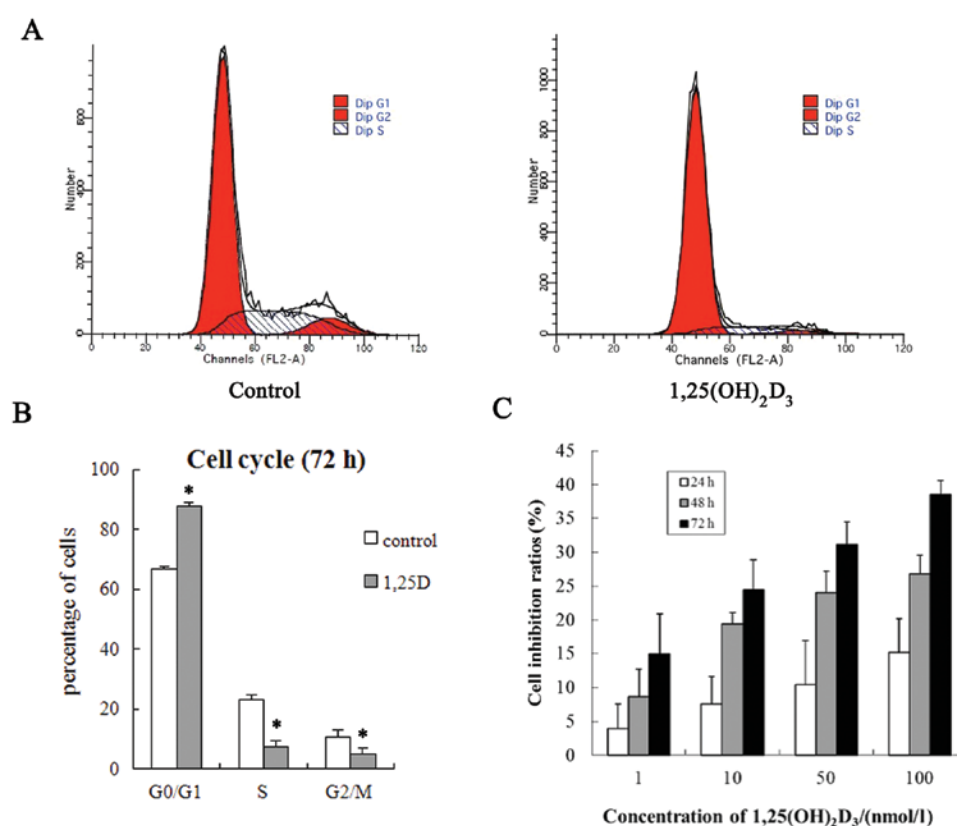

Figure 2. The 1,25(OH)$_2$D$_3$ effects on MCF-7 cell proliferation and cell cycle progression. (A) and (B) Cell cycle analysis by flow cytometry: MCF-7 cells were treated with 100 nmol/l 1,25(OH)$_2$D$_3$ for 72 h. Cell cycle analysis by flow cytometry of treated and untreated cells was carried out as described in Materials and methods. Data are presented as the relative fluorescence intensity of cell sub-populations in the two-dimensional profile (A) or bar diagram (B) (*P<0.05). (C) Comparative analysis of the antiproliferative effect of 1,25(OH)$_2$D$_3$ on MCF-7 cells. MCF-7 cells were treated with various concentrations (1-100 nmol/l) of 1,25(OH)$_2$D$_3$ for 24, 48 and 72 h, respectively. The MTT assay was carried out as described in Materials and methods. Experiments were performed in triplicates; data are expressed as the mean of the triplicate determinations of a representative experiment in % cell viability of untreated cells (100%).

expression and 73.8% of the tumors were postive for CYP1B1 expression. As demonstrated in Fig. 1, abundance of COX-2 and CYP1B1 were perdominantly observed in cell cytoplasm of breast cancer tissues, whereas p-ERα(ser118) displayed nuclear staining. Thus three groups were formed, COX-2 protein expression correlated significantly with p-ERα(ser118) (P<0.001) and CYP1B1 (P<0.01), the expression of p-ERα(ser118) correlated to CYP1B1 (P<0.05) (Table II).

Inhibitive effect of 1,25(OH)$_2$D$_3$ on the growth of MCF-7 cells. The results of the MTT assay showed that 1,25(OH)$_2$D$_3$ significantly inhibited the proliferation of MCF-7 cells, and the inhibitive effect was time- and dose-dependent (Fig. 2C). The average growth inhibition ratios were 14.9, 24.4, 31.1 and 38.5% after the cells were treated with 1,25(OH)$_2$D$_3$ of 1, 10, 50 and 100 nmol/l for 72 h. The ratios increased with the increase of 1,25(OH)$_2$D$_3$ concentration.
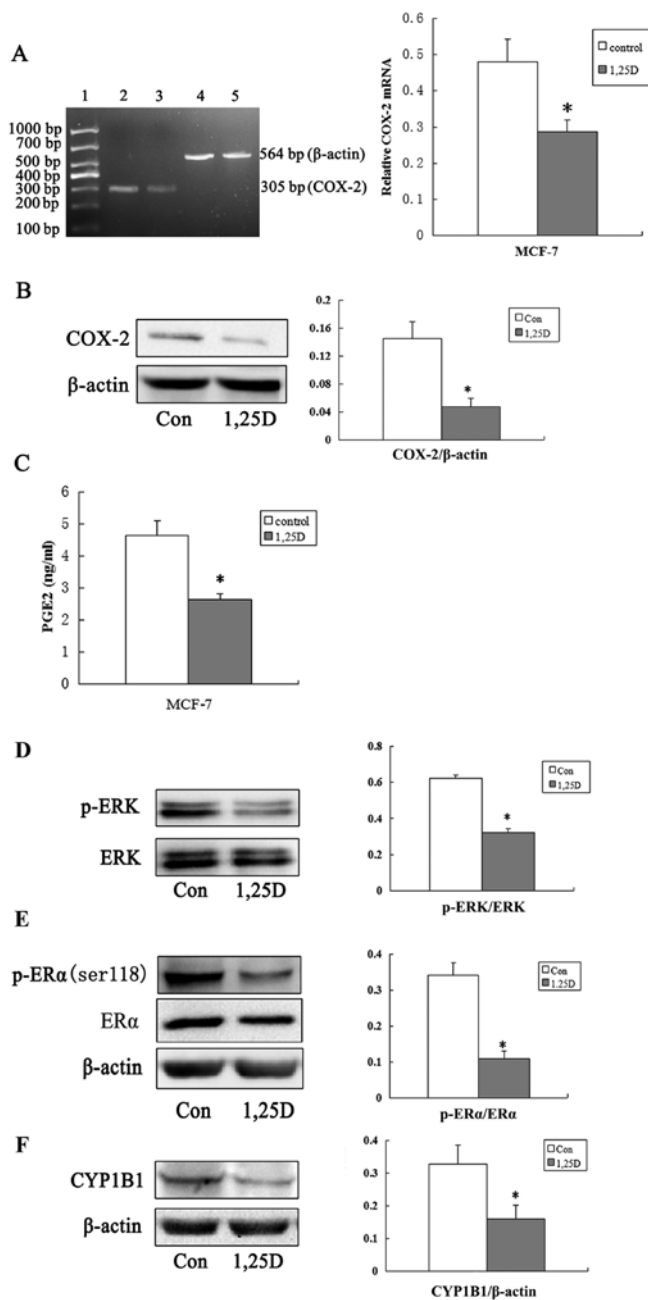
Figure 3. Interference with the COX-2/PGE2 pathway molecules and down-regulation of CYP1B1 by 1,25(OH)₂D₃. (A) The 1,25(OH)₂D₃ decreases COX-2 mRNA levels in MCF-7 cells. MCF-7 cells were treated with 1,25(OH)₂D₃ as described in Fig. 2A and COX-2 mRNA levels were determined. (*P<0.05). Expression levels of COX-2 in control (lane 2) and 1,25(OH)₂D₃ group (lane 3) were measured using RT-PCR. β-actin levels were used as internal positive controls (lanes 4 and 5). Lane 1 is DNA marker ladder. (B) 1,25(OH)₂D₃ decreases COX-2 protein levels. Western blot analysis showing COX-2 expression in MCF-7 cells treated with 100 nmol/l 1,25(OH)₂D₃ (1,25D) or vehicle (control) for 72 h. β-actin was used to normalise relative expressions among groups. The y-axis represents the relative protein expression level (ratio of protein/β-actin) (*P<0.05). (C) 1,25(OH)₂D₃ decreases PGE2 levels in MCF7 cells. MCF-7 cells were treated with 1,25(OH)₂D₃ as described in Fig. 2A. PGE2 levels in medium were measured (*P<0.05). (D-F) Effect of 1,25(OH)₂D₃ on the levels of phosphorylated ERK, phosphorylated p-ERα and CYP1B1 proteins in MCF-7 cells. Cells were treated with 100 nmol/l 1,25(OH)₂D₃ for 72 h. Cells were lysed and immunoblotted. The blot was probed with β-actin antibody for normalization and with (D)anti-ERK and anti-phospho-ERK antibodies, (E) anti-phospho-ERα-ser118 antibody and (F) anti-CYP1B1 antibody. Each blot representative of three independent experiments. *P<0.05 significant difference between control and 1,25(OH)₂D₃ group.

1,25(OH)₂D₃ induces MCF-7 cells arrest in the G0/G1 phase. MCF-7 cells were treated for 72 h with 100 nmol/l 1,25(OH)₂D₃, the percentage of G0/G1 phase cells increased from 66.65±0.77 to 87.64±1.23, (P<0.001), but S phase decreased from 22.94±1.79 to 7.49±1.98 (P<0.01), whereas G2/M phase decreased from 10.40±2.57 to 4.87±2.11 (P<0.05). These results suggest that 1,25(OH)₂D₃ blocked the cells at G0/G1 phase (Fig. 2A and B).

Suppression of the COX-2/PGE2 pathway by 1,25(OH)₂D₃ in MCF-7 cells. 1,25(OH)₂D₃ regulation of the expression of COX-2, the key enzymes involved in PGE2 metabolism. After 72 h, 100 nmol/l 1,25(OH)₂D₃ significantly decreased total COX-2 mRNA levels (Fig. 3A) and COX-2 protein levels (Fig. 3B) in MCF-7 cells. As the result of 1,25(OH)₂D₃ reduced COX-2 protein levels, 1,25(OH)₂D₃ significantly decreased PGE2 levels secreted into the medium from MCF-7 cells (Fig. 3C).

1,25(OH)₂D₃ reduces CYP1B1 expression through decreased PGE2-induced ERK and ERα activation. Based on the previous demonstration, PGE2-induced CYP1B1 expression is mediated by PGE2-induced phosphorylation of the ERα at serine residues 118 via the activation of ERK signaling pathway (6). MCF-7 cells were treated with 100 nmol/l 1,25(OH)₂D₃ for 72 h and 1,25(OH)₂D₃ decreased CYP1B1 protein levels (Fig. 3F). To evaluate the upstream signal for CYP1B1 expression after 1,25(OH)₂D₃ treatment, we examined the activation of ERK kinases and phosphorylated ERα(ser118) in MCF-7 cells. Western blot assay showed that as 1,25(OH)₂D₃-reduced PGE2 levels, phosphorylated ERK (Fig. 3D) and phosphorylated ERα(ser118) (Fig. 3E) decreased. Similar results were observed through immunofluorescence (Fig. 4).

Discussion

Inflammation is one of the hallmarks of a tumor (8). COX-2 is a key initiation factor in inflammatory response, stimulated to expression by inflammatory cytokines, growth factors, oncogenes and other stimuli, and plays an pivotal role in inflammation response and tumor development. Elevated COX-2 expression in breast cancer is associated with higher histological grade, much greater angiogenesis, increased invasion and metastasis, and decreased overall survival time (9). This phenomenon is mainly considered to be mediated via PGE2, the predominant product of COX-2. High expression of COX-2/PGE2 in breast cancer has been shown to correlate with the expression of enzymes such as aromatase and 17β-hydroxysteroid dehydrogenase (17β-HSD), the estrogen synthetases in human breast cancer (10), consequently resulted in high levels of estrogen in breast cancer and improved the risk of this disease. Recently, Han et al (6) found that in the estrogen receptor (ER)-positive breast cancer cells, estrogen α receptor (ERα) was phosphorylated by PGE2 in mutiple loci through activated multiple signaling pathways, and then phosphorylated ERα (p-ERα) combined with the estrogen receptor response element (ERE) on CYP1B1 promoter for transcriptional activation the expression of CYP1B1. In our study, serine residue 118, a vital phosphorylated sites on ERα, was used as a target for detection of p-ERα. Both MCF-7 cell line and tumor samples were analyzed. We found that all of COX-2, p-ERα and CYP1B1 were expressed *in vivo* and
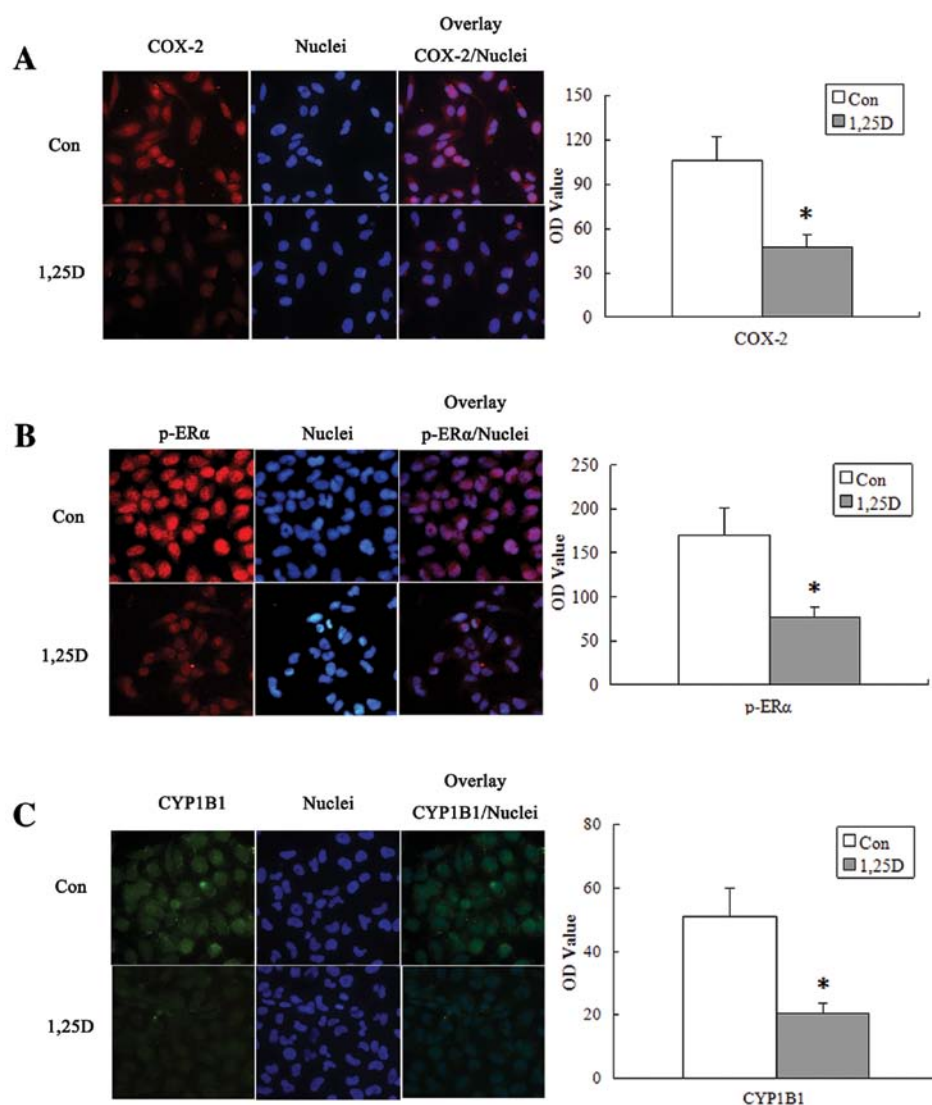
Figure 4. Immunofluorescence analysis of the effect of 1,25(OH)$_2$D$_3$ on the expression of COX-2, p-ERα(ser118) and CYP1B1 in MCF-7 cells. Cells were treated with 100 nmol/l 1,25(OH)$_2$D$_3$ (1,25D) or vehicle (control) for 72 h. Then, cells were permeabilized and immunostained with (A) anti-COX-2 antibody (red) characterized by perinuclear cytoplasmic immunofluorescence; with (B) anti-phospho-ERα-ser18 antibody (red) characterized by nuclear immunofluorescence; with (C) anti-CYP1B1 antibody (green) characterized by perinuclear cytoplasmic immunofluorescence. Cell nuclei were visualized with DAPI (blue). Original magnification, x400. The columns indicate the mean fluorescence intensity of each protein expression levels. *P<0.05 compared with control.

in vitro. There were significant positive correlation between any two of them in human breast cancer tissues, displaying that the mechanism of COX-2/PGE2 pathway regulate CYP1B1 expression may exist *in vivo*. We verified this mechanism in breast cancer cell line MCF-7. COX-2/PGE2 pathway that regulate estrogen-metabolizing enzyme CYP1B1 expression level influences the carcinogenic effects of estrogen, possibly playing a considerable role in breast cancer tumorigenesis.

Vitamin D is a steroid hormone mediating biological effect via its active metabolite form of 1,25-dihydroxyvitamin D$_3$ [1,25(OH)$_2$D$_3$] in the body. 1,25(OH)$_2$D$_3$ is the active metabolite form of vitamin D. 1,25(OH)$_2$D$_3$ has anti-inflammatory and anticancer effect, which is closely related to its inhibition function in COX-2/PGE2 pathway. Krishnan *et al* have reported that in human prostate cancer cells (11) and breast cancer cells (12), 1,25(OH)$_2$D$_3$ downregulated COX-2 both in transcription and translation levels, accompanied with the decline of PGE2 levels, followed by inactivation of downstream signaling

cascade reaction by PGE2 (13). Consistent with the previous report, our experiments demonstrated that 1,25(OH)$_2$D$_3$ downregulated the COX-2 both in gene and protein levels, therewith the decline of PGE2 levels in MCF-7 cell line. Further more, MTT assay confirmed that, within the 1-100 nmol/l range, 1,25(OH)$_2$D$_3$ inhibited MCF-7 cell proliferation in an time-and dose-dependent manner, while arrested the cell cycle in G0/G1 phase.

PGE2 is an inflammatory factor secreted in autocrine or paracrin manner, contributing to stimulation of cancer cell growth through a number of distinct ways. It stimulates cell proliferation through G protein-coupled pathway; activating the nuclear peroxisome proliferator-activated receptor (PPAR); activating several proliferation stimulation signaling pathways such as RAS/MAPK and PI3K/AKT (14). Among those pathways, ERK activation pathway in MAPK family is the most representative and indispensable. We found that in MCF-7 cells, when treated with 1,25(OH)$_2$D$_3$, there was a

significant reduction on PGE2 level as well as phosphorylated ERK, indicating that 1,25(OH)$_2$D$_3$ downregulated the activity of MAPK pathway. ERα is a transcription factor, which facilitates its function by phosphorylating the serine residues in the N-terminal region. The phosphorylation can be either estrogen-dependent or estrogen-independent. In the absence of estrogen, three serine residues in the N-terminal region of ERα can be phosphorylated: 118, by ERK (15); 167, by AKT (16); and 305, by PKA (17). To further investigate whether 1,25(OH)$_2$D$_3$ downregulates the phosphorylation of ERα at Ser118 [p-ERα(ser118)] via ERK pathway, MCF-7 cell line was selected for its high expression of ERα; estrogen-free serum and phenol red-free medium was chosen to exclude the estrogen and estrogen-like activity of phenol on the phosphorylation effect of ERα. Results show that, 1,25(OH)$_2$D$_3$ inhibited the expression of PGE2 and its downstream ERK pathway, then reduced the protein level of p-ERα(ser118). Thus, 1,25(OH)$_2$D$_3$ interfered with COX-2/PGE2 pathway reduced ERK activity, and then downregulated the transcription factor activity of ERα, leading to the negative adjustment of CYP1B1 expression.

CYP1B1, a member of the cytochrome P450 enzyme family, an extrahepatic enzyme in tumor initiation, promotion and drug resistance. It catalyzes the hydroxylation of estradiol in the C4 site, then the metabolites 4-hydroxy-estradiol (4-OH-E2) is futher oxidized into estradiol-3,4-quinone (E2-3,4-Q). E2-3,4-Q can reacted with the purine on DNA forming apurinic adducts, finally leading to DNA mutations (18). The oxygen free radicals, which were generated from the process of 4-OH-E2 oxidated into estradiol-3,4-quinone, could cause DNA damage. CYP1B1 itself can activate other carcinogens and metabolize a number of anticancer drugs, which lead to the development of drug resistance in tumors (19). CYP1B1 gene expression is mainly through activation of the phosphorylation of ERα (20). As CYP1B1 is important in tumor initiation and progression, and highly expressed on numerous tumors, thus, it is a good target for cancer prevention, diagnosis and treatment. Our research suggested that, in estrogen receptor-positive breast cancer cells, COX-2/PGE2 pathway may play a vital role on the control of CYP1B1 expression. 1,25(OH)$_2$D$_3$ interferes with the COX-2/PGE2 pathway, inhibits the activity levels of the phosphorylation of ERK and ERα, then downregulates the expression of CYP1B1, consequently inhibiting the proliferation of breast cancer cells. 1,25(OH)$_2$D$_3$ restrained the expression of CYP1B1, which is significant for its anti-breast cancer effects.

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