Influence of Calcitriol on Prostaglandin- and Vitamin D-metabolising Enzymes in Benign and Malignant Breast Cell Lines

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Abstract. *Background: Cyclooxygenase-2 (COX-2) is a potential molecular prognostic factor for breast cancer, and calcitriol [1,25(OH)$_2$D$_3$], the biologically active form of vitamin D, is a promising target in breast cancer therapy. Materials and Methods: The influence of calcitriol on the proliferation and the effects of calcitriol on the expression of prostaglandin- and vitamin D-metabolising enzymes were examined in benign and malignant breast cells. Results: Calcitriol inhibited the proliferation of MCF-10F and MCF-7 cells but not of invasive MDA-MB-231 cells and reduced the expression of COX-2 and 15-hydroxyprostaglandin dehydrogenase (15-PGDH) in the benign breast cell line MCF-10F. Furthermore, dysregulation in vitamin D-metabolising proteins was detected, especially in MDA-MB-231 cells. Conclusion: These results suggest dysregulation of vitamin D metabolism and a lack of a possible influence of calcitriol on the metabolism of prostaglandins in the malignant breast cell lines.*

Apart from its classic function in controlling calcium metabolism, vitamin D interacts with tumor biology in malignancies. The biologically active form of vitamin D (calcitriol, 1,25-dihydroxycholecalciferol, 1,25(OH)$_2$D$_3$) is considered to be a possible relevant factor in tumor prevention and therapy because of its antiproliferative effects in tumor cells (1). 1,25(OH)$_2$D$_3$ has been identified as a

potent inducer of cell differentiation in a number of established cancer cell lines and it suppresses tumor growth, inhibits metastasis and prolongs survival *in vivo* in animal models (2). As large epidemiologic studies have demonstrated an association between a lack of vitamin D and prostrate, colon and breast cancer (3, 4), a carcinoprotective effect has been attributed to vitamin D.

In breast cancer, the concept that inflammation is an important component of tumor progression has been thoroughly investigated regarding the molecular processes. Cyclooxygenase-2 (COX-2) catalyses conversion of arachidonic acid to prostaglandin E$_2$ (PGE$_2$), and is the rate-limiting step in prostaglandin (PG) and thromboxane biosynthesis involved in inflammatory reactions (5). Furthermore, COX-2 has a prognostic value as tumor cells with elevated COX-2 levels are highly resistant to apoptosis (6), and show increased proliferation (7), invasion and migration (7-9). Therefore, activity of the COX system in breast cancer is under intense evaluation (10) and evidence for a carcinoprotective effect of selective or non-selective COX-2 inhibitors is growing (11, 12).

Emphasis must be placed on the interaction between calcitriol and PG metabolism in cancer since calcitriol influences the synthesis of PGE$_2$, thus, the metabolism of calcitriol and PG synergistically influences tumor cell proliferation.

Moreno and co-workers observed synergistic growth inhibition of a prostate cancer cell line by combining calcitriol and inhibitors of PG anabolising enzymes (13). Moreover, our recent published data suggest a possible link between these two types of metabolism in breast cancer cell lines and tissue (14, 15).

In the present study, we describe the effect of calcitriol on PG- and vitamin D-metabolising enzymes in several benign and malignant breast cancer cell lines and with regard to their respective grade of invasiveness.

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Materials and Methods

Cell culture. The human non-tumorigenic breast cell line MCF-10F (ATCC No. CRL-10318) and the breast cancer cell lines MCF-7 (ATCC No. HTB-22) and MDA-MB-231 (ATCC No. HTB-26) were purchased from the LGC Standards GmbH (Wesel, Germany). MCF-7 and MDA-MB-231 cells were grown in RPMI1640 medium (25 mM HEPES, 1% L-glutamine; Invitrogen, Karlsruhe, Germany) supplemented with 10% foetal bovine serum (FBS). MCF-10F cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's F12 medium with 0.04 mM final calcium concentration, supplemented with 20 ng/ml epidermal growth factor (EGF), 100 ng/ml cholera toxin, 0.01 mg/ml insulin, 500 ng/ml hydrocortisone and 5% horse serum (Invitrogen).

Proliferation assay. To assess the influence of calcitriol on the proliferation of breast cell lines, 10^4 cells/well were plated on a 96-well plate and incubated overnight before treatment with different concentrations of calcitriol (1-10 nM) (Sigma, Deisenhofen, Germany) was performed for 72 h under standard growing conditions. Media and calcitriol were changed daily. The number of proliferating cells was determined with the BrdU Cell Proliferation ELISA (Roche, Mannheim, Germany) with colorimetric detection at 450 nm.

RNA isolation. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer´s protocol. Subconfluently grown cells were trypsinated and pelleted before use. The isolated RNA was quantified spectrophotometrically and its quality was verified by agarose gel electrophoresis. Possible genomic DNA contamination was eliminated using the RNase-Free DNase Set followed by a QIAquick PCR Purification Kit (both from Qiagen).

Reverse transcription and real-time PCR. 1 µg total RNA was added to cDNA synthesis with SuperScript™ II reverse transcriptase and random primers (Invitrogen). 2 µl of a 1:10 dilution of the cDNA were used for quantitative real-time PCR with Platinum® SYBR® Green qPCR SuperMix-UDG (Invitrogen). QuantiTect primer assays were purchased from Qiagen for human cyclooxygenase-2 (COX-2), 15-hydroxyprostaglandin dehydrogenase (15-PGDH) and vitamin D receptor (VDR) with the Cat.-No. QT00013454, QT00040586 and QT01010170, respectively. The primers for the genes of 1-α-hydroxylase (*CYP27B1*) and 24-hydroxylase (*CYP24*) as well as the reference genes TATA box binding protein (*TBP*) and porphobilinogen deaminase (*PBDG*) had the following sequences: *CYP24* forward: 5'-GCA GCC TAG TGC AGA TTT-3', reverse: 5'-ATT CAC CCA GAA CTG TTG-3'; *CYP27B1* forward: 5'-TGT TTG CAT TTG CTC AGA-3', reverse: 5'-CCG GGA GAG CTC ATA CAG-3'; *TBP* forward: 5'-CCA CTC ACA GAC TCT CAC AAC-3', reverse: 5'-CTG CGG TAC AAT CCC AGA ACT-3'; *PBDG* forward: 5'-AGC TAT GAA GGA TGG GCA AC-3', reverse: 5'-TTG TAT GCT ATC TGA GCC GTC TA-3'. Amplification of a single product was verified in a melting curve analysis. A pool of MCF-7 cDNA was used for the experimental calculation of each primer pairs´ efficiencies and as calibrator in different runs. The differences in gene expression were calculated according to Pfaffl (16). The experiments were performed in triplicate for each gene and repeated twice.

Western blotting. Total protein samples were extracted after washing the detached cells with Laemmli-buffer [125 mM Tris, 8% SDS, 30%
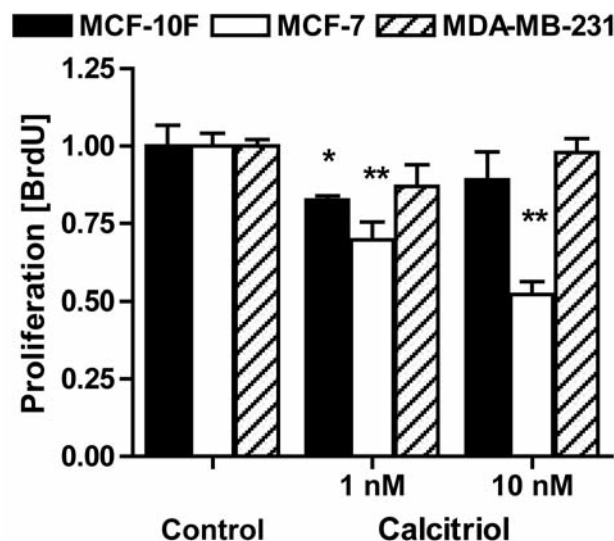

Figure 1. *Influence of calcitriol on the proliferation of MCF-10F, MCF-7 and MDA-MB-231 breast cell lines. The cell growth was measured using BrdU ELISA. Significantly different at *p<0.05, **p<0.01.*

glycerine and complete mini protease inhibitor cocktail (Roche, Mannheim, Germany)]. The lysate was heated for 5 min at 95°C, cooled on ice, and centrifuged at 13000 rpm for 15 min at 4°C. The supernatant contained the total protein extract. Protein concentrations were determined with the BCA Assay (Pierce, Bonn, Germany). The protein (40 µg) was applied onto 10% SDS-PAGE and blotted onto nitrocellulose membranes (Schleicher Schuell, Dassel, Germany). After blocking in 5% non-fat dry milk in PBST (phosphate buffered saline, pH 7.2, 0.1% Tween-20) for 1 h at room temperature, the membranes were incubated in blocking reagent overnight at 4°C with the primary antibodies for human COX-2 and 15-PGDH (IBL, Hamburg, Gerrmany) both at a dilution of 1:1000; the VDR antibody (clone 9A7; Dianova, Hamburg, Germany) at 1:10000; the CYP-24 antibody (S-20; SantaCruz, Heidelberg, Germany) and CYP27-B1 antibody (Antibodies-online, Aachen, Germany) both at 1:2000; and a beta-actin antibody (SantaCruz) were used for normalisation at a dilution of 1:100000. Three washing steps in PBST of 5 min each were followed by incubation with the corresponding horseradish peroxidise conjugated antibodies (Amersham Biosciences, Freiburg, Germany) for 1 h (at a dilution of 1:8000, COX-2, 15-PGDH, CYP27-B1, CYP24; 1:20000, VDR; and 1:100000, beta-actin). The ECL detection system (Amersham Biosciences, Freiburg, Germany) was used to visualise the signals, which were densitometrically analysed in relation to the beta-actin signal.

Statistical analysis. The statistical analysis of quantitative real-time RT-PCR (q-PCR) and Western blot results was performed with Student's *t*-test.

Results

Influence of calcitriol on the proliferation of breast cell lines. All tested cell lines were reduced in proliferation by applying calcitriol, however, the growth reduction in MDA-MB-231
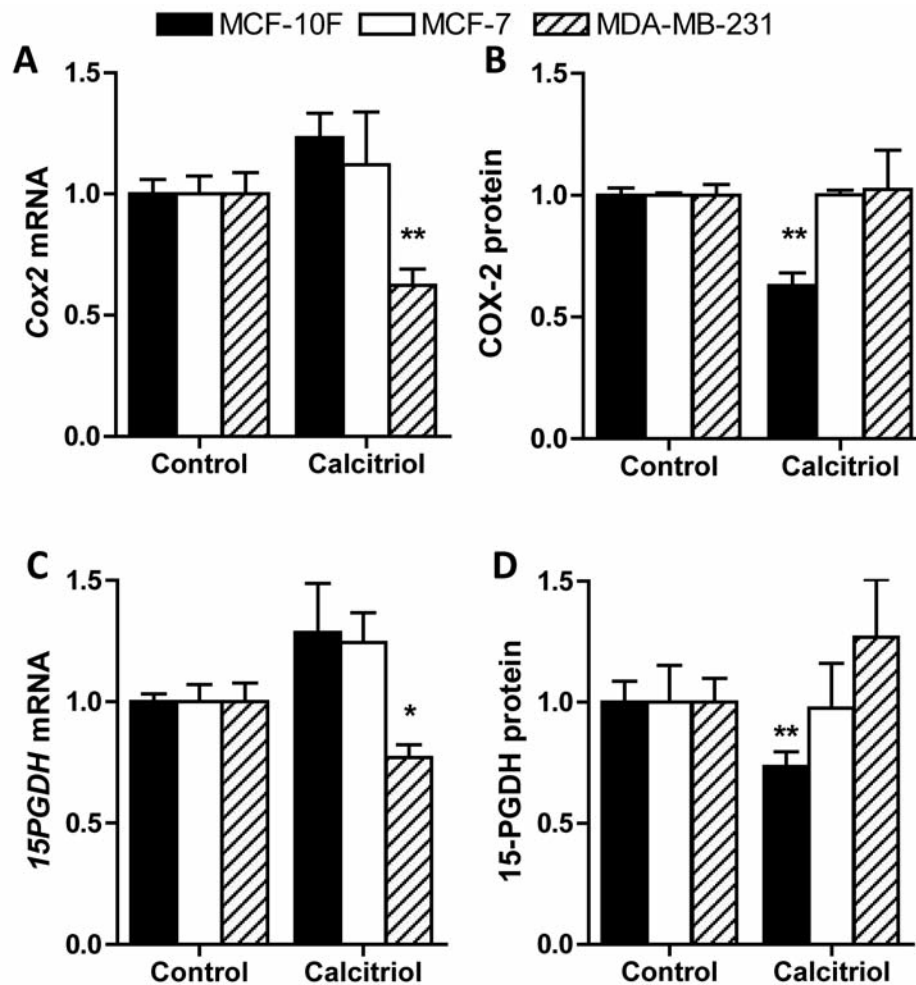
Figure 2. *Influence of calcitriol on the expression level of cyclooxygenase-2 (COX2) and 15-hydroxyprostaglandin dehydrogenase (15PGDH) mRNA (A, B) and protein (C,D) in MCF-10F, MCF-7 and MDA-MB-231 breast cell lines after treatment with 1 nM calcitriol for 72 h. mRNA level was quantified using real-time PCR and protein expression was densitometrically analysed and normalised to that of β-actin as loading control. Differences are significant at *p<0.05, **p<0.01.*

cells to 87% (±7%) at 1 nM calcitriol was not statistically significant. In MCF-10F cells 1 nM calcitriol inhibited proliferation up to 81% ($p<0.05$) compared to the solvent control, and in MCF-7 cells calcitriol the effect was dose-dependent, with 70% and 52% inhibition ($p<0.01$) at 1 nM and 10 nM, respectively (Figure 1).

Influence of calcitriol on PG-metabolising enzymes in breast cell lines. After treatment using 1 nM calcitriol for 72 h, we detected different results in q-PCR and western blot analyses. Compared to the control group (1.00±0.09 and 1.00±0.05) containing the solvent, only the *COX-2* mRNA expression significantly decreased after calcitriol treatment and then only in MDA-MB-231 cells (0.62±0.07; $p<0.01$). In the western blot analysis only the level of COX-2 protein in MCF-10F cells (0.63±0.05; $p<0.01$) was reduced by calcitriol. A significant

decrease in *15PGDH* expression was detected in q-PCR compared to the control group in the MDA-MB-231 cells (0.77±0.05; $p<0.05$) after 72 h treatment with 1 nM calcitriol, while the *15PGDH* mRNA was insignificantly increased in the malignant cell lines. In the western blot analysis, the 15-PGDH expression significantly decreased in the MCF-10F cells (0.74±0.06; $p<0.01$). In the other cell lines, no statistically significant effect of calcitriol was apparent (Figure 2).

Influence of calcitriol on VDR and vitamin D-metabolising enzymes in MCF-10F, MCF-7 and MDA-MB-231 cells. The mRNA for the *VDR* expression exclusively decreased in the MCF-10F cells (0.71±0.04; $p<0.01$) compared to the control group (1.00±0.02) on the treatment with calcitriol. In the western blot analysis, the VDR expression in the malignant cell lines was too low to compare their expression levels after

treatment with calcitriol. In the benign cell line MCF-10F, we detected a significant increase in the VDR expression (1.86±0.23, control group=1.02±0.01). The *CYP27B1* mRNA expression after 72 h of 1 nM calcitriol treatment significantly decreased in MCF-10F cells (0.48±0.14; $p<0.05$) and MCF-7 cells (0.71±0.09; $p<0.05$) compared to the control group, whereas the detected decrease in expression in MDA-MB-231 cells was not statistically significant. In the Western blot analysis, calcitriol treatment had a significant reducing effect only in the MCF-10F cells (0.78±0.06; $p<0.05$). The *CYP24* mRNA expression was influenced by calcitriol treatment in the MCF-10F cells, with a 3000-fold increase (1 301.25±46.78; $p<0.001$) and expression was also increased in the MCF-7 (33-fold) (33.74±8.48; $p<0.01$) and the MDA-MB-231 cells (13.96±3.00; $p<0.01$). In the western blot analysis, a sufficient level for the comparison between treated and untreated cells with the CYP24 antibody was only found in MCF-10F cells. Here, a significant increase of CYP24 after calcitriol treatment was found in accordance with the increase of the mRNA (Figure 3).

Discussion

In the present study, we analysed for the first time the influence of calcitriol on PG- and vitamin D-metabolising enzymes in breast cell lines. Results suggest a possible dysregulation of vitamin D metabolism in malignant breast cells.

Colston and Hansen summarised several studies and clarified that calcitriol can influence the cell cycle, cell differentiation, invasion and apoptosis of breast cancer cells *in vitro* (17). In most of the studies, the experiments were conducted using MCF-7 cells. In this study, we additionally used the invasive breast cancer cell line MDA-MB-231 and the benign cell line MCF-10F to demonstrate the effects of calcitriol in a range of different breast cell types.

In the current study, calcitriol had a significant antiproliferative effect after 72 h at a concentration of 1 nM and 10 nM in the MCF-10F and MCF-7 cell lines. In contrast, there was no significant effect on the proliferation of the invasive MDA-MB-231 cell line. These findings are in line with those of other studies (18, 19).

Our experiments regarding the effect of calcitriol on the PG-metabolising enzymes showed that 1 nM of calcitriol led to a significant reduction of *COX-2* and *15PGDH* mRNA expression only in the MDA-MB-231 cell line, and in the western blot analysis, a significant decrease of COX-2 and 15-PGDH protein expression was detectable in the MCF-10F cell line. Studies using benign and malignant prostate cell lines have illustrated that calcitriol inhibits the PG-dependent proliferation of prostate cancer cells (13). Moreover, the authors showed that a combination of calcitriol with a COX-2 inhibitor had a synergistic effect on the growth inhibition of prostate cancer cells (13).

In our other experiments (not shown in this publication, but published in this issue), we found very sparse protein expression of VDR in malignant breast cell lines. Therefore, it was not possible to evaluate the effect of calcitriol on the VDR expression. However, a difference between the two malignant cell lines was found: the protein expression was lower in the MDA-MB-231 than in the MCF-7 cell line. Similar data have been published by Swami and co-workers (19).

The regulation of the vitamin D-metabolising enzymes is liable to a dynamic feedback mechanism. The VDR plays a role both in the activation of *CYP24* and in the deactivation of *CYP27B1*. The expression of CYP27B1 is suppressed by calcitriol that binds to the VDR and *CYP24* is regulated positively. Therefore, high calcitriol levels lead to an inhibition of calcitriol synthesis and to an induction of calcitriol activation in terms of classic negative feedback. We would have expected a lower CYP27B1 or 1-α-hydroxylase expression under the influence of calcitriol, however, in our present experiments we verified this effect only in the MCF-10F cell line at the protein level and, additionally, in the MCF-7 cell line at the mRNA level. The invasive MDA-MB-231 cells were unaffected by calcitriol. Based on recently published data (14) and other data of our group (published in this issue), we think that increasing invasiveness in breast cell lines leads to a significant reduction of or practically no expression of VDR. For this reason, we suggest a dysregulation of vitamin D metabolism in malignant breast tissue and breast cells.

To evaluate the effect of calcitriol on the induction of *CYP24*, an insufficient positive feedback regulation was expected because of the predescribed results of our experiments. Concerning the low and accordingly sparse VDR expression in the malignant cell lines, the effect of calcitriol on *CYP24* mRNA expression was significantly different and was dependent upon the invasiveness of the cell line. In comparison to the control group, a significant increase in *CYP24* mRNA expression was detected in every cell line but was greatest in the malignant cell lines. Similar results were shown by Swami and co-workers (19). In a recently published study, Dhawan and co-workers observed that the poor response of the MDA-MB-231 cell line to calcitriol was associated with the lack of CCAAT enhancer-binding protein α (C/EBPα) expression, a central regulator of cell metabolism that is inducible by calcitriol in MCF-7 cells, as well as the VDR (20). The extent of VDR expression correlates with the antiproliferative effects of calcitriol in breast cancer cell lines (19, 21). Thus, the results by Dhanan and co-workers (20) are evidence that the induction of C/EBPα by calcitriol and the increase of *VDR* transcription by C/EBPα are an important mechanism by which calcitriol inhibits growth of breast cancer cells.

Based on several studies, summarised in a recent published review by Ma and co-workers, which have described the antiproliferative and proapoptotic effect of calcitriol, calcitriol
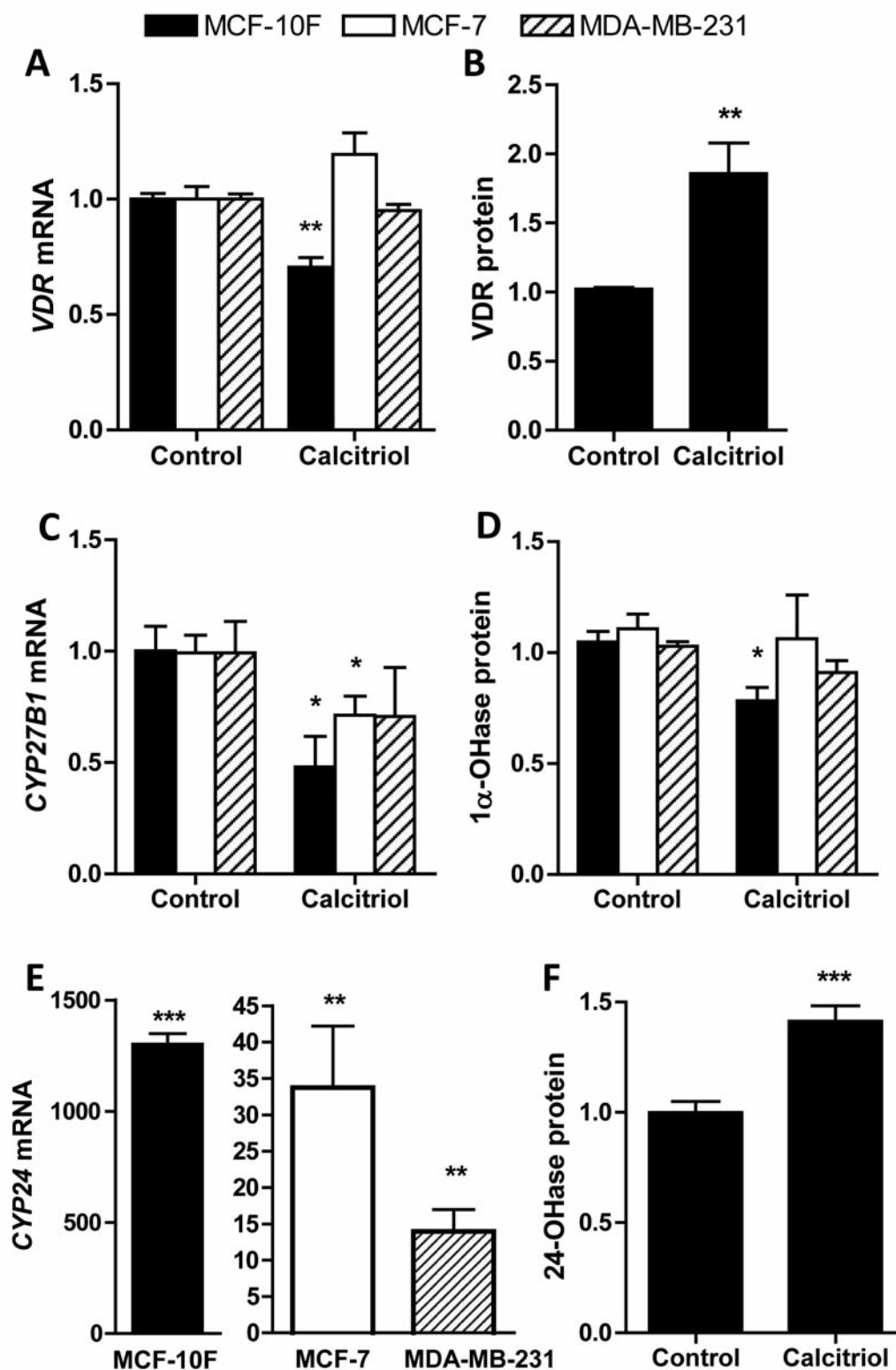
Figure 3. *Influence of calcitriol on the expression level of vitamin D receptor (VDR), 1-α-hydroxylase (CYP27B1) and 24-hydroxylase (CYP24) mRNA (A, C, E) and protein (B, D, F) in MCF-10F, MCF-7 and MDA-MB-231 breast cell lines after treatment with 1 nM calcitriol for 72 h. mRNA level was quantified using real-time PCR and protein expression was densitometrically analysed and normalised to β-actin as loading control. Statistically significant at *p<0.05, **p<0.01, ***p<0.001.*

depicts a promising approach for the treatment of cancer (22). Calcitriol controls genes involved in PG metabolism, up-regulates the expression of mitogen-activated protein kinase phosphatase-5 and therefore supports anti-inflammatory effects, such as a decreased expression of pro-inflammatory cytokines (23). Furthermore, it influences the activation of nuclear factor-kappaB (NFκB), a transcription factor that regulates several genes that are involved in both inflammatory and immune processes, and in cell proliferation (24). It is likely that NFκB also plays a key role in the development from inflammation to carcinogenesis (25). Furthermore, the antiproliferative effects of COX-2 inhibitors on cancer cells *in vitro* and *in vivo* are well known (26, 27). It is obvious that a combination of calcitriol and nonsteroidal anti-inflammatory drugs (NSAIDs) have a potentially synergistic inhibiting effect on the tumorigenesis of breast cancer. Several studies have revealed that a combination of calcitriol and NSAIDs might be a useful chemopreventive and/or therapeutic strategy in prostate cancer (13, 28). However, since calcitriol has a potential hypercalcaemic impact, application is very restricted and vitamin D analogues should be preferred (29-31). Data regarding the use of calcitriol is limited. In the ASCENT I trial (AIPC Study of Calcitriol Enhancing Taxotere), patients with pre-treated and advanced prostate cancer were treated with docetaxel and calcitriol, but the study arm treatment was associated with shorter survival than the control arm (32). There are more, yet inconsistent, data regarding the clinical use of COX-2 inhibitors in breast cancer (33-35).

Based on the actual data, we conclude that the response of breast cancer cells to calcitriol is dependent on the presence or absence of the VDR; however, the presence of VDR is also dependent on other factors, such as hormone receptor status or invasiveness of the cancer cells. Because of the sparse VDR expression in the malignant breast cell lines, we suggest more a chemopreventive role rather than a therapeutic one alone for calcitriol, as well as in its combination with a COX-2 inhibitor.

Conflict of Interest Statement

The Authors declare no conflict of interest relevant to this article.

Acknowledgements

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