Modulation of MAPK ERK1 and ERK2 in VDR-positive and -negative Breast Cancer Cell Lines

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Abstract. 1α,25-Dihydroxyvitamin D₃ (calcitriol), the biologically active metabolite of vitamin D, is known to regulate calcium and phosphate levels in bone metabolism. It is also known to influence proliferation and differentiation in carcinoma cells mediated by the vitamin D receptor (VDR). The antiproliferative effects of calcitriol are believed to be mediated by the nuclear pathway via binding the activated receptor to vitamin D-responsive elements. This induces the vitamin D-responsive genes. Another possible pathway might be the MAPK-cascade or rapid response pathway. The interaction of calcitriol and the MAP-kinase-cascade was evaluated on VDR-positive MCF-7 cells and VDR-negative MDA-MB-231 breast cancer cells. The cells were incubated with calcitriol solution at 10⁻⁷ M and 10⁻⁹ M, or ethanol as controls, for up to 48 h. The effects of calcitriol were measured by semi-quantitative Western blotting. Calcitriol stimulated the MAP-kinases ERK1 and ERK2. A biphasic activation was found for calcitriol in VDR-positive cells after incubation for 5 to 20 min and from 2 to 24 h. However, early activation of ERK1 and ERK2 was also demonstrated in VDR-negative cells. In the controls, ethanol also induced the MAPK-cascade at 5 to 10 min. Calcitriol induction was demonstrated after incubation from 2 to 24 h. In conclusion, it seems that the early induction of the MAPK-cascade was independent of the VDR. A calcitriol-induced MAPK activation was shown after 4 h, which may have been caused by activation of the nuclear receptor pathway.

1α,25-Dihydroxyvitamin D₃ (calcitriol) is a secosteroid hormone which regulates calcium and phosphate metabolism.

These effects are essentially modulated by the vitamin D receptor (VDR). Vitamin D metabolism is also known to modulate cell proliferation and differentiation (1-4).

Breast cancer is the most common cancer in women in Western countries. In Germany, 43,000 cases of breast cancer are diagnosed each year (5). New chemotherapy protocols are evaluated to reduce the risk of recurrence after primary therapy and new compounds, such as vitamin D analogs, are being evaluated and combined with chemotherapy. There might be an option of additional hormonal therapy in VDR-positive cancers. Eighty percent of breast cancer cells tested VDR-positive after surgery and, although no prognostic relevance is known to date, further studies are necessary to evaluate these effects.

The results of two studies have indicated the antiproliferative effects of calcitriol as a result of alteration of the expression of cyclin-dependent kinases (CdKs) or p21 (8, 9). Apoptosis, mediated by Bcl-2 proteins, seems to be another effect of calcitriol not linked to the VDR (10, 23).

The effects of calcitriol are mediated by the nuclear VDR (nVDR). The VDR is a steroid receptor like the estrogen and thyroid receptors. VDRs either develop homodimers with other VDRs or heterodimers with retinoid-x receptors (RXR), which have a high affinity to vitamin D-response elements (VDREs). These elements regulate the expression of vitamin D-related genes by activating RNA-polymerase II. These genes code products such as transforming growth factor-β, epidermal growth factor, Bel-2 and the cell cycle regulator proteins p21 and p27 (9, 11-14).

It is believed that the effects of calcitriol are also mediated by a fast non-nuclear VDR-mediated pathway, although these effects cannot be linked to one receptor at this point. One theory involves the MAP-kinase-cascade (mitogen-activated proteins), which is found in almost every cell population. The RAS protein, located in the cell membrane, is the link to the membrane receptors or ion channels. It binds to the serine/threonine-specific protein kinase Raf. Through GTP and MEK, a protein kinase, the extracellular-regulated MAP-kinases ERK1 and ERK2 are

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phosphorylated. The MAP-kinases are regulated by receptor-thyrosine-kinases (RTKs) or G-protein-linked receptors. Other groups have shown that there might be an interaction between calcitriol and the MAPK-cascade (15).

The aim of this study was to evaluate the stimulation of the MAP-kinases ERK1 and ERK2 by calcitriol in VDR-positive (VDR+) and -negative (VDR–) cell lines and to find an indication for a second membrane-linked rapid pathway (16-18, 24).

Materials and Methods

To evaluate the expression of the MAP-kinases ERK1 and ERK2, the breast cancer cell line MCF-7 with overexpressed VDR was used; VDR-negative (VDR–) MDA-MB231 cells served as the control. The VDR was detected by a VDR antibody (rat monoclonal antibody by ABR).

The cell cultures were incubated with ethanol and dissolved calcitriol at physiological (10⁻⁹ M) and supraphysiological (10⁻⁷ M) concentrations. The cell activation was evaluated after 0, 5, 10, 15, 20, 30, 45 and 60 min and 2, 4, 8, 24 and 48 h. After incubation, the cells were washed with 1 ml phosphate-buffered saline (PBS), centrifuged in RIPA and transferred to an Eppendorf tube. PMFS (1.5 µl) solution was added, followed by 45-min incubation. After centrifuging for 10 min, the lysate was separated and frozen at –80°C. Total protein concentration was measured in this lysate.

The samples were equally concentrated and diluted between 10 µg/µl and 20 µg/µl and the tubes were then filled with 25% diluted treatment buffer. After 5 min of denaturation at 99°C in the Thermomixer (Eppendorf, Germany) followed by centrifuging, the samples were stored at –20°C for the gel electrophoresis experiments. To separate the proteins, a 10% SDS-gel was used at 25 V for 30 min and 50 V for 60 min. The proteins were transferred to the membrane (0.45 µm Optitran BA-S 85, Schleicher & Schuell) at 400 mA for 60 min. The blots were then washed with PBS and blocked with 5% skim milk (Becton Dickinson Corp.) and PBS and incubated with the primary antibody (Santa Cruz Corp.) overnight. After washing, the membrane was incubated with the diluted secondary antibody for 1 h at room temperature. Detection was performed with the chemiluminescence solution (Amersham Bioscience) and visualized on a hyperfilm (Amersham Bioscience).

Semi-quantitative evaluation of the hyperfilm was done by EasyWin (Herolab GmbH). The effect of calcitriol on ERK1 and ERK2 by phosphorylation was related as follows:

\[ V(ERK1) = \frac{pERK1}{ERK1} \quad \text{or} \quad V(ERK2) = \frac{pERK2}{ERK2} \]

Equation 1

\[ V(ERK1/2) = \frac{\frac{pERK1}{ERK1}}{\frac{pERK2}{ERK2}} \]

Equation 2

Stripping of the blots was performed after evaluation. The primary antibodies were extracted after a 15-min incubation in Re-blot solution (1/10 solution in H₂O) (Chemicon Corp., International). Ten-min incubation in Re-blot solution was repeated twice. The blot was then incubated with a different primary antibody. This was necessary because ERK1 and ERK2 have similar molecular weights (ERK1/phosphorylated ERK1: 44 kDa, ERK2/phosphorylated ERK2: 42 kDa) and cannot be distinguished.

The VDR was detected by conventional PCR in VDR+ MCF-7 cells.

Results

Vitamin D-positive cell lines. The results in Figure 1 indicate the relationship between phosphorylated pERK1 or pERK2 and non-phosphorylated ERK1 or ERK2. The graph shows the percentage of pERK1 and ERK1 in the VDR+ cell line MCF-7 from 0 min to 48 h. The three graphs represent the
values of $V(ERK1)$ in all stimulations (ethanol, calcitriol $10^{-7}$ M and calcitriol $10^{-9}$ M).

The cells stimulated by physiological concentrations of calcitriol had an initial activation of 10% at 0 min, which increased to 21% after 20 min. No activation of $ERK1$ was measurable at 30 min and 2 h. A second peak of activation was seen after 4 h of incubation (35%), followed by a drop to 9% and a third peak after 24 h (30%). The activation finally dropped to 5%.

The induction of $ERK1$ in MCF-7 cells stimulated by supraphysiological calcitriol concentrations followed a similar development. The activation increased after 5 min to 25% and dropped to 11%. After 20 min, 22% of activated $ERK$ was measured. No activation was measurable between 30 min and 2 h. After 4 h, p$ERK1$ increased to 23% with a maximum, after 8 h, of 27% (Figure 1).

The control, ethanol, induced a similar course of induction of the $ERK1$ MAPK. The activation increased after 5 min to 25% and dropped continuously to 0% after 30 min. An increase to 12% of activation was also seen at 4 h. After 8 h, the activation of $ERK$ was found to be 4%, rising again to 24% after 48 h of stimulation (Figure 3).

The graphs of $ERK2$ in all three stimulations are provided in Figure 2. The physiological stimulations of $ERK2$ revealed an activation of 17% (0 min) to 54% after 5 min. The activation remained at a level of 47% after 10 min, 48% after 20 min and dropped to approximately 20% between 30 min and 1 h. An increase was measurable from 2 to 24 h, with values of almost 100% between 4 and 24 h.

Supraphysiological stimulation of MCF-7 cells induced a comparable development of $ERK2$ MAPK expression. After 5 min, an activation of 73% was measured. After a drop in activation to 50% (10 min) and an increase to about 70%, the values decreased to a minimum of 12% at 45 min. The maximum of 105% was measured after stimulation for 4 h. Subsequently, $V(ERK2)$ decreased continuously to 42% after 48 h (Figure 2).

Ethanol was found to stimulate $ERK$ to similar activation levels. A 5-min peak was measured at 90% p$ERK2$. A decrease of activation to 32% and a second increase to 65% after 20 min were also seen. The minimum of 5% after 45 min correlated with the calcitriol stimulations. A second activation after 4 h to 64%, followed by a decrease at 8 h and a final rise to 90% after 48 h, were measured (Figure 2).

$Vitamin D$-negative cell lines. The physiological stimulations of $ERK1$ in MDA-MB-231 cells, as shown in Figure 3, increased rapidly after 10 min to 200% and decreased to 0% after 20 min. After 2 h and 48 h, two maxima were measured at 160% and 190%, respectively. A second minimum was seen after 8 h (Figure 3).

The activation of cells stimulated by ethanol increased constantly to 380% after 15 min, with a first minimum of activation being measured after 20 min. A second increase of activation to 130% after 1 h was followed by a second minimum after 8 h. After 48 h, the p$ERK1$ levels were at 120% (Figure 3).

The supraphysiological stimulations produced similar graphs as the cells stimulated by ethanol. The levels of p$ERK1$ increased to 380% after 15 min and a minimum activation was measured after 20 min. A second minimum of activation was seen after 8 h, followed by increasing activation to 160% and 120% after 48 h, for all three stimulation conditions (Figure 3).

The activation of $ERK2$ was very similar to that of $ERK1$. All stimulations led to an increase of about 250% after 10 min. In stimulations with calcitriol at physiological concentrations,
the activation decreased to 210% and dropped to 0% after 20 min, whereas supraphysiological calcitriol and ethanol stimulations increased the phosphorylation to a maximum of almost 350%. All stimulations dropped to an activation of zero after 20 min (Figure 4).

After 30 min, an increase of pERK2 in all the stimulation conditions was measured. At 2 h, all the cells had reached an activation of 150%. A second minimum of activation after 8 h for all stimulations was measured at values around 90%. After 48 h, the activation measured 160% (Figure 4).

Equation 2 represents the pure calcitriol effects shown in Figures 5 and 6. The graphs reveal only one peak in each cell line after 1 h in VDR+ cells and after 8 h of stimulation in the VDR− cells.

Discussion

The results for the VDR+ MCF-7 cells after all three stimulations are provided in Figure 1. The graphs are remarkably similar, with a constant minimum after stimulations from 30 min to 2 h. In these stimulations, no activated ERK1 was measured. After 5 to 20 min and 4 to 48 h, more activated ERK1 than non-stimulated was measured in cells. A comparable biphasic graph was found for pERK2 in the VDR+ cells. The maxima were measured at more than 100%, whereas the minima were detected at around 10 to 20%, compared to activated ERK1. All stimulations had a biphasic development whether physiological, supraphysiological or ethanol-stimulated, whereas ERK2 had higher percentages of activation.

The slow activation after 4 to 48 h seems to be mediated by the nVDR.

Calcitriol and ethanol in VDR− cells had similar effects on ERK1 and ERK2. The values in all three stimulations increased rapidly to 350% and higher within 15 min, but decreased abruptly to 0% within the following 5 min. The similar development of the graphs continued after a minimum of 0% of activation at 20 min. The activation brought about by all three stimulation conditions increased slowly to an average of 104%. Compared to the VDR+ MCF-7 cells, ERK1 and ERK2 of the MDA-MB-231 cells seemed to show a higher response on stimulation with calcitriol. The activation was four-fold higher than the activation in MCF-7 (~100% vs. ~400%). The steady increase in activation after the minimum cannot be interpreted as a second peak as shown in the MCF-7 cells. This activation seems to be monophasic. In summary, we can assume that the VDR− cells are stimulated rapidly, possibly by the rapid response pathway. There is no evidence of stimulation of the nuclear receptor pathway by calcitriol.

To eliminate the ethanol effects, calculations (Equation 2) were performed. The results are shown in Figure 6 at supraphysiological concentrations of calcitriol for ERK1/2. The early peaks seen in Figures 1-4 are not present in Figures 5 and 6, the calculations having eliminated the ethanol effect. In Figures 5 (VDR+ cells) and 6 (VDR− cells), the pure calcitriol effect is shown. It seems that the early stimulations described above were induced by ethanol. ERK1, stimulated by physiological concentrations of calcitriol, seem to be activated after 4 h. This might be caused by the activation of the nuclear receptor pathway.

In the VDR− cell line MDA-MB-231, no activation after 4 h was detected. The activation of the MAPK was not significantly different. The induction of the MAPK-cascade was more an ethanol effect than an induction of calcitriol. The first activation between 5 to 20 min of ERK1 and ERK2 might have been caused by the rapid response pathway (19-22).
Conclusion

For both cell lines, a rapid activation of the MAP-kinases ERK1 and ERK2 was found. In the VDR+ cells, a biphasic activation was measured, leading to two different activation pathways, possibly the rapid response pathway and the nuclear receptor pathway. In the VDR- cells, activation was only measured in early stimulations (5 to 15 min), which might have been caused by ethanol and the nuclear receptor pathway. The results showed that there might be rapid response and nuclear receptor pathways in these cell lines. In the VDR+ cells, the induction caused by calcitriol seemed to be mediated by the nuclear pathway, whereas early activation appeared to be caused by ethanol, possibly mediated by the rapid response pathway.

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