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Molecular Pathways Involved in the Anti-Apoptotic Effect of 1,25-Dihydroxyvitamin D_3 in Primary Human Keratinocytes

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Abstract We previously reported that 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] protects primary human keratinocytes against ultraviolet (UV)B-induced apoptosis. Here, we confirmed the anti-apoptotic effect of 1,25$(OH)_2D_3$ in keratinocytes, using cisplatin and doxorubicin as apoptotic triggers. We further showed that 1,25$(OH)_2D_3$ activates two survival pathways in keratinocytes: the MEK/extracellular signal regulated kinase (ERK) and the phosphatidylinositol 3-kinase (PI-3K)/Akt pathway. Activation of ERK and Akt by 1,25$(OH)_2D_3$ was transient, required a minimal dose of 10^{-9} M and could be blocked by actinomycin D and cycloheximide. Moreover, inhibition of Akt or ERK activity with respectively a PI-3K inhibitor (LY294002) or MEK inhibitors (PD98059, UO126), partially or totally suppressed the anti-apoptotic capacity of 1,25$(OH)_2D_3$. Finally, 1,25$(OH)_2D_3$ changed the expression of different apoptosis regulators belonging to the Bcl-2 family. Indeed, 1,25$(OH)_2D_3$ treatment increased levels of the anti-apoptotic protein Bcl-2 and decreased levels of the pro-apoptotic proteins Bax and Bad in a time- and dose-dependent way. Induction of Bcl-2 by 1,25$(OH)_2D_3$ was further shown to be mediated by ERK and, to a lesser extent, by Akt. In conclusion, 1,25$(OH)_2D_3$ clearly protects keratinocytes against apoptosis (1) by activating the MEK/ERK and the PI-3K/Akt survival pathways and (2) by increasing the Bcl-2 to Bax and Bad ratio. J. Cell. Biochem. 93: 951–967, 2004. © 2004 Wiley-Liss, Inc.

Key words: 1,25-dihydroxyvitamin D_3; anti-apoptosis; Akt; extracellular signal regulated kinase; Bcl-2 family

1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the active metabolite of vitamin D_3, is essential for normal bone structure and the maintenance of serum calcium homeostasis. At the cellular level, 1,25$(OH)_2D_3$ behaves as a steroid hormone and exerts most of its effects via the vitamin D receptor (VDR), which belongs to the large superfamily of nuclear receptors. The liganded VDR heterodimerizes with the retinoid X receptor (RXR) and binds to vitamin D response elements (VDREs) in the promoter regions of target genes and modulates their transcription [reviewed in Bouillon, 2000]. In addition, 1,25$(OH)_2D_3$ can also influence cellular functions via a non-transcriptional, non-genomic pathway either through the activation of a, yet unidentified, membrane receptor, or through the localization of the nuclear VDR near the membrane. These non-genomic effects include the opening of voltage gated calcium and chloride channels and the activation of several signaling molecules, including protein kinase C (PKC), Raf, Src kinases, mitogen activated protein kinases (MAPKs) and phosphatidylinositol 3-kinase (PI-3K) [Norman et al., 2004].

Apart from its classical effects, 1,25$(OH)_2D_3$ also exerts pleiotropic effects on tissues and

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cells that do not participate in calcium metabolism [Bouillon, 2000; Sutton and MacDonald, 2003]. The epidermis is such a non-classical vitamin D target tissue that has attracted a lot of attention: epidermal keratinocytes not only synthesize vitamin D by a photochemical process [Bouillon, 2000], they also convert it to the active metabolite $1,25(OH)_2D_3$ [Lehmann et al., 2001]. In addition, keratinocytes contain the VDR and respond to $1,25(OH)_2D_3$ with growth arrest and differentiation [Segaert et al., 1997; Bikle et al., 2003], properties that have been successfully applied in the use of vitamin D analogues for the therapy of hyperproliferative skin diseases, especially psoriasis [van de Kerkhof, 1998]. Although the antiproliferative and pro-differentiating capacities of $1,25(OH)_2D_3$ in keratinocytes have been extensively studied, less is known about its effect on apoptosis in these cells. Nevertheless, apoptosis is critical for epidermal homeostasis. Keratinocyte proliferation is balanced by terminal differentiation that leads to nuclear fragmentation [Polakowska et al., 1994] and caspase activation [Weil et al., 1999], two hallmarks of apoptosis. Apoptotic keratinocyte cell death also results in the formation of the outermost epidermal layer, the stratum corneum [Ishida-Yamamoto et al., 1999]. In addition to these homeostatic functions in normal skin, keratinocyte apoptosis also represents an important response to environmental stress such as ultraviolet (UV)-irradiation [Kulms et al., 2002]. Until now, conflicting data exist about the effect of $1,25(OH)_2D_3$ on apoptosis in keratinocytes: three independent studies report $1,25(OH)_2D_3$ as a pro-apoptotic agent [Benassi et al., 1997; Bektas et al., 2000; Muller-Wieprecht et al., 2000], while two other groups describe an anti-apoptotic effect of $1,25(OH)_2D_3$ in keratinocytes [Manggau et al., 2001; Meineke et al., 2003].

Apoptotic cell death is a complex biological process that occurs through the activation of cysteine proteases (caspases), which results in specific cleavage of vital cytoskeletal and nuclear proteins and subsequent disassembly of the cell. The pathways activating caspases may be divided into two main groups, those involving death receptors (extrinsic pathway) and those involving the mitochondrium (intrinsic pathway) [reviewed by Adams, 2003]. A well-documented mitochondrial system involves the Bcl-2 family, in which there are both anti-apoptotic (e.g., Bcl-2 and Bcl-X_L) and pro-apoptotic (e.g., Bax and Bad) members. The net balance between these family members is important, since it appears to determine the susceptibility of cells to the induction of the death program [Cory and Adams, 2002]. The apoptotic process is further regulated by different cellular signaling cascades [Cross et al., 2000], including the Raf/MEK/extracellular signal regulated kinase (ERK) [Bonni et al., 1999; Chang et al., 2003] and the PI-3K/Akt pathways [Vivanco and Sawyers, 2002]. Activation of the latter two pathways will promote cell survival by modulating the activity or the expression of different apoptosis-regulating molecules.

We recently demonstrated a protective effect of $1,25(OH)_2D_3$ against UVB-induced apoptosis in primary human keratinocytes [De Haes et al., 2003]. In this study, we confirmed the anti-apoptotic role of $1,25(OH)_2D_3$ in keratinocytes and showed that two different survival signaling pathways and the Bcl-2 protein family are involved.

MATERIALS AND METHODS

Materials

$1,25(OH)_2D_3$, a gift from Dr. J. P. van de Velde (Solvay, Weesp, The Netherlands), was used from a stock in absolute ethanol. Cis-platinum(II)diammine dichloride, doxorubicin hydrochloride, and cycloheximide were purchased from Sigma (St. Louis, MO) and dissolved in PBS. Actinomycin D (Sigma) was used from an ethanol stock solution. LY294002, PD98059 (Biomol, Plymouth Meeting, PA), and UO126 (Promega, Madison, MI) were added from a 1,000-fold concentrated stock in DMSO. Control cultures received similar amounts of ethanol or DMSO only. Final ethanol or DMSO concentrations did not exceed 0.1%. IGF-1 and EGF were obtained from Sigma and reconstituted in 10 mM acetic acid containing 0.1% BSA to a final stock concentration of 50 and 200 μg/ml, respectively.

Cell Culture

Human keratinocytes, derived from the foreskin of young donors (<6 years), were isolated and cultured as described previously [De Haes et al., 2003] in keratinocyte serum free medium (keratinocyte-SFM; Invitrogen, Paisley, UK) supplemented with bovine pituitary extracts (BPE; 50 μg/ml) and human epidermal growth

factor (EGF; 5 ng/ml). Third- to fifth-passage cells were used in all experiments. During the course of the experiments cells never exceeded 80% confluence.

UVB-Irradiation

Prior to irradiation, cells were washed twice with PBS and irradiated through a thin film of PBS. After irradiation cells were provided fresh medium. The UVB-source was a parallel bank of three Philips TL 20W12 tubes with a peak output around 310 nm. Output was measured with an IL700 radiometer (International Light, Newburyport, MA).

Western Blot Analysis

At the indicated time points, cells were scraped in lysis buffer (25 mM HEPES pH 7.5, 0.3 mM NaCl, 1.5 mM $MgCl_2$, 20 mM β-glycerolphosphate, 2 mM EDTA, and 2 mM EGTA) containing 1% Triton X-100, 10% glycerol, 1 mM Na_3VO_4, 0.5 mM dithiothreitol, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml antipain. For the analysis of poly (ADP-ribose) polymerase (PARP)-cleavage, cells were lysed in a different lysis buffer, containing 50 mM Tris-Cl pH 8.0, 150 mM NaCl, 0.1% SDS, 1% Nonidet P-40, 0.5% Na deoxycholate, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 μg/ml antipain. Extracts were incubated on ice and centrifuged at 14,000 rpm for 20 min. Protein concentration was determined using the BCA Protein Assay Reagent (Pierce Chemical Company, Rockford, IL). Equal amounts of protein were separated by SDS–PAGE using precast NuPAGE 4-12% Bis-Tris gels (Invitrogen) and transferred to Hybond-C super membrane (Amersham Biosciences Corp., Piscataway, NJ). The membrane was subsequently blocked and probed with a commercially obtained antibody. Immunoreactive proteins were visualized using a chemiluminescence detection system (NEN; Amersham Biosciences Corp.). The antibodies used were: anti-cleaved PARP (Cell Signalling Technology; Beverly, MA); anti-Akt (Biosource International; Camarillo, CA); anti-S^{473}-phosphorylated Akt (Biosource International); anti-ERK1/2 (Promega); anti-active MAPK (pTEpY), specifically detecting the dually phosphorylated form of ERK1 and ERK2 (Promega); anti-Bcl-2 (BD Pharmingen, San Diego, CA); anti-Bcl-x_L (Transduction Laboratories; San Diego, CA); anti-Bax (Santa Cruz Biotechnology); anti-Bad (R&D Systems; Minneapolis, MN); and anti-β-actin (Sigma). Peroxidase conjugated goat anti-rabbit and rabit anti-mouse (Dako A/S, Glostrup, Denmark) were used as secondary antibodies.

Cell Death Detection ELISA

For a quantitative analysis of apoptosis, we used cell death ELISA (Roche Molecular Biomedicals, Indianapolis, IN). The assay is based on a sandwich enzyme immunoassay using monoclonal antibodies against histones and single- and double-stranded DNA, and thus allows a specific determination of mono- and oligo-nucleosomes in the cytoplasmic fraction of cell lysates. After incubation in 100-mm dishes with various conditions, cells were trypsinized and counted, using a Bürker chamber. For each condition, 5×10^4 cells were resuspended with 500 μl of incubation buffer, mixed thoroughly and incubated for 30 min at 4°C to obtain complete lysis. These cell lysates were further processed according to the manufacturer's instructions. Colorimetric measurements of the reaction were performed in a 96-well plate reader at 405 nm against substrate solution as blank. Averages were made from the double absorbance measurements (optical densities) of the samples. Subsequently, the specific enrichment of mono- and oligo-nucleosomes released into the cytoplasm (=the enrichment factor) was calculated using the following formula: the average optical density of the sample (treated cells) divided by the average optical density of the control (untreated cells).

Quantitative Real-Time PCR

RNA (5 μg) was reverse transcribed at 42°C for 80 min using 150 ng random primers and 200 U Superscript II (Invitrogen). PCR reactions on the resulting cDNA were performed in triplicate on an ABI PRISM 7700 sequence detector according to the manufacturer's protocol (Applied Biosystems, Foster City, CA). The primers and probes with fluorescent dye (FAM) and quencher (TAMRA) for Bcl-2 and Bax were a gift from L. Verlinden and were described in Verlinden et al. [2000]. Primers and dual-labeled detection probe for Bad were purchased from Sigma and sequences were as follows: CGCCCCCCAACCTCTG for the forward primer, CTCTTCGGGCGAGGAAGTC for the reverse primer, and CACAAACTCGTCACT-CATCCTCCGGAG for the detection probe.

Expression levels of these genes were normalized for the hypoxanthine–guanine phosphoribosyltransferase (*HPRT*) gene [Maes et al., 2002]. For quantification, standard curves for each gene were used, consisting of serial dilutions of the respective standard clone, containing defined numbers of input copies.

Statistics

Data were analyzed by a two-tailed Student's t-test for unpaired samples, using a statistical software program (NCSS, Kaysville, UT). $P < 0.05$ was accepted as significant.

RESULTS

$1,25(OH)_2D_3$ Protects Keratinocytes Against Doxorubicin- and Cisplatin-Induced Apoptosis

We previously described a protective effect of $1,25(OH)_2D_3$ against UVB-induced apoptosis in keratinocytes. This protective effect required a minimal dose $1,25(OH)_2D_3$ of 10^{-8} M and a pre-incubation period of at least 8 h [De Haes et al., 2003]. However, the question remained whether the anti-apoptotic activity of $1,25(OH)_2D_3$ in keratinocytes was restricted to UVB-induced apoptosis or could be extended to other apoptotic stimuli. We therefore studied the influence of $1,25(OH)_2D_3$ on doxorubicin- and cisplatin-induced apoptosis. Both DNA-damaging drugs are well-known apoptosis-inducing agents in different cell types [Sherman et al., 1985; Dunkern et al., 2003]. Keratinocytes were exposed to increasing doses doxorubicin (20–80 μM) during 24 h or cisplatin (20–60 μg/ml) during 1 h. As shown in Figure 1A,C, these treatments readily induced PARP-cleavage, a marker of caspase 3 activity and of apoptosis. The induction of apoptosis by both drugs was confirmed and further quantified with a cell death detection ELISA (Roche) detecting DNA-fragmentation (Fig. 1B,D). Pretreatment of the keratinocytes with $1,25(OH)_2D_3$ 10^{-6} M during 24 h, strongly suppressed the drug-induced PARP-cleavage (Fig. 1A,C) and reduced doxorubicin- and cisplatin-caused DNA-fragmentation with more than 75 and 55%, respectively (Fig. 1B,D), indicating a clear anti-apoptotic action of $1,25(OH)_2D_3$.

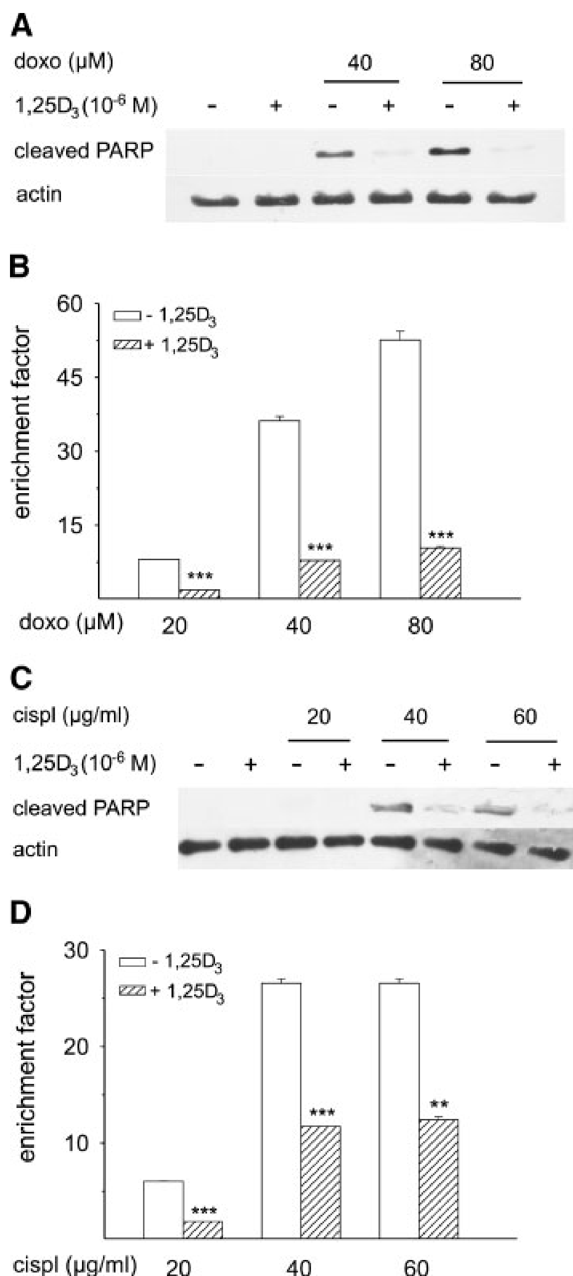


Fig. 1. Protective effect of 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] against doxorubicin- and cisplatin-induced apoptosis in human keratinocytes. Human keratinocytes were pretreated with vehicle or $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$) for 24 h. Subsequently, apoptosis was induced by addition of increasing doses doxorubicin (doxo) for 24 h (**A**, **B**) or cisplatin (cispl) for 1 h (**C**, **D**). After 24 h, cells were harvested either for the analysis of PARP-cleavage by Western blot (A, C) or for the quantification of apoptosis with a cell death detection ELISA (B, D). With the ELISA, rate of apoptosis is indicated by the enrichment factor (optical density of the sample cells/optical density of the control cells) shown on the Y axis (mean ± SD of triplicate samples). $^{**}P < 0.01$; $^{***}P < 0.001$ when comparing $1,25(OH)_2D_3$-pretreated cells with vehicle-pretreated cells.

$1,25(OH)_2D_3$ Stimulates Akt and ERK Activation in Primary Human Keratinocytes

In search for mechanisms underlying the anti-apoptotic effect of $1,25(OH)_2D_3$, we found

that $1,25(OH)_2D_3$ stimulates Akt and ERK activation in keratinocytes. The phosphorylation status of Akt and ERK was examined by Western blotting, using phospho-specific antibodies. This showed a moderate, but consistent, phosphorylation of Akt and ERK in the control cells, which can probably be ascribed to both keratinocyte secreted and exogenously supplemented growth factors in the medium. Treatment of the keratinocytes with $1,25(OH)_2D_3$ transiently augmented this basal phosphorylation level of Akt and ERK. Increased Akt and ERK activation was already seen 2 h posttreatment, sustained for several hours and disappeared after 24 h (Fig. 2A,B). $1,25(OH)_2D_3$-induced Akt and ERK activation was also concentration-dependent, requiring a minimal dose of 10^{-9} M and showing a maximal induction at 10^{-7} and 10^{-6} M (Fig. 2C,D). Treating keratinocytes with $1,25(OH)_2D_3$ 10^{-6} or 10^{-7} M induced Akt activation more than 2.5-fold and ERK activation more than 5-fold. These levels of phospho-Akt and phospho-ERK induction were comparable to those seen with insulin-like growth factor 1 (IGF-1) and EGF, both well-known activators of the PI-3K/Akt and the MEK/ERK

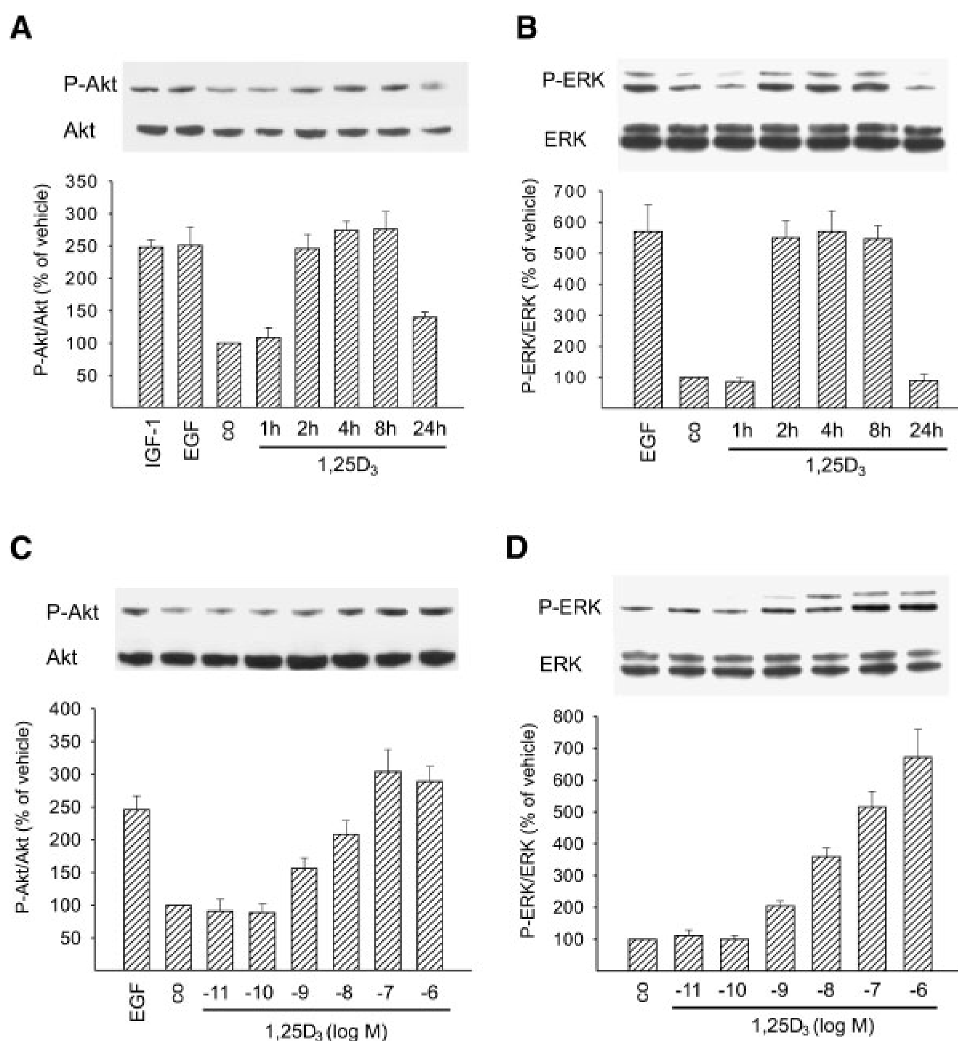


Fig. 2. $1,25(OH)_2D_3$ time- and dose-dependently stimulates the activation of both Akt and extracellular signal regulated kinase (ERK). Keratinocytes were incubated with vehicle (co) or $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$) for different time periods (**A**, **B**) or with increasing concentrations $1,25(OH)_2D_3$ for 4 h (**C**, **D**). Lysates of the cells were then subjected to Western blot analysis using a phosphospecific Akt antibody (P-Akt), a total Akt antibody (Akt), a phosphospecific ERK antibody (P-ERK), and a total ERK antibody (ERK). Stimulation with insulin-like growth factor 1 (IGF-1) 10 ng/ml or epidermal growth factor (EGF) 10 ng/ml for 20 min, was used as positive control. Blots from at least two independently performed experiments were scanned and densitometric values were plotted as a ratio of phosphorylated protein/total protein as percentage of vehicle controls (mean $\pm$ SD).

pathways [Danielsen and Maihle, 2002; Vincent and Feldman, 2002]. The total protein levels of Akt and ERK were not influenced by $1,25(OH)_2D_3$ treatment. We further demonstrated that the use of actinomycin D (1–10 μg/ml) and cycloheximide (1–10 μg/ml) completely abolished $1,25(OH)_2D_3$-stimulated Akt and ERK phosphorylation (Fig. 3A,B), suggesting that $1,25(OH)_2D_3$-induced activation of Akt and ERK requires transcription and protein synthesis.

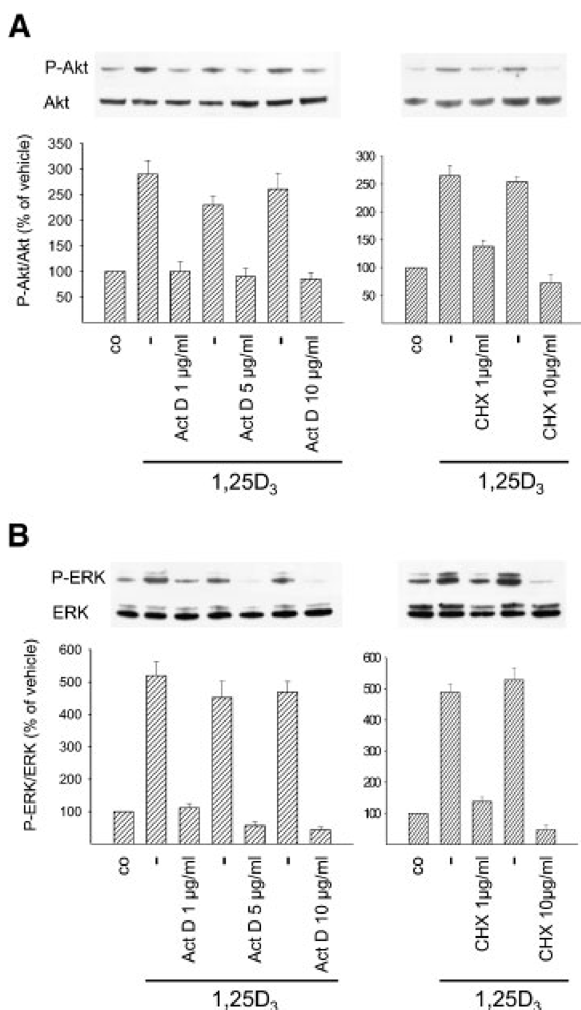


Fig. 3. $1,25(OH)_2D_3$-induced Akt and ERK activation is inhibited by actinomycin D or cycloheximide. Keratinocytes were treated with $1,25(OH)_2D_3$ 10^{-6} M for 4 h in the absence or the presence of increasing concentrations actinomycin D (Act D) or cycloheximide (CHX). Proteins were analyzed by Western blotting using a phosphospecific Akt antibody (P-Akt) and a total Akt antibody (Akt) (**A**) or, a phosphospecific ERK antibody (P-ERK) and a total ERK antibody (ERK) (**B**). Representative results of at least three separate experiments are shown and densitometric data are plotted as a ratio of phosphorylated protein/total protein as percentage of vehicle controls (mean ± SD).

PI-3K/Akt and the MEK/ERK Signaling Pathways Are Both Involved in the Anti-Apoptotic Effect of $1,25(OH)_2D_3$

$1,25(OH)_2D_3$-induced phosphorylation of Akt was efficiently inhibited by LY294002, an inhibitor of PI-3K (Fig. 4A); whereas PD98059 and UO126, both inhibitors of MEK, completely blocked $1,25(OH)_2D_3$-induced phosphorylation of ERK (Figs. 5A and 7A). Conversely, there was no influence of LY294002 on ERK activation and PD98059 and UO126 did not alter Akt phosphorylation (results not shown). To investigate whether the activation of Akt and ERK by $1,25(OH)_2D_3$ is functionally relevant for its anti-apoptotic capacity, we incubated our keratinocytes with LY294002, PD98059, or UO126, 30 min before and during the whole $1,25(OH)_2D_3$ incubation period. Subsequently, apoptosis was induced with three different stimuli: UVB 32 mJ/cm^2, cisplatin 60 μg/ml, or doxorubicin 60 μM. Both PARP-cleavage and a cell death detection ELISA (Roche) were used to study apoptotic cell death (Figs. 4–7). Adding LY294002 15 μM to the cells partially (±30%) inhibited the anti-apoptotic effect of $1,25(OH)_2D_3$ (Fig. 4B,C). Likewise, pre-incubation with PD98059 40 μM incompletely (±35%) suppressed $1,25(OH)_2D_3$-mediated protection against apoptosis (Fig. 5B,C). Combining LY294002 15 μM and PD98059 40 μM had an additive effect, reversing the anti-apoptotic effect of $1,25(OH)_2D_3$ by more than 65% (Fig. 6A,B). With UO126 10 μM we saw a complete abrogation of the anti-apoptotic capacity of $1,25(OH)_2D_3$, independent of the apoptotic stimulus (UVB, cisplatin, doxorubicin) used (Fig. 7B,C). Taken into account that UO126 may not be entirely selective and affects multiple signal transduction pathways [Fukazawa and Uehara, 2000; Swinnen et al., 2000], these results indicate that both, the PI-3K/Akt and the MEK/ERK pathway, contribute to the anti-apoptotic effect of $1,25(OH)_2D_3$.

$1,25(OH)_2D_3$ Increases Bcl-2 Expression and Decreases Bax and Bad Expression

To further elucidate the molecular mechanisms involved in the anti-apoptotic role of $1,25(OH)_2D_3$ in keratinocytes, we also studied its effect on the expression of different Bcl-2 family members. More specifically, we used Western blot analysis to assess the expression of the anti-apoptotic members Bcl-2 and $Bcl\text{-}X_L$

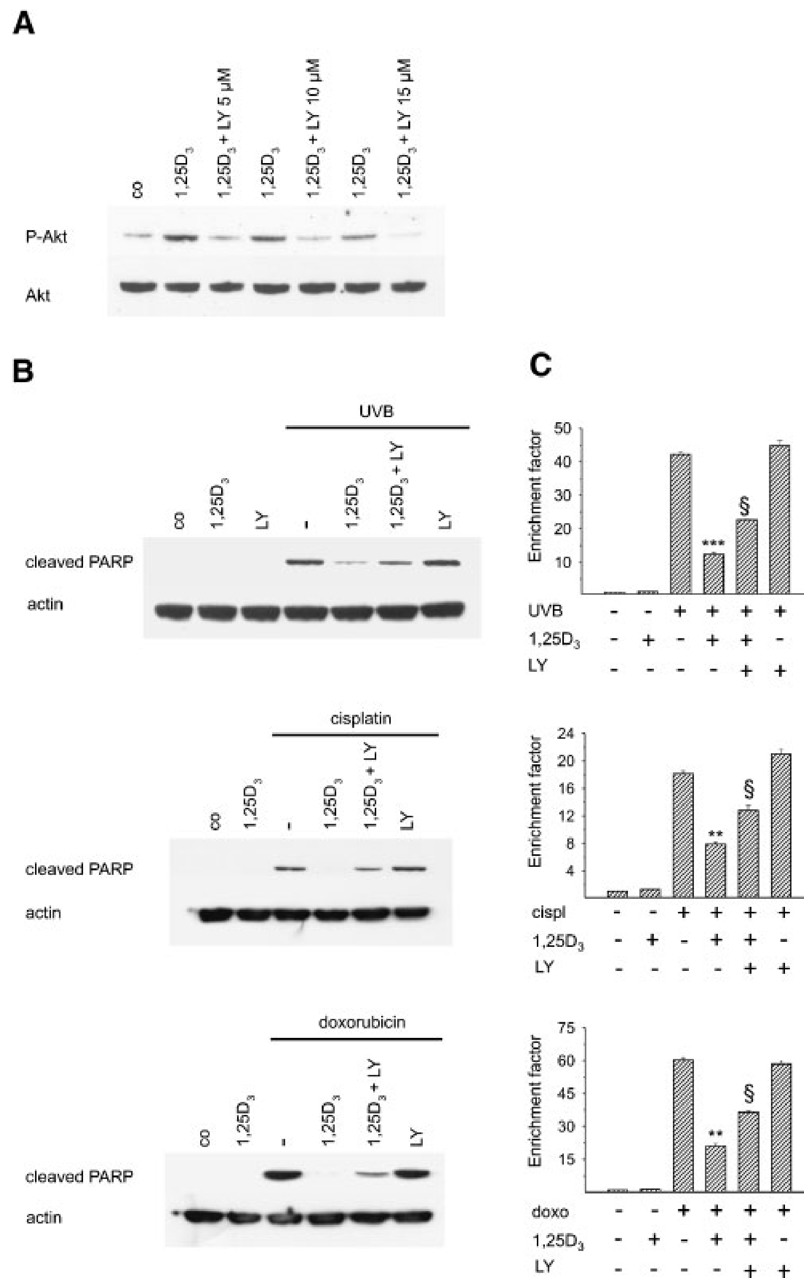


Fig. 4. LY294002, a phosphatidylinositol 3-kinase (PI-3K) inhibitor, partially inhibits the anti-apoptotic effect of $1,25(OH)_2D_3$. **A**: Keratinocytes were incubated with $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$) for 4 h in the absence or the presence of indicated doses LY294002 (LY). Lysates of the cells were then subjected to Western blot analysis using a phosphospecific Akt (P-Akt) and a total Akt (Akt) antibody. A representative result of at least three separate experiments is shown. **B**, **C**: Keratinocytes were treated for 24 h with vehicle (co), $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$), LY294002 15 µM (LY), or $1,25(OH)_2D_3$ + LY294002. In the latter condition, LY294002 15 µM was added 30 min before and during the whole $1,25(OH)_2D_3$ incubation period. Then apoptosis was induced with ultraviolet (UV)B 32 mJ/cm^2, cisplatin 60 µg/ml, or doxorubicin 60 µM. After 24 h, cells were harvested either for the analysis of PARP-cleavage by Western blot (B) or for the quantification of apoptosis with a cell death detection ELISA (C). With the ELISA, rate of apoptosis is reflected by the enrichment factor shown on the Y axis (mean ± SD of triplicate samples). $^{**}P < 0.01$, $^{***}P < 0.001$ when comparing induction of apoptosis with or without $1,25(OH)_2D_3$ pretreatment; $^{\S}P < 0.05$ when comparing the effect of LY294002 + $1,25(OH)_2D_3$ with $1,25(OH)_2D_3$ on the induction of apoptosis.

and of the pro-apoptotic molecules Bax and Bad (Fig. 8A,B). This revealed that $1,25(OH)_2D_3$ increased Bcl-2 protein expression by more than 400% when incubated for at least 8 h at 10^{-9}–10^{-6} M. Conversely, treatment of the keratinocytes with $1,25(OH)_2D_3$ inhibited the expression of Bax and Bad, in a time- and concentration-dependent manner. Bax protein expression was

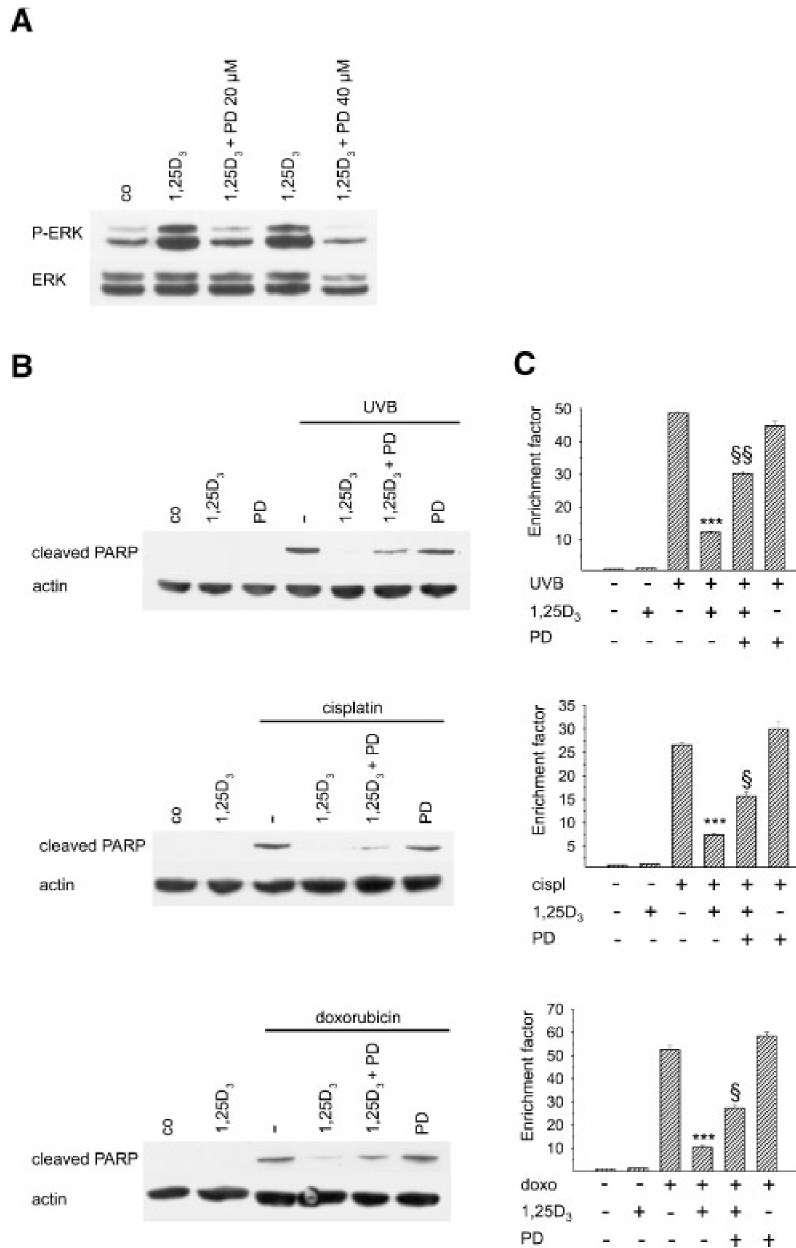


Fig. 5. PD98059, a MEK inhibitor, partially inhibits the anti-apoptotic effect of $1,25(OH)_2D_3$. **A**: Keratinocytes were incubated with $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$) for 4 h in the absence or the presence of indicated doses PD98059 (PD). Lysates of the cells were then subjected to Western blot analysis using a phosphospecific ERK (P-ERK) and a total ERK (ERK) antibody. A representative result of at least three separate experiments is shown. **B**, **C**: Keratinocytes were treated for 24 h with vehicle (co), $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$), PD98059 40 µM (PD), or $1,25(OH)_2D_3$ + PD98059. In the latter condition, PD98059 40 µM was added 30 min before and during the whole $1,25(OH)_2D_3$ incubation period. Then apoptosis was induced with UVB 32 mJ/cm^2, cisplatin 60 µg/ml, or doxorubicin 60 µM. After 24 h, cells were harvested either for the analysis of PARP-cleavage by Western blot (B) or for the quantification of apoptosis with a cell death detection ELISA (C). With the ELISA, rate of apoptosis is reflected by the enrichment factor shown on the Y axis (mean ± SD of triplicate samples). $^{***}P < 0.001$ when comparing induction of apoptosis with or without $1,25(OH)_2D_3$ pretreatment; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$ when comparing the effect of PD98059 + $1,25(OH)_2D_3$ with $1,25(OH)_2D_3$ on the induction of apoptosis.

inhibited to more than 70% when cells were pretreated for 24 h with $1,25(OH)_2D_3$ 10^{-8}–10^{-6} M. The expression of Bad showed a maximal inhibition of 50–65% with $1,25(OH)_2D_3$ 10^{-6} M, whereas $1,25(OH)_2D_3$ 10^{-8} M only decreased Bad levels by 16 ± 6%. The levels of Bcl-X_L were not affected by $1,25(OH)_2D_3$ treatment. Furthermore, the effects of $1,25(OH)_2D_3$ on Bcl-2, Bax, and Bad could be reproduced on the mRNA level by using quantitative RT-PCR (Fig. 8C).

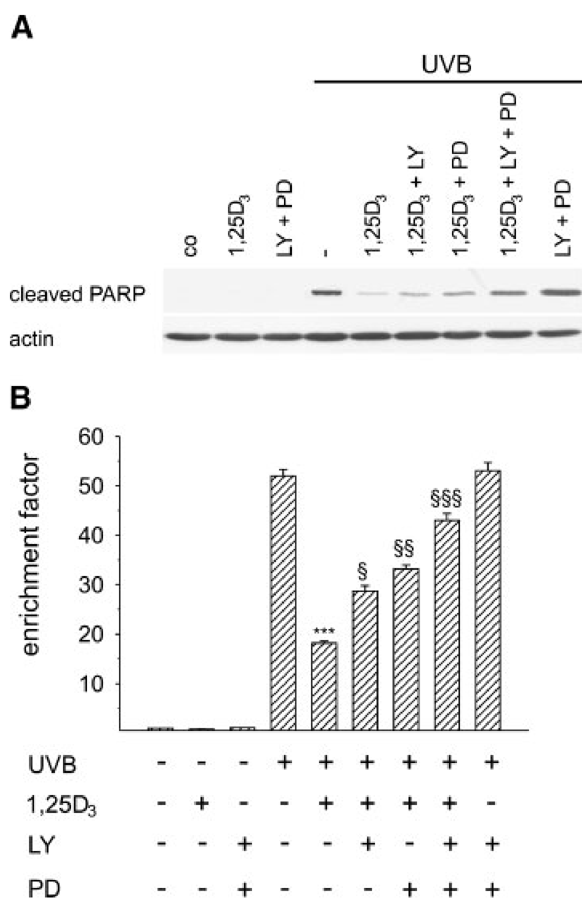


Fig. 6. Combining LY294002 and PD98059 has an additive inhibitory effect on the anti-apoptotic capacity of $1,25(OH)_2D_3$. Keratinocytes were treated for 24 h with vehicle (co), $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$), LY294002 15 μM (LY) + PD98059 40 μM (PD), or $1,25(OH)_2D_3$ + LY294002 + PD98059. In the latter condition, LY294002 15 μM and PD98059 40 μM were added 30 min before and during the whole $1,25(OH)_2D_3$ incubation period. Apoptosis was subsequently induced with UVB 32 mJ/cm^2 and studied by analyzing the cleavage of PARP (**A**) or using a cell death detection ELISA (**B**). With the ELISA, rate of apoptosis is reflected by the enrichment factor shown on the Y axis (mean ± SD of triplicate samples). $^{***}P < 0.001$ when comparing induction of apoptosis with or without $1,25(OH)_2D_3$ pretreatment; $^{\S}P < 0.05$, $^{\S\S}P < 0.01$, $^{\S\S\S}P < 0.001$ when comparing the effect of LY294002 + PD98059 + $1,25(OH)_2D_3$ with $1,25(OH)_2D_3$ on the induction of apoptosis.

Taken together, treating keratinocytes with $1,25(OH)_2D_3$ significantly increased the ratio of Bcl-2 to Bax and Bad in a time- and concentration-dependent manner.

$1,25(OH)_2D_3$-Induced Bcl-2 Expression Is Mediated by the MEK/ERK and the PI-3K/Akt Pathways

We have shown that $1,25(OH)_2D_3$ activates Akt and ERK and influences the expression of different Bcl-2 family proteins. A possible link between both was further investigated by studying the effect of LY294002, PD98059, or UO126 on $1,25(OH)_2D_3$-mediated changes in Bcl-2, Bax, and Bad expression. As shown by Western blot (Fig. 9A) and quantitative RT-PCR (Fig. 9B), $1,25(OH)_2D_3$-induced Bcl-2 protein and mRNA expression was slightly, but reproducibly attenuated by LY294002 15 μM and significantly, but also partially reduced by PD98059 40 μM. Combining both inhibitors further generated an additive inhibitory effect on $1,25(OH)_2D_3$-induced Bcl-2 expression. As for the anti-apoptotc effect of $1,25(OH)_2D_3$, UO126 10 μM almost completely reversed $1,25(OH)_2D_3$-mediated Bcl-2 expression. Taken together, activation of ERK and, to a lesser extent, Akt seems to be involved in $1,25(OH)_2D_3$-mediated Bcl-2 expression. Conversely, $1,25(OH)_2D_3$-mediated suppression of Bax and Bad expression was not influenced by either of the inhibitors used, therefore other mechanisms must be involved.

DISCUSSION

Active vitamin D_3 metabolites have been reported to modulate the process of apoptosis in different cell types. In breast cancer [Narvaez and Welsh, 2001], colon cancer [Diaz et al., 2000], prostate cancer [Guzey et al., 2002], and other cancer cell lines [McGuire et al., 2001; Galbiati et al., 2003; Wagner et al., 2003], $1,25(OH)_2D_3$ has been shown to induce apoptotic cell death. In these cancers, the activation of apoptotic pathways has been implicated as an additional mechanism underlying $1,25(OH)_2D_3$-mediated growth suppression. Induction of apoptosis is however not a universal response to $1,25(OH)_2D_3$ treatment. In human leukemic cells [Wang and Studzinski, 1997; Ketley et al., 2000], in normal human thyrocytes [Wang et al., 1999], and in human pancreatic islet cells [Riachy et al., 2002] $1,25(OH)_2D_3$ offers protection against apoptosis.

In primary human keratinocytes as well as in HaCaT cells, active vitamin D and analogues have been shown to induce apoptosis, when used at very high doses ($\geq 10^{-5}$ M) or during very long periods (≥3 days) [Benassi et al., 1997; Bektas et al., 2000]. In contrast, we recently demonstrated a time- and dose-dependent protective effect of $1,25(OH)_2D_3$ against UVB-induced apoptosis in primary human keratinocytes. Maximal protection was obtained when

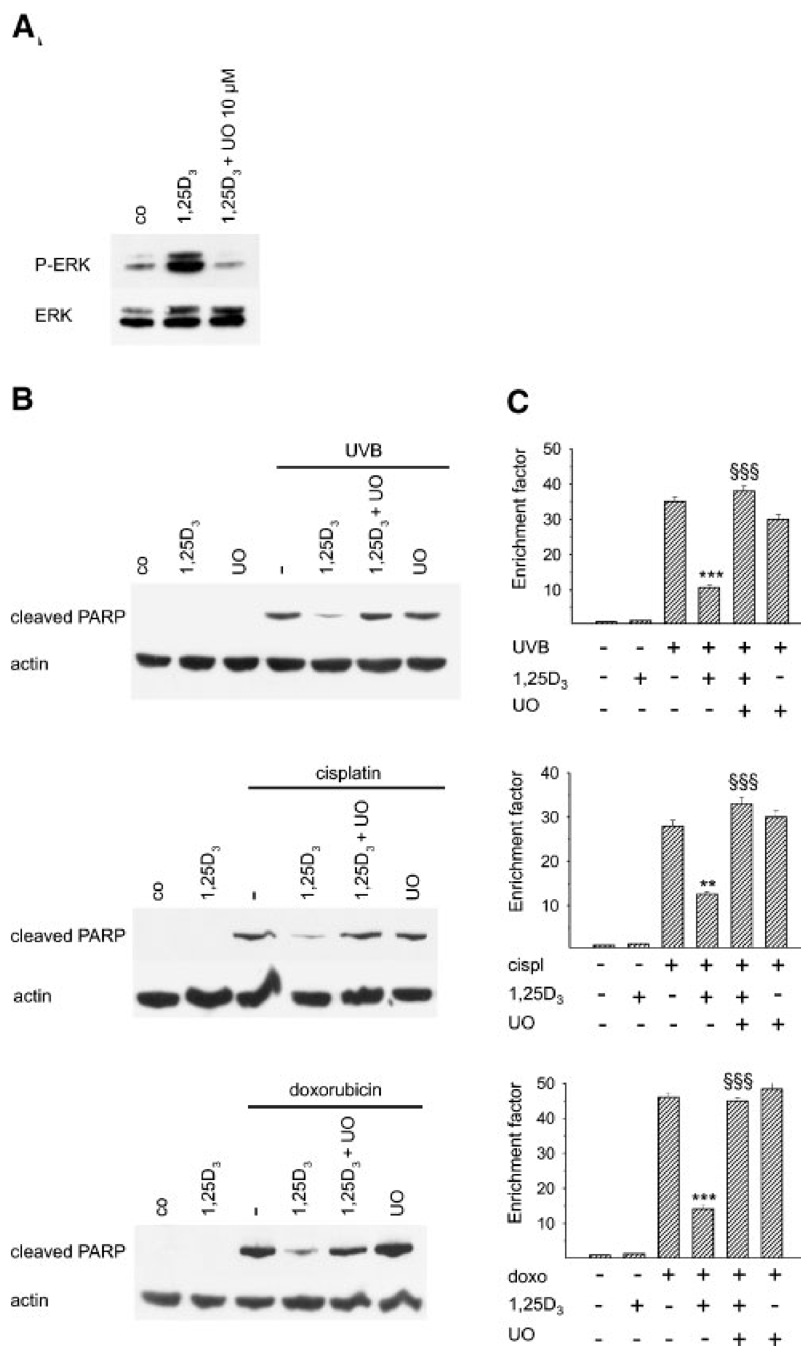


Fig. 7. UO126, another MEK inhibitor, completely abrogated the anti-apoptotic capacity of $1,25(OH)_2D_3$. **A**: Keratinocytes were incubated with $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$) for 4 h in the absence or the presence of indicated doses UO126 (UO). Lysates of the cells were then subjected to Western blot analysis using a phosphospecific ERK (P-ERK) and a total ERK (ERK) antibody. A representative result of at least three separate experiments is shown. **B**, **C**: Keratinocytes were treated for 24 h with vehicle (co), $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$), UO126 10 µM (UO), or $1,25(OH)_2D_3$ + UO126. In the latter condition, UO126 10 µM was added 30 min before and during the whole $1,25(OH)_2D_3$ incubation period. Then apoptosis was induced with UVB 32 mJ/cm^2, cisplatin 60 µg/ml, or doxorubicin 60 µM. After 24 h, cells were harvested either for the analysis of PARP-cleavage by Western blot (B) or for the quantification of apoptosis with a cell death detection ELISA (C). With the ELISA, rate of apoptosis is reflected by the enrichment factor shown on the Y axis (mean ± SD of triplicate samples). $^{**}P < 0.01$, $^{***}P < 0.001$ when comparing induction of apoptosis with or without $1,25(OH)_2D_3$ pretreatment; $^{§§§}P < 0.001$ when comparing the effect of UO126 + $1,25(OH)_2D_3$ with the effect of $1,25(OH)_2D_3$ on the induction of apoptosis.

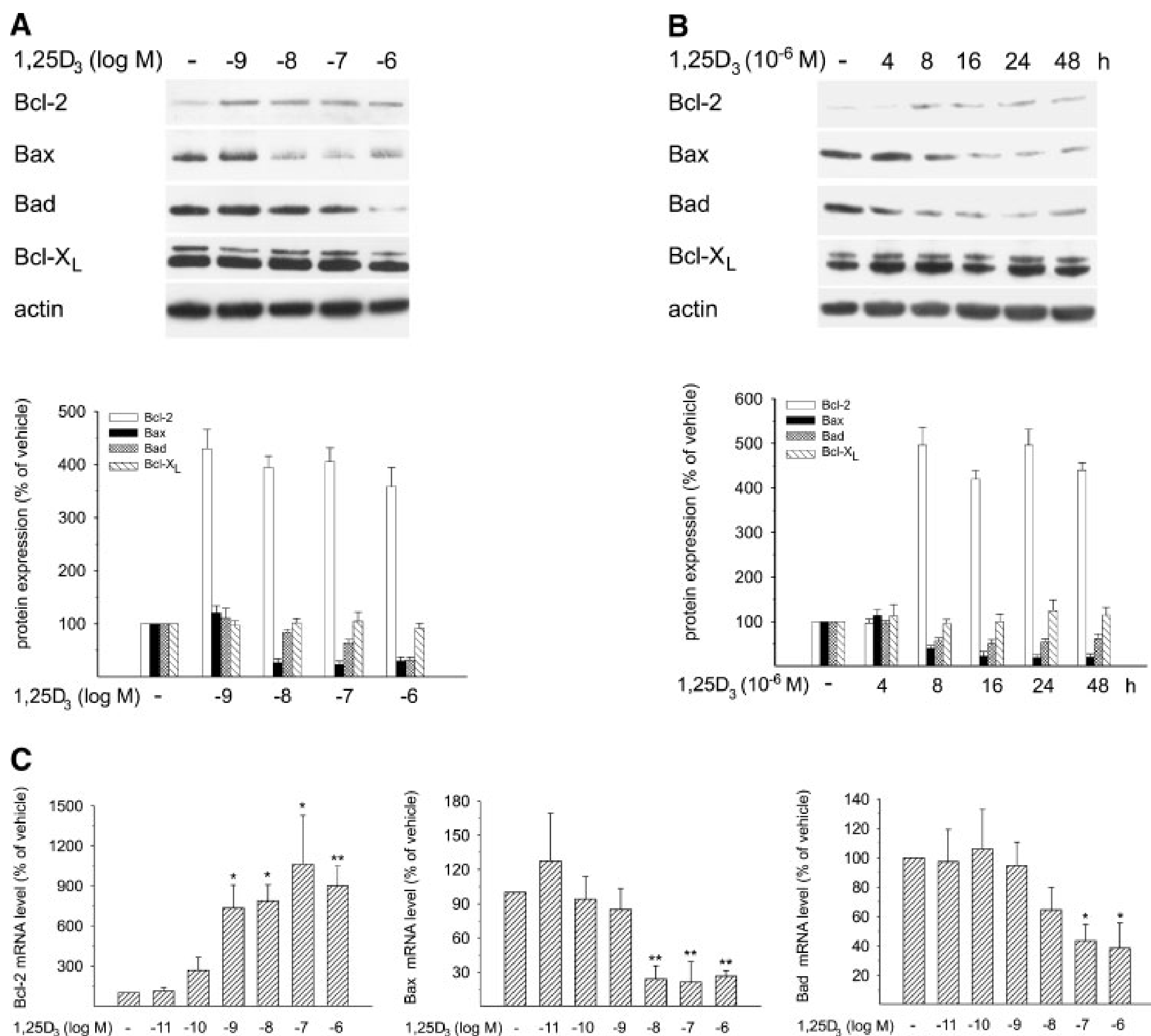
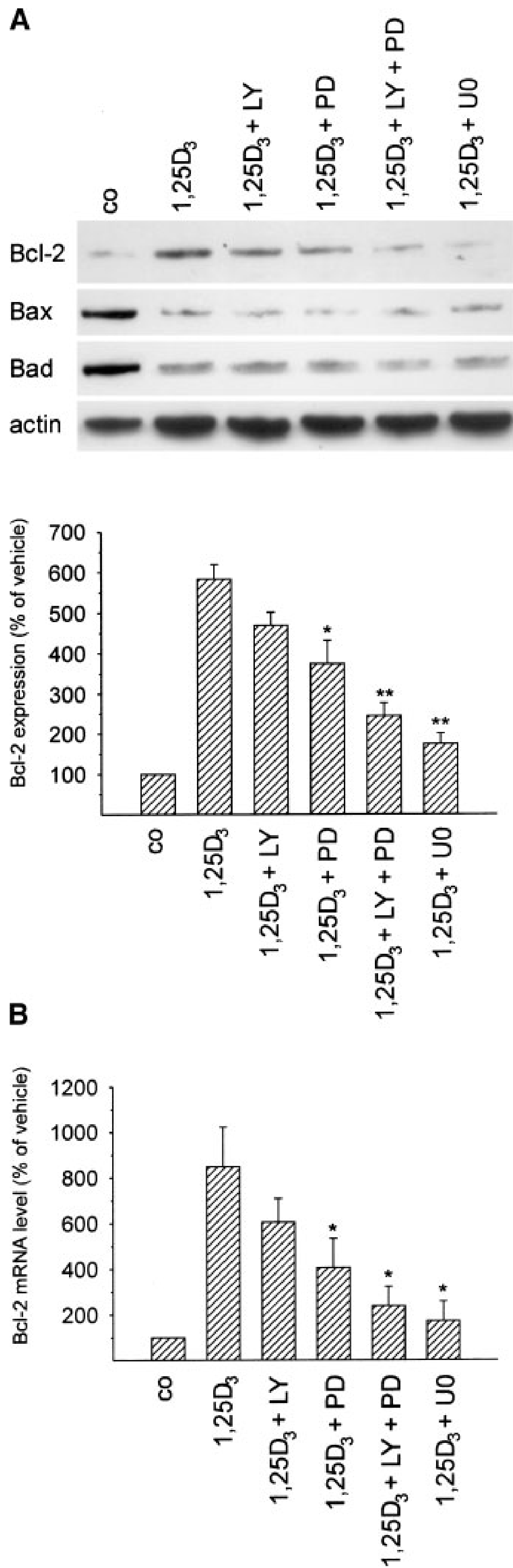


Fig. 8. Treatment of keratinocytes with $1,25(OH)_2D_3$ increases Bcl-2 expression and decreases Bax and Bad expression. **A**, **B**: Keratinocytes were treated with increasing doses $1,25(OH)_2D_3$ for 24 h (A) or with $1,25(OH)_2D_3$ 10^{-6} M during different incubation periods (B). Western blots were performed with antibodies for Bcl-2, Bax, Bad, and Bcl-X_L. The bands are representative results from triplicate experiments. Blots from three independently performed experiments were scanned and densitometric values, corrected for β-actin were plotted as percentage of vehicle controls as mean ± SD. **C**: qRT-PCR analyzes of Bcl-2, Bax, and Bad mRNA expression. Keratinocytes were treated with increasing doses $1,25(OH)_2D_3$ for 24 h. Target gene RNA levels were measured, normalized to HPRT RNA levels, and expressed as percentage of vehicle controls as mean ± SD of two independent experiments performed in triplicate. $^*P < 0.05$, $^{**}P < 0.01$ for $1,25(OH)_2D_3$-treated versus vehicle-treated cells.

$1,25(OH)_2D_3$ was pre-incubated for 24 h or more at a concentration of 10^{-6} M [De Haes et al., 2003]. Accordingly, Manggau et al. [2001], who concentrated on ceramide-induced apoptosis, also reported an anti-apoptotic role of $1,25(OH)_2D_3$ in primary human keratinocytes with comparable time- and dose-response curves. A recent report of Meineke et al. [2003] evidenced an inhibition of X-ray-induced apoptosis by $1,25(OH)_2D_3$ in HaCaT cells. Finally, we described in this study that $1,25(OH)_2D_3$ protects keratinocytes against cisplatin- and doxorubicin-induced apoptosis. Taken together, there has been some controversy about the influence of $1,25(OH)_2D_3$ on apoptosis in keratinocytes. However, our data support the more recent findings [Manggau et al., 2001; Meineke et al., 2003] that $1,25(OH)_2D_3$ efficiently protects keratinocytes against the induction of apoptosis, regardless of the apoptosis-triggering stimulus used.

We further elucidated possible mechanisms underlying the anti-apoptotic action of $1,25(OH)_2D_3$ in keratinocytes. First, we



demonstrated that $1,25(OH)_2D_3$ activates the PI-3K/Akt and the MEK/ERK survival pathways in keratinocytes and this with similar kinetics. To our knowledge, there has been no report mentioning an activation of Akt by $1,25(OH)_2D_3$, although the PI-3 kinase, which activates Akt, is known to be involved in $1,25(OH)_2D_3$-induced differentiation of myeloid leukemia cells [Hmama et al., 1999] and in $1,25(OH)_2D_3$-stimulated ERK and JNK activation in keratinocytes [Johansen et al., 2003]. In contrast, in different cancer cell lines and in tumor-derived endothelial cells, $1,25(OH)_2D_3$ has been shown to inhibit Akt as a mechanism for its pro-apoptotic and anti-proliferative effects in these cells [McGuire et al., 2001; Bernardi et al., 2002]. Activation of ERK by $1,25(OH)_2D_3$ has been reported previously in keratinocytes [Gniadecki, 1996; Johansen et al., 2003]. In these studies a very rapid (within minutes) and short (less then 1 h) activation of ERK by $1,25(OH)_2D_3$ is described via a transcription-independent way. In our keratinocyte culture system, however, an incubation period with $1,25(OH)_2D_3$ of at least 2 h was required for activation of ERK or Akt and this activation was clearly inhibited by cycloheximide and actinomycin D, suggesting a transcription-dependent mechanism and the involvement of a yet unidentified factor. Possible candidates might be found in the epidermal growth factor receptor (EGFR) and/or insulin-like growth factor receptor (IGFR) signaling system, since both the PI-3K/Akt and the MEK/ERK cascades are typically activated via these growth factor receptors [Danielsen and Maihle, 2002; Vincent and Feldman, 2002] and $1,25(OH)_2D_3$ interferes

Fig. 9. $1,25(OH)_2D_3$-induced Bcl-2 expression is mediated by ERK and Akt. **A**: Keratinocytes were treated for 24 h with vehicle (co), $1,25(OH)_2D_3$ 10^{-6} M ($1,25D_3$), $1,25(OH)_2D_3$ + LY294002 15 μM (LY), $1,25(OH)_2D_3$ + PD98059 40 μM (PD), $1,25(OH)_2D_3$ + LY294002 + PD98059, or $1,25(OH)_2D_3$ + UO126 10 μM (UO). LY294002, PD98059, and UO126 were added 30 min before and during the whole $1,25(OH)_2D_3$ incubation period. A: Western blots were performed with antibodies for Bcl-2, Bax, and Bad. The bands are representative results from duplicate experiments. Blots of Bcl-2, from two independently performed experiments, were scanned and densitometric values, corrected for β-actin, were plotted as percentage of vehicle controls as mean ± SD. **B**: qRT-PCR analyzes of Bcl-2 mRNA expression. Bcl-2 RNA levels were measured, normalized to HPRT RNA levels, and expressed as percentage of vehicle controls as mean ± SD of two independent experiments performed in triplicate. $*P < 0.05$, $**P < 0.01$ for the effect of LY294002, PD98059, or UO126 on $1,25(OH)_2D_3$-induced Bcl-2 expression.

with the EGFR and the IGFR signaling systems in different cell types [Falette et al., 1989; Boisseau-Garsaud et al., 1996; Garach-Jehoshua et al., 1999; Xie et al., 1999; Akutsu et al., 2001; Kveiborg et al., 2001; Sprenger et al., 2001; Gonzalez et al., 2002]. Although less likely, we can not totally exclude from our results that the inhibition of $1,25(OH)_2D_3$-mediated activation of Akt and ERK by actinomycin D and cycloheximide results from an inhibition of the biosynthesis of components of the PI-3K/Akt and the MEK/ERK pathways, rather than from an inhibition of the $1,25(OH)_2D_3$ effect. There was no consistent reduction of the levels of total Akt and ERK by actinomycin D or cycloheximide, however, a reduction of other components of these pathways can not be excluded. Therefore, a non-genomic mechanism might still be involved in the $1,25(OH)_2D_3$-induced activation of Akt and ERK.

Using chemical inhibitors, we further demonstrated a functional role for the ERK and the Akt pathways in the anti-apoptotic effect of $1,25(OH)_2D_3$. Inhibition of ERK or Akt activity with respectively a MEK (PD98059) or a PI-3K (LY294002) inhibitor, partially abrogated the anti-apoptotic capacity of $1,25(OH)_2D_3$. Combining both inhibitors generated an additive effect, inducing a strong, but still not complete reversal of $1,25(OH)_2D_3$-mediated protection against apoptosis. The complete inhibition of the anti-apoptotic effect of $1,25(OH)_2D_3$ by UO126 is probably due to aspecific actions of this inhibitor. Although synthesized as a selective MEK inhibitor [Chang et al., 2003], UO126 has been shown to inhibit other kinases as well [Fukazawa and Uehara, 2000; Swinnen et al., 2000] and this may explain its potent effect on the anti-apoptotic activity of $1,25(OH)_2D_3$. The importance of ERK activation for inhibition of apoptosis has further been shown for two other steroid hormones, 17β-estradiol and 5α-dihydrotestosterone. These sex steroids were shown to activate a Src/Shc/ERK signaling pathway and attenuate apoptosis via a transcription-independent, non-genomic way [Kousteni et al., 2001].

Next we demonstrated that $1,25(OH)_2D_3$ has clear effects on the expression of different members of the Bcl-2 family. In agreement with Manggau et al. [2001], we showed that $1,25(OH)_2D_3$ induced the expression of the anti-apoptotic Bcl-2 protein in keratinocytes. In fact, it seems that Bcl-2 is a more general target of $1,25(OH)_2D_3$, as it is downregulated by $1,25(OH)_2D_3$ in prostate carcinoma [Guzey et al., 2002], breast carcinoma [Narvaez and Welsh, 2001], and retinoblastoma cells [Wagner et al., 2003] and upregulated in normal thyrocytes [Wang et al., 1999]. $1,25(OH)_2D_3$ has also been identified to influence other Bcl-2 members in a variety of cell types [Wang and Studzinski, 1997; Diaz et al., 2000; Guzey et al., 2002; Galbiati et al., 2003]. Moreover, in addition to upregulating Bcl-2, we showed that $1,25(OH)_2D_3$ suppresses the levels of the pro-apoptotic proteins Bax and Bad. Thus, treatment of the keratinocytes with $1,25(OH)_2D_3$ for several hours shifts the balance between the members of the Bcl-2 family towards promotion of cell survival. Finally, we demonstrated that $1,25(OH)_2D_3$-induced upregulation of the Bcl-2 protein is mediated by the ERK and, to a lesser extent, the Akt pathway. The Bcl-2 protein has previously been identified as one of the downstream targets of the ERK and the Akt pathways involved in their anti-apoptotic effect. Indeed, activation of the ERK and Akt pathways can lead to phosphorylation and activation of the nuclear transcription factor cAMP response element-binding protein (CREB), which positively regulates the Bcl-2 gene expression [Bonni et al., 1999; Pugazhenthi et al., 2000; Chang et al., 2003].

Taken together, our results illustrate that activation of Akt and ERK, together with a modified expression of different Bcl-2 family members, contribute to the overall anti-apoptotic effect of $1,25(OH)_2D_3$ in keratinocytes (Fig. 10). In addition, we [De Haes et al., 2003] and others [Caelles et al., 1997; Ravid et al., 2002] previously described that $1,25(OH)_2D_3$ decreases stress-induced activation of c-Jun NH_2-terminal kinase (JNK). JNK, a member of the MAPK family, is known to be involved in the induction of apoptosis and therefore, inhibition of JNK by $1,25(OH)_2D_3$ might also contribute to its anti-apoptotic effect. Finally, Manggau et al. [2001] demonstrated the involvement of sphingosine kinase and sphingosine-1-phosphate in the anti-apoptotic effect of $1,25(OH)_2D_3$ in keratinocytes. The relative contribution of these molecular mechanisms to the anti-apoptotic capacity of $1,25(OH)_2D_3$ is however unclear, but it is possible, and even likely, that some of these pathways are linked. Indeed, sphingosine-1-phosphate has been

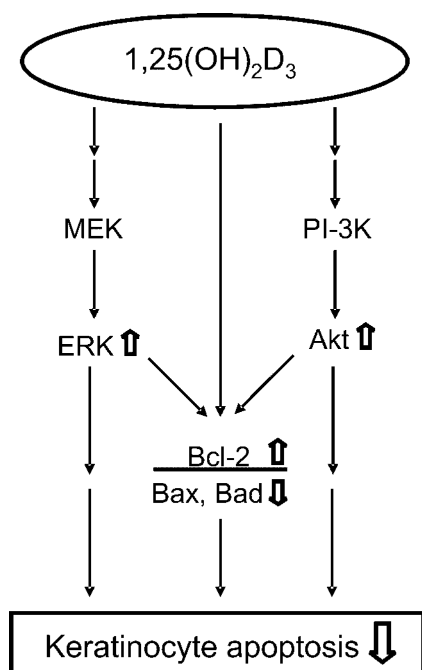


Fig. 10. Identified molecular mechanisms for the anti-apoptotic effect of $1,25(OH)_2D_3$ in human keratinocytes. $1,25(OH)_2D_3$ inhibits the induction of apoptosis both by activating the MEK/ERK and the PI-3K/Akt survival pathways and by increasing the Bcl-2 to Bax and Bad ratio. Induction of Bcl-2 occurs via the ERK and the Akt pathways, providing a link between both mechanisms.

recognized as a positive regulator of the ERK pathway [Wu et al., 1995], a suppressor of Bax protein [Goetzl et al., 1999] and an inducer of Bcl-2 protein [Manggau et al., 2001].

The implications of our findings are complex. It is clear that the anti-apoptotic effects of $1,25(OH)_2D_3$ in keratinocytes require pharmacological doses. Therefore, it is unlikely that physiological concentrations of active vitamin D_3, produced locally in the skin, will influence the process of apoptosis. In some cancer cells $1,25(OH)_2D_3$-induced growth arrest is at least in part mediated by the induction of apoptotic cell death. Treatment of normal keratinocytes with pharmacological doses $1,25(OH)_2D_3$ that are needed to inhibit cell growth, did however not induce apoptosis. Therefore, induction of apoptosis does not appear to contribute to the growth inhibitory effects of $1,25(OH)_2D_3$ in keratinocytes. In addition, we showed that pretreating keratinocytes with $1,25(OH)_2D_3$ protects them from the induction of apoptosis. Apoptosis is however considered as a mechanism ensuring the removal of irreversibly damaged and cancer precursor cells. Suppression of apoptosis by $1,25(OH)_2D_3$ in keratinocytes may therefore facilitate skin carcinogenesis, especially since two well-known oncogenes, Bcl-2 and Akt, [Cory and Adams, 2002; Vivanco and Sawyers, 2002] are upregulated or activated. However, epidemiologic studies suggest that active vitamin D metabolites protect against different cancers [Studzinski and Moore, 1995]. In addition, certain in vitro data support the existence of a protective role of $1,25(OH)_2D_3$ against UV-induced skin cancer. Indeed, it has been shown in keratinocytes that $1,25(OH)_2D_3$ induces metallothionein [Karasawa et al., 1987; De Haes et al., 2004], a radical scavenging protein that protects keratinocytes against oxidative-mediated UV-injury [Wang et al., 2004]. Moreover, $1,25(OH)_2D_3$ is said to protect keratinocytes against UVB-induced direct DNA-damage [own unpublished results and Wong et al., 2004]. In vivo studies on mouse skin carcinogenesis are inconclusive: $1,25(OH)_2D_3$ impaired the carcinogenesis process in some cases [Wood et al., 1983; Hershberger et al., 1999; Kensler et al., 2000] but it did not alter [Pence et al., 1991] or even facilitated the transformation process in other studies [Wood et al., 1985]. In VDR(−/−) mice, an enhanced sensitivity to chemically induced skin carcinogenesis has been described [Zinser et al., 2002]. Taken together, it is not sure yet whether $1,25(OH)_2D_3$ promotes or protects against skin carcinogenesis.

In conclusion, we showed that $1,25(OH)_2D_3$ promotes the survival of keratinocytes and inhibits the induction of apoptosis (1) by activating the MEK/ERK and the PI-3K/Akt survival pathways and (2) by increasing the Bcl-2 to Bax and Bad ratio. The implications of these findings need to be further evaluated, preferably using in vivo studies.

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