A vitamin D analogue KH 1060 activates the protein kinase C–c-fos signalling pathway to stimulate epidermal proliferation in murine skin

R Gniadecki
Department of Dermatological Research, Leo Pharmaceutical Products, 2750 Ballerup, Denmark
(Requests for offprints should be addressed to R Gniadecki, Department of Endocrinology, Polish Academy of Sciences, Banachala, 02097 Warsaw, Poland)

Abstract

The cellular signalling pathways of a potent 20-epi-22-oxa vitamin D₃ analogue (KH 1060) were examined in vivo in a hairless mouse model. Seventy two hours after a single topical application of KH 1060 a thickening of the epidermis (from 24.8±1.2 µm at 0-01 pmol/cm² KH 1060 to 124.2±6.8 µm at 5 pmol/cm² KH 1060, P<0.001) was elicited due to epidermal hyperproliferation. This effect could be blocked by topical 2.5 µmol/cm² sphingosine, an inhibitor of protein kinase C. Two hours after topical application of 2.5 pmol/cm² KH 1060 a translocation of protein kinase C activity from cytoplasm to the membrane fractions was observed. Moreover, using a reverse-transcription polymerase chain reaction technique, a transient upregulation of c-fos gene expression was seen 2 hours after topical treatment with KH 1060. The expression of c-fos was dependent on protein kinase C activation, since after pretreatment with the protein kinase C blocker sphingosine, c-fos messenger RNA was not detected. These findings strongly suggest that KH 1060 stimulates epidermal growth through activation of the protein kinase C – c-fos signalling axis in vivo.

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Introduction

1,25-dihydroxyvitamin D₃ (1,25-(OH)₂D₃) and its synthetic analogues are potent modulators of growth and differentiation for a variety of cells (Bikle 1992, Walters 1992). These effects are believed to be mediated by an activation of a vitamin D receptor which, together with auxiliary receptor proteins, binds the vitamin D responsive element in a gene promoter region and thus induces messenger RNA transcription (Carlberg et al. 1993). However, it is not known what kind of biochemical events related to cell growth are activated by this mechanism.

With respect to the regulation of cell proliferation and differentiation, a 20-epi analogue of 1,25-(OH)₂D₃, KH 1060, is one of the most potent vitamin D analogues (Binderup et al. 1991). Its mechanism of action is considered to be the same as that of the natural hormone. Studies of Lützow-Holm et al. (1992), Thieroff Ekerdt et al. (1994) and our own work (Gniadecki et al. 1994) demonstrated in a hairless mouse model that 1,25-(OH)₂D₃, KH 1060, and another vitamin D analogue, calcipotriol (MC 903) induce growth of normal murine epidermis in a dose-dependent manner after a topical application in vivo. These results were surprising because both 1,25-(OH)₂D₃ and KH 1060 have been considered to be inhibitors of cellular growth: they have been shown to downregulate proliferation of cancer cells and keratinocytes in vitro (Hosomi et al. 1983, Binderup et al. 1991), and 1,25-(OH)₂D₃ and calcipotriol have been used in the treatment of psoriasis to inhibit excessive epidermal proliferation (Bikle 1992). To explain these findings it is essential to obtain insight into the mechanism of growth regulation by 1,25-(OH)₂D₃ and its analogues. Since protein kinase C (PKC) activation is a strong stimulus for keratinocyte proliferation (Elder et al. 1992), and as there is evidence for activation of PKC by vitamin D derivatives in some cell lines (reviewed by Walters 1992), we investigated whether activation of the PKC signalling pathway could be involved in the stimulation of epidermal growth by KH 1060 in vivo.

Material and Methods

Drugs and solutions

KH 1060 and 1,25-(OH)₂D₃, were obtained from the Chemical Research Department, Leo Pharmaceutical Products, Ballerup, Denmark. Synthesis and receptor binding studies of KH 1060 are described elsewhere (Binderup et al. 1991, Hansen et al. 1991). Sphingosine and other reagents were purchased from Sigma (St Louis, MO, USA). Solutions were made in 1:1:2 w/w isopropyl alcohol/water mixture buffered with 1 g/l sodium citrate dihydrate (pH 8.9). This buffer was also used as placebo.
Animals
We used hairless C3H mice (females, strain hr/hr C3H/Tif Bom, weight approximately 20 g) that are homozygotic mutants in the recessive hr locus (Mann 1977). Hairless mice are widely used for the investigation of normal and pathologic proliferation of the epidermis (Lowe et al. 1985).

Determination of epidermal thickness
KH 1060 in a dose range 0.01–5.0 pmol/cm² was applied on the dorsal skin, 3 min after pretreatment with 2.5 µmol/cm² of sphingosine or placebo, as described previously in detail (Gnadecki et al. 1994). After 72 hours the mice were sacrificed by cervical dislocation, 2 punch biopsies (8 mm in diameter) were obtained, and after fixation in 10% formaldehyde sections were stained with haematoxylin and cosin. The epidermal and dermal thickness were measured in quintuplicate from each sample.

Determination of PKC activity
Skin was treated with 2.5 pmol/cm² KH 1060 and 2 hours later mice were sacrificed, skin removed and homogenized in a 50 mM Tris HCl pH 7.5 containing 5 mM EDTA, 10 mM EGTA, 0.3% w/v β-mercaptoethanol, 10 mM benzamidine and 50 µg/ml phenylmethylsulphonyl fluoride. Cytosol and membrane fractions were obtained as described (Arruda & Ho 1992) and PKC activity was determined with a protein kinase C enzyme assay system (Amersham International plc, Amersham, Bucks, UK).

c-fos mRNA detection
A 8 mm punch biopsy from the treated area was obtained, frozen in liquid nitrogen and homogenized. Polyadenylated RNA was extracted with a QuickPrep Micro mRNA Purification Kit (Pharmacia, Milwaukee, WI, USA) and dissolved in 0.4 ml low salt buffer provided in the kit. Two µl aliquots were reverse transcribed with c-fos antisense primer 5' AGCCTACGAGCACCTCCAG 3' and amplified by polymerase chain reaction using GeneAmp RNA PCR kit (Perkin Elmer, Norwalk, CT, USA) with both antisense and sense (5' CAAGTGCAG GAATCGGAGGAG 3') primers. The amplified fragment of the predicted size (233 base pairs) was detected with anion-exchange liquid chromatography using TSK-Gel DEAE-NPR column (Toso Haas, Stuttgart, Germany) as described (Gaus et al. 1993). The identity of the amplification product was confirmed by direct sequencing. Fifty µl of the crude PCR product was sequenced using Taq polymerase by the method of Innis et al. (1988).

Figure 1. Epidermal thickness 72 h after topical application of KH 1060 (○, n=3) and KH 1060 with 2.5 µmol/cm² sphingosine (●, n=3). Values are means±s.e.m. Note the reduction of hyperplastic response to KH 1060 by sphingosine (P<0.001, two-way analysis of variance).

Statistical analysis
Differences between groups were analyzed with a t-test for independent samples. For the dose-response experiments a two-way analysis of variance was performed.

Results
Inhibition of KH 1060-induced epidermal thickening by sphingosine
Sphingosine has been shown previously to inhibit PKC activity in vitro, and the inhibitory effect has been also demonstrated in vivo after topical application of the compound (Gupta et al. 1988). In the preliminary experiments we confirmed that 2.5 µmol/cm² of sphingosine blocked epidermal thickening caused by 12-0-tetradecanoylphorbol-13-acetate, a direct activator of PKC. The application of sphingosine alone did not have any influence on epidermal thickness (data not shown). KH 1060 stimulated epidermal thickening in a dose-dependent manner (Fig. 1). When different concentrations of KH 1060 were applied following skin
pretreatment with 2.5 µmol/cm² of sphingosine a statistically significant inhibition of epidermal thickening was found (P<0.001; Fig. 1).

**Activation of PKC by KH 1060**

To test whether KH 1060 actually causes activation of PKC, we estimated PKC activity in the cytosol and membrane fractions of the skin homogenate. In the control homogenates activity of PKC was approximately the same in both fractions, whereas 2 hours after KH 1060 treatment we detected a decreased activity in the cytosolic fraction (P<0.001) with a simultaneous increase of activity in the membrane fraction (P<0.001) (Fig. 2).

**c-fos expression**

To investigate whether KH 1060 can stimulate the PKC−dependent expression of c-fos we amplified the c-fos specific mRNA by the polymerase chain reaction, after its reverse transcription to cDNA (Fig. 3). In normal skin c-fos specific product of the polymerase chain reaction was not detected, whereas a strong signal was detected 2 hours after application of 2.5 pmol/cm² KH 1060. The c-fos expression mRNA was no longer detectable 12 hours after application of a drug. When KH 1060 was applied together with sphingosine c-fos gene was not expressed.
Discussion

It has been demonstrated recently that 1,25-(OH)₂D₃ affects various intracellular messenger systems formerly not assumed to be related to steroid hormone action, such as calcium and other ion channels (Bittiner et al. 1991, Binswanger et al. 1993, Sorensen et al. 1993, Jones & Sharpe 1994), cyclic GMP (Chenay Marchais & Frish 1993, Khare et al. 1993), cyclic AMP (Masheimer & de Boland 1992), arachidonic acid metabolites (de Boland & Boland 1993), and PKC. 1,25-(OH)₂D₃ affects PKC activity in various target cells. In HL-60 leukaemia cells and U937 human monoblastoid line, 1,25-(OH)₂D₃ increased the PKC levels in both the cytosolic and membrane fractions (Martell et al. 1987b, Wais et al. 1987). Differentiation of HL-60 cells induced by 1,25-(OH)₂D₃ could be suppressed by PKC inhibitors (Martell et al. 1987a). In contrast, Mezzetti et al. (1987) did not find evidence for PKC involvement in 1,25-(OH)₂D₃-induced differentiation of U937 cells. In myoblasts inositol phosphates and diacylglycerol are generated upon treatment with 1,25-(OH)₂D₃ (Morelli et al. 1993). It has been reported that PKC is also activated by 1,25-(OH)₂D₃ in classical targets, e.g. ROS 17/28 osteoblast line (Van Leeuwen et al. 1992), normal rat colonic epithelium (Wali et al. 1990), and bovine kidney cell line (Simboli-Campbell et al. 1992). However, the significance of these alternative signalling pathways in the modulation of epidermal proliferation in vivo have not been elucidated.

KH 1060-induced epidermal hyperproliferation could be inhibited by the PKC inhibitor sphingosine. Furthermore, KH 1060 promoted PKC translocation from cytoplasm to the membranes. These findings together indicate that PKC is functionally involved in mediating epidermal growth stimulation after application of KH 1060 in vivo. Several issues, however, need further elucidation. By using whole skin homogenates we were not able to determine which cell population(s) respond to KH 1060 by PKC translocation and c-fos induction. Sphingosine is not a selective inhibitor of PKC, and to a lesser extent it blocks the activity of other protein kinases. We could not, however, use more selective peptide blockers of PKC, since they are not active upon topical administration. Further studies in pure keratinocyte populations cultured in vitro are needed to clarify these points.

Our results are in accordance with the observations of Yada et al. (1989) who documented translocation of PKC in cultured keratinocytes. However, Koizumi et al. (1991) were unable to detect PKC activation after topical application of 1,25-(OH)₂D₃. The reason for this discrepancy may be the different experimental system they used (pig vs murine skin). In fact, we have observed (unpublished) that the epidermis of minipigs does not proliferate readily after a single application of KH 1060 and 1,25-(OH)₂D₃, as is seen in murine epidermis. Moreover, Koizumi et al. (1989) did not study PKC translocation but its activity in total epidermal homogenate. Since PKC activation depends primarily on its translocation to the membranes or nucleus, the total PKC activity may remain normal despite enzyme activation.

In response to PKC activation, an upregulation of c-fos gene expression takes place (Angel & Karin 1991). The c-fos mRNA is subsequently translated to Fos protein that dimerizes with another protein Jun to form a transcription factor AP-1. AP-1, and possibly other transcription factors (AP-2, NF-kB), bind to a consensus sequence in gene promoters and stimulate the expression of genes regulating cellular growth and differentiation (Housey et al. 1988, Angel & Karin 1991). In our experiments, KH 1060 upregulated c-fos gene expression. It was clearly a PKC-dependent phenomenon, as c-fos induction by KH 1060 was abrogated by treatment with sphingosine.

1,25-(OH)₂D₃ and analogues inhibit cell proliferation in vitro and have been used successfully for the treatment of hyperproliferative diseases such as psoriasis and cancer (Bikle 1992). It is not clear whether PKC stimulation mediates both epidermal proliferation in vivo and suppression of keratinocyte growth in vitro. The stimulatory effect appears to be relatively rapid, being detectable after a single topical application. In contrast, the vitamin D compound must be applied for prolonged periods (3–7 days) to obtain significant growth inhibition. It remains to be seen whether activation of the PKC-c-fos signalling axis is involved in the antiproliferative and therapeutic effect of the vitamin D analogues in hyperplastic skin disorders such as psoriasis.

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