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Liposomal Incorporation Changes the Effect of 1.25-Dihydroxyvitamin D_3 on the Phospholipase C Signal Transduction Pathway and the Eicosanoid Cascade on Keratinocytes *in Vitro*

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ABSTRACT. 1.25-dihydroxyvitamin D_3 is of clinical importance (e.g. in the treatment of psoriasis) given its ability to regulate the proliferation and differentiation of human keratinocytes. 1.25-Dihydroxyvitamin D_3 mediates its action via genomic and nongenomic pathways. The nongenomic actions begin with the activation of phospholipase C and the subsequent rapid rise in calcium within the cells. We incorporated 1.25-dihydroxyvitamin D_3 in liposomes of varying compositions in an attempt to improve their effect/negative side effect ratio. The influence of empty liposomes (1 mM) and free and liposomally incorporated 1.25-dihydroxyvitamin D_3 (10 nM) on the rapid release of sulfidoleucotrien and inositole 1,4,5 triphosphate was examined in keratinocytes *in vitro*. Free 10 nM 1.25-dihydroxyvitamin D_3 provoked a rapid rise in sulfidoleucotriens within 30 seconds, followed by a swift decrease in sulfidoleucotrien and inositole 1,4,5-triphosphate concentration after 10 minutes. Empty liposomes and liposomal-incorporated 1.25-dihydroxyvitamin D_3 did not show such a strong effect. These results suggest the occurrence of specific binding sites for 1.25-dihydroxyvitamin D_3 on the membrane level that are incapable of recognizing 1.25-dihydroxyvitamin D_3 trapped within liposomal membrane. BIOCHEM PHARMACOL 51;3:247–252, 1996.

KEY WORDS. 1.25-dihydroxyvitamin D_3; liposome, phospholipase C; inositole 1,4,5-triphosphate; sulfidoleucotrien; keratinocytes

Liposomes have been widely used as drug carriers [1–3]. Liposomal encapsulation allows the improved specificity of drug effect while minimizing negative side effects. In dermatological applications this means that a drug will remain confined to the skin, thereby avoiding systemic effects.

1.25-Dihydroxyvitamin D_3 is known to regulate the proliferation and differentiation of human keratinocytes. Therefore, it is used for the treatment of psoriasis, a disease of the skin marked by hyperproliferation, disregulation of the cytokine network, and inflammation of the skin. The lesional keratinocytes follow an altered differentiation pathway, leading to disturbed barrier functions of the skin.

Vitamin D compounds have been proven to exert antipsoriatic effects, as shown by the decrease in the proliferative activity of epidermal keratinocytes, normalization of the differentiation program, and anti-inflammatory capacity [4–6].

The mode of action of the steroid hormone vitamin D_3 can be subdivided into genomic and nongenomic effects [7]. The former are mediated by a specific nuclear receptor (VDR), which binds on promoter regions and regulates transcription of genes, thereby influencing proliferation and differentiation [8–10]. A 1.25-dihydroxyvitamin D_3-dependent phosphorylation of the VDR, probably by protein kinase C, is necessary for the transcriptional activity of the VDR [11]. When the inositole 1,4,5-triphosphate signal pathway is activated, calcium is delivered from intracellular depots [12]. In chicken embryo myoblasts, 1.25-dihydroxyvitamin D_3 induces a calcium-dependent arachidonic acid mobilization caused partially by activation of phospholipase A_2 [13]. As shown in our previous paper [14], the incorporation of different vitamin D_3 analogues in liposomes of various composition did not enhance the proliferation-inhibiting effect of the free vitamin D_3 analogues on human keratinocytes. In some cases this effect was even diminished. However, in no case was the liposomal incorporation cytotoxic. We were able to provide evidence that the uptake of liposomes by keratinocytes occurs within four hours, probably by endocytosis.

We suggested that the additional activation of phospholipase C and phospholipase A_2 by phospholipids, added as liposomes, may cause changes or cumulative effects in pathways responsible for proliferation and differentiation of human keratinocytes. In the present study we addressed the question whether liposomal incorporation of 1.25-dihydroxyvitamin D_3 causes changes in signal and metabolic pathways as compared to the free drug.

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† *Abbreviations:* 1.25 D_3, 1 alpha,25-dihydroxyvitamin D_3; DMEM, Dulbecco's modified Eagle's medium; DMPC, 1,2-dimyristoyl-sn-glycero-3-phosphocholine; eggPC, egg lecithin; DOPC, 1,2-dioleyl-sn-glycero-3-phosphocholine.

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MATERIALS AND METHODS

Materials

Dimyristoylphosphatidylcholine, dioleylphosphatidylcholine, and egg lecithin were obtained from SIGMA. 1.25-Dihydroxyvitamin D_3 was generously provided by Drs. Schönecker, Gliesing, and Reichenbächer (Faculty of Chemistry at the University of Jena). It was dissolved in ethanol.

Preparation of the Liposomes and Liposomal Encapsulations

The empty liposomes were prepared by a standard procedure. The different lipids were dissolved in pure chloroform to obtain stock solutions. A determined sample of lipid stock solution was placed in a flash and the organic solvents removed by rotary evaporation. The thin lipid film in the flask was dried, resuspended in bi-distilled water, and strongly shaken. The multilamellar liposomes formed by this procedure were degraded to smaller oligolamellare liposomes by ultrasonication for 10–20 minutes. Usually, smaller liposomes with a diameter of 200 nm were used in concentrations of approximately 2 mg lipid per 1 mL water.

After ultrasonic treatment, the liposome suspensions were filtered through a 200 nm sterile filter (Sartorius). The exact amount of lipid was determined by phosphorus analysis after sterile filtration. The lipid concentration of the liposomes used was 1 mM in 10 mM LiCl, supplemented Hank's saline (buffered with 10 mM HEPES; HBSS). Liposomes loaded with 1.25-dihydroxyvitamin D_3 were prepared as follows: 30 mM of 1.25-dihydroxyvitamin D_3 stock solution in methanol was added to 1 M of lipid in a flask. Loaded liposomes were prepared as described above for empty liposomes. Unincorporated drug was removed by chromatography on a Sephadex G 50 (Pharmacia) column. The concentrations of both lipid and drug were determined after sterile filtration. Encapsulated drug concentration was measured by HPLC on a C8 or C18 column (Kramer). A mixture of acetonitril/water in different ratios was used as eluting agent [15].

The concentration of free and liposomally-incorporated 1.25-dihydroxyvitamin D_3 used was 10 nm in 10 mM LiCl supplemented HBSS.

Cell Cultures

Human HaCaT keratinocytes [16] propagated in 175 mL tissue culture flasks (Falcon) with weekly passage in DMEM† (Gibco) containing 10% fetal calf serum (Gibco), 2% glutamine (Gibco), and 1% antimycotic/antibiotic (Gibco) were kept under sterile conditions in humidified atmosphere with 6% CO_2 at 37°C [17]. Cells were harvested by 0.25% trypsin/EDTA digestion, which was stopped after 15 min with DMEM; the suspension was then centrifuged for 10 min at 1000 g and resuspended.

Phospholipase C Activity

The cells were plated at a density of 10^5 cells/well in 6-well macrotiterplates (Greiner) in DMEM. After 48 hours, the cells were washed twice with PBS and incubated for 30 minutes in 10 mM LiCl-supplemented HBSS at 37°C. Thereafter, the cells were treated with liposomes, free and liposomally-incorporated 1.25-dihydroxyvitamin D_3 for 30 seconds, 2 minutes, and 10 minutes, respectively. The supernatants were collected for the determination of sulfidoleucotrien and prostaglandin E_2 release, and cells were fixed by adding ice-cold trichloric acid. Inositole 1,4,5-triphosphate was extracted from the cells by incubation with trichloric acid for 30 minutes at 4°C. The solution was neutralized using 1.5 M NH_3. The neutral sample was analyzed according to the manufacturer's advice supplied with the inositole 1,4,5-triphosphate kit (Amersham).

Sulfidoleucotrien

The amounts of these compounds were determined in the supernatants of the cultures that had been treated as described above (Bühlmann Laboratories AG).

Statistics

The median was determined by using the results from two independent experiments with two parallels in each case. The significance of differences between treated and control cell cultures was examined using Student's *t*-test.

RESULTS

Phospholipase C

EMPTY LIPOSOMES. Empty liposomes induced the formation of inositole 1,4,5-triphosphate in accordance with their composition (Table 1).

Contrary to DOPC liposomes, liposomes made of DMPC and egg PC clearly induced phospholipase C activity after thirty seconds. We used a lithiumchloride-containing buffer to

TABLE 1. Time-dependent formation of inositole 1,4,5-triphosphate after treatment of HaCaT cells with liposomes (lipid concentration 1 mM; mean ± SD, results from two independent experiments with two parallels each)

	30 seconds	2 minutes	10 minutes
Control	15 ± 7.1 pmol/tube	40 ± 21.7 pmol/tube	50 ± 2.8 pmol/tube
DMPC liposomes	50 ± 2.8 pmol/tube*	>50 pmol/tube	>50 pmol/tube
DOPC liposomes	25 ± 1.4 pmol/tube	25 ± 1.4 pmol/tube	>50 pmol/tube
Egg PC liposomes	50 pmol/tube*	>50 pmol/tube	>50 pmol/tube

* Significant difference from control $P < 1\%$.

TABLE 2. Time-dependent formation of inositole 1,4,5-triphosphate after treatment of HaCaT cells with 10 nM 1.25-dihydroxyvitamin D_3 (mean ± SD, results from two independent experiments with two parallels each)

	30 seconds	2 minutes	10 minutes
Control	15 ± 7.1 pmol/tube	40 ± 21.7 pmol/tube	50 pmol/tube
1.25 D_3	>50 pmol/tube	>50 pmol/tube	16 ± 2.1 pmol/tube*

* Significant difference from control $P < 1\%$.

stabilize and accumulate inositole-phosphates. The concentration of inositole 1,4,5-triphosphate in the buffer-treated controls exceeded the limit of measurability within 10 minutes. The spontaneous increase of inositole 1,4,5-triphosphate in the control was due to the use of the lithium-containing buffer.

1.25-DIHYDROXYVITAMIN D_3. 1 nm 1.25-dihydroxyvitamin D_3 induced a clear enhancement of inositole 1,4,5-triphosphate formation after 30 seconds. After 10 min the concentration of inositole 1,4,5-triphosphate was below control levels (Table 2).

LIPOSOMAL INCORPORATED 1.25-DIHYDROXYVITAMIN D_3. Depending on the lipid composition of the liposomes containing 10 nM 1.25-dihydroxyvitamin D_3, phospholipase C was activated by 1.25-dihydroxyvitamin D_3 in DMPC and DOPC liposomes (Table 3).

Sulfidoleucotriens

EMPTY LIPOSOMES. The formation of sulfidoleucotriens by the 5-lipoxygenase was only weakly influenced by liposomal phospholipids (Table 4).

1.25-DIHYDROXYVITAMIN D_3. The effect of 10 nm 1.25-dihydroxyvitamin D_3 on the expression of sulfidoleucotriens was very impressive. After 30 seconds, 500 ± 280 pg/mL sulfidoleucotriens were detectable in the supernatant of treated cells; after 2 minutes 125 ± 99.7 pg/mL; and after 10 minutes, 60 ± 86.3 pg/mL (Fig. 1).

LIPOSOMAL-INCORPORATED 1.25-DIHYDROXYVITAMIN D_3. The liposomal-incorporated 1.25-dihydroxyvitamin D_3 used in the same concentration as described above clearly showed different behavior (Table 5).

DISCUSSION

There is an increasing body of evidence that steroid hormones exert nongenomic effects on target tissues in addition to the known direct genomic effects mediated through nuclear receptors [24]. 1.25-Dihydroxyvitamin D_3 was the first steroid with clear nongenomic actions. Various tissues and cell types are targets for 1.25-dihydroxyvitamin D_3, and respond to 1.25-dihydroxyvitamin D_3 treatment with a rapid intracellular rise in calcium. Inositole 1,4,5-triphosphate mobilized calcium from intracellular depots. Osteogenic sarcoma cells responded to 10^{-9} M 1.25-dihydroxyvitamin D_3 treatment with Ca^{2+} rise and threshold inositole 1,4,5-triphosphate release [18]. The drug also increased intracellular calcium in osteoblast-like cells [19]. In confluent mouse myoblasts, phospholipase C was activated by 1.25-dihydroxyvitamin D_3, 24.25-dihydroxyvitamin D_3, and 25-hydroxyvitamin D_3 within seconds [20]. In growth zone cell cultures of chondrocytes, 10 to 0.1 nm 1.25-dihydroxyvitamin D_3 induced proteinkinase C activity beginning at three minutes in a cell maturation-specific manner [21]. Phospholipase A_2 was activated by 1.25-dihydroxyvitamin D_3 in embryonic chicken myoblasts [13]. A rise in intracellular free calcium [22, 23] and the increased production of inositole 1,4,5-triphosphate [12] was seen after treatment of keratinocytes with 1.25-dihydroxyvitamin D_3. The swift nongenomic effects of 1.25-dihydroxyvitamin D_3 likely are primarily mediated through activation of phospholipase C.

Phospholipase C catalyzes the hydrolysis of phosphatidylinositol 4,5 biphosphate to inositole 1,4,5-triphosphate and diacylglycerol. Inositole 1,4,5-triphosphate mediates the release of calcium from intracellular depots. The catabolism of

TABLE 3. Time-dependent formation of inositole 1,4,5-triphosphate after treatment of HaCaT cells with liposomally-incorporated 10 nM 1.25-dihydroxyvitamin D_3 (mean ± SD, results from two independent experiments with two parallels each)

	30 seconds	2 minutes	10 minutes
Control	15 ± 7.1 pmol/tube	40 ± 21.7 pmol/tube	50 pmol/tube
1.25 D_3 in DMPC	50 ± 1.4 pmol/tube*	>50 pmol/tube	50 pmol/tube
1.25 D_3 in DOPC	50 ± 12.0 pmol/tube*	>50 pmol/tube	>50 pmol/tube
1.25 D_3 in egg PC	35 ± 13.4 pmol/tube	50 ± 2.8 pmol/tube	34.5 ± 24.7 pmol/tube*

* Significant from control $P < 1\%$.

TABLE 4. Time-dependent expression of prostaglandin after treatment of HaCaT cells with liposomes of various composition (mean ± SD, results from two independent experiments with two parallels each)

	30 seconds	2 minutes	10 minutes
Control	0	0	0
DMPC liposomes	0	30 ± 26.2 pg/mL*	0
DOPC liposomes	0	31 ± 43.1 pg/mL	0
EggPC liposomes	0	0	140 ± 199.4 pg/mL

* Significant difference from control $P < 1\%$.

diacylglycerol leads to arachidonic acid, the substrate of PLA_2, and precursor of leucotriens and prostaglandins.

We expected cumulative or synergistic effects on phospholipase C after liposomal incorporation of the hormone.

Liposomal incorporation of 1.25-dihydroxyvitamin D_3 activated phospholipase C and the eicosanoid cascade in a different manner in comparison to the free drug, probably via the activation of phospholipase C. This difference was clearly seen by comparing the release of sulfidoleucotriens after treatment with free 1.25-dihydroxyvitamin D_3 and its liposomal incorporations.

The free drug caused a typical increase in sulfidoleucotrien release within 30 seconds and a rapid decrease after two minutes. The observed actions of 1.25-dihydroxyvitamin D_3 are likely nongenomic due to the swift time course of the observed events. The release of inositole 1,4,5-triphosphate after 1.25-dihydroxyvitamin D_3 exposure returned to a significantly lower level in comparison to the control after 10 minutes, suggesting strong enzymatic degradation activities typical after second messenger activation.

The hypothesis that there exists a specific membrane-binding site for 1.25-dihydroxyvitamin D_3 is supported by the different behavior of liposomal incorporations of 1.25-dihydroxy-vitamin D_3. A peak in sulfidoleucotrien release is not recognizable. The values of inositole 1,4,5-triphosphate and sulfidoleucotrien release are rather comparable with the results of empty liposomes, suggesting that the different sulfidoleucotrien-releasing effect arises from free 1.25-dihydroxyvitamin D_3.

Because of its amphiphilicity, 1.25-dihydroxyvitamin D_3 is incorporated within the liposome membrane [15]. A part of the drug is probably released before the liposomes enter the cell and acts as the free drug does. The major part is endocytosed and released within the cell, where it can develop its genomic actions. This part does not bind to membrane-binding sites for 1.25-dihydroxyvitamin D_3 incapable of recognizing 1.25-dihydroxyvitamin D_3 trapped within liposome membrane. This observation supports the notion that nongenomic actions of steroid hormones are mediated through membrane-binding sites [24]. Further experiments with 1.25-dihydroxyvitamin D_3 immobilized in the outer layer of the liposome membrane with appropriate spacer molecules will reveal whether the means of presenting the steroid affects membrane action of 1.25-dihydroxyvitamin D_3.

It is likely that the genomic and nongenomic responses are mediated by distinct steroid-binding structures. The 6-s-cis

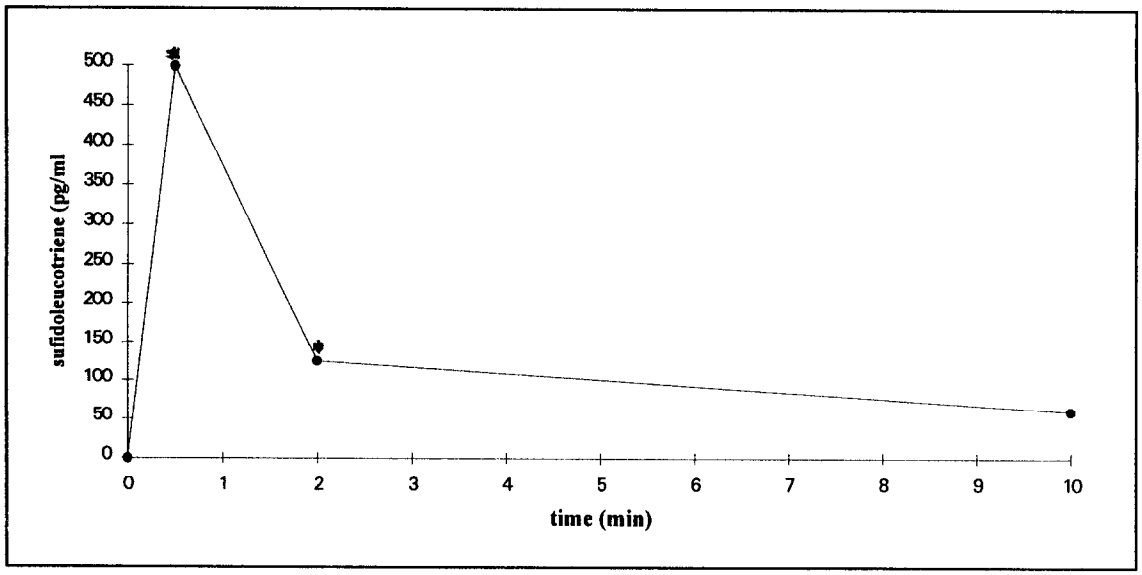

FIG. 1. Time-dependent expression of sulfidoleucotriens after treatment of HaCaT cells with 1.25-dihydroxyvitamin D_3 (*significant difference from control P < 1%; results from two independent experiments with two parallels each).

TABLE 5. Time-dependent expression of sulfidoleucotriens after treatment of HaCaT cells with liposomally-incorporated 1.25-dihydroxyvitamin D_3 (mean ± SD, results from two independent experiments with two parallels each)

	30 seconds	2 minutes	10 minutes
Control	0	0	0
1.25 D_3 in DMPC	0	81 ± 103.2 pg/mL	0
1.25 D_3 in DOPC	0	11 ± 7.1 pg/mL*	0
1.25 D_3 in egg PC	33.5 ± 47.4 pg/ml	26.5 ± 14.8 pg/mL*	0

* Significant difference from control $P < 1\%$.

steroid-like form of 1.25-dihydroxyvitamin D_3 may be selectively responsible for its nongenomic functions [25].

Such a receptor was found in the basal-lateral membrane of vitamin D-replete chick intestinal epithelium with $K_D = 0.72 \times 10^{-9}$ M [26] and in membrane preparations from ROS 24/1 cells (osteoblast-like rat osteosarkoma cells) with $K_D = 0.81 \times 10^{-8}$ M [27]. Further studies are needed to address the question whether keratinocytes possess such a membrane receptor.

Phospholipids of steroid-free liposomes are likely to interact with keratinocyte membranes *in vitro*. They influence the formation of inositole 1.4.5 triphosphate and sulfidoleucotriens in a different manner. DMPC and DOPC liposomes cause a very small release of sulfidoleucotriens after two minutes, whereas release after treatment of human keratinocytes with egg PC liposomes occurs only after ten minutes and is without significance. The activation of phospholipase A_2 could be nonspecific.

Phospholipase C is clearly activated by DMPC and egg PC liposomes. The proliferation-inhibiting properties of these liposomes in a concentration of 1 mM lipid could be due to the activation of phospholipase C and following pathways such as proteinkinase C and calcium release from intracellular depots, both of which play important roles in the differentiation and proliferation of human keratinocytes.

The liposomal incorporation of steroid hormones could specifically prevent negative side effects caused by nongenomic membrane actions. The previously observed impairment of the effects of steroid hormones and other drugs could be explained by the prevention of membrane interaction by the free drug.

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