The ethanolamine requirement of keratinocytes for growth is not due to defective synthesis of ethanolamine phosphoacylglycerols by the decarboxylation pathway

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We have investigated whether the growth requirement of keratinocytes for ethanolamine is due to defective synthesis of ethanolamine phosphoacylglycerols (EPG) via decarboxylation of serine phosphoacylglycerols. Proliferating keratinocytes readily incorporated [^3H]ethanolamine into phosphatidylethanolamine (PE) and [^3H]serine into phosphatidylserine (PS) and PE. Non-proliferating keratinocytes in ethanolamine-free medium incorporated [^3H]glycerol into phosphatidylcholine (PC), PS and PE in decreasing order of label incorporated. The order of decreasing incorporation of glycerol after addition of ethanolamine to the medium was PC > PE > PS. Incubation of non-proliferating keratinocytes with [^3H]serine resulted in incorporation of label into PS and PE. The extent of incorporation of [^3H]serine into PS in non-proliferating keratinocytes was not less than that in proliferating cells. Addition of ethanolamine to the medium of non-proliferating keratinocytes did not change the quantity of label incorporated into PS, but resulted in a decrease of label incorporated into PE. When cells were prelabelled overnight with [^3H]serine and subsequently incubated in medium containing ethanolamine, the loss of label from PS was inhibited relative to that of control cells incubated in medium without

ethanolamine. The activity of PS decarboxylase activity in keratinocyte mitochondria was inhibited by phosphoethanolamine and PE, but not by ethanolamine or CDP-ethanolamine. Both proliferating and non-proliferating keratinocytes incorporated [^3H]serine into ether-linked ethanolamine phospholipids. Taken together, the above results suggest that (1) both proliferating and non-proliferating keratinocytes are able to synthesize PE and ether-linked ethanolamine phospholipids from serine, and therefore the ethanolamine-requirement of the cells is not due to a defective decarboxylase pathway; (2) any inability of the decarboxylase pathway to meet cellular EPG requirement is not due to decreased synthesis of serine phospholipids; (3) synthesis of PE via decarboxylation, the major route in non-proliferating keratinocytes, appears to decrease when ethanolamine is made available and the CDP-ethanolamine pathway is functioning; (4) phosphoethanolamine and increased PE produced from the CDP-ethanolamine pathway may inhibit PS decarboxylase activity in the cells and provide a means of coordinating the synthesis of PE by the two pathways to prevent excess production.

INTRODUCTION

Phosphatidylethanolamine (PE) is a major phospholipid of mammalian cell membranes, accounting for 20–30% of total phospholipid. Two major pathways have been described for the synthesis of PE, one the CDP-ethanolamine pathway, in which ethanolamine is sequentially converted into phosphoethanolamine and CDP-ethanolamine, followed by transfer of the phosphoethanolamine group from the latter to a diacylglycerol to form the phospholipid. The other major pathway involves decarboxylation of phosphatidylserine (PS) to form PE in a reaction catalysed by the mitochondrial enzyme PS decarboxylase. A third route of PE synthesis, via base-exchange reactions, is thought to be of minor importance in tissues [1].

There is a widespread perception that cells growing in culture do not require ethanolamine in the medium, because they can satisfy their PE requirement from serine via decarboxylation of PS [2,3]. However, because most cells are cultured in media with serum, which contains ethanolamine [4], one cannot rule out the possibility that serum contains sufficient ethanolamine to meet the cell's requirement. Advances in tissue culture now permit the growth of some cells in defined medium under very low concentrations or in the complete absence of serum. Under such conditions it has been established that some cells require

ethanolamine or phosphoethanolamine for growth. These include normal human keratinocytes [5–7], 64-24 mammary carcinoma cell line [8,9], normal human melanocytes [10], normal human mammary epithelial cells [11] and normal human bronchial epithelial cells [12]. Ethanolamine and phosphoethanolamine were identified as the mitogens in bovine pituitary extract that stimulated the growth of the 64-24 cancer cell line [8,9]. In the development of a completely defined serum-free medium for the growth of primary keratinocytes, ethanolamine and phosphoethanolamine replaced bovine pituitary extract in the formulation [5]. This ethanolamine requirement of keratinocytes was evident in spite of the high concentration of serine in the basal medium [13]. Although the role of ethanolamine in the proliferation of keratinocytes is not known [10], studies with the 64-24 cell line suggested that they were probably utilized solely for PE synthesis [9]. Indeed, as far as is known, the cellular role of ethanolamine is the synthesis of PE or glycosylphosphatidylinositol anchors [14]. The ethanolamine requirement could be due to an inability to synthesize PE via decarboxylation of PS, or it may signify an inability to synthesize ether subclasses of ethanolamine phosphoacylglycerols (EPG) via the decarboxylase pathway. In either case the cells would be required to obtain their EPG complement solely via the CDP-ethanolamine pathway. It is not known whether keratinocytes have a functional or defective PS de-

Abbreviations used: PS, phosphatidylserine; PE, phosphatidylethanolamine; PC, phosphatidylcholine; HBSS, Hepes-buffered saline solution; EPG, ethanolamine phosphoacylglycerol.

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carboxylase pathway. As a first step to elucidating the role of ethanolamine in the proliferation of normal human keratinocytes, we have investigated the synthesis of EPG via the decarboxylase pathway in proliferating and non-proliferating keratinocytes.

MATERIALS AND METHODS

Cryopreserved primary human foreskin keratinocytes, keratinocyte basal medium, keratinocyte defined medium (ethanolamine/phosphoethanolamine-free), growth additives (cortisol, insulin, epidermal growth factor, bovine pituitary extract), trypsin, trypsin inhibitor and Hepes-buffered saline solution (HBSS) were purchased from Clonetics (San Diego, CA, U.S.A.). L-[3-³H]Serine, [1-³H]ethanolamine and L-3-phosphatidyl-L-[3-¹⁴C]serine (1,2-dioleoyl) were obtained from Amersham. [1,2,3-³H]Glycerol was purchased from Du Pont. Ethanolamine (free base), phosphoethanolamine, CDP-ethanolamine, EDTA and Triton X-100 were obtained from Sigma (St. Louis, MO, U.S.A.). Phospholipase C (*Bacillus cereus*) was a product of Boehringer Mannheim (Laval, Quebec, Canada). Ecolite was obtained from ICN Biomedicals (St.-Laurent, Quebec, Canada). PS (bovine brain) and PE (pig liver) were the products of Serdary Research Laboratories (London, Ontario, Canada). All tissue-culture ware (Falcon) and chromatographic material were purchased from Baxter Canlab (Winnipeg, Manitoba, Canada). Solvents and reagents (ACS grade) were obtained from Fisher Scientific (Winnipeg, Manitoba, Canada).

Cell culture and labelling studies

Keratinocytes were grown in complete serum-free growth medium (keratinocyte basal medium with all the growth additives as specified by Clonetics) until the cells were 80–90% confluent. The keratinocytes were passaged by trypsin treatment and seeded into the appropriate ware for each experiment. All the experiments in this study utilized tertiary cultures of keratinocytes from a single strain (strain 289).

In experiments with proliferating keratinocytes, the cells were seeded at a density of 4000 cells/cm² in 60 mm dishes and incubated with complete growth medium until cells had grown to about 70% confluency. On the day of the experiment the medium was replaced with 6 ml of fresh medium, followed by addition of [³H]serine (60 µCi/dish) or [³H]ethanolamine (20 µCi/dish). At the end of each incubation period, the cells were washed twice with HBSS, detached with trypsin and transferred to 30 ml centrifuge tubes. After dispersion with a 21-gauge needle, samples were taken for determination of the cell number with a Coulter ZM counter (Coulter Electronics). The rest of the cells were pelleted by centrifugation and the lipids were extracted as previously described [15]. Phospholipid markers were added to the samples, and the phospholipid classes were separated by t.l.c. on activated Whatman K6 plates [16]. Radioactivity associated with phosphatidylcholine (PC), PS and PE were determined by scintillation counting on a Beckman LS3801 scintillation counter with Ecolite as the scintillant.

To obtain non-proliferating keratinocytes, secondary cultures of keratinocytes were grown to 90% confluency. The cells were trypsin-treated and seeded into the appropriate tissue culture ware with ethanolamine/phosphoethanolamine-free keratinocyte basal medium, without bovine pituitary extract but containing cortisol, epidermal growth factor and insulin. The medium was changed every 2–3 days and the cells were used for experiments after 7 days.

In experiments to investigate the effect of ethanolamine on the incorporation of [³H]glycerol into phospholipids of non-

proliferating keratinocytes, the cells were seeded at a density of 1×10^5 cells/well into 6-well plates. After 7 days in ethanolamine/phosphoethanolamine-free medium, the experiment was initiated by replacement with fresh medium (2 ml), followed by addition of 20 µl of ethanolamine (10 mM) to give a final concentration of 0.1 mM in experimental wells. Control wells received an identical amount of water. [³H]Glycerol (30 µCi/well) was added and the cells were placed in an incubator at 37 °C, containing air/CO₂ (19:1). At the end of each incubation period, the cells were washed twice with HBSS. The cells were harvested by trypsin, and keratinocytes from two wells were combined for each analysis. The determination of cell number, lipid extraction and separation of lipids were as described above for the proliferating cells.

To investigate the effect of ethanolamine on incorporation of [³H]serine into non-proliferating keratinocyte phospholipids, the cells were plated in 100 mm dishes at a seeding density of 8000 cells/cm² and incubated with ethanolamine/phosphoethanolamine-free medium. After 7 days the medium was replaced with 8 ml of fresh medium, and 60 µl of ethanolamine (10 mM) was added to experimental wells to give a final concentration of 0.1 mM. An identical volume of water was added to control dishes. [³H]Serine (60 µCi/dish) was added and the dishes were placed in a CO₂ incubator at 37 °C (air/CO₂, 19:1). At the end of each incubation period, the cells were treated as described above.

In all experiments with [³H]serine, a correction for recycling of label was made by separating the phospholipids, extracting the lipids from t.l.c. plates [17], and hydrolysing with phospholipase C [16] to determine the proportion of label associated with the base.

Distribution of radiolabel in diacyl and ether subclasses of ethanolamine phospholipids

Lipids were extracted from cells incubated with [³H]serine or [³H]ethanolamine and purified by t.l.c. as described above. The plates were sprayed with dichlorofluorescein and the bands were revealed by u.v. light. The EPG bands were extracted by the method of Arvidson [17]. Samples of the purified lipids were subjected to mild alkaline hydrolysis and the reaction products were separated by t.l.c. [18]. The quantity of radiolabel in the lyso-PE fraction representing the combined ether fraction was determined.

Isolation of mitochondria

Proliferating keratinocytes were grown to 80% confluency in 150 mm dishes and subsequently harvested by trypsin treatment. The cells were washed in HBSS and dispersed in buffer composed of 0.25 M sucrose, 10 mM Tris/HCl, pH 7.4, and 1 mM EDTA. The cells were disrupted in a Kontes Mini-bomb cell disruptor at a N₂ pressure of 4.8 MPa (700 lb/in²) for 30 min. Mitochondria was isolated by differential centrifugation [19]. The protein content was determined by the method of Lowry et al. [20].

PS decarboxylase assay

PS decarboxylase activity in the mitochondrial fraction was measured by determining the rate of formation of PE from phosphatidyl-L-[3-¹⁴C]serine. The assay was modified from the procedure of Voelker and Frazier [21]. Tris/HCl (0.1 M), pH 6.8, replaced KH₂PO₄ without affecting the enzyme activity. PS stock solution (0.8 mM, sp. radioactivity 0.33 µCi/µmol) was prepared by adding water to a dried mixture of radiolabelled and unlabelled

lipid in a boiling tube and sonicating in a water-bath sonicator until the solution was clear. The final concentration of PS in the assay was 102 μM . Mitochondrial protein content of 100 μg was used for each assay in a total volume of 250 μl . All other assay conditions were as previously described [21]. The reaction was terminated by addition of 3 ml of chloroform/methanol (2:1, v/v), followed by 1.25 ml of 0.9% KCl. The tubes were thoroughly mixed and centrifuged. The upper phase was removed by suction, and reaction products in portions of the lower phase were separated by t.l.c. in chloroform/methanol/water/acetic acid (35:15:2:1, by vol.) as the development solvent. The bands were revealed by iodine vapour, and the radioactivity associated with the PE band was determined by scintillation counting in a Beckman LS3801 scintillation counter with Ecolite as the scintillant.

Determination of lipid phosphorus

Lipids were extracted from keratinocytes and separated into phospholipid classes as described above. The lipid classes were extracted from silica gel by the method of Arvidson [17]. The lipid phosphorous content in samples of each class was measured by a procedure using Malachite Green [22].

RESULTS AND DISCUSSION

Analysis of the phospholipid composition of proliferating keratinocytes showed that 26% of phospholipids contained ethanolamine as the base. Of this, 87% was the diacyl form, with the remainder composed of the ether (6% alkenyl + 7% alkyl) subclasses. Choline- and serine-containing lipids comprised 42% and 8% respectively of the total glycerophospholipids.

In this study, the incorporation of [^3H]ethanolamine was used to monitor the CDP-ethanolamine pathway, and incorporation of [^3H]serine was used to monitor the PS decarboxylase pathway [16]. If keratinocytes have a functional CDP-ethanolamine pathway, they would be expected to incorporate [^3H]ethanolamine into PE. When the cells were incubated with [^3H]ethanolamine, there was increasing incorporation of label into PE for up to 24 h (Table 1), an indication that proliferating keratinocytes are able to synthesize PE from exogenous ethanolamine. Because PE can be methylated to PC, the amount of label associated with PC was determined. After 24 h, the ratio of label in PE/PC was 218, hence very little of the PE synthesized from ethanolamine was methylated to PC.

To investigate whether keratinocytes have a functional PS decarboxylase pathway, proliferating keratinocytes were incubated with [^3H]serine and the incorporation of label into PS, PE and PC was determined at different times (1–24 h; Table 1). Since label from [^3H]serine can be recycled into the diradyl-glycerol moiety of phospholipids, phospholipids were hydrolysed by phospholipase C to determine the proportion of label associated with the base. In the PS fraction 97–99% of label was found in the base at all times. In the PE fraction the proportion of radiolabel in the base ranged from 74 to 76%; only 1–5% of the label in the PC fraction was associated with the base. The results in Table 1 show only the radioactivity associated with the base in each phospholipid. A progressive increase in incorporation of label into PS and PE from [^3H]serine was observed at all times. The ratio of distribution of label in PS/PE was 6.4 after 1 h, and had fallen to 2.8 after 24 h. These results indicate that proliferating keratinocytes are capable of incorporating serine into PS and subsequently into PE, presumably via the decarboxylation of PS. Very little label from [^3H]serine was found in the PC fraction. The ratio of label in PE/PC after 24 h

was about 85. These results confirm the lack of an active methylation pathway in these cells.

The above experiments clearly demonstrate that proliferating keratinocytes are capable of synthesizing PE from both serine and ethanolamine. We next investigated whether this was also the case for non-proliferating keratinocytes. Because non-proliferating keratinocytes were obtained by incubating cells in ethanolamine-free medium, [^3H]ethanolamine could not be used to monitor PE synthesis via the CDP-ethanolamine pathway; therefore [^3H]glycerol was used instead. Glycerol is incorporated into PC and PE by the CDP-choline and CDP-ethanolamine pathways respectively [1]. Glycerol is incorporated into PS via base exchange of serine with phospholipids, PC or PE [1], synthesized by the CDP-base pathways. We therefore used [^3H]glycerol to examine the relative synthesis of these phospholipids by the above pathways in non-proliferating keratinocytes.

Non-proliferating keratinocytes were obtained by incubating

Table 1 Incorporation of [^3H]serine or [^3H]ethanolamine into phospholipids of proliferating keratinocytes

[^3H]Serine or [^3H]ethanolamine was added to proliferating cultures of human foreskin keratinocytes. At selected intervals, the cells were washed and treated with trypsin, and samples were taken for determination of cell number. Lipids were extracted from the remainder, the phospholipid classes were separated by t.l.c. as described in the Materials and methods section, and the amounts of label associated with PC, PE and PS were determined. The results are expressed as d.p.m./ 10^6 cells and represent means $\pm$ S.D. of four independent determinations.

Time (h)	[^3H]Ethanolamine		[^3H]Serine		
	PE	PC	PS	PE	PC
1	2930 $\pm$ 143	34 $\pm$ 5	3860 $\pm$ 246	602 $\pm$ 47	29 $\pm$ 3
3	8463 $\pm$ 174	46 $\pm$ 4	7037 $\pm$ 78	1378 $\pm$ 69	52 $\pm$ 2
6	24271 $\pm$ 1648	109 $\pm$ 9	13092 $\pm$ 366	2985 $\pm$ 132	92 $\pm$ 7
12	54422 $\pm$ 3057	224 $\pm$ 14	22809 $\pm$ 695	6436 $\pm$ 355	76 $\pm$ 6
24	97749 $\pm$ 1888	448 $\pm$ 18	40788 $\pm$ 1196	14249 $\pm$ 362	168 $\pm$ 7

Figure 1 Effect of ethanolamine-, phosphoethanolamine- and bovine-pituitary-extract-deficient medium on keratinocyte proliferation

Keratinocytes were seeded into 6-well plates with growth medium. After 2 days, the medium in half the wells was replaced with fresh growth medium (●), and the medium in the other half was replaced with one without ethanolamine, phosphoethanolamine or bovine pituitary extract (■). The cell numbers were counted electronically every 24 h. The results represent the means of three independent determinations, with S.D. of less than 7%.

Table 2 Effect of ethanolamine on the incorporation of [³H]glycerol into phospholipids of non-proliferating keratinocytes

Ethanolamine was added to non-proliferating keratinocytes incubated in ethanolamine-free medium to give a final concentration of 0.1 mM. [³H]Glycerol was added to cells with and without (controls) ethanolamine. At selected intervals, the cells were harvested and the lipids were extracted and analysed as described in the Materials and methods section. The amount of label associated with PC, PS or PE is expressed as d.p.m./10⁶ cells. The results represent means $\pm$ S.D. of three independent determinations.

Time (h)	No ethanolamine			+ Ethanolamine		
	PC	PS	PE	PC	PS	PE
1	7953 $\pm$ 580	922 $\pm$ 154	522 $\pm$ 55	6102 $\pm$ 156	751 $\pm$ 50	3746 $\pm$ 554
3	23 979 $\pm$ 2924	3177 $\pm$ 449	1476 $\pm$ 192	22 850 $\pm$ 2783	3380 $\pm$ 684	12 424 $\pm$ 1806
6	47 377 $\pm$ 7713	5770 $\pm$ 370	2094 $\pm$ 340	36 175 $\pm$ 9950	5889 $\pm$ 1038	13 669 $\pm$ 2236
12	71 551 $\pm$ 10 538	7321 $\pm$ 911	3541 $\pm$ 657	72 501 $\pm$ 1138	10 750 $\pm$ 842	19 461 $\pm$ 2102
24	162 645 $\pm$ 13 376	21 439 $\pm$ 2648	11 891 $\pm$ 886	150 522 $\pm$ 20 911	22 439 $\pm$ 2443	38 792 $\pm$ 416

Table 3 Effect of ethanolamine on the incorporation of [³H]serine into phospholipids of non-proliferating keratinocytes

Non-proliferating keratinocytes incubated in ethanolamine-free medium were divided into two groups. Ethanolamine was added to one group to a final concentration of 0.1 mM, and the other (controls) received an identical amount of water. [³H]Serine was added to cells of both groups, and after incubation for 12 or 24 h the cells were harvested and the lipids were extracted as described in the Materials and methods section. The amount of label associated with PS or PE was determined and expressed as d.p.m./10⁶ cells. The results represent means $\pm$ S.D. of four independent determinations.

Time (h)	No ethanolamine		+ Ethanolamine	
	PS	PE	PS	PE
12	75 654 $\pm$ 3731	35 809 $\pm$ 2046	69 328 $\pm$ 10 860	22 615 $\pm$ 3875
24	162 714 $\pm$ 10 440	119 828 $\pm$ 6520	141 230 $\pm$ 14 396	68 829 $\pm$ 5180

cells with medium without ethanolamine, phosphoethanolamine and bovine pituitary extract. As shown in Figure 1, 6–7 days after incubation in the deficient medium the cells had stopped proliferating even though they were not confluent. The cells remained viable as judged by exclusion of Trypan Blue dye. In all subsequent experiments, cells were incubated in the above medium for 7 days before labelling studies. In these non-proliferating cells the amount of PE as a proportion of the total phospholipids fell to 19% from 26%, while the relative proportion of PC increased to 49% from 42%. The proportion of PS was unchanged. A similar decrease in PE content was observed in 62–64 cells cultured in the absence of ethanolamine [9]. Table 2 shows the distribution of [³H]glycerol into PC, PS and PE of non-proliferating keratinocytes incubated with or without ethanolamine (0.1 mM). In the absence of ethanolamine, the relative incorporation of label into three phospholipids at all times was PC > PS > PE. In mammalian cells, the only known synthetic pathway for PS formation from glycerol is via serine exchange with the base of preformed phospholipids [1]. In the absence of ethanolamine, PE is synthesized via decarboxylation of PS. The relative distribution of glycerol in PC, PS and PE above suggests that the PS is derived from PC by base exchange, and PE is synthesized by decarboxylation of PS. When ethanolamine was present in the medium, the relative distribution of label in the phospholipids at all times (1–24 h) was now PC > PE > PS. Thus the availability of ethanolamine results in greater incorporation of label into PE compared with PS. We did not observe significant differences in the quantity of [³H]glycerol incorporated into PC and PS in the presence or absence of ethanolamine. How-

ever, the incorporation of glycerol into PE increased 3–8-fold at different times as a consequence of the addition of ethanolamine. These results suggest that, in non-proliferating keratinocytes cultured in media with serine but no ethanolamine, the synthesis of PE occurs by the PS decarboxylase pathway. However, when ethanolamine is added, synthesis of PE via the CDP-ethanolamine pathway becomes the major route for the synthesis of the phospholipid.

Further insight into the PE biosynthetic process in non-proliferating keratinocytes was obtained in studies with [³H]serine (Table 3). When cells were cultured in ethanolamine-free medium, the distribution of label from [³H]serine in PS was greater than that in PE, as was the case with [³H]glycerol. This result is consistent with an initial synthesis of PS from serine followed by the synthesis of PE by decarboxylation of PS. In contrast with the results obtained with glycerol, the addition of ethanolamine did not cause a reversal of incorporation of label from [³H]serine into the two phospholipids. The labelling in PS still remained higher than that in PE. These results provide evidence that the pathways for the synthesis of PE from glycerol or serine in non-proliferating keratinocytes are similar (i.e. via PS decarboxylation) in the absence of ethanolamine, but the biosynthetic pathways from the two precursors are clearly different when ethanolamine is present in the medium. Comparison of the incorporation of [³H]serine into PS of proliferating keratinocytes (Table 1) and non-proliferating keratinocytes (Table 3) shows that the absence of active PE synthesis by the CDP-ethanolamine pathway and the relative decrease of PE content (from 26 to 19%) do not inhibit the synthesis of PS. On the contrary, there was greater incorporation of [³H]serine into PS in non-proliferating keratinocytes relative to that of proliferating cells. This indicates that serine exchange with the ethanolamine base of PE is unlikely to be a major route of PS synthesis in these cells, and supports the suggestion from the [³H]glycerol-labelling studies (Table 2) that PS is derived primarily from base exchange of serine with choline of PC. The implication of these observations is that any inability to synthesize sufficient quantities of PE via decarboxylation cannot be attributed to decreased synthesis of PS in the absence of ethanolamine.

In non-proliferating keratinocytes, the addition of ethanolamine to cells incubated in ethanolamine-free medium did not significantly change the extent of [³H]serine incorporation into PS (Table 3); however, the incorporation of label into PE decreased by about 40% as a consequence of the inclusion of ethanolamine in the medium. A possible reason for the above is that the presence of ethanolamine may have resulted in decreased synthesis of PE from PS. The loss of [³H]serine from PS in the presence of ethanolamine was therefore investigated. Non-

Table 4 Effect of ethanolamine on loss of [^3H]serine from PS in non-proliferating keratinocytes

Non-proliferating keratinocytes were incubated with [^3H]serine for 18 h. The cells were washed twice and incubated with ethanolamine-free medium with or without 0.1 M ethanolamine. The cells were harvested at the indicated times, and samples were taken for determination of cell number. Lipids were extracted from the remainder and the label associated with PS was determined. The results are expressed as d.p.m./ 10^6 cells and represent the means $\pm$ S.D. of four independent determinations.

Time (h)	No ethanolamine	+ Ethanolamine
0	112858 $\pm$ 12268	112858 $\pm$ 12268
6	52187 $\pm$ 2885	77291 $\pm$ 6311
12	39159 $\pm$ 6447	58448 $\pm$ 5193

Table 5 Effect of metabolites of the CDP-ethanolamine pathway on the activity of keratinocyte mitochondrial PS decarboxylase

PS decarboxylase activity was assayed in the presence of ethanolamine, phosphoethanolamine, CDP-ethanolamine and PE at the concentrations indicated. The assays were performed as described in the Materials and methods section. Control tubes did not contain any of the above metabolites. The specific activity of the enzyme in control tubes was 18.6 ± 1.9 nmol of PE formed/h per mg of protein. The values represent means $\pm$ S.D. of two experiments each performed in triplicate.

Addition	Activity (% of control)
None	100
5 mM Ethanolamine	105 $\pm$ 5
5 mM CDP-ethanolamine	106 $\pm$ 12
1 mM Phosphoethanolamine	92 $\pm$ 7
2 mM Phosphoethanolamine	87 $\pm$ 4
3.5 mM Phosphoethanolamine	70 $\pm$ 3
5 mM Phosphoethanolamine	54 $\pm$ 7
25 μM PE	85 $\pm$ 6
50 μM PE	69 $\pm$ 9
100 μM PE	62 $\pm$ 4

proliferating keratinocytes were incubated with [^3H]serine for 18 h followed by washing and incubation in medium with or without ethanolamine. The label associated with the PS fraction was determined at different times. The results (Table 4) show that the presence of ethanolamine in the incubation medium inhibited the loss of label from PS relative to control cells in ethanolamine-free medium. Since ethanolamine did not inhibit the synthesis of PS (Tables 2 and 3), this cannot be the cause for the observed decrease in loss of [^3H]serine from PS. The results support the idea that PE synthesis via decarboxylation of PS is inhibited when ethanolamine is included in the incubation medium. The implication of the above is that the synthesis of PE by the CDP-ethanolamine and the decarboxylase pathways may be regulated in a co-ordinated fashion in keratinocytes. To investigate how this might be achieved, experiments were conducted to determine whether metabolites of the CDP-ethanolamine pathway could inhibit the activity of PS decarboxylase. The rationale was that, although these metabolites would be absent from non-proliferating keratinocytes incubated in ethanolamine-free medium, their concentration would increase significantly upon activation of the CDP-ethanolamine pathway after addition of ethanolamine. The effects of ethanolamine, phosphoethanolamine, CDP-ethanolamine and PE on the activity of keratinocyte mitochondrial PS decarboxylase activity are displayed in Table 5. Neither ethanolamine nor CDP-ethanolamine at concentrations of up to 5 mM had any effect on the activity of PS decarboxylase.

In contrast, phosphoethanolamine significantly and progressively inhibited the activity of the enzyme by about 8% at a concentration of 1 mM and by about 50% at 5 mM. Although relatively high concentrations of phosphoethanolamine are required to inhibit PS decarboxylase activity *in vitro*, this does not necessarily imply that similar concentrations would be needed to inhibit the enzyme activity in the cells. In the assay, Triton X-100 (0.5 mg/ml) is added to disrupt the membranes, since PS decarboxylase is localized in the mitochondrial inner membrane [23]. The loss of membrane organization could conceivably lead to inefficient interactions of compounds with the enzyme. It is worth noting that inhibition by phosphoethanolamine was relatively specific, since neither ethanolamine nor CDP-ethanolamine had an effect on the enzyme activity. Initiation of PE synthesis by the CDP-ethanolamine pathway in non-proliferating keratinocytes would be expected to increase significantly the concentration of phosphoethanolamine in the cells, as the CTP:phosphoethanolamine cytidyltransferase is the rate-limiting step in the pathway [1]. Phosphoethanolamine is also the first committed metabolite of the pathway. These considerations, together with the observed inhibition *in vitro*, make phosphoethanolamine a prime candidate to act as the metabolite that inhibits PS decarboxylase *in vivo*, thereby linking the two pathways for PE synthesis.

PE was also quite inhibitory to PS decarboxylase in our assays. At a concentration of 100 μM , PE inhibited the activity by 40%. Although this may reflect product inhibition with no physiological significance, it could signify another level for co-ordinating the synthesis of PE by the CDP-ethanolamine and PS decarboxylase pathways. Production of PE by the CDP-ethanolamine pathway in non-proliferating keratinocytes would ultimately result in increased PE content in cellular membranes from the 19% in non-proliferating cells to normal levels of 26% present in proliferating cells. Since PE formed by decarboxylation is not utilized exclusively in the mitochondria, but may be preferentially exported to the endoplasmic reticulum [23], it would be desirable to regulate the output of PE from PS decarboxylation, in line with the total cellular requirement, when the CDP-ethanolamine pathway is active. The intracellular mechanism by which PE would inhibit PS decarboxylase activity is unclear; however, since the mitochondria import lipids, it can be envisaged that any increase in PE content resulting from active synthesis by the CDP-ethanolamine pathway would be reflected in the composition of lipids that flow to the mitochondria from the endoplasmic reticulum. The increased PE content of lipids flowing to the mitochondria could inhibit the activity of the PS decarboxylase and consequently decrease the flow of PE from the mitochondria to the endoplasmic reticulum.

The above studies have demonstrated that both proliferating and non-proliferating keratinocytes are capable of synthesizing PE from ethanolamine via the CDP-ethanolamine pathway and from serine via the PS decarboxylase pathway. The presence of a functional decarboxylase pathway in keratinocytes implies that the mitogenic effect of ethanolamine in these cells cannot be explained by an inability to synthesize PE from serine. Why do these cells require ethanolamine for proliferation? One simple explanation could be that the synthesis of PE by decarboxylation is quantitatively inadequate for the proliferative needs of the cells. Thus a PE content of 19% may be inadequate for the proliferative needs of these cells. Another possible reason could be an inability to synthesize ether-linked EPG by the decarboxylase pathway. In most cells, the CDP-ethanolamine pathway is the major pathway for the synthesis of ether-linked ethanolamine phospholipids [24], although some cells are capable of synthesizing these lipids via the decarboxylase pathway [25]. We

Table 6 Distribution of [³H]serine in ether subclasses of ethanolamine phospholipids in non-proliferating and proliferating keratinocytes

Proliferating or non-proliferating keratinocytes were incubated with [³H]serine. At selected times, lipids were extracted from the cells and the EPG fraction was purified by t.l.c. The phospholipid was extracted and samples were subjected to mild alkaline hydrolysis as described in the Materials and methods section. The quantity of radiolabel associated with the ether fraction was determined and expressed as a percentage of the total label associated with the EPG fraction. The results are means $\pm$ S.D. of four independent determinations.

Time (h)	Distribution of [³ H]serine in ether-EPG (% of total EPG)		
	Non-proliferating		Proliferating
	No ethanolamine	+ Ethanolamine	
12	7.39 $\pm$ 0.43	5.23 $\pm$ 0.27	5.73 $\pm$ 0.68
24	6.46 $\pm$ 0.72	3.41 $\pm$ 0.38	7.53 $\pm$ 0.52

therefore compared the incorporation of [³H]serine into ether-linked ethanolamine phospholipids in proliferating and non-proliferating cells. After incubating cells with [³H]serine, the ethanolamine phospholipid fraction was separated by lipid extracts and subjected to mild alkali hydrolysis to convert the ether lipids into the lyso-derivatives. The results obtained are displayed in Table 6. In non-proliferating keratinocytes incubated with [³H]serine in ethanolamine-free medium, about 7% of the total label found in the EPG fraction was associated with the ether subclasses after 12 h and 24 h. In medium containing ethanolamine, the corresponding values found in ether analogues after 12 and 24 h were 5 and 3.4% respectively. Similar experiments with proliferating cells in complete growth medium revealed a distribution of label in the ether EPG similar to those obtained for non-proliferating cells incubated in the absence of ethanolamine. The above results suggest that keratinocytes are capable of synthesizing ether-linked EPG via the decarboxylase pathway. We cannot, however, rule out the possibility of incorporation of [³H]serine into PE by decarboxylation of PS, followed by release of the labelled ethanolamine through base-exchange reactions and its utilization for ether-linked EPG synthesis via the CDP-ethanolamine pathway. In view of the limited extent of base-exchange reactions involving PE discussed above, the observed incorporation of label from [³H]serine into ether-linked EPG is likely to be from decarboxylation of serine phosphoacylglycerols, but definitive proof will require the demonstration of precursor-product relationship between ether-linked serine phosphoacylglycerols and ether-linked EPG. The results displayed in Table 6 also indicate that incubation in medium without ethanolamine does not appear to affect the rate of synthesis of ether EPG relative to the synthesis of diacyl-EPG by decarboxylation of serine phosphoacylglycerols. However, the experiments do not indicate whether the quantities synthesized by this pathway are sufficient for cellular requirements in either proliferating or non-proliferating keratinocytes. Indeed, in proliferating keratinocytes incubated with [³H]ethanolamine for 12 or 24 h, we observed that about 15% of the total label in the EPG fraction was associated with the ether subclasses. This

suggests that most of the ether-linked ethanolamine lipids are probably synthesized by the CDP-ethanolamine pathway in these cells. Whether a lack of synthesis of sufficient quantities of ether lipids by decarboxylation lies at the root of the ethanolamine requirement deserves further study.

Another possibility that cannot be ruled out is the requirement of ethanolamine for the synthesis of glycosylphosphatidylinositol compounds, which anchor a large number of proteins to the plasma membrane. Although other cellular functions for these compounds have yet to be established, there is a report of its involvement in the activation of T cells [26]. Finally, one can also not discount the possibility that the water-soluble ethanolamine metabolites, particularly phosphoethanolamine, may have specific growth-promoting functions other than as intermediates in the synthesis of EPG or glycosylphosphatidylinositol anchors [27]. Further studies are therefore needed to discriminate between the above possibilities.

This study was supported by funds from the University of Manitoba Research Development Fund and a Medical Research Council of Canada scholarship to G.A.

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