ETHANOLAMINE REQUIREMENT AND CELL PROLIFERATION

by

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A Thesis Submitted to the Faculty of Graduate Studies

in Partial Fulfillment of the Requirement

for the Degree of

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ABSTRACT

Phosphatidylethanolamine a major phospholipid of mammalian cell membranes is synthesized from ethanolamine or serine. Studies have shown that in cells of epithelial origin, the absence of ethanolamine in the growth medium results in reduced cell proliferation even though serine is present. The mechanism(s) responsible for this growth inhibition have yet to be elucidated. The hypothesis that ethanolamine deficiency alters the membrane phospholipid composition to such an extent that transduction of growth factor signals is inhibited was examined in two ethanolamine-responsive normal human cell lines, epidermal keratinocytes and mammary epithelial cells.

Incubation of keratinocytes and human mammary epithelial cells in media without ethanolamine resulted in a 55.2% and 53.1% reduction in cell proliferation respectively. Further studies with mammary epithelial cells showed that in the absence of ethanolamine in the growth medium, incorporation of [^{3}H]-thymidine into DNA was reduced by 5-fold. In keratinocytes, ethanolamine significantly enhanced the stimulatory effects of insulin and epidermal growth factor on DNA synthesis by 69% and 40.5% respectively. Insulin, in particular, was critical for the optimal proliferation of keratinocytes. Addition of ethanolamine to the growth media of mammary epithelial cells enabled a normal progression of cells through the cell cycle. In contrast, ethanolamine-deficient cells accumulated in the G0/G1 phase of the cell cycle. Ethanolamine but not dimethylethanolamine or monomethylethanolamine was mitogenic for quiescent ethanolamine-deficient cells. Proliferation of cells incubated in dimethylethanolamine and monomethylethanolamine was

55.5% and 47.2%, respectively, of cells incubated in ethanolamine-sufficient media.

The incorporation of [^{3}H]-glycerol into phosphatidylethanolamine in ethanolamine-deficient keratinocytes and mammary epithelial cells was significantly reduced by 58.5% and 64.3%, respectively, compared to controls. The incorporation of [^{3}H]-glycerol into other glycerophospholipids in ethanolamine-sufficient and ethanolamine-deficient cells were unchanged. Insulin stimulation of quiescent keratinocytes and mammary epithelial cells in the presence of ethanolamine activated mitogen-activated protein kinase. In the absence of ethanolamine, activation of mitogen-activated protein kinase was significantly inhibited.

Taken together, these observations suggest that a deficiency of ethanolamine alters the membrane phospholipid composition that interferes with signaling events upstream of mitogen-activated protein kinase and this may be involved in the inhibition of cell proliferation.

DEDICATION

"Science is nothing but perception."
(Plato)

This thesis is dedicated to Bernice, Ruth, Evangeline, Jennifer and Ezra
and to my parents, brothers and sisters.

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LIST OF ABBREVIATIONS

Etn	Ethanolamine
PE	Phosphatidylethanolamine
PI	Phosphatidylinositol
PS	Phosphatidylserine
EGP	Ethanolamine glycerophospholipid
GPI	Glycosyl phosphatidylinositol
LPE	Lysophosphatidylethanolamine
ER	Endoplasmic reticulum
ET	CTP : Phosphoethanolamine cytidylyltransferase
CT	CTP : Phosphocholine cytidylyltransferase
CHO	Chinese hamster ovary
Diacyl-PS	1,2-diacyl-*sn*-glycero-3-phosphoserine
DAG	1,2-diacylglycerol
PA	Phosphatidic acid
P-Etn	Phosphoethanolamine
IGF-1	Insulin growth factor-1
GPEA	Glycerophosphorylethanolamine
PDB	Phorbol-12, 13-dibutyrate
PKC	Protein kinase C
IRS-1	Insulin receptor substrate-1

SH2	Src homology-2
PI 3-Kinase	Phosphatidylinositol 3-kinase
Grb-2	Growth factor receptor binding-2
Sos	Son of sevenless
DME	Dimethylethanolamine
MME	Monomethylethanolamine
Diacyl-GPE	1,2-diacyl-*sn*-glycero-3-phosphoethanolamine
Alkylacyl-GPE	Alkylacyl-*sn*-glycero-3-phosphoethanolamine
Alkenylacyl-GPE	Alkenylacyl-*sn*-glycero-3-phosphoethanolamine
NHEK	Normal human epidermal keratinocyte
HME	Human mammary epithelial
TNS	Trypsin neutralizing solution
BSA	Bovine serum albumin
MBP	Myelin basic protein
EDTA	Ethylenediamnetetraacetic acid
BHT	Butylated hydroxy toluene
DDW	Distilled deionised water
DTT	Dithiothreitol
EGTA	[Ethylenebis(oxyethylenenitrilo)]tetra acetic acid
PMSF	Phenylmethylsulfonylfluoride
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
BPE	Bovine pituitary extract

KGM	Keratinocyte growth medium
KBM	Keratinocyte basal medium
KDM	Keratinocyte defined medium
KGF	Keratinocyte growth factor
EGF	Epidermal growth factor
INS	Insulin
MEGM	Mammary epithelial growth medium
MEDM	Mammary epithelial defined medium
TLC	Thin layer chromatography
HBSS	HEPES-buffered saline solution
PBS	Phosphate-buffered solution
SDS	Sodium dodecyl sulfate
SD	Standard deviation
CI	Confidence interval
ddH_2O	Distilled deionised water
hr	Hour
NP-40	Nonidet P-40
PKC	Protein kinase C
MAP	Mitogen-activated protein
KDa	Kilodalton
MEK	MAPK/extracellular signal-regulated kinase
GTP	Guanosine triphosphate

KCl	Potassium chloride
HCl	Hydrochloric acid
AEBSF	Aminoethylbenzenesulfonyl fluoride
MAPKAP-K2	MAPK-activated protein kinase 2
PLC	Phospholipase C
$cPLA_2$	Cytosolic phospholipase A_2
DNA	Deoxyribonucleic acid
UV	Ultraviolet
nm	Nanometer
pg.	Page
CDP	Cytidine diphosphate

1. LITERATURE REVIEW

1.0 Introductory Remarks

Phosphatidylethanolamine (PE), a major component of cell membranes, is gradually gaining the attention of researchers because of the realization that either PE or its water soluble metabolites may have growth regulatory effects hitherto unknown. Results of studies over the years indicate that various rat and human cells appear to require ethanolamine (Etn) to proliferate. The reasons for this Etn requirement are not known. Solving this puzzle will contribute to our knowledge and understanding of how cell proliferation is regulated.

To help the reader gain an understanding of this study on Etn requirement and cell proliferation, an overview of the membrane and its lipid components is presented followed by a discussion on the synthesis and degradation of PE. Evidence that implicates Etn and/or its analogues in cell proliferation is also discussed. Some hypotheses to explain why certain cells appear to require Etn to proliferate are examined. In the last section of the introduction, the hypothesis to be tested and the objectives of this study are presented. Finally, evidence will be presented in this thesis to show that Etn may play important regulatory roles in cell proliferation.

1.1 The Membrane

Biological membranes of living organisms are composed of lipids, proteins and glycoproteins. Proteins either equal or exceed lipids in most membranes [Martin *et al.*, 1985;

see Fig.1, pg. 4]. Some proteins are integral components of the membrane and are referred to as intrinsic proteins while others are loosely associated with the membrane. The latter are referred to as extrinsic proteins. Extrinsic proteins can be dissociated from the membrane by treatment with solutions of low ionic strength. Intrinsic proteins on the other hand require treatment with detergents or organic solvents in order to dissociate them. Membranes serve a wide range of functions. As a selective permeable barrier, membranes control the transport of various substances between the external and internal environment of the cell. Membranes also delineate the various intracellular organelles such as the nucleus, endoplasmic and sarcoplasmic reticula, mitochondria, lysosomes, chloroplasts, vacuoles in plants, and the golgi apparatus. In eucaryotes, the presence of internal membranes partitions the interior of cells into various functionally distinct compartments allowing for different biochemical reactions to occur [Alberts *et al.*, 1994]. The membrane thus provides support for a variety of enzymes and receptors thereby exerting control over cellular metabolism.

1.2 Membrane Lipids

The lipid portion of membranes consists of a diverse group of compounds that vary between species and organelles. The main lipid groups present include, phosphosphingolipids, sterols, glycosphingolipids and glycerophospholipids. Glycosphingolipids differ from phosphosphingolipids in having a sugar moiety attached to the primary hydroxyl group of sphingosine rather than phosphorylcholine as in sphingomyelin. Cholesterol, the most common sterol in animals, is predominantly localized

to the plasma membrane and together with unsaturated fatty acids influence the fluidity of membranes.

Phospholipids are the most abundant membrane lipids. A notable feature of their structure is the presence of a hydrophilic or polar head group and a hydrophobic hydrocarbon tail. Phospholipids are thus amphipathic and tend to form bilayers in which their polar head groups are oriented toward the aqueous environment and their hydrophobic hydrocarbon tails buried within the bilayer. This critical function of phospholipids is essential for the integrity of the membrane and survival of cells. Membrane proteins are embedded in the lipid bilayer as shown in the fluid mosaic model of membrane structure [Singer and Nicolson, 1972; see Fig.2, pg. 5]. The fatty acyl components of phospholipids can serve as energy stores to be oxidized in the mitochondria under certain extreme conditions such as starvation [Harwood and Gurr, 1991]. Another function of phospholipids is the generation of second messengers; eicosanoids, diacylglycerols and inositol (1,4,5) triphosphate [Vance, 1991].

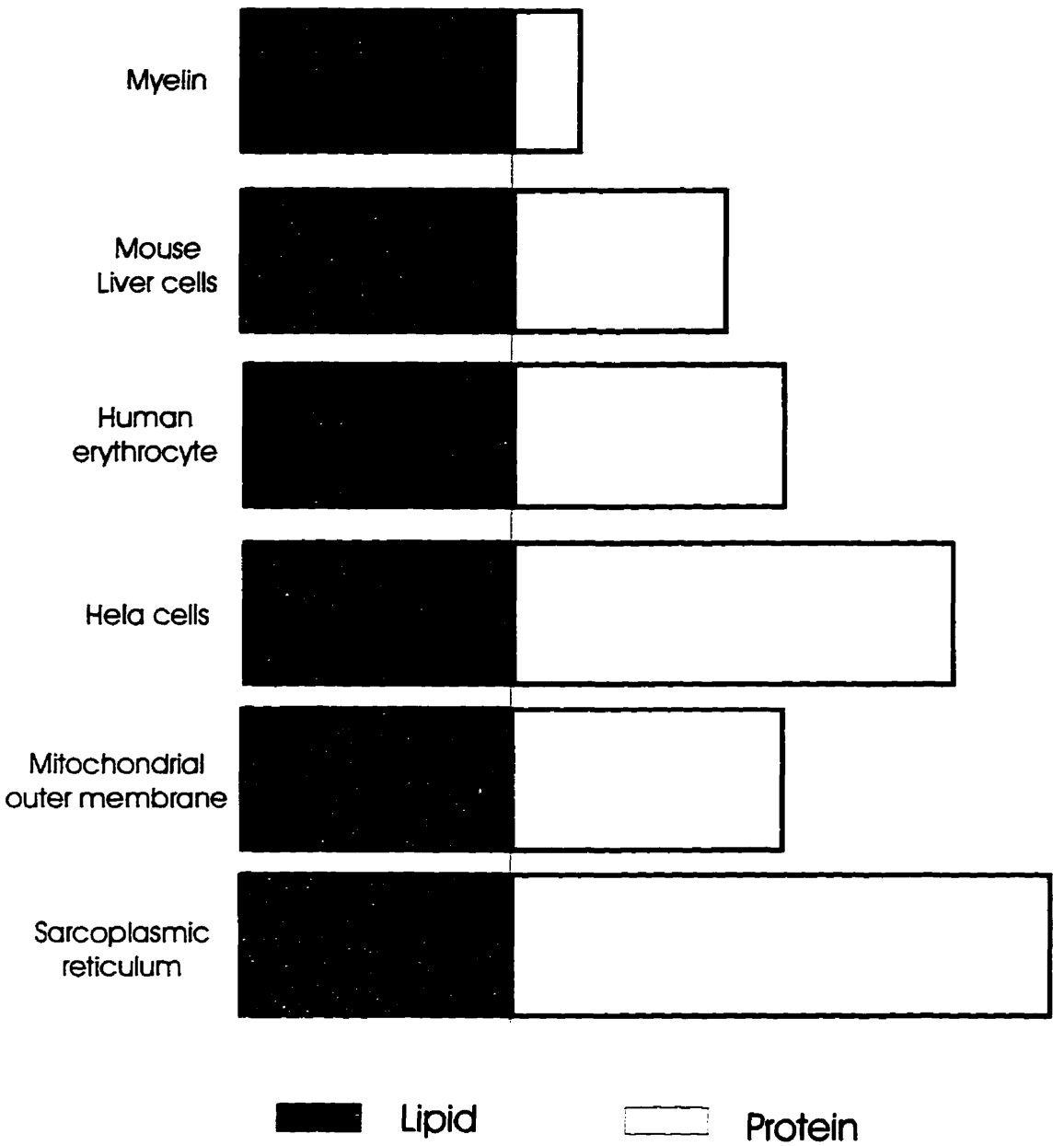


Figure 1. Ratio of Protein to Lipid in Membranes.
Adapted from Martin *et al.*, (1985)

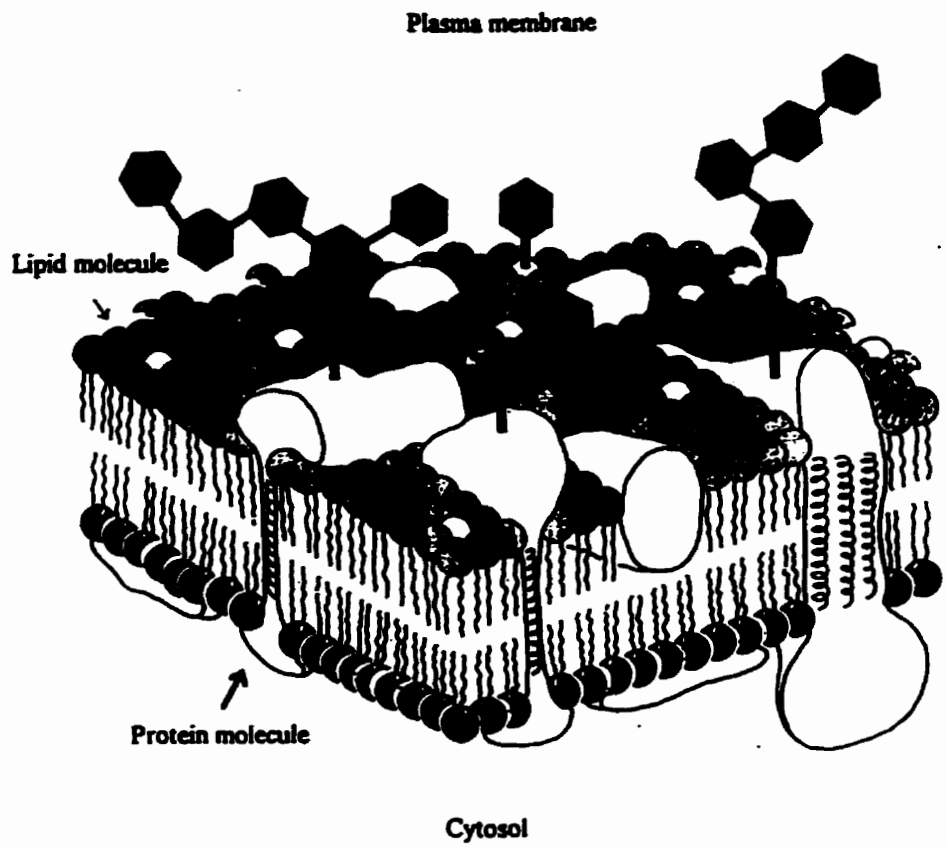


Figure 2. The Fluid Mosaic Model of Membrane Structure

1.3 The Structure and Biosynthesis of Phosphatidylethanolamine

The major classes of glycerophospholipids are shown in Table 1 (pg. 9). Phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are the major glycerophospholipids in most cell membranes [Table 2, pg. 10]. Phosphatidylserine (PS), PE and cardiolipin are located on the cytoplasmic half of the lipid bilayer while PC, sphingomyelin and glycolipids are located predominantly on the exterior.

Three types of Etn glycerophospholipids (EGP) are found in mammalian cells namely diacyl-, alkenylacyl- and alkylacyl-*sn*-glycero-3-phosphoethanolamine with their proportions varying depending on the cells and tissues of origin. 1,2-diacyl-*sn*-glycero-3-phosphoethanolamine (Diacyl-GPE) or PE consists of a glycerol backbone to which fatty acyl groups are esterified at the carbons-1 and -2 positions. The fatty acid in the carbon-1 position is usually saturated (e.g., stearic acid (18:0) and palmitic acid (16:0)) and that in the 2-position is normally unsaturated (e.g., oleic acid (18:1), linoleic acid (18:2) and arachidonic acid (20:4)). These are long chain fatty acids that mostly consist of even-numbered carbon molecules. Etn is linked via a phosphodiester bond to a phosphate group which is in turn linked via a phosphodiester bond to carbon-3 of the glycerol backbone. In alkenylacyl-*sn*-glycero-3-phosphoethanolamine (alkenylacyl-GPE) or Etn plasmalogens (e.g., 1-alk-1'-enyl-2-GPE), the unsaturated alkyl radical is attached to carbon-1 of the glycerol backbone via an ether linkage instead of the normal ester linkage found in most acylglycerols. Alkylacyl-*sn*-glycero-3-phosphoethanolamine (alkylacyl-GPE) also consists of a hydrocarbon side chain attached via an ether linkage to glycerol. However, unlike the alkenylacyl-GPE, the alkyl

radicals or hydrocarbon side chains are saturated. Plasmalogens are present in both procaryotes and eucaryotes. In the adult man, plasmalogens constitute about 18.7% of the total phospholipids [Horrocks and Sharma, 1982]. Etn plasmalogens and alkyacyl-GPE are present, amongst others, in the heart and skeletal muscle, adipose, testis and brain tissue of various mammals.

Etn, an amino-containing compound, is a precursor for the biosynthesis of PE and is also present in glycophosphatidyl inositol (GPI) anchors that target certain proteins to the surface of membranes [Englund, 1993]. Exogenous Etn is taken up into cells by high- and low-affinity transport systems and is also present as the free unbound form in the circulation of animals with a concentration range of between 5 and 50 μM [Shiao and Vance, 1995]. For example, the concentration of Etn in fetal bovine serum is approximately 25 μM [Houweling *et al.*, 1992].

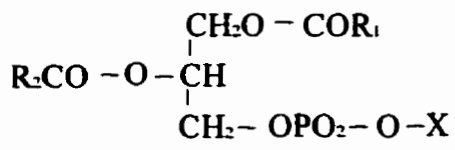
Four distinct pathways have been described for the biosynthesis of PE [Fig.3, pg. 11]. These are (a) from the decarboxylation of phosphatidylserine (PS) [Dennis and Kennedy, 1972], (b) incorporation of Etn into PE via CDP-Etn pathway [Kennedy and Weiss, 1956], (c) via Ca^{2+}- dependent base exchange reactions involving Etn and an existing phospholipid such as PS or PC [Borkenhagen *et al.*, 1961], and (d) acylation of lysophosphatidylethanolamine (LPE) catalyzed by LPE-acyltransferases. Synthesis of PE unlike the majority of phospholipids occurs at two intracellular sites. The decarboxylation of PS occurs primarily in the mitochondria [Dennis and Kennedy, 1972] while synthesis via the CDP-Etn pathway occurs in the endoplasmic reticulum [Vance and Vance, 1988]. A soluble phospholipid transfer protein has been proposed to transport newly synthesized PE

from the ER to the plasma membrane [Trotter and Voelker, 1994].

The enzyme catalyzing the rate-limiting step in the CDP-Etn or *de novo* pathway is CTP : phosphoethanolamine cytidylytransferace (ET) which is distinct from the analogous enzyme, CTP : phosphocholine cytidylytransferase (CT), in the CDP-choline pathway [Vance, 1991; Ansell and Spanner, 1982). ET has been purified to homogeneity from rat liver cytosol with a subunit molecular weight of 50-KDa [Vermeulen *et al.*, 1993]. Initial studies suggested that ET is localized to the cytosol with no lipid requirement for activity [Vance, 1991]. However, studies by van Hellemond *et al.* (1994) indicate that the enzyme is enriched in the regions of the rough endoplasmic reticulum suggesting that ET might have a weak affinity for membranes [Kent, 1995]. Further support for this comes from evidence that in castor bean endosperm, ET is primarily associated with the mitochondrial membrane [Wang and Morres, 1991].

The relative importance of the *de novo* and PS decarboxylase pathways in the synthesis of PE *in vivo* has not only been puzzling but generated a lively debate [Kent, 1995; Ansell and Spanner, 1982]. In rat liver, the *de novo* synthesis pathway is thought to be the major route for the biosynthesis of PE [Tijburg *et al.*, 1989]. However, in yeast and several mammalian cell lines, the PS decarboxylase pathway appears to be the primary path for PE biosynthesis [Kent, 1995]. In fact, in cultured baby hamster kidney fibroblasts and Chinese hamster ovary (CHO) cells, the decarboxylation of PS provides the bulk of PE even though the cells are supplemented with Etn [Miller and Kent, 1986; Kuge *et al.*, 1986]. Thus, for a long time it was thought that cells in culture obtained their PE solely from the decarboxylase pathway and had no requirement for Etn.

Table 1. Major Classes of Glycerophospholipids



R_1 and R_2 = Fatty acyl substituents

x = Polar head group

Precursor of X	Formula of X	Glycerophospholipid
Water	- H	Phosphatidic acid
Choline		Phosphatidylcholine
Ethanolamine		Phosphatidylethanolamine
Serine		Phosphatidylserine
Glycerol		Phosphatidylglycerol
Myo-inositol		Phosphatidylinositol

Adapted from: Martin *et al.* (1985)

Table 2. Lipid Composition of Various Biological Membranes (weight % of total lipid)

Lipid	Erythrocyte[a]	Myelin[a]	Mitochondria[b] (inner and outer membrane)	Endoplasmic Reticulum
Cholesterol	23	22	3	6
Phosphatidylethanolamine	18	15	35	17
Phosphatidylcholine	17	10	39	40
Sphingomyelin	18	8	-	5
Phosphatidylserine	7	9	2	5
Cardiolipin	-	-	21	-
Glycolipid	3	28	-	-
Others	13	8	-	27

[a]Human sources [b]Rat liver Source: Cullis and Hope (1991)

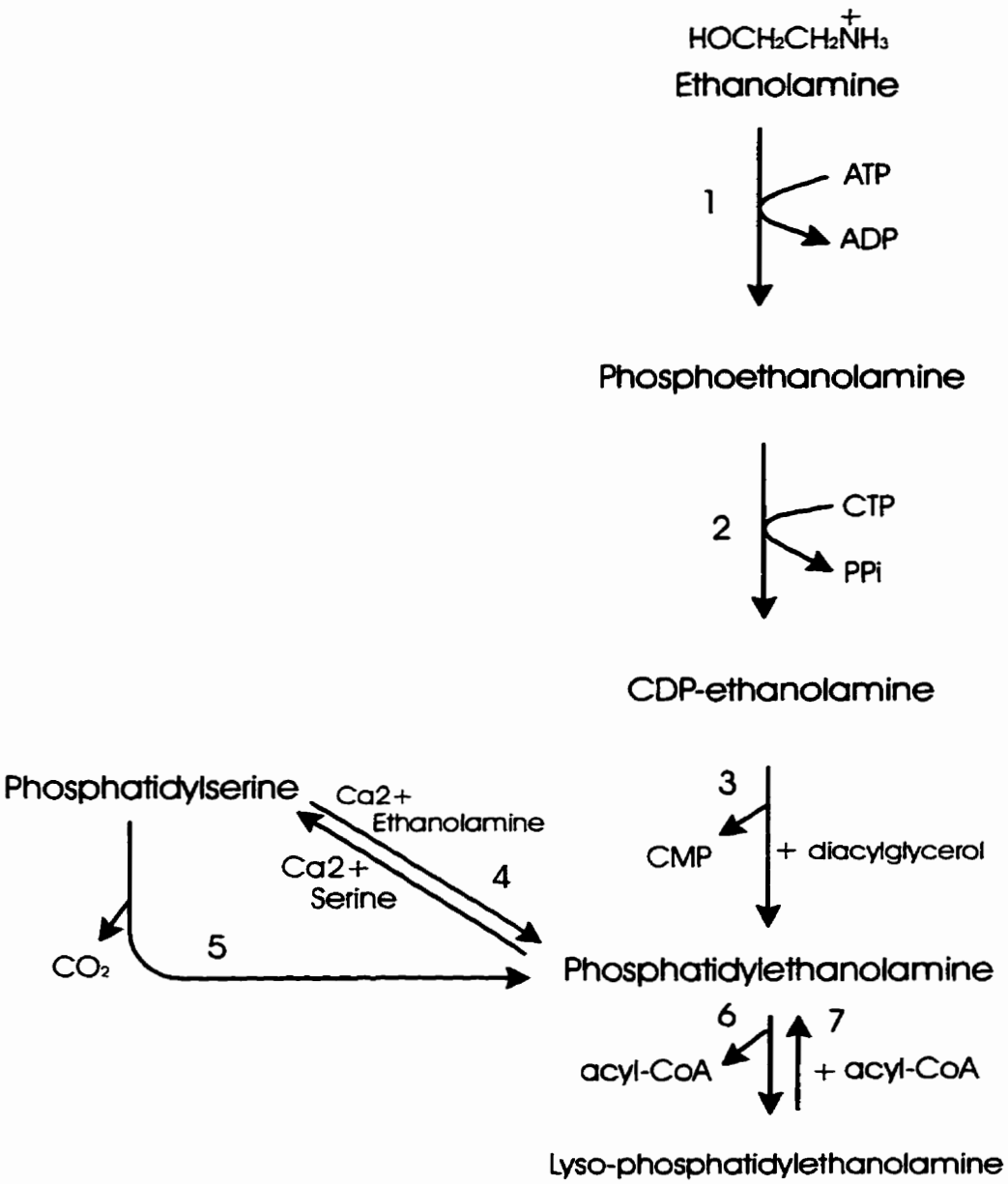


Figure 3. Pathways for the Biosynthesis of Phosphatidylethanolamine. The numbers indicate the enzymes involved: **1**. Ethanolamine kinase: **2**. CTP:ethanolaminephosphate cytidylyltransferase: **3**. CDP-ethanolamine:1,2-diacylglycerol ethanolamine phosphotransferase: **4**. Phosphatidylserine synthase: **5**. Phosphatidylserine decarboxylase: **6**. Phospholipase A_2: **7**. acyl-CoA:lysophosphatidylethanolamine. Adapted from: Vance (1991)

Some mammalian cells have however been shown to require Etn for growth [Kano-Sueoka and King, 1987]. It has been suggested that a possible role of the CDP-Etn pathway may be the biosynthesis of PE and Etn plasmalogen [Kent, 1995]. Labeling of three rat tissues *in vivo* showed that [^{3}H]-serine was incorporated mainly into diacyl-PS and diacyl-PE fractions while [^{14}C]-Etn was incorporated into both PE and Etn plasmalogen [Arthur and Page, 1991]. Similar studies with human retinoblastoma cells also showed that labeled Etn and phosphate are largely incorporated into PE and Etn plasmalogen at comparable rates [Yorek *et al.*, 1985; Kent, 1995]. According to Kent (1995), the CDP-Etn pathway in some cells may function to augment PE levels while in others, it may be used for the biosynthesis of distinct pools of Etn phospholipids.

1.4 Degradation of Phosphatidylethanolamine

PE is susceptible to the hydrolytic activity of phospholipases [Kiss and Anderson, 1990]. Phospholipase A_1 degradation of PE at the carbon-1 position generates saturated fatty acids (e.g., stearic acid) while phospholipase A_2 degradation results in lyso-GPE and unsaturated fatty acids such as arachidonic acid. Phospholipases C and D generate 1,2-*sn*-diacylglycerol (DAG) and phosphoethanolamine (P-Etn), and phosphatidic acid (PA) respectively. The degradative products of phospholipase activity such as DAG and arachidonic acid are involved in phospholipid mediated signaling in cells [Exton, 1994]. Sequential methylation of PE to produce PC catalyzed by phosphatidylethanolamine-N-methyltransferase, and base-exchange reactions involving PE and serine (to generate PS) are

the other routes for the degradation of PE [Vance, 1991]. The existence of an Etn cycle has been proposed in which the Etn moiety is continuously released from PE and re-cycled back into PE [Shiao and Vance, 1995].

1.5 Ethanolamine as a Growth Promoting Factor

Studies on the growth characteristics of the 64-24 rat mammary carcinoma cell line in culture led to the purification and identification of P-Etn as the growth promoting substance in crude pituitary extract [Kano-Sueoka *et al.*, 1979]. Further experiments with 64-24 rat mammary carcinoma cells showed that other compounds structurally related to P-Etn such as monomethylethanolamine, 1-amino-2-propanol, 2-amino-1-propanol and Etn were also growth stimulatory [Kano-Sueoka and Errick, 1981; see Table 3, pg. 15]. Etn in particular had a significantly higher growth activity compared to P-Etn. These studies also showed that in 64-24 rat mammary carcinoma cells, P-Etn and Etn were taken up in a dose dependent manner and incorporated into PE. The effective dosage of P-Etn and Etn was found to be as low as 10^{-6} M indicating that P-Etn and Etn may be acting as modulators to stimulate cell growth rather than as nutrients [Kano-Sueoka, 1981]. A new classification of cells was thus proposed in which cells were either classified as Etn-responsive or Etn-nonresponsive [Table 4, pg. 16]. In particular cells of epithelial origin appear to require Etn to proliferate optimally under low serum or serum-free conditions since serum contains sufficient Etn to support their growth [Kano-Sueoka and King, 1987]. These included normal mammary epithelial cells [Hammond *et al.*, 1984], Keratinocytes [Tsao *et al.*, 1982],

mammary carcinoma cells as well as other cell types.

It has also been reported by Tomono *et al.* (1995) that choline phosphate and Etn enhanced the stimulatory effects of insulin and insulin-like growth factor I (IGF-I) in NIH 3T3 fibroblasts. The mitogenic effect was more pronounced when both compounds were used together.

In a recent report, glycerophosphorylethanolamine (GPEA), a breakdown product of PE, has been shown to stimulate growth of hepatocytes in conjunction with certain hepatocyte growth factors. An interesting observation made was that the Etn moiety of GPEA was critical for growth stimulation in serum-free cultures of hepatocytes [Nelson *et al.*, 1996].

Table 3. Growth Stimulation by Compounds Related to Phosphoethanolamine

Compound	Amount added (nmol/ml)	Cell no.	Relative Growth
No addition[1]	-	9.7×10^4	1.0
Phosphoethanolamine	5.0	3.3×10^5	3.4
Ethanolamine	5.0	5.8×10^5	6.0
Monomethylethanolamine	5.0	2.5×10^5	2.5
No addition[2]	-	1.3×10^5	1.0
Ethanolamine	4.0	1.3×10^6	9.9
1-Amino-2-propanol	40.0	8.3×10^5	6.4
2-Amino-1-propanol	8.0	9.2×10^5	7.1

64-24 rat mammary carcinoma cells were plated in DME containing Fetal calf Serum (FCS) and the compounds as indicated. Five days after plating the cells were counted.
[1] Contained 1% FCS. [2] Contained 2% FCS. Source: Kano-Sueoka and Errick (1981).

Table 4. Ethanolamine-Responsive and -Nonresponsive Cells

Ethanolamine-responsive cells
Primary culture of rat mammary epithelial cells
Primary culture of human mammary epithelial cells
Rat mammary carcinoma cell lines: 64-24 and MT9/PL
Human breast carcinoma cell line: T47D
Human epidermal keratinocytes
Human lung carcinoma cells
Human bronchial epithelial cells
Rat esophageal epithelial cells
Mouse plasmacytoma and mouse & rat hybridoma cells
Ethanolamine-nonresponsive cells
Rat mammary carcinoma cell lines: 22-1 and WRK-1
Human breast carcinoma cell lines: MDAMB-231, MCF-7 and HBL-100
Mouse, rat and Chinese hamster fibroblasts
Rat neuronal and glial cell lines: RT4D, RT4E, B103 and B50

Source: Kano-Sueoka and King (1987)

1.6 Ethanolamine Deficiency in Cells

Studies on the effects of fasting on the levels of water soluble metabolites of PE in rats indicates that fasting for 48-hrs resulted in a decreased liver Etn and PE levels [Tijburg *et al.* 1988]. Earlier on, studies with an Etn-requiring rat 64-24 cell line showed that cell proliferation is significantly reduced in the absence of exogenous Etn. Under such conditions, cells tended to become Etn-deficient with a characteristic decrease in cellular PE levels [Kano-Sueoka and King 1987]. Thus changes in dietary conditions or the absence of Etn in the growth medium of cells caused changes in the intracellular pools of PE and its precursors.

In the absence of exogenous Etn, the PS decarboxylase pathway becomes the major biosynthetic route for the synthesis of PE in mammalian cells [Dennis and Kennedy, 1972]. Serine, a precursor for the biosynthesis of PS is present in sufficient quantities in the growth medium and can be synthesized from glycine and 3-phosphoglyceric acid. Cells should therefore be able to meet their PE requirements by synthesizing PS from serine and subsequently decarboxylating it to PE, but clearly some cells are unable to do this. It has been suggested that the drop in PE levels observed in Etn-deficient cells may be due to the inability of the PS decarboxylase pathway to meet cellular requirements for PE; a consequence of low PS supply since there was no difference in the activity of PS decarboxylase in crude cell lysates of Etn-requiring 64-24 and Etn-nonrequiring 22-1 rat mammary carcinoma cell lines [Kano-Sueoka and King, 1987]. Evidence to support this suggestion has come from experiments with Chinese hamster ovary (CHO) cells in which

mutants defective in PS synthase activities required either PS or Etn to grow normally [Voelker and Frazier, 1986]. The efficiency of this pathway varied between different cell types with less efficient cells having a slower growth in the absence of Etn [Nelson *et al.* 1996; Kano-Sueoka and King, 1987]. Comparative studies between Etn-requiring 64-24 and Etn-nonrequiring 22-1 rat carcinoma cell lines indicated that both cell types were capable of synthesizing PS from serine in the growth medium although the rate of synthesis in the Etn-requiring cells was slower than in the Etn-nonrequiring cells due to a slower serine-Etn base exchange activity. However, no differences in the *in vitro* activity of the base exchange enzymes or PS levels was observed [Kano-Sueoka and King, 1987]. Furthermore, experiments with keratinocytes (an Etn-requiring cell line) showed that both proliferating and non-proliferating cells were able to synthesize PE by the decarboxylation of PS and also via the *de novo* pathway. Addition of Etn to the growth medium of non-proliferating cells stimulated PE synthesis presumably via the *de novo* pathway while synthesis via the PS decarboxylase pathway declined [Arthur and Lu, 1993]. These observations suggested that synthesis of PE via the *de novo* pathway may be curtailed by the lack of Etn so that cells have to rely on the PS decarboxylase pathway which although functional could not meet the PE demands of the cell. The resultant low levels of PE may be responsible for the inhibition of cell proliferation.

1.7 Ethanolamine Requirement in Cells: Some Hypotheses

The reason(s) why certain cells require Etn to proliferate while others do not is poorly

understood. As mentioned in the previous section, if the PS decarboxylase pathway cannot meet the cellular requirement for PE in the absence of Etn, this could lead to a reduced cellular PE content. Experiments with rat 64-24 mammary carcinoma cells showed that inhibition of cell proliferation could be correlated with reduced PE content. In fact, membrane PE content in Etn-deficient cells dropped to between 30-50% while that of PC increased by 30% compared to Etn-sufficient cells [Kano-Sueoka *et al.*, 1983].

1.7.0 Membrane Lipid Hypothesis

Fisk and Kano-Sueoka (1992) proposed that since a proper membrane lipid environment is required for cell proliferation, an alteration in the membrane phospholipid composition may affect membrane-associated functions and the transduction of extracellular growth signals into the interior of cells. It can be envisaged that changes in the membrane phospholipid composition induced by Etn deficiency may affect functions involving the membrane such as membrane biogenesis and fluidity, transport of molecules across the membrane and interaction of various proteins with the membrane. As noted by Horrobin (1995), the membrane lipid milieu can affect the activity of proteins; for instance, affinity of receptors for their ligands, generation of second messengers from the fatty acid component of membrane lipids and the association of proteins with the membrane.

In support of this hypothesis, it was demonstrated by Kano-Sueoka and King (1988) that there are small but significant differences in the binding properties of a phorbol ester, phorbol-12,13-dibutyrate (PDB), in Etn-sufficient and -deficient rat 64-24 cells. PDB

stimulated growth of Etn-sufficient rat 64-24 cells but not Etn-deficient cells. PDB binds to and activates protein kinase C (PKC) in the presence of PS. Studies have shown that both Etn-deficient and Etn-sufficient cells have a similar PS content in their membranes [Kano-Sueoka and King, 1987]. Since PE rather than PC enhances the *in vitro* activity of PKC [Kikuchi *et al.*, 1981; Kano-Sueoka and Nicks, 1993], it was concluded that PE deficiency may influence the binding of PDB and the subsequent activation of PKC [Kano-Sueoka and King, 1988]. PKC is an important protein kinase involved in intracellular signal transduction and inhibition of its activation as a consequence of an altered membrane phospholipid composition, as occurs in Etn-deficient cells, could interfere with the growth response of Etn-deficient cells. However, PDB is not a growth factor and whether the changes do in fact affect growth factor-induced PKC activation is not known.

In another study with rat 64-24 mammary carcinoma cells, PDB was shown to cause a two-fold elevation in the activity of membrane-bound CTP : phosphorylcholine cytidylyltransferase (CT), a regulatory enzyme in PC biosynthesis, in Etn-sufficient cells but not in Etn-deficient cells [Fisk and Kano-Sueoka, 1992]. Both PC and PE can be hydrolyzed by phospholipases in response to extracellular signals generating second messengers such as arachidonic acid and DAG [Cook *et al.*, 1989; Kiss and Anderson, 1990]. DAG activates and causes the translocation of CT to the membrane [Wright *et al.*, 1985]. Thus, the effect of PDB on CT activity was an indirect one; a result of the slow accumulation of DAG due to hydrolysis of PC or PE [Fisk and Kano-Sueoka, 1992].

Differences in the binding characteristics of epidermal growth factor (EGF) to their receptors in an Etn-requiring rat 64-24 cell line have also been noted [Kano-Sueoka *et al.*,

1990]. In these studies, EGF displayed a weaker but significant affinity for its receptors in Etn-deficient cells compared to cells that were Etn-sufficient. Although the physiological relevance of this small difference in binding of EGF is not known, the binding effect was observed to be more pronounced when cells were pre-treated with a phorbol ester, PDB. Pre-treatment with PDB caused a loss of high-affinity sites in Etn-sufficient cells. Binding in Etn-deficient cells however became refractory to PDB pre-treatment with a 25% decrease in the internalization of bound EGF.

In a subsequent experiment to determine the effect of refractoriness to phorbol ester on PKC activity, it was noted that in Etn-deficient cells PKC-α had an abnormal function. In these cells, there was little translocation of PKC to the membrane upon activation with a resultant lower activity compared to Etn-sufficient cells where the majority of PKC disappeared from the cytosol. The abnormal function of PKC was attributed to its inability to associate with the membranes in Etn-deficient cells [Kano-Sueoka and Nicks, 1993].

Bazzi *et al* (1992) have noted that the strategic location of PE in the interior of the membrane may be important for membrane-protein interactions. Using artificial membranes of various phospholipid compositions, they have shown that PKC and other cytoplasmic proteins (with molecular weight range of 22 - 62 KDa) bound selectively to membranes composed of PE with a lower requirement for calcium compared to membranes composed of PC. Membranes composed of a 20% PS / 60% PE provided optimum conditions for binding compared to membranes composed entirely of PS. This evidence provided further proof that the membrane lipid environment may be important for the activity of biomolecules. Taken together, these studies provide indirect evidence that Etn deficiency

interferes with phospholipid metabolism and activity of membrane-associated proteins that in turn affects cell proliferation.

The response of a cell to extracellular growth signals is mediated by various proteins associated with the internal surface of the membrane. For example, activation of the intracellular kinase domains of receptor tyrosine kinases by the binding of a ligand such as EGF causes dimerisation of the receptor and activation of the receptor tyrosine kinase activity [Malarkey *et al.*, 1995; Fig. 4, pg. 24]. Auto phosphorylated receptors then bind directly to proteins containing SH2 domains. However, not all receptors with intrinsic tyrosine kinase activity bind directly to SH2-containing proteins [White and Kahn, 1994; Malarkey *et al.*, 1995]. The receptors for insulin and insulin-like growth factor-1 (IGF-1) are activated by tyrosine auto phosphorylation during ligand stimulation. But, unlike the EGF receptor, the activated insulin receptor phosphorylates its principal substrate, insulin receptor substrate-1(IRS-1), on multiple tyrosine residues [White and Kahn, 1994]. IRS-1 in turn binds to various SH2-containing proteins including PI 3-kinase, Nck, SH-PTP and Grb-2 [Lee and Pilch, 1994; Fig.5, pg. 25]. Grb-2 serves as an adapter molecule to link the guanine nucleotide exchange factor for Ras, son-of-sevenless (Sos), to phosphotyrosine-containing proteins such as the EGF receptor and IRS-1 [White and Kahn, 1994]. This promotes the binding of Ras with GTP forming an active Ras that is associated with the membrane. Ras then recruits Raf-1 to the membrane where the latter phosphorylates MEK. MEK in turn phosphorylates and activates mitogen-activated protein (MAP) kinase. Activated MAP kinase translocates into the nucleus where it phosphorylates its downstream substrates resulting in the activation of nuclear transcription factors and turning on of specific genes

[Cobb and Goldsmith, 1995; Fig. 4, pg. 24]. MAP kinases transduce signals from a variety of growth factors thus playing a critical role in the proliferation and differentiation of cells [Malarkey *et al.*, 1995]. Since the transduction of growth factor signals is important for cell growth, perturbations of membrane phospholipid composition could affect the transduction of the signals which could translate into reduced cell proliferation as occurs in Etn-deficient cells.

1.7.1 Other Hypothesis

It is generally agreed that phospholipid precursors are required for growth because each mitotic cycle requires that cells double their phospholipid mass in order to form daughter cells [Jakowski, 1996]. This led to the suggestion that phospholipid precursors are required for growth primarily because of the increased need to synthesize phospholipids [Warden and Freidkin, 1984]. However, Kiss and Crilly (1996) have proposed that the mechanism by which Etn and its analogues regulate cell growth is unrelated to their role as phospholipid precursors since they were able to demonstrate that Etn and its analogues enhanced DNA synthesis in NIH 3T3 fibroblasts without a corresponding increase in PE synthesis. Dimethylethanolamine (DME) in particular was mitogenically more potent than monomethylethanolamine (MME) and Etn at concentrations between 0.5 to 1.0 mM. The high concentrations of Etn analogues required to exert a maximal mitogenic effect may be explained by the fact that fibroblasts are Etn-nonresponsive and hence it is not clear how relevant these observations and suggestions are to Etn-requiring cells such as epithelial cells.

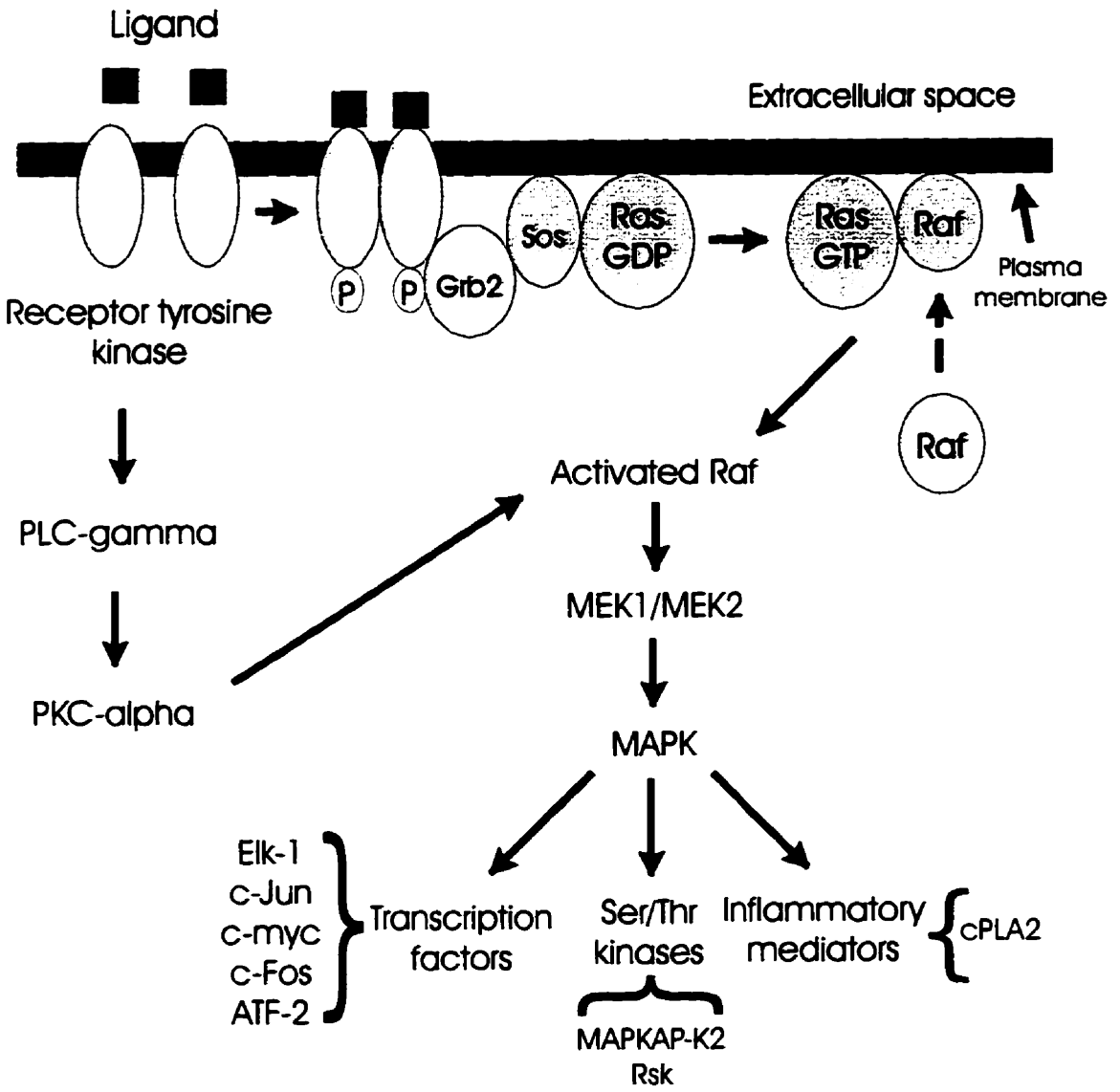


Figure 4. Signaling via the Ras/Raf/MEK/MAP Kinase Pathway

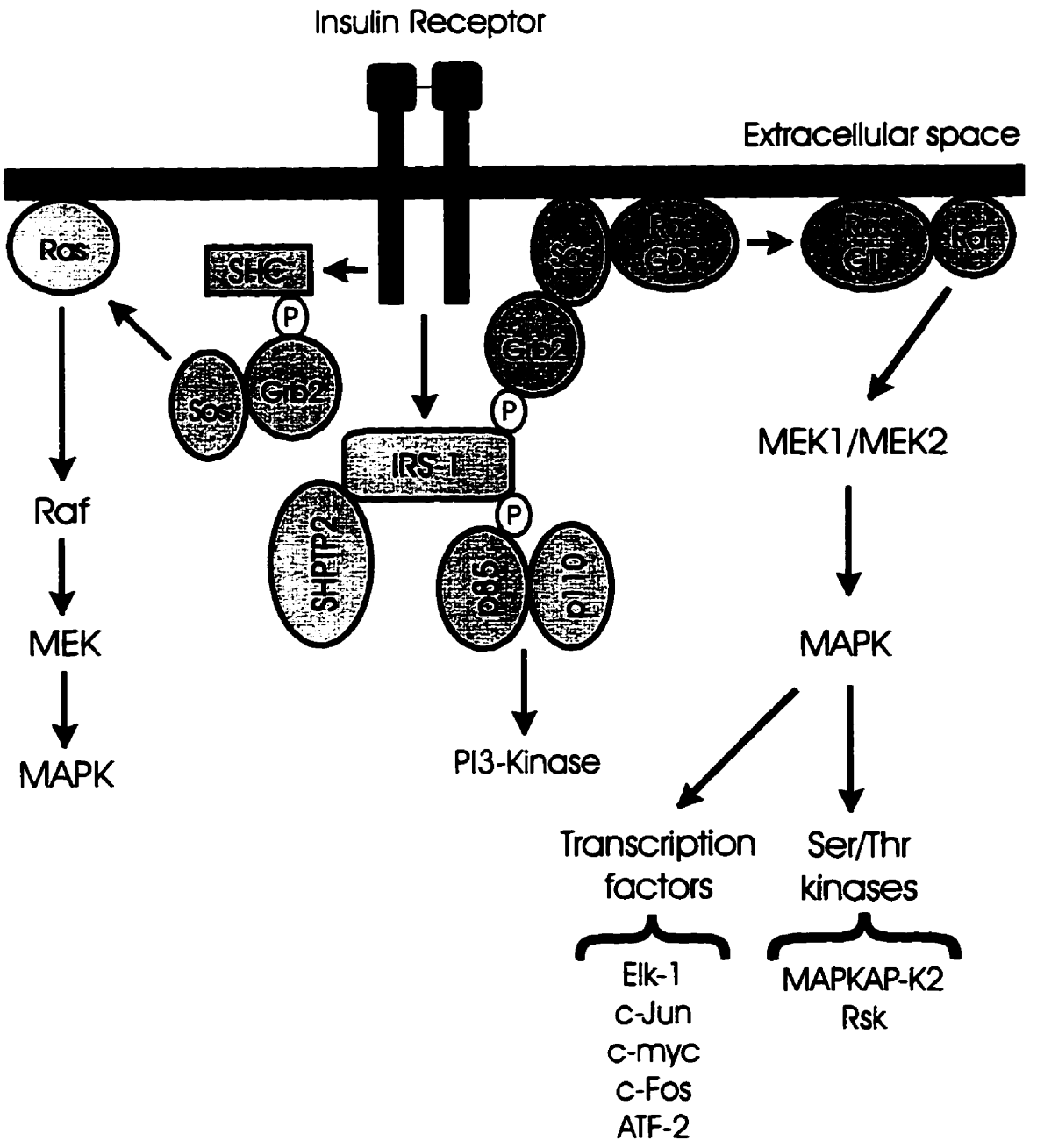


Figure 5. Signaling via the Insulin Receptor

2. WORKING HYPOTHESIS AND OBJECTIVES

The mechanism(s) by which Etn and its analogues regulate cell growth is not known. Most of the studies on the Etn requirement of cells have been carried out with rat mammary carcinoma cells. For these observations to be relevant to humans, further studies have to be conducted using human cell systems. Also, contributions by unknown factor(s) present in the serum used to supplement the growth media in the initial studies cannot be ruled out. In our studies on the Etn requirement of cells, we hypothesize that in the absence of an exogenous supply of Etn, the membrane phospholipid composition becomes altered to such an extent that interaction of key signaling proteins with the membrane is perturbed. This in turn interferes with intracellular transduction of growth factor signals to the nucleus which leads to an inhibition of cell proliferation.

OBJECTIVES

The objective of this study was to investigate the basis for the Etn requirement of cells. Specifically, we examined the effect of Etn on:

(1) cell proliferation,

(2) membrane phospholipid composition,

(3) DNA synthesis, and

(4) transduction of growth factor signals via the MAP kinase pathway.

3. MATERIALS AND METHODS

3.0 Cell Models:

Two cell lines, normal human epidermal keratinocytes (NHEK) and normal human mammary epithelial (HME) cells were used in this study. Both cell types are of epithelial origin and have been reported to require Etn for optimal growth [Kano-Sueoka and King, 1987], and have also been well characterized with respect to their growth in completely defined media (serum-free). The growth factors that affect their growth have also been characterized. Growth in completely defined media is important for our studies. Since the individual components in serum-free media are defined, the composition of the lipid precursors and growth factors in the media can be controlled. In this way, the individual components of the growth media can be manipulated and the effects on cell growth monitored. NHEK and HME cells are also widely used as cell models in research on lipid metabolism.

3.1 Materials and Chemicals

Fungizone, trypsin-EDTA, HEPES and myelin basic protein (MBP) were products of Gibco BRL (Burlington, ON). Gentamicin sulfate, penicillin-streptomycin, amphotericin B, insulin, trypsin neutralizing solution, bovine serum albumin (BSA), calmidazolium, sodium orthovanadate and protease inhibitors were purchased from Sigma Chemical Co., St. Loius,

MO. Ethanolamine, dimethylethanolamine and monomethylethanolamine were obtained from Aldrich Chemical Company Inc., Milwaukee. [^{3}H]-thymidine, [γ-^{32}P] ATP and Ecolite were from ICN Biomedicals, Montreal. [^{3}H]-glycerol and [^{3}H]-Etn were purchased from Amersham Corp., Canada. Protein A-agarose was obtained from Santa Cruz Biotech. (Santa Cruz, CA). Mouse anti-phosphotyrosine monoclonal antibody was obtained from Transduction Laboratories (Lexington, KY). Silica gel 60 TLC plates (20 x 20 cm, 250 μm thickness) were purchased from Merck (Darmstadt, Germany). Cell culture media, growth media supplements, normal human epidermal keratinocytes and normal human mammary epithelial cells were purchased from Clonetics (San Diego, CA). KGF was a product of Boehringer Mannheim. Coomassie protein assay reagent was procured from Pierce (Rockford, IL). Chloroform, methanol, acetic acid, butylated hydroxy toluene (BHT), 70% perchloric acid ($HCLO_4$), and 80% H_3PO_4 were obtained from Fisher Scientific (Nepean, Ontario). Plastic culture ware, Whatman P81 and #2 filter papers were procured from VWR Scientific Ltd, Canada. All chemicals and reagents were of analytical grade.

3.2 Solutions and Buffers

3.2.0 Coomassie Gel Staining and De-staining Solutions.

COOMASSIE GEL STAIN: 1.0g Coomassie Blue dye, 450 ml methanol, 100 ml glacial acetic acid, 450 ml ddH_2O was added to bring the final volume to 1-litre.

COOMASSIE GEL DE-STAIN: 100 ml methanol, 100 ml glacial acetic acid and 800

ml ddH_2O.

3.2.1 Preparation of Solutions for Malachite Green Assay

SOLUTION A (0.3% malachite green base in 1.5 M HCl w/v): The appropriate amount of malachite green base (3.0g) was mixed with 700 ml of 1.5 M HCl and vigorously stirred with a stir bar on a magnetic stirrer for at least 30 minutes. A sufficient amount of 1.5 M HCl was then added to bring the final volume to 1-litre. The solution was then filtered using a Whatman #2 filter paper.

SOLUTION B (4.2% ammonium molybdate in 1.5 M HCl w/v): The desired amount of ammonium molybdate (21.0g) was weighed and gradually dissolved in 1.5 M HCl with stirring until the solution was clear. A sufficient amount of 1.5 M HCl was then added to bring the final volume to 500 ml.

SOLUTION C (4.0% Tween 20 v/v): A 500 ml solution was prepared by dissolving 20 ml of Tween 20 in 300 ml of ddH_2O. Sufficient ddH_2O was then added to bring the final volume to 500 ml.

WORKING SOLUTION: To 3 volumes of Solution A was added one volume of Solution B followed by mixing with a stir bar on a magnetic stirrer for at least 10 minutes. Solution C was then added to bring the final concentration of Tween 20 in the working solution to 0.008%. The solution was mixed vigorously for at least 5 minutes followed by filtration using a Whatman #2 filter paper. The filtrate is the working solution for the determination of inorganic phosphorus.

3.2.2 HEPES-Buffered Saline Solution (HBSS)

HBSS consisted of the following: 10 mM glucose, 3.0 mM KCL, 130 mM NaCl, 1.0 mM Na_2HPO_4, 30 mM HEPES, and 0.0033 mM phenol red. The pH of the buffer was adjusted to 7.4 with 1 M HCl followed by sterile filtration by passage through a 0.2 μm membrane filter.

3.2.3 Preparation of MAP Kinase Extraction Buffer

The extraction buffer consisted of the following: 100 mM β-glycerophosphate, 2 mM EGTA, 1 mM Na_3VO_4, 1 mM DTT, 20 mM Tris-HCl (pH 7.4) 10 μg/ml aprotinin, 10 μg/ml Leupeptin, 0.1 mM PMSF, 0.1 mM AEBSF, 0.2 mM benzamidine, 10% Triton X-100 (v/v), and 10% NP-40 (v/v).

3.2.4 MAP Kinase Assay Buffer

SOLUTION A : 250 μl of 1 M Tris-HCl (pH 7.4), 50 μl of 2 mg/ml BSA, 50 μl of 1 M $MgCl_2$ and 150 μl of ddH_2O to bring the final volume to 500 μl.

SOLUTION B: 30 μl of 20 μM PKI peptide (inhibits cAMP-dependent protein kinases), 20 μl of 125 μM Calmidazolium (inhibits calcium-calmodulin protein kinases) and 30 μl of solution A.

SOLUTION C (Myelin Basic Protein (MBP)): 2 mg/ml of Solution C was prepared

by dissolving the appropriate amount of myelin basic protein in ddH_2O.

SOLUTION D: To prepare a 100 µl solution, the following were pipetted: 5µl of cold 10mM ATP, 20 - 40 µl (20 - 40 µCi) of [γ-^{32}P] ATP, ddH_2O to bring the final volume to 100 µl. The final concentration of cold ATP in the solution was 100 µM.

3.2.5 RNase - Propidium Iodide Staining Solution (RNase - PI)

RNase - PI staining solution was prepared by dissolving the appropriate amounts of the following in ddH_2O: 0.05 mg/ml propidium iodide, 1 mg/ml sodium citrate, 1 mg/ml Triton X-100, and 0.1 mg/ml RNase A

3.2.6 10X Hanks Balanced Salt Solution

Component (g/L): KCl 4.0g, KH_2PO_4 0.6g, NaCl 80.0g, $Na_2HPO_4.7H_20$ 0.9g and D-glucose 10.0g

3.2.7 Immunoprecipitation Buffer

1% Triton X-100 (v/v), 150 mM NaCl, 10 mM Tris (pH 7.4), 1 mM EDTA, 1 mM EGTA, 0.2 mM Na_3VO_4, 0.2 mM PMSF and 0.5% NP-40 (v/v).

3.2.8 Preparation of Bovine Pituitary Extract (BPE)

Bovine pituitary glands were obtained from Pelfreez (Rogers, Ark.) and prepared as described in Tsao *et al.* (1982). 105g of bovine pituitaries were homogenized in 250 ml cold 0.15 M NaCl for 10 minutes in a Waring blender. The homogenate was then transferred to a cold beaker and stirred for 90 minutes at 4°C followed by centrifugation at 9800 x g for 10 minutes at 4°C. The pellet was discarded and the supernatant divided into aliquots and stored at -20°C. When required, the aliquots were thawed and centrifuged at 9800 x g for 15 minutes at 4°C. The supernatant was first filter sterilized by passing sequentially through 0.8 μm and 0.45 μm membrane filters under non-sterile conditions, and then sterilized by passage through a 0.2 μm membrane filter under sterile conditions.

4. METHODS

4.0 Cell Culture and Media

Keratinocytes were routinely cultured in keratinocyte growth media (KGM) which comprised keratinocyte basal media (KBM) supplemented with 0.1 ng/ml EGF, 0.5 μg/ml hydrocortisone, 5.0 μg/ml insulin, 50 μg/ml gentamicin and 50 ng/ml amphotericin or penicillin/streptomycin and fungizone, and 2 ml bovine pituitary extract (BPE). The concentration of choline and Etn in this media was 10^{-4} M.

Mammary epithelial cells were cultured in mammary epithelial growth media (MEGM) which consisted of mammary epithelial basal media (MEBM) supplemented with growth factors, BPE, Etn, choline, and antibiotics at the same levels as in KGM except for EGF which was supplemented at 10 ng/ml. For experiments requiring completely defined medium, keratinocytes were incubated in keratinocyte defined media (KDM) and mammary epithelial cells in mammary epithelial defined media (MEDM). KDM differs from KGM in having KGF in place of BPE. BPE was omitted from MEGM to form MEDM.

For routine culture, cryopreserved cells were thawed in a water bath at 37°C and quickly suspended in pre-warmed growth media. Cells were seeded into T 75 tissue culture flasks at a density of 2500 cells/cm^2 and incubated at 37°C in a humidified incubator with 5% CO_2 / 95% air. After 6 - 9 days, when the cells were 60 - 80% confluent, they were passaged by detaching with trypsin. Trypsin was neutralized with trypsin neutralizing solution (TNS) and the cells were subcultured into 6- or 24-well plates, 100-mm or 150-mm tissue culture

dishes. Third or fourth generation keratinocytes and ninth generation mammary epithelial cells were used in all experiments.

4.1 Effect of Ethanolamine on Cell Proliferation

Cells were subcultured into 6- or 24-well plates as described above. The growth media of log-phase keratinocytes and mammary epithelial cells was removed by aspiration. Traces of BPE-containing growth media was washed off with HEPES buffered saline solution (HBSS) and the cells made quiescent by incubating in basal media supplemented with 0.05% fatty acid-free BSA without growth factors for 24-hrs. The cells were then stimulated with defined growth media in the presence or absence of Etn. At selected times, the cells were washed with HBSS, detached with trypsin and transferred into a cuvette containing 10 ml Isoton II, a balanced electrolyte solution. The wells were rinsed once to remove all detached cells and the cell numbers determined using an electronic cell counter (Coulter ZM Counter).

4.2 Uptake of Ethanolamine

The growth media of log-phase cells was replaced with Etn deficient defined media. After 72-hrs of incubation, the defined media was aspirated with a sterile pasteur pipette, washed twice with HBSS and incubated in basal media supplemented with 0.05% BSA for 24-hrs. Quiescent cells were then stimulated with defined growth media containing 1 μCi/ml

were air-dried and the individual phospholipids visualized by exposing the TLC plates to iodine vapor. The relative position (Rf values) of each phospholipid was determined by comparison with standard phospholipids. The individual spots were scraped into scintillation vials and the radioactivity determined using a liquid scintillation counter (Model LS3801, Beckman Instruments, Fullerton, CA).

4.3 Cell Cycle Analysis by Flow Cytometry

Cells were split into 100-mm culture dishes and cultured in growth media containing BPE. The media of log-phase cells was replaced with basal media with or without Etn and supplemented with 0.05% fatty acid-free BSA. After incubation for 24-hrs, the cells were stimulated with defined growth media with or without Etn. At selected times, the growth media was aspirated and the cells washed with HBSS. The cells were harvested by trypsinization and aliquots taken for cell number determination. After centrifuging at 1000 rpm for 5 minutes, the pellet was washed three times with cold PBS/2 mM EDTA (PBS-EDTA) buffer. Cell pellets were resuspended in 750 µl cold PBS-EDTA buffer, vortexed and 2 ml cold 95% ethanol added drop-wise to fix the cells. The fixed cells were pelleted by centrifugation and washed twice with cold PBS-EDTA solution. Finally, the fixed cells were suspended in 500 µl RNase-PI staining solution and incubated in the dark for at least 1-hr at 4°C. Prior to analysis, the cells were passed through a 41-µ mesh size macro porous filter to remove large cell clumps. Cell cycle analysis was performed using a flow cytometer (EPICS 753, Coulter Electronics Inc.).

4.4 Incorporation of [^{3}H]-Thymidine into DNA

The growth media of cells growing in 6-well plates was aspirated with a sterile pasteur pipette. After washing with pre-warmed HBSS, cells were incubated in Etn deficient defined media. At selected periods, cells were made quiescent by incubating in basal media supplemented with 0.05% fatty acid-free BSA with or without Etn for 24-hrs followed by stimulation with defined growth media. 1.5 μCi/well of [^{3}H]-thymidine was added to each well in the last 6-hrs of incubation. Cells were washed twice with buffer and solubilized with pre-warmed 1% sodium dodecyl sulfate (SDS) in 0.3 N NaOH. The radioactivity incorporated was determined using a liquid scintillation counter.

4.5 Incorporation of [^{3}H]-Glycerol into Phospholipids

Log-phase cells were incubated in Etn-deficient defined growth media for periods of up to 72-hrs. Cells were then incubated in basal media supplemented with 0.05% fatty acid-free BSA with or without Etn for 24-hrs. The quiescent cells were then incubated with defined growth media with or without Etn in the presence of 5 μCi/ml [^{3}H]-glycerol for 24-hrs. The cells were washed twice with pre-warmed HBSS and detached with trypsin. Further washing of the cells was done by repeatedly mixing with a pipette and pelleting by centrifugation at 1000 rpm for 10 minutes. After dispersing with a syringe, an aliquot was taken for cell number determination. Cell pellets were resuspended in 20 ml chloroform/methanol (1:1 v/v), a few grains of BHT were added followed by vortexing. After

centrifugation, the chloroform/methanol (1:1 v/v) mixture was decanted into large boiling tubes and the solvents dried under nitrogen. 5 ml of chloroform/methanol (2:1 v/v) was added to the pellets, vortexed and centrifuged. This extraction step was repeated two more times. The supernatants were pooled and dried under nitrogen. After drying, 8 ml chloroform/methanol (2:1 v/v), 6 ml 0.9% KCl and 4 ml chloroform/methanol (2:1 v/v) were added. The aqueous and organic phases were allowed to separate followed by centrifugation at 2000 rpm for 5 minutes. The lower organic phase was transferred into screw capped tubes and stored at -20°C. Aliquots of the organic phase were taken to determine total radioactivity incorporated. To determine the amounts of [^{3}H]-glycerol incorporated into the various phospholipid classes, 1 - 2 ml of the sample was pipetted into test tubes and dried under nitrogen. Lipids were then re-suspended in 120 µl of chloroform/methanol (2:1 v/v) and spotted onto pre-activated TLC plates. Phospholipids were separated using a chloroform/methanol/acetic acid/water (50 : 37.5 : 3.5 : 2 v/v/v/v) solvent system. Individual phospholipids were identified by comparing the Rf values to standard phospholipids. Exposure of the TLC plates to iodine vapor enabled the visualization of the individual phospholipids. The marked spots were then scraped into scintillation vials and the radioactivity incorporated determined using a liquid scintillation counter.

4.6 Phosphorus Determination

Phospholipids were quantitated by assaying for inorganic phosphate following digestion by perchloric acid ($HClO_4$). Phosphorus was quantitated using the malachite green

method [Zhou and Arthur, 1992]. Phospholipids were extracted and separated as described above. Aliquots (100 - 200 µl) of the lipid extract were pipetted into test tubes and dried under nitrogen. 1 - 2 ml of 70% perchloric acid was added and the lipids digested at 110°C until the solution was clear. After cooling, 100 µl was transferred to fresh test tubes and de-ionized distilled water added to bring the final volume to 400 µl. 2 ml of working solution was added and the absorbance of the dye-phosphomolybdate complex formed determined at 660-nm using a UV-visible spectrophotometer (Hitachi U-2000 spectrophotometer). Blanks were also set up to correct for background. Total phosphorus and hence lipid content of the samples was determined from a calibration curve constructed using inorganic phosphate. 10 - 20 µg of lipid from the cell extracts was spotted on pre-activated silica gel TLC plates and after resolving, the bands corresponding to PC, PS, PI, PE and PA were scraped off into glass tubes and digested followed by phosphorus assay as described above.

4.7 MAP Kinase Assay

Log-phase cells were incubated in Etn deficient defined growth media for selected periods. Incubation was then continued in basal media supplemented with 0.05% fatty acid-free BSA with or without Etn for 24-hrs. Quiescent cells were stimulated with 5 µg/ml insulin for various periods. At the end of the stimulation period, cells were washed with ice-cold 1X concentrated Hanks buffer and 0.5 - 1.0 ml of ice-cold extraction buffer was added. The cells were quickly scraped on ice with a rubber policeman, transferred into centrifuge tubes with a pipette and sonicated for 10 seconds (3-times). Soluble and particulate fractions

were separated by centrifugation at 100,000 x g for 30 minutes at 4°C. The supernatant was transferred into micro centrifuge tubes. An aliquot was taken for protein determination and the rest of the supernatant stored at -70°C. MAP kinase activity was assayed as follows: To each micro test tube was added 8 µl of Solution B, 6 µl of Solution C and 6 µl of Solution D. Kinase reaction was initiated by the addition of 10 µl (1 µg) cytosol to bring the total reaction volume to 30 µl. Blank tubes contained ddH_2O instead of myelin basic protein (MBP). Tubes were incubated at 30 ± 1°C for 10 - 12 mins in a water bath with shaking and the kinase reaction stopped by pipetting 20 µl of assay mixture onto Whatman P81 paper. The P81 papers were washed thoroughly with 1.28% H_3PO_4, air-dried and the radioactivity incorporated determined using a liquid scintillation counter.

4.8 Immunoprecipitation of Tyrosine-Phosphorylated Proteins

To a 1.5 ml capacity micro centrifuge tube was added total cell lysate containing 250 µg total protein and 500 µl of immunoprecipitation buffer. Immunoprecipitation buffer was added to bring the final volume to 1.0 ml followed by the addition of 20 µl protein A-agarose, vortexed and incubated at 4°C for 1.5-hours with end-to-end rotation. Lysates were centrifuged at 14,000 rpm for 3 minutes and the supernatant transferred into fresh micro centrifuge tubes. 6 µg anti-phosphotyrosine antibody (PY 20,) was added and incubated overnight at 4°C. 20 µl of protein A-agarose was added and the incubation continued for another 2-hrs after which the immunoprecitates were collected by centrifugation and washed three times with immunoprecipitation buffer. The pellets were resuspended in 30 µl of 2X

concentrated SDS-sample buffer, boiled for 5 minutes and centrifuged at 14,000 rpm for 50 seconds. 30 µl of each boiled sample was loaded onto 10% SDS-polyacrylamide gel and subjected to electrophoresis at 150V. The gels were stained with coomassie blue dye for 15-minutes. After de-staining and drying the gel, protein bands were quantitated by densitometric analysis with a high resolution scanner (PDI 325oe; Huntington Station, NY) using the ImageMaster scanning program (Pharmacia LKB Biotechnology Inc., Piscataway, NJ)

4.9 Protein Determination

Pierce coomassie protein reagent was used for the determination of the protein content of all samples. 10 µl of the sample was pipetted into test tubes and ddH_2O added to bring the final volume to 1.0 ml. 2 ml of the coomassie reagent was added, vortexed and the absorbance at 595 nm determined using a UV-visible spectrophotometer. The concentration of protein was determined from a calibration curve constructed using bovine serum albumin (BSA) as the standard protein.

4.10 Statistical Analysis

Statistical differences were evaluated using unpaired student t-test. The statistical analysis was carried out using GraphPad Prism software (GraphPad Software, Inc., San Diego, CA).

5. RESULTS

5.0 Effect of Ethanolamine on Cell Proliferation

Both normal human epidermal keratinocytes and mammary epithelial cells have been reported to require Etn for growth [Kano-Sueoka and King, 1987]. Since our objectives were to investigate the role of Etn in cell proliferation, it was essential for us to confirm that the cells were indeed Etn-requiring. Keratinocytes and HME cells were grown in the presence or absence of Etn as described in the methods section and the cell proliferation monitored.

Comparison of the effect of Etn on the proliferation of keratinocytes and mammary epithelial cells indicates that cells incubated in Etn-deficient media had a significantly reduced cell proliferation compared to those incubated in media with Etn. Incubation of keratinocytes for 72-hrs in Etn-deficient media resulted in a 55.2% decrease in cell proliferation [Fig.6, pg. 47]. A 47.4% decrease in cell proliferation was observed as early as 24-hrs and 65.7% after 48-hrs of incubation in Etn-deficient media. A similar effect was also observed in human mammary epithelial cells in which cell proliferation was reduced by 53.1% [Fig.7, pg. 48]. There was a 10.5% and 41.8% decrease in cell proliferation after 24- and 48-hrs of incubation, respectively, in Etn-deficient media. The results clearly showed that both keratinocytes and HME cells were Etn-requiring.

Experiments were then conducted to investigate if a minimum concentration of Etn could be established below which cell proliferation could not be sustained. These studies were conducted with keratinocytes. The cells were incubated with varying Etn concentrations

and the increase in cell number determined daily. Cells incubated in 10^{-4} M Etn served as controls. This concentration of Etn had been reported to be optimal for the proliferation of cells [Tsao *et al.*, 1982]. As shown in Fig.8 (pg. 49), proliferation of cells incubated for 24-hrs in the absence of Etn and also in the presence of 10^{-6} M Etn was 59.9% and 64.2% of controls, respectively. The rate of proliferation of cells incubated in 10^{-5}, 10^{-7} and 10^{-8} molar Etn for 24-hrs was comparable to control cells. Proliferation of cells after 72-hrs incubation in 10^{-7} and 10^{-8} molar Etn was 89.0% and 84.3% of controls, respectively, while proliferation of cells incubated in 10^{-6} M increased to 81.7%. The rate of proliferation of cells incubated in 10^{-6} M Etn for 96- and 120-hrs was similar to control cells while the rate of proliferation of cells incubated in 10^{-7} M and 10^{-8} M Etn for 120-hrs was 62.4% and 59.2% of control cells, respectively. While the rate of proliferation of cells incubated in 10^{-5} M Etn was comparable to control cells, the proliferation of cells incubated in the absence of Etn decreased at all times. In fact, after 120-hrs of incubation in Etn-deficient media, cell proliferation had decreased to 33.0% of controls. These results showed that Etn concentrations as low as 10^{-6} M could sustain the long term proliferation of keratinocytes. The results also confirmed our observations that incubation of cells in the absence of Etn resulted in a significantly reduced cell proliferation.

Since there have been suggestions that methylated analogues of Etn rather than Etn itself are responsible for mitogenesis, the effect of mono- and -dimethylated analogues of Etn on cell proliferation was examined in HME cells. In this experiment, HME cells were incubated in media containing 10^{-4} M DME or MME for 72-hrs. For comparison, cells were also incubated with Etn (10^{-4} M) for 72-hrs. As displayed in Fig.9 (pg. 50), Etn significantly

enhanced the proliferative potential of HME cells compared to the Etn analogues. After 24-hrs of incubation, proliferation of cells incubated in the presence of DME and MME was 81.2% and 76.2% respectively, of cells incubated in the presence of Etn. However, when cells were incubated with DME and MME for 48-hrs, cell proliferation as compared to control cells decreased to 57.4% and 48.4%, respectively. The rate of proliferation in the presence of DME and MME after 72-hrs of incubation was 55.5% and 47.2% of cells incubated in Etn-containing media. Proliferation of cells incubated in DME was higher compared to cells incubated in MME over the same period. This suggests that Etn is a more potent mitogen compared to either of its methylated derivatives.

5.1 Effect of Ethanolamine on DNA Synthesis

To investigate the effect of Etn on DNA synthesis, the growth media of log-phase cells was replaced with basal media with or without Etn supplemented with 0.05% fatty acid-free BSA and incubated for 24-hrs. Quiescent Etn-sufficient and -deficient HME cells were stimulated with defined growth media with or without Etn in the presence of [^{3}H]-thymidine. The radioactivity incorporated into DNA was determined with a liquid scintillation counter. The results are displayed in Fig.10 (pg. 51). Incubation of HME cells in Etn-deficient media for 24-hrs resulted in a 91.5% decrease in the incorporation of [^{3}H]-thymidine into DNA as compared to cells incubated with Etn. After 48-hrs of incubation, there was a 5-fold increased incorporation of label into DNA compared to the levels after 24-hrs in cells in Etn-deficient media but this only represented 33.2% of the radioactive label in Etn-sufficient

cells. These results clearly demonstrated that Etn was mitogenic for HME cells.

In keratinocytes, Etn significantly enhanced the mitogenic effects of insulin and EGF [Fig.11, pg. 52]. Stimulation of cells with insulin and EGF in the presence of Etn resulted in a 69% and 40.5% respectively, increased incorporation of [^{3}H]-thymidine into DNA [compare Fig.11, I, III & IV]. The presence of insulin and EGF enabled cells to overcome the inhibitory effect of Etn-deficiency on DNA synthesis. In the absence of growth factor stimulation, addition of Etn induced a 29.3% increased incorporation of label into DNA [see Fig.11, I]. Stimulation of cells with all three growth factors in the presence of Etn also induced about 21% incorporation of [^{3}H]-thymidine into DNA [Fig.11, II]. These results provided further evidence of the mitogenic potential of Etn.

The effect of methylated analogues of Etn on DNA synthesis in HME cells was also examined. In these experiments, quiescent cells were stimulated with growth media containing 10^{-4} M DME, MME and Etn in the presence of [^{3}H]-thymidine. The results which are displayed in Fig.12 (pg. 53) showed that after 24-hrs of incubation, DME and MME did not significantly promote incorporation of label into DNA as compared to cells incubated with Etn. Incorporation of label into DNA was only 16.8%, 17.9% and 18.4% of controls (Etn-sufficient) in DME, MME and Etn-deficient cells, respectively, after 24-hrs of incubation. However, after 48-hrs of incubation, DME and MME stimulated a 77.6% and 66.0% increase in DNA synthesis respectively. In Etn -deficient cells, incorporation of label into DNA was only 42.2% of controls. Taken together, the order of increasing mitogenic potency of Etn analogues in HME cells was: Etn > DME > MME. These results confirm our observations that Etn is a much more potent mitogen compared to its analogues since

stimulation of DNA synthesis was enhanced in the presence of Etn rather than with DME or MME.

5.2 Effect of Ethanolamine on Cell Cycle Progression

The requirement of Etn for normal progression through the cell cycle was investigated in HME cells incubated in the presence or absence of Etn for 96-hrs and analyzed by flow cytometry. As shown in Fig.13 (pg. 54), 79% of Etn-deficient HME cells were in the G0/G1 phase of the cell cycle compared to 63% of Etn-sufficient cells. In addition, fewer Etn-deficient cells were in S (10%) and G2/M (11%) phases of the cell cycle compared to 18% and 19% respectively, of control cells. In a subsequent experiment, cells growing in media with or without Etn were made quiescent by incubating in basal media for 24-hrs. Cells were then released by stimulating with growth media in the presence or absence of Etn followed by flow cytometric analysis. As displayed in Fig.14 (pg. 55), supplementation of the growth media with Etn resulted in a normal progression through the cell cycle in Etn-sufficient cells whereas Etn depleted cells tended to progress much slowly. 12-hrs after stimulation, only about 55% of Etn-sufficient cells were in the G1/G0 phase. A higher percentage of these cells had progressed into the S (≥29%) and G2/M (≥15%) phases. The progression of Etn-deficient cells after stimulation was however less rapid with greater than 75% of the cells still in G1/G0 and a lower percentage in S (≥15%) and G2/M (≥8%). These results showed that a deficiency of Etn inhibited the distribution and cell cycle progression of cells hence the accumulation of cells in the G1/G0 phase of the cell cycle.

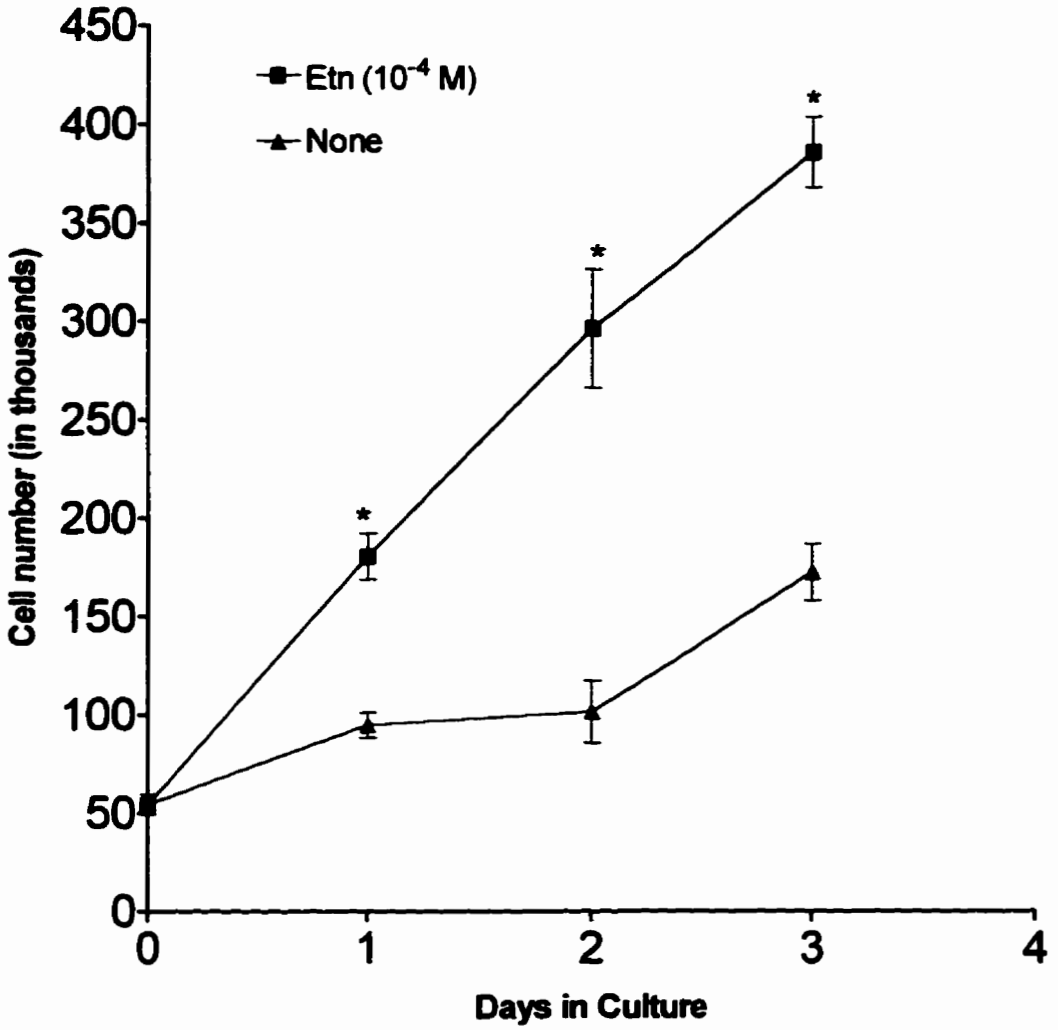


Figure 6. Effect of Ethanolamine on the Proliferation of Keratinocytes. Quiescent cells were stimulated with growth media (Day 0) in the presence or absence of Etn for 72-hrs. The increase in cell number was determined at 24-hr intervals with an electronic cell counter. Values are expressed as mean ± S.D. of a single experiment with four determinations.
*$P < 0.05$

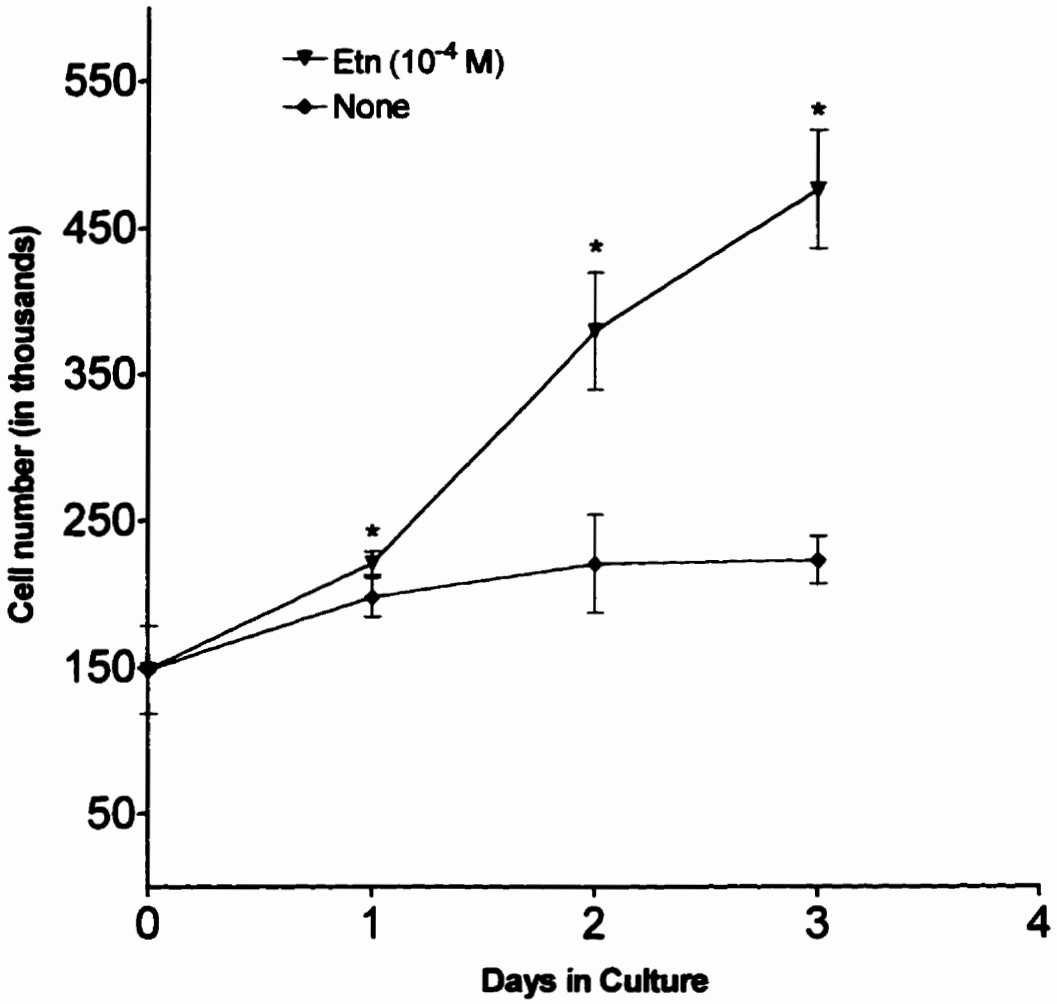


Figure 7. Effect of Ethanolamine on the Proliferation of Human Mammary Epithelial Cells. Quiescent cells were stimulated with growth media (Day 0) in the presence or absence of Etn for 72-hrs. Cell number was determined at 24-hr intervals with an electronic cell counter. The results are the mean ± S.D. of two experiments each with six determinations. *$P < 0.05$

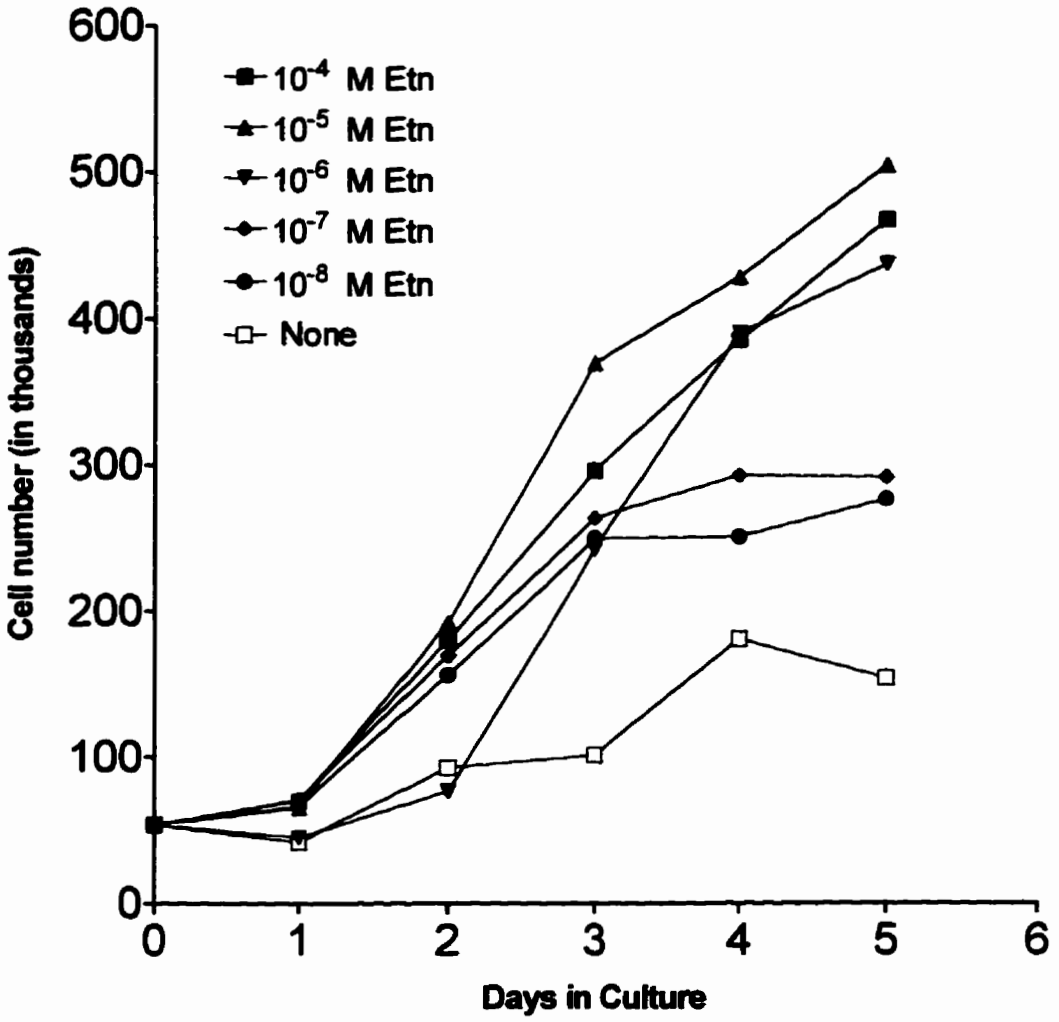


Figure 8. Dose Response Growth of Keratinocytes to Ethanolamine. Quiescent cells were stimulated (Time = 0) with media containing varying concentrations of Etn. Control cells were incubated in 10^{-4} M Etn. At 24-hr intervals, cells were washed and detached with trypsin followed by cell number determination using an electronic cell counter. Data are the mean of a single experiment performed in quadruplicate. Standard deviations were less than 13%. For clarity, error bars have been omitted.

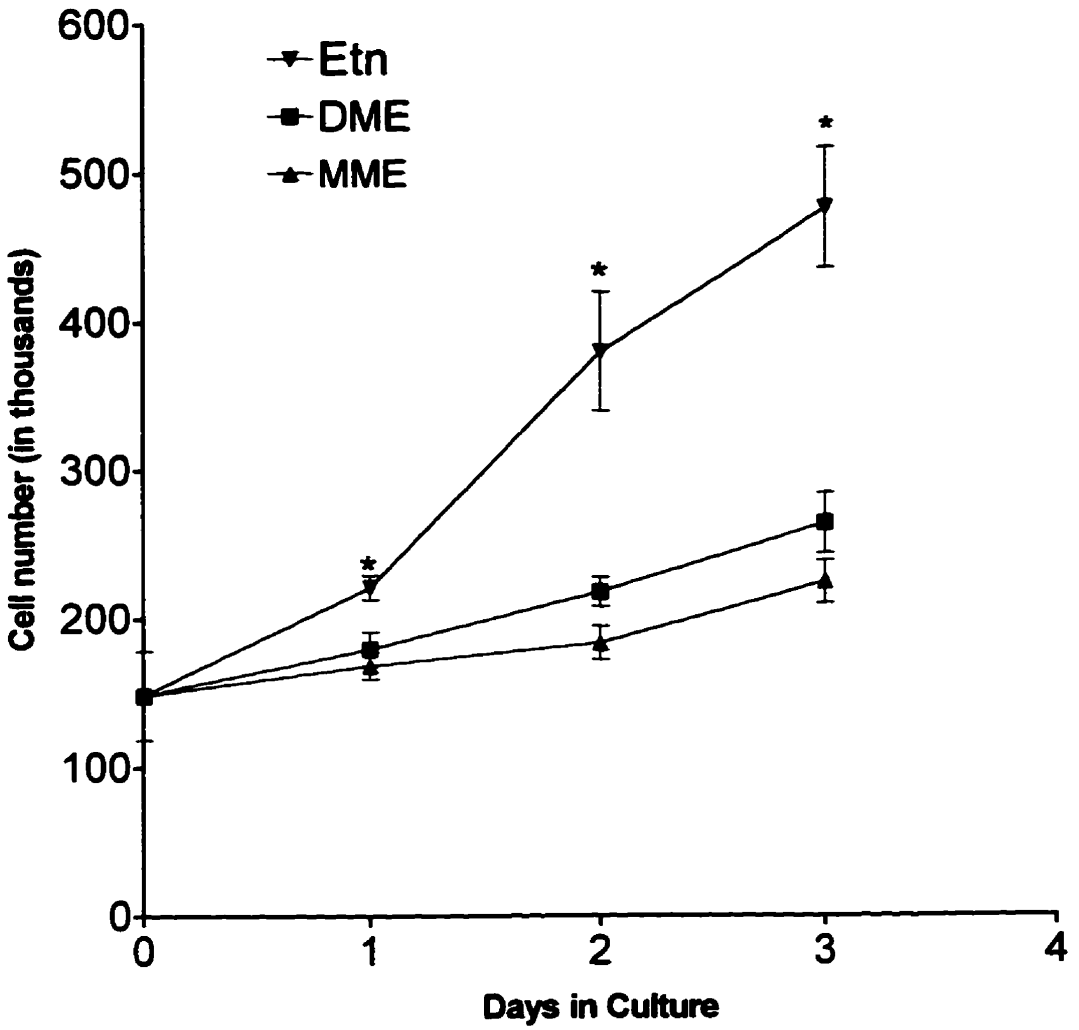


Figure 9. Growth Response of Mammary Epithelial Cells to Ethanolamine Analogues. Quiescent cells were stimulated with media containing 10^{-4} M dimethylethanolamine (DME), monomethylethanolamine (MME) and Etn for 3-days. The increase in cell number was determined using an electronic cell counter at 24-hr intervals. Values represent the mean ± S.D. of two experiments each with six determinations. Two independent experiments also showed similar results. *$P < 0.05$

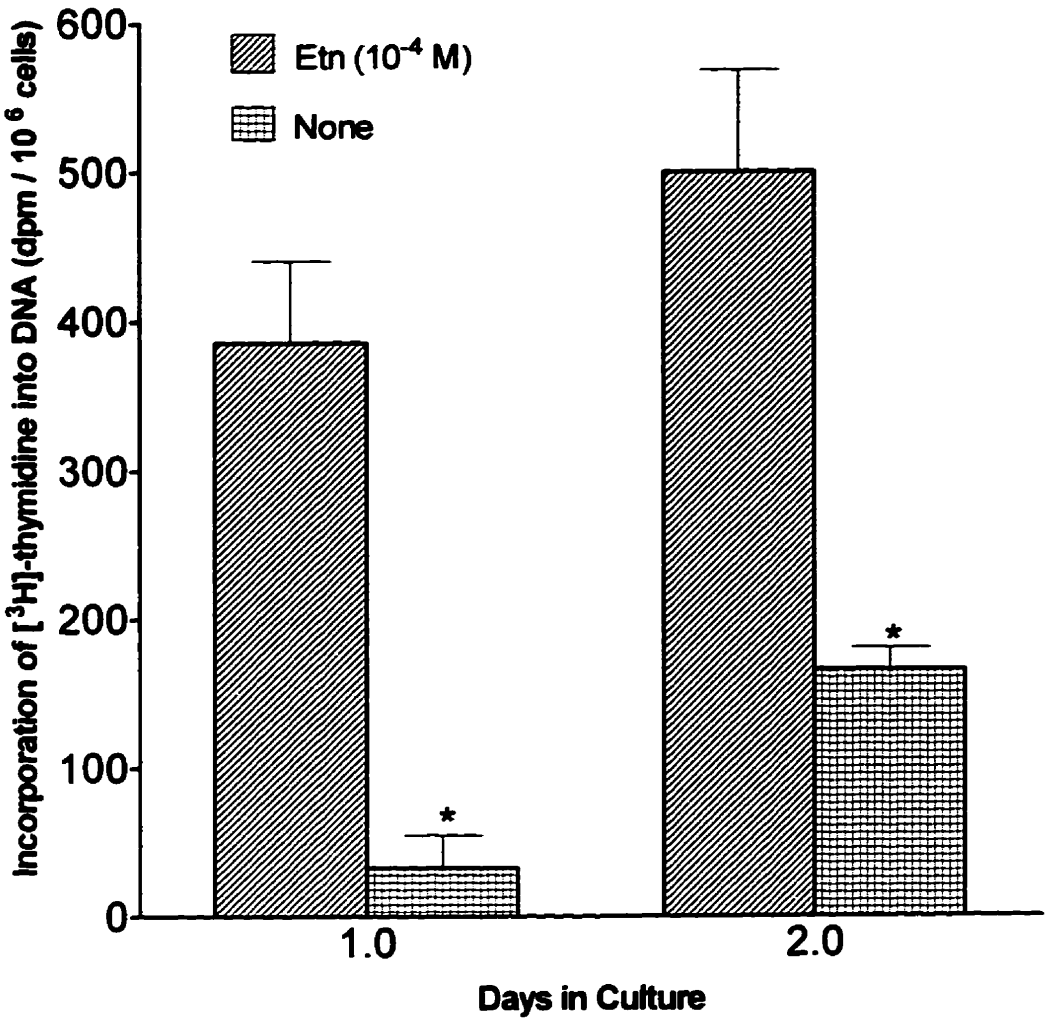


Figure 10. Effect of Ethanolamine on Incorporation of [^{3}H]-Thymidine into DNA in HME cells. Log-phase cells were incubated in basal medium for 24-hrs. Quiescent cells were then stimulated with media in the presence (10^{-4} M) or absence of Etn for 48-hrs. 5 μCi/ml of [^{3}H]-thymidine was added in the last 6-hrs of incubation. After washing, cells were solubilized and radioactivity incorporated into DNA determined as described in the methods. Data represent the mean ± S.D. of two experiments each with six determinations. *$P < 0.05$

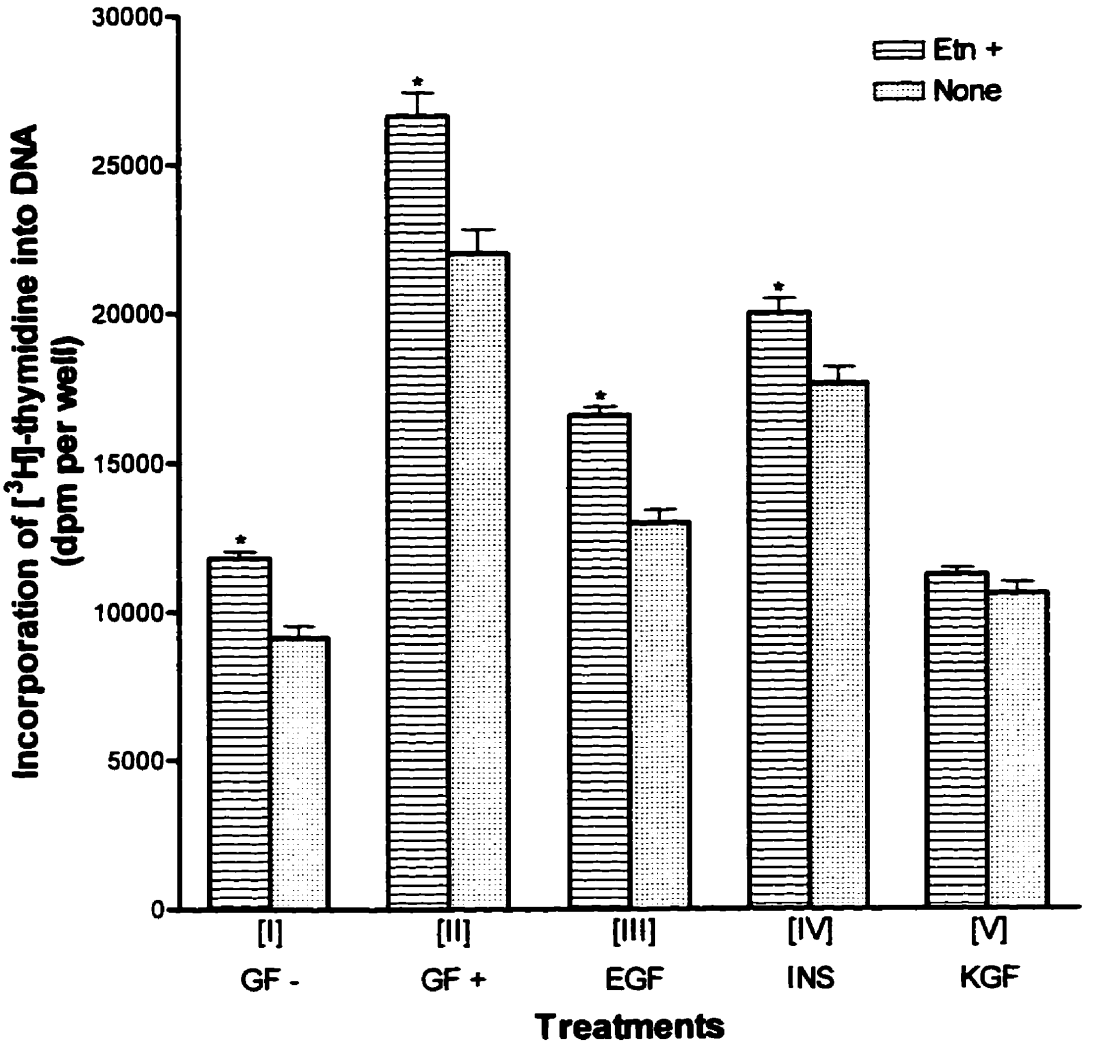


Figure 11. Effect of Ethanolamine on Growth Factor Stimulation of Keratinocytes. Quiescent cells were stimulated with 5 μg/ml insulin, 0.1 ng/ml EGF, 10 ng/ml KGF for 18-hrs and pulsed with 0.5 μCi/ml [^{3}H]-thymidine for 6-hrs. Cells were washed and solubilized as described in the methods. Radioactivity incorporated into DNA was determined using a liquid scintillation counter. GF- = No growth factor added, GF+ = Growth factors (INS, EGF, and KGF) added . Data represent the mean ± S.D. of two experiments each with six determinations. *$P < 0.05$

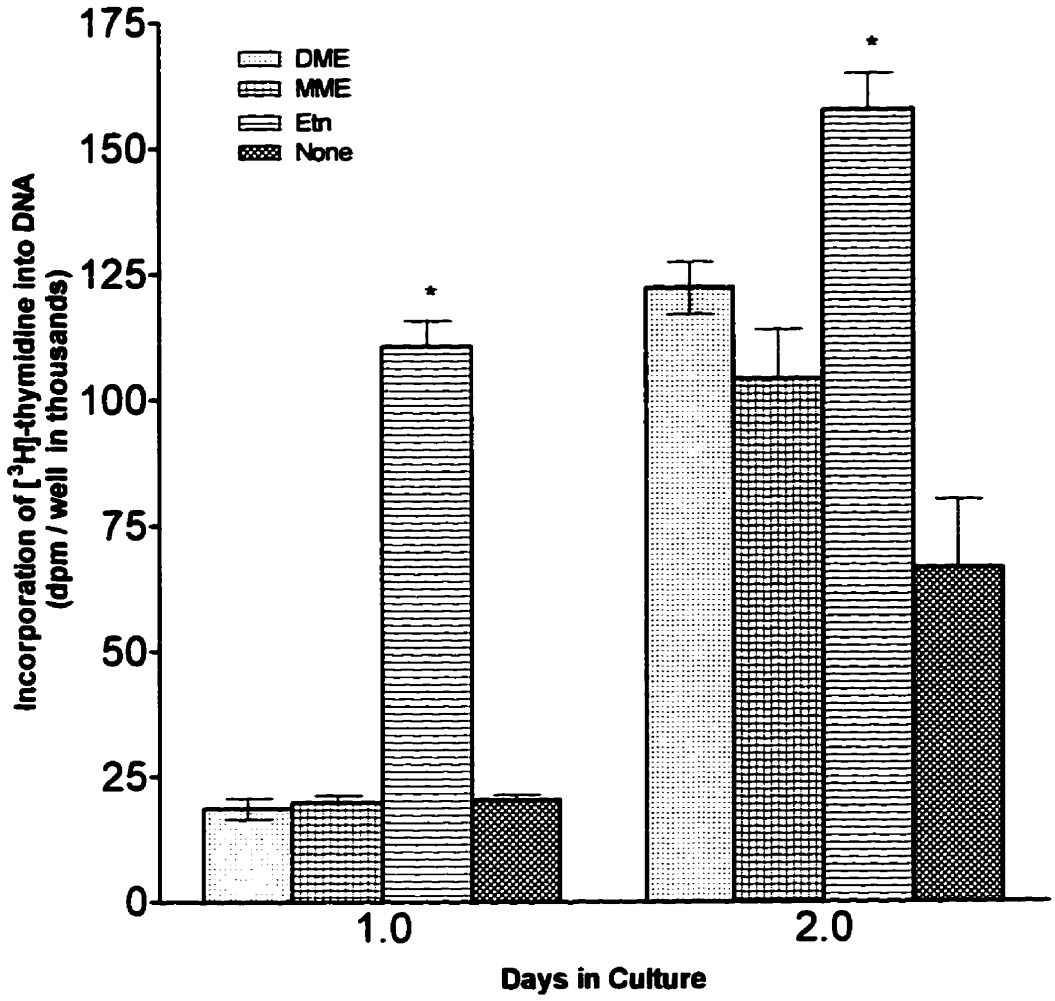


Figure 12. Effect of Ethanolamine Analogues on Incorporation of [^{3}H]-Thymidine into DNA in Mammary Epithelial Cells. Quiescent cells were stimulated with 10^{-4} M DME, MME, Etn and in the absence of Etn. Experiments were performed as described in the legend to Figure 14. Results represent the mean ± S.D. of two experiments each with six determinations. *P < 0.05

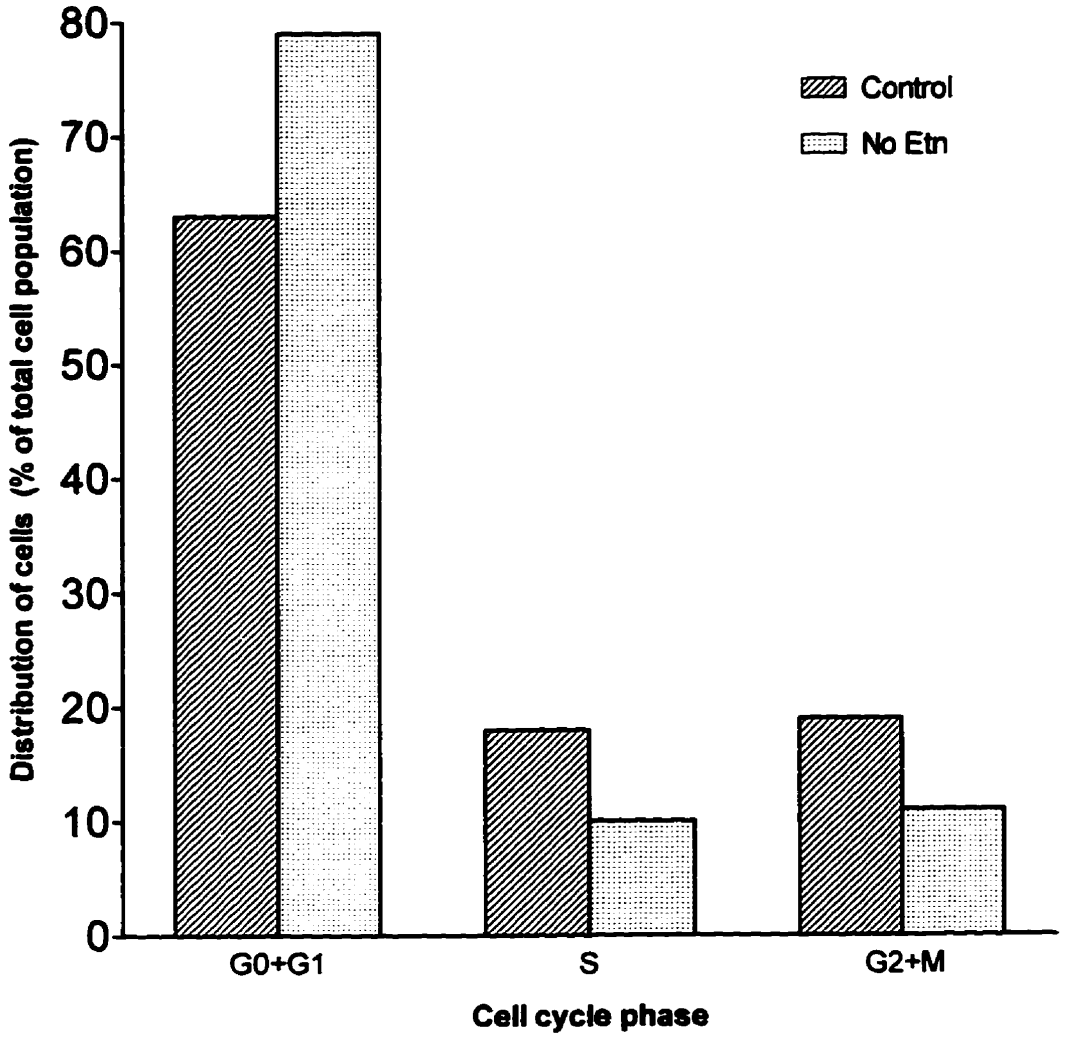


Figure 13. Cell Cycle Distribution of Ethanolamine-sufficient and -deficient Mammary Epithelial Cells. The media of cells growing in 100-mm dishes was replaced with basal medium for 24-hrs. Quiescent cells were then stimulated with defined media in the absence or presence of 10^{-4} M Etn (Control) for 4-days. Cells were prepared for flow cytometric analysis as described in the methods. The results represent the fraction (%) of cells in each phase of the cell cycle.

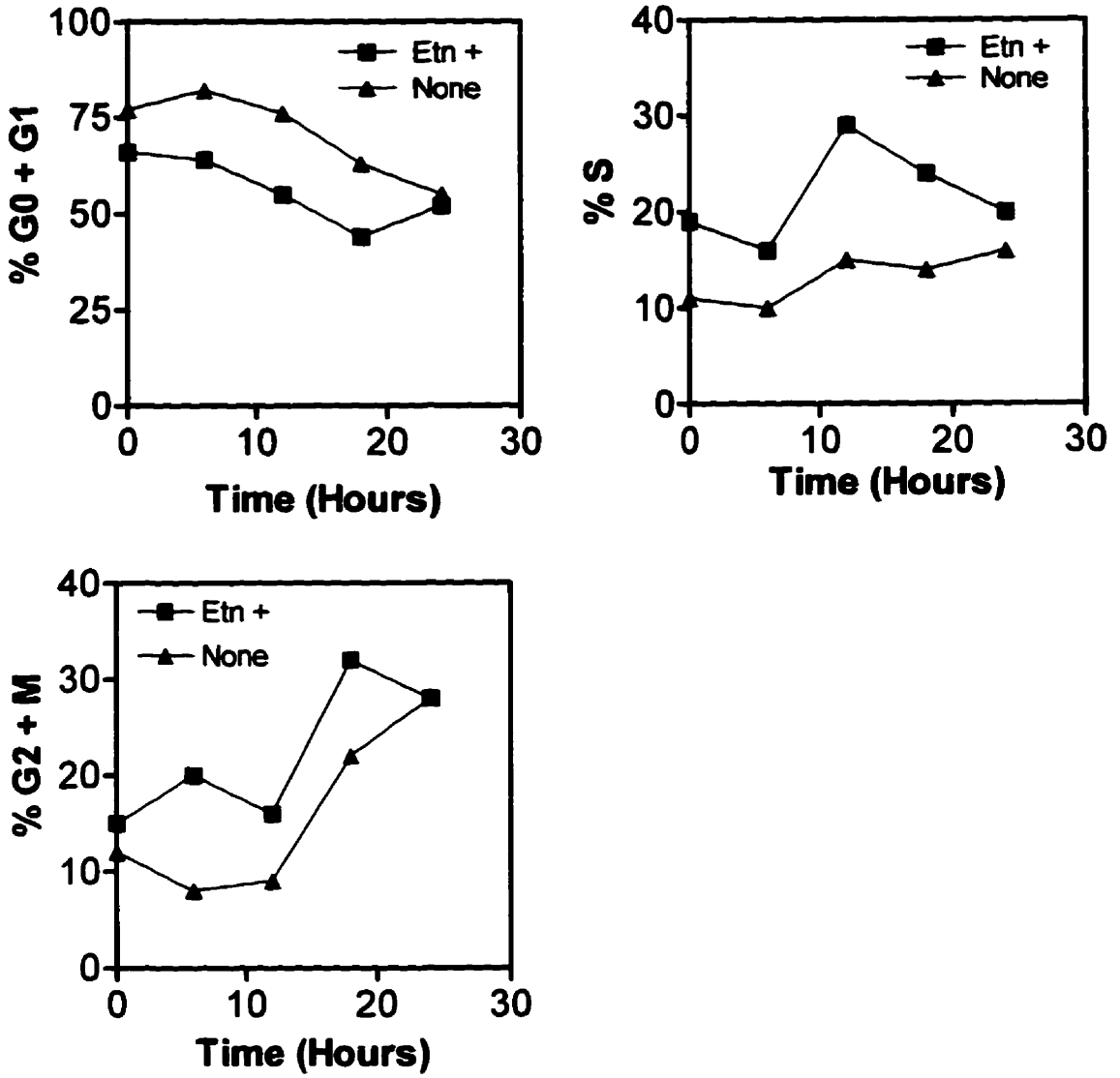


Figure 14. Effect of Ethanolamine on Cell Cycle Progression of Mammary Epithelial Cells. Cells growing in media with or without Etn for 24-hrs were made quiescent by incubating in basal medium for 24-hrs. Cells were then released by stimulating with growth media in the presence or absence of Etn followed by flow cytometric analysis as described in the methods. Results represent the fraction (%) of cells in each phase of the cell cycle.

5.3 Membrane Phospholipid Synthesis and Composition in Ethanolamine-Deficient cells.

Previous studies with rat 64-24 and 22-1 mammary carcinoma cells [Kano-Sueoka *et al.*, 1983; Kano-Sueoka and King, 1987] suggested that extracellular Etn is incorporated into water soluble metabolites of PE and phospholipids. To investigate the incorporation of exogenously supplied Etn into HME cells, the media of cells growing in Etn-deficient defined media for 72-hrs was replaced with basal media (without Etn) supplemented with 0.05% BSA for 24-hrs. Quiescent cells were then stimulated with defined media (without Etn) in the presence of [^{3}H]-Etn. The radioactivity incorporated into water soluble metabolites and PE was determined at periodic intervals. As displayed in Fig.15 (pg. 62), there was a continuous uptake and incorporation of [^{3}H]-Etn into water soluble metabolites of PE. The Etn aqueous pool increased linearly with time with no saturation observed. Similarly, the incorporation of label into PE was linear over the 24-hr period [Fig.16, pg. 63]. The amount of [^{3}H]-Etn incorporated into PE was higher at all times compared to the amount of label in the aqueous fraction. Although the pool sizes of the water soluble precursors of PE such as Etn, P-Etn and CDP-Etn were not determined in this experiment, it can be inferred from the results that Etn-deficient HME cells take up Etn and rapidly incorporate it into PE. The results also suggest that incubating cells in Etn-deficient media depleted the cells of PE hence the rapid incorporation of Etn in order to restore intracellular levels of PE.

Since Etn is utilized for the biosynthesis of PE, the effect of an Etn-deficiency on phospholipid synthesis was investigated in keratinocytes and HME cells. Cells were initially

5.4 Effect of Ethanolamine on the Activation of MAP Kinase

Activation of MAP kinase in cells is crucial for the transmission of growth factor signals into the nucleus to initiate events that culminate in proliferation [Cobb and Goldsmith, 1995]. Since the receptors for the growth factors in the serum-free media for both keratinocytes and HME cells were receptor tyrosine kinases that transduced signals via the MAP kinase cascade [Marshall, 1995; Cobb and Goldsmith, 1995], the decreased proliferation observed in Etn-deficient cells could result from changes in the cells that perturb signaling via this pathway. We therefore investigated the effect of Etn on the activation of MAP kinase in the cells. Prior to the determination of MAP kinase activity in cells, experiments were set up to determine the growth factors and conditions required for stimulation. This experiment was conducted with keratinocytes. The growth media of cells was replaced with defined growth media containing 10 ng/ml EGF, 10 ng/ml KGF and 5 μg/ml insulin. These concentrations of growth factors had previously been shown to be optimal for the proliferation of keratinocytes [Tsao *et al.*, 1982]. Fig.20 (pg.67) shows the effect of incubation of keratinocytes in media containing EGF, KGF and insulin. A combination of 0.1 ng/ml EGF and 5 μg/ml insulin produced the highest cell numbers. Insulin, in particular, was critical for the optimal proliferation of the cells since its omission from the media inhibited cell proliferation. This observation together with results on the stimulatory effects of insulin on DNA synthesis [Fig.11, pg. 52] showed that the order of increasing mitogenic potency for keratinocytes was: Insulin > EGF > KGF. Thus, insulin was routinely used to stimulate cells.

To determine the effect of Etn deficiency on MAP kinase activation, cells growing in defined media without Etn for 48-hrs were made quiescent by incubation in basal media supplemented with 0.05% BSA for 24-hrs in the presence or absence of Etn. Incubation in Etn-containing media ensured that control cells were Etn sufficient prior to stimulation. Cells were then stimulated with 5 μg/ml insulin in the presence or absence of Etn and MAP kinase activity in the cytosol determined by measuring the incorporation of ^{32}P into MBP, a MAP kinase substrate. As shown in Fig.21 (pg. 68), MAP kinase activity in both Etn-sufficient (control) and Etn-deficient HME cells was optimal after 10 minutes of stimulation with insulin. However, MAP kinase activity in control cells was 1.4 times higher than the activity in Etn-deficient cells. The magnitude of MAP kinase activation after 3 and 15 minutes of stimulation was 2.5- and 2.4-fold greater in cells incubated with Etn. After stimulation for 30 minutes, little or no MAP kinase activity could be detected in Etn-deficient cells compared to control cells incubated in the presence of Etn in which MAP kinase activity was 217.6 pmol/min per mg protein. Thus, the activation of MAP kinase in HME cells incubated with Etn was higher and sustained for a longer period compared to Etn-deficient cells.

The results of a similar experiment in keratinocytes are displayed in Fig.22 (pg. 69). MAP kinase activity in control cells peaked after 10 minutes whereas activity in Etn-deficient cells peaked after 3 minutes of stimulation and then declined very rapidly. MAP kinase activity after 10 minutes of stimulation was 2.5-fold greater in control cells than in Etn-deficient cells. After 3 and 7 minutes of stimulation, MAP kinase activity was 1.7- and 1.9-fold higher in cells incubated with Etn. Unlike in control cells, little or no MAP kinase activity was observed in Etn-deficient cells 15 and 30 minutes after stimulation. However,

in control cells, MAP kinase activity was 380 and 441 pmol/min/mg protein after 15- and 30-mins of stimulation with insulin indicating a greater magnitude and sustained activation of MAP kinase.

Generally, in both keratinocytes and HME cells, the rates of activation of MAP kinase were slower and the levels of activation were also lower in cells incubated without Etn. These results demonstrate clearly that Etn deficiency inhibited the magnitude and duration of activation of MAP kinase activity and hence the transduction of growth factor signals.

Studies have shown that phosphorylation on tyrosine residues and the subsequent activation of various protein kinases play a central role in intracellular signaling [Malarkey *et al.*, 1995]. Results from our studies described in the previous sections showed that in Etn-deficient cells membrane phospholipid was altered and the activation of MAP kinase inhibited. Since the signaling events required in the activation of protein kinases, for example MAP kinase, involves phosphorylation on tyrosine residues [Cobb and Goldsmith, 1995], we examined the effect of Etn-deficiency on the levels of tyrosine phosphorylated proteins. This experiment was conducted with HME cells. Cells growing in defined growth media without Etn were made quiescent by incubating in basal media supplemented with 0.05% BSA with or without Etn for 24-hrs. The cells were then stimulated with 5 μg/ml insulin for various periods. Tyrosine phosphorylated proteins were immunoprecipitated from the cytosol as described in the methods using a phosphotyrosine specific antibody. Although the method employed in this study does not provide information with regard to the individual tyrosine phosphorylated proteins, as illustrated in Fig.23 (pg. 70), there was a nearly 3-fold reduction

in phosphorylation of a 30 - 33 KDa protein in Etn-deficient cells stimulated for 7 mins. This preliminary result suggests that cells incubated without Etn had decreased levels of tyrosine phosphorylated proteins.

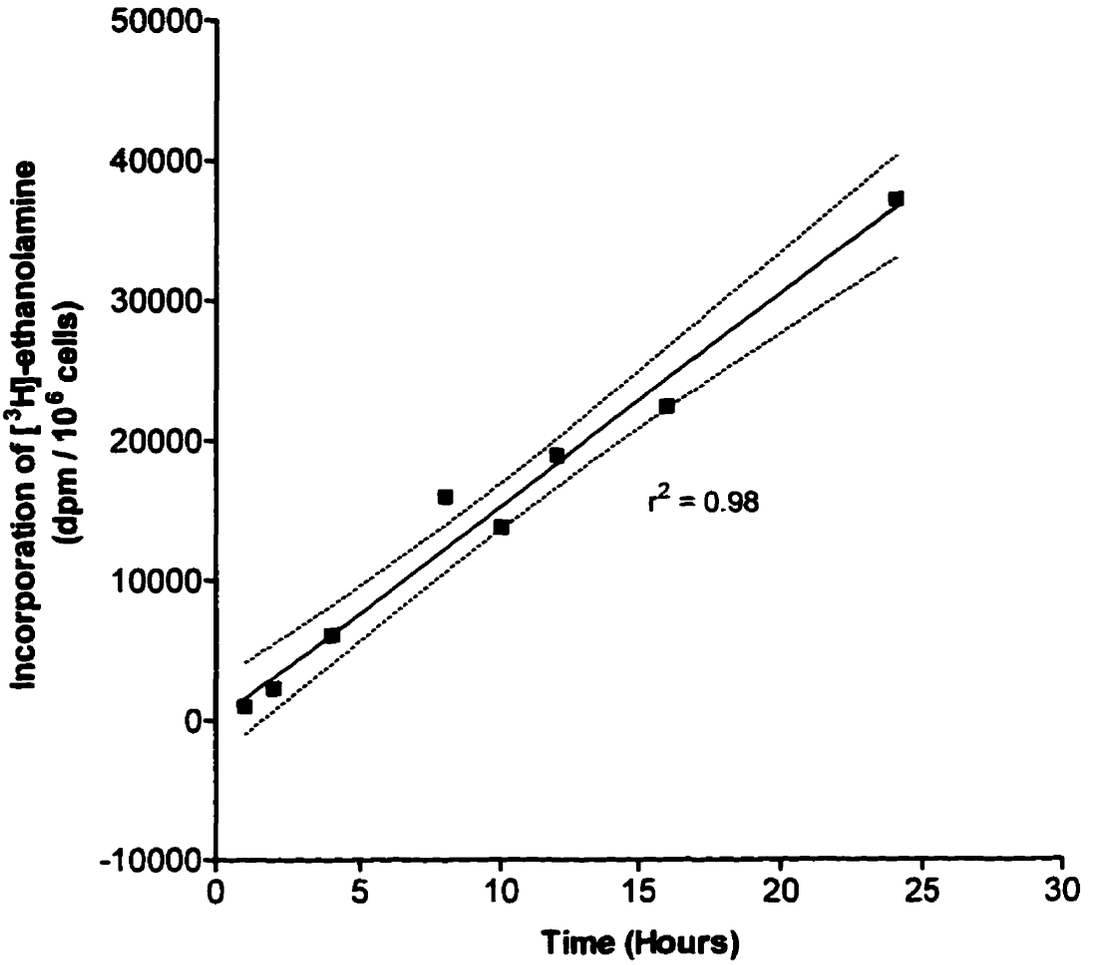


Figure 15. Incorporation of [^{3}H]-Ethanolamine into Water-Soluble Metabolites of PE in Mammary Epithelial Cells. Etn-deficient quiescent cells were stimulated with growth media in the presence of [^{3}H]-Etn (Time = 0). At periodic intervals, cells were washed and extracted to yield a bi-phasic solution as described in the methods. Radioactivity incorporated into water-soluble metabolites was determined with a liquid scintillation counter. The results of a single experiment with six determinations are shown. Data were analyzed by linear regression with 95% confidence interval (CI).

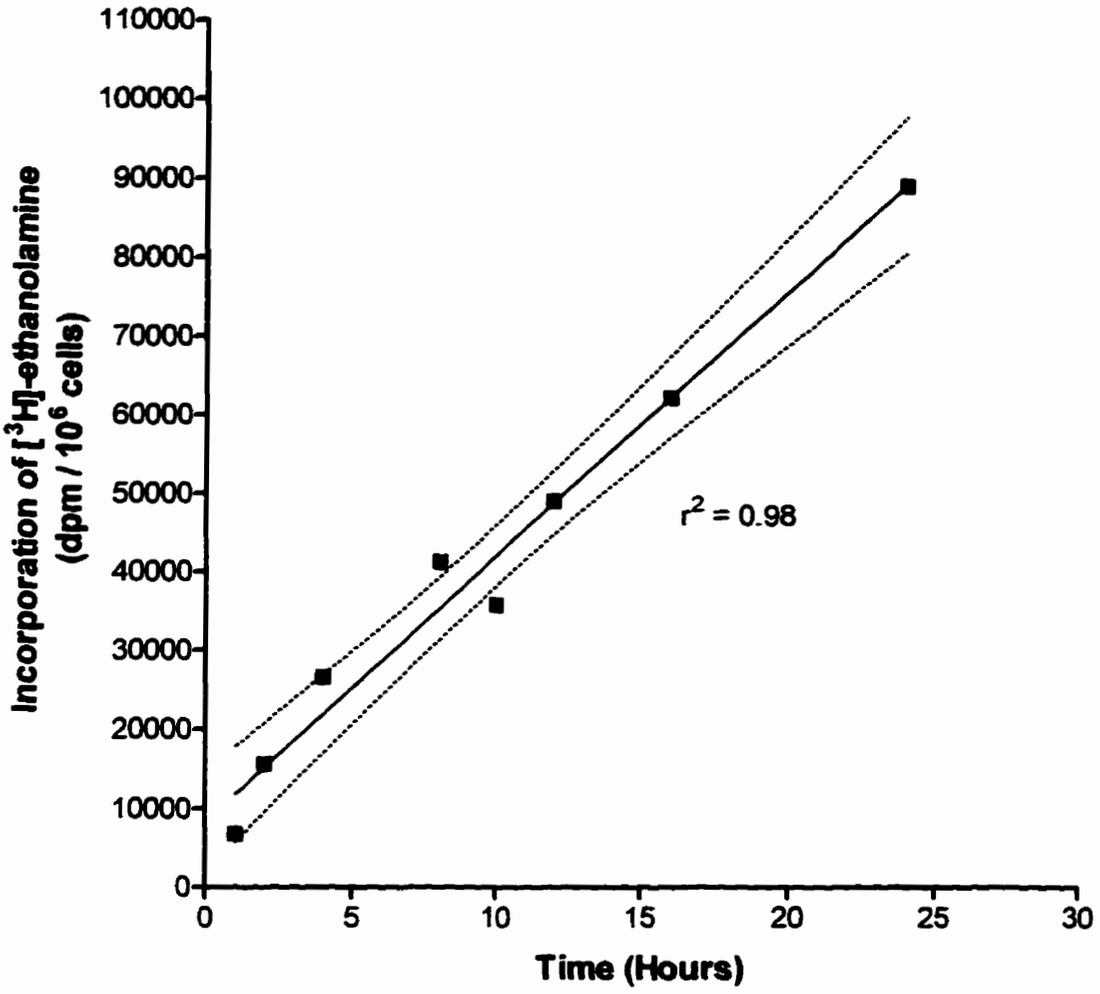


Figure 16. Incorporation of [^{3}H]-Ethanolamine into PE in Mammary Epithelial Cells. Etn-deficient quiescent cells were stimulated with growth media in presence of [^{3}H]-Etn (Time = 0). At periodic intervals, cells were washed and extracted to yield a bi-phasic solution as described in the methods. Radioactivity incorporated into PE was determined with a liquid scintillation counter. The results of a single experiment with six determinations are shown. Data were analyzed by linear regression with 95% confidence interval (CI).

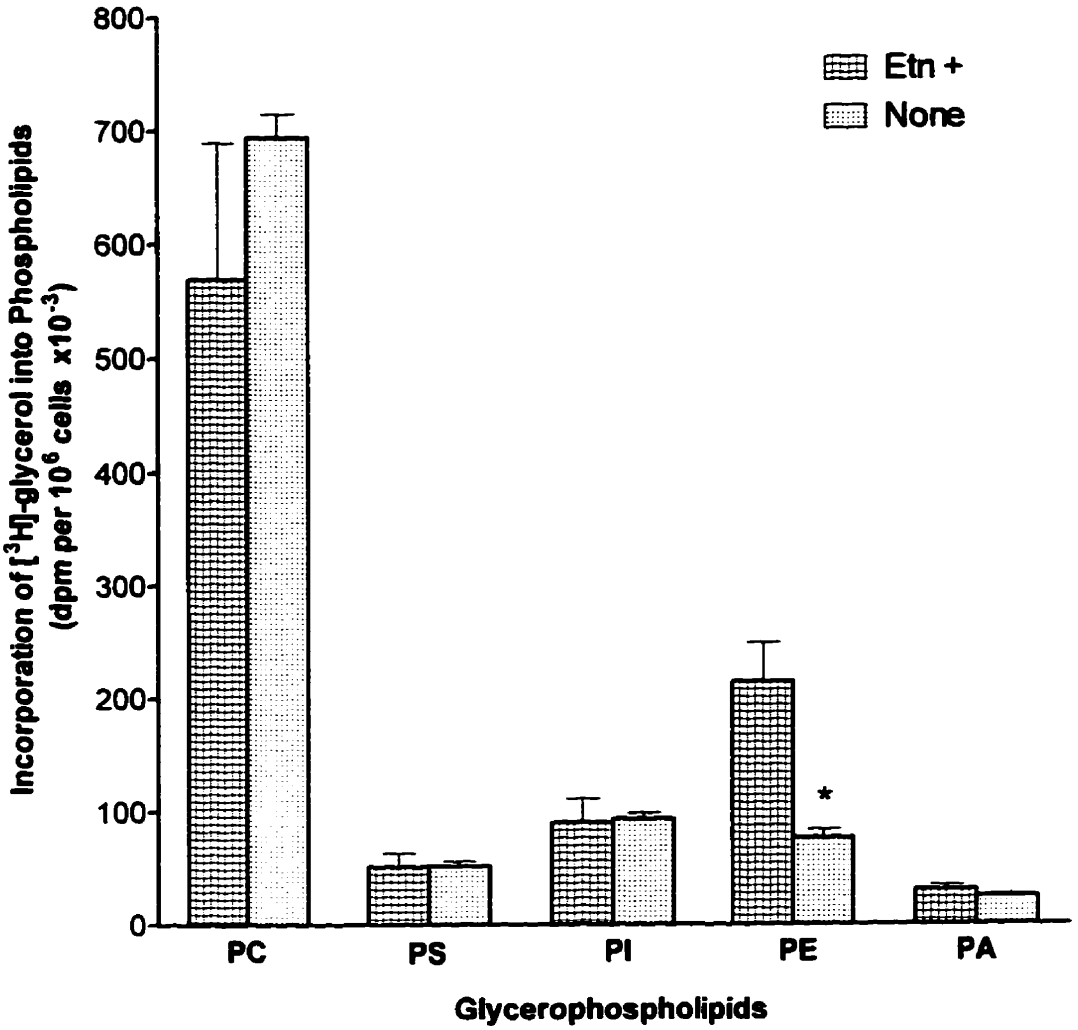


Figure 17. Incorporation of [^{3}H]-Glycerol into Phospholipids in Mammary Epithelial Cells. Cells growing in Etn-deficient media for 3-days were made quiescent by incubating in basal media for 24-hrs. Subsequently, cells were stimulated with growth media with or without Etn, and also in the presence of [^{3}H]-glycerol. After washing, lipids were extracted and the radioactivity incorporated determined as described in the methods. Results are the mean ± S.D. of a single experiment performed in duplicate. The experiment was repeated once with similar results.

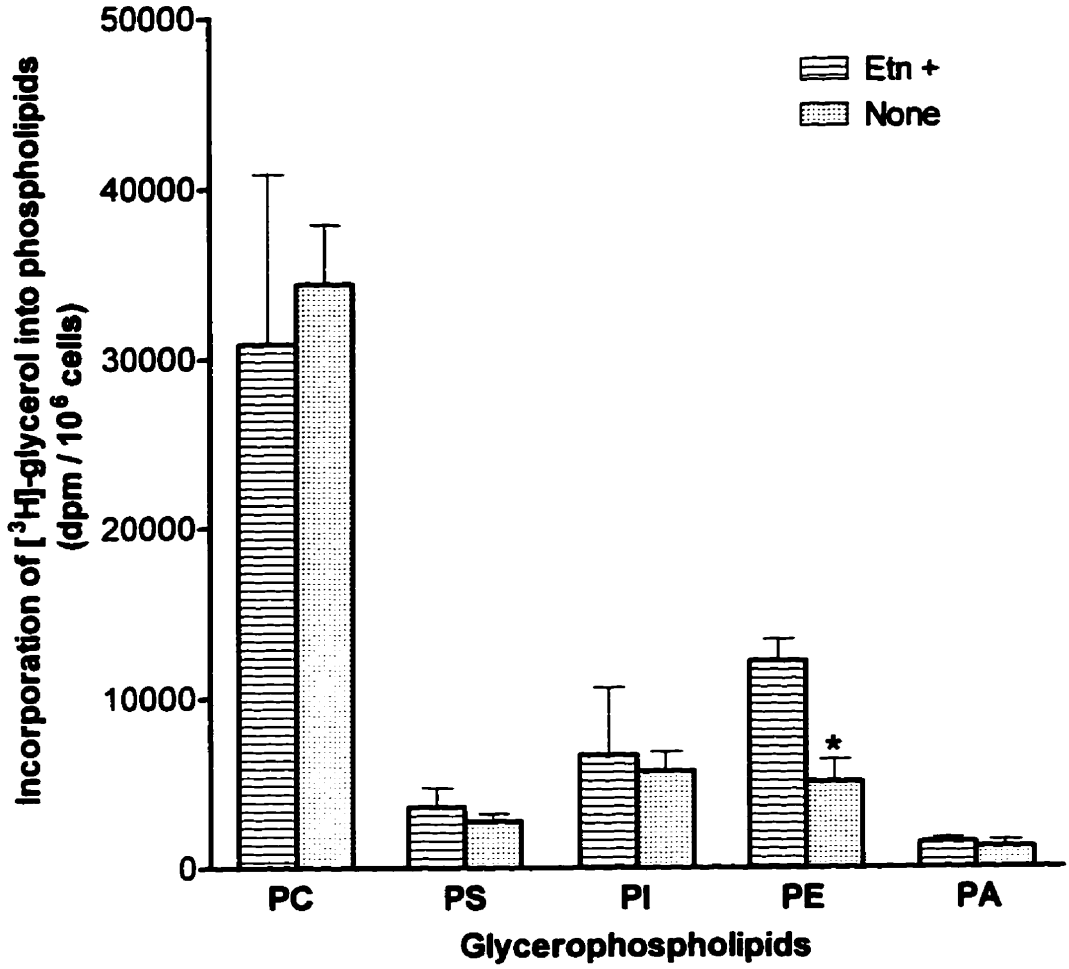


Figure 18. Incorporation of [^{3}H]-Glycerol into Phospholipids in Keratinocytes.

Experiments were performed as described in the legend to Figure 17. Results are the mean ± S.D. of a single experiment performed in duplicate. *P < 0.05

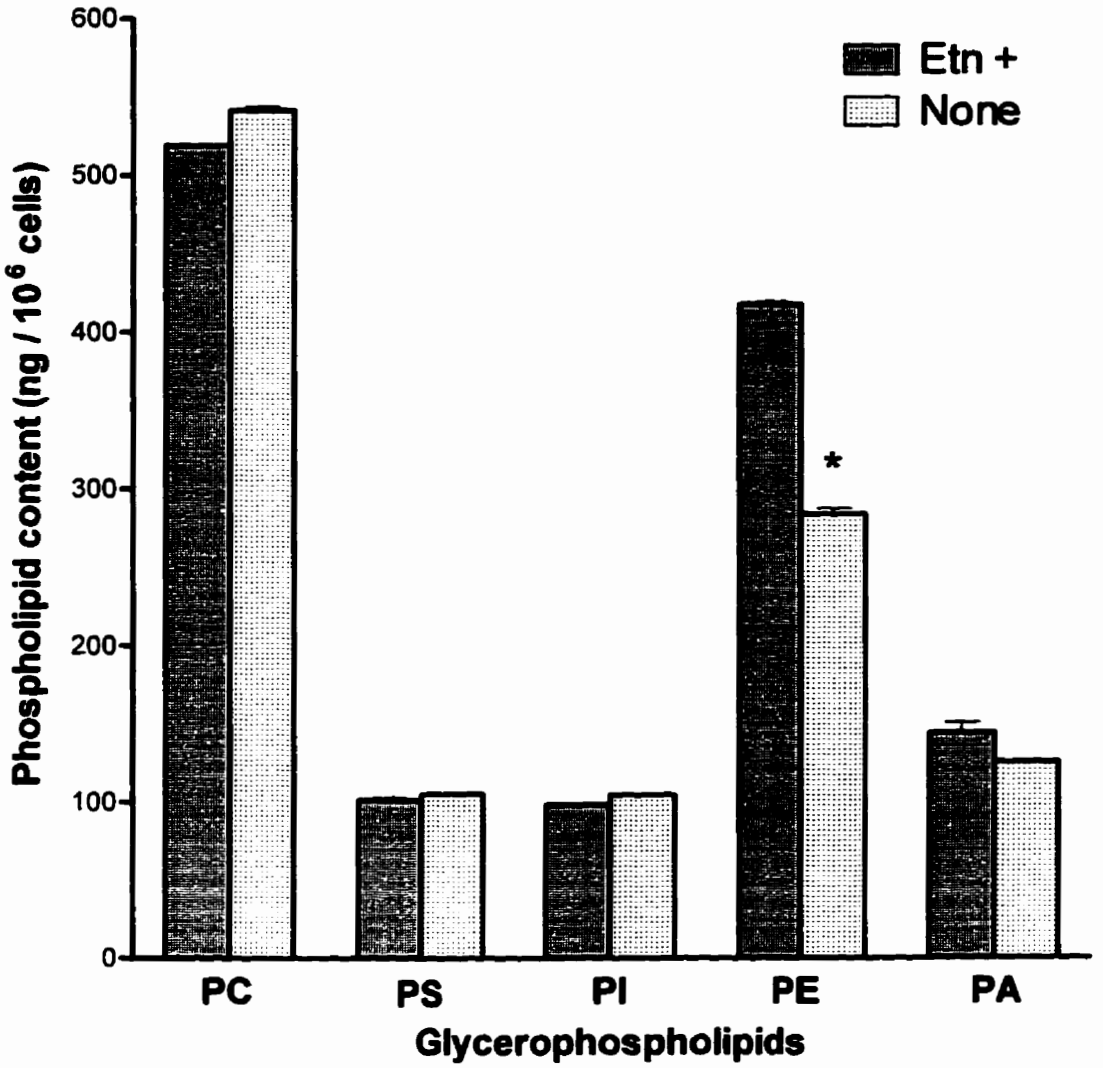


Figure 19. Effect of Ethanolamine on Membrane Phospholipid Content in Keratinocytes. Quiescent cells were stimulated with growth media in the presence or absence of Etn for 48-hrs. Extraction and quantitation of phospholipids was performed as described in the methods. Phosphorus was determined using the malachite green method. Results represent the mean ±S.D. of a single experiment performed in triplicate. In some instances error bars are too small to be seen. *P < 0.05

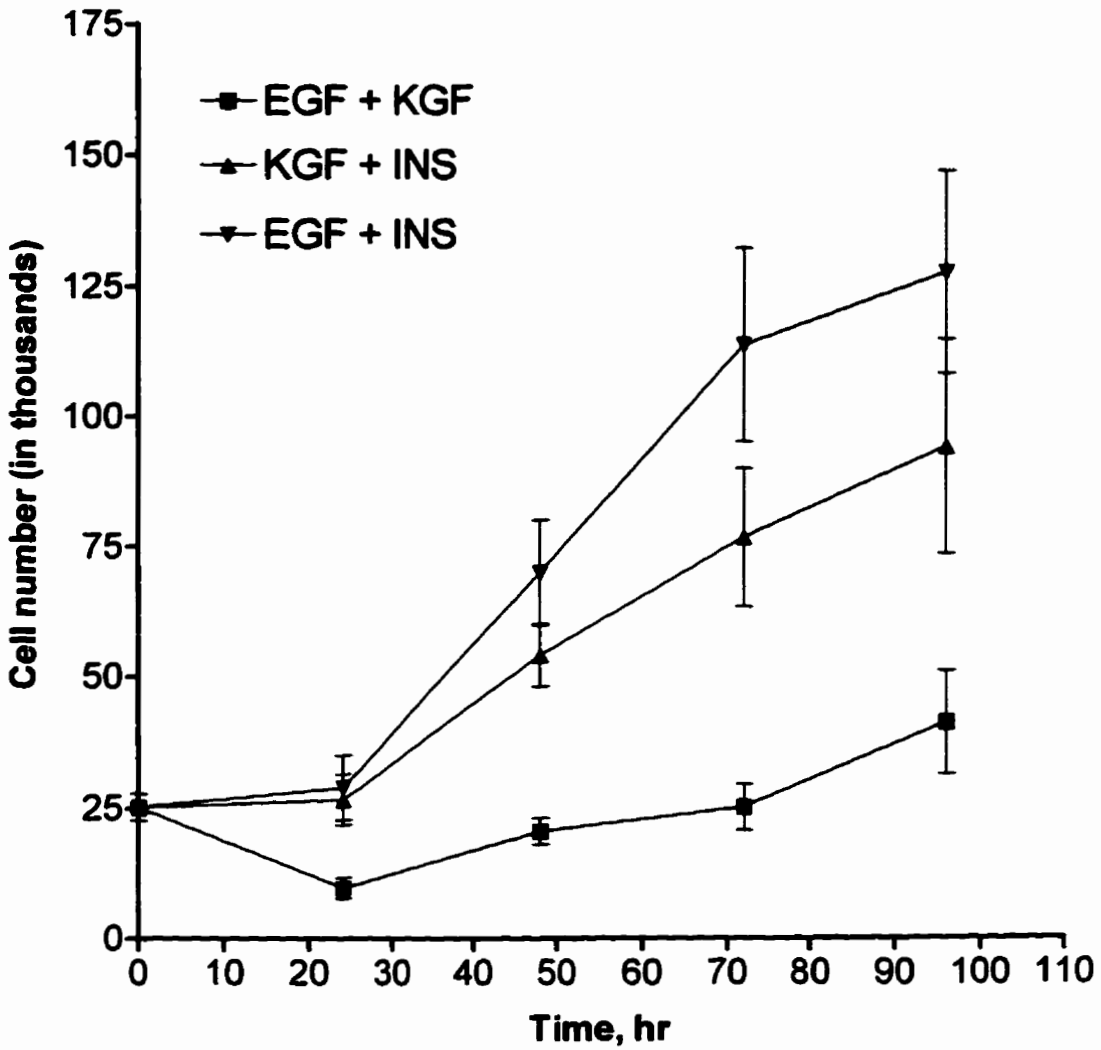


Figure 20. Response of Keratinocytes to Growth Factor Stimulation. The growth media of cells was replaced (Day 0) with one containing combinations of growth factors and incubated for 5-days. The increase in cell number was determined at 24-hr intervals as described in the methods. The results are the mean of a single experiment with six determinations. Standard deviations were less than 20%.

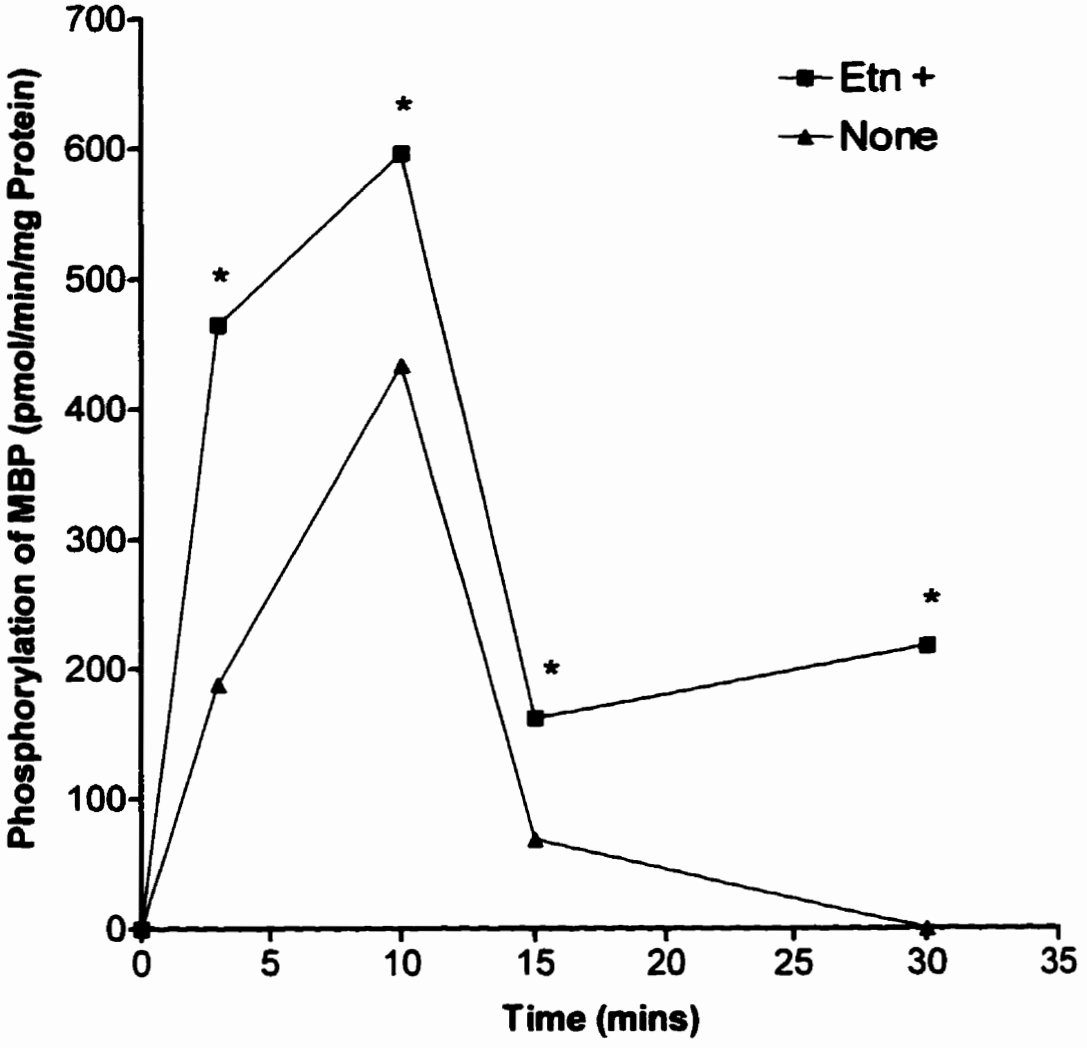


Figure 21. Effect of Ethanolamine on Activation of MAP Kinase in Mammary Epithelial Cells. Cells were incubated in defined media with or without Etn for 48-hrs. Quiescent cells were stimulated with 5 μg/ml insulin for various periods. MAP kinase activity was determined as described in the methods. Results are from a single experiment. Values are the mean of three determinations. This experiment was repeated twice with reproducible results. *$P < 0.05$

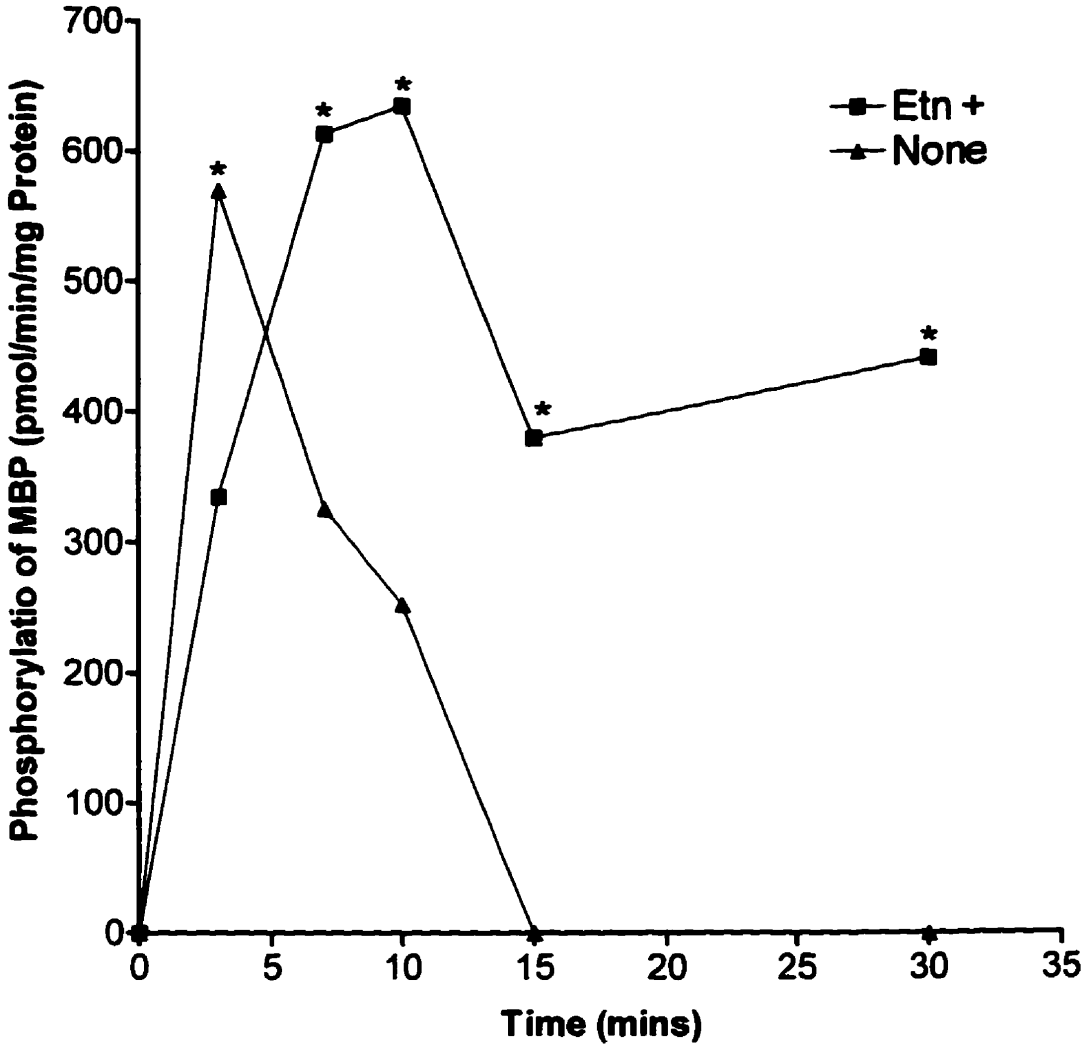


Figure 22. Effect of Ethanolamine on the Activation of MAP Kinase in Keratinocytes. Experiments were performed as described in the legend to Figure 21. Values are the mean of three determinations. *P < 0.05

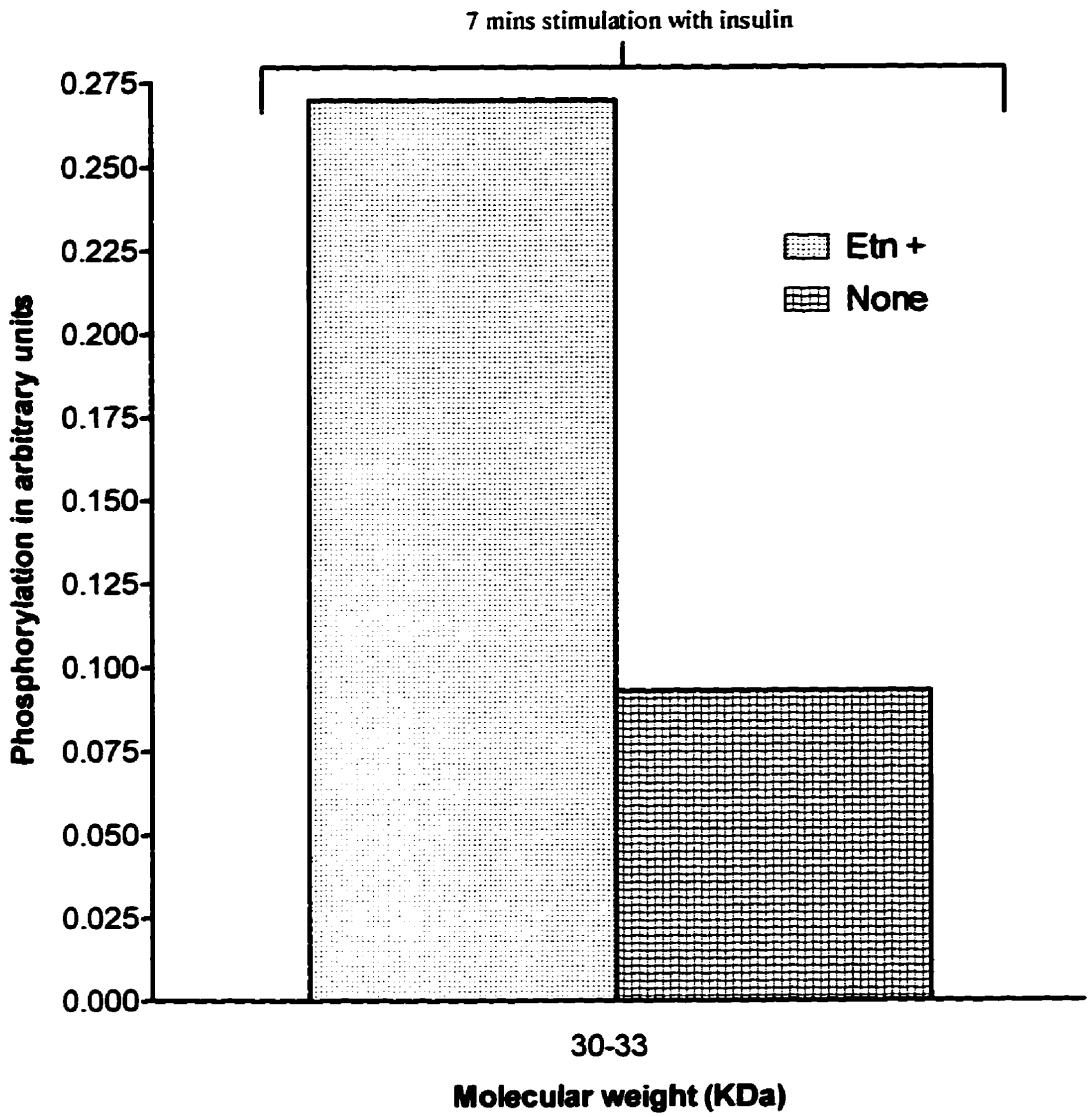


Figure 23. Effect of Ethanolamine on Tyrosine Phosphorylated Proteins in Mammary Epithelial Cells. Experiments were conducted as described in the legend to Figure 21. Cytosol was obtained from control and Etn-deficient cells and tyrosine phosphorylated proteins immunoprecipitated with mouse anti-PY20 mAb. Proteins were visualized by staining with coomassie blue after electrophoresis on 10% SDS-PAGE. Immunoblots were quantitated by densitometric analysis.

6. DISCUSSION

The effects of Etn on cell proliferation was studied in two Etn-requiring human cell lines, keratinocytes and mammary epithelial cells. Results obtained show that Etn is mitogenic for quiescent keratinocytes and HME cells. In the absence of Etn in the growth media (a) cell proliferation and DNA synthesis is inhibited, (b) normal progression through the cell cycle is impaired with the resultant accumulation of cells in G0/G1 phase of the cell cycle, (c) membrane phospholipid composition becomes altered with a decrease in synthesis and cellular PE content, and (d) activation of MAP kinase is inhibited.

The mechanism by which Etn regulates cell growth is not known. Most of the pioneering studies on the Etn requirement of cells was carried out in rat cell lines [Kano-Sueoka, 1981]. Although useful information was provided using rat cells, differences between cells of different species makes it necessary to obtain accurate information that reflects the cellular requirements of cells in tissues of human origin. Also, the masking effect of residual Etn in serum used to supplement the media cannot be ruled out. Thus in our studies, a radically different approach was employed; that is, human cell lines that grow optimally in serum free media were used. Our results showed that in these human cell lines Etn promoted cell proliferation. The proliferative rate of these cells varied with the Etn concentration in a dose-dependent manner. Stimulatory effects were observed at concentrations as low as 10^{-6} M. These results are consistent with observations by Kano-Sueoka and King (1987). The Etn requirement is a phenomenon that is observed in normal and non-transformed epithelial cells at physiological concentrations of Etn found in the

serum and is thus a physiological phenomenon [Kano-Sueoka and King, 1987]. Thus, keratinocytes and human mammary epithelial cells are good cell models for the study of Etn metabolism.

Our results have demonstrated that contrary to the report by Kiss *et al.* (1996), Etn is a far more potent mitogen than its analogues since the Etn requirement of cells could not be replaced by DME or MME. In our opinion fibroblasts may not be the most appropriate cell model to investigate the Etn requirement of cells in view of the high (mM) concentrations required to generate any proliferative responses as opposed to the lower (μM) doses required for epithelial cells.

We have clearly demonstrated that Etn deficiency resulted in the accumulation of cells in G0/G1 phase of the cell cycle similar to the effect of choline deficiency in C3H/10T1/2 fibroblasts [Terce *et al.*, 1994]. Phospholipid synthesis occurs in all phases of the cell cycle with accumulation in the S-phase [Jakowski, 1994] hence the G1/G0 blockage was unexpected. Provision of Etn in the growth media enabled Etn-deficient cells to progress into the S-phase albeit at a slower rate compared to Etn-sufficient cells. The incorporation of [^{3}H]-thymidine into DNA in Etn-deficient cells was also markedly reduced. These results suggested that the cells were not responding to growth factors in the medium in the absence of Etn. Since duplication of DNA is necessary for progression through the cell cycle [Cornell *et al.*, 1977], it will be expected that inhibition of DNA synthesis as seen in Etn-deficient cells may inhibit cell division. Also, the observation that Etn deficiency inhibited the stimulatory effects of insulin and EGF on DNA synthesis suggests that perturbations in signaling via their respective receptors may be involved in the mechanisms by which Etn

inhibits cell proliferation.

From our results, a correlation can be established between Etn availability, PE synthesis and cell proliferation. Our studies showed that there was a relatively linear incorporation of [^{3}H]-Etn into water soluble metabolites and into PE in Etn-deficient cells. The incorporation of [^{3}H]-glycerol into PE in Etn-deficient cells was significantly reduced. Decreased PE synthesis has also been noted in rat 64-24 mammary carcinoma cells [Kano-Sueoka and Errick, 1981]. Since PE is a major phospholipid of mammalian membranes, it can be envisaged that the inability to synthesize PE would ultimately lead to an inability of the cells to produce sufficient membranes to permit cell division. What are the characteristics of PE that could allow it to fulfill the postulated role in cell proliferation? PE unlike PC has been postulated to be involved in forming non-lamellar phases in membranes which may be important in accommodating the integral proteins in the lipid bilayer [Stier *et al.*, 1978]. PE is also a major source of polyunsaturated fatty acids in most cells [Ansell and Spanner, 1982]. This distribution may be important in cell proliferation since the pharmacological inhibition of the transfer of arachidonic acid from PC to PE in HL60 cells which resulted in the accumulation of fatty acid into PC resulted in apoptosis [Surette *et al.*, 1996]. The localization of PE in the cytoplasmic half of the lipid bilayer may play a key role in cell proliferation. Recent reports have suggested that the transduction of signals from a large number of extracellular growth factors such as insulin and epidermal growth factor requires the translocation and association of cytoplasmic signaling proteins with the membrane [Cantley *et al.*, 1991; Pouyssegur and Seumen, 1992]. The observed increase in synthesis of PE in Etn-sufficient cells dispels the notion that cell proliferation is unrelated

to increased phospholipid synthesis. In addition, the fact that membrane phospholipid composition is altered in Etn-deficient cells suggests that the availability of PE may be necessary for maintaining membrane homeostasis and cell growth.

The asymmetric localization of PE in the plasma membrane may also be important in that PE can serve as a substrate for intracellular phospholipases to generate lipid signal mediators such as DAG, arachidonate and phosphatidic acid. Hydrolysis of PE by phospholipase C (PLC) to generate DAG occurs in many cells [Exton, 1994]. One function of DAG is to activate PKC [Berridge, 1993] that in turn can phosphorylate and activate protein kinases that are components of phosphorylation cascades, e.g. Raf-1, $MAPK^{p42/p44}$, and S6 kinase $^{p70/p90rsk}$ [Exton, 1994; Azzi et al., 1992; Erikson, 1991]. MAP kinase when activated can phosphorylate a number of transcription factors to initiate gene expression. It can therefore be envisioned that decreased synthesis of PE could deprive cells of DAG and hence affect the transduction of growth factor signals. In fact, it has been demonstrated that DAG accumulates in Ha-ras transformed NIH 3T3 cells with a sustained activation of PKC activity. Accumulation of DAG was attributed to an increase in P-Etn and phosphocholine due to the action of a phospholipase C on PE and PC respectively [Wolfman and Macara, 1987; Lacal *et al.*, 1987; Exton, 1994]. PE together with PC and PI are major sources of arachidonic acid (AA) and its eicosanoid metabolites. These can be generated by agonist activation of phospholipase A_2 in cells [Exton, 1990]. AA together with other unsaturated long chain fatty acids can activate isozymes of PKC [Murakami and Routtenberg, 1985; Sekiguchi *et al.*, 1987; Exton, 1994] which as mentioned above can activate the Raf-1 protein.

To test our hypothesis that an altered membrane phospholipid composition induced by Etn deficiency interferes with the interaction of proteins involved in the transduction of growth factor signals, the activation of MAP kinase was determined in Etn-sufficient and -deficient cells. MAP kinase was used as an indicator of the transmission of growth factor signals since it serves to integrate signals from a variety of growth factors [Marshall 1995]. The activation of the MAP kinase pathway involves the translocation of cytosolic molecules to the membrane [Cobb and Goldsmith, 1995]. We have clearly demonstrated that the activation of MAP kinase in Etn-deficient mammary epithelial cells and keratinocytes was considerably reduced and this correlated with inhibition of cell proliferation. This suggests that the lack of Etn perturbs the molecular interactions that are required in transmitting growth factor signals to the interior of the cell thus inhibiting the activation of the kinase cascade. Differences in the levels of tyrosine phosphorylated proteins in Etn-sufficient and Etn-deficient cells was also observed. This is not surprising since transduction of growth factor signals involves an elaborate network or cascade in which various protein kinases are activated by phosphorylation on tyrosine residues. The effect of Etn deficiency on MAP kinase activity also suggests that the lesion responsible for this growth inhibition may be upstream of MAP kinase. Future studies should examine whether the effect of Etn deficiency on MAP kinase activation is restricted to the insulin pathway or is a general phenomena involving other growth factors such as EGF and KGF in Etn-requiring cells. Since the magnitude and duration of activation of MAP kinase is important in determining the capability of cells to proliferate [Marshall, 1995], we believe these results are the first to provide a clear-cut link between the inhibition of a pathway involved in cell proliferation and

Etn deficiency.

7. CONCLUSION

Results from our studies substantiates the hypothesis that a deficiency of PE perturbs membrane phospholipid composition to the extent that transduction of growth factor signals into cells is inhibited. This conclusion is supported by evidence that cell proliferation, DNA synthesis, progression through the cell cycle, synthesis of PE and activation of MAP kinase in Etn deficient cells is inhibited.

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