Vitamin E enhances the acylation of 1-*O*-alkyl-*sn*-glycero-3-phosphocholine in human endothelial cells

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1-*O*-Alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC) is the precursor of platelet-activating factor. It is formed via the CoA-independent transacylase reaction, which transfers the polyenoyl acyl group from the *sn*-2 position of a diacyl phospholipid to the *sn*-2 position of 1-*O*-alkyl-*sn*-glycero-3-phosphocholine (alkyl-GPC). We have reported previously that vitamin E alters phospholipid turnover in the endothelial cells by increasing arachidonic acid release and prostacyclin synthesis. In the present study, the role of vitamin E in the formation of alkylacyl-GPC was investigated. Incubation of endothelial cells with vitamin E resulted in an increase in the formation of [^{3}H]alkylacyl-GPC from [^{3}H]alkyl-GPC. The effect of vitamin E was dose-dependent at concentrations below 23 μM. However, vitamin E did not have a direct effect on the transacylase activity. When endothelial cells were incubated with vitamin E, the CoA-independent transacylase activity in the cell homogenate was found to be enhanced. Kinetic analysis of the transacylase activity in the pre-incubated cells showed that the enhancement of enzyme activity was at the enzyme–substrate level. When endothelial cells were incubated with vitamin E analogues (Trolox, tocol and tocopherol acetate), only limited enhancement of the transacylation process was detected. It is clear that vitamin E enhanced the synthesis of alkylacyl-GPC from alkyl-GPC in a very specific manner by an indirect stimulation of the CoA-independent transacylase activity. The regulation by vitamin E of the formation of alkylacyl-GPC may mediate the transfer of arachidonate from the diacyl phospholipid pool into the ether-linked phospholipid pool.

INTRODUCTION

1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine (alkylacyl-GPC), one of the subclasses of diradylphosphatidylcholine, is the precursor of two types of potent lipid mediator: platelet-activating factor (PAF; 1-*O*-alkyl-2-acetyl-*sn*-glycero-3-phosphocholine) and eicosanoids [1–3]. The syntheses of these two lipid mediators are now known to be coupled. 1-*O*-Alkyl-2-arachidonoyl-GPC is initially hydrolysed by phospholipase A_2 to yield alkyl-GPC and arachidonic acid. Alkyl-GPC is converted to PAF by acetyltransferase, while arachidonic acid is converted to leukotrienes by lipoxygenase or to prostaglandins by cyclo-oxygenase. Subsequent to the mediation of the cellular event, PAF is inactivated by enzymic conversion into alkyl-GPC. Alkyl-GPC may be acylated back to alkylacyl-GPC, which is the PAF precursor, by a specific acylation process. This acylation reaction is catalysed by a CoA-independent transacylase [4–6], which is distinct from the CoA-dependent acyltransferase that acylates acyl-GPC to diacyl-GPC [7–9]. The CoA-independent transacylase has been shown to selectively transfer polyunsaturated fatty acids, especially arachidonic acid, from diacyl phospholipids to ether-linked lysophospholipids [4–6,8–13].

The conversion of alkyl-GPC back to alkylacyl-GPC is one of the most important steps in the overall conservation of the PAF precursor pool. In addition, this acylation process would serve to prevent the accumulation of lysophospholipids, which are cytolytic at high concentrations [14]. The acylation of alkyl-GPC is very active in neutrophils [11] and platelets [5,6] but is less active in other cell types, including endothelial cells [15–18]. It is not clear whether the lower acylation rate found in endothelial cells plays a role in the regulation of the alkyl-GPC content in these cells.

It has been shown that vitamin E, a lipid-soluble chain-breaking antioxidant, can potentiate prostacyclin (PGI_2) synthesis in endothelial cells [19–21]. We have reported previously that vitamin E enhances the release of arachidonic acid and the subsequent synthesis of PGI_2 [19,22]. The redistribution of arachidonic acid may activate an arachidonoyl-specific phospholipase A_2, which hydrolyses both alkylarachidonoyl-GPC and acylarachidonoyl-GPC [23,24]. Vitamin E may also affect the redistribution of arachidonic acid between phospholipid pools, thus changing the levels of eicosanoid and PAF formation during cell activation. In the present study, the effect of vitamin E on the acylation of alkyl-GPC in endothelial cells was determined. Our study shows that the conversion of alkyl-GPC to alkylacyl-GPC in endothelial cells is potentiated by incubation with a physiological concentration of vitamin E.

MATERIALS AND METHODS

Materials

1-*O*-[1,2-^{3}H]Alkyl-*sn*-glycero-3-phosphocholine (52 Ci/mmol) was from New England Nuclear (Lachine, PQ, Canada). Arachidonyl-CoA, linoleoyl-CoA, heparin, collagenase type IV, gentamycin sulphate, all rac-α-tocopherol acetate and all standard lipids were from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Medium 199, penicillin G sodium (10000 units/ml), streptomycin sulphate (10000 μg/ml), Fungizone (250 μg of amphotericin B and 205 μg of deoxycholate/ml), trypsin-EDTA, heat-inactivated fetal bovine serum and all tissue-culture plasticware were from

Abbreviations used: acyl-GPC, 1-acyl-*sn*-glycero-3-phosphocholine; alkylacyl-GPC, 1-*O*-alkyl-2-acyl-*sn*-glycero-3-phosphocholine; alkyl-GPC, 1-*O*-alkyl-*sn*-glycero-3-phosphorylcholine; HBS, Hepes-buffered solution; PAF, platelet activating factor; PGI_2, prostacyclin; α-tocopherol, 2,5,7,8-tetramethyl-2-(4′,8′,12′-trimethyltridecyl)-6-chromanol; Trolox, 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid.

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Gibco (Burlington, Ontario, Canada). Endothelial cell growth supplement was from Collaborative Research (Bedford, MA, U.S.A.). *RRR*-α-Tocopherol was donated by the Vitamin E Research Information Services (La Grange, IL, U.S.A.); tocopherol analogues were gifts from Eisai Co. Ltd. (Tokyo, Japan); Trolox was from Aldrich Inc. (Milwaukee, WI, U.S.A.). Thin layer plates (silica gel 60A) were from Whatman. All solvents used were h.p.l.c. grade and were purchased from BDH Chemicals (Toronto, Ontario, Canada).

Cell culture

Endothelial cells were isolated from human umbilical veins according to the method of Jaffe [25] and cultured as previously described [26]. Briefly, human umbilical veins were enzymically digested with 0.2% collagenase, and effluents were plated on gelatin-precoated tissue-culture dishes. Cells were incubated at 37 °C in a 5% CO_2/95% air incubator in medium 199 containing fetal bovine serum (10%, v/v), endothelial cell growth supplement (30 μg/ml), heparin (90 μg/ml), Hepes (25 mM), gentamicin sulphate (40 μg/ml), penicillin G sodium (100 units/ml), streptomycin sulphate (100 μg/ml) and Fungizone (amphotericin B, 2.5 μg/ml). Endothelial cells were identified by confirming the expression of von Willebrand factor antigen, as previously reported [25,26]. Cells from passages 1–3 were used in these experiments.

Tocopherol enrichment of endothelial cells

Enrichment of cells with vitamin E or its analogues was carried out as described previously [26]. Briefly, vitamin E or one of its analogues was first dissolved in dimethyl sulphoxide (DMSO) (final concentration not more than 0.2%), and an aliquot was added into fetal bovine serum which was then incubated at 37 °C for 10 min. Medium 199 and antibiotic/antimycotic solution were added into the vitamin E-enriched serum, and the culture medium was further incubated for 10 min at 37 °C before it was added to cell monolayers. In these studies, cells were routinely incubated for 4 h with medium enriched with vitamin E or its analogues.

Alkylacyl-GPC formation in endothelial cells

To study the effect of vitamin E on the formation of alkylacyl-GPC, confluent endothelial monolayers in 35 mm dishes were first incubated with vitamin E (0–92 μM) for 4 h in culture medium containing 10% fetal bovine serum. Cells were rinsed three times with 10 mM Hepes-buffered solution (HBS) followed by the addition of [^{3}H]alkyl-GPC in serum-free medium for 1.5 h. After washing three times with HBS, cells were scraped into 1 ml of ice-cold methanol/HCl (50:1, v/v) plus 1 ml of HBS. Cellular lipids were extracted by the method of Bligh and Dyer [27] in the presence of 20 μg of alkylacyl-GPC and alkyl-GPC as carrier. Alkyl-GPC ($R_F = 0.28$) and alkylacyl-GPC ($R_F = 0.67$) were separated by t.l.c. in a solvent system consisting of chloroform/methanol/acetic acid/water (50:30:8:5, by vol.). After exposure to iodine vapour, the spots corresponding to alkyl-GPC and alkylacyl-GPC were scraped into scintillation vials and radioactivity was determined by liquid-scintillation spectroscopy.

Acyl-CoA acyltransferase and CoA-independent transacylase assays

The activities of these two enzymes were determined in cell homogenates by measuring the production of [^{3}H]alkylacyl-GPC from [^{3}H]alkyl-GPC in the presence or absence of acyl-CoA. After being enriched or not with vitamin E for 4 h, cells from 100 mm dishes were scraped off and washed three times with ice-cold HBS. The cell pellet was resuspended in ice-cold Tris/HCl buffer (75 mM), pH 8.5, and 1 mM EDTA, and the cell suspension was sonicated with an ultrasonic cell disruptor for 3 × 30 s each at 70% output. Acyl-CoA acyltransferase activity was determined in an assay mixture containing 0.2–0.3 μCi of [^{3}H]alkyl-GPC, 40 μM of linoleoyl-CoA or arachidonoyl-CoA, 60–160 μg of homogenate protein and 1 mM EDTA, in a final volume of 0.35 ml of 75 mM Tris/HCl, pH 8.5. The reaction was started by the addition of [^{3}H]alkyl-GPC, and the mixture was incubated for 15 min at 37 °C. The reaction was terminated by the addition of 0.5 ml of methanol/acetic acid (9:1, v/v). Total lipids were extracted by chloroform/methanol; alkylacyl-GPC was separated by t.l.c. and radioactivity was quantified as described above. Determination of transacylase activity was similar to the method for acyl-CoA acyltransferase, except that it was conducted in the absence of acyl-CoA. Protein was determined by the method of Lowry et al. [28] using BSA as standard.

RESULTS

Effect of vitamin E on the formation of alkylacyl-GPC from alkyl-GPC

Endothelial cells were incubated with [^{3}H]alkyl-GPC, and total uptake of radioactivity and the labelling of alkylacyl-GPC were determined. Since the confluence and metabolic state of the cells were not identical from one cellular preparation to another, the uptake of radioactivity and the formation of alkylacyl-GPC were also different between cellular preparations. In order to obtain consistent results, cells from the same preparation were used for a given set of experiments. The validity of the findings obtained

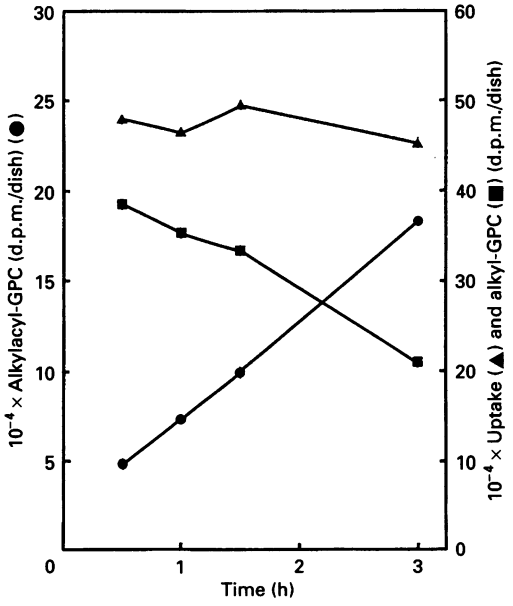

Figure 1 Time course of the formation of alkylacyl-GPC

Cell monolayers were incubated with 0.67 μCi of [^{3}H]alkyl-GPC (17 nM) for the indicated times. Lipid analysis and cellular radioactivity determination were performed as described in the Materials and methods section. The radioactivity associated with total uptake (▲), alkyl-GPC (■) or alkylacyl-GPC (●) is expressed as d.p.m./dish. Each point is the mean value obtained from three separate dishes. The results were confirmed in two additional sets of experiments.

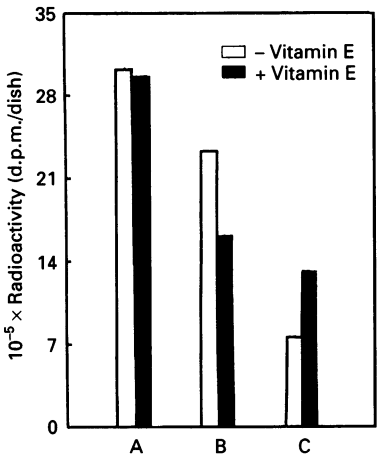

Figure 2 Effect of vitamin E on the total cellular radioactivity and the synthesis of alkylacyl-GPC from alkyl-GPC

Cell monolayers were incubated with and without vitamin E (23 μM) for 4 h followed by labelling with 4.8 μCi of [^{3}H]alkyl-GPC (50 nM) for 1.5 h. Lipid analysis and cellular radioactivity determination were performed as described in the Materials and methods section. The mean values for total radioactivity (A), alkyl-GPC (B) and alkylacyl-GPC (C) were obtained from three separate dishes. The results were confirmed in six additional sets of experiment.

Table 1 Effect of vitamin E on alkylacyl-GPC formation after different incubation periods with alkyl-GPC

Cell monolayers were incubated with or without vitamin E (23 μM) for 4 h followed by labelling with 0.27 μCi of [^{3}H]alkyl-GPC (7 nM) for the additional indicated times. Lipid analysis and cellular radioactivity were determined as described in the Materials and methods section. The study was repeated four times and the results of a typical study are depicted. Each value is the mean of three dishes.

Time (h)	10^{-4} × Alkylacyl-GPC (d.p.m./dish)	
	– Vitamin E	+ Vitamin E
0.5	4.84	8.44
1.0	7.26	11.41
1.5	10.04	13.61

from one set of experiments was confirmed by reproducing the result in a qualitative manner in at least two consecutive sets of experiments. The number of experimental sets done for each study is provided in the Figure and Table legends.

The total uptake of radioactivity by the endothelial cells was found to reach a maximum by 30 min and then remained unchanged up to 3 h of incubation (Figure 1). The formation of [^{3}H]alkylacyl-GPC was linear with incubation time, with a comparable decrease in [^{3}H]alkyl-GPC. When endothelial cells from vitamin E-treated (23 μM, 4 h) and non-treated groups were incubated with [^{3}H]alkyl-GPC for 1.5 h, there was no significant difference in the total uptake of radioactivity (Figure 2). However, vitamin E caused an increase in the formation of alkylacyl-GPC, with a corresponding decrease in the labelling of alkyl-GPC. It is clear that the acylation of alkyl-GPC in endothelial cells was enhanced by vitamin E.

The effect of vitamin E on the time course of [^{3}H]alkylacyl-GPC formation was examined. Endothelial cells were incubated with 23 μM vitamin E for 4 h. [^{3}H]Alkyl-GPC was added and the

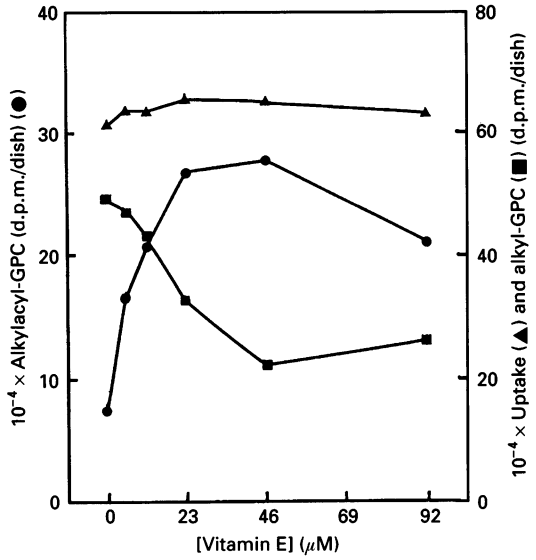

Figure 3 Dose-dependent stimulation by vitamin E of the synthesis of alkylacyl-GPC from alkyl-GPC

Cell monolayers were incubated with the indicated concentrations of vitamin E for 4 h followed by labelling with 0.67 μCi of [^{3}H]alkyl-GPC (17 nM) for 1.5 h. The radioactivity associated with total uptake (▲), alkyl-GPC (■) and alkylacyl-GPC (●) is expressed as d.p.m./dish. Each point is the mean obtained from three separate dishes. The results were confirmed in two additional sets of experiment.

cells were incubated for the prescribed time periods. Table 1 shows that vitamin E significantly enhanced the formation of [^{3}H]alkylacyl-GPC at all time points. The effect of vitamin E concentration on the formation of labelled alkylacyl-GPC was also studied. Endothelial cells were incubated with 6–92 μM vitamin E for 4 h prior to labelling with alkyl-GPC for 1.5 h. Vitamin E had no effect on total uptake of radioactivity. The results in Figure 3 show that vitamin E potentiated the formation of labelled alkylacyl-GPC in a dose-dependent manner at low concentrations (< 23 μM), with a concomitant decrease in labelled alkyl-GPC. The maximum rate of alkylacyl-GPC formation was achieved at 23 μM vitamin E, and no further increase was detected at higher vitamin E concentrations. It appears that the enhancement of alkylacyl-GPC formation was not mediated by a differential uptake of alkyl-GPC. It is interesting to note that, at 92 μM vitamin E, the activation of alkylacyl-GPC synthesis was less than that observed at lower concentrations (23 and 46 μM) of vitamin E. The reason for the decrease in enhancement of alkylacyl-GPC synthesis at a high vitamin E concentration is not known.

Effect of vitamin E on the enzyme responsible for the acylation process

The activities of acyl-CoA acyltransferase and the CoA-independent transacylase activity for alkyl-GPC in the cell homogenate were determined. The addition of vitamin E to both assay mixtures did not elicit any changes in enzyme activity. Cells pretreated or not with vitamin E were homogenized, and the acyltransferase and transacylase activities in the homogenate were determined in the presence and absence of acyl-CoA. As depicted in Table 2, vitamin E caused a 2-fold increase in the formation of alkylacyl-GPC, but the acylation of alkyl-GPC was independent of added acyl-CoA. This is not surprising, since alkyl-GPC has been shown to be quickly acylated by a CoA-

Table 2 Effects of vitamin E on acyltransferase activities in cell homogenates

Cell monolayers were incubated with or without vitamin E (23 μM) for 4 h. The acyltransferase activities of homogenates containing approx. 160 μg of protein were determined in assay mixture containing 0.25 μCi of [^{3}H]alkyl-GPC (50 nM) in the presence or absence of either 40 μM linoleoyl-CoA (18:2) or 40 μM arachidonyl-CoA (20:4) after 15 min of incubation at 37 °C. The study was repeated three times and the results of a typical study are depicted. Each value is the mean of four separate determinations.

	Activity (pmol/min per mg)	
Acyl-CoA	−Vitamin E	+Vitamin E
None	1.87	3.10
18:2	1.82	3.08
20:4	1.88	2.80

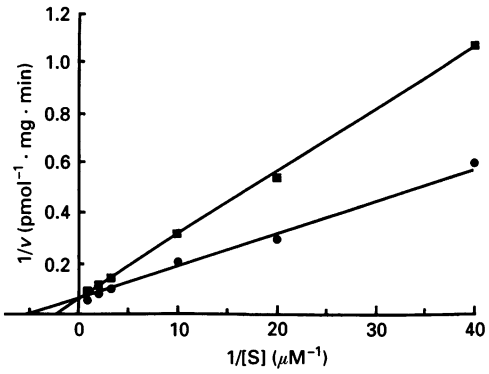

Figure 4 Double-reciprocal plot of transacylase activity from cells pre-incubated with or without vitamin E

Cell monolayers were incubated with (●) or without (■) vitamin E (23 μM) for 4 h. Cell homogenates (approx. 60 μg of protein) were incubated for 5 min at 37 °C with various concentrations of [^{3}H]alkyl-GPC (0.2 μCi). Transacylase activity was determined as described in the Materials and methods section. Each point represents the mean of two separate determinations.

Table 3 Effect of vitamin E and its analogues on alkylacyl-GPC formation

Cell monolayers were incubated with or without vitamin E or its analogues (23 μM) for 4 h followed by labelling with 0.30 μCi of [^{3}H]alkyl-GPC (7.8 nM) for 1.5 h. Lipid analysis and cellular radioactivity were determined as described in the Materials and methods section. The study was repeated three times and the results of a typical study are depicted. Each value is the mean obtained from three dishes.

Analogue	Radioactivity in alkylacyl-GPC (% of control)
None	100
Trolox	112
Tocol	142
α-Tocopherol	200
Tocopherol acetate	106

independent transacylase. This enzyme has the ability to directly transfer arachidonoyl or other polyenoyl groups from a diacyl phospholipid to an ether-linked lysophospholipid, such as alkyl-GPC [4–6,8–13]. The results show that vitamin E has the ability to enhance the CoA-independent transacylase process in an indirect manner.

The mechanism by which vitamin E exerts its effect on transacylase activity was explored. The effect of vitamin E on the CoA-independent transacylase activity was studied at various concentrations of alkyl-GPC. Endothelial cells were pre-treated with vitamin E (23 μM) for 4 h and the homogenate was used to assay for CoA-independent transacylase activity at 25–1000 nM substrate concentrations. A double-reciprocal plot of substrate concentration versus rate of product formation revealed that vitamin E caused the activation of enzyme activity by altering the affinity at the enzyme–substrate level. Vitamin E caused a change in the K_m for alkyl-GPC, but the reaction displayed similar $V_{max.}$ values in the vitamin E-treated and control cells (Figure 4).

Structural specificity of vitamin E for the enhancement of alkylacyl-GPC formation

Since both the hydroxyl group and the anchorage site (phytyl side chain and methyl groups) of vitamin E play a crucial role for its biological activity, the significance of these groups towards the formation of alkylacyl-GPC was examined. Endothelial cells were incubated with vitamin E or its analogues for 4 h, followed by labelling the cells with [^{3}H]alkyl-GPC for 1.5 h. Table 3 shows that the formation of alkylacyl-GPC in the cells was increased 2-fold by vitamin E treatment. Tocol, a vitamin E structural analogue without the methyl groups on the chromanol ring, enhanced alkylacyl-GPC formation by only 42%. Trolox, a vitamin E analogue lacking the phytyl tail, and tocopherol acetate, the hydroxyl group of which was blocked by acetylation, did not exert any significant effect on alkylacyl-GPC formation. Therefore the presence of the free hydroxyl group, the phytyl side chain and the methyl groups in the vitamin E molecule are required for the maximum enhancement of alkylacyl-GPC formation.

DISCUSSION

It is generally accepted that PAF is inactivated by acetylhydrolase to form alkyl-GPC, which is then transacylated to form the PAF precursor molecule. In endothelial cells, agonist stimulation induces the formation of at least two PAF subclasses in which the 1-acyl analogue predominates over the 1-*O*-alkyl analogue [29], because most of the choline phosphoacylglycerol in endothelial cells is the diacyl subclass [30]. Thus PAF degradation will generate both alkyl-GPC and acyl-GPC. The relative amounts of these lyso-PAF subclasses are not know at present. In the present study, we show for the first time that the acylation of alkyl-GPC is enhanced by vitamin E. A similar effect of vitamin E on the acylation of acyl-GPC to diacyl-GPC was also detected in endothelial cells (results not shown). Our findings suggest a new role for this vitamin in the regeneration of PAF precursors in endothelial cells.

It is clear from this study that the activity of the CoA-independent transacylase was stimulated by vitamin E. The stimulation was caused by a change in the affinity of the substrate for the enzyme. However, the enhancement of enzyme activity did not result from a direct action of vitamin E on the enzyme, since the addition of vitamin E to the assay mixture did not cause any enhancement of enzyme activity. One possible explanation is that the stimulation of enzyme activity was caused by the direct action of a vitamin E metabolite(s). Alternatively, it is plausible that the activation of enzyme activity was mediated via a membrane-associated mechanism induced by vitamin E. We have demonstrated in a previous study that vitamin E enhances the release of arachidonic acid from phosphatidylcholine in

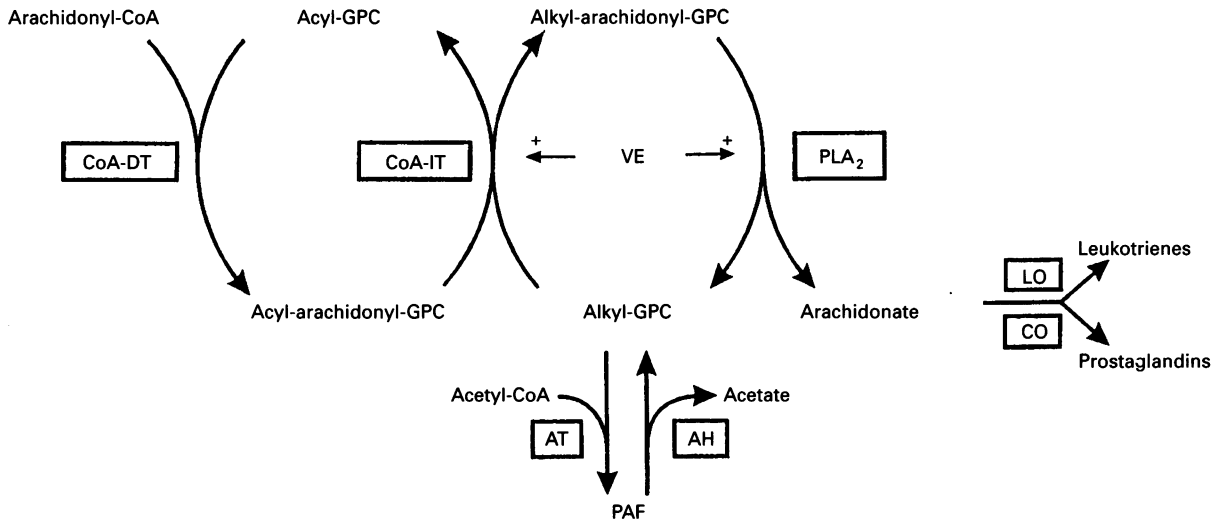

Figure 5 Regulation of choline phosphoglyceride metabolism by vitamin E in human endothelial cells

Abbreviations used in the diagram are: CoA-DT, CoA-dependent acyltransferase; CoA-IT, CoA-independent transacylase; PLA_2, phospholipase A_2; AT, acetyl-CoA transferase; AH, acetylhydrolase; LO, lipoxygenase; CO, cyclo-oxygenase.

endothelial cells [19,22]. The stimulation of the phospholipase A_2 was also found to be mediated via an indirect action of vitamin E. The role of vitamin E in the modulation of alkylacyl-GPC metabolism and its relationship with the metabolism of other choline glycerophospholipids are depicted in Figure 5.

The characteristics of the transacylase identified in the present study are consistent with previous reports in other cells [10,13]. For instance, this enzyme does not require acyl-CoA, ATP or bivalent cations such as Ca^{2+} or Mg^{2+}. It has been shown that the selective transfer of arachidonate by the CoA-independent transacylase from a diacylphospholipid to the ether-linked lysophospholipids is a significant regulatory event which has major implications in pathophysiology [2–6,8–13,23]. This selective transfer results in arachidonate enrichment of the ether phospholipid pool, which is the source of arachidonic acid release for leukotriene synthesis in human neutrophils [2,3]. The selective transfer of arachidonate has also been shown to regulate PAF biosynthesis [23,24]. Therefore the redistribution of arachidonic acid between phospholipid pools in endothelial cells may determine the level of PGI_2 formation. The effective dosage of vitamin E for the stimulation of alkylacyl-GPC formation bears close similarity with our previous reports on the enhancement of arachidonic acid release [22] and PGI_2 synthesis [19] in endothelial cells. It is also clear that the intact vitamin E molecule is required for the regulation of acyl group transfer between the diacyl and the alkylacyl subclasses.

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