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# **VITAMIN E: INVOLVEMENT IN PHOSPHOLIPID METABOLISM AND REGENERATION**

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Thesis submitted to  
the School of Graduate Studies and Research  
in partial fulfilment of the requirements for the degree of  
Doctor of Philosophy

Department of Biochemistry  
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Ottawa, Ontario, Canada  
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Khai T. Tran, Ottawa, Canada, 1993



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## ABSTRACT

Vitamin E is a well known chain-breaking antioxidant that protects membrane from radical-induced damage. Among the naturally occurring form of vitamin E,  $\alpha$ -tocopherol possessed highest biological activity. Our goal is to determine whether vitamin E, especially  $\alpha$ -tocopherol, has any role in the regulation of membrane phospholipid metabolism and whether it can be recycled after being oxidized. The involvement of vitamin E in phospholipid metabolism in human endothelial cells was supported by the findings that vitamin E enhances the acylation processes of cholinephosphoglycerides, that vitamin E regulates the levels of diacylglycerol and phosphatidic acid in response to thrombin, and that vitamin E regulates protein kinase C activity in both resting and phorbol ester-stimulated cells. The mechanisms of vitamin E regeneration were delineated from the study using human platelet as a machinery to induce endogenous vitamin E oxidation and regeneration.

The involvement of vitamin E in the regulation of acylation processes was implicated from studies in which human endothelial cells were preincubated with vitamin E and then labelled with [ $^3$ H]-alkyl-GPC and [ $^{14}$ C]-acyl-GPC.  $\alpha$ -Tocopherol, was found to increase the formation of [ $^3$ H]-alkylacyl-GPC and [ $^{14}$ C]-diacyl-GPC, whereas other vitamin E analogs showed only limited effect. Enzyme characterization revealed that vitamin E enhanced the formation of alkylacyl-GPC by an indirect stimulation of the CoA-independent transacylase, whereas the increased acylation of acyl-GPC by vitamin E occurred at a level other than CoA-dependent acyltransferase. Since cis-unsaturated fatty acids, especially arachidonic acid, are preferentially incorporated and transferred via these acylation pathways, this finding suggests a novel role of vitamin E in the mobilization of arachidonic acid between phospholipid pools.

The regulation of vitamin E on the cellular diacylglycerol and phosphatidic acid levels in thrombin-stimulated endothelial cells was studied by determination of radiolabelled lipid products, by the quantitation of diacylglycerol mass and by the quantitation of enzyme activity.  $\alpha$ -Tocopherol incubation was found to attenuate the transient accumulation of diacylglycerol with a concomitant increase in the formation of phosphatidic acid. This vitamin E effect on diacylglycerol and phosphatidic acid formation was resulted from an activation of diacylglycerol kinase which convert diacylglycerol to phosphatidic acid. Other enzyme pathways contributing to diacylglycerol and phosphatidic acid formation, including phospholipase C, phospholipase D and phosphatidate phosphohydrolase, were unaffected by vitamin E. This finding implicates a role of vitamin E in the signalling processes by regulating the formation of two important second messengers, diacylglycerol and phosphatidic acid.

Vitamin E was also found to modulate membrane protein kinase C (PKC) activity in both resting and phorbol ester (PMA)-stimulated endothelial cells. Incubation of endothelial cells with  $\alpha$ -tocopherol caused an increase in PKC activity in the membrane fraction. In response to PMA stimulation, the increase in PKC activity in the membrane was attenuated by vitamin E. By contrast, duroquinone, an oxyradical-generating substance, had a completely opposite effect on membrane PKC in both resting and PMA-stimulated cells. In addition, the effect of duroquinone on membrane PKC was totally abolished by vitamin E. Both vitamin E and duroquinone had no effect on cytosolic PKC. These results showed that vitamin E regulates resting and PMA-stimulated PKC by at least two different mechanisms. Due to the differential effects of vitamin E and duroquinone on PKC it is therefore suggested that the intracellular levels of antioxidants and oxidants are most likely involved in the regulation of PKC activity in vascular endothelial cells.

The mechanisms of vitamin E regeneration were elucidated from studies using human platelet vitamin E as endogenous substrate.  $\alpha$ -Tocopherol in human platelet homogenates was readily oxidized by arachidonic acid (AA) via the 12-lipoxygenase pathway. This AA-induced oxidized tocopherol can be reduced back to tocopherol by water soluble reductants such as nordihydroguaiaretic acid (NDGA), ascorbate (vitamin C) and reduced glutathione (GSH). Differential analysis revealed that ascorbate and NDGA induced tocopherol regeneration by a non-enzymic mechanism whereas GSH recycled tocopherol via an enzymic pathway. In addition, tocopherol regeneration was negatively correlated with oxidation time induced by AA, suggesting that the intermediate molecule is a short-lived tocopheroxyl radical. This finding provides direct evidence from mass analysis that vitamin E can be regenerated in human cells. This finding also indicates that both membrane and cytosolic antioxidants operate in concert to maximally protect cell membrane from radical induced injury.

Taken together, the above findings demonstrated a definite role of vitamin E in phospholipid metabolism and the ability of vitamin E to be regenerated after being oxidized; this regeneration event may help to increase the efficiency of vitamin E in cell membrane.

**DEDICATED TO**

**MY FATHER - THANH-SON, TRAN**

for the uncompromising principles that guided his life,

**MY MOTHER - KIM-THIEN, TRAN**

for leading her children into intellectual pursuits,

**MY WIFE - KHUE-TU, NGUYEN**

for her magnificent devotion to her family,

**MY SON - MINH-LUAN, TRAN**

for making everything worthwhile,

and

**MY BELOVED BROTHER - TUAN-DAT, TRAN (deceased)**

for helping me to better understand the meaning of life

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## LIST OF ABBREVIATIONS

12-HETE	(12S)-12-hydroxy-5,8,10,14-eicosatetraenoic acid
12-HPETE	(12s)-12-hydroperoxy-5,8,10,14-eicosatetraenoic acid
AA	arachidonic acid
Acyl-GPC	1-acyl-sn-glycero-3-phosphorylcholine
AH	acetylhydrolase
Alkyl-GPC	1-0-alkyl-sn-glycero-3-phosphorylcholine
Alkylacyl-GPC	1-0-alkyl-2-acyl-sn-glycero-3-phosphorylcholine
AT	acetyltransferase
ATP	adenosine triphosphate
BSA	bovine serum albumin
CO	cyclooxygenase
CoA-DT	coenzyme A-dependent acyltransferase
CoA-IT	coenzyme A-independent transacylase
DAG	diacylglycerol
Diacyl-GPC	1,2-diacyl-sn-glycero-3-phosphorylcholine
DMSO	dimethylsulfoxide
EDRF	endothelial derived relaxing factor
EDTA	ethylenediaminetetraacetic acid
EGTA	ethylene glycol-bis( $\beta$ -aminoethyl ether) N,N,N',N'-tetraacetic acid
ETYA	5,8,11,14-eicosatetraenoic acid
FA	fatty acid
GSH	reduced glutathione
HBS	hepes-buffered saline
HBSS	Hank's balanced salt solution
HDL	high-density lipoprotein
HEPES	(N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid])
HPLC	high pressure liquid chromatography
IP	inositol phosphate
IP <sub>2</sub>	inositol bisphosphate
IP <sub>3</sub>	inositol trisphosphate
LDL	low-density lipoprotein
LO	lipoygenase
lysoPC	lysophosphatidylcholine
NDGA	nordihydroguaiaretic acid
PA	phosphatidic acid
PAF	platelet activating factor
PC	phosphatidylcholine
PE	phosphatidylethanolamine
PEt	phosphatidylethanol
PGI <sub>2</sub>	prostacyclin
PI	phosphatidylinositol

PKC	.....	protein kinase C
PLA <sub>2</sub>	.....	phospholipase A <sub>2</sub>
PLC	.....	phospholipase C
PLD	.....	phospholipase D
PMA	.....	phorbol 12-myristate 13-acetate
PS	.....	phosphatidylserine
TG	.....	triglyceride
TLC	.....	thin layer chromatography
VLDL	.....	very low-density lipoprotein

## GENERAL INTRODUCTION

Atherosclerosis, a disease caused by lipid deposition in the blood vessel wall and narrowing of vessels lumen, remains the leading cause of morbidity and death in North America. This pathological condition is initiated by injury of endothelial cells as the result of some unknown mechanisms.

Free radical is believed to be one of the factors associated with atherosclerosis since vitamin E, a naturally-occurring and membrane-bound antioxidant, can protect endothelial cells from oxidative injury. However, little is known at present whether vitamin E, due to its unique location within cellular membrane, may modulate phospholipid metabolism of endothelial cells, thus leading to the maintenance of endothelial integrity and to the protection against atherogenesis.

This thesis is therefore focused on the involvement of vitamin E in phospholipid metabolism in human endothelial cells. In addition, to explain the high efficiency of vitamin E despite its low ratio to membrane phospholipid, this thesis is also concerned with its *in vivo* regeneration by other physiological reductants.

In order to appreciate better the present investigation, it is helpful to begin with some information about vitamin E and phospholipid metabolism.

## **I. VITAMIN E**

### **1. History**

In 1922, Herbert Evans and Katherine Bishop discovered that laboratory rats failed to reproduce when they were fed with a rancid lard diet unless lettuce or whole wheat were supplemented (Evans and Bishop, 1922). That unknown dietary factor present in the lettuce or whole wheat was first called factor "X", a vitamin-like substance. It is a fat-soluble substance and the inadequacy of which in the diet resulted in fetal death and resorption in rat. The letter E was given to that vitamin-like substance to include in the family of already known vitamins, after vitamin D.

After isolation and purification of vitamin E from wheat germ oil, Evans and other scientists found an alcohol with chemical formula ( $C_{29}H_{50}O_2$ ) having marked biological activity of vitamin E. Thus, they proposed the name tocopherol from the Greek tokos, meaning childbirth, and the verb phero, meaning to bring forth. The ol indicated the alcohol nature of the substance. Now, vitamin E is a family of both tocopherols and tocotrienols isolated from plant sources.

### **2. Structure and Nomenclature**

The vitamin E family consists of at least eight different forms having been isolated and purified to homogeneity from plant oil. These are  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocopherols and  $\alpha$ -,  $\beta$ -,  $\gamma$ -,  $\delta$ -tocotrienols. The tocopherols possess a general tocol

structure having a "head", 2-methyl 6-chromanol, and a 16-carbon isoprenoid side chain called the phytyl tail attached at the carbon numbered two of the chromanol ring. The  $\alpha$ -,  $\beta$ -,  $\gamma$ - and  $\delta$ -forms differ from each other in the number and position of methyl groups on the aromatic ring of the "head". For instance, the  $\alpha$ -form has three methyl groups on positions 5, 7 and 8, whereas  $\beta$ - and  $\gamma$ -compounds have only two methyl groups on positions 5, 8 and 7, 8, respectively. The  $\delta$ -form has only one methyl group on position 8. Tocotrienols have unsaturated tails (double bonds at carbons 3', 7' and 11'). The structures of tocopherols and tocotrienols are shown in Figure 1.

The tocol structure of tocopherols contain three asymmetric (chiral) carbons at the numbered 2-position of the ring and at the 4' and 8'-positions of the tail. Thus each tocopherol consists of  $2^3$  or eight possible stereoisomers. These are RRR, RSR, RRS, RSS, SSS, SRS, SSR and SRR-isomers which can be grouped into four racemic mixtures, each contains two enantiomers. The four racemic mixtures are RRR/SSS, RSR/SRS, RRS/SSR and RSS/SRR. Synthetic tocopherol possesses all of the four racemic mixtures of these stereoisomers and is termed, for instance, all-rac- $\alpha$ -tocopherol for the  $\alpha$ -form. The natural occurring form of tocopherol has only one geometrical structure which is the RRR-stereoisomer, and the most biologically active compound of vitamin E is  $\alpha$ -tocopherol. Thus, in this thesis, the naturally occurring form of vitamin E is synonymous with RRR- $\alpha$ -tocopherol.

### **3. Occurrence**

All eight forms of tocopherols and tocotrienols are widely distributed in nature (Bauernfeind, 1980). Despite the difference in the distribution,  $\alpha$ -tocopherol has the highest biological activity of vitamin E. The richest sources of vitamin E are foods of plant origin such as vegetable oils, nuts and whole grains. Fruits also contain moderate levels of vitamin E. The lowest sources of vitamin E are foods of animal origin in which  $\alpha$ -tocopherol accounts for almost all vitamin E activity.

The natural tocopherols are not very stable. All foods containing tocopherol significantly lose their vitamin E activity during refining, processing and storage, particularly upon exposure to heat and oxygen. Hence,  $\alpha$ -tocopherol acetate, a modified and stable form of vitamin E, is usually found in the pill form.

### **4. Absorption and Tissue Deposition**

The absorption of vitamin E in the intestine is incomplete (30-50%) and is maximally enhanced by dietary fats, bile acid and pancreatic juice (Gallo-Torres, 1980; Kayden and Traber, 1993). Vitamin esters must be hydrolysed by duodenal mucosal esterases prior to absorption. Mixed micelles of vitamin E, dietary fats and bile acids passively penetrate the brush-border membrane of the absorptive cells of the small intestine. After uptake into intestinal cells, vitamin E is secreted in chylomicrons and transported to the circulatory system via the mesenteric lymphatic ducts. The intestine secretes chylomicrons containing various forms of vitamin E (RRR- and SSR- $\alpha$ -tocopherols,  $\gamma$ -tocopherol).

Transfer of vitamin E to tissues begins to take place during chylomicron catabolism. Chylomicrons are catabolized in the circulation by an endothelial-bound enzyme, lipoprotein lipase, which transfers tocopherols along with fatty acids to tissues. Chylomicrons is now converted to chylomicron remnants which can transfer tocopherols to high density lipoprotein (HDL) and acquire apolipoprotein E from HDL during the exchange of excess surface. The chylomicron remnants are then taken up by the liver for catabolism.

Following chylomicron remnants uptake, the liver secretes vitamin E along with newly absorbed dietary lipids in very low density lipoproteins (VLDL) into the circulation. This process selectively incorporates RRR- $\alpha$ -tocopherol into nascent VLDL (Traber et al., 1990) probably mediated by a hepatic tocopherol-binding protein. About half of the VLDL are catabolized by lipoprotein lipase and hepatic triglyceride lipase and are converted to low density lipoprotein (LDL), while the remainder is partially delipidated in the circulation and returned to the liver. Therefore, some  $\alpha$ -tocopherol can be transferred to HDL during lipolysis, some can travel with VLDL core during the conversion to LDL, and some can return to the liver as in VLDL remnants. Schematic representation for the absorption and circulation of vitamin E is presented in Figure 2.

LDL uptake by tissues through LDL receptors may be one of the mechanisms by which tissues obtain  $\alpha$ -tocopherol, as demonstrated in vivo using fibroblasts with and without LDL receptors (Traber and Kayden, 1984). Tissues with high LDL receptor activity including adrenal glands, ovaries, adipose tissue, liver and intestine may obtain tocopherol through this mechanism. Alternatively,

other mechanisms are also possible for the delivery of  $\alpha$ -tocopherol to tissues since Watanabe rabbits, which have defective LDL receptor activity, have normal tissue  $\alpha$ -tocopherol levels (Cohn and Kuhn, 1989).

A technique for the incorporation of vitamin E into cultured endothelial cells in vitro has been developed in our laboratory (Chan and Tran, 1990). Endothelial cells were found to incorporate  $\alpha$ -tocopherol in a dose- and time-dependent manner. The incorporation of  $\alpha$ -tocopherol into endothelial cells was linear up to 8 hr of incubation, after which tocopherol levels were unchanged.  $\alpha$ -Tocopherol was found to associate mainly with the membrane fractions of the cells. Similarly,  $\gamma$ -tocopherol was also readily incorporated into endothelial cells (Tran and Chan, 1992). However,  $\gamma$ -tocopherol was found to have a higher turnover rate and a lower  $t_{1/2}$  (the time to deplete half) than  $\alpha$ -tocopherol, resulting in a low retention of  $\gamma$ -tocopherol in the cells. Thus, the highest biological activity of  $\alpha$ -tocopherol may be due to the discrimination of the retention of tocopherols inside the cells.

Being a hydrophobic and fat-soluble molecule, vitamin E tends to associate with cellular fractions rich in membranes (Burton and Ingold, 1986). Under normal level, approximate one molecule of  $\alpha$ -tocopherol per 2000 molecules of phospholipids are found in the membranes. Once incorporated into the membrane, the phytol tail of the tocopherol molecule is inserted into the lipid bilayers whereas the chromanol "head" bearing the hydroxyl group stays near the carbonyl group of the phospholipids. It is believed that the tocopherol molecule can move vertically and move laterally in the lipid bilayers to maximally perform its function.

## 5. Function(s) of Vitamin E

The question "How does vitamin E work as an antioxidant?" cannot be adequately answered if a sufficient knowledge about free radicals, the principal targets of vitamin E, is lacking. It is therefore necessary to begin with some general understandings about free radicals occurring in living systems before considering the biological functions of vitamin E.

a) Free Radicals (for more details please see review of Halliwell and Gutteridge, 1989)

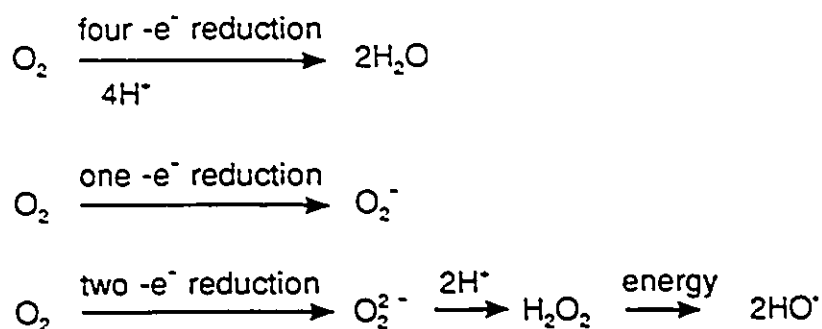
### i) Definition

By definition, "a free radical is any species capable of independent existence that contains one or more impaired electrons". If two atoms A and B are covalently bonded (A-B), homolytic fission of the A-B molecule will generate A-radical (A $\cdot$ ) and B-radical (B $\cdot$ ).



During aerobic respiration, oxygen molecule (O<sub>2</sub>) is a good oxidizing agent that accepts four electrons and four protons to become two molecules of water (H<sub>2</sub>O). In addition, the term "oxygen toxicity" usually implicates the toxic effect of oxygen toward living organisms, being attributed by the formation of free oxygen radicals. Upon one electron reduction, oxygen is converted to superoxide (O<sub>2</sub> $\cdot^-$ ) which is, by definition, an oxygen radical. Addition of two electrons to oxygen will give the peroxide ion (O<sub>2</sub> $^{2-}$ ) and, in the presence of protons, O<sub>2</sub> $^{2-}$  will be protonated to hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>). Either O<sub>2</sub> $\cdot^-$  or H<sub>2</sub>O<sub>2</sub> is not a free radical. However, due to the relatively weak O-O bond, H<sub>2</sub>O<sub>2</sub> decomposes easily to give the hydroxyl

radical (HO<sup>•</sup>). To summarize:

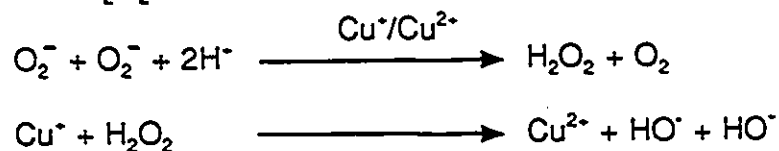


ii) Generation of Free Radicals

Superoxide radical (O<sub>2</sub><sup>-</sup>) is usually generated by phagocytes in the defense mechanism against foreign invaders. Salts of transition metals such as iron (II)/iron(III) (Fe<sup>2+</sup>/Fe<sup>3+</sup>) and copper (I)/copper (II) (Cu<sup>+</sup>/Cu<sup>2+</sup>) are found to involve in the formation of radicals, especially hydroxyl radical (HO<sup>•</sup>). For instance, hydrogen peroxide in the presence of iron salts will give HO<sup>•</sup> according to the Fenton reactions.



In addition, the copper salt is involved in the formation of H<sub>2</sub>O<sub>2</sub> from O<sub>2</sub><sup>-</sup> or in the formation of HO<sup>•</sup> from H<sub>2</sub>O<sub>2</sub>.



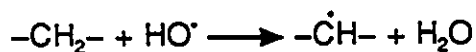
Other free radicals, normally generated in many biological systems are thiyl radicals (RS<sup>•</sup>) which are sulfur-centred radicals formed by the homolytic fission of disulfide bond in proteins, carbon-centred radicals ( $-\overset{\cdot}{\underset{|}{\text{C}}}$ ) which react rapidly with O<sub>2</sub> to give peroxy radicals ( $-\overset{\cdot}{\underset{|}{\text{C}}}-\text{O}_2$ ) and nitrogen-centred radicals ( $-\overset{\cdot}{\text{N}}-$ ) formed

during oxidation of phenylhydrazine by erythrocytes. Among the radicals presented, hydroxyl radicals is the most aggressive species in damaging cellular DNA and membranes. Factors involved in the production of hydroxyl radicals are ionizing radiation of high energy such as X-rays,  $\gamma$ -rays or ultrasound, and the overload of transition metals such as iron or copper. In addition, ozone ( $O_3$ ), an important product of photochemical air pollution, is a powerful oxidizing agent that can directly attack many biological molecules or through the production of hydroxyl radicals.

In summary, any biological system generating superoxide radical ( $O_2^-$ ) will produce hydrogen peroxide ( $H_2O_2$ ). Hydrogen peroxide can readily cross the cell membranes whereas superoxide cannot. Inside the cells, hydrogen peroxide reacts with iron (II) or probably Cu (I) to form hydroxyl radical ( $HO^\bullet$ ) which is the origin of many toxic effects, including DNA single-strand breaks and lipid peroxidation of cellular membranes.

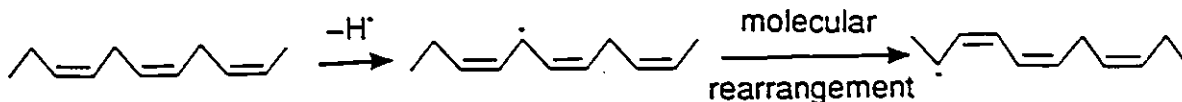
iii) Lipid Peroxidation

Lipid peroxidation is a radical chain reaction in which a reactive species such as hydroxyl radical ( $HO^\bullet$ ) initially attack a polyunsaturated fatty acid in the membrane bilayer by abstracting a hydrogen atom from a methylene ( $-CH_2-$ ) group. This process is called first-chain initiation:

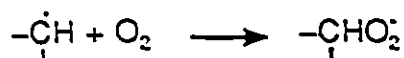


The methylene ( $-CH_2-$ ) group attacked by  $HO^\bullet$  is usually adjacent to the double bond in the fatty acid. The presence of the double bond weakens the C-H bond of the adjacent methylene group and therefore makes the H removal easier.

Hydrogen abstraction results in the formation of a carbon-centred radical. This carbon radical is stabilized by a molecular rearrangement to form a conjugated diene which can be detected by UV absorbance at 234 nm.

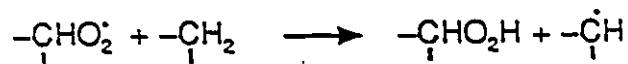


Under aerobic conditions,  $O_2$  combines with the carbon radical to form peroxy radical ( $ROO\cdot$  or  $RO_2\cdot$ ).



The peroxy radical is capable of abstracting hydrogen from another fatty acid to become itself a lipid hydroperoxide ( $ROOH$ ) and to generate a new carbon radical.

This is the propagation stage of lipid peroxidation:



Furthermore, the presence of  $Fe(II)/Fe(III)/O_2$  complexes inside the cells will decompose lipid hydroperoxides ( $ROOH$ ) to peroxy radicals ( $ROO\cdot$ ) or alkoxyradicals ( $RO\cdot$ ). Together, these result in accelerating the propagation stage.

The propagation stage can, however, be terminated when the two radicals ( $L\cdot$ ) combine with each other. This is the termination stage of lipid peroxidation:



The combination of the radicals in the termination stage could result in the cross-linking between fatty acid molecules. From this point of view, either propagation or termination stage in the lipid peroxidation will severely damage the biological membranes.

There are different defence mechanisms to protect against free radical-induced damage to the organism. Any substance participating in these defence mechanisms are termed antioxidants. As far as lipid peroxidation is concerned, antioxidants can act at different levels:

- Reducing  $O_2$  concentrations
- Preventing the first-chain initiation by scavenging  $HO^\bullet$
- Binding metal ions ( $Fe^{2+}/Fe^{3+}$ ), and thus preventing them to form  $HO^\bullet$
- Decomposing hydroperoxides to alcohols, non-radical products
- Chain-breaking

The last defence mechanism is performed by substances called chain-breaking antioxidants which are often phenols or aromatic amines. These antioxidants scavenge the intermediate radicals such as peroxy ( $ROO^\bullet$ ) or alkoxy ( $RO^\bullet$ ) radicals, and therefore prevent further hydrogen abstraction. Among the phenol bearing compounds, vitamin E is classified as a powerful chain-breaking antioxidant.

b) Function of Vitamin E

The conventional function of vitamin E is its ability to scavenge peroxy radical produced in lipid peroxidation as described above. Vitamin E, especially  $\alpha$ -tocopherol (TOH), acts as a reducing agent by donating its phenolic hydrogen atoms to the peroxy radical ( $ROO^\bullet$ ) and becoming itself a radical called tocopheroxy radical ( $TO^\bullet$ ). Tocopheroxy radical ( $TO^\bullet$ ) is a stable, unreactive species due to the delocalization of unpaired electron on the oxygen atom into the aromatic ring. Upon accepting a hydrogen atom, peroxy radical ( $ROO^\bullet$ ) is thus

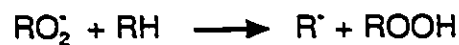
converted to peroxides (ROOH) which will be removed by cellular repairing mechanisms. Therefore, by scavenging the peroxy radical (ROO<sup>•</sup>), vitamin E interrupts and terminates the chain reaction of lipid peroxidation, and thus it is considered as a chain-breaking antioxidant.

To summarize:

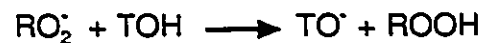
Initiation:



Propagation:



Antioxidant reaction:



Tocopheroxyl radical (TO<sup>•</sup>) is eventually removed from the system by reacting with another radical, forming a relatively stable non-radical product. The process is called termination:

Termination:



or, after being oxidized, tocopheroxyl radical can be reduced back to the tocopherol molecule by other reducing agents. The process is termed "Regeneration of Tocopherol".



Radicals formed from these water soluble reducing agents will be removed or converted back to its original form by an enzyme reaction. The regeneration of vitamin E will be explored in detail in the last chapter of this thesis.

As an antioxidant *in vivo*, vitamin E has been shown to interfere with the free-radical-mediated reactions of both the lipoxygenase and the cyclooxygenase pathways of arachidonic acid metabolism (Panganamala and Cornwell, 1982). Vitamin E was found to suppress thromboxane (TXA<sub>2</sub>) synthesis in platelets (Hamelin and Chan, 1983) and inhibit the formation of 5-hydroxyeicosatetranoic acid (5-HETE) in neutrophils (Chan et al., 1989), due to inhibition of phospholipase A (Douglas et al., 1986; Cao et al., 1987) and lipoxygenase activity (Goetzi, 1980). By contrast, enrichment of vitamin E in endothelial cells was found to enhance arachidonic acid release (Tran and Chan, 1988) and prostacyclin (PGI<sub>2</sub>) synthesis (Tran and Chan, 1990). The role of vitamin E in modulating these processes has recently been summarized (Chan, 1993).

## II. PHOSPHOLIPID METABOLISM

### 1. The CoA-Dependent and CoA-Independent Transacylation Processes

The incorporation and mobilization of fatty acids in cellular membrane are important in the remodelling of phospholipids in mammalian cells. Acylation of fatty acids into membrane phospholipids is catalyzed by both the coenzyme A (CoA)-dependent and the CoA-independent transacylation systems (Figure 3). Incorporation of free fatty acids into membrane phospholipids is mediated via the CoA-dependent transacylation system which requires the presence of CoA (Irvine and Dawson, 1979; Kramer et al., 1984a). This system catalyzes the transfer of fatty acyl-CoA to various lysophospholipids. A wide range of different fatty acids and lysophospholipids are substrates of the CoA-dependent transacylation system.

The CoA-independent transacylation system, on the other hand, was first described by Kramer and Deykin for human platelets (Kramer and Deykin, 1983; Kramer et al., 1984b). Later, similar enzyme was also found in several mammalian tissues and cells (Reddy and Schmid, 1986; Robinson et al., 1985; Sugiura et al., 1987). This system catalyzes the transfer of C<sub>20</sub> and C<sub>22</sub> polyunsaturated fatty acids to the ether-linked lysophospholipids as acceptor. Both C<sub>20</sub> and C<sub>22</sub> of either n-6 or n-3 polyunsaturated fatty acids have to be first esterified at the sn 2-position of diradyl phospholipids, especially diacylglycerophosphocholine (diacyl-GPC) which serves as the preferred donor phospholipids. Diacylglycerophosphoethanolamine (diacyl-GPE) can also be the donor

phospholipid whereas diacylglycero-phosphoinositol (diacyl-GPI) is not as effective as a donor phospholipid. As for the acceptor lysophospholipids, 1-alkyl-GPC is most rapidly acylated than other choline-containing lysophospholipids (1-alkenyl-GPC, 1-acyl-GPC). Ethanolamine-containing lysophospholipids (1-alkenyl-GPE, 1-alkyl-GPE, and 1-acyl-GPE) can also be acylated with arachidonic acid transferred from diacyl-GPC. However, 1-acyl-GPI and 1-acylglycerophosphoserine (1-acyl-GPS) are not effective acceptors.

It has been demonstrated that arachidonic acid is preferentially transferred from diacyl-GPC to ether-containing phospholipids (alkyl-GPC) in several inflammatory cells, endothelial cells and testis (Blank et al., 1973; Rittenhouse et al., 1977; Sugiura et al., 1984; Sugiura et al., 1985; Colard et al., 1984; Chilton and Murphy, 1986; Chilton et al., 1987). Other C<sub>16</sub> or C<sub>18</sub> fatty acids including palmitic (16:0), stearic (18:0), oleic (18:1) and linoleic (18:2) acids are transferred at very low rates, if any. Free fatty acids once liberated from the donor phospholipids cannot be incorporated into phospholipids by the CoA-independent transacylation process. Thus, the CoA-independent transacylation system appears to have high specificity in mobilizing polyunsaturated fatty acids especially arachidonic acid from diacyl-GPC into alkyl-GPC. This system may be an effective mechanism for disposing of intracellularly generated ether-linked lysophospholipids to form non-toxic diradyl phospholipids. It may also act to enrich arachidonic acid into the alkylacyl-GPC which is a precursor of platelet activating factor (PAF) (Braquet et al., 1987). Liberation of arachidonic acid from alkylacyl-GPC has been shown to be involved in the formation of prostaglandins and leukotrienes (Chilton

et al., 1991). A schematic representation for the incorporation and mobilization of arachidonic acid between choline-containing phospholipids is presented in Figure 3.

## 2. Enzymes Responsible for the Generation of Lipid Mediators

Many  $\text{Ca}^{2+}$ -mobilizing agonists including hormones, neurotransmitters and growth factors exert their effect in various number of cells and tissues by causing profound changes in lipid metabolism. These changes are initiated by receptor-mediated stimulation of at least three main phospholipases. These are phospholipase  $A_2$  ( $\text{PLA}_2$ ), phospholipase C (PLC) and phospholipase D (PLD) (for review, see Dennis et al., 1991). In addition, enzymes such as phosphatidate phosphohydrolase, diacylglycerol kinase and protein kinase C will also be reviewed due to their pivotal role in signal transduction.

### a) Phospholipase $A_2$

Many cell-types release arachidonic acid esterified at the sn-2 position of membrane phospholipids upon cell stimulation. Although arachidonic acid can potentially be released by a number of phospholipases,  $\text{PLA}_2$  is generally regarded as the major generator of arachidonic acid, for at least the biosynthesis of potent extracellular mediators, prostaglandins and leukotrienes (Dennis, 1987). The other product of  $\text{PLA}_2$  is lysophospholipid which is a biological detergent lytic to cell membrane at high concentrations. When ether-containing phosphatidylcholine (alkylacyl-GPC) is the substrate of  $\text{PLA}_2$ , the lysophospholipid (alkyl-GPC) upon acetylation forms platelet activating factor (PAF, alkylacetyl-GPC), another potent

lipid mediator (for review, see Braquet et al., 1987).

Previous studies on PLA<sub>2</sub> were mainly focused on the extracellular PLA<sub>2</sub> from pancreas, snake venoms and secretory granules of neutrophils and platelets (Davidson and Dennis, 1990). All secreted PLA<sub>2</sub> are composed of single polypeptide chain, about 120 amino acids, containing 10-14 cys, all in disulfide pairs. These PLA<sub>2</sub>'s required Ca<sup>2+</sup> and alkaline pH for optimum activity.

It is only in the last few years that serious attempts have been made to identify intracellular PLA<sub>2</sub>. Dennis and coworkers have successfully purified and characterized a membrane-associated PLA<sub>2</sub> from macrophage-like cell line, the P388D<sub>1</sub> cell line (Ulevitch et al., 1988; Lister et al., 1988). The enzyme has a molecular mass of about 18 kDa, requiring Ca<sup>2+</sup> and high pH for optimum activity.

Recently, PLA<sub>2</sub> has also been identified in the cytosol of several cell types and tissues including human monocytic cell line U937 (Clark et al., 1991; Clark et al., 1990; Diez and Mong, 1990; Kramer et al., 1991) and macrophage cell line RAW 264.7 (Channon and Leslie, 1990; Leslie, 1991). The enzyme has a molecular mass of 90-110 kDa and shares no homology with the secreted forms of PLA<sub>2</sub>. The most interesting feature of the cytosolic PLA<sub>2</sub> is its ability to translocate to the membranes in a Ca<sup>2+</sup>-dependent manner due to the presence of a Ca<sup>2+</sup>-dependent phospholipid binding motif which is also found in protein kinase C, GTPase activating protein (GAP) and PLC- $\gamma$ 1 (Clark et al. 1991). Thus, the cytosolic PLA<sub>2</sub> may be a target of Ca<sup>2+</sup>-mobilizing agonists and is responsible for the release of arachidonic acid when cells are activated.

The preferred substrates of PLA<sub>2</sub> are phospholipids containing arachidonic acid esterified at the sn-2 position. Arachidonic acid is present in phosphatidylethanolamine (PE), phosphatidylcholine (PC) and phosphatidylinositol (PI). The dominance of one pool containing more arachidonic acid than the other is dependent on different cell types. Thus, all main phospholipid classes are possible substrates of PLA<sub>2</sub>, and the specificity of the enzyme does not appear to be determined by the phospholipid head group.

b) Phosphoinositide-Specific Phospholipase C

Extensive studies had focussed on the identification of the phosphatidylinositol (PI)-specific phospholipase C which catalyzes the hydrolysis of inositol-containing glycerophospholipids, generating intracellular second messengers, inositol phosphates and diacylglycerol. Inositol phosphates mobilize intracellular Ca<sup>2+</sup>, while diacylglycerol is an activator of protein kinase C. Many different PLC isoforms has been identified and cloned from a variety of mammalian tissues. Due to their difference in amino acid sequences and immunological reactivity, mammalian PLC can be divided into four types - PLC- $\alpha$ , PLC- $\beta$ , PLC- $\gamma$  and PLC- $\delta$  - which are products of distinct genes (Rhee et al., 1989). All these four types are single polypeptides with molecular mass of 62-68 kDa for PLC- $\alpha$ , 150-154 kDa for PLC- $\beta$ , 145-148 kDa for PLC- $\gamma$  and 85-88 kDa for PLC- $\delta$ . In addition, each of PLC type ( $\beta$ ,  $\gamma$  and  $\delta$ ) consists of several different family members designated with arabic numerals as subscripts of the Greek letters. For example, PLC- $\gamma_1$  can be found in most mammalian tissues and its amino acid sequence is highly conserved (greater than 95%) in different species and tissues.

Despite the difference in their structures, all four isoenzymes have similar catalytic properties that they hydrolyze three common phosphoinositides, PI, PI-4P and PI-4,5P<sub>2</sub>, and their catalytic activities are dependent on concentration of Ca<sup>2+</sup>. Many of the PLC forms have been purified from the cytosol with the exception of PLC-β<sub>1</sub>, which is tightly associated with membranes. Thus, recruitment of the cytosolic enzymes to the membrane must in part rely on the presence of a Ca<sup>2+</sup>-dependent translocation domain.

c) Phospholipase D

During signal transduction, hydrolysis of phosphatidylcholine by phosphodiesterases, PLC and PLD, makes a major contribution to the formation lipid mediators such as diacylglycerol (DAG) and phosphatidic acid (PA). The products of PLC activity would be DAG and cholinephosphate, whereas PLD activity would produce PA and choline. The products of these phosphodiesterases are readily interconvertible by specific kinases and phosphatases. For example, DAG and PA are interconverted by DAG kinase and PA phosphohydrolase. Similarly, choline can be phosphorylated by choline kinase and cholinephosphate can be dephosphorylated by cholinephosphate phosphatase. Thus, direct identification and quantitation of these products cannot determine exactly which phosphodiesterases are involved in the hydrolysis of PC. Recently, numerous evidence indicated the ubiquitous distribution of PLD activity in a variety of cells and tissues whereas PC-specific PLC was found to be restricted in a few cell types (Billah and Anthes, 1990). Unfortunately, unlike PLA<sub>2</sub> and PI-specific PLC, purification of mammalian PLC and PLD that act on PC is not yet successful. In

this section, a brief overview of the catalytic property of PLD will be presented.

PLD catalyzes the catalytic cleavage of the terminal phosphate diester bond of glycerophospholipids, resulting in the formation of PA and the corresponding water-soluble headgroup. PLD was originally discovered in plants, bacteria and fungi (Heller, 1978). The presence of PLD activity in mammalian tissues was first discovered by Kanfer and his colleagues through their studies on the recycling of choline to acetylcholine in the brain (Hattori and Kanfer, 1985).

Phosphatidylcholine appears to be the major substrate of PLD (Billah and Anthes, 1990), even though there is evidence for the presence of PI-specific PLD in the cytosol of human neutrophils (Balsinde et al., 1989). In human neutrophils, distribution of ether and ester linkages at the sn-1 position of PA was found to be similar to PC but not PE or PI (Billah et al., 1989; Agwu et al., 1989). Thus, this finding forms the basis to believe that PC is the main substrate of PLD.

One unique characteristic of PLD is its ability to catalyze transphosphatidyl transfer reaction between PC and primary alcohols. In the presence of ethanol, PLD transfers the phosphatidyl moiety to ethanol to form phosphatidylethanol. Thus, PLD activity in intact cells can be simply detected by monitoring the formation of phosphatidylethanol. In addition, Billah and coworkers have developed a second approach to determine PLD in neutrophils. It involves the labelling of endogenous phospholipids with  $^{32}\text{P}$  in the absence of [ $^{32}\text{P}$ ]ATP (Billah et al., 1989). Cells were incubated with lyso[ $^{32}\text{P}$ ]PC which was readily acylated to form membrane associated [ $^{32}\text{P}$ ]PC. Thus, during stimulation, the formation of [ $^{32}\text{P}$ ]PA must occur only from PLD action.

PC hydrolysis was found to contribute a major part of the accumulation of intracellular DAG. Upon stimulation by specific agonists, many cells generate DAG in a biphasic manner. The early phase is transient and quantitatively small, peaks within 30 s of stimulation, whereas the delay phase is sustained and large, reaches maximum within 2-15 min of stimulation. The first peak of DAG coincides with PI hydrolysis by PI-PLC whereas PC hydrolysis is the major source of sustained DAG levels. In certain cell types (MDCK-D1 kidney cells, A10 smooth muscle cells and 3T3-L1 fibroblasts), PC hydrolysis by PLC appears to be the major route of DAG formation, whereas in other cell types including neutrophils, sustained accumulation of DAG is formed by dephosphorylation of PA, product of PLD (for specific references, see Billah and Anthes, 1990; Exton, 1990).

d) Phosphatidic Acid Phosphohydrolase

Phosphatidic acid phosphohydrolase catalyzes the conversion of PA to DAG by dephosphorylation reaction. PA has long been known to be the precursor of DAG which serves as intermediate for the synthesis of major glycerolipids in animal cells. Recently, PA has been implicated as an important cellular second messenger. PA has been shown to affect several key functional proteins including protein kinase C (Epand and Stafford, 1990), GTPase-activating protein (Tsai et al., 1989) and phosphatidylinositol-4-phosphate kinase (Moritz et al., 1992). Thus PA phosphohydrolase is likely to be an important regulatory enzyme in glycerolipid metabolism and cell signalling.

PA phosphohydrolase has been successfully purified from yeast membranes (Morlock et al. 1991), whereas early attempts to purify the enzyme from animal

tissues was unsuccessful. Recent works (Taylor et al., 1993; Aridor-Piterman et al., 1992; Jamal et al., 1991) using crude enzyme preparations of animal tissues showed that PA phosphohydrolase consists of at least two isoforms which are distinguished from each other with respect to subcellular localization, cations dependencies and sensitivity to various inhibitors. For instance, in neutrophils (Taylor et al., 1993) PA phosphohydrolase activity was detected mainly in the membrane and about 20% in the cytosol.  $Mg^{2+}$  potentiated the activity of the cytosolic form but not the activity of the membrane form, whereas  $Ca^{2+}$  moderately inhibited both forms. Enzyme activity was markedly inhibited by NaF, a universal phosphatase inhibitor, but slightly attenuated by propranolol, a known PA phosphohydrolase inhibitor in intact cells. In addition, the cytosolic form of PA phosphohydrolase was sensitive to thio-reactive reagents such as N-ethylmaleimide (NEM) whereas the plasma membrane associated form was NEM-insensitive (Jamal et al., 1991).

Using gel filtration and anion-exchange chromatography, Day and Yeaman have successfully separated two distinct forms of PA phosphohydrolase from rat liver (Day and Yeaman, 1992). A cytosolic form ( $M_r = 540$  kDa) was sensitive to NEM and could be translocated to the membrane by oleate. The other form ( $M_r = 240$  kDa) was NEM-insensitive and mainly associated with plasma membrane, only 10% of the total cellular activity was located in endoplasmic reticulum.

Recently, Kanoh and coworkers were also successful in the purification of a membrane-bound PA phosphohydrolase from porcine thymus (Kanoh et al., 1992). The enzyme was purified to homogeneity characterized by a single band

of Mr = 83 kDa on sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The enzyme activity was insensitive to NEM and was independent of  $Mg^{2+}$ , but was inhibited by other cations such as  $Ca^{2+}$ ,  $Mn^{2+}$  and  $Zn^{2+}$ . The enzyme was inhibited by 1,2-DAG, but not by 1- or 2-monoacylglycerol and triacylglycerol. It was also inhibited by sphingosine and chlorpromazine, but less potently by propranolol. Although the enzyme is likely an integral membrane protein, the enzyme activity was independent upon the addition of major membrane phospholipids. This was in contrast to the observation that the cytosolic form of PA phosphohydrolase from rat liver was stimulated by PE, but inhibited by PG, PS and PI (Humble and Berglund, 1991). This discrepancy may account for the difference between enzyme isoforms.

Certainly, the present available information provides a basis for future studies of PA phosphohydrolase at the molecular level. Other enzyme affecting the levels of PA and DAG is DAG kinase, the action of which is totally opposite to that of PA phosphohydrolase. With respect to the importance of DAG and PA in lipid metabolism, the actions of PA phosphohydrolase and DAG kinase are likely to be regulated by hitherto unknown mechanisms in order to maintain a normal flow for glycerolipid biosynthesis and to regulate the cellular signalling. Information pertaining to DAG kinase will be presented in the following section.

e) Diacylglycerol Kinase

Diacylglycerol kinase converts diacylglycerol (DAG) to phosphatidic acid (PA). Similar as PA phosphohydrolase, much attention has also been focused on this enzyme since DAG is a known biological activator of protein kinase C. DAG

kinase may therefore function as a critical modulator of the intracellular concentration of DAG accumulated from multiple sources including PI-PLC, PC-PLC and PLD/PA phosphohydrolase during agonist stimulation. In addition, the enzyme may act to initiate the resynthesis of phosphoinositides which are cleaved by PI-PLC following receptor activation.

A DAG kinase with an apparent molecular weight of 80 kDa was first successfully purified from the cytosol of porcine brain (Kano et al., 1983). Later, the enzyme was found to be abundant in porcine and human lymphocytes (Yamada et al., 1989). Primary structure of the 80 kDa DAG kinase (Sakane et al., 1990) revealed the presence of EF-hand motifs typical of calmodulin and other  $Ca^{2+}$ -binding proteins, and the presence of zinc finger-like sequences that are similar to those in protein kinase C.

Immunological and enzyme purification studies show that there are several DAG kinase isozymes (Yamada and Kano, 1988). At least two DAG kinase isozymes (80 and 150 kDa) have been purified from porcine thymus (Sakane et al., 1989), rat brain (Kato and Takenawa, 1990) and human platelets (Yada et al., 1990). The physiological significance of these isozymes is not yet clear, but the presence of multiple forms of DAG kinase may explain the complex nature of DAG metabolism in stimulated cells.

DAG kinase activity is ubiquitously found in many cell types and tissues, and it has been detected in both soluble and various membrane fractions of the cells (Yamada et al., 1989; Kano et al., 1990). Recently, an arachidonoyl-DAG-specific DAG kinase was found to be exclusively associated with the membranes

whereas the non-specific-DAG kinase was a soluble form present in the cytosol (MacDonald et al., 1988a; MacDonald et al., 1988b; Lemaitre et al., 1990). It has been suggested that translocation of cytosolic DAG kinase to membranes may occur in an analogous manner to that of protein kinase C. Translocation of cytosolic DAG kinase to the membranes has been so far observed in several cell types including rat brain (Besterman et al., 1986), human neutrophils (Ishitoya et al., 1987), amoeba (Jimenez et al., 1988) and NIH 3T3 cells (Kato et al., 1988). However, in Swiss 3T3 cells, DAG kinase translocation from cytosol to membranes was not observed when cells were stimulated with platelet-derived growth factor (MacDonald et al., 1988a; MacDonald et al., 1988b), but it was partially detected when the cells were treated with phorbol ester (Maroney and Macara, 1989). The reason for this discrepancy is unknown, but it may be attributed to the presence of multiple DAG kinase isozymes in fibroblasts.

In addition, it is still unclear about the mechanisms of DAG kinase action. *In vitro* study has shown that phosphorylation of 80 kDa DAG kinase by protein kinase C is linked to its membrane association (Kanoh et al., 1989). Alternatively, membrane-bound DAG kinase in parotid glands could be activated via phosphorylation by cAMP-dependent and  $Ca^{2+}$ /calmodulin-dependent protein kinases (Soeling et al., 1989). Recently, it was found that  $Ca^{2+}$  alone may be sufficient to cause the 80 kDa DAG kinase of porcine thymus cytosol to associate with the membrane phospholipids (Sakane et al., 1991). Although it is not yet known about the relative importance of the enzyme phosphorylation and  $Ca^{2+}$  binding in the activation of DAG kinase, it seems that this enzyme is operating

under multiple control mechanisms.

f) Protein Kinase C

An overwhelming amount of information about protein kinase C (PKC) has been accumulated over the past decade. Numerous references have been compiled in recently detailed reviews (Bell and Burns, 1991; Nishizuka, 1992; Azzi et al., 1992, Hug and Sarre, 1993). A brief summary of information related to the structure and distribution of PKC will be presented in this section (for detailed references, please see the above reviews).

PKC is a serine/threonine kinase consisting of a single polypeptide chain of 77-83 kDa. Purification and biochemical studies revealed that PKC represents a protein family of at least eight isozymes which can be divided into two major groups: the  $\text{Ca}^{2+}$ -dependent or conventional PKCs (cPKCs) and the  $\text{Ca}^{2+}$ -independent or novel PKCs (nPKCs). The PKC isoforms  $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$  belong to the  $\text{Ca}^{2+}$ -dependent group, while the PKC isoforms  $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$  and  $\theta$  belong to the  $\text{Ca}^{2+}$ -independent group.

The cPKC isozymes ( $\alpha$ ,  $\beta$ I,  $\beta$ II and  $\gamma$ ) have four functional domains ( $C_1$ - $C_4$ ) separated by five variable regions ( $V_1$ - $V_5$ ). The amino terminal half ( $V_1$ ,  $C_1$ ,  $V_2$ ,  $C_2$ ) forms the regulatory domain which is linked with the catalytic domain ( $C_3$ ,  $V_4$ ,  $C_4$ ,  $V_5$ ) by a hinge region ( $V_3$ ). The nPKC isozymes ( $\delta$ ,  $\epsilon$ ,  $\zeta$ ,  $\eta$  and  $\theta$ ) have same primary structure as that of cPKCs but lack the  $C_2$  region which is a  $\text{Ca}^{2+}$ -binding domain of cPKCs. The  $C_1$  domain consists of two zinc fingers (except  $\zeta$  isoform contains only one zinc finger and does not bind DG or phorbol esters), a pseudosubstrate sequence motif, and binding sites for phosphatidylserine (PS) and

DAG or phorbol esters. In the carboxy-terminal half (catalytic domain), C<sub>3</sub> region contains the ATP binding site, and C<sub>4</sub> region contains the substrate binding site and phosphate transfer region. Inhibitors of PKC can interact at either the regulatory or catalytic regions; calphostin and sphingosine interact at the regulatory domain, while H-7 and staurosporin interact at the catalytic region. Structures of PKC isoforms are presented in Figure 4.

Activation of PKC is thought to be involved in the translocation of cytosolic PKC to the membranes induced by elevation of intracellular Ca<sup>2+</sup> concentrations. Of course, as mentioned above, Ca<sup>2+</sup> is not required for activation of the nPKC group. DAG (or phorbol ester) acts as activator and PS as cofactor of activation. In many cell types, prolonged treatment with phorbol esters results in almost complete depletion of cellular PKC, termed "PKC downregulation" possibly due to proteolytic cleavage. However, translocation and downregulation of each PKC isoform appear to be dependent on the cell type and specific stimulus. For instance, significant differences in PMA sensitivity between PKC isozymes were observed in various cells; and therefore prolonged treatment with PMA would not be necessary to deplete all of the cellular PKC. In addition, distribution of PKC within the resting (unstimulated) cells is dependent on the PKC isoforms and on the cell type. The  $\alpha$ ,  $\beta$ ,  $\gamma$  and  $\zeta$ -isoforms seem to be present as cytosolic enzymes whereas PKC  $\delta$  and  $\epsilon$  are cytosolic in certain cell types but membrane-associated in the others. PKC  $\eta$  has been found to be present specifically in the cell nucleus.

Studies on tissue distribution of PKC isozymes has been conducted by Northern blot analyses and, more recently, by Western blot using isozyme-specific

antibodies. PKC  $\alpha$ ,  $\beta$ I/II,  $\delta$ ,  $\epsilon$  and  $\zeta$  seem to be ubiquitously distributed in many cell types and tissues. PKC  $\gamma$  is strictly present in central nervous system, e.g. brain; PKC  $\eta$  is mainly expressed in lung, skin and heart; and PKC  $\theta$  is the major isoenzyme in skeletal muscle. In addition to tissue-specific patterns of expression, the amount and number of PKC isozymes is also varied in a given tissue depending on its development stages. Thus, this indicates that a certain set of PKC isozymes is necessary for the maintenance of the characteristics and functions of a given tissue at a certain stage.

### **3. Interactions Between Various Pathways in Signal Transduction**

Extracellular signals are transmitted across the cellular membrane by agonist/receptor-mediated activation of different phospholipases which hydrolyse various membrane phospholipids. Are these phospholipases independently activated or is there a hierarchy of activation in the signalling pathways? What are the biochemical mechanisms of interactions between various enzymic pathways in the signal transduction? These questions are now partially answered from many independent studies on the temporal formation of various products during cell activation.

A general correlation of the time course of various signalling molecules generation is illustrated in Figure 5 (from Nishizuka, 1992). In most stimulated cells, hydrolysis of phosphatidylinositol 4,5-bisphosphate ( $\text{PIP}_2$ ) by PI-PLC was transient, resulting in a rapid accumulation of inositol 1,4,5-trisphosphate ( $\text{IP}_3$ ) and DAG which peak within the first 30 s of stimulation. The increase in intracellular

$\text{Ca}^{2+}$  is well correlated with  $\text{IP}_3$  formation, a  $\text{Ca}^{2+}$  secretagogue. The transient peak of DAG is followed by a second phase of DAG formation which probably results from hydrolysis of PC by either PLC or PLD/PA phosphohydrolase (Billah and Anthes, 1990). DAG formed from these phosphoinositide-independent pathways may contribute to the sustained activation of PKC.

$\text{Ca}^{2+}$  mobilization and PKC activation are believed to act synergistically to activate  $\text{PLA}_2$ . Most of the  $\text{PLA}_2$  are active in the presence of  $\text{Ca}^{2+}$  and at alkaline pH. Based upon a very close temporal relationship between receptor occupancy,  $\text{IP}_3$  production,  $\text{Ca}^{2+}$  increases, fatty acid release and lysophospholipid formation, a general hypothesis for the sequential nature of PLC- $\text{PLA}_2$  activation after agonist challenge has been proposed (Jaffe et al., 1987). Initial increase of  $\text{Ca}^{2+}$  from the action of  $\text{IP}_3$  may be responsible for the translocation of cytosolic  $\text{PLA}_2$  to membranes in a similar mechanism found for PKC, GAP and PLC- $\gamma$  (Clark et al., 1991). However, the intracellular concentration of  $\text{Ca}^{2+}$  increases only transiently, whereas  $\text{PLA}_2$  activation persists long after the  $\text{Ca}^{2+}$  concentration returns to its basal level. Studies with intact cells using phorbol esters and membrane-permeant DAGs have suggested that  $\text{PLA}_2$  activity can be regulated by PKC (Ho and Kleine, 1987). PKC may augment  $\text{PLA}_2$  activity by lowering its requirement for cytosolic  $\text{Ca}^{2+}$  (Carter et al., 1989). Other mechanisms by which PKC may enhance  $\text{PLA}_2$  activity includes an inhibition in the activity of an anti- $\text{PLA}_2$  proteins known as lipocortins (Hirata et al., 1981) or activation of the  $\text{Na}^+/\text{H}^+$  counterporter (Kitazono et al., 1989). In addition, increases in intracellular  $\text{Ca}^{2+}$  and sustained activation of PKC may synergistically activate PLD (Billah and Anthes, 1990) and may induce

the translocation of cytosolic DAG kinase to membranes (Kano et al., 1989).

Products of PLA<sub>2</sub> including free fatty acids and lysophospholipids have been shown to act as intracellular regulators of other signalling pathways. Lysophosphatidylcholine and cis unsaturated fatty acids including oleic, linoleic, linolenic, arachidonic, and docosahexaenoic acids were found to enhance PKC activation induced by DAG or phorbol esters (Shinomura et al., 1991; Chen and Murakami, 1992; Yoshida et al., 1992; Asaoka et al., 1992). These cis unsaturated fatty acids also activate PLD (Kobayashi and Kanfer, 1987), DAG kinase (Kelleher and Sun, 1989) and PA phosphohydrolase (Siddiqui and Exton, 1992). Unsaturated fatty acid especially oleic acid was found to augment PA phosphohydrolase activity by enhancing the redistribution of cytosolic form to the membrane (Aridor-Piterman et al., 1992). Other signalling molecules including Ca<sup>2+</sup>, DAG, and sphingosine, a breakdown product sphingolipids, were found to activate DAG kinase (Sakane et al., 1991; Lemaitre et al., 1990; Sakane et al., 1989) but inhibit PA phosphohydrolase (Kano et al., 1992; Aridor-Piterman et al., 1992).

Many signalling pathways including phospholipases C, D and A<sub>2</sub> are linked to the cell surface receptors by GTP-binding proteins known as G-proteins (Bourne et al., 1991). Substantial evidence have shown that the PI-specific PLC is coupled to the receptor via a G-protein, G<sub>p</sub> (Cockcroft and Gomperts, 1985). More recent evidence suggests that activation of PLA<sub>2</sub> and PLD may also be under the control of G-proteins (Cockcroft, 1992) even though the mechanism of how agonist-receptor binding leads to the action of these enzymes has yet to be firmly

established. It is likely that an agonist-receptor complex could interact with more than one type of G-protein and that an activated G-protein could interact with more than one effector system (Neer and Clapham, 1988).

Several reports have described the inhibitory effects of PKC activators on receptor-mediated  $\text{PIP}_2$  turnover and increase in intracellular  $\text{Ca}^{2+}$ , suggesting a role of PKC in the termination of signal transduction (Zavoico et al., 1985; Krishnamurthi et al., 1989). This event was shown by a rapid return to basal levels of the initial rise of  $\text{IP}_3$  and  $\text{Ca}^{2+}$  after the early phase of cell stimulation (Figure 5). The exact mechanism by which PKC exerts a negative feedback regulation on the agonist-induced increases in intracellular  $\text{Ca}^{2+}$  and inositol phosphate formation is incompletely understood. There are suggestions that PKC may directly modulate PLC enzyme or may exert its effect on PLC activity via inhibition of Gp or activation of GTPase.

In general, it is clear that many signalling pathways are interrelated to each other with respect to PKC activation and their product formation. Schematic representation of agonist-induced membrane phospholipid degradation is illustrated in Figure 6. A bacterial toxin-insensitive G-protein (Gp) couples a receptor occupied by an agonist to a PI-specific-PLC whose activation is possibly an early event in cell activation resulting in  $\text{PIP}_2$  hydrolysis,  $\text{IP}_3$  and DAG formation. PLD is activated by both DAG-mediated PKC activation and  $\text{IP}_3$ -induced  $\text{Ca}^{2+}$  mobilization, and it generates PA, a source of DAG via PA phosphohydrolase. PKC activation, in turn, exerts a feedback inhibition of PI-PLC resulting in a decrease in  $\text{Ca}^{2+}$  release. The early rise in  $\text{Ca}^{2+}$  mediated by PI-PLC is a key

event in PLA<sub>2</sub> activation generating free unsaturated fatty acids and lysophospholipids which are potentiators of PKC activation. PKC, despite its negative effect on the Ca<sup>2+</sup> release, subsequently promotes PLA<sub>2</sub> activity by probably increasing its Ca<sup>2+</sup> sensitivity. Thus, PKC activated by DAG derived from both PIP<sub>2</sub> hydrolysis by PLC and PC hydrolysis by PLD has distinct regulatory effects on the three phospholipases C, D and A<sub>2</sub>. The mechanism by which PKC regulates these phospholipases is not clear. PKC may exert a direct effect on the enzyme or the G-proteins such as G<sub>p</sub>, G<sub>PLA2</sub> and G<sub>PLD</sub> even though the evidence for G<sub>PLA2</sub> and G<sub>PLD</sub> remains speculative.

#### **4. Phospholipid Metabolism in Endothelial Cells**

##### **a) Endothelial Cells**

Endothelial cells lie inside of the entire vascular system. The endothelium is now regarded as a distinct metabolic and endocrine organ of diverse capacities. First, endothelium contributes to regulation of vasomotor tone by secreting two well known vasodilator substances, prostacyclin (PGI<sub>2</sub>) and endothelial derived relaxing factor (EDRF). Endothelial cells also secrete endothelium dependent vasodilators such as ATP, acetylcholine and substance P, and at least one peptide vasoconstrictor substance, endothelin. These vasoactive substances are important in mediating the influence of flow on vasomotor tone, vascular geometry and angiogenesis.

A second function of endothelium is regulation of intravascular thrombosis. The endothelial cells secrete various platelet adhesion proteins including

thrombospondin, fibronectin, collagen and von Willebrand factor. On the other hand, PGI<sub>2</sub> and EDRF secreted by endothelial cells synergistically inhibit platelet activation. In addition, endothelium promotes coagulation through expression of tissue factor and secretion of factor V and plasminogen activator inhibitor. By contrast, endothelium inhibits coagulation through secretion of thrombomodulin and tissue plasminogen activator.

A third function of endothelium is the regulation of the extravasation of leukocytes by expression of adhesion proteins and secretion of interleukin-1 and platelet activating factor which is relevant to atherogenesis and to inflammatory response. In addition, endothelial secretion of mitogens and growth inhibitors of vascular smooth muscle cells may also be important in atherogenesis.

b) Phospholipid Composition of Endothelial Cells

Lipid analysis of endothelial cells was elaborately studied by Kito and coworkers (Takamura et al., 1990). These authors carefully compared the distribution of phospholipid molecular species between endothelial cells derived from artery and vein of human umbilical cord. Only a minor difference has been observed for phospholipid compositions of artery and vein endothelial cells. In these two kinds of cells, PC was the predominant phospholipid class (49 - 51%), followed by PE (26 - 28%), PS (9%), sphingomyelin (6 - 7%), PI (6%) and cardiolipin (2%).

Subclass composition studies revealed that PC consisted mostly as the diacyl form (91 - 97% of total PC). The alkylacyl subclass of PC which is the precursor of platelet activating factor was about three time higher in artery

endothelial cells (7%) than in vein endothelial cells (3%). The main subclasses of PE were diacyl and alkenylacyl forms. Equal amount of diacyl- and alkenylacyl-forms of PE were found in artery endothelial cells, whereas in vein endothelial cells, the diacyl subclass of PE was about twice that of the alkenylacyl subclass. PI, PS and cardiolipin were mainly in diacyl forms.

Artery endothelial cells had higher amounts of phospholipid classes and subclasses containing arachidonic acid than in vein endothelial cells. The relative percentages of total phospholipids containing arachidonic acid were 24% and 14% in artery and in vein endothelial cells, respectively. In artery endothelial cells, arachidonic acid was highest in alkenylacyl PE, followed by diacyl PC, diacyl PE and PI, whereas in vein endothelial cells, arachidonic acid was highest in PI, followed by diacyl PE, diacyl PC and alkenylacyl PE. The higher amount of phospholipid molecular species containing arachidonic acid in artery endothelial cells may be related to the functional differences between artery and vein, e.g. higher formation of prostacyclin was found in artery than in vein endothelial cells (Johnson, 1980).

c) Molecular Mechanisms of Thrombin-Induced Endothelial Cell Activation

Thrombin, a key regulatory protein of hemostasis, is a potent stimulus of endothelial cell activation which is involved in multiple pathological vascular disorders such as ischemia, thrombosis, inflammation and atherosclerosis (Fenton, 1988). Activation of thrombin receptor is mediated by a novel mechanism by which, after thrombin binding, a specific cleavage of the receptor by thrombin occurs at the arginine residue 41 (R41) carboxyl to the amino terminus, releasing

an extracellular portion of the receptor (Vu et al., 1991). Proteolysis of the receptor generates a new amino-terminus which functions as a built-in activating ligand.

Thrombin-induced receptor cleavage is linked with PI-PLC activation, an early event associated with a transient rise of intracellular  $\text{Ca}^{2+}$  from the secretagogue  $\text{IP}_3$  (Brock and Capasso, 1988). PLC activation by thrombin is directly associated with a GTP binding protein (termed  $G_p$  or  $G_{PLC}$ ). This was confirmed from the studies using  $\text{GTP}_{\gamma}\text{S}$ , a G-protein activator. Addition of  $\text{GTP}_{\gamma}\text{S}$  to permeabilized human endothelial cells caused an increase in  $\text{IP}_3$  formation (Brock et al., 1988; Brock and Capasso, 1989). In addition, preincubation of endothelial cells with  $\text{GDP}_{\gamma}\text{S}$ , a G-protein inhibitor, markedly attenuated both thrombin and NaF-induced  $\text{Ca}^{2+}$  increase but did not inhibit the rise in  $\text{Ca}^{2+}$  induced by  $\text{Ca}^{2+}$  ionophore A23187 (Garcia et al., 1991), providing further support that activation of PI-PLC by thrombin is directly coupled with a G-protein. This GTP-binding protein ( $G_p$ ) coupled thrombin receptor to PLC was found to be insensitive to bacterial toxin including botulinum toxin C, cholera toxin, and pertussis toxin (Garcia et al., 1991; Brock and Capasso, 1989).

Kinetic analysis of the formation of  $\text{IP}_3$ , the increase in intracellular  $\text{Ca}^{2+}$  and the release of  $\text{PGI}_2$  suggested a sequential activation of PLC and  $\text{PLA}_2$  by thrombin (Jaffe et al., 1987). Thrombin-induced activation of  $\text{PLA}_2$  was tightly linked with the increase of intracellular  $\text{Ca}^{2+}$  since chelation of intracellular  $\text{Ca}^{2+}$  attenuated  $\text{PGI}_2$  synthesis, whereas chelation of extracellular  $\text{Ca}^{2+}$  only mildly inhibited  $\text{PGI}_2$  production induced by thrombin (Jaffe et al., 1987; Hallam et al.,

1988). Thrombin-induced activation of PLA<sub>2</sub> also leads to the formation of another potent lipid autocoid, platelet activating factor (PAF) (Prescott et al., 1984). Synthesis of PAF by endothelial cells was found to couple with G-protein activation and entry of extracellular Ca<sup>2+</sup> through membrane Ca<sup>2+</sup> channel (Whatley et al., 1990).

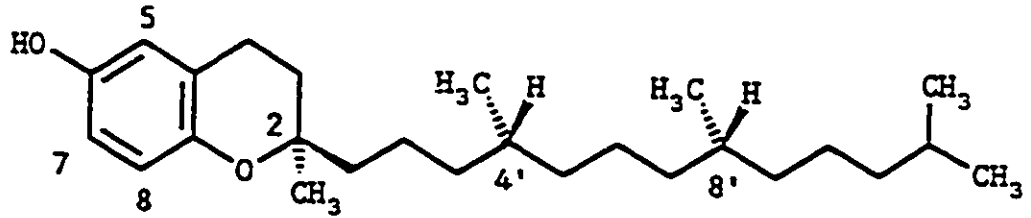
Thrombin-induced hydrolysis of PIP<sub>2</sub> in endothelial cells generates another product, DAG, a known endogenous activator of PKC (Brock and Capasso, 1988). Receptor activation with thrombin or direct G-protein activation with NaF induces a rapid accumulation and translocation of cytosolic PKC to the membranes (Heller et al., 1991; Garcia et al., 1992a). Moreover, activation of PKC by phorbol ester was found to inhibit both thrombin-mediated and NaF-mediated IP<sub>3</sub> formation, and to attenuate thrombin-induced Ca<sup>2+</sup>-mobilization, suggesting that thrombin exerts a feedback inhibition on PI-PLC activity. Despite the effect of PKC activation in lowering the intracellular Ca<sup>2+</sup> concentrations, PKC was found to enhance PLA<sub>2</sub> activity characterized by arachidonate release, PGI<sub>2</sub> and PAF synthesis induced by thrombin, NaF or Ca<sup>2+</sup> ionophore A23187 (Whatley et al., 1989; Heller et al., 1991; Garcia et al., 1992a). Thus, PKC activation appears to up-regulate PLA<sub>2</sub> activity possibly through an increase in Ca<sup>2+</sup> sensitivity of the enzyme (Carter et al., 1989), although additional mechanisms may exist.

Similar to PLA<sub>2</sub>, both the increase in Ca<sup>2+</sup> and activation of membrane PKC are required for thrombin-stimulated PLD activity (Garcia et al., 1992b). PLD activity in endothelial cells was identified by a marked increase in phosphatidylethanol formation induced by thrombin in the presence of ethanol.

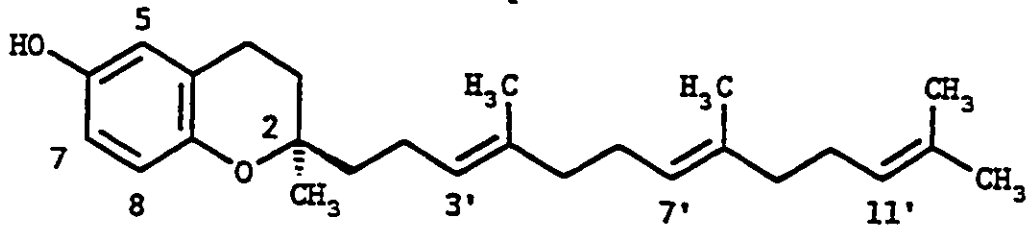
Chelation of intracellular  $Ca^{2+}$ , inhibition of PKC by staurosporin or PKC down-regulation by prolonged incubation with PMA significantly reduced thrombin-induced phosphatidylethanol formation. In addition, it appears that  $G_s$  - a G-protein subunit regulated cyclic AMP formation - is responsible for the coupling of thrombin receptor to PLD, since cholera toxin, forskolin, 8-bromo cyclic AMP and dibutyryl cyclic AMP were found to enhance  $\alpha$ -thrombin-stimulated PLD activity.

The interaction of thrombin with endothelial cell surface receptor and the resultant increase in intracellular  $Ca^{2+}$  and PKC activation are correlated with the induction of cellular proliferation (Moscat et al., 1987), the generation of protein C (Esmon and Owen, 1981), the synthesis of membrane-associated platelet activating factor (Prescott et al., 1984), the synthesis and release of tissue plasminogen activator (Levine et al., 1984), platelet derived growth factor (Harlan et al., 1986) and various coagulant proteins (Galdal et al., 1985; Levine et al., 1982). Thrombin was also found to stimulate leukocyte-endothelial cell adhesion (Zimmerman et al., 1985; Bizios et al., 1988), and to cause endothelial cell contraction leading to barrier dysfunction and increase in vascular permeability (Lynch et al., 1990; Stasek and Garcia, 1992). Thus, it seems that perturbation of endothelial cells functions mediated through agonist-receptor activation is associated with amplification of inflammatory responses and atherogenesis.

**Figure 1 - Structures of tocopherols and tocotrienols with vitamin E activity  
(from Machlin, 1991).**



*Tocopherol*



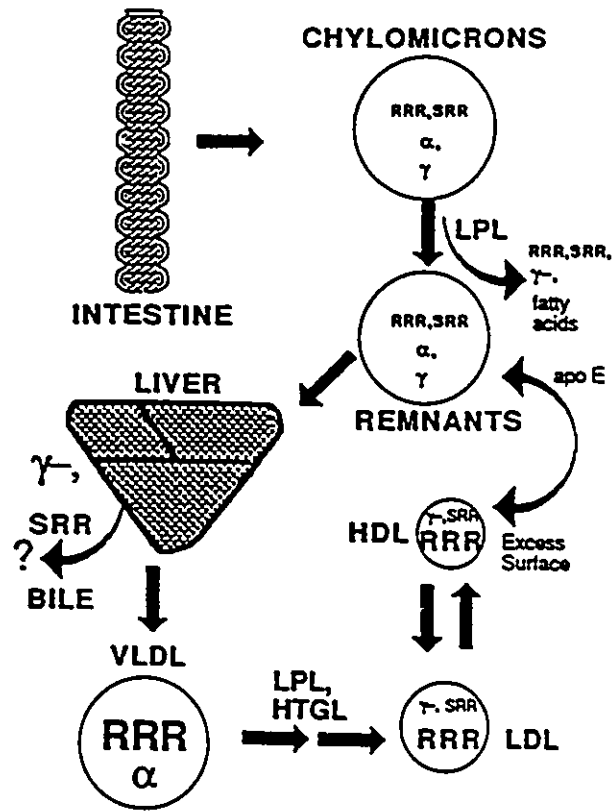
*Tocotrienol*

Position of Methyl Groups	Trivial Names (Abbreviations)	
	Tocopherols	Tocotrienols
5, 7, 8	$\alpha$ -tocopherol ( $\alpha$ -T)	$\alpha$ -tocotrienol ( $\alpha$ -T-3)
5, 8	$\beta$ -tocopherol ( $\beta$ -T)	$\beta$ -tocotrienol ( $\beta$ -T-3)
7, 8	$\gamma$ -tocopherol ( $\gamma$ -T)	$\gamma$ -tocotrienol ( $\gamma$ -T-3)
8	$\delta$ -tocopherol ( $\delta$ -T)	$\delta$ -tocotrienol ( $\delta$ -T-3)

**Figure 2 - Schematic representation for the absorption and circulation of vitamin E (from Kayden and Traber, 1993).**

Various forms of vitamin E: RRR- $\alpha$ -, SRR- $\alpha$ -, RRR- $\gamma$ - or SRR - $\gamma$ -tocopherols.

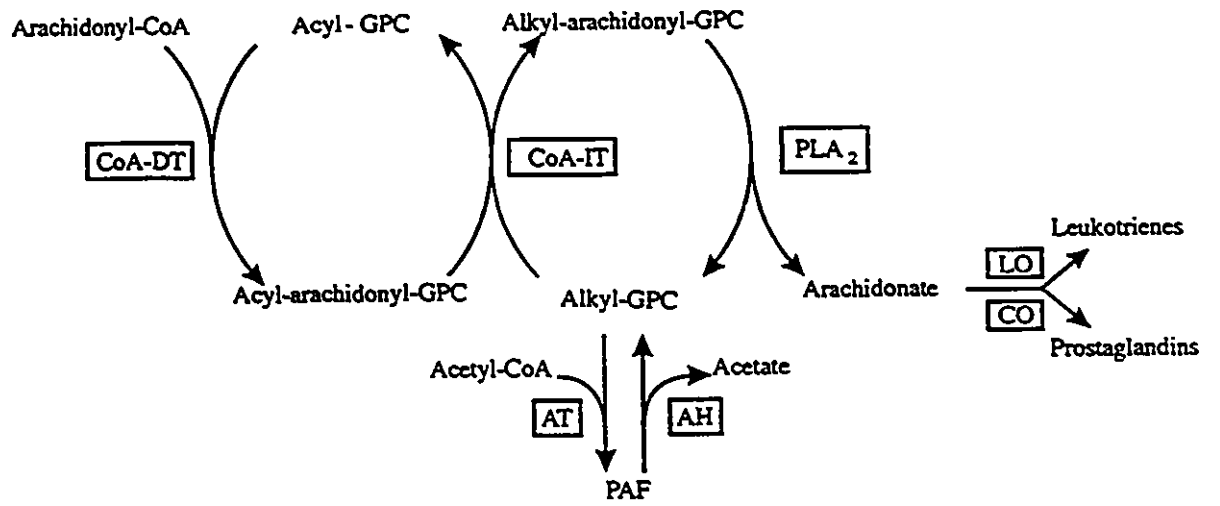
LPL:	lipoprotein lipase
HDL:	high-density lipoprotein
VLDL:	very-low-density lipoprotein
LDL:	low-density lipoprotein
HTGL:	hepatic triglyceride lipase
apoE:	apolipoprotein E



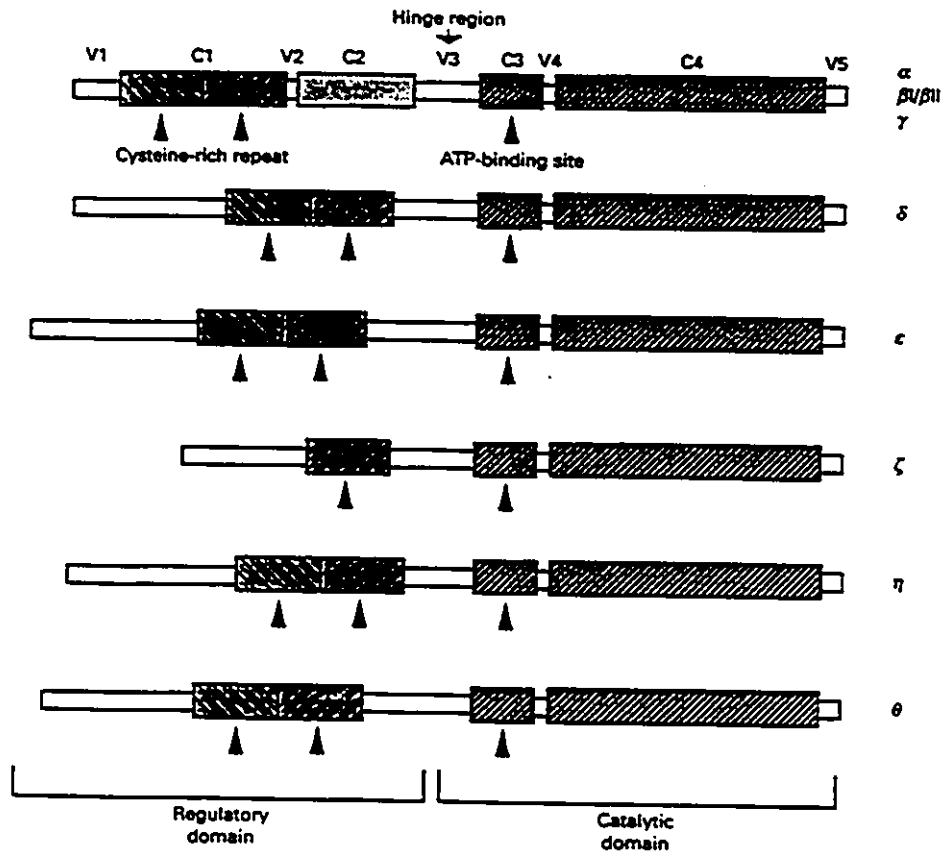
**Figure 3 - Scheme of the incorporation and mobilization of arachidonic acid**

**(from Tran et al., 1993a).**

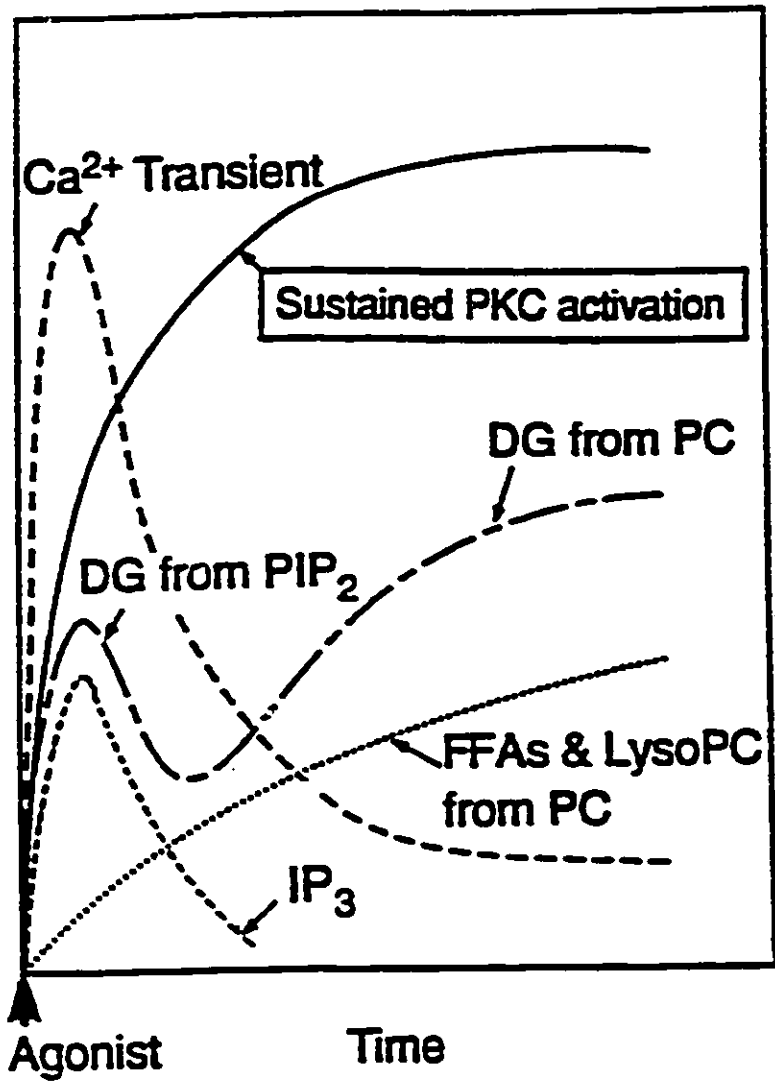
CoA-DT:	CoA-dependent acyltransferase
CoA-IT:	CoA-independent transacylase
PLA <sub>2</sub> :	phospholipase A <sub>2</sub>
LO:	lipoxygenase
CO:	cyclooxygenase
AT:	acetyltransferase
AH:	acetylhydrolase



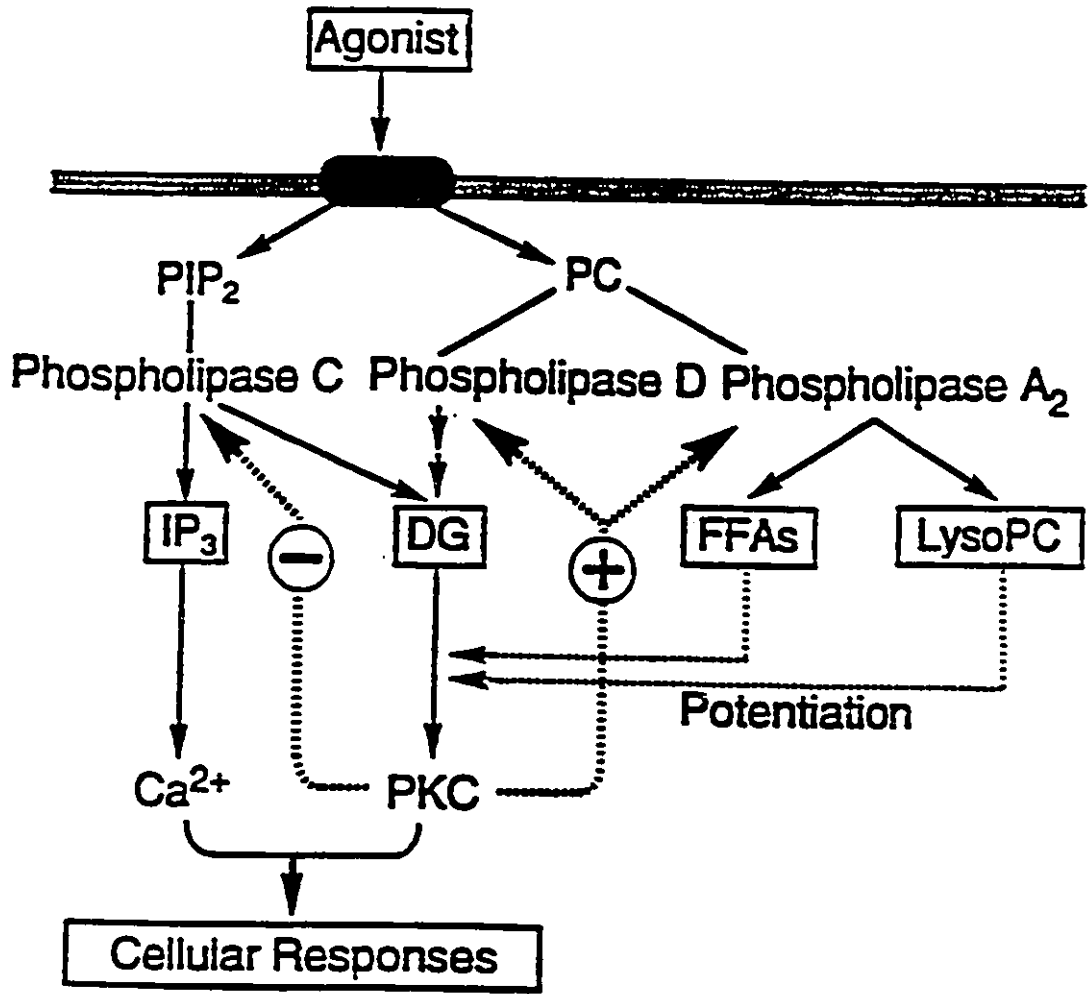
**Figure 4 - Structures of PKC isoforms (from Hug and Sarre, 1993).**



**Figure 5 - Time course of various signalling molecules generation (from Nishizuka, 1992).**



**Figure 6 - Schematic representation of agonist-induced membrane phospholipid degradation (from Nishizuka, 1992).**



## OBJECTIVES

- (1) To investigate the effect of vitamin E on the acylation processes of cholinephosphoglycerides in human vascular endothelial cells.
  
- (2) To study the role of vitamin E in the formation of cellular signalling molecules particularly diacylglycerol and phosphatidic acid in thrombin-stimulated endothelial cells.
  
- (3) To study the involvement of vitamin E in controlling protein kinase C activity in resting and phorbol ester-stimulated endothelial cells.
  
- (4) To delineate the mechanisms of vitamin E regeneration by water soluble reductants using endogenous vitamin E from human platelets as substrate and exogenous arachidonic acid as oxidant.

## CHAPTER 1

# VITAMIN E ENHANCES THE ACYLATION OF 1-ALKYL-sn-GLYCERO-3-PHOSPHORYLCHOLINE AND 1-ACYL-sn-GLYCERO-3-PHOSPHORYLCHOLINE IN HUMAN ENDOTHELIAL CELLS

### I. SUMMARY

The acylation of 1-acyl-sn-glycero-3-phosphorylcholine (acyl-GPC) and 1-0-alkyl-sn-glycero-3-phosphorylcholine (alkyl-GPC) is mediated by the CoA-dependent acyltransferase and CoA-independent transacylase reactions, respectively. These acylation processes lead to mobilization and enrichment of arachidonic acid into specific phospholipid pools. We reported earlier that vitamin E altered the phospholipid turnover in the endothelial cells by increasing the arachidonic acid release and prostacyclin synthesis (Tran and Chan, 1988; 1990). In this study, the role of vitamin E on the acylation of alkyl-GPC and acyl-GPC was investigated. Incubation of endothelial cells with vitamin E resulted in an increase in the formation of [<sup>3</sup>H]-alkylacyl-GPC and [<sup>14</sup>C]-diacyl-GPC from [<sup>3</sup>H]-alkyl-GPC and [<sup>14</sup>C]-acyl-GPC, respectively. The effect of vitamin E was dose-dependent at concentrations below 23 μM. When endothelial cells were incubated with vitamin

E analogs, only limited enhancement of the acylation process was detected. However, vitamin E did not have any direct effect on both enzyme activities. When endothelial cells were incubated with vitamin E, the CoA-independent transacylase activities in cell homogenate were found to be enhanced. Kinetic analysis of the transacylase activity in the vitamin E pre-incubated cells showed that the enhancement of the enzyme activity was at the enzyme-substrate level. By contrast, there was no enhancement of the CoA-dependent acyltransferase activity by vitamin E. It is suggested that vitamin E enhanced the acylation of alkyl-GPC in a very specific manner by an indirect stimulation of the CoA-independent transacylase activity, whereas the increased acylation of acyl-GPC by vitamin E might occur at the level other than CoA-dependent acyltransferase. These results indicate that the regulation of vitamin E in the formation of alkylacyl-GPC and diacyl-GPC may alter the transfer of arachidonate from diacylphospholipid pool to the ether-linked phospholipid pool.

## II. INTRODUCTION

The turnover of the existing cellular phospholipids involves the deacylation and reacylation process that results in the mobilization and turnover of fatty acids in the biological membranes. Agonist-induced activation of phospholipase A<sub>2</sub> (PLA<sub>2</sub>) results in the release of the acyl groups, usually polyunsaturated fatty acids, from the sn-2 position of a glycerophospholipid. Cholineglycerophospholipids are the main substrates of PLA<sub>2</sub> and consist of two subclasses which are 1-O-alkyl-2-acyl-sn-glycero-3-phosphorylcholine (alkylacyl-GPC) and 1,2-diacyl-sn-glycero-3-phosphorylcholine (diacyl-GPC). Hydrolysis of these cholineglycerophospholipids by PLA<sub>2</sub> releases arachidonate and generates lysophospholipids (alkyl-GPC or acyl-GPC). Arachidonate release is the rate limiting step for eicosanoid synthesis (Waite, 1985), whereas acetylation of alkyl-GPC results in the formation of platelet activating factor (PAF) which has diverse pathophysiological functions (Braquet et al., 1987; Snyder, 1990; Prescott et al., 1990). Inactivation of PAF by acetylhydrolase results in the regeneration of alkyl-GPC. Both alkyl-GPC and acyl-GPC are cytolytic at high concentrations due to their detergent property (Weltzien, 1979; Herrmann and Newmann, 1986). It has been found that these two lysophospholipids are readily acylated back to their precursors by two different mechanisms. Acylation of acyl-GPC involves the action of acyl-CoA-dependent acyltransferase which uses acyl-CoA and acyl-GPC as substrates (Lands and Merkl, 1963; Robinson et al., 1985). By contrast, acylation of alkyl-GPC is

catalyzed by acyl-CoA-independent acyltransferase which directly transfers an acyl group from a diacylphospholipid to alkyl-GPC (Robinson et al., 1985; Kramer et al., 1984b; Malone et al., 1985). Both acyl-CoA-dependent and independent acyltransferase were found to prefer polyenoyl groups such as arachidonoyl (Robinson et al., 1985; Kramer et al., 1984a, 1984b; Malone et al., 1985; Wilson et al., 1982, Chilton and Murphy, 1986; Sugiura et al., 1987; Kramer and Deykin, 1983; Chilton et al., 1983; Masuzawa et al., 1989; Winkler et al., 1991). These reacylation processes therefore result in the mobilization of arachidonic acid between phospholipid pools and thus provide arachidonate available for eicosanoid synthesis in the next stimulation (Chilton et al., 1991).

Vascular endothelial cells regulate the vascular tone and homeostasis through synthesis of various potent lipid mediators such as PAF and prostacyclin ( $\text{PGI}_2$ ) (Prescott et al., 1990; Moncada et al., 1976). PAF has been found to act as a chemotactic factor that recruits neutrophils to bind to the endothelium (Zimmerman et al, 1990). On the other hand,  $\text{PGI}_2$ , a potent vasodilator and platelet antiaggregator, is synthesized from the conversion of arachidonate by  $\text{PGI}_2$  synthetase in the cyclooxygenase pathway (Moncada et al., 1976).

We have recently reported that vitamin E, a membrane soluble chain breaking antioxidant, enhances arachidonate release and  $\text{PGI}_2$  synthesis in human endothelial cells (Tran and Chan, 1988; 1990). This was explained by the ability of vitamin E to potentiate the hydrolysis of phosphatidylcholine by  $\text{PLA}_2$  (Tran and Chan, 1990). However, it is still unknown whether vitamin E has any effect in the acylation processes that may enrich arachidonic acid in the cholineglycero-

phospholipids, thus increasing arachidonate availability for PGI<sub>2</sub> synthesis. In the present study, the effect of vitamin E on the acylation of alkyl-GPC and acyl-GPC in human endothelial cells was determined. Our study showed that the conversion of alkyl-GPC to alkylacyl-GPC and acyl-GPC to diacyl-GPC in endothelial cells was potentiated by incubation with a physiological concentration of vitamin E.

### III. MATERIALS AND METHODS

1-O-[alkyl-1,2-<sup>3</sup>H]-sn-glycero-3-phosphorylcholine (52 Ci/mmol) and 1-[palmitoyl-<sup>14</sup>C]-sn-glycero-3-phosphorylcholine (55 mCi/mmol) were from New England Nuclear (Lachine, PQ, Canada). 1-O-alkyl-sn-glycero-3-phosphorylcholine, 1-palmitoyl-sn-glycero-3-phosphorylcholine, arachidonoyl-CoA, linoleoyl-CoA, heparin, collagenase type IV, gentamicin sulfate, all rac- $\alpha$ -tocopherol acetate and all standard lipids were from Sigma Chemical Co. (St. Louis, MO, USA). Medium 199, penicillin G sodium (10,000 units/ml), streptomycin sulfate (10,000  $\mu$ g/ml), fungizone (250  $\mu$ g Amphotericin B and 205  $\mu$ g deoxycholate/ml), trypsin-EDTA, heat inactivated fetal bovine serum and all tissue culture plasticware were from Gibco Ltd. (Burlington, ON, Canada). Endothelial cell growth supplement was from Collaborative Research (Bedford, MA, USA). RRR- $\alpha$ -tocopherol was donated by the Vitamin E Research Information Services (La Grange, IL, USA); tocol,  $\beta$ -,  $\gamma$ - and  $\delta$ -tocopherol were gifts from Eisai co. Ltd. (Tokyo, Japan); Trolox<sup>R</sup> was from Aldrich (Milwaukee, WI, USA). Thin layer chromatography plates (silica gel 60A) were from Canlab (Mississauga, ON, Canada). All solvents used were h.p.l.c. grade and were purchased from BDH Chemical Inc. (Toronto, ON, Canada). All glassware was silanized before use.

#### 1. Culture Medium

Medium 199 (pH 7.4) was supplemented with heparin (90  $\mu$ g/ml), Hepes (25

mM), gentamicin sulfate (40 µg/ml), sodium penicillin G (100 units/ml), streptomycin sulfate (100 µg/ml), fungizone (2.5 µg amphotericin B / ml) and heat-inactivated fetal bovine serum (10%). The endothelial cell growth supplement (30 µg/ml) was added into the culture dishes after each feeding.

The stock medium 199 (pH 7.4) containing heparin, Hepes and gentamicin sulfate was prepared in large quantity and stored after being sterilized by filtering through a 0.22 µm filter purchased from Millipore (Bedford, MA, USA).

The culture medium supplemented with antibiotic/antimycotic and fetal bovine serum was freshly prepared and was not stored for more than two weeks.

The stock of endothelial cell growth supplement (ECGS) was prepared by dissolving lyophilized ECGS in serum-free medium 199 to a concentration of 3 mg/ml. The ECGS solution was stored in small volume at -20°C. At each time of changing new medium, 10 µl of stock ECGS was added to each ml of medium to obtain a final concentration of 30 µg/ml.

## **2. Culture of Endothelial Cells**

Endothelial cells were isolated and cultured from human umbilical veins according to the method of Jaffe (Jaffe, 1984). Fresh human umbilical cords were obtained from the Case Room of the Ottawa General Hospital (Ottawa, ON). The portions of the cords having any clamp marks or needle holes were removed. Cord vein, largest in diameter as compared with the two cord arteries, was cannulated with the tubings of butterfly needles and was flushed with 50 ml of warm phosphate-buffered saline (PBS), pH 7.4, to remove any residual blood. The

vein was then filled with 2-5 ml of 0.2% collagenase (type IV) in PBS, and was incubated in the tissue culture incubator for 15 min. The collagenase/cell mixture was flushed with PBS into a 50 ml plastic conical centrifuge tube containing 10 ml of culture medium. The cells were sedimented by light centrifugation at 1000 rpm (Beckman TJ-6) for 10 min; the cell pellet was resuspended in 10 ml of culture medium and plated on a 0.2% gelatin-precoated 100 mm petri dish. Maximum attachment of the cells to the bottom of the dish was obtained after overnight incubation in a cell culture incubator (5% CO<sub>2</sub> / 95% air); unattached cells and any contaminated red blood cells were removed by rinsing the dish with warmed PBS and 10 ml of fresh medium plus 100 µl of stock ECGS was added. The medium was changed every 2-3 days.

Gentle massage to the cord after incubation with collagenase increased cell yield and confluency was reached in 5-7 days.

Cells were detached by using trypsin-EDTA and subcultured in a 1:3 ratio. Briefly, the culture dish was rinsed twice with warm PBS, and the cell monolayer was incubated with 2 ml of 0.05% trypsin - 0.53 mM EDTA for 3-5 min at 37°C. About 10 ml of culture medium was added to stop trypsin digestion, the cells were collected, sedimented by centrifugation and subcultured as described above. Cells used in all experiments were at passage 1. The cells were identified as endothelial origin by the appearance of cobblestone shape using phase contrast microscopy and by the presence of factor VIII-related antigen by immunofluorescent microscopy (Chan and Tran, 1990).

### **3. Tocopherol Enrichment**

Medium enriched with  $\alpha$ -tocopherol or its analogues was prepared as follows. A microliter volume of  $\alpha$ -tocopherol or its analogues carried in DMSO was added into appropriate volumes of heat-inactivated fetal bovine serum. Final concentrations of DMSO and the serum present in the cultured medium were 0.2% and 10%, respectively. The mixture was vortexed and incubated in a 37°C waterbath for 10 min in the dark. Medium 199 and antibiotic/antimycotic solution were added into the vitamin E-enriched serum, and the medium was further incubated for 10 min before adding to the cells.

### **4. Incorporation of Alkyl-GPC and Acyl-GPC**

To study the effect of vitamin E on the acylation processes, cell monolayers were first enriched with vitamin E and then incubated with either [ $^3\text{H}$ ]-alkyl-GPC or [ $^{14}\text{C}$ ]-acyl-GPC. The acylation processes were determined by the formation of corresponding radiolabelled diradyl-GPC.

Cell monolayers in 35 mm dishes were first incubated with vitamin E at indicated concentrations for 4 h in culture medium containing 10% fetal bovine serum. Cells were rinsed three times with serum-free medium 199 and were further incubated with [ $^3\text{H}$ ]-alkyl-GPC or [ $^{14}\text{C}$ ]-acyl-GPC in a serum-free medium. After 1.5 h of incubation, cells were rinsed three times with 10 mM HEPES-buffered solution (HBS) containing 0.1% BSA and were scraped in 1 ml of ice-cold methanol/HCl (50:1, v/v). Cellular lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959) in the presence of 20  $\mu\text{g}$  of either alkylacyl-GPC

/alkyl-GPC or diacyl-/acyl-GPC as carrier. Alkyl-GPC or acyl-GPC ( $R_f = 0.23$ ) and alkylacyl-GPC or diacyl-GPC ( $R_f = 0.67$ ) were separated by thin layer chromatography using a solvent system consisting of chloroform/methanol/acetic acid/water (50:30:8:5, by volume). After being visualized by iodine vapour, the spots corresponding to authentic standards were scraped into scintillation vials and their radioactivities were determined by liquid-scintillation spectroscopy.

## **5. CoA-Dependent Acyltransferase and CoA-Independent Transacylase Assays**

The activities of these two enzymes in cell homogenates were determined by the conversion [ $^3\text{H}$ ]-alkyl-GPC or [ $^{14}\text{C}$ ]-acyl-GPC into [ $^3\text{H}$ ]-alkylacyl-GPC or [ $^{14}\text{C}$ ]-diacyl-GPC, respectively, in the presence or absence of acyl-CoA. After being enriched with indicated concentrations of vitamin E for 4 h, cells in 100 mm dishes were scraped in ice-cold PBS, pH 7.4. Cells were sedimented and cell pellets were resuspended in ice-cold Tris-HCl buffer (75 mM, pH 8.5, 1 mM EDTA). Cell suspensions were sonicated for three times at 10 s each using an ultrasonic cell disruptor regulated at 50% output of energy. Acyl-CoA acyltransferase activity was determined in an assay mixture containing 0.2  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]-acyl-GPC, 40  $\mu\text{M}$  of linoleoyl-CoA or arachidonoyl-CoA, 60-130  $\mu\text{g}$  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 [ $^{14}\text{C}$ ]-acyl-GPC, and the mixture was incubated for 5-15 min at 37°C. The reaction was terminated by the addition of 0.5 ml of methanol/acetatic acid (9:1, v/v). Total lipids were extracted by

chloroform/methanol and separated by thin layer chromatography. [<sup>14</sup>C]-Diacyl-GPC radioactivity was quantitated as described above. Determination of the transacylase activity was similar of that of acyl-CoA acyltransferase except that it was conducted using [<sup>3</sup>H]-alkyl-GPC as substrate and in the absence of acyl-CoA. Protein was determined by the method of Lowry et al. (Lowry et al., 1951).

## **6. Statistics**

Student's t test was used for statistical analysis. The level of significance was set at  $p < 0.05$ .

#### IV. RESULTS

##### 1. $\alpha$ -Tocopherol Enhances the Formation of Alkylacyl-GPC and Diacyl-GPC

In this study, we sought to determine the effects of vitamin E on the formation of alkylacyl-GPC and diacyl-GPC from its lyso-compounds (missing an acyl group at sn-2 position) such as alkyl-GPC and acyl-GPC, respectively. Our previous study (Chan and Tran, 1990) showed that endothelial cells incorporated  $\alpha$ -tocopherol linearly up to 8 h of incubation. Thus, we routinely enriched the cells with  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h, at which time it was convenient to work and provided sufficient  $\alpha$ -tocopherol enrichment. Cells from either  $\alpha$ -tocopherol treated or non-treated groups were incubated with [ $^3$ H]-alkyl-GPC or [ $^{14}$ C]-acyl-GPC for indicated times.

The acylation process of [ $^3$ H]-alkyl-GPC and [ $^{14}$ C]-acyl-GPC in endothelial cells were dependent on the time of incubation. Figure 1-1A and 1-1B showed that at different times of incubation with [ $^3$ H]-alkyl-GPC or [ $^{14}$ C]-acyl-GPC, there was a time dependent increase in the formation of [ $^3$ H]-alkylacyl-GPC or [ $^{14}$ C]-diacyl-GPC, respectively. This was accompanied by a corresponding decrease of incorporated labelled substrates (lyso-compounds). Determination of radioactivity for total cellular uptake revealed that maximum incorporation of [ $^3$ H]-alkyl-GPC and [ $^{14}$ C]-acyl-GPC into endothelial cells was reached at 30 min, after which there was no change in the total radioactivity inside the cells (data not presented). This

indicated that the fast incorporation of lyso-compounds into endothelial cells is followed by their removal processes. One of these processes involves the acylation reaction which is time dependent.

Figure 1-2A and 1-2B showed that no difference in the total radioactivity was observed in both cells treated with or without  $\alpha$ -tocopherol. The interference of total cellular radioactivity by non-specific binding of [ $^3$ H]-alkyl-GPC or [ $^{14}$ C]-acyl-GPC could be ruled out since the cells were washed with 0.1% BSA in HBS before lipid extraction was conducted. These results indicate that the enrichment of  $\alpha$ -tocopherol in cellular membrane did not interfere with the incorporation of [ $^3$ H]-alkyl-GPC and [ $^{14}$ C]-acyl-GPC. In addition, the formation of [ $^3$ H]-alkylacyl-GPC and [ $^{14}$ C]-diacyl-GPC from their corresponding substrates were found to be enhanced in cells preenriched with  $\alpha$ -tocopherol. The increase in formation of diradyl-GPC induced by  $\alpha$ -tocopherol was concomitant with the decrease in the corresponding intracellular lyso-compounds (Figures 1-2A and 1-2B).

The incorporation of [ $^3$ H]-alkyl-GPC and [ $^{14}$ C]-acyl-GPC and their conversion into corresponding diradyl-GPC were dependent on the concentration of  $\alpha$ -tocopherol in the medium (Figure 1-3A and 1-3B). Our previous studies (Chan and Tran, 1990) showed that  $\alpha$ -tocopherol uptake by endothelial cells was dose-dependent and maximum incorporation of  $\alpha$ -tocopherol into cellular membrane was attained at 92.8  $\mu$ M (or 40 mg/dl) which was four times higher than normal physiological concentration found in human plasma (23.2  $\mu$ M or 10 mg/dl). In these studies, cells were enriched with indicated concentrations of  $\alpha$ -tocopherol and then incubated with [ $^3$ H]-alkyl-GPC or [ $^{14}$ C]-acyl-GPC in order to determine the

$\alpha$ -tocopherol concentration that will elicit maximum effect. Figures 1-3A and 1-3B showed that the formation of [ $^3\text{H}$ ]-alkyl-GPC and [ $^{14}\text{C}$ ]-acyl-GPC was attained maximally at 23  $\mu\text{M}$  of  $\alpha$ -tocopherol, respectively. However, the increase in the formation of both diradyl compounds began to reach a plateau at 23  $\mu\text{M}$  of  $\alpha$ -tocopherol. The increase in the formation of diradyl compounds with respect to  $\alpha$ -tocopherol concentration shown in Figures 1-3A and 1-3B was concomitant with the decrease in their corresponding incorporated substrates as previously observed in Figures 1-2A and 1-2B.

## **2. $\alpha$ -Tocopherol has Highest Effect Among its Analogues on Alkylacyl-GPC and Diacyl-GPC Formation.**

Both the hydroxyl group and the anchorage sites (phytyl side chain and methyl groups) of tocopherol play a crucial role for its biological activity in the membrane. In this study, the effect of different tocopherol analogues in the formation of alkylacyl-GPC and diacyl-GPC was tested in order to determine the structural specificity of the tocopherol molecule required for this activity. Endothelial cells were incubated with  $\alpha$ -tocopherol or its analogues (23  $\mu\text{M}$ ) for 4 h followed by labelling with [ $^3\text{H}$ ]-alkyl-GPC or [ $^{14}\text{C}$ ]-acyl-GPC for 1.5 h. Table 1-1 showed that the formation of alkyl-GPC and diacyl-GPC were highest in the cells enriched with  $\alpha$ -tocopherol.  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols which lack one methyl group on the chromanol ring, only partially induced the increase in the formation of alkylacyl-GPC and diacyl-GPC when compared with  $\alpha$ -tocopherol. Tocol, a tocopherol analogue lacking all the three methyl groups on the chromanol ring, had

similar effect as  $\beta$ -,  $\gamma$ -, and  $\delta$ -tocopherols. When the phytyl side chain of the tocopherol molecule was missing, as of Trolox, or when the hydroxyl group on the chromanol ring was quenched by an acetyl group, as of tocopherol acetate, incubation of cells with these molecules resulted in a complete abolishing of the tocopherol effect on the formation of alkyl-GPC and diacyl-GPC. Taken together, results in Table 1-1 indicated that the hydroxyl group, the methyl groups and the phytyl side chain were all necessary for the tocopherol molecule to maximally perform its effect.

### **3. The Effects of $\alpha$ -Tocopherol on Alkylacyl-GPC and Diacyl-GPC Formation are Dependent on the Concentrations of Substrates**

Although the above studies clearly indicate  $\alpha$ -tocopherol enhanced diradyl-GPC synthesis in endothelial cells, results from some experiments showed that the enhancing effect of  $\alpha$ -tocopherol was essentially abolished when cells were incubated with high concentrations of alkyl-GPC or acyl-GPC. We therefore conducted the following experiments to find out the range of substrate concentrations at which the effect of  $\alpha$ -tocopherol was still observable. When endothelial cells were exposed to increasing concentrations of alkyl-GPC (from 5 to 5000 nM) or acyl-GPC (from 2-50  $\mu$ M), there was a proportional increase in the formation of alkylacyl-GPC or diacyl-GPC, respectively in both vitamin E-treated and non-treated groups (Tables 1-2a and 1-2b). However, the potentiating effect of vitamin E on alkylacyl-GPC or diacyl-GPC formation was gradually diminished with increasing concentrations of their corresponding substrates. The vitamin E

effect was completely abolished at 5  $\mu\text{M}$  of alkyl-GPC or at 25  $\mu\text{M}$  of acyl-GPC as shown in Table 1-2a and 1-2b.

We therefore conducted further studies to find out whether increasing  $\alpha$ -tocopherol concentrations could restore its potentiating effect on diradyl-GPC formations when the cells were exposed to high concentrations of substrates. Endothelial cells were first enriched with increasing concentrations of  $\alpha$ -tocopherol (from 0 to 93  $\mu\text{M}$ ) and then the cells were labelled with 2  $\mu\text{M}$  of [ $^3\text{H}$ ]-alkyl-GPC or 20  $\mu\text{M}$  of [ $^{14}\text{C}$ ]-acyl-GPC. Results from Table 1-3 showed that the formation of alkylacyl-GPC and diacyl-GPC by high substrate concentrations was unchanged irrespective to different levels of  $\alpha$ -tocopherol.

Taken together, results from the above studies indicated that  $\alpha$ -tocopherol at either physiological or pharmacological concentrations lost its potentiating effect on the formation of diradyl-GPC when the cells were exposed to more than 2  $\mu\text{M}$  of alkyl-GPC or 20  $\mu\text{M}$  of acyl-GPC.

#### **4. $\alpha$ -Tocopherol is an Activator of the CoA-Independent Transacylase but not CoA-Dependent Acyltransferase**

To investigate whether  $\alpha$ -tocopherol had any effect on the enzyme responsible for the acylation process, the next studies dealt with enzyme assays and enzyme kinetics of the CoA-independent acyltransferase, also called transacylase, and the CoA-dependent acyltransferase. Cell homogenates obtained from cells enriched with or without  $\alpha$ -tocopherol were used to measure the activities of these enzymes in the presence and absence of acyl-CoA such as

linoleoyl-CoA (18:2-CoA) or arachidonoyl CoA (20:4-CoA). Results from Table 1-4a showed that  $\alpha$ -tocopherol caused a two-fold increase in the formation of alkylacyl-GPC, and the acylation process of alkyl-GPC was independent of added acyl-CoA. In other words, the activity of the enzyme responsible for the formation of alkylacyl-GPC was increased almost 2-fold by  $\alpha$ -tocopherol irrespective to the presence or absence of exogenously added linoleoyl-CoA or arachidonoyl-CoA. This enzyme is called CoA-independent acyltransferase or transacylase which is responsible for transfer of arachidonoyl or polyenoyl group from a diacylphospholipid to an ether-linked lysophospholipid, such as alkyl-GPC (Kramer and Deykin, 1983). By contrast, the activity of the enzyme responsible for the acylation of acyl-GPC to diacyl-GPC was dependent on the presence of acyl-CoA. This enzyme is termed CoA-dependent acyltransferase (Robinson et al., 1985). Results from Table 1-4b showed that the activity of the enzyme was significantly increased when linoleoyl-CoA or arachidonoyl-CoA was present in the assay mixture. Surprisingly, the activity of CoA-dependent acyltransferase was not affected by  $\alpha$ -tocopherol. Table 1-4b showed that there was no difference in the enzyme activities of both  $\alpha$ -tocopherol treated or non-treated cell homogenates.

We next examined the effect of  $\alpha$ -tocopherol on the kinetics of these enzymes. Assays were performed by using cell homogenates as the source of enzyme obtained from cells previously enriched with or without  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h. The activities were measured as function of low concentrations of substrates ranging from 25 to 1000 nM for alkyl-GPC and from 2 to 20  $\mu$ M for acyl-GPC. Results of the double reciprocal plot in Figure 1-4A showed that  $\alpha$ -

tocopherol activated the CoA-independent transacylase activity by the same mechanism as that of in the competitive inhibition in which the enzymes from  $\alpha$ -tocopherol treated and non-treated cell homogenates had different  $K_m$  (measurement of enzyme-substrate affinity), but same  $V_{max}$ . The similarity in  $V_{max}$  observed in the enzyme kinetics study of transacylase enzyme was in agreement with the above observation that  $\alpha$ -tocopherol began to lose its effect on alkylacyl-GPC formation when substrate concentrations approached micromolar concentration range (Table 1-2a). In addition, increasing  $\alpha$ -tocopherol concentrations also failed to generate any effect on the enzyme activities in the presence of high substrate concentrations (Table 1-5).

Kinetic analysis of the CoA-dependent acyltransferase, on the other hand, showed that the enzyme activity was not affected by  $\alpha$ -tocopherol. Figure 1-4B showed that there was no difference in the enzyme activities of both  $\alpha$ -tocopherol treated or non-treated cell homogenates throughout all of the tested concentrations of acyl-GPC.

In summary,  $\alpha$ -tocopherol enhanced the formation of alkylacyl-GPC and diacyl-GPC from alkyl-GPC and acyl-GPC, respectively. The presence of the free hydroxyl group, the phytyl side chain and the methyl groups on the tocopherol molecules were all required for its maximum effect. Enzyme kinetic analysis revealed that the enzyme CoA-independent acyltransferase was responsible for the formation of alkylacyl-GPC from alkyl-GPC, whereas the enzyme CoA-dependent acyltransferase was responsible for the formation of diacyl-GPC from acyl-GPC; and,  $\alpha$ -tocopherol activated the activity of CoA-independent acyltransferase but not of CoA-dependent acyltransferase activity.

## V. DISCUSSION

It is generally accepted that lysophospholipids are intermediates of phospholipid deacylation/reacylation processes. Alkyl-GPC and acyl-GPC, two lysophospholipids of the cholinephosphoglyceride, can be generated by either PLA<sub>2</sub> or PAF acetylhydrolase that hydrolyzes the corresponding diradyl-GPC. In endothelial cells, phospholipid analysis revealed that these exist both in alkylacyl-GPC and diacyl-GPC, in which diacyl subclass is the most abundant (Takamura et al., 1990). Similarly, it was found that agonist stimulated endothelial cells produced at least two PAF subclasses in which the 1-acyl analog is predominantly higher than the 1-O-alkyl analog (Whatley et al., 1992). Thus, hydrolysis at the sn-2 position of these choline phosphoglycerides in endothelial cells will obviously generate both alkyl-GPC and acyl-GPC. However, the relative amount of these lysophospholipids is not known at present. In this study, we are the first to show that the acylation processes of alkyl-GPC and acyl-GPC are enhanced by vitamin E. Our study suggests a new role of this vitamin in the regeneration of the precursors of PAF and the synthesis of eicosanoids 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 the change in affinity of the substrate to the enzyme. However, the enhancement of the enzyme activity might not have resulted from the direct effect 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 facile explanation is that the stimulation of enzyme activity was caused by the direct action of 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.

Vitamin E was found to potentiate the acylation process of acyl-GPC without any effect on the CoA-dependent acyltransferase. It is plausible that the increase in acylation process of acyl-GPC is mediated via a membrane-associated mechanism by which the enhancement of the enzyme activity occurs only in intact cells but not after homogenization. Alternatively, the increased acylation of acyl-GPC by vitamin E could result from an increase in acyl-CoA formation. It is speculated that vitamin E alters the acyl-CoA synthetase activity, resulting in increased acyl-CoA formation and, thus, leading to increased acylation of acyl-GPC. Whether vitamin E has any effect on the acyl-CoA synthetase awaits further investigation.

It was recently found that both CoA-dependent and CoA-independent acylation processes were stimulated by activators of protein kinase C such as phorbol esters (Kanzaki et al., 1989; Breton and Colard, 1991; Ninio et al., 1991). It was suggested that these enzymes are modulated by protein kinase C. Therefore, it is plausible that there is a link between vitamin E and protein kinase C in the regulation of these processes.

The acylation of both acyl-GPC and alkyl-GPC has major implications in pathophysiology of the cells due to the ability to incorporate and mobilize arachidonate between phospholipid pools. It has been shown that CoA-dependent

acyltransferase prefer arachidonyl CoA as substrate among the acyl-CoAs tested (Robinson et al., 1985; Wilson et al., 1982), and the CoA-independent transacylase selectively transfers arachidonate from a diacylphospholipid to an ether-linked lysophospholipid (Kramer et al., 1984b; Winkler et al. 1991). In human neutrophils, this selective transfer was found to result in arachidonate enrichment of the ether-phospholipid pool which is the precursor of PAF and the source of arachidonate release for leukotriene synthesis (Chilton et al., 1984; Chilton and Connell, 1988; Chilton, 1989). Therefore, the redistribution of arachidonate between phospholipid pools in endothelial cells may determine the level of PGI<sub>2</sub> formation.

We have demonstrated in a previous study that vitamin E enhances the release of arachidonic acid and the synthesis of PGI<sub>2</sub> (Tran and Chan, 1988; 1990). This was in part explained by the ability of vitamin E to alter PLA<sub>2</sub> activity, resulting in increased AA release for PGI<sub>2</sub> synthesis. Alternatively, the finding in this study suggests that the increased acylation processes by vitamin E may lead to the mobilization of arachidonate into specific phospholipid pools that is selectively hydrolyzed by an arachidonate-specific PLA<sub>2</sub> (Suga et al., 1990; Garcia et al., 1991).

The effective dosage of vitamin E on the stimulation of alkylacyl-GPC and diacyl-GPC formation bears some similarity with our previous reports on the enhancement of arachidonate release and PGI<sub>2</sub> synthesis in endothelial cells (Tran and Chan, 1988; 1990). Maximum effective concentration of vitamin E observed in all studies was similar to normal physiological concentration found in plasma (23 μM or 10 mg/dl). In addition, the structural specificity of the α-tocopherol molecule

to elicit the biological effect lends further support to the notion that vitamin E is a physiological factor for the regulation of the acylation and for transfer of the acyl groups between phospholipid pools.

## VI. CONCLUSION

The finding suggests a novel role in vitamin E in the regulation of phospholipid turnover. The increased formation of diradyl-GPC by vitamin E via the acylation process reveals the importance of vitamin E in the incorporation and redistribution of the arachidonate in membrane phospholipids. This provides a second explanation to how vitamin E enhances PGI<sub>2</sub> synthesis in endothelial cells apart from its ability to regulate PLA<sub>2</sub>. Whether vitamin E exerts its effects via its antioxidant property or via a membrane-associated mechanism is still open for further investigation.

**Table 1-1 - Effect of  $\alpha$ -tocopherol and its analogues on alkylacyl-GPC and diacyl-GPC formation.**

Cell monolayers were incubated with or without  $\alpha$ -tocopherol or its analogues (23  $\mu$ M) for 4 h followed by labelling with either 0.30  $\mu$ Ci of [ $^3$ H]-alkyl-GPC (7.8 nM) or 0.12  $\mu$ Ci of [ $^{14}$ C]-acyl-GPC (2  $\mu$ M) for 1.5 h. Lipid analysis and cellular radioactivity were determined as described in "Materials and Methods". Values are means of three dishes from a representative experiment out of three. Variations on S.D. were less than 10% of the means in all treatments.

Analogues	Radioactivity (% control)	
	Alkyl-acylGPC	Diacyl-GPC
Control	100	100
$\alpha$ -Tocopherol	200	163
$\beta$ -Tocopherol	140	128
$\gamma$ -Tocopherol	150	127
$\delta$ -Tocopherol	130	130
Tocol	142	120
Trolox	112	100
Tocopherol acetate	106	88

**Table 1-2a - Effects of  $\alpha$ -tocopherol enrichment on alkylacyl-GPC formation in intact cells at different alkyl-GPC concentrations.**

Cell monolayers were incubated with or without  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h followed by labelling for 1.5 h with 0.4  $\mu$ Ci of [ $^3$ H]-alkyl-GPC with varying amounts of unlabelled alkyl-GPC to give the indicated concentrations. Cells were subjected to lipid extraction and phospholipids were separated by thin layer chromatography as described under "Materials and Methods". Values are means  $\pm$  S.D. of four dishes. ND (no difference).

Alkyl-GPC (nM)	Alkylacyl-GPC Formation (pmol/dish)		% Difference
	Tocopherol ( $\mu$ M): 0	23	
5	0.81 $\pm$ 0.09	1.46 $\pm$ 0.17	+ 80
25	2.67 $\pm$ 0.20	4.73 $\pm$ 0.53	+ 77
50	4.71 $\pm$ 0.25	8.07 $\pm$ 0.45	+ 71
100	7.70 $\pm$ 0.52	11.10 $\pm$ 1.36	+ 44
200	14.55 $\pm$ 1.56	20.68 $\pm$ 2.48	+ 42
5000	310 $\pm$ 43	273 $\pm$ 20	ND

**Table 1-2b - Effects of  $\alpha$ -tocopherol enrichment on diacyl-GPC formation at different acyl-GPC concentrations.**

Cell monolayers were incubated with or without  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h followed by labelling for 1.5 h with 0.1  $\mu$ Ci of [ $^{14}$ C]-acyl-GPC with varying amounts of unlabelled acyl-GPC to give the indicated concentrations. Cells were subjected to lipid extraction and phospholipids were separated by thin layer chromatography as described under "Materials and Methods". Values are means  $\pm$  S.D. of four dishes. ND (no difference).

Acyl-GPC ( $\mu$ M)	Diacyl-GPC Formation (nmol/dish)		
	Tocopherol ( $\mu$ M): 0	23	% Difference
2	0.16 $\pm$ 0.02	0.27 $\pm$ 0.03	+ 69
5	0.46 $\pm$ 0.01	0.65 $\pm$ 0.04	+ 41
10	0.8 $\pm$ 0.07	1.24 $\pm$ 0.10	+ 40
25	1.91 $\pm$ 0.20	1.71 $\pm$ 0.15	ND
50	3.85 $\pm$ 0.35	3.60 $\pm$ 0.40	ND

**Table 1-3 - Formation of alkylacyl-GPC and diacyl-GPC in response to high concentrations of substrates and indicated levels of  $\alpha$ -tocopherol.**

Cell monolayers were enriched with indicated concentrations of  $\alpha$ -tocopherol for 4 h. The medium was removed and the cells were labelled with either 0.3  $\mu$ Ci of [ $^3$ H]-acyl-GPC (2000 nM) or 0.1  $\mu$ Ci of [ $^{14}$ C]-acyl-GPC (20  $\mu$ M). After 1.5 h, cells were subjected to lipid extraction and phospholipid separation as described under "Materials and Methods". Values are means  $\pm$  S.D. of three dishes.

<b>Tocopherol (<math>\mu</math>M)</b>	<b>Alkylacyl-GPC (pmol/dish)</b>	<b>Diacyl-GPC (nmol/dish)</b>
0	251 $\pm$ 41	2.42 $\pm$ 0.16
23	247 $\pm$ 16	2.49 $\pm$ 0.22
46	233 $\pm$ 10	2.64 $\pm$ 0.11
93	261 $\pm$ 17	2.41 $\pm$ 0.03

**Table 1-4a - Effects of  $\alpha$ -tocopherol on the activities of CoA-independent acyltransferase (transacylase) in cell homogenate.**

Cell monolayers were incubated with or without  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h. Transacylase activities of approximately 130  $\mu$ g protein homogenate were determined in an assay mixture containing 0.25  $\mu$ Ci of [ $^3$ H]-alkyl-GPC (50 nM) in the presence or absence of either 40  $\mu$ M of linoleoyl-CoA (18:2) or arachidonyl-CoA (20:4) for 15 min at 37°C. Values are means  $\pm$  S.D. of four determinations, from one out of three representative experiments.

Acyl CoA	Activities (pmol/mg/min)	
	Tocopherol ( $\mu$ M)	
	0	23
none	1.87 $\pm$ 0.20	3.10 $\pm$ 0.24*
18:2	1.82 $\pm$ 0.14	3.08 $\pm$ 0.18*
20:4	1.88 $\pm$ 0.23	2.80 $\pm$ 0.14*

\*p < 0.05

**Table 1-4b - Effects of  $\alpha$ -tocopherol enrichment on the activity of CoA-dependent acyltransferase in cell homogenates.**

Cell monolayers were incubated with or without  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h. CoA-dependent acyltransferase activities of approximate 130  $\mu$ g protein homogenates were determined in an assay mixture containing 0.14  $\mu$ Ci of [ $^{14}$ C]-acyl-GPC (5  $\mu$ M) in the presence or absence of either 40  $\mu$ M of linoleoyl-CoA (18:2) or arachidonyl-CoA (20:4) after 15 min of incubation at 37°C. Values are means  $\pm$  S.D. of four determinations, from one out of three representative experiments.

Acyl CoA	Activity (nmol/mg/15 min)	
	Tocopherol ( $\mu$ M)	
	0	23
none	0.54 $\pm$ 0.05	0.69 $\pm$ 0.03*
18:2	3.62 $\pm$ 0.40	3.91 $\pm$ 0.20*
20:4	1.30 $\pm$ 0.12	1.65 $\pm$ 0.10*

\*No difference

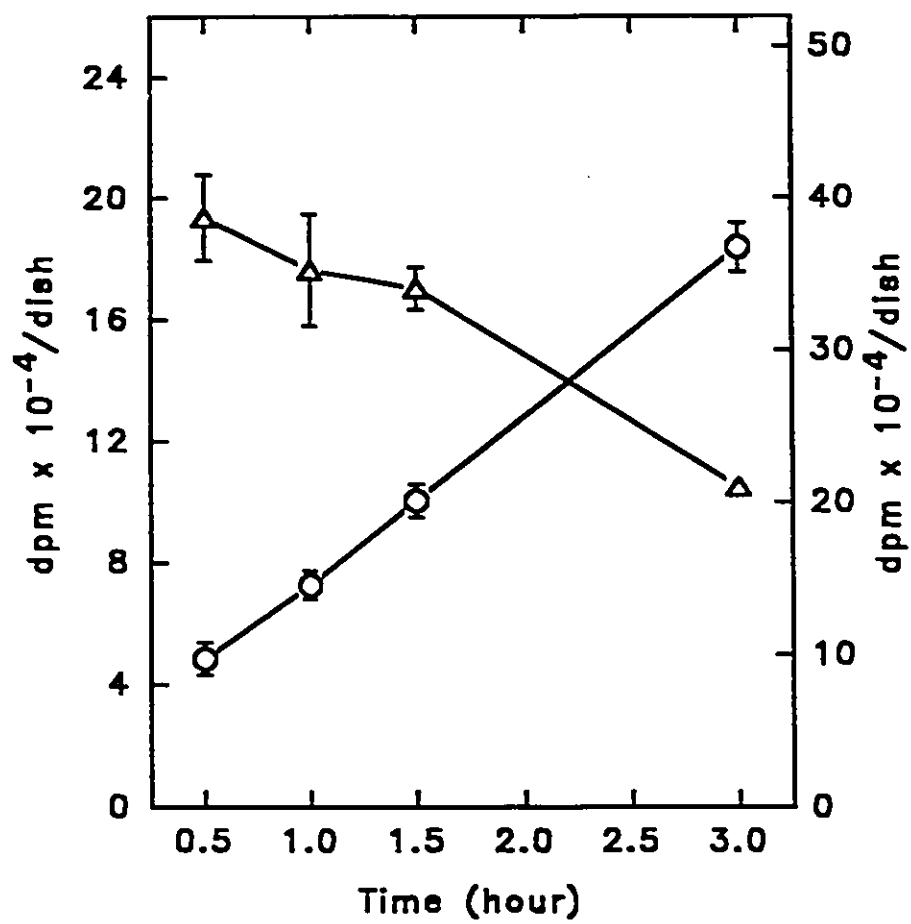
**Table 1-5 - Effect of high concentrations of substrates on the activities of transacylase and CoA-dependent acyltransferase from cells enriched with various concentrations of  $\alpha$ -tocopherol.**

Cell monolayers were enriched with indicated concentrations of  $\alpha$ -tocopherol for 4 h followed by sonication. Cell homogenates of approximately 60  $\mu$ g protein were incubated for 15 min at 37°C with 0.3  $\mu$ Ci of [ $^3$ H]-alkyl-GPC (2000 nM) or 0.1  $\mu$ Ci of [ $^{14}$ C]-acyl-GPC (20  $\mu$ M). Transacylase and CoA-dependent acyltransferase activities were determined by the formation of [ $^3$ H]-alkylacyl-GPC and [ $^{14}$ C]-diacyl-GPC, respectively, as described under "Materials and Methods". Values are means  $\pm$  S.D. of triplicates from a representative experiment out of two.

<b>Tocopherol (<math>\mu</math>M)</b>	<b>Transacylase (nmol/15 min/mg)</b>	<b>Acyltransferase (nmol/15 min/mg)</b>
0	3.49 $\pm$ 0.13	7.73 $\pm$ 0.18
23	2.98 $\pm$ 0.08	6.65 $\pm$ 0.17
46	2.85 $\pm$ 0.23	7.03 $\pm$ 0.22
93	3.27 $\pm$ 0.15	7.23 $\pm$ 0.54

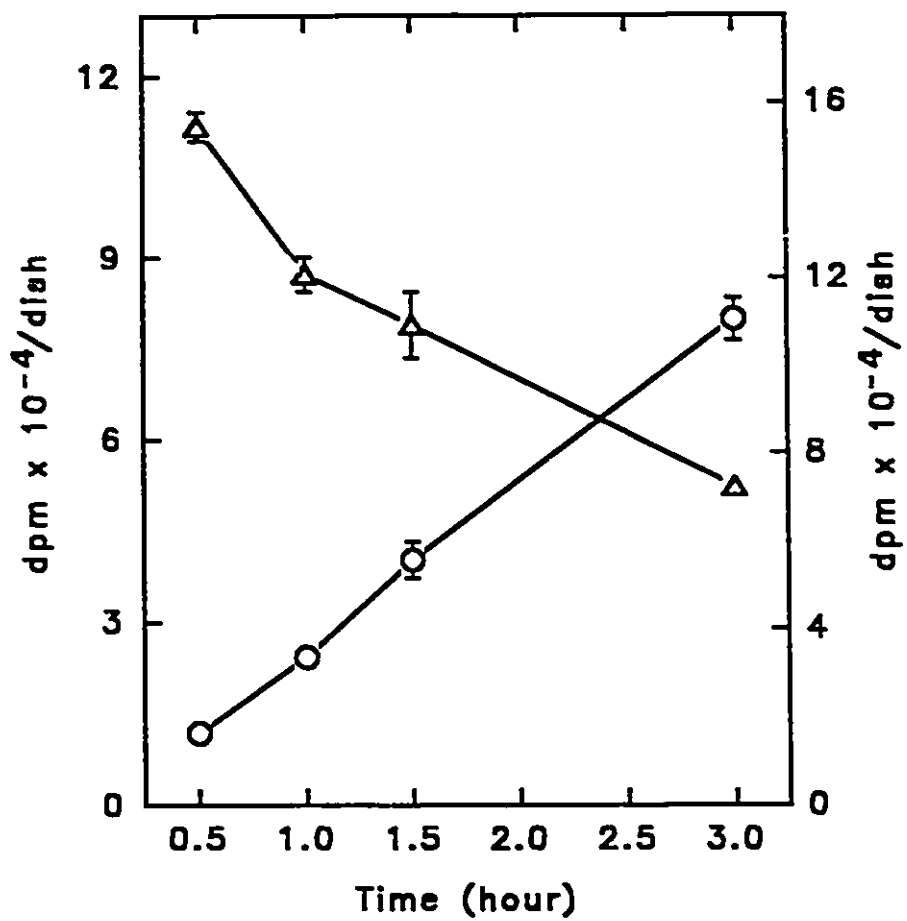
**Figure 1-1A - Time course for the incorporation of alkyl-GPC.**

Cell monolayers were incubated with 0.67  $\mu\text{Ci}$  of [ $^3\text{H}$ ]-alkyl-GPC (17 nM) for indicated times. Lipids analysis and cellular radioactivity determination were performed as described in "Materials and Methods". Radioactivities associated with alkyl-GPC ( $\Delta$ , right axis) and alkylacyl-GPC (O, left axis) were expressed as dpm/dish of cells. Values are means  $\pm$  S.D. of three dishes, from one out of two representative experiments.



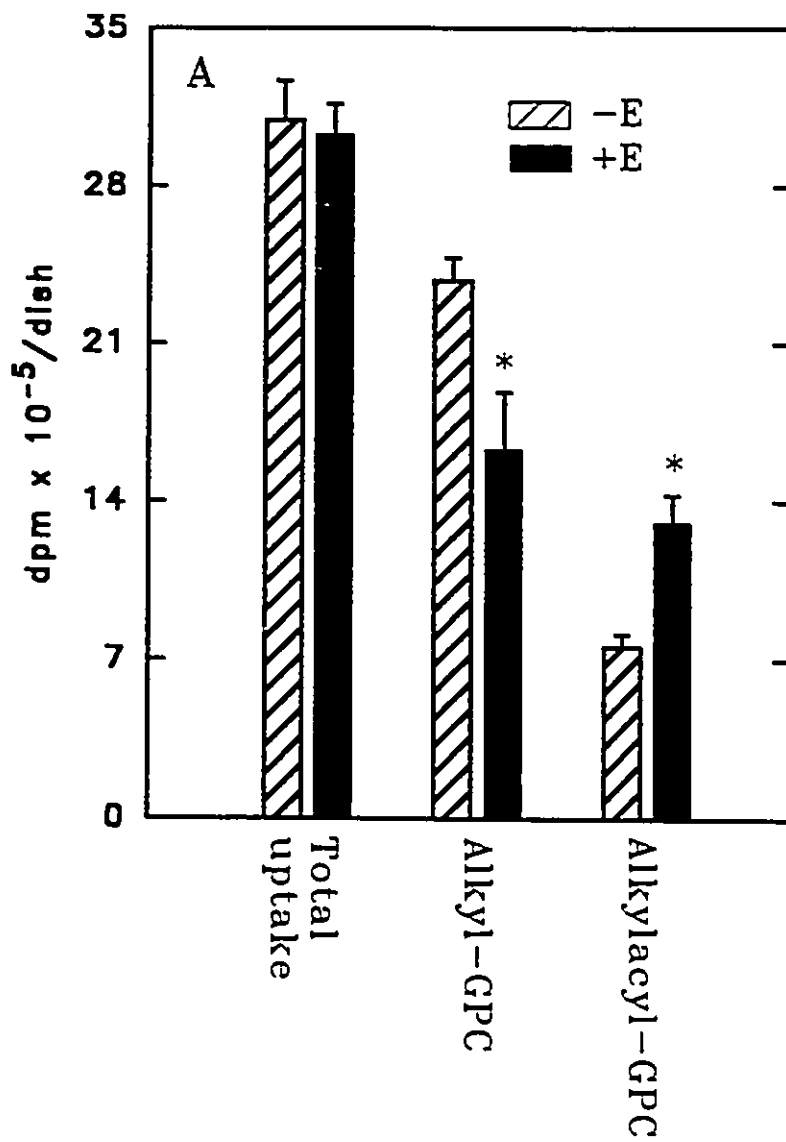
**Figure 1-1B - Time course for the incorporation of acyl-GPC.**

Cell monolayers were incubated with 0.13  $\mu\text{Ci}$  of [ $^{14}\text{C}$ ]-acyl-GPC (2  $\mu\text{M}$ ) for indicated times. Lipid analysis and cellular radioactivity determination were performed as described in "Materials and Methods". Radioactivities associated with acyl-GPC ( $\Delta$ , right axis) and diacyl-GPC (O, left axis) were expressed as dpm/dish of cells. Values are means  $\pm$  S.D. of three dishes, from one out of two representative experiments.



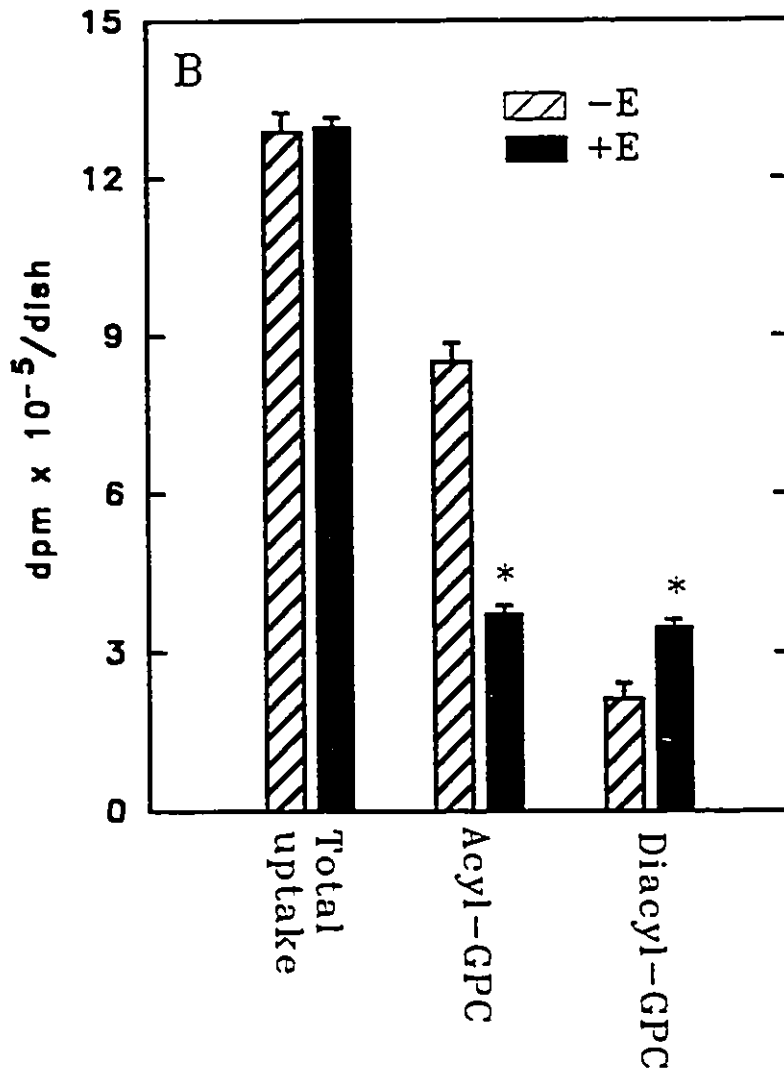
**Figure 1-2A - Effect of  $\alpha$ -tocopherol on the total cellular radioactivity and the synthesis of alkylacyl-GPC from alkyl-GPC.**

Cell monolayers were incubated with (+E) or without (-E)  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h followed by labelling with 4.8  $\mu$ Ci of [ $^3$ H]-alkyl-GPC (50 nM) for 1.5 h. Lipid analysis and cellular radioactivity determination were performed as described in "Materials and Methods". Values are means  $\pm$  S.D. of three dishes from one out of six representative experiments. \*p < 0.05.



**Figure 1-2B - Effect of  $\alpha$ -tocopherol on the total cellular radioactivity and the synthesis of diacyl-GPC from acyl-GPC.**

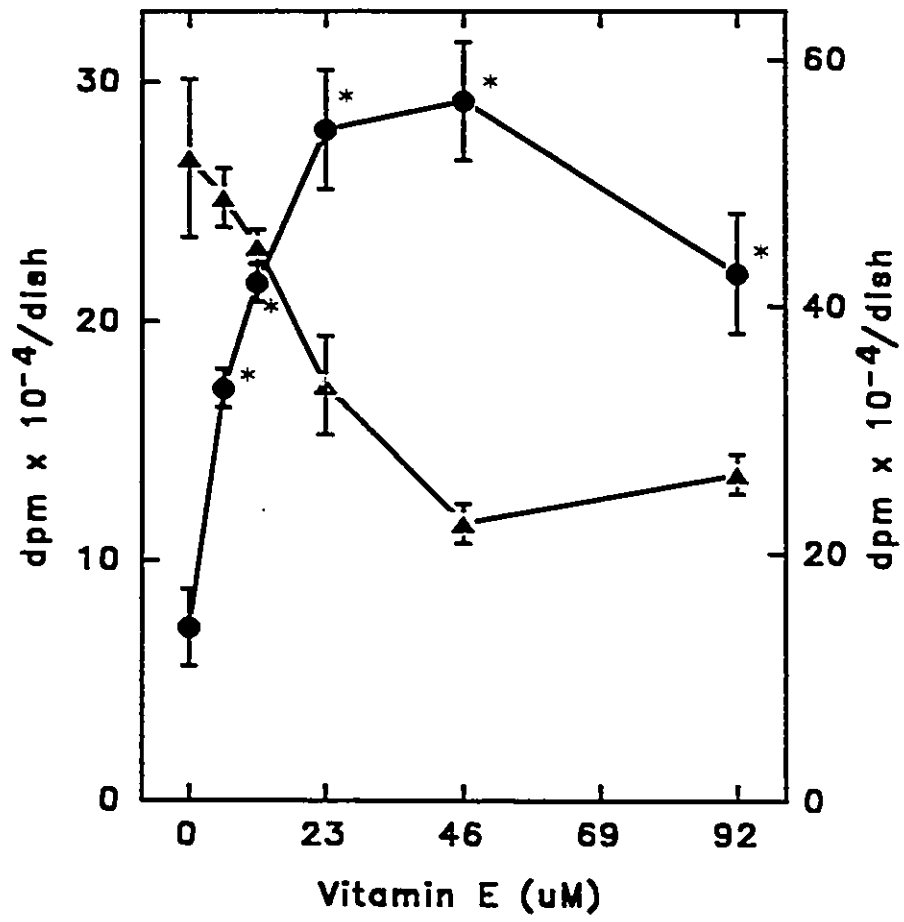
Cell monolayers were incubated with (+E) or without (-E)  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h followed by labelling with 0.1  $\mu$ Ci of [ $^{14}$ C]-acyl-GPC (2  $\mu$ M) for 1.5 h. Lipid analysis and cellular radioactivity determination were performed as described in "Materials and Methods". Values are means  $\pm$  S.D. of three dishes from one out of six representative experiments. \*p < 0.05.



**Figure 1-3A - Dose-dependent stimulation of  $\alpha$ -tocopherol on the formation of alkylacyl-GPC from alkyl-GPC.**

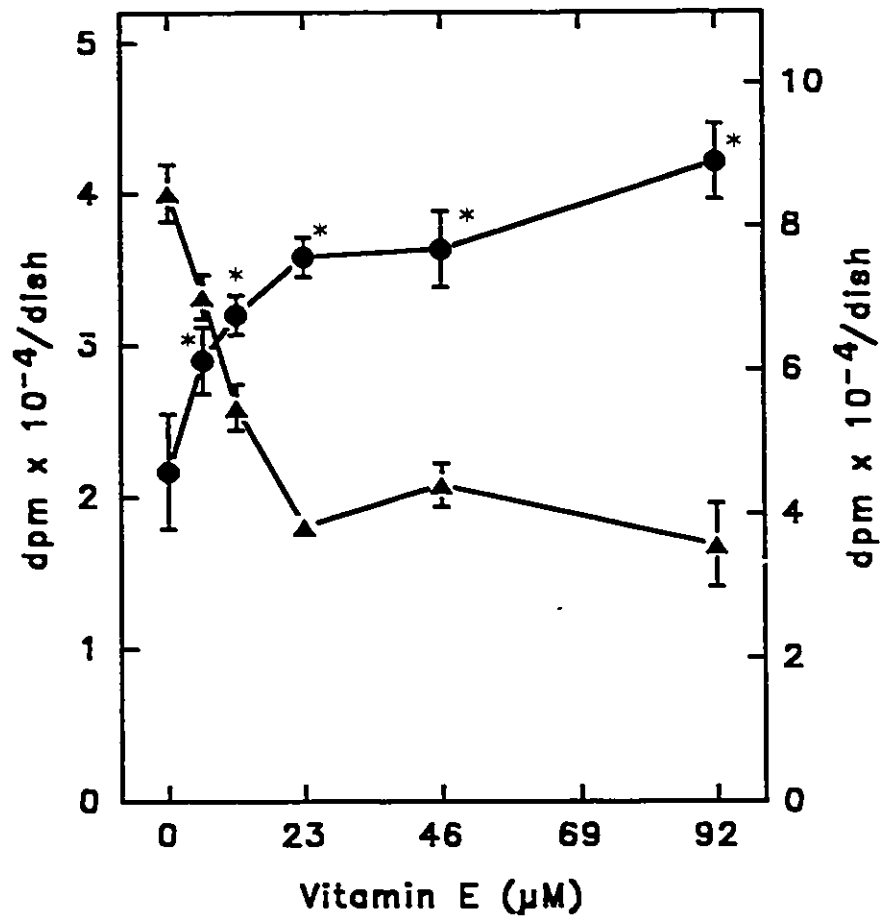
Cell monolayers were incubated with indicated concentrations of  $\alpha$ -tocopherol for 4 h followed by labelling with 0.67  $\mu$ Ci [ $^3$ H]-alkyl-GPC (17 nM) for 1.5 h. Lipid analysis and cellular radioactivity determination were performed as described in "Materials and Methods". Radioactivities associated with alkyl-GPC ( $\blacktriangle$ , right axis) and alkylacyl-GPC ( $\bullet$ , left axis) were expressed as dpm/dish of cells. Values are means  $\pm$  S.D. of three dishes from one out of three representative experiments.

\*p < 0.05.



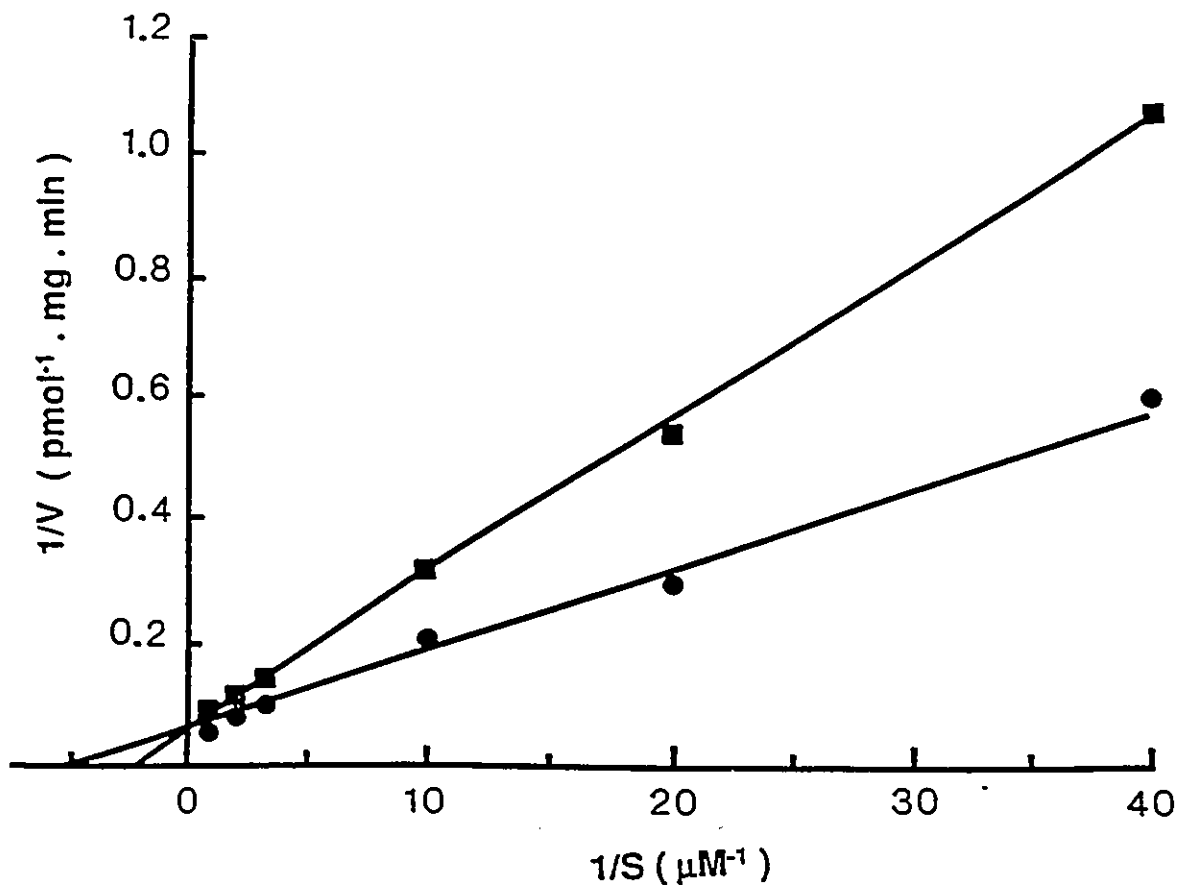
**Figure 1-3B - Dose-dependent stimulation of  $\alpha$ -tocopherol on the formation of diacyl-GPC from acyl-GPC.**

Cell monolayers were incubated with indicated concentrations of  $\alpha$ -tocopherol for 4 h followed by labelling with 0.08  $\mu$ Ci of [ $^{14}$ C]-acyl-GPC (1.4  $\mu$ M) for 1.5 h. Lipid analysis and cellular radioactivity determination were performed as described in "Materials and Methods". Radioactivities associated with acyl-GPC ( $\blacktriangle$ , right axis) and diacyl-GPC ( $\bullet$ , left axis) were expressed as dpm/dish of cells. Values are means  $\pm$  S.D. of three dishes from one out of three representative experiments. \* $p < 0.05$ .



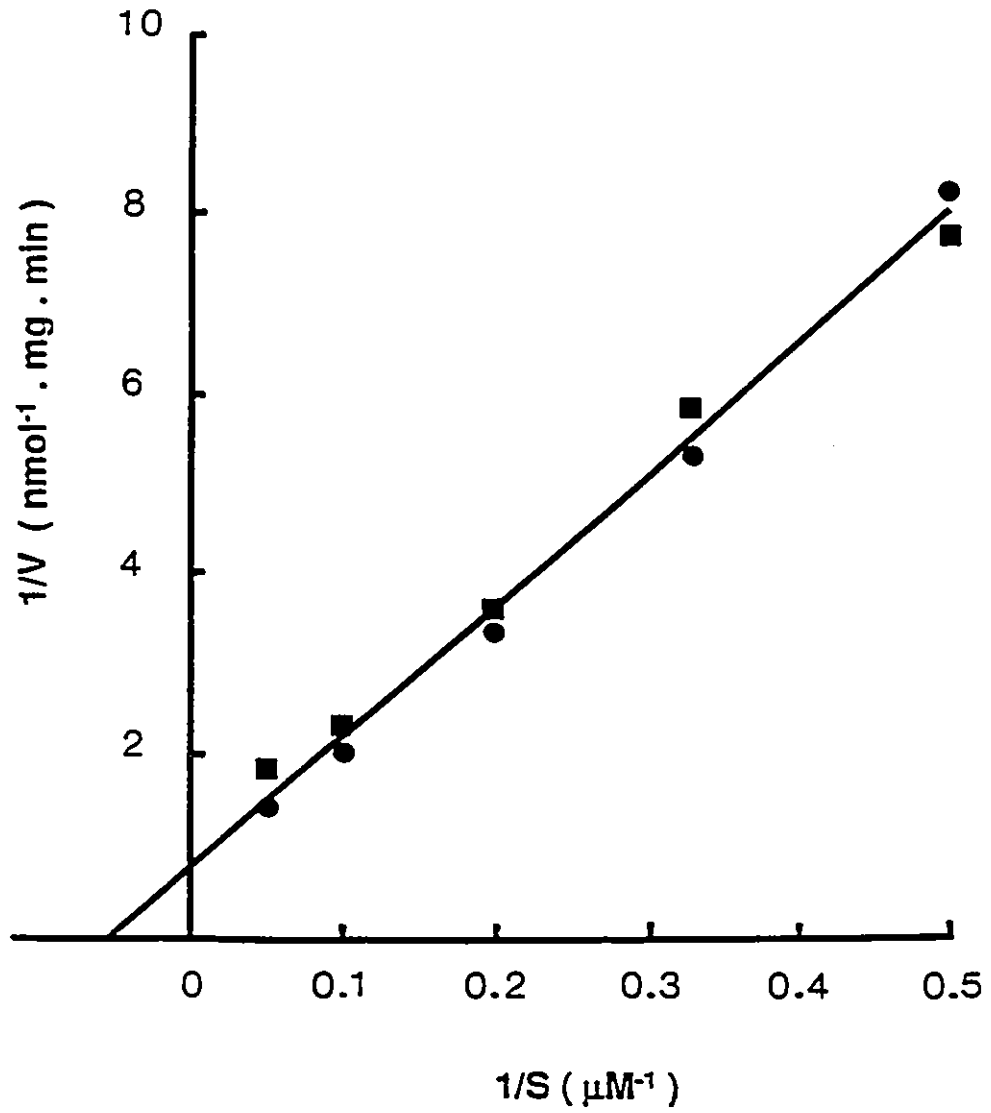
**Figure 1-4A - Double-reciprocal plot of CoA-independent transacylase activity from cells preincubated with or without  $\alpha$ -tocopherol.**

Cell monolayers were incubated with (●) or without (■)  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h. Cell homogenates (approx. 60  $\mu$ g protein) were incubated for 5 min at 37°C with varying concentrations of 0.2  $\mu$ Ci of [ $^3$ H]-alkyl-GPC. Transacylase activity was determined as described in "Materials and Methods". Each point represents the mean of two separate determinations, each performed in duplicate.



**Figure 1-4B - Double-reciprocal plot of CoA-dependent acyltransferase activity from cells preincubated with or without  $\alpha$ -tocopherol.**

Cell monolayers were incubated with (●) or without (■)  $\alpha$ -tocopherol (23  $\mu$ M) for 4 h. Cell homogenates (approx. 60  $\mu$ g protein) were incubated for 5 min at 37°C with varying concentrations of 0.04  $\mu$ Ci of [ $^{14}$ C]-acyl-GPC. Acyltransferase activity was determined as described in "Materials and Methods". Each point represents the mean of two separate determinations, each performed in duplicate.



**CHAPTER 2**

**VITAMIN E REGULATES THE LEVELS OF**

**DIACYLGLYCEROL (DAG) AND PHOSPHATIDIC ACID (PA)**

**IN THROMBIN-STIMULATED ENDOTHELIAL CELLS**

**I. SUMMARY**

The present study has examined the role of vitamin E in the production of diacylglycerol (DAG) and phosphatidic acid (PA) in thrombin-stimulated human endothelial cells. Cellular lipids were labelled with [<sup>3</sup>H]-myristate and stimulation was induced with thrombin. The incorporation and distribution of [<sup>3</sup>H]-myristate into cellular lipids was not affected by  $\alpha$ -tocopherol. However, in response to thrombin stimulation, more PA and less DAG formation were found in  $\alpha$ -tocopherol enriched cells as compared with control cells. Results from thrombin stimulation time course indicated that  $\alpha$ -tocopherol reduced the sustained DAG levels without affecting the transient accumulation of DAG. Direct determination of DAG mass further confirmed the role of  $\alpha$ -tocopherol in suppressing the accumulation of DAG induced by thrombin. In the presence of ethanol, the formation of [<sup>3</sup>H]-phosphatidylethanol (PEt) in [<sup>3</sup>H]-myristate labelled cells stimulated by thrombin was unaffected by  $\alpha$ -tocopherol. DL-propranolol, a PA phosphohydrolase inhibitor, caused an accumulation of PA, but did not affect DAG formation in both  $\alpha$ -tocopherol-treated and untreated cells. This indicated that the increase in PA and

decrease in DAG in  $\alpha$ -tocopherol treated cells was not due to a stimulation of phospholipase D or an inhibition of PA phosphohydrolase. Determination of inositol phosphates formation in response to thrombin showed that the change of sustained DAG levels elicited by  $\alpha$ -tocopherol was independent of phospholipase C-induced hydrolysis of inositol phospholipids. However, direct analysis of DAG kinase activity showed that  $\alpha$ -tocopherol enrichment enhanced the activity of the enzyme in both resting and thrombin-stimulated endothelial cells. Taken together, these data indicated that, in endothelial cells, the formation of DAG and PA in response to thrombin was derived from the activation of both phospholipase C and phospholipase D, and  $\alpha$ -tocopherol caused an increase conversion of DAG to PA by enhancing DAG kinase activity without any effect on phospholipase D, PA phosphohydrolase or phospholipase C.

## II. INTRODUCTION

Many human pathophysiological conditions such as aging, cardiovascular diseases and certain forms of cancers are related to oxidative stress and free radical induced damage. Nowadays, there is increased tendency to have recourse to natural antioxidants as a mean to alleviate or delay the onset of the above disorder conditions. Among the natural antioxidants, vitamin E has been widely recognized as a lipid-soluble chain breaking antioxidant and a membrane stabilizer (Burton et al., 1983; Burton and Ingold, 1986). The naturally occurring form of vitamin E is RRR- $\alpha$ -tocopherol. We and others have recently found that, after scavenging peroxy radical, oxidized  $\alpha$ -tocopherol can be reduced back to  $\alpha$ -tocopherol by vitamin C or reduced glutathione (Niki, 1987; McCay et al., 1989; Chan et al., 1991; Chan and Tran, 1993).

In addition to its unique role in protecting biological membranes from injury induced by free radicals, vitamin E was found to influence other biological processes such as the cyclooxygenase pathways of arachidonic acid metabolism (Panganamala and Cornwell, 1982; Stuart, 1982; Chan, 1993). We have also observed that  $\alpha$ -tocopherol suppressed the release of thromboxane  $A_2$  (TXA<sub>2</sub>) from thrombin-stimulated rat platelets (Hamelin and Chan, 1983) and potentiated the synthesis of prostacyclin (PGI<sub>2</sub>) from vascular endothelium (Chan and Leith, 1981; Tran and Chan, 1990). It was speculated that the regulation of TXA<sub>2</sub> and PGI<sub>2</sub> synthesis by vitamin E occurred at the level of arachidonic acid release catalyzed

by phospholipase A<sub>2</sub> (PLA<sub>2</sub>). This speculation was derived from the observations that α-tocopherol inhibited PLA<sub>2</sub> activity in platelets (Douglas et al., 1986; Cao et al., 1987) and increased arachidonic acid release from endothelial cells (Tran and Chan, 1988). However, it was still unclear how α-tocopherol could simultaneously regulate TXA<sub>2</sub> and PGI<sub>2</sub> synthesis in a completely opposite manner if it was simply mediated by PLA<sub>2</sub> activity. It is possible that the effect of α-tocopherol on PLA<sub>2</sub> results from its antioxidant property or from other mechanisms yet to be determined. Due to its unique property in specifically remaining in the membranes of most tissues, there is an increased tendency towards investigating the role of vitamin E in signal transduction processes usually occurred in plasma membranes.

Current literature generally accepted that the binding of hormones or neurotransmitters to specific receptors of many cell types and tissues trigger multiple processes of signal transduction operating in a tightly regulated manner to release various second messengers for many cellular responses (for review see Dennis et al., 1991). For instance, the binding of thrombin to its receptors in endothelial cells leads to the activation of phospholipase C (PLC), phospholipase D (PLD) and PLA<sub>2</sub> (Brock and Capasso, 1988; Garcia et al., 1992b; Whatley et al., 1989). Activation of endothelial phosphatidylinositol-specific PLC by thrombin hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP<sub>2</sub>) resulting in the accumulation of inositol 1,4,5-trisphosphate (IP<sub>3</sub>) and sn 1,2-diacylglycerol (DAG). However, there is now strong evidence that DAG is not solely generated from PIP<sub>2</sub>, but also from phosphatidylcholine (PC) in many cell types (Billah and Anthes, 1990; Exton, 1990) including endothelial cells (Ragab-Thomas et al., 1987). Both

PC-specific PLC and PLD activities were detected in endothelial cells and were responsible for the hydrolysis of PC to generate DAG and PA, respectively (Martin et al., 1987; Martin, 1988). Moreover, DAG and PA are readily interconverted by DAG kinase and PA phosphohydrolase via phosphorylation and dephosphorylation reaction. DAG is responsible for activating a large family of protein kinase C (PKC) isoenzymes (Nishisuka, 1992; Bell and Burns, 1991) while  $IP_3$  is a calcium secretagogue (Mitchell, 1986). The increase in intracellular calcium and PKC activation are intimately related to  $PLA_2$  activity which is a rate-limiting step in the synthesis of platelet activating factor (PAF) and  $PGI_2$  (Whatley et al., 1989; Garcia et al., 1992a; Jaffe et al., 1987).

In the previous chapter, vitamin E, was found to enhance the acylation processes in resting endothelial cells. One of these processes is the transacylation reaction in which  $\alpha$ -tocopherol activates the CoA-independent transacylase resulting in the enhanced formation of alkylacyl-GPC, a presumed precursor of PAF and  $PGI_2$ . This provides an explanation for the potentiating effect of vitamin E in  $PGI_2$  synthesis by endothelial cells. In this chapter, we examined the role of vitamin E in the regulation of second messengers formation in agonist-receptor mediated stimulation of endothelial cells.

It was recently reported that  $\alpha$ -tocopherol inhibited PKC in smooth muscle cells leading to the inhibition of cell proliferation (Boscoboinik et al., 1991a and 1991b). We also obtained similar observations in a different smooth muscle cell line and the exact mechanism was still undetermined although the antioxidant property of  $\alpha$ -tocopherol might play an essential role in the regulation of PKC

activity (Ho et al., 1993).

We reported here that  $\alpha$ -tocopherol suppressed the sustained level of DAG and increased PA formation in cultured human endothelial cells stimulated by thrombin. The observations were derived from the determination of [ $^3$ H]-myristate labelled products and from the direct quantitation of DAG mass. Indirect studies ruled out the involvement of  $\alpha$ -tocopherol in the modulation of PLC, PLD or PA phosphohydrolase activities. However,  $\alpha$ -tocopherol was found to augment the activity of DAG kinase which was responsible for the increased conversion of DAG to PA.

### III. MATERIALS AND METHODS

#### 1. Materials

[<sup>3</sup>H]-Myristic acid (60 Ci/mmol) was from American Radiolabelled Chemicals, Inc. (St. Louis, MO, USA). [<sup>3</sup>H]-Myo-inositol (50.2 Ci/mmol) and [ $\gamma$ -<sup>32</sup>P]-ATP (30 Ci/mmol) were from New England Nuclear (Lachine, PQ, Canada). sn-1,2-Diacylglycerol (DAG) assay reagents system was from Amersham Canada, Ltd. (Oakville, ON, Canada). Human thrombin (3000 NIH units/mg protein), heparin, gelatin, collagenase type IV, gentamicin sulfate, Hepes, DL-propranolol, dithiothreitol and octylglucoside were from Sigma Chemicals, Co. (St. Louis, MO, USA). Medium 199, inositol free-Dulbecco's modified eagle medium, lyophilized antibiotic-antimycotic, trypsin-EDTA, heat-inactivated fetal bovine serum and all tissue culture plasticware were from Gibco, Ltd. (Burlington, ON, Canada). Endothelial cell growth supplement was from Collaborative Research (Bedford, MA, USA). Phosphatidylethanol was from Avanti Polar Lipids, Inc. (Alabaster, AL, USA) and all other standard lipids were from Serdary Research Laboratories, Inc. (London, ON, Canada). Dowex (AG-1X8 resin, formate form) and plastic chromatography columns were from BioRad Laboratories, Ltd. (Mississauga, ON, Canada). RRR- $\alpha$ -tocopherol was donated by the vitamin E Research Information Services (LaGrange; IL, USA). Thin layer plates (silica gel 60A) were from Canlab (Mississauga, ON, Canada). All solvents used were h.p.l.c. grade and were purchased from BDH Chemicals, Inc. (Toronto, ON, Canada). Glassware were

silanized before use.

## **2. Culture of Endothelial Cells**

Human endothelial cells were isolated from fresh umbilical veins after incubation with 0.2% collagenase and seeded on 100 mm petri dishes precoated with 0.2% gelatin as previously described (Tran and Chan, 1988; Jaffe, 1984). The cells were grown to confluence in medium-199 (pH 7.4) supplemented with heat-inactivated fetal calf serum (10%), endothelial cell growth supplement (30  $\mu\text{g/ml}$ ), heparin (90  $\mu\text{g/ml}$ ), HEPES (25 mM), gentamicin sulfate (40  $\mu\text{g/ml}$ ), antibiotic-antimycotic (100 units/ml penicillin G sodium, 100  $\mu\text{g/ml}$  streptomycin sulfate and 0.25  $\mu\text{g/ml}$  amphotericin B). The endothelial monolayer was detached using 0.05% trypsin-EDTA and centrifuged at 100 g for 10 min. The cells were subcultured in a 1:3 ratio on 35 mm or 100 mm petri dishes. Cells of the first passage were routinely used in all experiments. Confluency was reached in 5 to 7 days. The cells were identified as endothelial origin by the appearance of cobblestone shape using phase contrast microscopy and by the presence factor VIII-related antigen by immunofluorescent microscopy (Chan and Tran, 1990).

## **3. [ $^3\text{H}$ ]-Myristate Labelling and Thrombin Stimulation**

Cell monolayers in 35 mm dishes were rinsed twice with warm phosphate buffered saline (PBS) and radiolabelled with 1  $\mu\text{Ci/ml}$  of [ $^3\text{H}$ ]-myristate in medium-199 containing 10% fetal calf serum. After 20 h of incubation, radiolabelled medium was removed, cells were rinsed twice with warm PBS and further

incubated for 4 h with vitamin E-enriched medium.

For thrombin stimulation, vitamin E-enriched medium was removed and the cells were rinsed twice with warm PBS. The stimulation was started by adding 1.5 units/ml of thrombin dissolved in Hanks' Balanced salt solution (HBSS), pH 7.4 containing 10 mM Hepes, 0.8 mM Mg SO<sub>4</sub> and 5 mM CaCl<sub>2</sub>. The same incubation buffer, but without thrombin, was added in the unstimulated control dishes. In some experiments, cells were incubated with DL-propranolol (inhibitors of phosphatidic acid phosphohydrolase) for 5 min before the addition of thrombin. In the experiments designed to determine the presence of phospholipase D activation in human endothelial cells, ethanol (0.1-2%) was included during thrombin stimulation. At the appropriate times, stimulation was stopped by adding 1 ml of ice-cold methanol/con HCl (100: 1, v/v). The cells were scraped and collected into test tubes containing 40 µg of lipid standards (lysoPC, PI, PC, PE, PA, DAG, TG or PEt). Total lipids were extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). The lower chloroform phase of the extraction was collected and stored at -20°C for lipid analysis.

#### **4. Myo-[<sup>3</sup>H]-Inositol Labelling**

Cells grown at about 80% confluence in 35 mm dishes were radiolabelled with 5 µCi/ml of myo-[<sup>3</sup>H]-inositol in inositol-free Dulbecco's modified Eagle medium (DME) containing 10% of dialyzed fetal calf serum. After 20 h of incubation, cells were rinsed three times with serum-free medium-199, reincubated with vitamin E-enriched medium-199 for 4 h and stimulated with thrombin in the presence of 10

mM LiCl. The stimulation was stopped with 1 ml of ice-cold methanol; cells were scraped and the inositol phosphates were extracted by chloroform/methanol as in the method of Bligh and Dyer. The upper aqueous phase of the extraction was collected and saved at -20°C for the determination of inositol phosphates.

## **5. Preparation of Vitamin E-Enriched Medium**

Vitamin E-enriched medium-199 containing 10% fetal calf serum was prepared as previously described (Tran and Chan, 1990). Appropriate amount of RRR- $\alpha$ -tocopherol dissolved in dimethyl sulfoxide (DMSO) was added in warmed fetal calf serum. The amount of DMSO added did not exceed 0.2% of the total volume. The mixture was vortexed vigorously and incubated at 37°C in the dark for 15 min. Medium-199 and antibiotics were added last and the mixture was incubated for another 15 min before feeding to the monolayers. Control medium contained the same amount of DMSO as the vitamin E-enriched medium.

## **6. Analysis of Lipids by Thin-Layer Chromatography**

The lower chloroform phase of the Bligh and Dyer extraction was evaporated under N<sub>2</sub> gas. The lipid was resuspended in chloroform/methanol (9:1, v/v) and spotted on precoated glass silica gel G plates. The plates were developed in a solvent system of chloroform/methanol/acetic acid/water (85:15:10:3, by volume) (system I). In this system, major phospholipids such as lysoPC ( $R_f = 0.05$ ), PI ( $R_f = 0.14$ ), PC ( $R_f = 0.20$ ), PE ( $R_f = 0.40$ ) and PA ( $R_f = 0.63$ ) are separated from each other and from neutral lipids. To separate neutral

lipids, the plates were developed in a solvent system of hexane/diethylether/acetic acid (70:30:1, by volume) (system II). In this system, DAG ( $R_F = 0.13$ ), free fatty acid ( $R_F = 0.36$ ) and triglyceride ( $R_F = 0.82$ ) were separated from major phospholipids ( $R_F = 0$ ). To separate PEt from other phospholipids, the plates were developed in the upper organic phase of ethylacetate/isooctane/acetic acid/water (110:50:20:100, by volume) (system III) in which PA ( $R_F = 0.07$ ) and PEt ( $R_F = 0.14$ ) were separated from PC, PE, PI and LysoPC (all of them have  $R_F = 0$ ). In this system, the neutral lipids migrated close to the solvent front. When it was necessary to simultaneously separate PA, PEt and DAG, the plates were first developed half-way in the solvent system III, dried and redeveloped to the top of the plates in the solvent system II (Billah et al., 1989). The individual spots comigrated with lipid standards were visualized by iodine vapor, scraped and quantitated by liquid scintillation spectrometry.

## **7. Analysis of Inositol Phosphates**

Inositol phosphates were separated by anion-exchange chromatography (Berridge et al., 1983). About two thirds of the aqueous phase of the Bligh and Dyer extract was applied to a commercial plastic column containing 1 ml of Dowex (AG 1 - x8; formate form). Free inositol was first eluted with 20 ml of water. Glycerophosphoinositol, inositolmonophosphate, inositolbisphosphate and inositoltrisphosphate were eluted with 10 ml of 5 mM disodiumtetraborate / 60 mM sodium formate, 10 ml of 0.2 M ammonium formate / 0.1 M formic acid, 10 ml of 0.4 M ammonium formate / 0.1 M formic acid and 5 ml of 1.0 M ammonium

formate / 0.1 M formic acid, respectively. A 1.0 ml portion of each fraction was used for liquid scintillation counting.

## 8. Assay of Diacylglycerol Kinase

Cells were harvested from 100 mm diameter dishes by scraping in ice-cold phosphate buffered saline, pH 7.4, containing 5 mM EDTA. Cells were sedimented at 800 x g for 5 min and the cell pellet was resuspended in the homogenizing buffer (50 mM Tris-HCl, pH 7.4, 10% glycerol, 0.5 mM EDTA and 2 mM dithiothreitol). Cell suspension was sonicated twice for 15 sec each at a power setting for 50% output using an ultrasonic cell disruptor. After removing the nuclei and unbroken cells from 800 x g centrifugation for 5 min, the postnuclear fraction was subjected to ultracentrifugation (100,000 x g, 30 min) to obtain the membrane and cytosolic fractions. The membrane pellet was resuspended in homogenizing buffer and disrupted with the aid of 1 ml syringe and 25 gauge needle.

Diacylglycerol kinase activity was determined by measuring the incorporation of  $^{32}\text{P}$  from  $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$  into DAG to form  $[\text{}^{32}\text{P}]\text{-PA}$ . The standard assay mixture was adopted with slight modifications from the previous works (Walsh and Bell, 1986; MacDonald et al., 1988a). To measure the activity of diacylglycerol kinase using exogenous DAG, the membrane or cytosolic fraction (10-30  $\mu\text{g}$  protein) was added in a total reaction mixture of 100  $\mu\text{l}$  containing 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, 15 mM  $\text{MgCl}_2$ , 1 mM EGTA, 2 mM dithiothreitol, 20 mM NaF, 6.8 mol % (2 mM) diolein, 3 mol % (0.9 mM) PS and

51 mM octyl glucoside. The reaction was started by adding [ $\gamma$ - $^{32}\text{P}$ ]-ATP (4000 cpm/100 pmol  $\text{Na}_2\text{ATP}$ ) and the mixture was incubated in 30°C waterbath. After 5 min of incubation, the reaction was terminated by adding 0.4 ml of methanol / chloroform (1:1, by volume) and 0.1 ml of 200 mM  $\text{CaCl}_2$  (Ford and Gross, 1990). The lower chloroform phase was washed three times with 1% of perchloric acid and spotted on silica gel G plates which were developed in the solvent system I. Radiolabelled phosphatidic acid spots were visualized by autoradiogram and quantitated by liquid scintillation spectrometry.

#### **9. Determination of DAG Mass**

DAG mass was enzymatically quantitated by using the assay kit purchased from Amersham. The principal idea of this assay was the conversion of cellular DAG to [ $^{32}\text{P}$ ]-PA by *E. coli* diglyceride kinase in the presence of [ $\gamma$ - $^{32}\text{P}$ ]-ATP (Kennerly et al., 1979; Preiss et al., 1986). Briefly, one half of the lipid extracts obtained from 35 mm diameter dishes was first solubilized with detergent solution (n-octylglucoside / cardiolipin) using a waterbath sonicator. The soluble lipid solution was treated with excess *E. coli* diacylglycerol kinase in the presence of [ $\gamma$ - $^{32}\text{P}$ ]-ATP (specific activity: 50,000 - 100,000 cpm/nmole). The [ $^{32}\text{P}$ ]-PA formed was separated by thin layer chromatography using solvent system I, identified by autoradiogram, and quantitated by liquid scintillation counting. The mass of [ $^{32}\text{P}$ ]-PA was calculated from the known specific activity of [ $\gamma$ - $^{32}\text{P}$ ]-ATP.

## 10. Data Presentation

In [<sup>3</sup>H]-myristate labelling experiments, the relative distribution of tritiated radioactivity in phospholipids and neutral lipids was presented as percentage of radioactivity among the bands on TLC: % of total = (dpm of a band / total dpm of the TLC lane) x 100.

In Figure 2-5, data were presented as percentage increase of DAG kinase activity induced by thrombin stimulation: % of increase from thrombin stimulation = [activity (+ thrombin) - activity (-thrombin) / activity (-thrombin)] x 100.

#### **IV. RESULTS**

In this chapter, the role of (RRR)- $\alpha$ -tocopherol in the regulation of thrombin-induced DAG and PA formation was investigated in cultured human endothelial cells. Due to the multiple sources of DAG, especially PC being considered as one of the main sources of the sustained level of DAG during agonist stimulation (Pelech and Vance, 1989), cells were therefore labelled with [ $^3$ H]-myristate which preferentially incorporated into PC (Martin and Michaelis, 1988).

Data in Table 2-1 shows that the incorporation of [ $^3$ H]-myristate into cellular lipids remained unaffected by  $\alpha$ -tocopherol enrichment. Total radioactivities incorporated into lipids of cells without and with  $\alpha$ -tocopherol pretreatment were  $1.83 \pm 0.10$  and  $1.70 \pm 0.20$  ( $\times 10^5$  dpm/ $10^5$  cells), respectively. Total incorporation of [ $^3$ H]-myristate by the cells was approximately 15% after 20 h incubation. The percentage of tritium incorporated into PC was highest (70%), followed by TG (11%), PI (5%), PE (3%). Tritium was incorporated the least into DAG and PA, suggesting the low steady state levels of these two lipid products.

##### **1. Effects of $\alpha$ -Tocopherol on the Change of Cellular Lipid Levels Induced by Thrombin**

In order to evaluate the role of  $\alpha$ -tocopherol in the regulation of lipid metabolism, we first investigated whether  $\alpha$ -tocopherol has any influence on the intracellular levels of DAG, PA, PI, PC and PE after thrombin stimulation. The data in Figure 2-1 show that  $\alpha$ -tocopherol caused a 40% decrease in the amount

of intracellular labelled DAG after 5 min of thrombin stimulation with a concomitant increase in the amount of cellular PA and PI. At the early time of stimulation (0-60 sec), thrombin elicited a rapid increase in DAG. The transient peak of DAG was followed by its sustained accumulation over the remaining period of stimulation (2.5 - 10 min). During this time period,  $\alpha$ -tocopherol caused about 40% reduction in DAG levels. On the other hand, PA generation was slowly induced and attained a maximum at 5 min after addition of thrombin. In contrast to the effect of  $\alpha$ -tocopherol on DAG formation, the level of PA accumulated during 2.5 - 5 min of thrombin stimulation was markedly higher in cells preenriched with  $\alpha$ -tocopherol than in the control cells. The transient increase of DAG, between 0-60 sec, was concomitant with a decrease in PI level. However, between 1 and 5 min of stimulation, PI began to increase to its steady level owing to its resynthesis from PA. Remarkably, the labelled PI level in the  $\alpha$ -tocopherol-enriched cells was 2-fold higher than that in the control cells. In addition, thrombin induced a gradual decrease of [ $^3$ H]-PC, a drop of about 4% after 5 min in both tocopherol treated and untreated cells. PE levels in both  $\alpha$ -tocopherol-treated and untreated cells were not affected by thrombin addition for up to 10 min (data not shown).

## **2. Effect of $\alpha$ -Tocopherol on the Accumulation of Cellular DAG Mass Induced by Thrombin**

Although the above study clearly demonstrated that  $\alpha$ -tocopherol caused a change in the radioactivities of DAG and PA, we sought further evidence to show that the reduction of DAG levels by  $\alpha$ -tocopherol has not resulted from the compartmentalization of radioactivity in specific lipid pools. Direct measurement

of cellular DAG mass was performed in thrombin-stimulated cells pretreated with various concentrations of  $\alpha$ -tocopherol. Data in Figure 2-2 show that  $\alpha$ -tocopherol dose-dependently suppressed the elevation of thrombin-stimulated DAG mass. The change of DAG level after 5 min of thrombin stimulation was detectable at 11.5  $\mu$ M of  $\alpha$ -tocopherol, and the inhibition was maximum (40-45%) when  $\alpha$ -tocopherol concentrations reached near 46-69  $\mu$ M. However,  $\alpha$ -tocopherol had no effect on the basal level of DAG mass in cells that were not treated with thrombin (Figure 2-2), suggesting the difference in the compartmentalization of DAG between the stimulated and unstimulated states.

### **3. The Increase in PA Formation by $\alpha$ -Tocopherol is not Caused by the Activation of PLD nor the Inhibition of PA Phosphohydrolase**

In endothelial cells, evidence for the presence of PLD activity that generates PA mainly from PC has been recently reported (Martin and Michaelis, 1989). To determine whether the increase in PA generation results from the enhancement of PLD activity by  $\alpha$ -tocopherol, we adopted the transphosphatidyl reaction, a unique characteristic of PLD, which transfers a primary alcohol to the phosphatidyl group of a phospholipid substrate to form phosphatidylalcohol (Bocckino et al., 1987). Therefore, [ $^3$ H]-myristate labelled cells enriched with or without  $\alpha$ -tocopherol were stimulated with thrombin in the presence of ethanol, and [ $^3$ H]-PEt formation was determined. The results in Figure 2-3A show a near linear increase of [ $^3$ H]-PEt induced by thrombin over 2 min of stimulation, and there was no difference in the formation of [ $^3$ H]-PEt in both  $\alpha$ -tocopherol treated and untreated cells. Similarly, thrombin-stimulated [ $^3$ H]-PEt formation was increased

with increasing concentration of ethanol, but was not affected by  $\alpha$ -tocopherol (Figure 2-3B). Analysis of the radiolabelled PA showed that  $\alpha$ -tocopherol enhanced [ $^3$ H]-PA formation at all tested concentrations of ethanol (Figure 2-3B). If PA was the only product of PLD, the increase of [ $^3$ H]-PEt was usually accompanied by a decrease in [ $^3$ H]-PA due to the competition of PLD hydrolysis and its transphosphatidylation. However, as noted by the unchanged levels of [ $^3$ H]-PA in Figure 2-3B, PA was therefore not only formed from the activation of PLD, but also generated from other sources, such as DAG kinase that converts DAG to PA. Taken together, the data indicated that the increase in PA formation elicited by  $\alpha$ -tocopherol was not resulted from an increase in PLD activity.

To determine whether  $\alpha$ -tocopherol inhibits PA phosphohydrolase that leads to the accumulation of PA, the generations [ $^3$ H]-DAG and [ $^3$ H]-PA of thrombin-stimulated endothelial cells preenriched with  $\alpha$ -tocopherol were examined in the presence of propranolol, a phosphohydrolase inhibitor (Pappu and Hauser, 1983; Koul and Hauser, 1987). This approach was intended to observe whether DAG formation is mainly derived from PLD/PA phosphohydrolase pathway which might be inhibited by vitamin E; and if it does, whether the effects of propranolol and vitamin E on the changes of DAG and PA levels are mimicking. Data in Table 2-2 showed that, in response to 5 min of thrombin addition, propranolol elicited a two-fold increase in [ $^3$ H]-PA formation in both  $\alpha$ -tocopherol treated and untreated cells, but it had no effect on the levels of [ $^3$ H]-DAG. Regardless of the presence or absence of propranolol,  $\alpha$ -tocopherol enrichment resulted in an increase in PA and a decrease in DAG formation. Thus, the results showed that DAG was not mainly formed via PLD/PA phosphohydrolase pathway since propranolol did not cause a

decrease in DAG levels. Also, the fact that propranolol did not mimic the effect of  $\alpha$ -tocopherol on the formation of DAG and PA ruled out the possibility that the increase in PA and the decrease in DAG formation was resulted from the inhibition of PA phosphohydrolase by  $\alpha$ -tocopherol.

#### **4. The Decrease in the Sustained Level of DAG by $\alpha$ -Tocopherol is not Caused by the Inhibition of PI-PLC**

Although the initial results showed in Figure 2-1 demonstrated that there was no difference in the transient accumulation of DAG between  $\alpha$ -tocopherol treated and untreated cells during the early time (0-60 sec) of stimulation, we sought whether  $\alpha$ -tocopherol had any effect on the hydrolysis of phosphoinositides by PLC that might affect the level of DAG. The data in Figure 2-4 shows a rapid generation of [ $^3$ H]-IP<sub>2</sub> and [ $^3$ H]-IP<sub>3</sub> induced by thrombin that peaked at 15 sec, while [ $^3$ H]-IP level increased in a near-linear fashion over a 5 min time course.  $\alpha$ -Tocopherol had no effect on the generation of both [ $^3$ H]-IP and [ $^3$ H]-IP<sub>2</sub>, but it seems to attenuate the transient elevation of [ $^3$ H]-IP<sub>3</sub> at 15 sec. At this time, the levels of [ $^3$ H]-IP<sub>3</sub> were ten times lower than those of other inositolphosphates. In addition,  $\alpha$ -tocopherol had no effect on the change in [ $^3$ H]-labelled PI induced by thrombin (data not shown). Taken together, these data suggested that the hydrolysis of PI and PIP by PLC mainly contributed to the formation of both transient and sustained levels of DAG in thrombin-stimulated endothelial cells, whereas PLC mediated hydrolysis of PIP<sub>2</sub> was not the main source for the transient accumulation of DAG which was found to be unaffected by  $\alpha$ -tocopherol (Figure 2-1). So far, it has been shown that PLD, PA phosphohydrolase and PI-

PLC pathways were not responsible for the  $\alpha$ -tocopherol-induced changes of DAG and PA levels, the remaining DAG kinase pathway which also contributes to the regulation of intracellular DAG and PA levels is likely to be affected by  $\alpha$ -tocopherol.

### **5. $\alpha$ -Tocopherol Enhances DAG Kinase Activity**

To further investigate whether  $\alpha$ -tocopherol affected the removing pathway of DAG, leading to the decrease in the sustained level of DAG, the activity of DAG kinase was determined in the total homogenate, membrane and the cytosolic fractions obtained from cells pretreated with and without  $\alpha$ -tocopherol. Diolein was used as the substrate and the standard assay conditions for DAG kinase was performed as described under "Experimental Procedures". In resting cells,  $\alpha$ -tocopherol was found to enhance the DAG kinase activity in both membrane and cytosolic fractions (Figure 2-5). Maximum effective concentration of  $\alpha$ -tocopherol was 46  $\mu$ M which caused 22% and 42% increase in the activity of DAG kinase in the membrane and cytosolic fractions, respectively. Upon stimulation by thrombin, DAG kinase activities were increased by about 40% in the membrane fractions and 30% in the cytosolic fractions as compared with unstimulated controls (Figure 2-6). In addition,  $\alpha$ -tocopherol was found to further potentiate the thrombin activation of DAG kinase, especially in the membrane fractions. Thus, the changes in the sustained accumulation of DAG and PA levels in  $\alpha$ -tocopherol enriched cells are likely to be mediated through the activation of DAG kinase.

## V. DISCUSSION

$\alpha$ -Tocopherol, a naturally occurring and most potent form of vitamin E, was found for the first time to regulate the levels of DAG and PA from human endothelial cells in response to thrombin stimulation. Our studies suggest that the effect of  $\alpha$ -tocopherol on the reduction of cellular DAG levels is mediated via an activation of DAG kinase since  $\alpha$ -tocopherol enrichment caused an increase in DAG kinase activity in both particulate and cytosolic fractions of endothelial cells, resulting in an accumulation of PA. Our results also showed that  $\alpha$ -tocopherol lowered the sustained cellular DAG levels, but it had no effect on the transient increase of DAG induced by brief exposure of thrombin. In addition, our studies suggested that DAG was derived from phosphodiesteric cleavage of both phosphoinositides and phosphatidylcholine by either phospholipase C or phospholipase D and PA phosphohydrolase; and neither of these enzymes was found to be affected by  $\alpha$ -tocopherol. These findings suggest a new role of vitamin E in the signal transduction by regulating the levels of important second messengers such as DAG and PA.

In response to various extracellular signals, there was an increased turnover of phosphoinositides and a transient accumulation of DAG in numerous cell systems (Nishisuka, 1992). For example, thrombin stimulates the hydrolysis of  $PIP_2$  by PI-specific PLC generating  $IP_3$  and DAG in human endothelial cells (Brock and Capasso, 1988; Jaffe et al., 1987). Consistently, we observed that thrombin

stimulated a transient increase of [ $^3\text{H}$ ]-DAG and caused a rapid decrease in radiolabelled-PI in [ $^3\text{H}$ ]-myristate-labelled cells with a concomitant peak of inositol phosphates in [ $^3\text{H}$ ]-inositol-labelled cells at the first 15-30 s of stimulation (Figures 2-1 and 2-4). However, hydrolysis of [ $^3\text{H}$ ]-myristate labelled PI was only transient whereas the level of DAG was maintained throughout the time course of stimulation (Figure 2-1). Recently, it was suggested that DAG could arise from phospholipids other than  $\text{PIP}_2$  due to the quantitative discrepancies between the levels of DAG generated from  $\text{PIP}_2$  hydrolysis and the total mass of DAG accumulated in agonist stimulated cells (Exton, 1990). PC, an important source of AA release and  $\text{PGI}_2$  synthesis in human endothelial cells, was found to be a substrate for DAG production in numerous cell types including endothelial cells (Ragab-Thomas et al., 1987; Billah et al., 1989, Bocckino et al., 1987; Price et al., 1989). A PC-specific PLC that was identified and characterized from bovine endothelial cells was considered as one pathway for the generation of PC-derived DAG (Martin et al., 1987). We observed that thrombin stimulated a gradual decrease of cellular radiolabelled PC, with not much change in PE. In comparing between the levels of DAG and PC changed in response to thrombin, our results confirmed that PC hydrolysis contributed to the maintenance of the sustained accumulation of DAG.

$\alpha$ -Tocopherol enrichment was found to markedly reduce the sustained levels of DAG with concomitantly increase PA and PI levels in response to prolonged exposure of thrombin (Figure 2-1). The effect of  $\alpha$ -tocopherol on DAG levels was further supported by direct measurement of DAG mass (Figure 2-2). The increase

in radiolabelled PI in  $\alpha$ -tocopherol enriched cells could be explained by its rapid resynthesis from PA which was increased by  $\alpha$ -tocopherol. In most cell systems, DAG undergoes rapid phosphorylation by DAG kinase to PA and the resynthesis of inositol phospholipids occurs by priming PA with CTP to form CDP-DAG (Mitchell, 1986). Thus, the first pathway that would be affected by  $\alpha$ -tocopherol is PLC followed by DAG kinase pathway that subsequently generates DAG and PA. However, it was recently discovered that many cell types, including endothelium possess phospholipase D activity catalyzing the hydrolysis of PC, yielding choline and PA (Martin, 1988; Martin and Michaelis, 1989; Pai et al., 1988; Cabot et al., 1988; Truett et al., 1988). PA formed by PLD activity was quickly removed by dephosphorylation reaction via PA phosphohydrolase, resulting in the generation of large amounts of DAG (Billah et al., 1989, Bocckino et al., 1987). Thus, a second pathway that would be affected by  $\alpha$ -tocopherol is PLD followed by PA-phosphohydrolase pathway.

Taken together, the increase in PA and the decrease in DAG levels could be due to an effect of any pathway responsible for their generation and conversion including PLD, PA phosphohydrolase, PLC or DAG kinase. First, we evaluated the effect of  $\alpha$ -tocopherol on phospholipase D pathway by employing the transphosphatidyl transfer reaction, a unique characteristic of this enzyme (Bocckino et al., 1987). The results suggested that  $\alpha$ -tocopherol did not affect PLD activity in endothelial cells since there was no difference of PEt formation between cells pre-enriched with and without  $\alpha$ -tocopherol. Accumulation of PEt in response to thrombin stimulation confirmed the occurrence of PLD activity which contributed

to the formation of PA and subsequently to DAG via PA phosphatase. Therefore, other possibility to account for the increase in PA levels by  $\alpha$ -tocopherol would be resulted from an effect at the level of PA phosphohydrolase. To test this possibility, we used DL-propranolol, a  $\beta$ -adrenergic receptor antagonist (Stiles et al., 1984) and a potent inhibitor of PA phosphohydrolase in intact cells (Pappu and Hauser, 1983; Koul and Hauser, 1987), to see whether the drug can mimic the effect of  $\alpha$ -tocopherol on the PA and DAG levels. The increase in PA formation in the presence of propranolol suggested that there was inhibition of PA phosphohydrolase. However, the lack of the interference of propranolol on the DAG levels in both  $\alpha$ -tocopherol treated and untreated cells suggested that the sequential activation of PLD/PA phosphohydrolase by thrombin in endothelial cells was not the primary pathway contributing to accumulation of DAG; the results also suggested that the elevation of PA and the reduction of DAG levels induced by  $\alpha$ -tocopherol were not resulted from an inhibition of PA phosphohydrolase. The next enzyme to be taken into consideration was PLC and, especially PI-specific PLC was chosen to investigate whether it was affected by  $\alpha$ -tocopherol.  $\alpha$ -Tocopherol appeared to selectively inhibit the hydrolysis of  $PIP_2$ , as showing from the attenuation of 15 s peak of  $IP_3$  in  $\alpha$ -tocopherol enriched cells, but did not demonstrate any discrimination towards the formation of IP and  $IP_2$  which were presumably derived from the hydrolysis of PI and PIP, respectively (Mitchell, 1986). However, the low level of  $PIP_2$  in the biological membrane as compared with other abundant substrates of PLC such as PI, PIP and PC, suggested that  $PIP_2$  hydrolysis by PLC was not primarily responsible for the transient accumulation of

DAG. In fact, we observed that there was no difference in DAG levels during the first 30 s of thrombin stimulation in both cells enriched with or without  $\alpha$ -tocopherol. The lack of apparent effect of  $\alpha$ -tocopherol on the transient elevation of DAG and on the hydrolysis of PC, PI and PIP suggested that modulation of PIP<sub>2</sub>-PLC by  $\alpha$ -tocopherol, if there is any, plays a negligible role in the regulation of sustained DAG levels. Whether  $\alpha$ -tocopherol exerts any effect on PIP<sub>2</sub>-PLC is under investigation. Therefore, the reduction of elevated DAG levels induced by thrombin found in this study was unlikely resulted from an inhibition of PLC by  $\alpha$ -tocopherol.

Finally, the effect of  $\alpha$ -tocopherol on DAG and PA levels could be explained entirely on the basis of an effect of  $\alpha$ -tocopherol on DAG kinase. By direct measurement of DAG kinase activity, we observed that  $\alpha$ -tocopherol significantly enhanced the basal activity of this enzyme in both membrane and cytosolic fractions. In addition, the synergistic activation of DAG kinase by thrombin and  $\alpha$ -tocopherol would be likely the key point in lowering the sustained levels of DAG. The findings that  $\alpha$ -tocopherol dose-dependently suppressed the accumulation of DAG mass induced by thrombin further substantiates this possibility.

DAG is considered to be an important second messenger in many cell types including endothelial cells, and it responds by activating and translocating Ca<sup>2+</sup>-phospholipid dependent protein kinase C (Nishisuka, 1992; Bell and Burns, 1991). Enhancement of PKC phosphorylation activities involved in the regulation of many agonist-induced endothelial cell events including platelet-activating factor and prostaglandins synthesis (Whatley et al., 1989; Garcia et al., 1992a; Heller et al., 1991; Zavoico et al., 1990; Carter et al., 1989), barrier dysfunction (Lynch et al.,

1990) and von Willebrand factor secretion (Carew et al., 1992). In addition, it was found that PKC participates in the regulation of DAG kinase activation by inducing translocation and protein phosphorylation (Kanoh et al., 1989). Other mechanisms for regulation of DAG kinase include the activation of the enzyme by free oleic acid and oleoyl-CoA (Kelleher and Sun, 1989), and by arachidonoyl-DAG (MacDonald et al., 1988b). In contrast, DAG kinase activity was found to be inhibited by lipoxygenase metabolite of arachidonic acid such as 15- and 12-hydroxyeicosatetraenoic acids (15-HETE and 12-HETE) (Setty et al., 1987). Thus, the enhanced DAG activation by  $\alpha$ -tocopherol may be a consequence of the effect of  $\alpha$ -tocopherol in controlling any of the above processes that occur in endothelial cells. We have recently reported that  $\alpha$ -tocopherol enhanced arachidonic acid release (Tran and Chan, 1988) and the formation alkylacylglycerophosphorylcholine (Tran et al., 1993a) in endothelial cells. We also observed that in endothelial cells,  $\alpha$ -tocopherol increased the PMA-unstimulated membrane PKC activity, but inhibited PMA-induced PKC translocation (Tran and Chan, 1993; and reported in the next chapter). More work is obviously needed to elucidate the relationship of these observations.

## VI. CONCLUSION

$\alpha$ -Tocopherol was found to suppress the accumulation of thrombin induced DAG levels via DAG kinase activation, resulting in an increase in PA formation in endothelial cells. Other enzymes such as PLD, PLC and PA phosphohydrolase was apparently unaffected by  $\alpha$ -tocopherol. While participating in the regulation of DAG levels,  $\alpha$ -tocopherol may regulate the uncontrolled activation of PKC associated with the overproduction of DAG induced by agonists. Thus, this would be a mechanism by which vitamin E exerts its role in preventing many pathological disorders.

**Table 2-1 - Distribution of radioactivity in lipids of human endothelial cells incubated with [<sup>3</sup>H]-myristate.**

Cell monolayers were labelled with [<sup>3</sup>H]-myristate (1 μCi/ml) for 20 h and then incubated in the absence (-E) or presence (+E) of α-tocopherol (46 μM) for 4 h. Total lipids were extracted and quantitated by TLC as described in "Materials and Methods". Values are means ± S.D. (n = 3 dishes) and they were presented as percentage of radioactivity among the bands on the TLC. Total radioactivities incorporated into lipids of cells without and with α-tocopherol pretreatment were 1.83 ± 0.10 and 1.70 ± 0.20 (x 10<sup>5</sup> dpm/10<sup>5</sup> cells), respectively.

Cellular lipids	Incorporation (% of total radioactivity)	
	-E	+E
LysoPC	1.43 ± 0.33	1.39 ± 0.48
PI	5.05 ± 0.17	5.16 ± 1.45
PC	69.80 ± 0.70	69.60 ± 1.40
PE	3.33 ± 0.12	3.62 ± 0.21
PA	0.46 ± 0.09	0.50 ± 0.02
DAG	0.73 ± 0.12	0.85 ± 0.05
FA	1.26 ± 0.26	0.95 ± 0.14
TG	10.96 ± 0.91	10.67 ± 0.50
Others	6.98 ± 0.20	7.26 ± 0.30

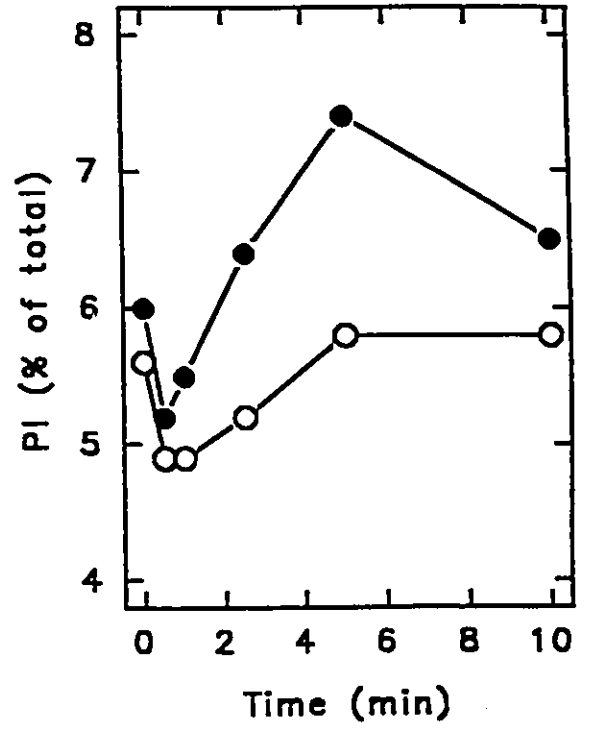
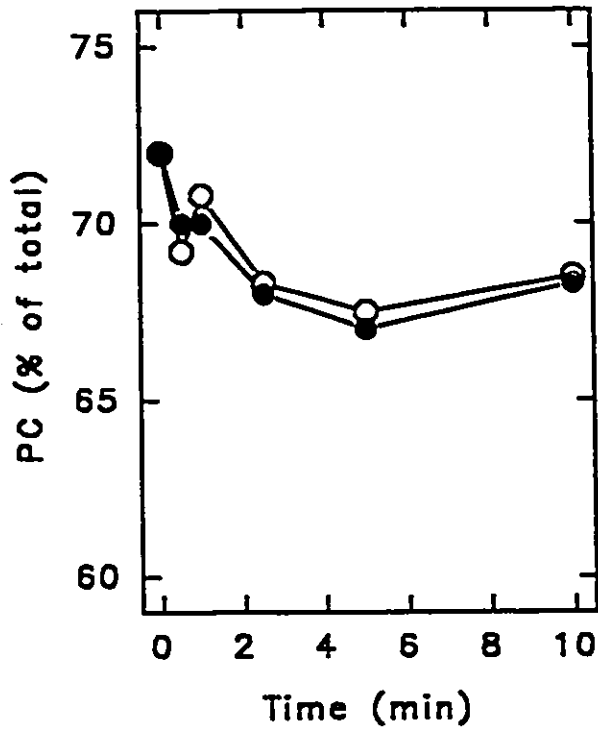
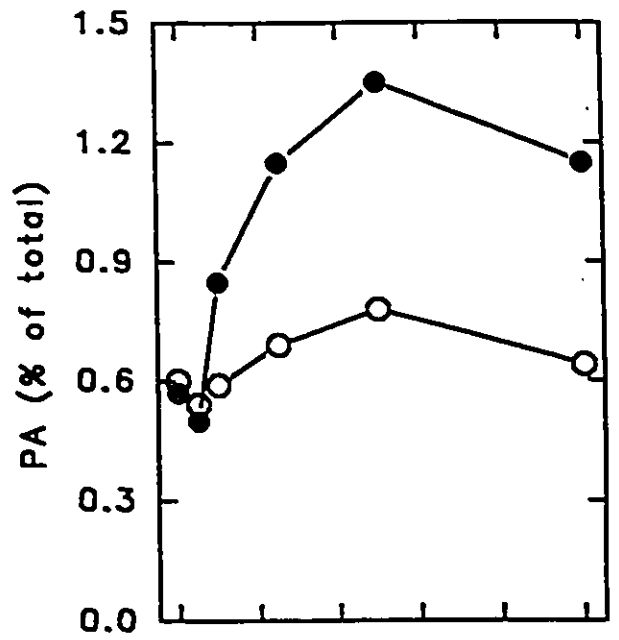
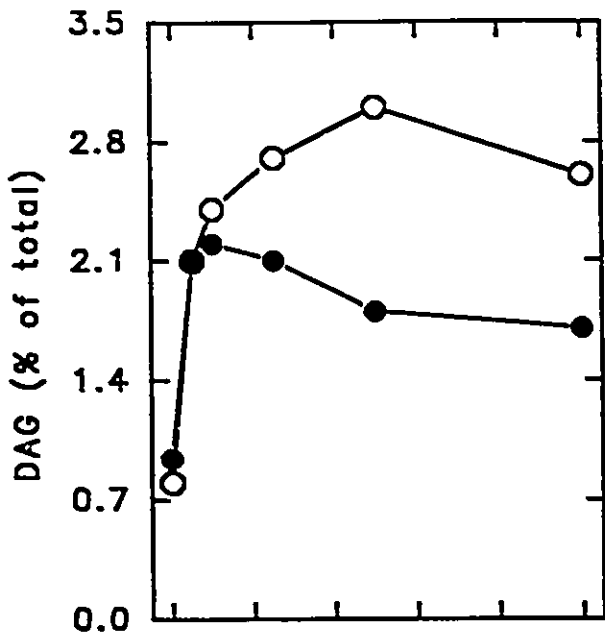
**Table 2-2 - Effects of propranolol on the formation of [<sup>3</sup>H]-DAG and [<sup>3</sup>H]-PA by thrombin-stimulated human endothelial cells preenriched with or without α-tocopherol.**

Cell monolayers were labelled with [<sup>3</sup>H]-myristate (μCi/ml) for 20 h and then incubated in the absence (-E) or presence (+E) of α-tocopherol (46 μM) for 4 h. Cells were rinsed and preincubated with propranolol (200 μM) in HBSS for 5 min before adding thrombin (1.5 U/ml) for an additional 5 min. [<sup>3</sup>H]-DAG and [<sup>3</sup>H]-PA were quantitated by TLC as described in "Materials and Methods". Values are means ± S.D. (n = 3 dishes) of one representative experiment out of three.

	<b>[<sup>3</sup>H] Radioactivity (% of total)</b>			
	<b>DAG</b>		<b>PA</b>	
	<b>-E</b>	<b>+E</b>	<b>-E</b>	<b>+E</b>
Control	0.76 ± 0.02	0.84 ± 0.03	0.40 ± 0.01	0.45 ± 0.04
Thrombin	2.80 ± 0.23	1.80 ± 0.10	0.47 ± 0.02	1.20 ± 0.04
Propranolol + Thrombin	2.71 ± 0.17	1.70 ± 0.16	0.78 ± 0.11	1.74 ± 0.01

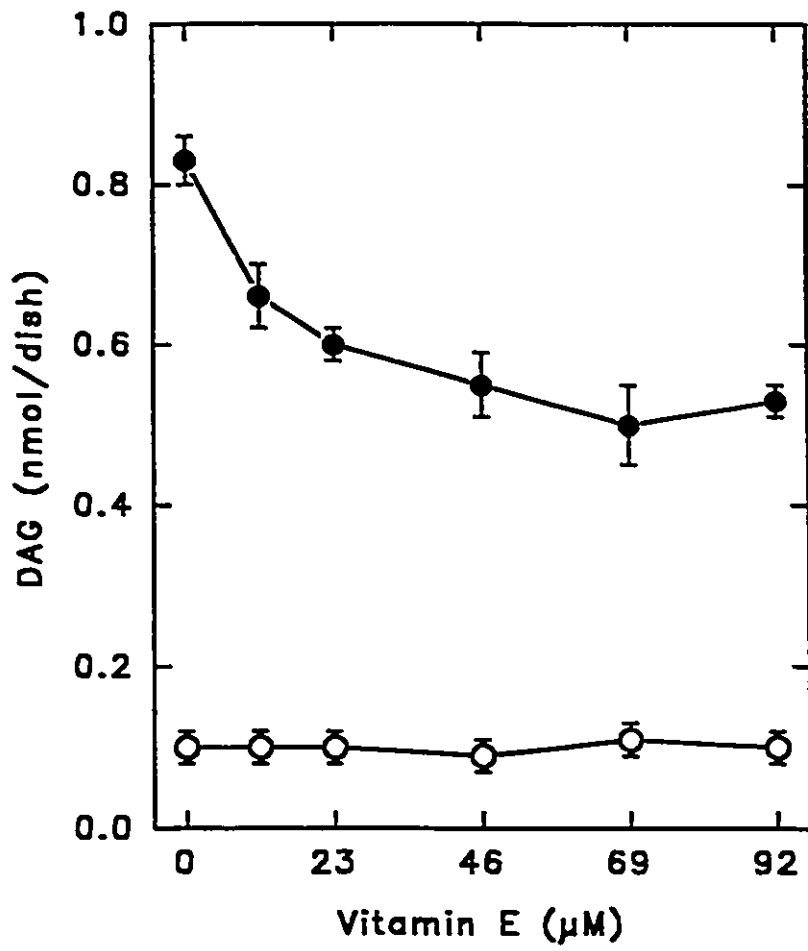
**Figure 2-1 - Time course of radiolabelled lipids change by thrombin-stimulated human endothelial cells preenriched with or without  $\alpha$ -tocopherol.**

Cell monolayers were labelled with [ $^3$ H]-myristate (1  $\mu$ Ci/ml) for 20 h and then incubated in the absence (O) or presence (●) of  $\alpha$ -tocopherol (46  $\mu$ M) for 4 h before being stimulated with thrombin (1.5 U/ml) for the indicated times. Tritiated DAG, PA, PI, and PC were quantitated by TLC as described in "Materials and Methods". Values are means of two dishes from one representative experiment out of three.



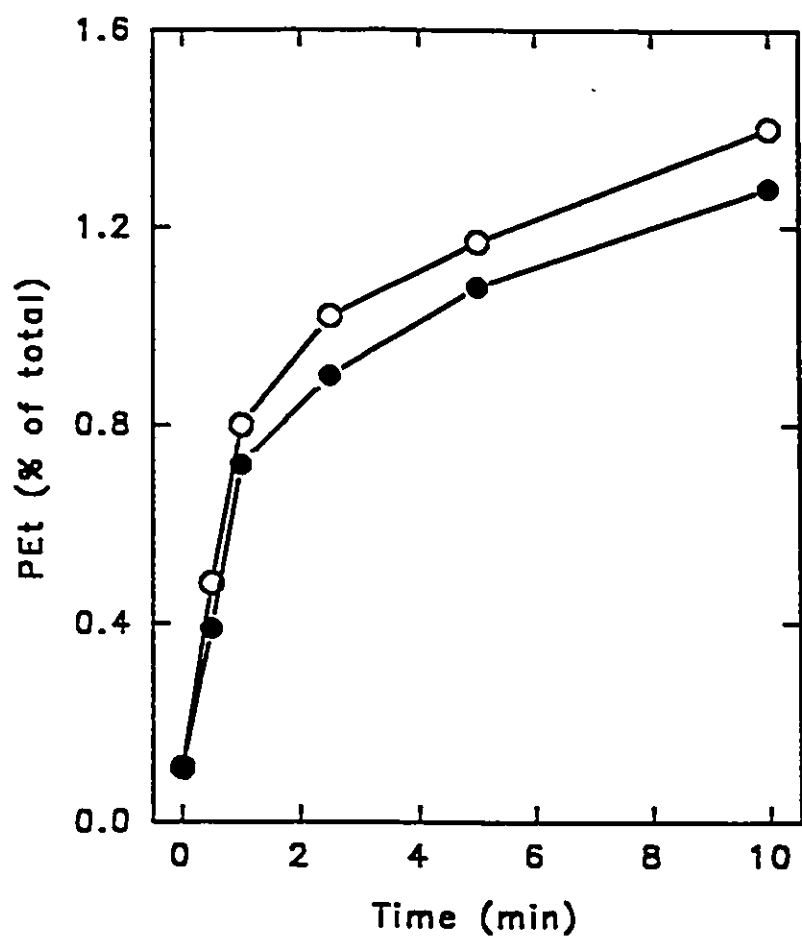
**Figure 2-2 - Effect of  $\alpha$ -tocopherol on the mass of DAG in thrombin-stimulated human endothelial cells.**

Cell monolayers were incubated with indicated concentrations of  $\alpha$ -tocopherol for 4 h. Cells were then treated with either 1.5 U/ml of thrombin (●) or buffer alone (○) for 5 min. The extracted lipids were subjected to DAG mass determination as described in "Materials and Methods". Values are means  $\pm$  S.D. (n = 5 dishes).



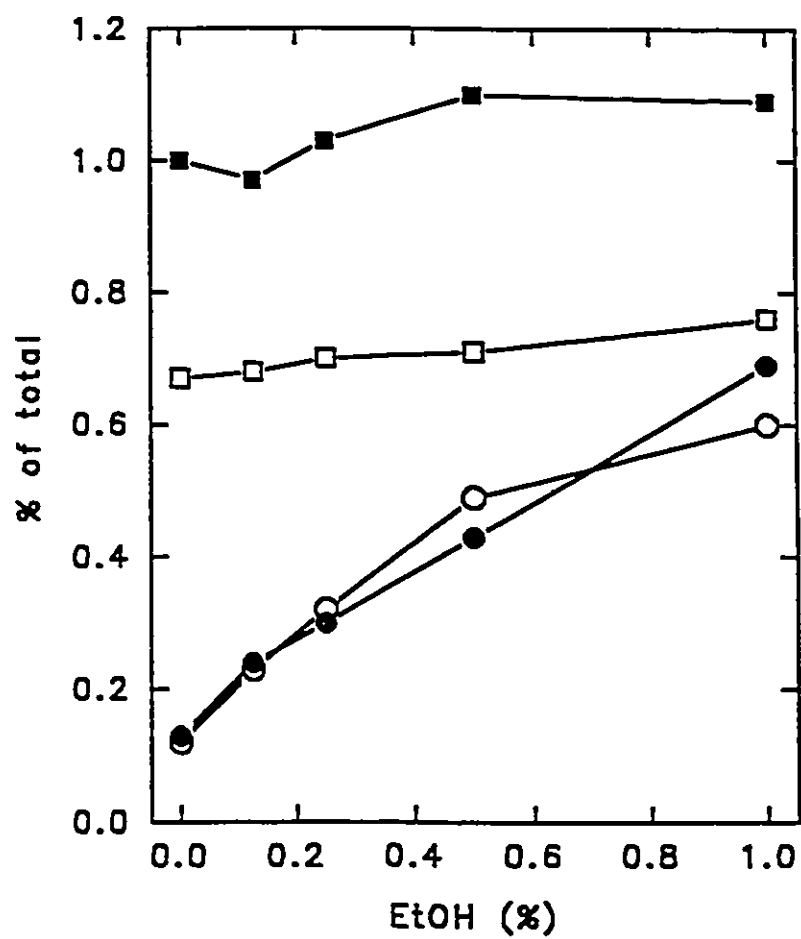
**Figure 2-3A - Time course of [<sup>3</sup>H]-PEt formation in cells preenriched with and without  $\alpha$ -tocopherol.**

Cell monolayers were labelled with [<sup>3</sup>H]-myristate (1  $\mu$ Ci/ml) for 20 h and then incubated in the presence (●) or absence of (○) of 46  $\mu$ M of  $\alpha$ -tocopherol for 4 h. Cells were stimulated with 1.5 U/ml thrombin in the presence of 2% of ethanol. At indicated times, the reaction was stopped and [<sup>3</sup>H]-PEt were quantitated by TLC as described in "Materials and Methods". Values are means of two dishes from one representative experiment out of three.



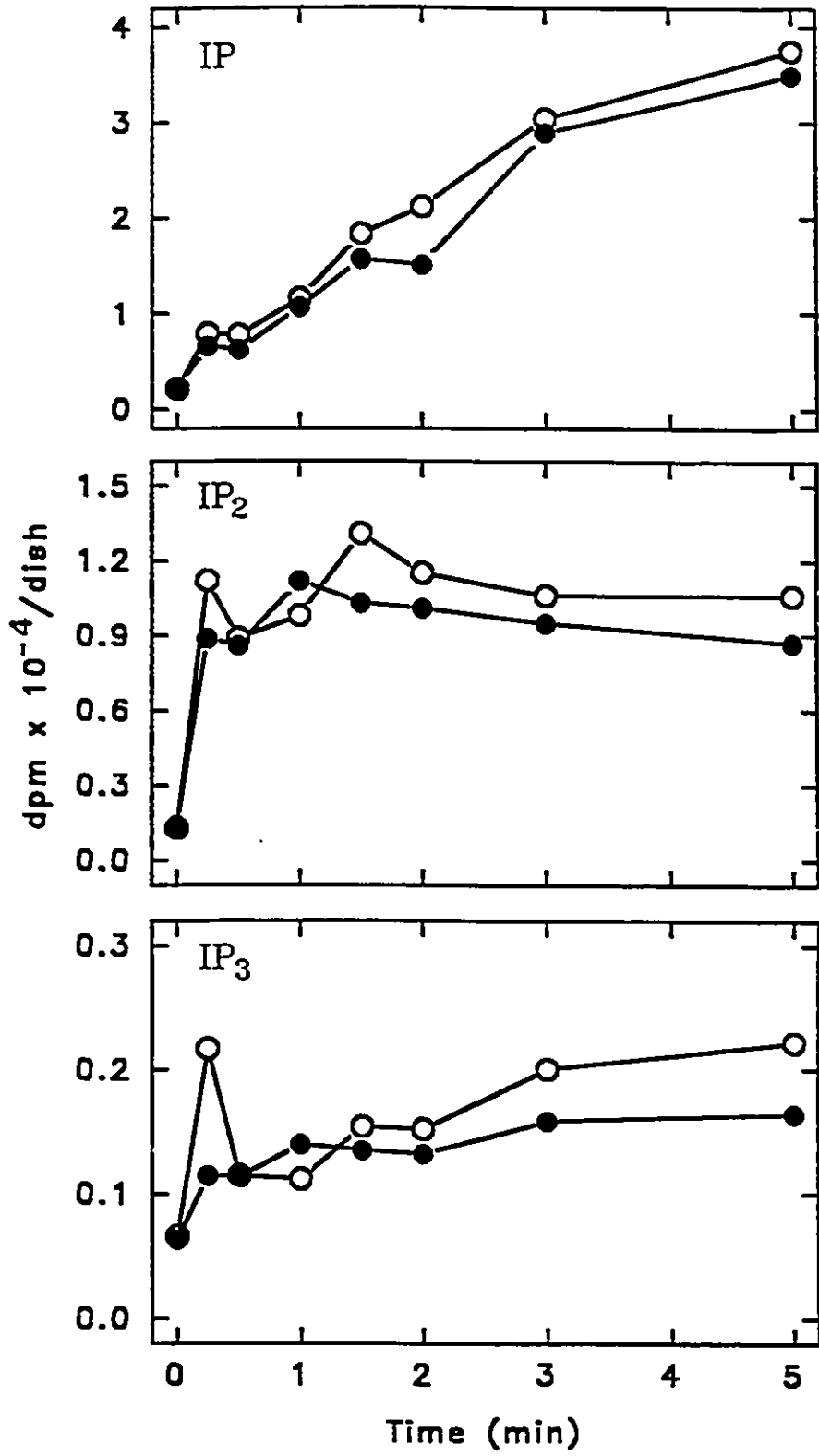
**Figure 2-3B - Effect of  $\alpha$ -tocopherol on the formation of [ $^3$ H]-PA and [ $^3$ H]-PEt in thrombin-stimulated endothelial cells.**

Cell monolayers were labelled with [ $^3$ H]-myristate (1  $\mu$ Ci/ml) for 20 h and then incubated in the absence (O, $\square$ ) or presence ( $\bullet$ , $\blacksquare$ ) of 46  $\mu$ M of  $\alpha$ -tocopherol for 4 h. Cells were stimulated with 1.5 U/ml thrombin for 5 min in the presence of indicated concentrations of ethanol. The reaction was stopped and [ $^3$ H]-PA ( $\square$ , $\blacksquare$ ) and [ $^3$ H]-PEt (O, $\bullet$ ) were quantitated by TLC as described in "Materials and Methods". Values are means of two dishes from one representative experiment out of three.



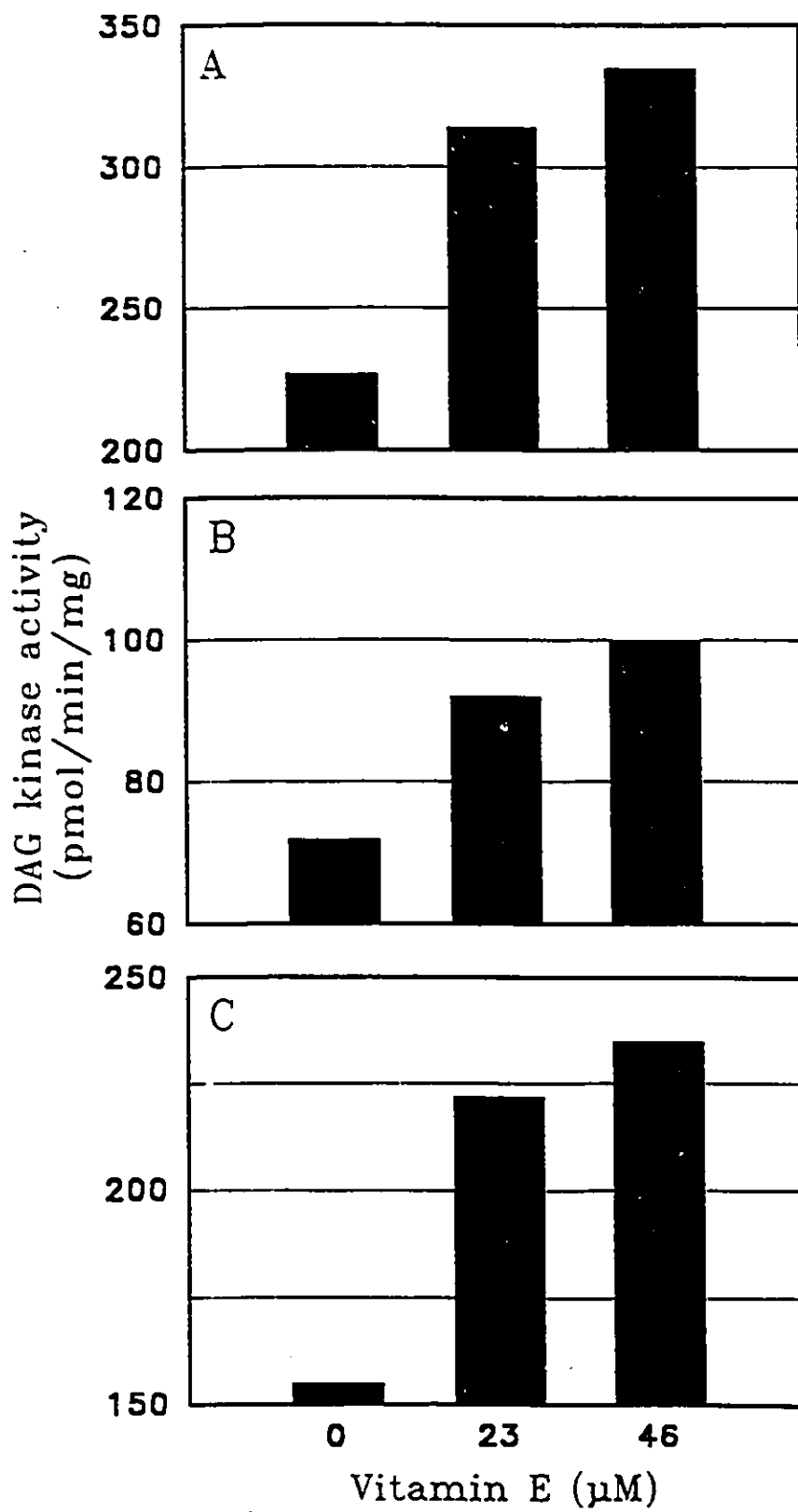
**Figure 2-4 - Time course of [<sup>3</sup>H]-inositol phosphates formation by thrombin-stimulated human endothelial cells preenriched with or without  $\alpha$ -tocopherol.**

Cells were labelled with myo-[<sup>3</sup>H]-inositol (5  $\mu$ Ci/ml) for 20 h and then incubated in the absence (○) or presence (●) of  $\alpha$ -tocopherol (46  $\mu$ M) for 4 h before being stimulated with 1.5 U/ml of thrombin for the indicated times. [<sup>3</sup>H]-Inositol phosphates were quantitated by anion exchanged chromatography as described in "Materials and Methods". Values are means of two dishes from one representative experiment out of two.



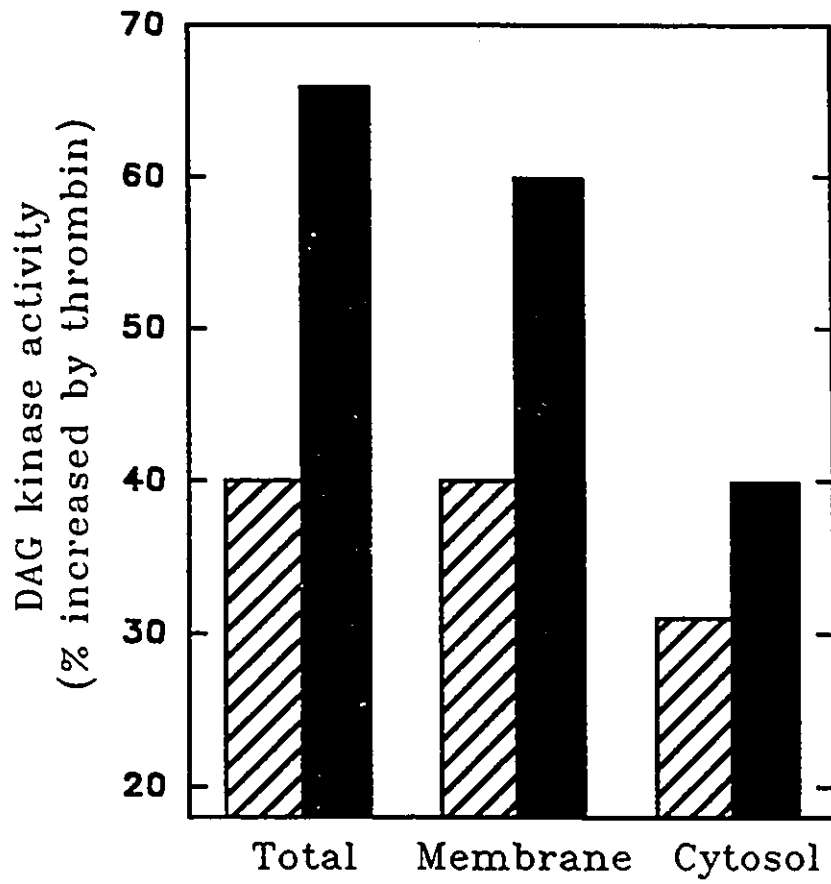
**Figure 2-5 - Effect of  $\alpha$ -tocopherol on the unstimulated activity of DAG kinase in human endothelial cells.**

Cell monolayers were incubated with indicated concentrations of  $\alpha$ -tocopherol for 4 h. Cells were scraped, homogenized and fractionated by ultracentrifugation into a 100,000 x g pellet (membrane fraction) and 100,000 x g supernatant (cytosolic fraction). DAG kinase activity in the total homogenate (A), membrane fractions (B) or cytosolic fractions (C) was determined as described in "Materials and Methods" and expressed as pmole of [ $\gamma$ - $^{32}$ P]-ATP transferred to exogenous DAG (diolein) per min per mg protein. Values are means of duplicate from a representative experiment out of four.



**Figure 2-6 - Effect of  $\alpha$ -tocopherol on thrombin-stimulated DAG kinase activity in human endothelial cells.**

Cells monolayers were incubated in the absence (hatched bar) or presence (closed bar) of  $\alpha$ -tocopherol (46  $\mu$ M) for 4 h followed by 5 min of thrombin (1.5 U/ml) stimulation. Cells were scraped, homogenized and fractionated by ultracentrifugation into a 100,000 x g pellet (membrane fraction) and supernatant (cytosolic fraction). DAG kinase activity was determined as described in "Materials and Methods". Values were presented as percent increase of DAG kinase activity stimulated by thrombin as compared with non-stimulated control. Values are means of duplicate from a representative experiment out of three.



## CHAPTER 3

# DIFFERENTIAL EFFECTS OF VITAMIN E AND DUROQUINONE ON PROTEIN KINASE C ACTIVITY IN HUMAN ENDOTHELIAL CELLS

### I. SUMMARY

In this study, the effect of an antioxidant (vitamin E) and an oxyradical generator (duroquinone) on the activation and translocation of protein kinase C (PKC) in endothelial cells was determined. Incubation of endothelial cells with vitamin E ( $\alpha$ -tocopherol), resulted in an increase in PKC activity in the membrane fractions. In response to phorbol 12-myristate 13-acetate (PMA) stimulation, PKC activity in the membrane was substantially increased with concomitant decrease in the cytosolic PKC activity. In vitamin E incubated cells, PMA induced a smaller increase in membrane PKC activity than vitamin E untreated cells. Thus, vitamin E exerted a dual effect on membrane PKC activity by increasing the resting membrane PKC activity, but inhibiting the PKC activation induced by PMA. By contrast, duroquinone which is known to generate oxygen radicals through a redox cycling reaction, had a completely opposite effect on PKC activity as compared with vitamin E in both resting and PMA-stimulated cells. The effect of duroquinone

on PKC activity was totally abolished in cells preincubated with vitamin E. Taken together, these results suggested that the intracellular ratio of oxidant/antioxidant may play a role in the regulation of PKC activity in endothelial cells.

## II. INTRODUCTION

$\alpha$ -Tocopherol, a naturally occurring form of vitamin E, is a potent lipid soluble chain-breaking antioxidant and membrane stabilizer (Burton and Ingold, 1986; Burton et al., 1983). It is recognized by its ability to effectively prevent lipid peroxidation induced by reactive oxygen species. We have previously observed that  $\alpha$ -tocopherol plays a role in the modulation of arachidonic acid and phospholipid metabolism in human endothelial cells. First,  $\alpha$ -tocopherol was found to enhance prostacyclin ( $\text{PGI}_2$ ) synthesis by altering the release of arachidonic acid presumably via phospholipase  $\text{A}_2$  ( $\text{PLA}_2$ ) (Tran and Chan, 1988; Tran and Chan, 1990). Second,  $\alpha$ -tocopherol was found to alter the CoA-independent acyltransferase activity, resulting in an increase in the transacylation of alkylglycerophosphorylcholine (Tran et al., 1993a), precursor of platelet activating factor and eicosanoids (Chilton et al., 1984; Chilton, 1989). Third, we observed that  $\alpha$ -tocopherol can regulate the levels of diacylglycerol (DAG) and phosphatidic acid (PA) by altering DAG kinase activity (Tran et al., 1993b). However, the exact mechanism of how vitamin E influences these processes is still poorly understood.

A novel role of vitamin E in signal transduction has been recently suggested by its ability to modulate protein kinase C (PKC) activity. Azzi and his coworkers found that, in smooth muscle cells,  $\alpha$ -tocopherol inhibits PKC activity, its translocation and PKC phosphorylation of its specific substrates, resulting in inhibition of cell proliferation (Boscoboinik et al., 1991a; Boscoboinik et al., 1991b;

Chatelain et al., 1993). In consistency to this finding, work in our laboratory also reported similar observation that the inhibition of PKC activity by  $\alpha$ -tocopherol in rat aortic smooth muscle (A10) cells is likely to be mediated by the antioxidant property of vitamin E (Ho et al., 1993). In addition, a suggestion for an antitumor promotion actions of vitamin E has been recently deduced from the ability of vitamin E to decrease the rate of PKC down-regulation induced by PMA and to decrease oxidative modification of PKC induced by oxidants (Gopalakrishna et al., 1993).

In endothelial cells, PKC activation is associated with agonist induced PGI<sub>2</sub> synthesis (Carter et al., 1989; Garcia et al., 1992) and platelet activating factors (Whatley et al., 1989; Zavoico et al., 1990; Heller et al., 1991). This was further supported by the contribution of PKC in the increase in phospholipase A<sub>2</sub> activity (Chakraborti et al., 1992), by either lowering the Ca<sup>2+</sup> requirement for PLA<sub>2</sub> (Carter et al., 1989) inactivating of anti-PLA<sub>2</sub> proteins known as lipocortins (Hirata et al., 1981), activating of the Na<sup>+</sup>/H<sup>+</sup> exchanger (Kitazono et al., 1989) or modulating the G-protein linked to PLA<sub>2</sub> (Kajiyama et al. 1989). In addition, PKC activation was found to link with increased translocation of DAG kinase (Kanoh et al., 1989) and increased activities of both CoA-dependent acyltransferase and CoA-independent transacylase that selectively incorporate and mobilize arachidonate between phospholipid pools (Kanzaki et al., 1989; Breton and Colard, 1991). Because of the similarities in the effect of vitamin E and PKC in modulating these processes, our objective is to determine whether there is any link between vitamin E and PKC activity, that may provide an explanation for vitamin E actions in our earlier findings.

In this study, we have examined the role of vitamin E in the basal activity of PKC and PKC translocation induced by phorbol ester, such as PMA, in human endothelial cells. In addition, incorporation of duroquinone, a substance known to intracellularly generate oxygen radicals through a redox cycling reaction (Rossie et al., 1986; Kappus, 1986) was used to explore further possible mechanism of PKC modulation by vitamin E.

### III. MATERIALS AND METHODS

Protein kinase C enzyme assay system and [ $\gamma$ - $^{32}$ P]-ATP (30 Ci/mmol) were purchased from Amersham Canada, Ltd. (Oakville, ON, Canada). Duroquinone,  $\beta$ -mercaptoethanol, benzamidine, phenylmethylsulfonylfluoride (PMSF), phorbol 12-myristate 13-acetate (PMA), leupeptin, soybean trypsin inhibitor, pepstatin A, heparin, gelatin, collagenase type IV, gentamicin sulfate and HEPES were from Sigma Chemicals, Co. (St. Louis, MO, USA). Medium 199, lyophilized antibiotic-antimycotic, trypsin-EDTA, heat inactivated bovine serum and all tissue culture plasticware were from Gibco, Ltd. (Burlington, ON, Canada). Endothelial cell growth supplement was from Collaborative Research (Bedford, MA, USA). RRR- $\alpha$ -tocopherol was donated by the Vitamin E Research Information Services (La Grange, IL, USA). All solvents used were h.p.l.c. grade and were purchased from BDH Chemicals, Inc. (Toronto, ON, Canada). Glassware were silanized before use.

#### 1. Culture of Endothelial Cells

Culture of endothelial cells isolated from veins of human umbilical cords and preparation of vitamin E-enriched culture medium were described in Chapters 1 and 2.

## 2. Protein Kinase C Assay

Protein kinase C enzyme activity in subcellular fractions was detected by using the assay system purchased from Amersham. The assay employed is a modification of a mixed micelle assay (Hannun, et al., 1985) activating enzyme with phorbol 12-myristate 13-acetate in the presence of  $Mg^{2+}$ ,  $Ca^{2+}$  and phosphatidylserine. The reaction is performed at pH 7.5 in a Tris (hydroxymethyl) aminomethane (Tris) buffer. Enzyme present in the samples will catalyze the transfer of  $\gamma$ -phosphate of adenosine-5'-triphosphate (ATP) to the threonine group of a peptide (truncated EGF receptor) which is specific for protein kinase C. Phosphorylated peptide is separated on binding paper. After washing the paper, the extent of phosphorylation can be detected by scintillation counting.

To study the effect of  $\alpha$ -tocopherol on PKC activity, a sample of the membrane or cytosolic fractions obtained from endothelial cells preenriched with or without  $\alpha$ -tocopherol was employed in the enzyme assay. Confluent endothelial cells of 1-3 passages in 100 mm-dishes were routinely used in all experiments. Cells were first enriched with indicated concentrations of  $\alpha$ -tocopherol in 10 ml of complete culture medium. After 4 h of incubation, the medium was removed and 10 ml of  $\alpha$ -tocopherol-free medium was added to the dishes. 20  $\mu$ l of phorbol 12-myristate 13-acetate or its carrier (DMSO) was then added to stimulate the cells for 5 min at 37°C. This medium was quickly removed and the cells were rinsed three times with ice-cold PBS containing 5 mM EDTA. Cells were scraped in the same ice-cold buffer and were sedimented by centrifugation (Beckman TJG) at 1800 rpm for 5 min. Cell pellet was resuspended in 0.5 ml of lysis buffer (50 mM

Tris-HCl buffer, pH 7.5 containing 5 mM EDTA, 10 mM EGTA, 0.3%  $\beta$ -mercaptoethanol, 10 mM benzamidine, 50  $\mu$ g/ml PMSF, 5  $\mu$ g/ml Leupeptin, 250  $\mu$ g/ml soybean trypsin inhibitor, 50  $\mu$ g/ml pepstatin A). Cells were disrupted by sonicating with one pulse at 10 s using an ultrasonic cell disruptor setting at 50% output. The mixture was vortexed and centrifuged for 5 min at 1800 rpm to sediment the nuclei and unbroken cells. 0.45 ml of postnuclear fraction was centrifuged for 15 min at 4°C using a microfuge. The pellet was washed, resuspended in 0.25 ml of lysis buffer, sonicated with one pulse at 10 s and the solution was considered as the membrane fraction. The supernatant was further ultracentrifuged at 100,000 x g in a tabletop ultracentrifuge (Beckman TL100) for 30 min at 4°C; the resulting supernatant was considered as the cytosolic fraction. 25  $\mu$ l of each fraction was used per assay.

The assay system contained 4 mM calcium acetate, 2 mole % phosphatidylserine, 6  $\mu$ g/ml phorbol 12-myristate 13-acetate, 225  $\mu$ M peptide, 7.5 mM dithiothreitol, 35  $\mu$ M ATP and 11 mM magnesium acetate in a total volume of 25  $\mu$ l of 50 mM Tris/HCl with 0.05% v/v sodium azide pH 7.5. 25  $\mu$ l of the membrane or cytosolic fraction was added to the assay system mixture and the reaction was started by the addition of 25  $\mu$ l of [ $\gamma$ -<sup>32</sup>P]-ATP (~ 25,000 cpm per tube). The mixture was incubated for 15 min in a waterbath at room temperature. The reaction was terminated by the addition of 100  $\mu$ l of a dilute reaction - quenching reagent supplied with the kit. 125  $\mu$ l of the terminated reaction mixture was pipetted onto a numbered square of binding paper. The solution was allowed to completely soak into the paper; the papers were washed three times with 5%

v/v acetic acid in separate weighing boats. At each washing, the papers were left for 10 min with intermittent gentle mixing in acetic acid solution. The papers were carefully lifted from their containers and were placed in scintillation vials for phosphorous-32 determination in liquid scintillation counter.

The blank tubes contained all the component mixture except the sample. The peptide, a specific substrate of protein kinase C, was omitted in the tubes used to control the phosphorylation of endogenous substrate. The actual protein kinase C activity was obtained after being subtracted from the blank and control values, and was expressed as pmoles of phosphate transferred per min per mg protein (pmoles/min/mg).

## **IV. RESULTS**

### **1. Characterization of PKC Activity in Human Endothelial Cells**

PKC activity in the membrane and cytosolic fractions of endothelial cells was determined from the ability of the enzyme to catalyze the incorporation of phosphorous-32 of adenosine-5'-triphosphate into the synthetic peptide. It was first required to standardize the methods for the preparation of the membrane and cytosolic fractions including sonication and differential centrifugation. Next, enzyme activity was measured as a function of protein concentrations; and finally, the dose and time of phorbol ester (Phorbol myristate acetate, PMA) stimulation to induce PKC translocation was determined.

The preparation of the membrane and cytosolic fractions involved the disruption of the cells by sonication in a hypotonic buffer followed by differential centrifugation. The purpose was to obtain the membrane and cytosolic fraction of which the enzyme activity was specific with respect to the synthetic peptide, an exogenously added specific substrate of PKC. The first procedure involved one pulse sonication for 10 s of the cell suspension in Tris buffer. Prolonged or multiple time of sonication abolished the specificity of the membrane enzyme for the peptide. Next, the nuclei and any unbroken cells had to be removed by light centrifugation (800 x g, 5 min). Failure of the separation of nuclei also interfered in the enzyme specificity of the membrane fraction. The membrane pellet was obtained by microfuge centrifugation of the previous supernatant for 15 min in an

Eppendorf-type centrifuge. If the postnuclear fraction was directly subjected to ultracentrifugation at 100,000 x g, the enzyme of the membrane fraction would be no longer specific toward the peptide. In addition, if the supernatant obtained from microfuge centrifugation was considered as the cytosolic fraction without further sedimentation (100,000 x g), the specificity of the PKC enzyme in the cytosolic fraction toward the peptide was reduced. In summary, to prepare the membrane and cytosolic fractions, we routinely sonicated the cell suspension once for 10 s followed by 800 x g centrifugation to sediment the nuclei fraction. The postnuclear fraction was centrifuged by microfugecentrifugation to obtain the membrane pellet and the supernatant which was further subjected to 100,000 x g ultracentrifugation. The supernatant of 100,000 x g ultracentrifugation was considered as the cytosolic fraction.

Figure 3-1 showed the activities of the PKC enzyme in the membrane and cytosolic fractions with respect to the presence or absence of  $\text{Ca}^{2+}$ , lipids (PS, PMA substituted for DAG) and peptide. PKC enzyme in the membrane fraction showed only moderate specificity toward exogenous peptide even though the procedures were optimized (Figure 3-1A). When both  $\text{Ca}^{2+}$  and lipids were present in the assay system, the rate of phosphorylation by enzyme in the membrane fraction was about 25% higher in the presence of exogenous peptide. Removing  $\text{Ca}^{2+}$  and lipids from the assay system markedly decreased the enzyme activity. This indicated that the membrane fraction contained a large amount of endogenous substrate for PKC enzyme, and  $\text{Ca}^{2+}$  and lipids were required to optimize the enzyme activity. In contrast, cytosolic PKC activity was quite specific

toward the peptide as shown in Figure 3-1B. When peptide or  $\text{Ca}^{2+}$  and lipids were removed from the assay system, the activity of the enzyme in the cytosolic fraction was significantly reduced. Thus, in both membrane and cytosol, the difference of activities between plus or minus peptide, but in the presence of both  $\text{Ca}^{2+}$  and lipids, was considered as the activities of PKC that were specific toward the peptide, an exogenously added substrate.

The levels of protein in the sample were also important in the determination of PKC activity. It was known that endogenous inhibitors of PKC enzyme might be present in the tissue sample, and therefore, high levels of proteins used in the assay would result in an inhibition of the enzyme activity. To determine the range of protein levels at which there was no inhibition, PKC activity was measured with graded levels of protein obtained from serial dilutions of the membrane and cytosolic fractions. Figure 3-2 showed that phosphorylation of the peptide was linearly proportional to the amount of protein in the enzyme fraction of both membrane and cytosol. The tested range of protein in the membrane was 5-70  $\mu\text{g}$  and in the cytosol was 5-50  $\mu\text{g}$ . Therefore, subsequent PKC assays were conducted with the enzyme protein levels within these ranges.

Finally, we sought to determine the dose and time of PMA to stimulate PKC translocation. PMA, a tumor promoting phorbol ester, was known to cause PKC to translocate from the cytosol to the membrane. As a result, the activity of PKC in the membrane was increased with a concomitant decrease in the cytosolic activity. As shown in Figure 3-3A and 3-3B, there was a three fold increase in the membrane enzyme activity with a complete disappearance of the cytosolic activity

after the cells were exposed with 200 nM PMA for 30 min. The translocation occurred less drastically in the brief exposure of the cells with PMA, i.e. 5 min, at which the cytosolic enzyme activity was still detectable. In addition, we observed that 200 nM was the optimum concentration of PMA that gave a maximum increase in the membrane enzyme activity. Therefore, in the following experiments wherever PKC translocation was studied, cells were routinely exposed with 200 nM of PMA for 5 min.

## **2. Effect of $\alpha$ -Tocopherol-Enrichment on PKC Activity**

The purpose of these studies was to investigate whether  $\alpha$ -tocopherol has any effect in PKC activity in the membrane and cytosol fractions from both PMA-stimulated and PMA-unstimulated endothelial cells. In these experiments, cells were preenriched with or without  $\alpha$ -tocopherol followed by brief exposure with PMA. In the unstimulated controls, cells were treated with carrier (DMSO). Tables 3-1 and 3-2 presented the PKC activities of the membrane and cytosolic fractions which were obtained from cells preenriched with or without  $\alpha$ -tocopherol and exposed with or without PMA. The results showed that basal PKC activity in the membrane fraction of resting cells was increased by 68% after the cells were enriched with 46  $\mu$ M of  $\alpha$ -tocopherol for 4 h (Table 3-1). Exposure of PMA to the cells caused a marked increase in membrane enzyme activity of both  $\alpha$ -tocopherol treated and non-treated cells. However, the membrane activity of PKC in  $\alpha$ -tocopherol enriched cells was 18% lower than that of control cells (without tocopherol) in response to PMA stimulation. Furthermore, to determine how much

PKC activities were increased by PMA, a comparison of activities between plus and minus PMA was made. As shown in Table 3-1, PMA induced elevation of PKC activity in the membrane by 281% and 87% of the control cells and  $\alpha$ -tocopherol-enriched cells, respectively. These indicated that  $\alpha$ -tocopherol stimulated the basal activity of membrane PKC, but it attenuated the elevation of PKC activity induced by PMA. In contrast,  $\alpha$ -tocopherol has no effect in the cytosolic PKC activity from both basal and PMA-stimulated cells (Table 3-2). Taken together, these results showed that  $\alpha$ -tocopherol had a dual effect in regulating PKC activity in the membrane from both resting and PMA-stimulated cells.

### **3. Effect of Duroquinone on PKC Activity - A Comparison with $\alpha$ -Tocopherol**

To determine the mechanism of  $\alpha$ -tocopherol in the regulation of PKC activity in the membrane of endothelial cells, a free radical generating substance was used to observe any counter effect on PKC activity done by free radicals as compared with  $\alpha$ -tocopherol. Duroquinone, a lipid soluble oxidant known to generate oxygen radicals through a redox cycling system once being incorporated in the membrane, was chosen for the purpose of study.

Figure 3-4A showed the basal and PMA-stimulated PKC activity in the membrane of the cells pretreated with  $\alpha$ -tocopherol or duroquinone. Consistent with the results in Table 3-1,  $\alpha$ -tocopherol stimulated the basal PKC activity, but attenuated the elevation of PKC activity induced by PMA. Conversely, when the

cells were treated with 0.5 mM of duroquinone for 1 h, there was about 40-50% reduction in the basal PKC activity in the membrane as compared with duroquinone untreated controls. In addition, when the cells were treated with duroquinone (0.5 mM, 1 h) and then exposed to PMA (200 nM, 5 min) membrane PKC activity was markedly increased and far more exceeded than that of duroquinone untreated cells. PMA induced an elevation of membrane PKC activities by 280%, 87% and 480% in the control,  $\alpha$ -tocopherol-treated and duroquinone-treated cells, respectively. However, duroquinone did not show any apparent effect in the cytosolic PKC activity in both basal and PMA-stimulated cells, which was similar to  $\alpha$ -tocopherol (Figure 3-4B). The dose and incubation time of duroquinone chosen in this study did not cause any change in the morphology of the cells as judged by phase contrast microscopy.

The above results showed the counter effect of  $\alpha$ -tocopherol and duroquinone on the PKC activity in the membrane of endothelial cells.  $\alpha$ -Tocopherol, an antioxidant, caused an increase in the basal PKC activity, but it inhibited PKC activation induced by PMA. Conversely, duroquinone, a free radical generating oxidant, exerted a complete reverse effect on membrane PKC activity by inhibiting the basal but stimulating the PMA-induced elevation of PKC.

#### **4. Interaction of $\alpha$ -Tocopherol and Duroquinone in Controlling PKC Activity**

To further explore the counter effect of  $\alpha$ -tocopherol and duroquinone on membrane PKC, the enzyme activity was measured in cells preenriched with  $\alpha$ -tocopherol followed by treatment with duroquinone. The purpose was to test whether the presence of  $\alpha$ -tocopherol in the membrane could reverse duroquinone effect on PKC activity.

Figures 3-5 and 3-6 illustrated membrane PKC activities of both basal and PMA-stimulated cells which were pretreated separately with  $\alpha$ -tocopherol or duroquinone, or  $\alpha$ -tocopherol followed by duroquinone. In the resting cells, duroquinone inhibited basal PKC activity as compared with its control (Figure 3-5). However, in the presence of  $\alpha$ -tocopherol, duroquinone lost its ability to inhibit basal PKC activity. In other words,  $\alpha$ -tocopherol prevented the decrease in PKC activity induced by duroquinone, which was confirmed by an increase of the enzyme activity in cells preenriched with  $\alpha$ -tocopherol followed by duroquinone treatment. When cells were exposed to PMA (Figure 3-6), there was marked increase in membrane PKC activity. In the presence of duroquinone, PMA induced a 500% increase in PKC activity in duroquinone treated cells as compared with 260% increase in duroquinone untreated controls. These increases were significantly inhibited by the presence of  $\alpha$ -tocopherol, in which PMA induced an increase in PKC activity by only 120% or 75% in cells preenriched with  $\alpha$ -tocopherol alone or cells preenriched with  $\alpha$ -tocopherol followed by duroquinone treatment, respectively.

In summary, results from the above studies showed that the PKC activity determined under the present experimental conditions could be regulated by the status of oxidant and antioxidant in the cells. This was demonstrated by the counter effect of  $\alpha$ -tocopherol and duroquinone on the PKC activity in both basal and PMA-stimulated conditions. In the absence of PMA,  $\alpha$ -tocopherol increased the membrane PKC activity which was inhibited by duroquinone. In the presence of PMA,  $\alpha$ -tocopherol suppressed the elevation of membrane PKC induced by PMA while duroquinone had the reverse effect. Furthermore, in the presence of  $\alpha$ -tocopherol, duroquinone lost its effect on PKC activity. Therefore, the ratio of oxidant/antioxidant inside the cells may play an important role in controlling PKC activity.

## V. DISCUSSION

The ability to activate or inhibit  $\text{Ca}^{2+}$ /phospholipid-dependent PKC plays an important role in the regulation of various transmembrane signalling processes. Our study demonstrated that  $\alpha$ -tocopherol stimulates PKC activity but inhibits PKC activation induced by PMA. To determine the mechanism of PKC regulation by  $\alpha$ -tocopherol, we employed the redox-cycling quinone, such as duroquinone known to intracellularly generate active oxygen species (Rossi et al., 1986). In this redox cycling process, quinone undergoes reduction by presumably NADPH-cytochrome P-450 reductase to form semiquinone radical which is oxidized back to quinone yielding mostly superoxide radical ( $\text{O}_2^{\cdot-}$ ) and subsequent metabolites such as hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) and hydroxyl radical ( $\text{HO}^{\cdot}$ ) (Kappus, 1986). The counter effect of  $\alpha$ -tocopherol and duroquinone on PKC activity suggests that  $\alpha$ -tocopherol modulates PKC via its antioxidant property even though other mechanisms such as the membrane-associated effect of vitamin E could not be precluded.

Current literature shows that oxidants can regulate PKC activity, and, depending on the types of oxidants and their mode of action, PKC can be either activated or inhibited. Gopalakrishna and Anderson (Gopalakrishna and Anderson, 1987, 1989) have shown that membrane-bound PKC is relatively susceptible to oxidative inactivation by  $\text{H}_2\text{O}_2$  at its catalytic domain. By contrast, selective oxidation of the regulatory domain of PKC results in an increase in  $\text{Ca}^{2+}$ /phospholipid independent kinase activity (Gopalakrishna, 1989). Recently,

Larsson and Cerutti reported a dual effect of oxidants on PKC in mouse epidermal JB6 cells (Larsson and Cerutti, 1988, 1989). These authors observed that high concentrations of H<sub>2</sub>O<sub>2</sub> induced PKC translocation to the membrane leading to a Ca<sup>2+</sup>-dependent phosphorylation of its specific substrate (Larsson and Cerutti, 1988), while mild oxidation modification of cytosolic PKC by low concentrations of superoxide resulted in a Ca<sup>2+</sup>-independent activation mechanism (Larsson and Cerutti, 1989). Our observation that duroquinone inhibited PKC activity in the endothelial membrane suggests an oxidative inhibition of the enzyme by oxygen radical as proposed by Gopalakrisna and Anderson (Gopalakrisna and Anderson), 1989). By contrast, our results showed that duroquinone stimulated PKC translocation induced by PMA. This could be due to the action of duroquinone generated oxidants which modified the cytosolic PKC to a form that was readily translocated to the plasma membrane upon PKC stimulation. Our results were consistent with those in the study by Kass et coworkers (Kass et al., 1989). These authors showed that, in hepatocytes, duroquinone stimulated the PMA-induced translocation of the activated cytosolic PKC to the membrane. Therefore, the effect of  $\alpha$ -tocopherol on PKC observed in our study is best explained by its ability to quench free radicals and lipid peroxidation, thus preventing oxidative modification of both membrane and cytosolic PKC. However, other mechanisms including the possible effect of vitamin E on the structure and integrity of the membrane that leads to the changing of PKC activity cannot be precluded. In addition, lysophosphatidylcholine and several cis unsaturated fatty acids including arachidonic acid, which are the primary products of phosphatidylcholine hydrolysis

catalyzed by phospholipase A<sub>2</sub>, were found to potentiate PKC activation (Shinomura et al., 1991; Seifert et al., 1988; Chen and Murakami, 1992; Yoshida et al., 1992). Thus, it is also plausible that vitamin E modulates PKC activity by affecting the formation of phospholipase A<sub>2</sub> products.

The effect of vitamin E on PKC was first studied by Azzi and coworkers who reported that  $\alpha$ -tocopherol inhibited the proliferation and PKC activity of aorta smooth muscle cells (A7r5) (Boscoboinik et al., 1991a; Boscoboinik et al., 1991b; Chatelain et al., 1993). These authors suggested that  $\alpha$ -tocopherol inhibited smooth muscle cells PKC activity and prevented PKC down-regulation upon prolonged exposure with PMA, by inhibiting PKC translocation. The inhibition of PKC was concomitant with the inhibition of cell proliferation by  $\alpha$ -tocopherol, whereas tocopherol acetate, Trolox C, and butylated hydroxytoluene (BHT) were ineffective (Boscoboinik et al., 1991a). A study from our laboratory also reported a similar observation in a different smooth muscle cell line (A-10) (Ho et al., 1993). However, in endothelial cells, we found that  $\alpha$ -tocopherol stimulated membrane PKC activity which appears to be in contradiction with the above observations. This discrepancy is probably due to the selective regulation by vitamin E of different PKC isoenzymes present in different cell types. Unfortunately, the knowledge of PKC isoenzymes in endothelial cells is not yet available. On the other hand, we found that  $\alpha$ -tocopherol inhibited PKC activation in response to brief phorbol ester treatment. This may be a mechanism for the action of vitamin E in decreasing the rate of PKC down-regulation by PMA as previously observed (Gopalakrishna et al., 1993; Boscoboinik et al., 1991a). We proposed that  $\alpha$ -

tocopherol plays a role in the control of optimal vascular endothelial cell functions by keeping PKC in a moderate active state in the resting condition, while preventing any functional deterioration from over-stimulation by inhibiting PKC translocation.

## VI. CONCLUSION

The effect of vitamin E on PKC implies a novel regulating mechanism of various processes in the signal transduction through the PKC pathway. Activation of membrane PKC by vitamin E provides a possible mechanistic explanation for our previous findings that vitamin E enhances PGI<sub>2</sub> synthesis via phospholipase A<sub>2</sub> (Tran and Chan, 1988; Tran and Chan, 1990), the transacylation processes catalyzed by CoA-independent transacylase (Tran et al., 1993a; chapter 1) and the conversion of diacylglycerol to phosphatidic acid via diacylglycerol kinase (Tran et al., 1993b; chapter 2), since these processes were all found to be potentiated by PKC activation (Carter et al., 1989; Garcia et al., 1992a; Whatley et al., 1989; Zavoico et al., 1990.; Heller et al., 1991; Chakraborti et al., 1992; Kanoh et al., 1989; Kanzaki et al., 1989; Breton and Colard, 1991). Moreover, the inhibition of PMA-induced PKC activation by vitamin E may serve as feedback downregulation of the above processes during sustained activation of PKC. The opposite effect of vitamin E and duroquinone on PKC activity suggests that the ratio of oxidant/antioxidant in living organism plays an important role in the regulation of different processes through the PKC pathway. In general, this finding provides a relevant implication of a micronutrient such as vitamin E that may be involved in the regulation of endothelial cells metabolism, particularly at the level of protein kinase C.

**Table 3-1 - Effect of  $\alpha$ -tocopherol on membrane PKC.**

Cell monolayers were incubated without (-E) or with (+E)  $\alpha$ -tocopherol (46  $\mu$ M) for 4h. Cells were rinsed and stimulated with or without PMA (200 nM) for 5 min. Subcellular fractionation and quantitation of PKC activities in the membrane were described in "Materials and Methods". Values are means  $\pm$  S.E. of three separate experiments, each performed in duplicate.

	PKC Activity (pmoles/min/mg)		% of Control
	-E	+E	
- PMA	19 $\pm$ 3	32 $\pm$ 6	68 $\pm$ 0.3
+ PMA	70 $\pm$ 8	59 $\pm$ 9	-18 $\pm$ 6
% increase by PMA	281 $\pm$ 15	87 $\pm$ 16	

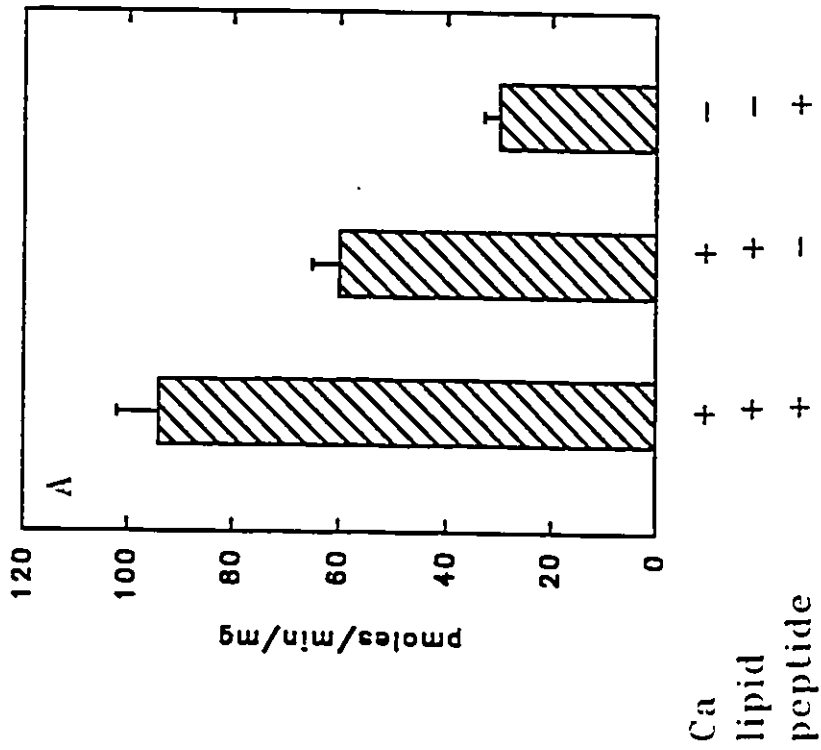
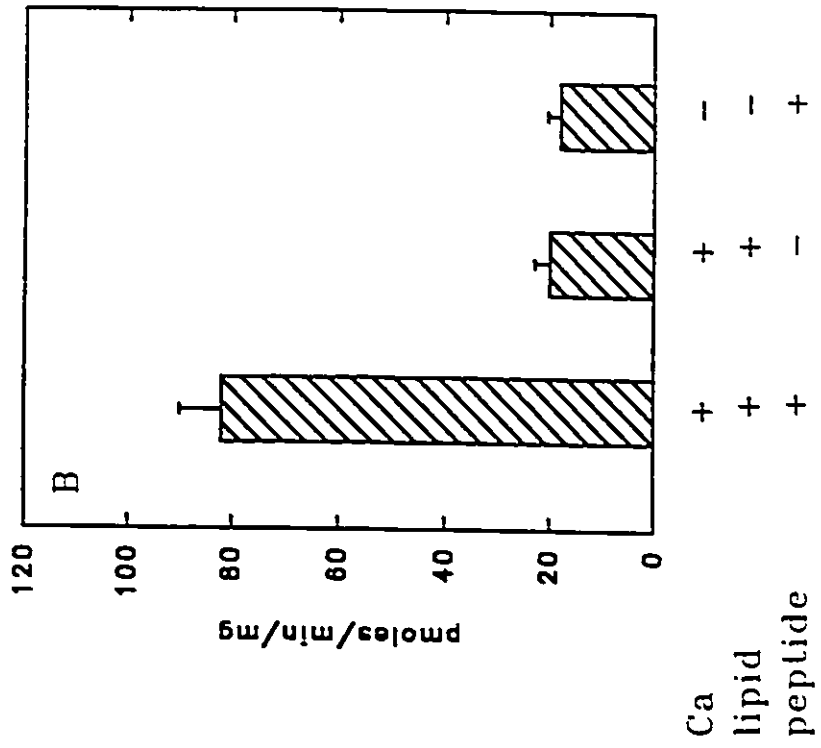
**Table 3-2 - Effect of  $\alpha$ -tocopherol on cytosolic PKC.**

Cell monolayers were incubated without (-E) or with (+E)  $\alpha$ -tocopherol (46  $\mu$ M) for 4h. Cells were rinsed and stimulated with PMA (200 nM) for 5 min. Subcellular fractionation and quantitation of PKC activities in the membrane were described in "Materials and Methods". Values are means  $\pm$  S.E. of three separate experiments, each performed in duplicate. ND - no difference.

	PKC Activity (pmoles/min/mg)		% of Control
	-E	+E	
- PMA	55 $\pm$ 7	57 $\pm$ 8	ND
+ PMA	7 $\pm$ 6	5 $\pm$ 4	ND
% decrease by PMA	88 $\pm$ 10	89 $\pm$ 6	

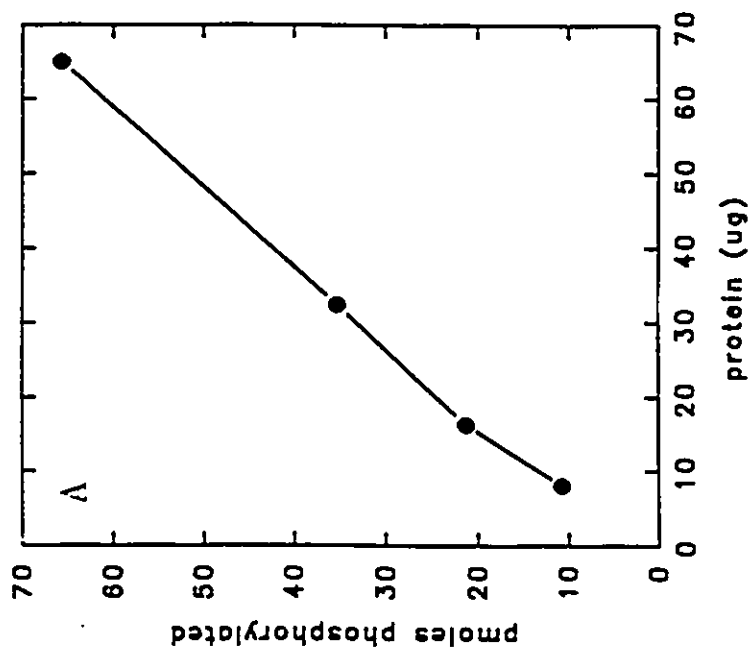
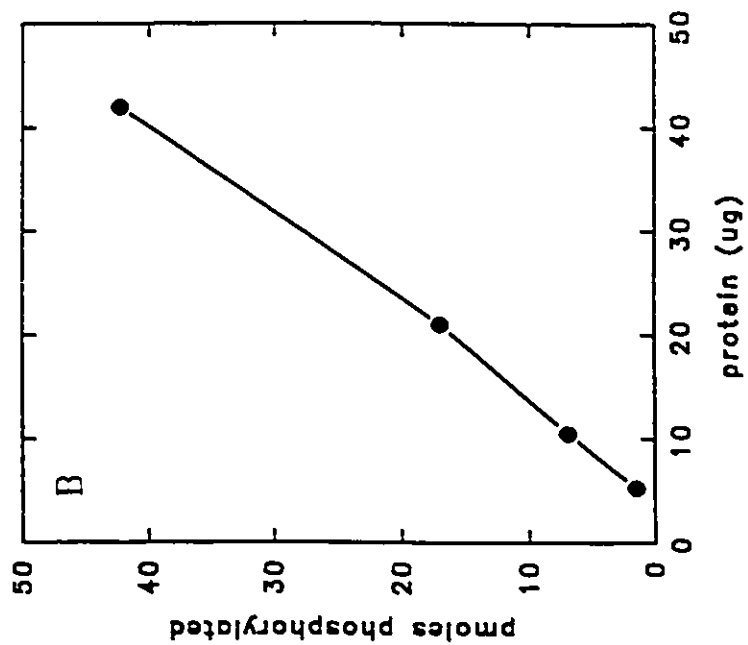
**Figure 3-1 - Membrane and cytosolic PKC activities.**

PKC activities from membrane (A) and cytosolic (B) fractions of human endothelial cells were quantitated in the presence (+) or absence (-) of either  $\text{Ca}^{2+}$ , lipid (PS, PMA) or peptide. Activity is expressed as pmoles/min/mg. Values are means  $\pm$  S.D. of triplicates.



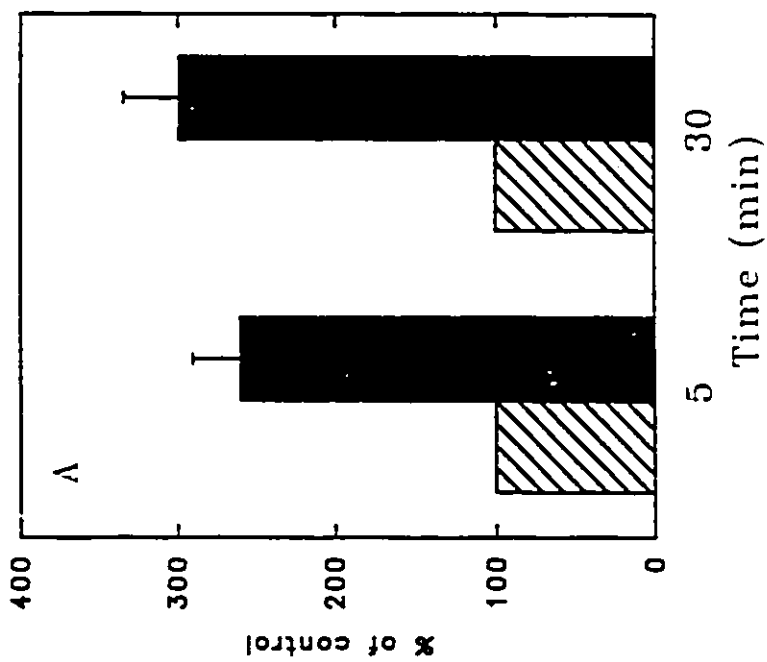
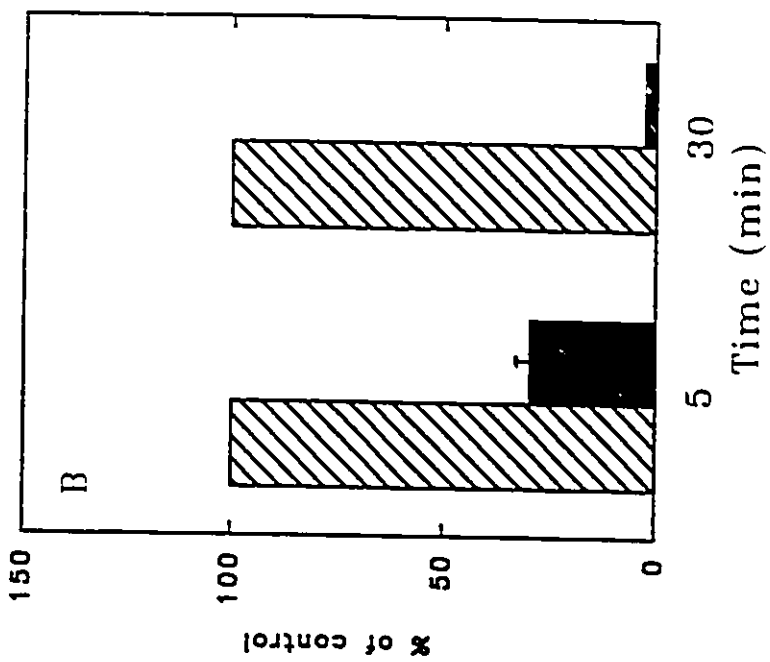
**Figure 3-2 - Dose response of PKC in human endothelial cells.**

PKC activity from membrane (A) and cytosolic (B) fractions of human endothelial cells were quantitated with increasing amounts of proteins. Activity is expressed as pmoles of ATP used to phosphorylate the peptide.



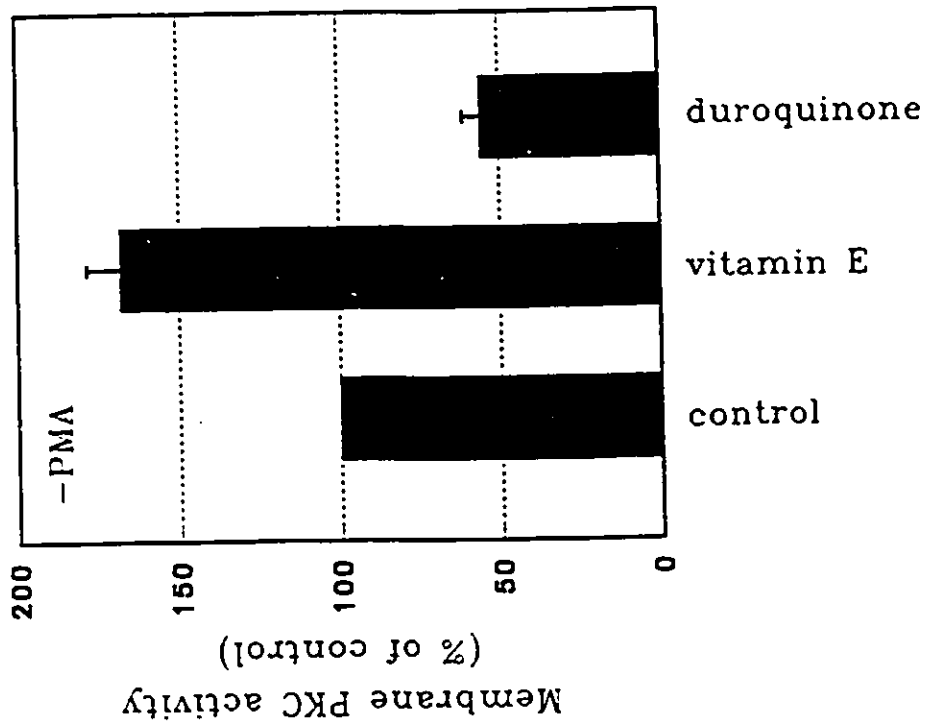
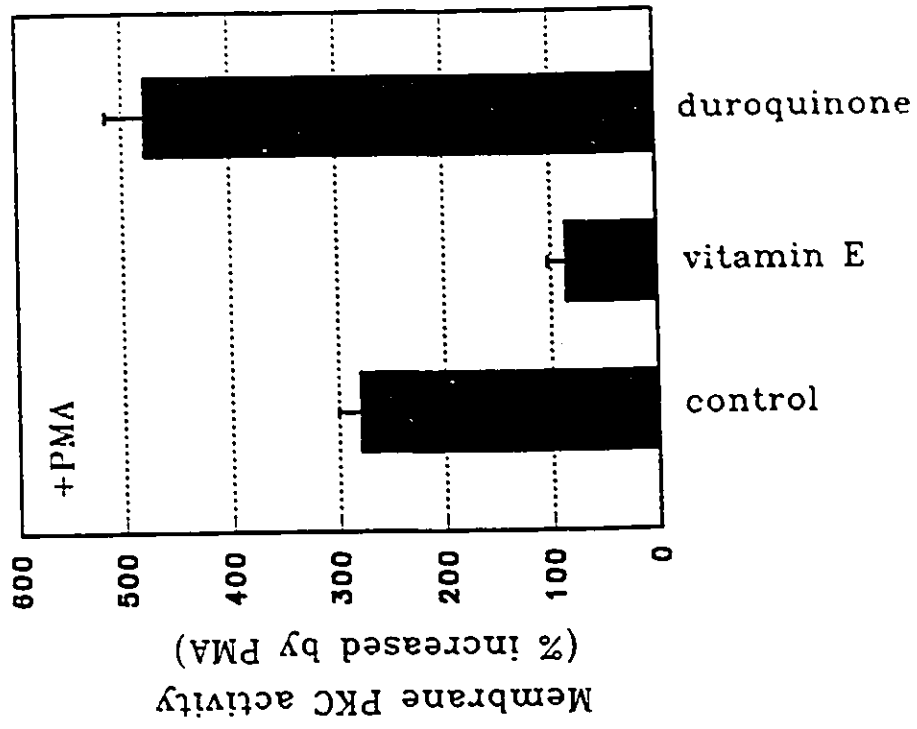
**Figure 3-3 - Effect of PMA on PKC activity.**

Cell monolayers were stimulated with PMA (200 nM) for 5 and 30 min. Subcellular fractionation and quantitation of PKC activities in the membrane (A) and cytosolic (B) fractions were described in "Materials and Methods". PMA-induced PKC activity (solid bars) was expressed as % of unstimulated control (hatched bars). Values are means  $\pm$  S.D. of triplicates.



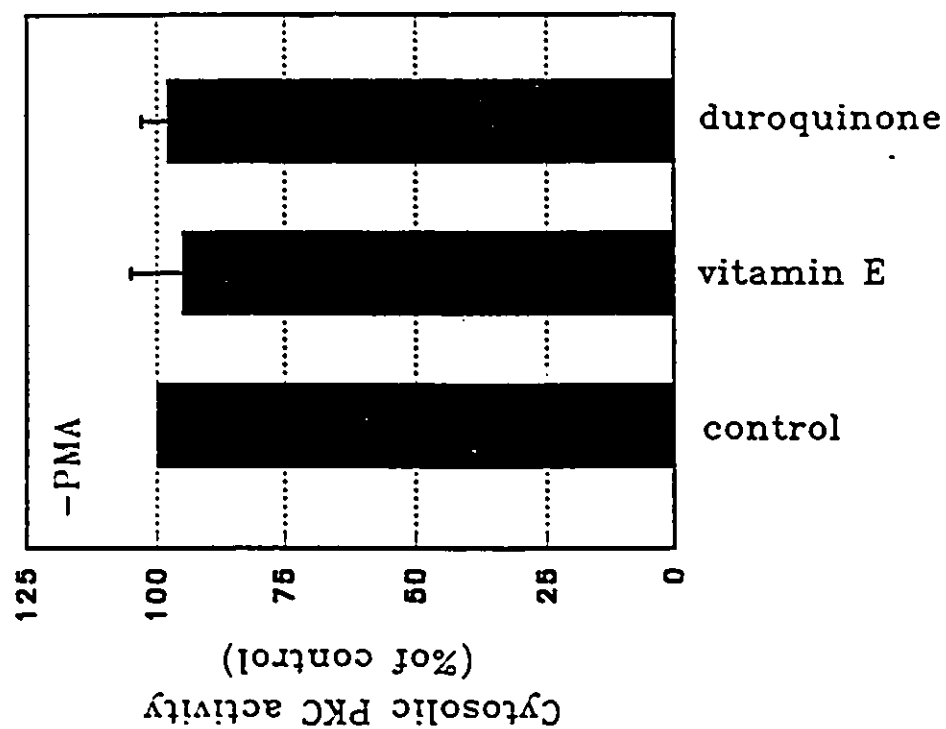
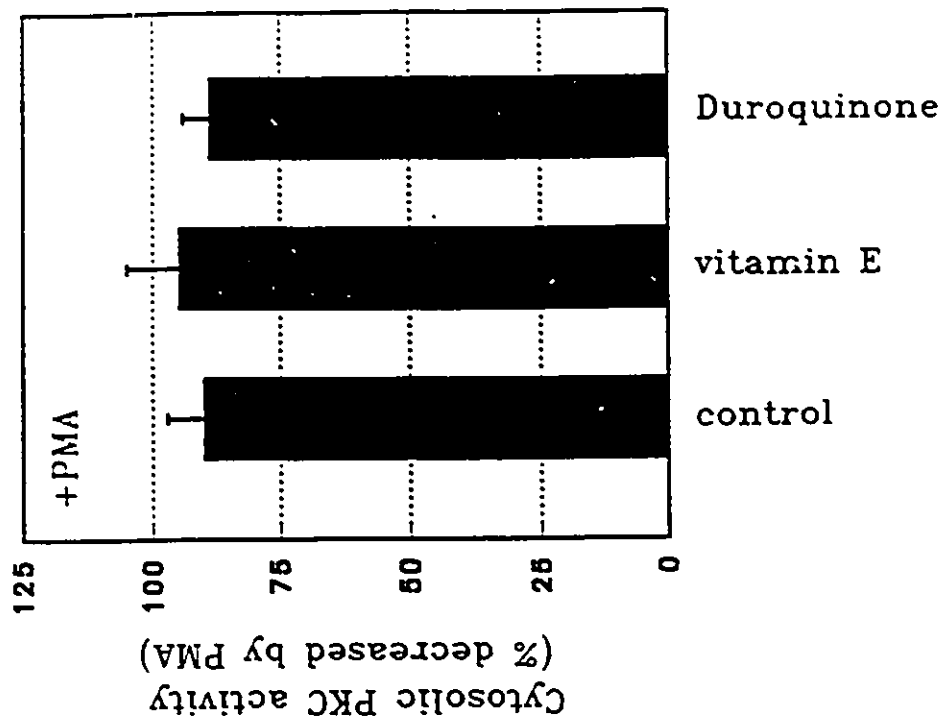
**Figure 3-4A - Effect of duroquinone on membrane PKC activity. A comparison with  $\alpha$ -tocopherol.**

Cell monolayers were incubated with duroquinone (0.5 mM) for 1 h or with  $\alpha$ -tocopherol (46  $\mu$ M) for 4 h. Cells were rinsed and stimulated with or without PMA (200 nM) for 5 min. Subcellular fractionation and determination of membrane PKC activity were described in "Materials and Methods". Values are means  $\pm$  S.E. of three separate experiments, each performed in duplicate.



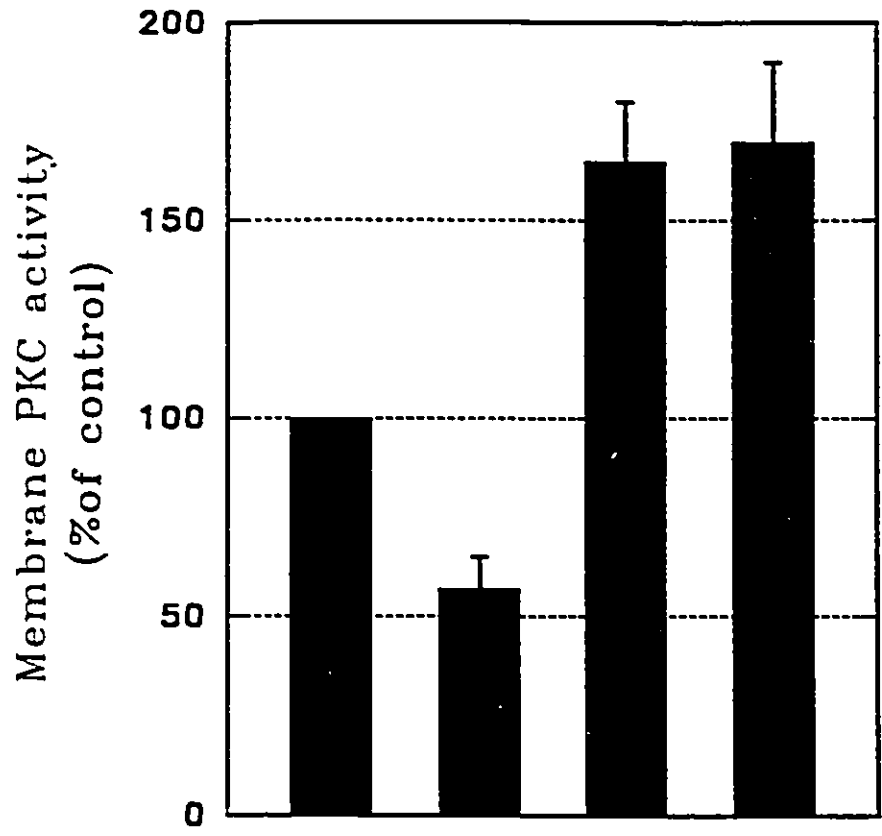
**Figure 3-4B - Effect of duroquinone on cytosolic PKC activity. A comparison with  $\alpha$ -tocopherol.**

Cell monolayers were incubated with duroquinone (0.5  $\mu$ M) for 1 h or with  $\alpha$ -tocopherol (46  $\mu$ M) for 4 h. Cells were rinsed and stimulated with or without PMA (200 nM) for 5 min. Subcellular fractionation and determination of membrane PKC activity were described in "Materials and Methods". Values are means  $\pm$  S.E. of three separate experiments, each performed in duplicate.



**Figure 3-5 - Effect of  $\alpha$ -tocopherol and duroquinone coincubation on membrane PKC activity in resting endothelial cells.**

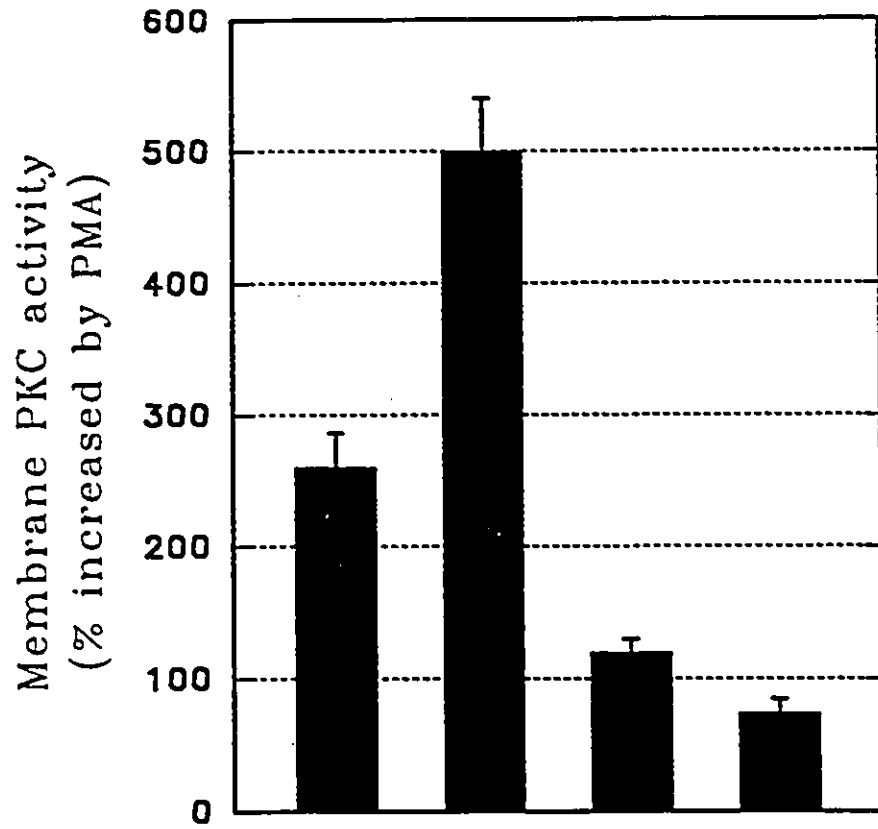
Cell monolayers were incubated with  $\alpha$ -tocopherol (46  $\mu$ M, 4 h) or duroquinone (0.5 mM, 1 h) or with  $\alpha$ -tocopherol (46  $\mu$ M, 4 h) followed by duroquinone (0.5 mM, 1 h). Subcellular fractionation and determination of membrane PKC activity were described in "Material and Methods". Values are means  $\pm$  S.E. of three separate experiments, each performed in duplicate.



vitamin E	-	-	+	+
duroquinone	-	+	-	+
PMA	-	-	-	-

**Figure 3-6 - Effect of  $\alpha$ -tocopherol and duroquinone coincubation on membrane PKC activity in PMA-stimulated endothelial cells.**

Cell monolayers were incubated with  $\alpha$ -tocopherol (46  $\mu$ M, 4 h) or duroquinone (0.5 mM, 1 h) or with  $\alpha$ -tocopherol (46  $\mu$ M, 4 h) followed by duroquinone (0.5 mM, 1 h). Cells were rinsed and stimulated with PMA (200 nM) for 5 min. Subcellular fractionation and determination of membrane PKC activity were described in "Materials and Methods". Values are means  $\pm$  S.E. of three separate experiments, each performed in duplicate.



vitamin E	-	-	+	+
duroquinone	-	+	-	+
PMA	+	+	+	+

## CHAPTER 4

### REGENERATION OF VITAMIN E

#### I. SUMMARY

Human platelets possess active lipoxygenase and cyclooxygenase, and when platelet homogenates were incubated with arachidonic acid (AA), there was a rapid consumption of platelet vitamin E ( $\alpha$ -tocopherol). Over half of AA and tocopherol were metabolized within 0.5 min of incubation. Mass formation of lipoxygenase products (12-HPETE and 12-HETE) or cyclooxygenase products (TXB<sub>2</sub> and HHT) exceeded the mass of tocopherol oxidized. Preincubation with eicosatetraenoic acid (ETYA), a lipoxygenase inhibitor, completely abolished this AA-induced tocopherol oxidation whereas aspirin and indomethacin, cyclooxygenase inhibitors, further potentiated tocopherol oxidation, indicating that this oxidation is closely linked with platelet 12-lipoxygenase activity. Incubation with lipoxygenase metabolites showed that only 12-HPETE caused a rapid tocopherol oxidation which was followed by a gradual tocopherol reappearance. By using nordihydroguaiaretic acid (NDGA), a lipoxygenase inhibitor which is also a strong reductant, over 60% of the AA-induced oxidized tocopherol was regenerated. Tocopherol regeneration declined with increasing oxidation time

induced by AA, and, after 30-60 min, virtually no regeneration could be observed, suggesting that the precursor molecule was unstable. We postulate that the precursor molecule is the tocopheroxyl radical. In the presence of ETYA, either ascorbate or GSH provided significant tocopherol regeneration. Kinetic studies showed that tocopherol regeneration after the addition of ascorbate was essentially completed by 1 min. By contrast, GSH caused a steady increase in tocopherol which peaked after 10 min of its addition. To determine whether this rapid regeneration is chemical or enzymic, regeneration was studied in the presence of chloroform and methanol. Comparison of various reductants in this denaturing condition for enzymes showed that ascorbate and NDGA afforded significant regeneration whereas GSH was ineffective, indicating that there are distinct enzymic and non-enzymic mechanisms for tocopherol regeneration. This study provides direct evidence from mass analysis that tocopherol can be regenerated in human cells. This finding also implies that maintenance of membrane tocopherol status may be an essential function of ascorbate and GSH which operate in concert to ensure maximum membrane protection against oxidative damage.

## II. INTRODUCTION

There are a number of cases, in which free-radical reactions are employed in living systems for useful purposes. One example is phagocytosis carried out by neutrophils and macrophages in the defense mechanism against bacterial invasion. The mechanism involves the increase in  $O_2$  uptake during the "respiratory burst" of these cells, leading to the formation of superoxide, hydrogen peroxide and hydroxyl radical which destroy bacteria or any foreign particle inside the phagocytic vacuoles. However, the uncontrolled radical production induced by endogenous or exogenous factors is linked to many pathophysiological conditions. Free radicals can be the primary cause, or they predispose the organism to a disease caused by other factors, or they make some conditions worse, or they may be an antagonist to the body's natural healing processes. A few general pathophysiological conditions related to free radical production are aging, atherosclerosis, ischaemia/reoxygenation injury, lung damage and cancer. Fortunately, there exist multiple cellular defense mechanisms against these radical-induced damages. These mechanisms, enzymic and non-enzymic, operate in concert for the purpose of maximal removal of radicals or terminating radical-initiated secondary products (e.g. peroxy or alkoxy radicals derived from lipid peroxides). Dietary components contain an array of micronutrients that are directly involved in the enzymic and non-enzymic means of free radical defense. Table 4-1 summarizes these micronutrients and shows the specific cellular

compartmentalization of these trace nutrients and their ligands. For instance,  $\beta$ -carotene and vitamin E are primarily present in cellular membranes whereas ascorbate (vitamin C) and glutathione (GSH/GSSG) are predominantly in the cytosol due to their different solubility in lipid and water.

Located in the biomembranes, vitamin E acts as a major peroxy radical scavenger with powerful chain-breaking property; and its protective role as a membrane stabilizer is well established (Burton et al., 1983; Burton and Ingold, 1986). Despite the very low ratio of vitamin E to phospholipids in the biomembrane, averaging about 1 to 2000, vitamin E effectively performs its role. In 1968, Tappel (Tappel, 1968) proposed that there must exist a recycling system to regenerate vitamin E from its oxidized form, and vitamin C may function as a substrate. Although it is difficult to conceive that this could occur *in vivo* due to distinct cellular locations of these two vitamins, a direct reduction of tocopheroxyl radical by vitamin C was detected *in vitro* in 1979 (Packer et al., 1979). Subsequent studies consistently revealed that the oxidation of vitamin E can be spared by vitamin C or glutathione in a number of pure and biological systems (Leung et al., 1981; Bascetta et al., 1983; Niki et al., 1982; Barclay et al., 1983; Niki et al., 1984; Scarpa et al., 1984; Barclay et al., 1985; Liebler et al., 1986; Niki, 1987; Frei et al., 1988; McCay et al., 1989; Mehlhorn et al., 1989; Vatassery et al., 1989; van den Berg et al., 1990; Sato et al., 1990). The protective role of glutathione in diminishing vitamin E oxidation has led McCay to propose a GSH-dependent reductase in hepatic microsomes which acts to recycle vitamin E (McCay, 1985). A number of recent studies from Packer's laboratory reported the

successful generation of tocopheroxyl radicals in rat hepatic organelles incubated with a combination of soy bean lipoxygenase and arachidonic acid (Packer et al., 1989; Maguire et al., 1989; Kagan et al., 1990). These studies not only permit a detailed analysis of the ESR decay kinetics of tocopheroxyl radicals, but also provide an additional repair mechanism for oxidized tocopherol that is closely linked with mitochondrial electron transport (Maguire et al., 1989). Collectively, these findings lend strong support to the hypothesis that repairing mechanisms for oxidized vitamin E do exist *in vivo*. In the present study, we have provided direct evidence from mass analysis, that in human platelet homogenates, vitamin E, after being oxidized by addition of exogenous arachidonic acid, can be regenerated by physiological relevant water soluble reductants such as vitamin C and reduced glutathione (GSH).

### III. MATERIALS AND METHODS

[1-<sup>14</sup>C]-Arachidonate (specific activity, 51.3 mCi/mmol) was from Du Pont New England Nuclear (Lachine, PQ, Canada). Arachidonate was from Nu Chek Prep (Elysian, MN, USA). 12-HPETE and 12-HETE were from Cayman (Ann Arbor, MI, USA). Reduced glutathione (GSH), glutathione peroxidase from bovine erythrocyte, NDGA, ascorbate and all rac- $\alpha$ -tocopherol acetate were from Sigma Chemical Co. (St. Louis, MO, USA). ETYA was donated by Dr. W.S. Powell, McGill University (Montréal, PQ, Canada). All glassware was silanized with dimethyldichlorosilane before use. Arachidonic was purified on a silicic acid column by eluting with 20% dimethyl ether in hexane on the day before using.

#### 1. Preparation of Platelets

Human platelets ( $1.30 - 2.00 \times 10^{12}$  cells/ml, on day 1-3 of preparation) were obtained from whole blood donated by healthy individuals at the Ottawa Blood Center of the Canadian Red Cross (Ottawa, ON, Canada). Residual red blood cells were removed by a 30 min centrifugation at 170 x g. Platelets in the supernatant were sedimented at 450 x g for 30 min. The platelet pellets were washed with calcium- and magnesium-free Ringer Tyrode's buffer, pH 7.4, containing 2 mM EDTA and 0.1% glucose. The washed platelets were suspended in the same buffer and were adjusted to a concentration of 5 -10 mg of cell protein/ml. Platelet protein was determined by the method of Lowry et al. (Lowry

et al., 1951) using bovine serum albumin as standard. Washed platelet suspensions were sonicated using a Microson ultrasonic cell disruptor setting at 50% output for 3 x 30 s immediately before conducting the experiments.

## **2. Quantitation of $\alpha$ -Tocopherol**

Total lipid was extracted by the method of Bligh and Dyer (Bligh and Dyer, 1959). Briefly, 0.4 ml of sonicated platelet solution was subsequently mixed and vortexed with 1 ml of methanol, 1 ml of chloroform and 0.4 ml of 1 M NaCl solution. All rac- $\alpha$ -tocopherol acetate (0.25  $\mu$ g) was added in the extraction mixture as an internal standard. The organic and aqueous layers were separated by brief centrifugation. The organic (chloroform) layer, at the bottom, was pooled and dried using nitrogen gas. The lipid droplet was dissolved in methanol and was filtered through 0.45  $\mu$ m filter paper (Millipore Co., Bedford, MA, USA).  $\alpha$ -Tocopherol was detected and quantitated by reversed phase HPLC equipped with a C-18 column using a solvent containing methanol / water / trifluoroacetic acid (99:1:0.1, by volume).

## **3. Determination of 12-Lipoxygenase and Cyclooxygenase Activities**

12-Lipoxygenase activity in platelets was determined from the conversion of exogenous arachidonic acid into 12-HPETE and 12-HETE; cyclooxygenase activity was represented by the conversion of arachidonic acid into thromboxane B<sub>2</sub> (TXB<sub>2</sub>) 12-hydroxy-5, 8, 10-heptadecatrienoic acid (HHT). Briefly, platelet homogenates were incubated at 37°C with 0.1  $\mu$ Ci of [1-<sup>14</sup>C]-arachidonate (20  $\mu$ M)

in a total volume of 0.5 ml of Ringer Tyrode's buffer, pH 7.4, containing 2 mM  $\text{CaCl}_2$ . At indicated times, the reaction was stopped by acidifying to pH 3.5 with 1 N formic acid. The remaining arachidonate and the products of lipoxygenase and cyclooxygenase were extracted twice with 6 volumes of ethyl acetate, and they were separated by thin-layer chromatography using a solvent system of chloroform / methanol / acetic acid / water (90: 8: 1: 0.8, by volume). Radiolabelled products were detected by autoradiography and the spots comigrated with authentic standards were scraped into scintillation vials for radioactivity determination. The sum of radioactivity associated with 12-HPETE and 12-HETE was considered as lipoxygenase activity whereas that associated with  $\text{TXB}_2$  and HHT was considered as cyclooxygenase activity.

## IV. RESULTS

### 1. Metabolism of Arachidonic Acid in Human Platelets and Oxidation of Vitamin E

Human platelets possess two major pathways for the enzymic oxygenation of arachidonic acid (Figure 4-1). The cyclooxygenase converts arachidonate to the prostaglandin endoperoxide  $\text{PGG}_2$  which is reduced to  $\text{PGH}_2$  by the peroxidase activity of the cyclooxygenase enzyme. Thromboxane synthetase in platelet utilized two molecules of  $\text{PGH}_2$  to generate thromboxane  $\text{B}_2$  ( $\text{TXB}_2$ ), 12-hydroxyheptadecatrienoic acid (HHT) and malondialdehyde (MDA). Reduction of  $\text{PGG}_2$  to  $\text{PGH}_2$  has been shown to generate tyrosyl radical which inactivates cyclooxygenase thereby limiting the amount of prostaglandins produced (Lassman et al., 1991). The lipoxygenase pathway in platelets convert arachidonate to 12-hydroperoxyeicosatetraenoic acid (12-HPETE) which is then reduced to 12-hydroxyeicosatetraenoic acid (12-HETE). Carbon-centered radicals and alkoxy radicals are formed as intermediate products along the lipoxygenase pathway.

When homogenates of washed human platelets were incubated for 5 min with increasing concentrations of arachidonic acid (AA), there was a dose-dependent disappearance of endogenous  $\alpha$ -tocopherol. Maximum tocopherol oxidation would be achieved at arachidonate concentrations above 20  $\mu\text{M}$  (Figure 4-2). To explore the mechanism of this AA-induced tocopherol oxidation, a time course of AA metabolites formation was monitored in parallel with the

disappearance of arachidonate and  $\alpha$ -tocopherol. Figure 4-3 shows that within 0.5 min after the addition of AA, over half of AA as well as  $\alpha$ -tocopherol were oxidized. The loss of AA was correlated with the formation of AA-metabolites of the cyclooxygenase pathway (TXB<sub>2</sub> and HHT) and the lipoxygenase pathway (12-HPETE and 12-HETE). At 0.5 min after the addition of AA, about 0.4 nmoles of  $\alpha$ -tocopherol was disappeared while 0.93 nmoles and 0.57 nmoles of AA metabolites were formed from the cyclooxygenase and the lipoxygenase pathway, respectively. These data indicate that oxyradicals generate by either the cyclooxygenase or lipoxygenase activity of platelets are sufficient to account for the mass of tocopherol oxidized. However, the bulk of AA loss exceeded the sum of lipoxygenase and cyclooxygenase products, indicating that a minor portion of AA was transformed by other enzymic or non-enzymic pathways under our experimental conditions.

To differentiate which pathway was involved in this AA-induced tocopherol oxidation, we employed eicosatetraynoic acid (ETYA), as lipoxygenase inhibitor, and indomethacin and aspirin, as cyclooxygenase inhibitors. Platelets were preincubated with these inhibitors prior to the addition of AA. Results in Table 4-2 shows that tocopherol oxidation was sensitive toward inhibitors of lipoxygenase but not of cyclooxygenase. AA-induced oxidation of tocopherol was completely abolished when platelet homogenates were preincubated with ETYA (100  $\mu$ M). However, preincubation with known cyclooxygenase inhibitors such as aspirin or indomethacin failed to prevent tocopherol oxidation induced by AA, but rather had a reverse effect. At 10  $\mu$ M of AA, pretreatment with cyclooxygenase inhibitors

consistently caused a greater amount of tocopherol oxidized at all time points tested. A similar trend was observed with a higher level of AA (20  $\mu$ M), but the effects of aspirin and indomethacin were only obvious at 0.5 min, beyond which the magnitude of tocopherol oxidation approached 90% irrespective of drug pretreatment. In addition to being potent inhibitors of cyclooxygenase, aspirin and indomethacin were also shown by Siegel et al. (Siegel et al., 1979a) to be strong inhibitors of platelet peroxidase, leading to the accumulation of 12-HPETE which in turn activated platelet 12-lipoxygenase (Siegel et al., 1979b). The differential effects of cyclooxygenase and lipoxygenase inhibitors observed herein strongly support the notion that AA-induced tocopherol oxidation in platelets is largely mediated by its 12-lipoxygenase activity.

To determine the mechanism by which lipoxygenase might mediate tocopherol oxidation, we studied the effects of different lipoxygenase products, 12-HPETE and 12-HETE, on the platelet tocopherol level. To avoid lipoxygenase activity from endogenous substrate, ETYA was co-incubated with these lipoxygenase products, and the addition of AA in the absence of ETYA served as positive control. Results in Figure 4-4 show that ETYA alone or in combination with 12-HETE had no effect on platelet tocopherol level whereas 12-HPETE induced a 30% loss of tocopherol in the presence of ETYA during the first minute of incubation. In the presence of  $Ca^{2+}$ , addition of AA alone or 12-HPETE plus ETYA resulted in a greater loss of tocopherol. This effect of  $Ca^{2+}$  may be mediated by metal catalyzed decomposition of 12-HPETE to its peroxy radical which causes more tocopherol to be oxidized. To our great surprise, however, at

2.5 and 5 min after the addition of 12-HPETE, we detected reappearance of tocopherol. These results revealed that when endogenous lipoxygenase is blocked by ETYA, 12-HPETE-induced oxidation of platelet tocopherol is reversible, and suggested the presence of endogenous reductant(s) which can be used to regenerate tocopherol. In contrast, in the absence of ETYA, AA induced a prolonged and sustained tocopherol disappearance without apparent regeneration. These data indicate that 12-HPETE but not 12-HETE can mediate the oxidation of tocopherol, and suggest that the amount of tocopherol regenerated is highly dependent on the levels of endogenous reductants. We speculate that AA could induce a sustained tocopherol oxidation by depleting endogenous reductants through the generation of carbon-centered arachidonyl radical generated during the formation of 12-HPETE (Hamberg and Hamberg, 1980). Collectively, data from Figures 4-3 and 4-4 and Table 4-2 show that AA-induced oxidation of  $\alpha$ -tocopherol was mediated mainly by 12-lipoxygenase activity; however, the possibility of other enzymic or non-enzymic effects of AA could not be precluded.

## **2. Regeneration of Tocopherol**

The above observation indicates that in order to systematically investigate tocopherol regeneration, it is necessary to create conditions to block the lipoxygenase activity right after the AA-induced oxidation of tocopherol is initiated, to provide a source of reductant, and to remove the added  $\text{Ca}^{2+}$  so as to avoid further metal-catalyzed formation of peroxy radicals during regeneration process. To achieve these goals, we have therefore devised a "regenerating system"

consisting of ethylenediaminetetracetic acid (EDTA) and nordihydroguaiaretic acid (NDGA). EDTA is a divalent cation chelator while NDGA is a lipoxygenase inhibitor and is also a strong reductant. Figure 4-5 shows that when platelet homogenates were incubated with 20  $\mu$ M of arachidonate for 5 min, 86% of tocopherol was oxidized. When identical tubes were further incubated with EDTA/NDGA solution for an additional 10 min, 60% of the oxidized tocopherol was regenerated. These data show that oxidized tocopherol can be regenerated under appropriate conditions.

The HPLC profile of tocopherol regeneration is illustrated in Figure 4-6, and it is interesting to note that  $\gamma$ -tocopherol appears to be more resistant to AA-induced oxidation than  $\alpha$ -tocopherol, an observation consistent with that reported by Niki and his group (Niki et al., 1986; Sato et al., 1990) whose work dealt with pure systems of tocopherols incorporated liposomes. To further investigate the stability of the precursor molecule, we incubated platelet homogenates with AA at various time points after which the mixtures were further incubated with the "regenerating system" for additional 30 min. Figure 4-7 clearly shows that tocopherol regeneration peaked at 2.5 min after AA addition, and thereafter, the capacity of regeneration steadily declined and remained only 10% by 30 min; and after 1 h of exposure with AA, regeneration capacity of tocopherol was completely lost. These data suggest that the precursor molecule for tocopherol regeneration was labile and rapidly decomposed between 0.5 to 1 h. This rate of decomposition coincides precisely with the ESR decay rate of tocopheroxyl radical detected by Packer's group (Mehlhorn et al., 1989). We therefore postulate that the precursor

molecule is the tocopheroxyl radical.

### **3. Kinetics and Requirements of Tocopherol Regeneration**

Although our "regenerating system" provided significant tocopherol-regenerating capacity, the presence of NDGA, a lipoxygenase inhibitor with strong reducing activity, did not allow us to evaluate other physiological relevant reductants that may be involved in the tocopherol-regenerating reaction. We therefore selected other lipoxygenase inhibitor, such as ETYA, which is devoid of reducing activity, and compared the kinetics of the regenerating reaction mediated by two physiological reductants, vitamin C (ascorbate) and reduced glutathione (GSH). Figure 4-8 illustrates the time course of regeneration after the addition of ascorbate or GSH in the presence of ETYA and EDTA. After 5 min of AA-induced tocopherol loss, the addition of ascorbate caused a rapid tocopherol regeneration which peaked at 1 min by which time 64% of oxidized tocopherol was regenerated. By contrast, the addition of GSH caused a steady increase of tocopherol level which reached maximum after 10 min, at which time the magnitude of regenerated tocopherol is the same as that afforded by ascorbate. The difference in the rate of tocopherol regeneration suggests that the mechanisms of tocopherol regeneration elicited by these two reductant may be different.

The rapid regeneration of tocopherol caused by ascorbate addition suggests that the mechanism of regeneration is chemical rather than enzymic. To test this hypothesis, regeneration of tocopherol was conducted in a protein denatured condition. Tocopherol oxidation was induced by incubating platelet homogenates

with 20  $\mu$ M of AA for 1 or 5 min at which time a mixture of chloroform and methanol was added to terminate any enzymic activity. This was immediately followed by the addition of various reductants, and tocopherol regeneration in this single phase mixture was allowed to proceed for 2 min. In this enzyme denaturing condition, both NDGA and ascorbate provided significant tocopherol regeneration, but not GSH. Table 4-3 shows that after platelet homogenates were incubated with AA for 1 or 5 min, 61-85% of oxidized tocopherol was regenerated by NDGA, and 42-60% by ascorbate. Virtually no regeneration was detected by the addition of GSH. These data suggest that tocopherol regeneration elicited by NDGA and ascorbate occurred via a chemical reaction. By contrast, the inability of GSH to regenerate tocopherol under these denaturing conditions suggests that tocopherol regeneration by GSH observed in Figure 4-8 proceeds with the aid of protein catalysis as proposed by McCay (McCay, 1985). Collectively, these data demonstrate that more than one mechanism is involved in the regeneration reaction.

## V. DISCUSSION

Results from the present study demonstrate that in human platelets, oxidation of tocopherol is closely linked with the turnover of arachidonic acid via the 12-lipoxygenase pathway. This AA-induced oxidation of tocopherol can be reversed by cytosolic soluble reductants such as ascorbate or GSH, indicating that the maintenance of membrane tocopherol status may be an essential function of cellular ascorbate and GSH which operate in concert to maximize membrane redox potential against radical induced damages. The AA-induced loss of platelet tocopherol is consistent with the recent report from Packer's laboratory (Packer et al., 1989; Maguire et al., 1989; Kagan et al., 1990) that tocopheroxyl radical was generated when rat hepatic microsomes were incubated with soybean lipoxygenase and arachidonate. Since the hydrolysis of arachidonate in platelets is a steady state phenomenon coupled with the remodelling of phospholipids, it is plausible that some tocopherol is continuously oxidized and recycled at the membrane-cytosol interface.

The regeneration of tocopherol under denaturing conditions for enzymes strongly suggests that the mechanisms of tocopherol regeneration afforded by ascorbate and NDGA is a chemical reaction (Table 4-3). In contrast, GSH failed to regenerate tocopherol under these enzyme-denatured conditions, indicating that its role in the regenerating reaction may be indirect or is closely linked to a GSH-dependent reductase system as proposed by McCay (McCay, 1985). In this

context, an alternative enzyme-catalyzed two-electron reduction of tocopherones proposed by Liebler and coworkers (Liebler et al., 1989) may also have relevance *in vivo*. Recent work from Packer's laboratory (Maguire et al., 1989) revealed that in mitochondria, the electron transport system is capable of reducing tocopheroxyl radical. Collectively, these data strongly support the existence of both enzymic and non-enzymic mechanisms for the repair of oxidized tocopherol in biomembranes.

The synergistic effect of ascorbic acid and tocopherol in preventing oxidative rancidity of dietary fats has been noted half a century ago when Golumbic and Mattill (Golumbic and Mattill, 1941) reported that addition of ascorbate reduces the rate of tocopherol consumption. Table 4-4 summarizes the historical development of the sequence of observation leading to the identification of vitamin E cycle in human cells. In 1968, Tappel proposed that one of the functions of ascorbate *in vivo* is to reduce oxidized tocopherol (Tappel et al., 1968). Eleven years later, Packer and coworkers (Packer et al., 1979) showed that tocopheroxyl radicals decay rapidly in the presence of ascorbate in organic solvent. Subsequently, Niki's (Niki et al., 1982; Niki et al., 1984), Barclay's (Barclay et al., 1983) and Scarpa's groups (Scarpa et al., 1984) reported that in liposomes, addition of ascorbate immediately quenched the oxidation of vitamin E. More recently, studies from Packer's laboratory have shown that reduction of tocopheroxyl radical can occur in rat hepatic organelle membranes and extended the effective reductants to include the pyridine nucleotide (Mehlhorn et al., 1989; Maguire et al., 1989). Our observation from mass analysis of vitamin E demonstrates that repair mechanisms

for oxidized tocopherol also exist in human cell homogenate (Chan et al., 1991). These findings illustrate the intricate dependency of membrane redox potential on cytosolic reductants and further reinforce the notion that, in estimating the vitamin E requirement in human, other dietary factors such as ascorbate, selenium (a component of GSH peroxidase) and unsaturated fatty acids should also be considered.

If one accepts the notion of nutrient requirement, the rate of turnover of a particular nutrient will be important in determining its need. In this regard, Figure 4-9 illustrates that the oxidation of vitamin E does occur by distinct enzymic and environmental factors. Experimental evidence from animals and man has long established that increasing of dietary polyunsaturated fats increases the requirement of vitamin E. Therefore, the turnover of membrane polyunsaturated fatty acids, a steady state in the remodelling process of membrane phospholipids, must consume vitamin E mainly via the lipoxygenase pathway. The rate of vitamin E consumption is expected to be much higher during inflammation in which lipoxygenase activity and oxyradical production are much higher in inflammatory cells such as leukocytes and platelets. We have already shown that oxidized vitamin E elicited by hemoglobin can also be regenerated in rat neutrophils (Ho and Chan, 1992).

Experimental design to study the synergistic interaction of vitamins E and C are limited to few animal species that are similar to humans in having a defective ascorbate synthetic pathway. However, evidence indicating that high dietary vitamin C elevates plasma tissue vitamin E levels in guinea pigs has been

reported (Bendich et al., 1984). The vitamin C sparing effect on vitamin E has similarly been reported recently to occur in a mutant strain of Wistar rat defective in vitamin C synthesis (Igarashi et al., 1991). Although direct quantitation of vitamin E regeneration reaction in human is difficult, cumulative direct and indirect experimental evidence strongly suggests that such reaction does occur *in vivo*. Whether ascorbate is the key reductant to this reaction, or other molecules such as glutathione or reduced ubiquinone (Figure 4-9) are equally important donors remained to be elucidated.

## VI. CONCLUSION

In protecting the biomembranes from oxidative injury, vitamin E has to be consumed and become itself a tocopheroxyl radical. The efficiency of the role of vitamin E relies on the ability to be regenerated from its oxidized form, and the presence of water soluble reductants in the cytosol warrants this regeneration reaction. Thus, the antioxidant role in the protection against radical induced damage is not limited to a particular micronutrient, nor any one is more important than the other, but they all work in concert to provide maximal and global protection in different environments. The regeneration of vitamin E also has other implications in the notion that it will strengthen the new role of vitamin E in the phospholipid metabolism presented in the previous chapters of this thesis.

**Table 4-1 - Micronutrients involved in free-radical defense.**

<b>Nutrients</b>	<b>Cellular Compartments</b>	<b>Functional Role</b>
$\beta$ -carotene	membranes, plasma	antioxidant
Vitamin E	membranes, plasma	antioxidant
Vitamin C	cytosol, plasma	antioxidant
Niacin, Tryptophan	cytosol, mitochondria	component of NADH/NADPH
Riboflavin	cytosol, plasma	GSH-reductase
Selenium	cytosol, membranes	GSH-peroxidase
Zinc/Copper	cytosol	superoxide dismutase
Manganese	mitochondria	superoxide dismutase

**Table 4-2 - Effects of aspirin and indomethacin on arachidonate-induced tocopherol oxidation.**

Platelet homogenates containing approximate 0.6 nmol of tocopherol were preincubated with aspirin or indomethacin for 15 min prior to the addition of 10 or 20  $\mu\text{M}$  of arachidonate in the presence of 2 mM of  $\text{Ca}^{2+}$ . At indicated times, the reactions were stopped with  $\text{CHCl}_3/\text{MeOH}$  and tocopherol was determined as described under "Materials and Methods". Each value represents the mean of two separate determinations.

Pretreatment	AA	Tocopherol Oxidized		
		0.5 min	2.5 min	5 min
	$\mu\text{M}$		%	
ETYA (100 $\mu\text{M}$ )	20	0	0	0
None	10	28	52	48
	20	54	88	87
Indomethacin (200 $\mu\text{M}$ )	10	31	71	86
	20	68	93	96
Aspirin (2 mM)	10	39	74	89
	20	81	94	95

**Table 4-3 - Effects of different reductants on the regeneration of tocopherol in a protein denaturing condition.**

Platelet homogenates containing approximately 0.6 nmol of tocopherol were incubated with 20  $\mu$ M of AA and 2 mM of  $Ca^{2+}$  for 1 or 5 min followed by addition of 3ml of  $CHCl_3/MeOH$  (1:2, v/v). NDGA, ascorbate or GSH was immediately added to the reaction mixture, and tocopherol regeneration was allowed to proceed for another 2 min. Tocopherol determination was described under "Materials and Methods". Levels of tocopherol after 1 or 5 min oxidation were 0.45 and 0.51, respectively. Each value represents the mean of two separate determinations

Reductants	Tocopherol Regeneration	
	1 min <sup>a</sup>	5 min <sup>a</sup>
	%	
NDGA (50 $\mu$ M)	85.4	61.0
Ascorbate (2 mM)	60.5	42.0
GSH (2 mM)	6.3	0.

<sup>a</sup>Time of incubation with AA.

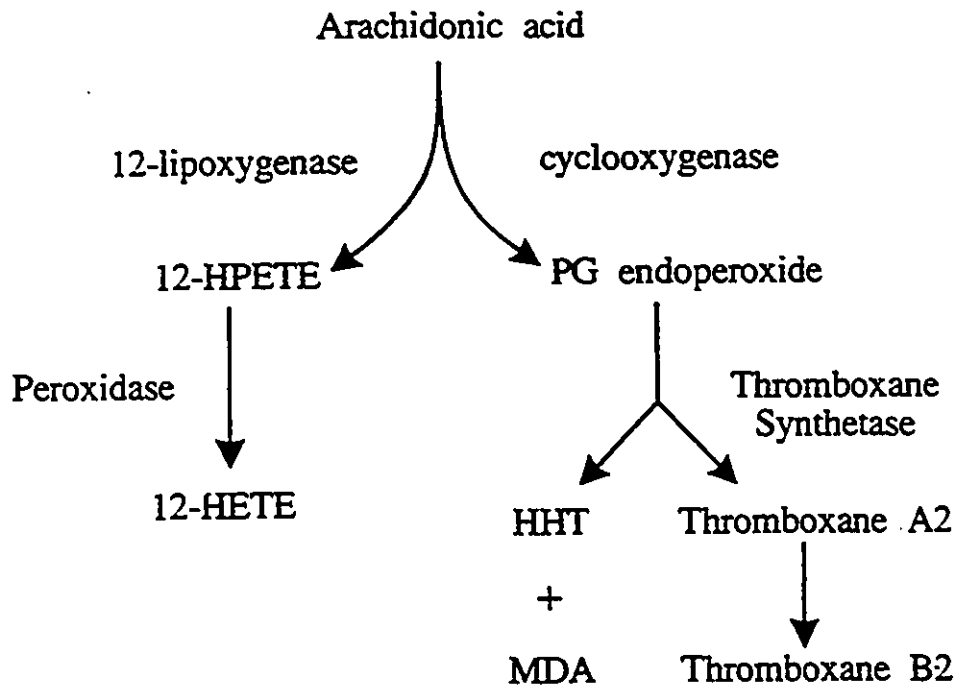
**Table 4-4 - Historical development of vitamin E and its cycle.**

1. Identification of vitamin E as an essential nutrient	Evans and Bishop, 1922
2. Suggestion for vitamin E regeneration from vitamin C	Tappel, 1968
3. Regeneration of vitamin E in organic solution	Packer et al., 1979 Niki et al., 1982 Barclay et al., 1983
4. Regeneration of vitamin E in rat liver mitochondria and microsomes	Mehlhorn et al., 1989 Maguire et al., 1989
5. Two different pathways of the regeneration of vitamin E in human platelets	Chan et al., 1991

**Figure 4-1 - Arachidonic acid metabolism in human platelets.**

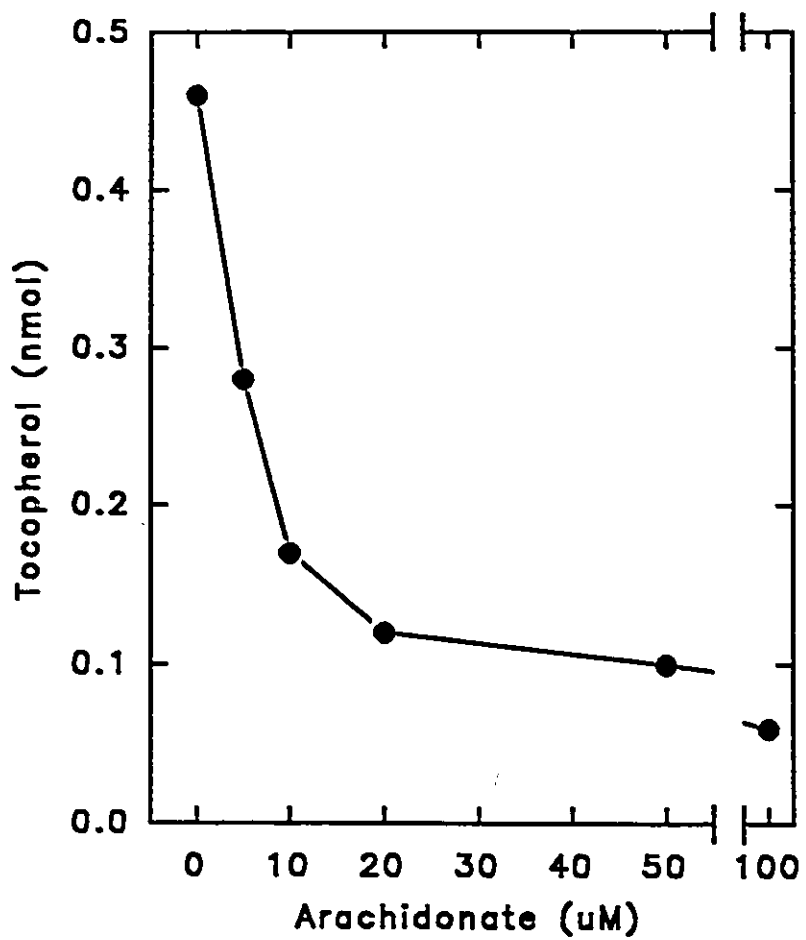
12-HPETE, 12-hydroperoxyeicosatetraenoic acid; 12-HETE, hydroxyeicosatetraenoic acid; PG, prostaglandin; HHT, hydroxyheptadecatrienoic acid; MDA, malondialdehyde.

Arachidonic Acid Metabolism in Human Platelets



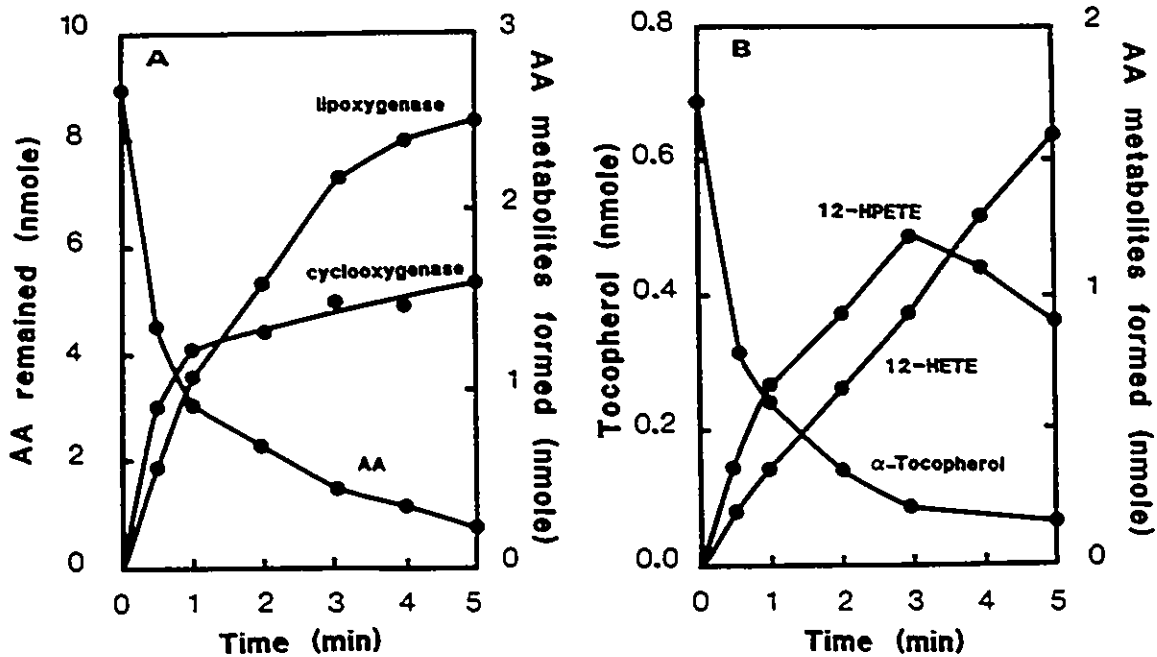
**Figure 4-2 - Oxidation of platelet tocopherol by arachidonic acid.**

Platelet homogenates were incubated for 5 min with indicated arachidonate concentrations. Tocopherol was determined as described in "Materials and Methods". Each point is the mean of triplicate from a representative experiment.



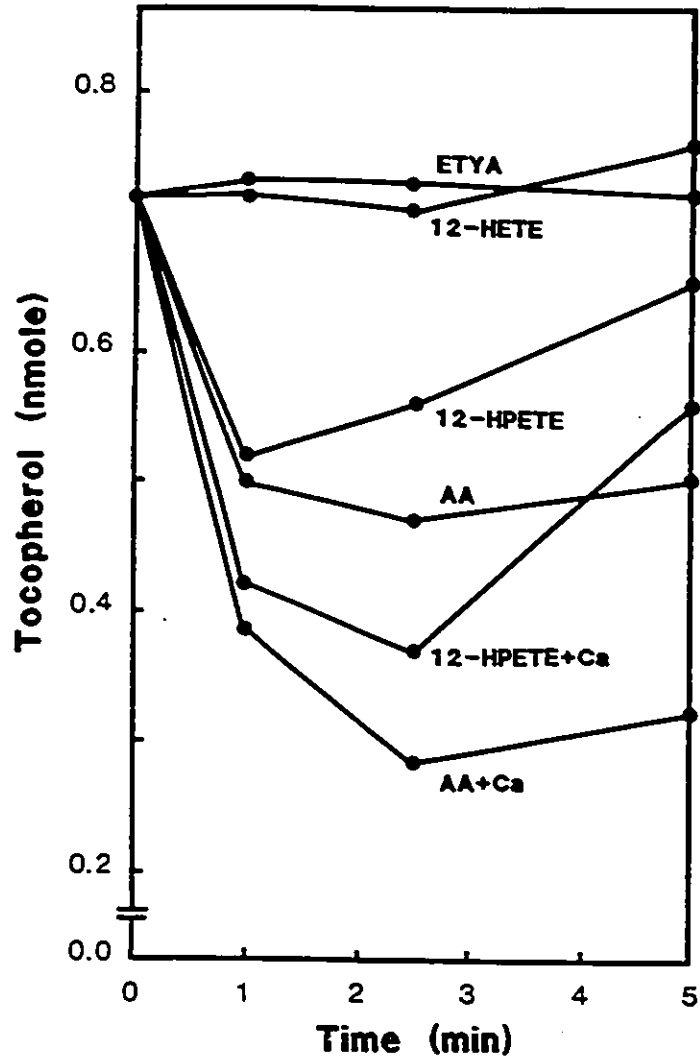
**Figure 4-3 - Time course of arachidonic metabolism; correlation with tocopherol oxidation.**

Platelet homogenates were incubated with 20  $\mu\text{M}$  of [ $1\text{-}^{14}\text{C}$ ] arachidonic acid (200,000 cpm) in the presence of 2 mM  $\text{Ca}^{2+}$  for indicated times. Determination of tocopherol and arachidonate metabolites are described in "Materials and Methods". Panel A, lipoxygenase products are the sum of 12-HPETE and 12-HETE, and cyclooxygenase products are the sum of thromboxane  $\text{B}_2$  and HHT. Panel B, the oxidation of tocopherol and the formation of 12-HPETE and 12-HETE. Each point represents the mean of two separate determinations.



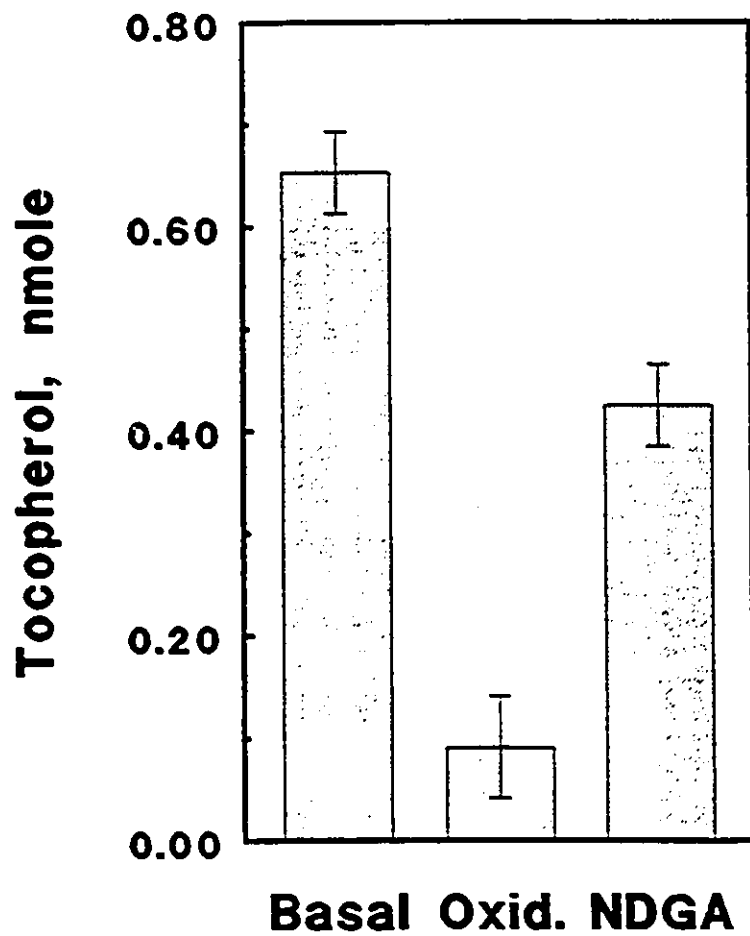
**Figure 4-4 - Effects of arachidonate and its 12-lipoxygenase products on the oxidation of tocopherol.**

Platelet homogenates were incubated with 20  $\mu\text{M}$  of tested compound for the indicated times. ETYA (100  $\mu\text{M}$ ) was present in reactions involving 12-HPETE and 12-HETE to inhibit lipoxygenase activity from endogenous substrate. In reactions where  $\text{Ca}^{2+}$  was present, the net concentration was 2 mM. Determination of tocopherol is described in "Materials and Methods". Values are means of two determinations with variation less than 5%.



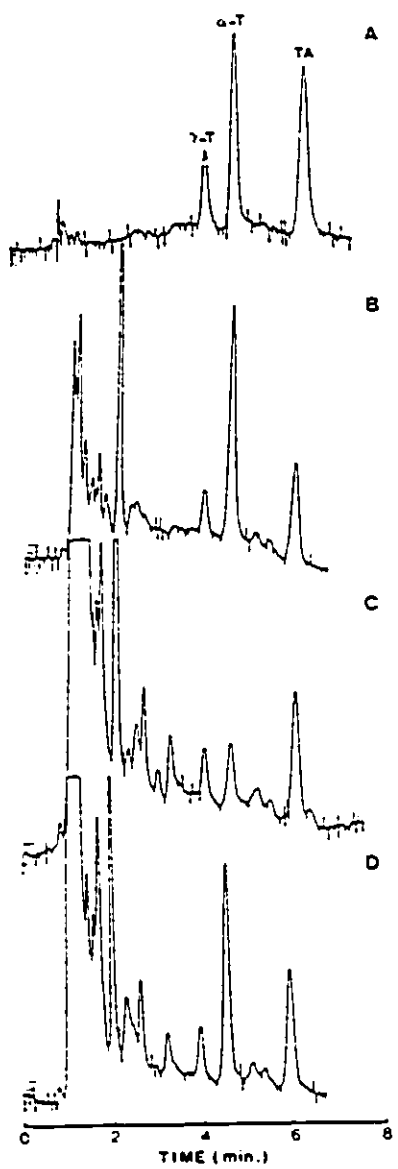
**Figure 4-5 - Regeneration of tocopherol by NDGA.**

Platelet homogenates containing approximately 0.60 nmol of tocopherol was incubated with 20  $\mu\text{M}$  of AA and 2 mM of  $\text{Ca}^{2+}$  for 5 min at 37°C (oxidation, Oxid.). Regeneration of tocopherol was assessed by adding 50  $\mu\text{M}$  of NDGA and 4 mM of EDTA immediately after 5 min of oxidation induced by AA; the reaction mixtures were further incubated for 10 min. Tocopherol was determined as described in "Materials and Methods". Values are means  $\pm$  S.D. of five platelet preparations obtained from healthy donors.



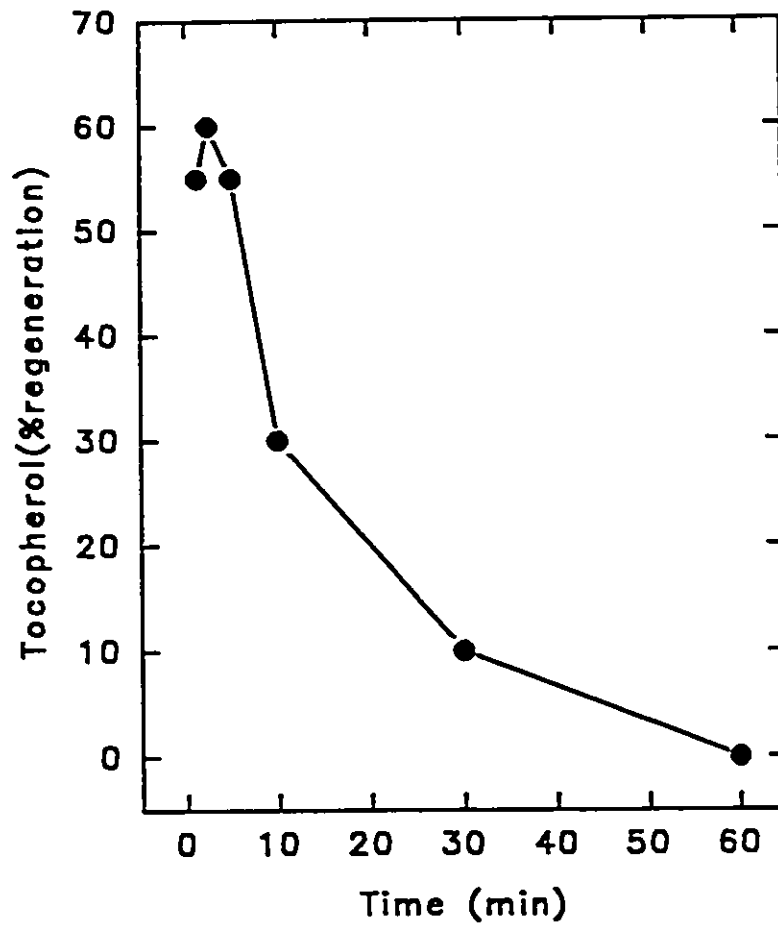
**Figure 4-6 - HPLC chromatograms indicating the regeneration of  $\alpha$ -tocopherol.**

A, elution profile of  $\gamma$ -tocopherol ( $\gamma$ -T),  $\alpha$ -tocopherol ( $\alpha$ -T) and tocopherol acetate (TA) standards. B, elution profile of lipid extract from platelet homogenate with TA as internal standard. C, elution profile of lipid extract from platelet homogenate incubated with 20  $\mu$ M of AA for 5 min. D, same treatment as in C but further incubated with 50  $\mu$ M of NDGA and 4 mM of EDTA for 10 min.



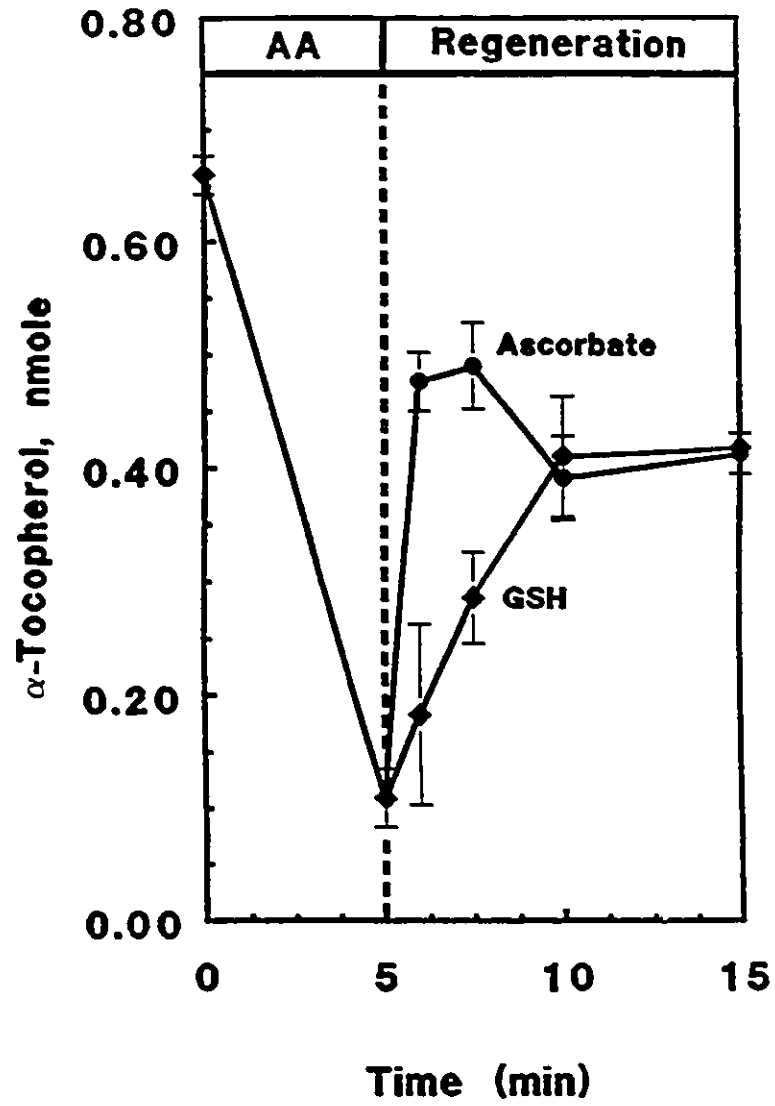
**Figure 4-7 - Regeneration of tocopherol as a function of oxidation time.**

Platelet homogenates were incubated with 20  $\mu\text{M}$  of AA for indicated times. The reaction was then either stopped with methanol or was proceeded further with the addition of 50  $\mu\text{M}$  NDGA and 4 mM EDTA and incubated for 30 min. Percent tocopherol regenerated = (mass of tocopherol regenerated) / (mass of tocopherol oxidized) x 100. Each value is the mean of triplicate from a representative experiment.



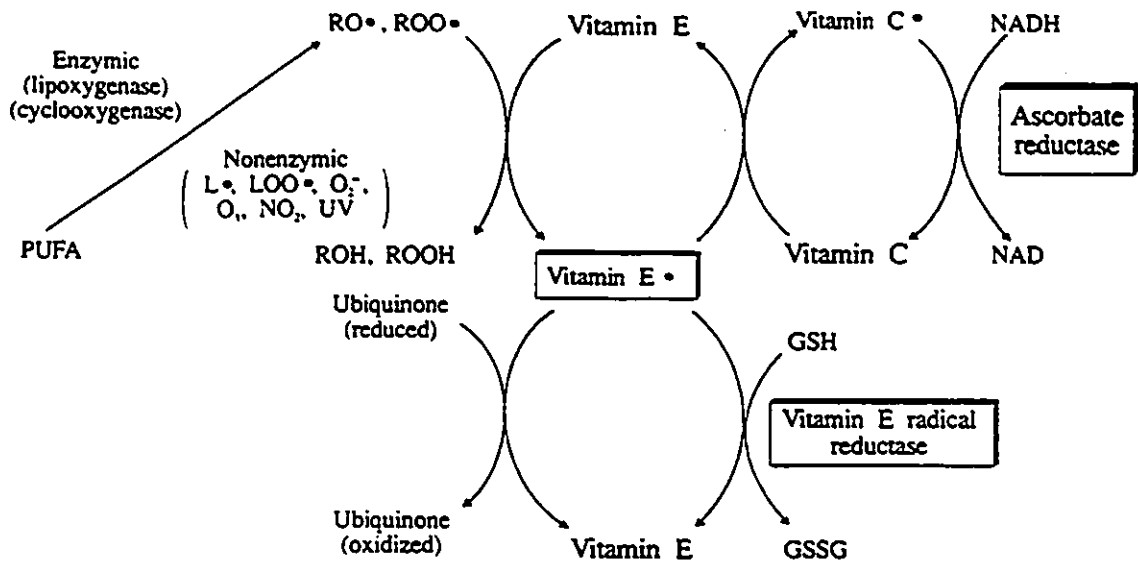
**Figure 4-8 - Time course of tocopherol regeneration.**

Platelet homogenates were incubated with 20  $\mu\text{M}$  of AA and 2 mM  $\text{Ca}^{2+}$  for 5 min. The reaction was either stopped or further incubated with 2 mM of GSH or ascorbate in the presence of 100  $\mu\text{M}$  ETYA and 2 mM EDTA. Tocopherol regeneration was monitored over indicated times. Values are means  $\pm$  S.D. of five platelet preparations obtained from healthy donors.



**Figure 4-9 - Pathways for the oxidation and regeneration of vitamin E.**

Peroxidation of membrane polyunsaturated fatty acid (PUFA) can be mediated by either enzymic or non-enzymic process, forming alkoxy ( $\text{RO}^\cdot$ ) and peroxy ( $\text{ROO}^\cdot$ ) radicals. Vitamin E converts these radicals into alcohol ( $\text{ROH}$ ) and hydroperoxide ( $\text{ROOH}$ ) and becomes vitamin E radical (vitamin  $\text{E}^\cdot$ ). Regeneration of vitamin E from vitamin  $\text{E}^\cdot$  is facilitated by other physiological reductants including reduced ubiquinone, vitamin C and GSH.



## GENERAL CONCLUSION

Our findings demonstrate for the first time the involvement of vitamin E in phospholipid metabolism in human vascular endothelial cells. The enhancing effect of vitamin E in the acylation processes may be related to the control of vascular tone and thrombosis via the synthesis of prostacyclin, a potent vasodilator and inhibitor of platelet aggregation. In addition, the ability of vitamin E to regulate protein kinase C as well as the formation of cellular signalling second messengers such as diacylglycerol and phosphatidic acid indicates that the functions of vitamin E in the protection of cellular damage may not be simply limited on its ability to reduce free radical-induced oxidation on cellular membrane, but it may also rely on its ability to keep many key cellular enzymes in phospholipid metabolism at their physiological levels. It is therefore likely that the fundamental role of this vitamin is to prevent the functional deteriorating processes by maintaining healthy physiological environmental condition of endothelial cells.

Of course, there is no single factor *in vivo*, such as vitamin E alone, that is capable of performing its role optimally. The finding that vitamin E can be regenerated by vitamin C and GSH after being oxidized again indicates that multiple factors are indispensable for optimal cellular functions and particularly the protection of cell membrane from injury requires the presence of multiple reductants in both membrane and cytosolic compartments. From this notion, it is tempted to extrapolate that vitamin E regeneration may be a phenomenon

necessary for its high efficiency in performing its function(s), such as its regulation of phospholipid metabolism in endothelial cells despite its low concentration in cellular membrane.

Current hypothesis for the mechanism of atherogenesis (Steinberg, 1991) proposed that low-density lipoprotein (LDL) undergoes oxidation and modification by radicals to a form which is recognized by the "scavenger" receptor on macrophages to create foam cells. The atherogenic effects of oxidized LDL also include its chemoattractant action circulating monocytes, its cytotoxicity to vascular endothelial cells and its ability to stimulate the release of growth factors and cytokines leading to the proliferation of smooth muscle cells and constriction of arteries. In an effort to prevent this pathogenesis, vitamin E was found to increase the resistance of LDL to oxidation when added to plasma (Esterbauer et al., 1991) and to inhibit the proliferation of smooth muscle cells *in vitro* (Boscoboinik et al., 1991a and 1991b; Chatelaine et al., 1993). For the same purpose, we think that our findings can contribute to this general effort by demonstrating that vascular endothelial cells metabolism is regulated by vitamin E. Although adequate data are not yet available to justify the use of vitamin E in atherosclerosis treatment, recent large-scale prospective studies reported in the New England Journal of Medicine showed that the use of vitamin E supplements for more than two years is associated with a significantly decreased risk of coronary heart disease in both men (Rimm et al, 1993) and women (Stampfer et al., 1993).

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