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Vitamin E and Arachidonic Acid
Metabolism in Cultured Human Endothelial Cells

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A Thesis submitted to the school of Graduate Studies and Research of the
University of Ottawa in partial fulfillment of the requirement for the degree of
Master of Sciences.

Department of Biochemistry
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Khai T. Tran, Ottawa, Canada, 1990



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ABSTRACT

A novel approach to study the metabolism of nutrients in human cells was developed. The incorporation and depletion of 2R,4'R,8'R- α -tocopherol (RRR- α -T), a natural form of vitamin E, and its subsequent effect on the metabolism of arachidonic acid were conducted on human umbilical vein endothelial cells (HUVECs) in culture. HUVECs incorporated physiological and pharmacological concentrations of RRR- α -T in a time- and dose-dependent manner. Incorporated tocopherol was found mainly associated with membrane fractions of the cell. At physiological concentrations (23.2 μ M), the uptake of RRR- α -T by HUVECs was dependent on the incubation time and was saturated at 8 h. In addition, HUVECs incorporated RRR- α -T proportionally to the concentrations of tocopherol present in the medium. However, when the cells were preincubated with 23.2 μ M of RRR- α -T for 4 h, followed by replacing the medium with tocopherol-free material, intracellular tocopherol decreased over time. Enrichment of HUVECs with RRR- α -T was found to potentiate agonist-induced arachidonic acid release as well as basal prostacyclin (PGI₂) synthesis. This stimulatory activity of tocopherol has an absolute structural requirement for both free hydroxyl moiety and the hydrophobic phytyl side chain, although the position and the presence of the methyl groups attached to the aromatic moiety are not required for its activity. Tocol, 2R,4'R,8'R-, β -, γ -, δ - and all-rac- α -tocopherols have similar potentiating activity, whereas phytol, Trolox^R and all-rac- α -tocopherol acetate failed to stimulate PGI₂ release. In order to elucidate the mechanism(s) involved, HUVECs were either incubated with RRR- α -T followed by labelling with [1-¹⁴C] arachidonic acid or they were labelled with arachidonate followed by incubation with RRR- α -T. Irrespective of the sequence of incubation with arachidonic acid or tocopherol, RRR- α -T-enriched cells

released significantly more labelled arachidonic acid when stimulated with thrombin or ionophore A23187. In order to elucidate the mechanism(s) by which tocopherol exerts its effect, similar studies were conducted in which HUVECs were labelled with [^3H]-methylcholine. Results showed a significant increase of [^3H]-lysophosphatidylcholine in tocopherol-enriched cells when they were stimulated with A23187 for 10 min. These observations suggested that the potentiating effect of PGI_2 synthesis is mediated by tocopherol at the level of arachidonic acid release. Direct analysis of enzymes involved in phospholipid metabolism indicated that tocopherol-enrichment caused an increase in phospholipase A_2 activity without affecting the activities of lysophospholipase or acylCoA-acyltransferase. It was also found that hydrogen peroxide when combined with α -tocopherol synergistically stimulated basal release of PGI_2 from endothelial cells. In addition, α -tocopherol potentiates PGI_2 synthesis when challenged with exogenous arachidonic acid. Therefore, the potentiating effect of α -tocopherol on PGI_2 synthesis can also be accounted for at the level of the cyclooxygenase system.

OBJECTIVES

General

To establish a human vascular cell model for the study of the effect of 2R,4'R,8'R- α -tocopherol on the metabolism of arachidonic acid.

Specific

- To study the uptake and depletion of cellular tocopherol in vascular human endothelial cells.
- To study the involvement of α -tocopherol in the metabolic turnover of arachidonic acid focusing on arachidonic acid release, prostacyclin synthesis, and enzymes that are involved in phospholipid metabolism.

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LIST OF ABBREVIATIONS

AA	Arachidonic acid
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
HBS	Hepes buffered saline
HDL	High density lipoprotein
Hepes	4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid
15-HETE	15-hydroxy-eicosatetraenoic acid
H ₂ O ₂	hydrogen peroxide
15-HPETE	15-hydroperoxy-eicosatetraenoic acid
HPLC	high performance liquid chromatography
HUVEC	human umbilical vein endothelial cell
6-keto-PGF ₁ α	6-keto-prostaglandin F ₁ α
LDL	Low density lipoprotein
LPC	lysophosphatidylcholine
NL	Neutral lipid
13-OHDE	13-hydroperoxy-9,11-octadecaenoic acid
PA	phosphatidic acid
PAF	platelet activating factor
PBS	phosphate buffered saline
PC	phosphatidylcholine
PDGF	platelet derived growth factor

PE	phosphatidylethanolamine
PGE ₂	prostaglandin E ₂
PGF _{2α}	prostaglandin F _{2α}
PGG ₂	prostaglandin G ₂
PGH ₂	prostaglandin H ₂
PGI ₂	prostacyclin
PHS	Prostaglandin H synthase
PLA ₂	Phospholipase A ₂
PUFA	polyunsaturated fatty acid
ROOH	fatty acid hydroperoxide
RT	Ringer-Tyrode
RRR- α -T	2R,4'R,8'R- α -tocopherol
TXA ₂	Thromboxane A ₂
TXB ₂	Thromboxane B ₂

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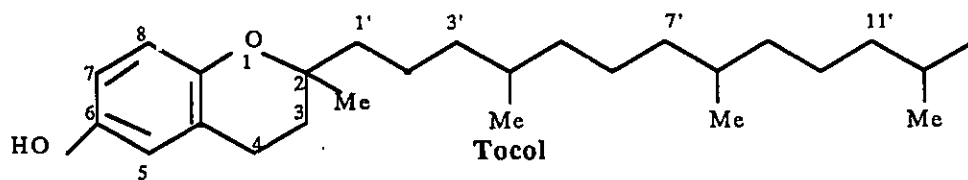
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INTRODUCTION

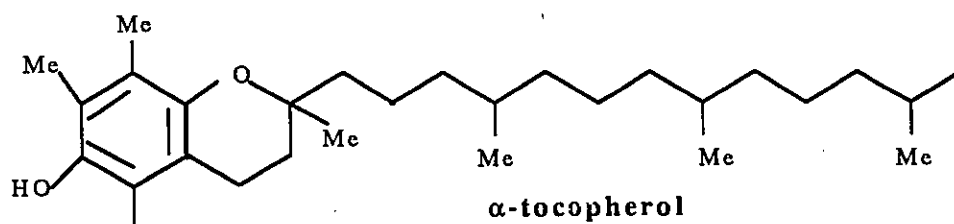
In 1922, Evans and Bishop (1) reported that the deficiency of an unknown "substance X" in the diet resulted in fetal death and resorption in laboratory rat. The letter E was given to that vitamin-like "substance X" to include in the family of already known vitamins, after vitamin D. Later, isolation and purification to homogeneous substances further subdivided the vitamin E family according to the position of methyl groups on the chromanol ring and they were designated as α -, β -, γ -, δ -tocopherol, in which α -tocopherol exhibited the highest biological activity. Tocopherols was found mainly in seed and vegetable oils, ie. wheat germ, cottonseed, lettuce, rice germ, soybean and other seed-germ oils.

Structure of natural tocopherols

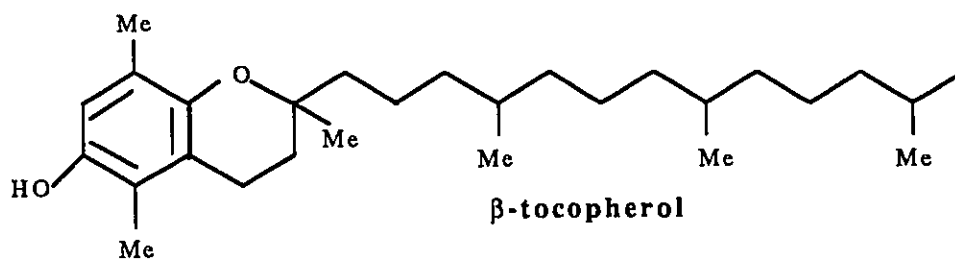
The tocopherols possess a general tocol structure consisting of 2-methyl-6-chromanol bearing a saturated C-16 isoprenoid chain attached at C-2. Tocopherols differ only on the degree of methylation on the aromatic ring at C-5, C-7, and C-8. The structures of natural tocopherols are shown in figure 1. The tocopherols are chiral molecules possessing three chiral centers at C-2, C-4' and C-8'. Therefore, a maximum of eight stereoisomers of each tocopherol can exist, depending on the substrates used in organic synthesis. The natural form of α -tocopherol has 2-R, 4'-R and 8'-R configuration, and thus, 2R,4'R,8'R- α -tocopherol is denoted for the natural α -form.



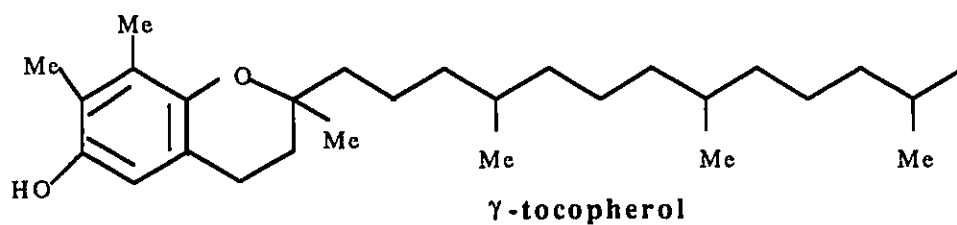
2-methyl-2-(4,8,12-trimethyltridecyl)-6-chromanol



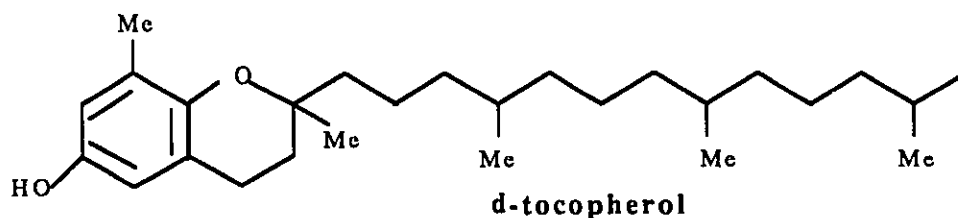
2,5,7,8-tetramethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol



2,5,8-trimethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol



2,7,8-trimethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol

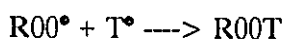
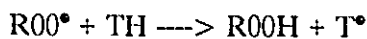


2,8-trimethyl-2-(4',8',12'-trimethyltridecyl)-6-chromanol

Figure 1 : Structures of natural tocopherols

Function(s) of the tocopherols

The biological function or functions of tocopherol is still a controversial issue and some aspects of its biologic role remain to be resolved. The antioxidant property of tocopherol is unquestioned. Many beneficial effects of tocopherol in a variety of clinical problems, such as retrolental fibroplasia, hemolytic anemia associated with iron supplementation, inhibition of tumor promotion, lesions in nervous systems and skeletal muscles can be explained on the basis of its ability to prevent oxidation of lipid fractions especially polyunsaturated fatty acids in the living organisms. Tocopherol is a hydrophobic chain-breaking antioxidant that traps the chain-propagating peroxy radicals, $\text{R}\ddot{\text{O}}\text{O}^\bullet$ (2). The mechanism of action is described below. A molecule of tocopherol (TH) donates the hydrogen from its phenolic hydroxyl group to the peroxy radicals, $\text{R}\ddot{\text{O}}\text{O}^\bullet$ and becomes itself a resonance-stabilized radical, $\text{T}^\bullet$ which can react with a second $\text{R}\ddot{\text{O}}\text{O}^\bullet$ to form $\text{R}\ddot{\text{O}}\text{O}\text{T}$.



Apart from its antioxidant property, many other investigators have proposed that vitamin E may stabilize the membrane bilayer by physico-chemical interactions. The work was pioneered by Diplock and Lucy (3). From their in vitro studies aimed at determining the influence of acyl unsaturation in phospholipids on the penetration of tocopherols, and the effect of α -tocopherol on the leakage of $[^{14}\text{C}]$ -D-glucose from liposomes, they proposed that the methyl groups located on the isoprenoid side chain of the tocopherol molecule might interact physico-chemically with Z pockets of arachidonyl residues in membrane phospholipids. However, no direct evidence has been

shown to support their theory. This theory has recently been challenged by Urano and Matsuo (4) who used fluorescence quenching and ^{13}C -NMR relaxation techniques in studying the interaction between tocopherol and a model membrane. In contrast to Diplock and Lucy's hypothesis, their results showed that the chromanol moiety of α -tocopherol interact with the double bonds of unsaturated fatty acids. In addition, at least two isoprene units from the phytol chain are necessary for anchorage in the liposomal membranes. Other works, including deuterium NMR and calorimetric and infrared spectroscopic studies, have shown that tocopherol is able to order and disorder acyl chain motion of the lipid bilayer depending on the operating temperature and thus, tocopherol appears to induce more than one phase in the bilayers at all temperatures (5,6). Therefore, the physical role of vitamin E on lipid bilayer that may directly or indirectly affect its biological functions is still debatable.

Another function of vitamin E is its ability to affect with the metabolic turnover of arachidonic acid. Goetzl (7) found an enhancement of 5-lipoxygenase activity in neutrophils enriched with a physiological concentration ($30\ \mu\text{M}$) of vitamin E. No inhibition was observed, as one would expect for an antioxidant, until millimolar concentration was used. There is also evidence that vitamin E suppresses the generation of thromboxane (TXB_2) from stimulated platelets, but stimulates prostacyclin (PGI_2) release in rabbit aorta (8,9). The question is whether there is a single general mechanism for vitamin E on the arachidonate cascade, or is its effect specific in different cell types. To answer this question, more work on the mechanism must be carried out. My project was chosen to examine the effect of vitamin E on the turnover of arachidonic acid in cultured endothelial cells isolated from human umbilical cord veins.

Endothelial cells and the metabolism of arachidonic acid

Endothelial cells

Endothelial cells line the inside of the entire vascular system. The body contains about 10^{12} endothelial cells that occupy a surface area of more than 1000 m^2 and weigh in excess of 100 grams. Thus, because of their location and immediate contact with the blood stream, endothelial cells regulate hemostasis and play a role in a variety of metabolic processes.

The endothelium is now suggested to be a distinct metabolic and endocrine organ of diverse capabilities (10,11). For instance, endothelial cells synthesize and secrete physiologically important molecules into the blood and/or into the subendothelial extracellular matrix that can influence the structural and functional integrity of the circulation. These molecules include glycoproteins such as fibronectin and heparan sulfate proteoglycans, interleukin 1, tissue plasminogen activator and inhibitors, prostacyclin, 13-hydroxy-9,11-octadecanoic acid (13-OHDE), endothelium-derived relaxing factor (EDRF) and platelet activating factor. The production of these substances contributes to a number of endothelium properties which regulate thrombosis (12). For example, the antithrombotic property of the endothelium is attributed to its release of prostacyclin, thrombomodulin and heparin sulfate. In contrast, von Willebrand factor and tissue factor are thrombogenic and they participate in the formation of platelet and fibrin thrombi.

Arachidonic acid metabolism in endothelial cells

Cyclooxygenase products

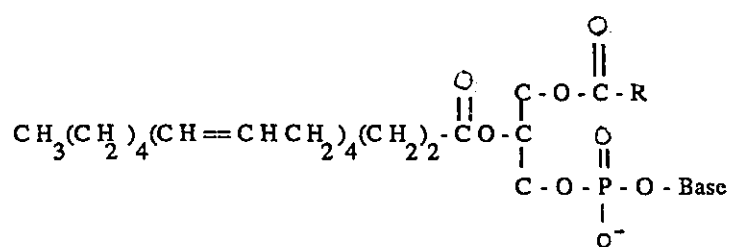
The synthesis of prostacyclin (PGI_2) begins with the liberation of arachidonic acid mediated by three possible mechanisms shown in figure 2. Most of the arachidonic acid involved in PGI_2 synthesis are primarily derived from the direct hydrolytic action of phospholipase A_2 on cellular phospholipids, although the initial rise of intracellular arachidonic acid was attributed to the sequential actions of phospholipase C and di- and mono-glyceride lipase. In addition, the sequential actions of phospholipase D and a phosphatidic acid-specific phospholipase A_2 also contribute to the release of endogenous arachidonic acid.

The cyclooxygenase cyclizes arachidonic acid and adds a 15-hydroperoxy group to form prostaglandin G_2 (PGG_2) which is reduced to prostaglandin H_2 (PGH_2) by the peroxidase activity of the enzyme. The endoperoxide, PGH_2 , is a key intermediate in the cyclooxygenase pathway. PGI_2 is formed from PGH_2 by PGI_2 synthase, an enzyme present in both endothelial and smooth muscle cells (13). The chemical half life of PGI_2 at physiological pH is about 3 minutes but its metabolic half life is less than one circulation time. PGI_2 is rapidly converted to 6-keto-prostaglandin $\text{F}_{1\alpha}$ (6-keto- $\text{PGF}_{1\alpha}$), a stable, inactive product. Small amounts of prostaglandin E_2 (PGE_2) and prostaglandin $\text{F}_{2\alpha}$ ($\text{PGF}_{2\alpha}$) are also produced by endothelial cells.

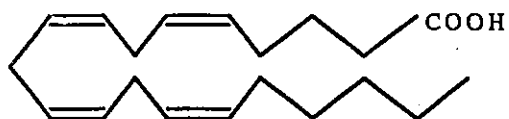
A number of endogenous and exogenous mediators can stimulate the production of PGI_2 in cultured endothelial cells. These include adenine nucleotides, angiotensin II, arachidonic acid, bradykinin, calcitonin, high density lipoproteins (HDL), histamine, interleukin-1, platelet derived growth factor (PDGF), PGH_2 , thrombin and trypsin. In

contrast, glucocorticoids, cyclooxygenase inhibitors, low density lipoproteins (LDL) and vitamin K₁ all inhibit PGI₂ synthesis. Testosterone reduces the PGI₂ formation whereas the female sex hormones, such as estradiol-17 β , have the opposite effect (14). Therefore, males are more susceptible to thrombotic and atherosclerotic injury than females. In addition, the capability of PGI₂ generation decreases with age.

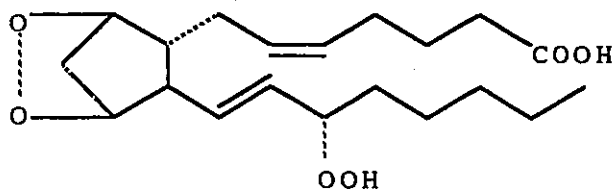
PGI₂ is an extremely potent vasodilator and inhibitor of platelet aggregation (15). The anti-aggregatory and vasodilating actions of PGI₂ are mediated by stimulation of adenylate cyclase in platelets or vascular smooth muscle, resulting in the elevation of cAMP (16). PGI₂ also possesses fibrinolytic and cytoprotective properties that are not mediated by cAMP. The thrombolytic effect of PGI₂ may be related to its potentiating effect on tissue plasminogen activator formation (17), whereas the cytoprotective action of PGI₂ against reperfusion damage is mediated by the reduction of oxygen radicals generated by polymorphonuclear leukocytes (18).



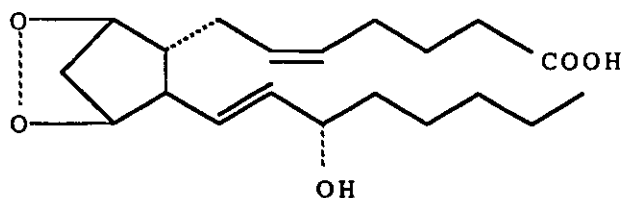
Phospholipid



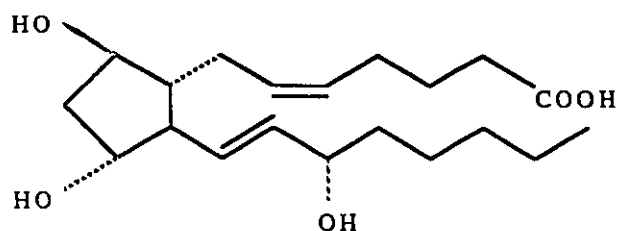
Arachidonic Acid



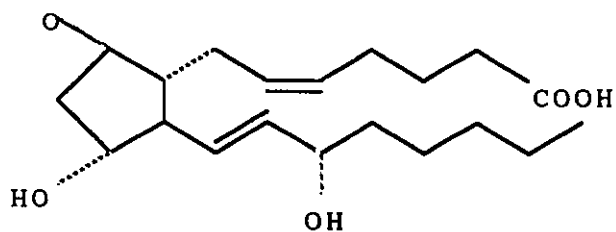
PGG₂ (Prostaglandin G₂)



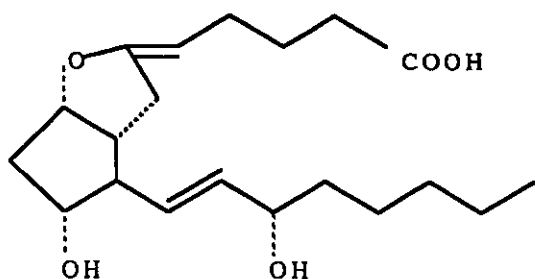
PGH₂ (Prostaglandin H₂)



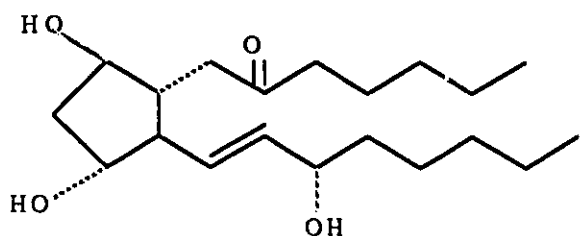
PGF₂ (Prostaglandin F₂)



PGE₂ (Prostaglandin E₂)



PGI₂ (Prostacyclin)



6-keto-PGF_{1α} (6-keto-prostaglandin F_{1α})

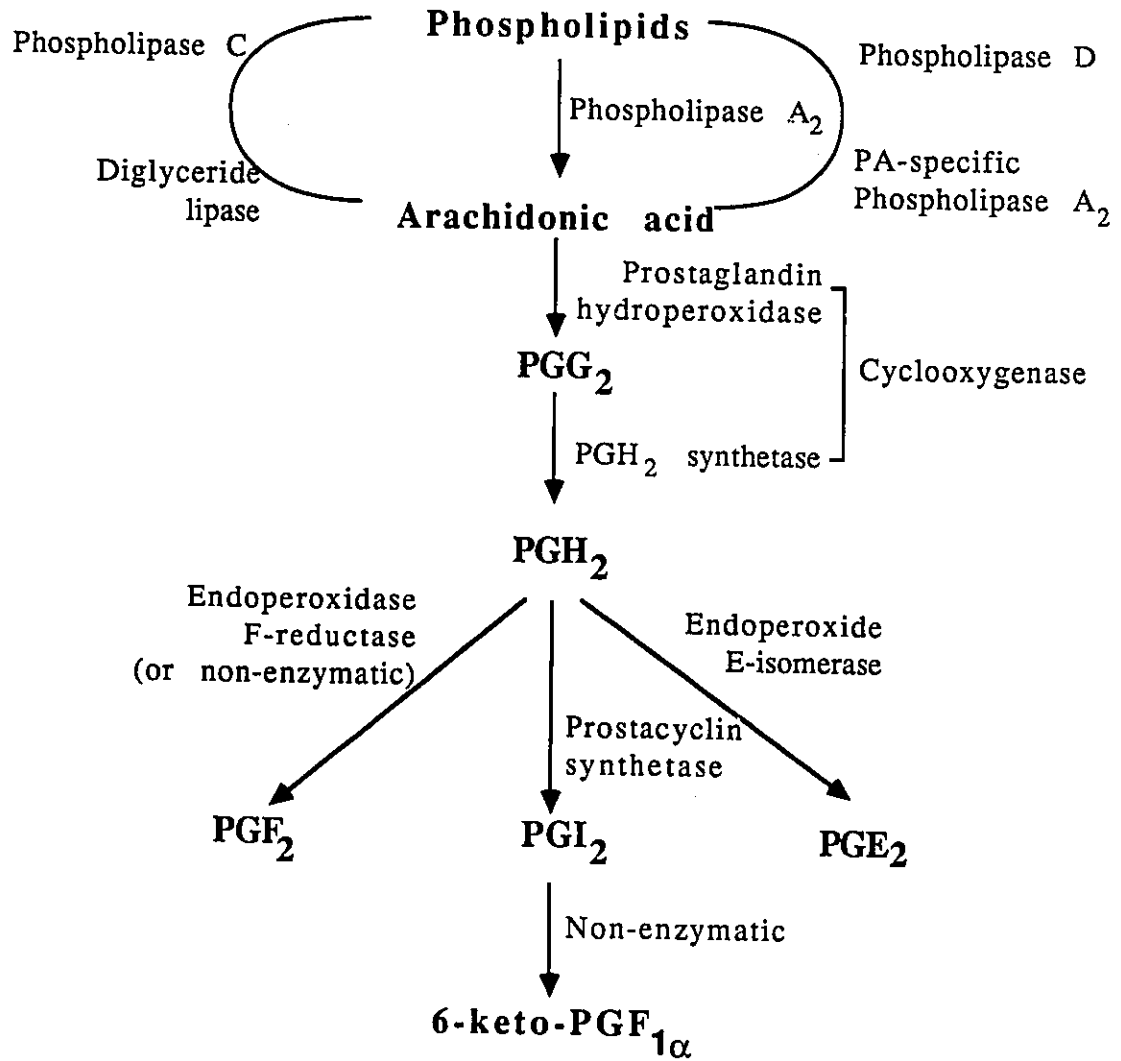


Figure 2 - Metabolism of arachidonic acid in endothelial cells

Lipoxygenase products

Cultured endothelial cells from human umbilical veins or bovine aorta contain a 15-lipoxygenase activity that converts arachidonic acid to 15-hydroxy-eicosatetraenoic acid (15-HETE) (19). However, the presence of 15-lipoxygenase in endothelial cells derived from human umbilical veins cannot be confirmed and the reported 15-lipoxygenase activity (19) is thought to be derived from macrophage contamination in the cell cultures (Chan, A.C. and Prescott, S., personal communication). Buchanan and his colleagues reported that cultured human endothelial cells synthesize a cytosol-associated lipoxygenase metabolite which was called LOX and was later identified by HPLC and GC-MS as 13-hydroxyoctadecadienoic acid (13-HODE) (20,21). It has been proposed that under basal conditions, 13-HODE is continuously formed and is an intracellular substance which maintains endothelial lining in a non-adhesive state. For further review, please see reference 22.

Many nutritional studies of vitamin E have been done on small laboratory animals. This is a limitation if one wants to interpret the results back to the human system. In order to better understand the turnover of tocopherol in man, cultured human endothelial cells isolated from umbilical vein were adopted for use as a model to study the uptake and disappearance of cellular tocopherol.

Alpha-tocopherol has been reported to influence the cyclooxygenase product profile in an array of mammalian tissues and the effect of tocopherol appears to be tissue specific (23,24). Recent studies have shown that α -tocopherol inhibits rat platelets and myocardial phospholipase A₂ activity (25,26) that leads to a decreased thromboxane A₂ synthesis in thrombin-stimulated rat platelets (27). In contrast, cyclooxygenase activity in skeletal muscle and PGI₂ synthesis by vascular tissues appeared to correlate proportionally with the amount of dietary tocopherol (23,28-30).

In the experiments described herein, the endothelial cell model was used to study the involvement of α -tocopherol in the metabolic turnover of arachidonic acid. The success of this model was largely due to the low (undetectable) amount of tocopherol present in the heat-inactivated fetal bovine serum as well as the method of incorporating tocopherol in the culture medium. The study was divided into four parts. First, endothelial cell culture was developed which was used to study the uptake and disappearance of tocopherol in these cells. Second, it will be demonstrated that vitamin E enhances the release of arachidonic acid from cellular lipids. Third, I will demonstrate that α -tocopherol stimulates PGI₂ release in cultured human endothelial cells at doses below and above physiologic concentration. This is the first time that vitamin E has been reported to enhance the release of PGI₂ under basal conditions. Lastly, an attempt was undertaken to understand the mechanism by which tocopherol stimulates PGI₂ release.

MATERIALS AND METHODS

Materials

Medium 199 (powder) with Hanks' salts and L-glutamine, heat-inactivated fetal bovine serum, sodium penicillin G (10,000 units/ml), streptomycin sulfate (10,000 μ g/ml), fungizone (250 μ g amphotericin B and 205 μ g sodium deoxycholate/ml), trypsin-EDTA, culture flasks and culture dishes were obtained from Gibco (Burlington, Ontario). All-rac- α -tocopheryl acetate, Hepes, collagenase type IV, thrombin (1000 NIH units/mg protein), gentamicin sulfate, heparin (180 units/mg), calcium ionophore A23187 and all lipid standards were from Sigma (St.Louis, MO.). Endothelial cell growth supplement (ECGS) was from Collaborative Research (Bedford, MA). [1-¹⁴C] Arachidonic acid (54.9 mCi/mmol), [methyl-³H]choline chloride (80 Ci/mmol), [³H] 6-keto-prostaglandin-F_{1 α} (150 Ci/mmol), [¹⁴C]-Lysophosphatidylcholine (55 mCi/mmol) and sn-1-palmitoyl-2-[1-¹⁴C]-arachidonylphosphatidylcholine (52 mCi/mmol) were purchased from Du Pont - New England Nuclear (Boston, MA). Glass plates (20x20 cm) for thin layer chromatography precoated with silica gel G were obtained from Fisher Scientific Co. Ltd. 2R,4'R,8'R- α -tocopherol, tocol and phytol were gifts from Eisai Co. (Tokyo, Japan) and Trolox^R was from Aldrich (Milwaukee, WI). HPLC grade solvents were from BDH Chemicals, Inc (Toronto, Ontario). All glassware was silanized prior to use.

Culture medium

The medium 199 (pH 7.4) was supplemented with heparin (90 $\mu\text{g/ml}$) Hepes (25 mM), gentamicin sulfate (40 $\mu\text{g/ml}$), sodium penicillin G (100 units/ml), streptomycin sulfate (100 $\mu\text{g/ml}$), fungizone (2.5 μg amphotericin B/ml) and heat-inactivated fetal bovine serum (10%). The endothelial cell growth supplement (30 $\mu\text{g/ml}$) was added into the culture dishes after each feeding.

The stock medium 199 (pH 7.4) contained heparin, Hepes and gentamicin sulfate was prepared and stored in large quantity after sterilized filtering through a 0.22 μm filter obtained from Millipore (Bedford, MA). The culture medium with antibiotics and fetal bovine serum was always freshly prepared and did not last more than a week.

Culture of endothelial cells

Endothelial cells were isolated from human umbilical cord veins by the method of Jaffe (31). The human umbilical cords were obtained freshly from the Case Room of the Ottawa Civic Hospital. The portion of the cords which had clamp marks or needle holes were removed. Cord veins were cannulated with tubing from butterfly needles and flushed with 50 ml of warm Hepes-buffered saline (HBS) (10 mM Hepes / 0.14 M NaCl/4mM KCl/11 mM glucose (pH/7.4)) to remove residual blood. The vein was then filled with 5 to 10ml of 0.2% collagenase (type IV) in HBS and incubated at 37°C for 15 min. The collagenase/cell mixture was flushed with HBS into a 50 ml plastic conical centrifuge tube containing an equal volume of culture medium and then centrifuged 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 T-75 flask. After 24 h in 5% CO₂ / 95% air incubator, the attached cells were rinsed with warm HBS and fresh cultured medium supplemented with 30 µg/ml endothelial cell growth supplement was added. Cell medium was changed every 2-3 days.

Gentle massage to the cord after incubation with collagenase will increase cell yield and confluency can be reached in about 5-7 days. The cells were passaged by using trypsin-EDTA. Briefly, the culture vessel was washed with warm HBS, the monolayer was incubated with 0.05 % trypsin - 0.53 mM EDTA for 5 min at 37 ° C, the detached cells were collected in an equal volume of culture medium, centrifuged, washed and split in a 1:2 or 1:3 ratio.

Immunofluorescence

Cultured human endothelial cells were identified by the presence of factor VIII-related antigen by immunofluorescent microscopy (32).

Enrichment of culture medium with tocopherol and related compounds

Before the monolayers were used for enrichment experiments, we routinely replaced the medium after 20 - 24 hours without the addition of endothelial cell growth supplement. A volume of tocopherol or its analogues dissolved in ethanol was pipetted into a 20 ml-conical centrifuge tube and ethanol was evaporated under N₂ gas. A volume of dimethyl sulfoxide carrier (DMSO) which represented 0.1% of the total medium volume was used as a solubilizer. Heat-inactivated fetal bovine serum (10% of

final medium volume) was added, the tube was vortexed vigorously and this mixture was incubated at 37 °C in the dark for 15 minutes. Medium 199 and other culture reagents were added last, and the medium was incubated at 37 °C for another 15 min before feeding to the monolayers. Control medium contained the same amount of carrier (DMSO) as the tocopherol-enriched medium. Recovery of tocopherol and its analogues carried out by this protocol was greater than 95% as determined by HPLC analysis.

Preparation of [1-¹⁴C] arachidonic acid-enriched medium

A volume of [1-¹⁴C] arachidonic acid stock in ethanol was dried under N₂ gas. A volume of DMSO was added as carrier vehicle (0.1%) and the mixture was vortexed before fetal bovine serum (10%), medium 199 and antibiotics were added.

Incorporation and determination of 2R,4'R,8'R- α -tocopherol

In all experiments, confluent cell monolayers were fed with fresh medium a day before being used. The medium was removed and the monolayer was rinsed with warm HBS. The tocopherol enriched-medium was added to the monolayer and the cells were incubated at 37°C in 5% CO₂ / 95% air incubator. At the indicated incubation times, the medium was quickly removed, the monolayer was washed three times with HBS containing 0.25% bovine serum albumin (BSA) to remove any tocopherol nonspecifically attached to the cell surface. The cells were obtained by trypsinization or by scraping. After centrifuging and washing, the cell pellet was resuspended in HBS

and an aliquot was taken for the determination of number of cells. The remaining cell suspension was extracted by the method of Bligh and Dyer (33) in the presence of all-rac- α -tocopherol acetate as internal standard. Tocopherol was quantitated by reverse phase HPLC, using a C-18 column with a solvent system which consisted of 99% methanol, 1% water and 0.1% trifluoroacetic acid, modified from the method originally described by Bieri et al (34).

Isolation of subcellular fractions

After incubation with 2R,4'R,8'R- α -tocopherol-enriched medium (23.2 μ M) for 21 hours, cells were harvested and homogenized in a tissue grinder homogenizer with Hepes buffer (10 mM Hepes, 0.25M sucrose, 0.2 mM EDTA, pH 7.5). An aliquot of the homogenate was taken for tocopherol determination. The homogenate was centrifuged at 1000xg for 10 min at 4 ° C to remove nuclei and cell debris, and the mitochondria pellet was obtained after centrifuging the post-nuclear supernatant at 10,000xg for 30 min in a Beckman L8-M centrifuge using a 70.1 Ti rotor. The postmitochondrial supernatant was centrifuged at 27,000 xg for 30 minutes in order to obtain the crude plasma membrane and finally, the endoplasmic reticulum was isolated from this supernatant after 100,000xg centrifugation for 1 h. The final post-endoplasmic reticulum supernatant was considered as cytosol. All subcellular pellets were resuspended in a small volume of phosphate buffered saline (PBS), pH 7.4 from which aliquots were taken for protein and tocopherol determination.

Thrombin and Ionophore-induced arachidonic acid release

After labelling with 0.25 μCi of $[1-^{14}\text{C}]$ arachidonic acid (54.9 mCi/mmol) for 5 or 20 h, cells were rinsed three times with HBS (0.25% BSA) to remove any nonspecific binding of arachidonic acid to the cells prior to agonist stimulation. Thrombin (2.5 units/ml) and ionophore A23187 (1 μM) were in Ringer-Tyrode buffer (137 mM NaCl, 2.7 mM KCl, 12 mM NaHCO_3 , 5 mM glucose, pH 7.4) containing 0.25% BSA and 1 mM Ca^{2+} . At the end of 10 min stimulation at 37 ° C, the medium was quickly removed and an aliquot was taken for determination of radioactivity by scintillation counting. The cells were washed with HBS (0.25% BSA) before being detached by trypsinization and scraping.

Lipid analysis

Detached cells were acidified with glacial acetic acid to pH 3.6 and total cellular lipids were extracted with $\text{CHCl}_3/\text{MeOH}$ (33) in the presence of appropriate lipid standards. The CHCl_3 phase was evaporated under N_2 gas and spotted onto glass silica gel G precoated plates. Lipids were separated by two thin layer chromatographic procedures using the solvent system consisting of chloroform/methanol/acetic acid/water (85:15:20:3.5, v/v) for phospholipids and hexane/diethyl ether/acetic acid (70:30:1, v/v) for neutral lipids. The spots comigrating with authentic standards were detected by autoradiography or visualized by I_2 vapor, and were directly scraped to detect radioactivity by scintillation counting.

Prostacyclin release studies

Cells used for prostacyclin studies were all in first passage. Confluent monolayers in 35-mm dishes were incubated with 2 ml of medium enriched with tocopherol or its analogues. After the indicated time of incubation, the medium was removed and the monolayers were rinsed with Ringer-Tyrode (RT) buffer. For prostacyclin (PGI₂) release studies under basal condition, the monolayers were incubated at 37 °C with RT-buffer containing 1 mM Ca²⁺. For PGI₂ release studies under agonist stimulation, the monolayers were incubated at 37 °C with agonists (ionophore A23187 or arachidonic acid, AA) carried in RT-buffer (1 mM Ca²⁺). At indicated times, the medium was collected, acidified to pH 3.6 with 1N HCl. All samples were centrifuged to remove cell debris and stored at -70 ° C for radioimmunoassay.

Radioimmunoassay

The levels of 6-keto-prostaglandin F_{1α} (6-keto-PGF_{1α}), a stable nonenzymic hydration product of PGI₂, were directly determined in the supernatant by radioimmunoassay as previously described (28).

Protein determination

Protein from cell homogenate was colorimetrically determined by the method of Lowry et al (35) using bovine serum albumin as standard.

Choline labelling experiments

Confluent monolayers were labelled with 10 μCi [^3H]-choline in 2 ml of culture medium for 20 h. Approximately 10% of [^3H]-choline was incorporated into phosphatidylcholine under these conditions. In experiment I, labelled monolayers were incubated with and without 2R,4'R,8'R- α -tocopherol (23.2 μM) for 4 hours. In experiment II, tocopherol was coincubated with [^3H]-choline for a total of 20 hours. Under these conditions, tocopherol was found not to affect cellular incorporation of [^3H]-choline. After the appropriate incubation time, cells were rinsed twice with RT-buffer followed by ionophore A23187 stimulation (1 μM , 10 minute). Stimulation was terminated by addition of 1 ml of MeOH. Phosphatidylcholine (PC) and lysophosphatidyl choline (LPC) were separated by thin layer chromatography after cellular lipids were extracted as described above. The solvent system contained $\text{CHCl}_3\text{:MeOH:HAc:H}_2\text{O}$ (85:15:10:3.5, v/v/v/v).

Determination of lysophosphatidylcholine acylCoA acyltransferase and lysophospholipase activities

After preincubation with and without 2R,4'R,8'R- α -tocopherol (23.2 μM) for 4 h, confluent endothelial monolayers were detached by trypsinization and the cells were sonicated in ice-cold buffer. AcylCoA acyltransferase activity was determined in an assay mixture (0.7 ml) which contained 110 nmole of linoleoyl-CoA, 100 nmole of [^{14}C]-lysophosphatidylcholine (specific activity 3.33 $\mu\text{Ci}/\mu\text{mole}$) and sonicated cell suspension (130 μg protein) in Tris-HCl buffer, 75 mM, pH 8.5 with 1 mM EDTA. The reaction was started with the addition of cell homogenate. After 30 min at 37 ° C, 1

ml of $\text{CHCl}_3\text{:MeOH}$ (1:1, v/v) was added to stop the reaction. Total lipids were extracted and [^{14}C]-labelled phosphatidylcholine and lysophosphatidylcholine were separated by thin layer chromatography as described above. Radioactive lipid spots were identified by autoradiography and the amount of phosphatidylcholine formed was determined by direct scraping from thin layer plates followed by scintillation counting.

Lysophospholipase activity was determined using [^{14}C]-lysophosphatidylcholine (3.33 $\mu\text{Ci}/\mu\text{mole}$) as substrate. The assay mixture (0.7 ml) contained 100 nmoles of labelled substrate in Tris-HCl buffer, 75 mM, pH 8.5 with 1 mM EDTA. The reaction was started with sonicated cell suspension (130 μg protein) at 37 ° C for 30 min, and 1 ml of $\text{CHCl}_3\text{:MeOH}$ (1:1,v/v) was added to terminate the reaction. After lipid extraction, the labelled free fatty acid released was separated on thin layer plates and radioactivity was quantitated by scintillation counting after identification by autoradiography.

Determination of phospholipase A₂ activity

Enzyme activity was estimated by measuring the release of [^{14}C]-arachidonic acid from sn-1-palmitoyl-2-[^{14}C]-arachidonylphosphatidylcholine as previously described (25). The assay mixture contained 0.1 M Tris-HCl (pH 9.3), 12.8 μM of sonicated substrate (236,000 dpm), bovine serum albumin (2 mg/ml), 60-70 μg cell protein and indicated amount of calcium in a final volume of 160 μl . After 1 h of incubation at 37°C, the reaction was stopped with 1 ml of Dole's reagent. The released arachidonate was extracted from the incubation medium by the method of Dole and Meinertz (36) and radioactivity was quantitated by scintillation counting.

RESULTS

The following results are in three parts:

In Part A, human umbilical cord vein endothelial cell in culture was developed as a model to study the uptake and disappearance of tocopherol. In this study, the cells were enriched with 2R,4'R,8'R- α -tocopherol at different concentrations or times, and cellular tocopherol was determined from HPLC analysis. Cellular tocopherol was also monitored over times in the tocopherol free medium in order to obtain the rate of disappearance and the half-life of tocopherol in the cells.

In Part B, the involvement of α -tocopherol in the metabolic turnover of arachidonic acid in human endothelial cells in culture will be presented. Two experiments were conducted in which cells were either incubated with 2R,4'R,8'R- α -tocopherol followed by labelling with ^{14}C arachidonic acid or labelled with arachidonic acid followed by incubation with tocopherol. The release of arachidonic acid upon stimulation with thrombin or ionophore A23187 was monitored by the radioactivity released in the medium and the corresponding decrease of radioactivity from cellular phospholipids.

In Part C, I will report that α -tocopherol stimulates PGI_2 release in cultured human endothelial cells at doses below and above physiological concentration. The activating effect of tocopherol in the PGI_2 release from cultured human endothelial cells was observed in both basal and stimulating conditions. The structural specificity of the tocopherol molecule required to potentiate PGI_2 synthesis was investigated by using various tocopherol analogues. Finally, to delineate the mechanism by which tocopherol acts as an activator of arachidonic acid metabolism in human endothelial cells, I will present an indirect study using ^3H -choline labelling of cellular phosphatidylcholine as well as direct analysis of phospholipase A_2 activity in tocopherol-enriched cells.

A. Study of tocopherol on human endothelial cells

In order to verify that cells isolated from collagenase digestion of umbilical cord veins were of endothelial origin, immunofluorescent microscopy against von Willebrand factor was performed on primary cell culture. Figure 3 clearly shows that intense fluorescent particles, stained for von Willebrand factor, were located throughout the cytoplasm of these cells, an observation in agreement with Jaffe (32) who demonstrated this unique characteristic of endothelial cells which expresses von Willebrand factor.

Uptake of tocopherol by endothelial cells

When incubated with physiological concentration of 2R,4'R,8'R- α -tocopherol (23.2 μ M or 1 mg %), the uptake of tocopherol by endothelial cells was dependent on the incubation time and was saturated at 8 hours (figure 4). There is, however, no difference between the incorporation of the natural form, 2R,4'R,8'R- α -tocopherol and the synthetic form, all-rac- α -tocopherol (data not shown).

Additional experiments were conducted in order to simulate plasma concentrations of tocopherol found in North Americans (23.2 μ M) and those who supplemented themselves with moderate or very high dose of tocopherol (46.4 and 92.8 μ M), respectively. After 20 h of incubation with these tocopherol concentrations, monolayers exhibited a dose-dependent uptake of tocopherol (figure 5). The dose and time dependence of tocopherol uptake by the cells was repeated and presented in Table I. At 92.8 μ M, tocopherol uptake by the cells was consistently 3-fold higher than at 23.2 μ M, irrespec-

tive of whether the incubation time was 5 or 20 h. Within the same concentration of tocopherol-enriched medium, cellular uptake of tocopherol was doubled in the 20 h when compared to the 5 h incubation (0.56 vs 0.32 nmoles at 23.2 μ M and 1.80 vs 0.98 nmoles at 92.8 μ M). The above observations indicated that cellular tocopherol content could be enriched by three-fold as the tocopherol concentration in the medium was increased by four-fold, and the tocopherol level in the cells reached maximum at 20 h irrespective of the doses. Supplementation of tocopherol in the culture medium appeared to have no effect on cell numbers (Figure 4, Table I) or cell viability as judged by trypan blue exclusion.

Figure 3 - Immunofluorescent microscopy of cells showing the presence of von Willebrand factor antigen, verifying that cells were of endothelial origin.



Figure 3

Figure 4 - Time course of incorporation of R,R,R- α -tocopherol by human endothelial cells. *Confluent monolayers were incubated for the indicated time periods with 23.2 μ M (10 μ g/ml) of R,R,R- α -tocopherol in complete culture medium. The cells were harvested by trypsinization. An aliquot of cell suspension was taken for cell number determination and the remainder was extracted for lipid analysis in the presence of all rac- α -tocopheryl acetate as internal standard. Tocopherol was determined by HPLC as described in Materials and Methods. Values are means $\pm$ S.D. of three dishes.*

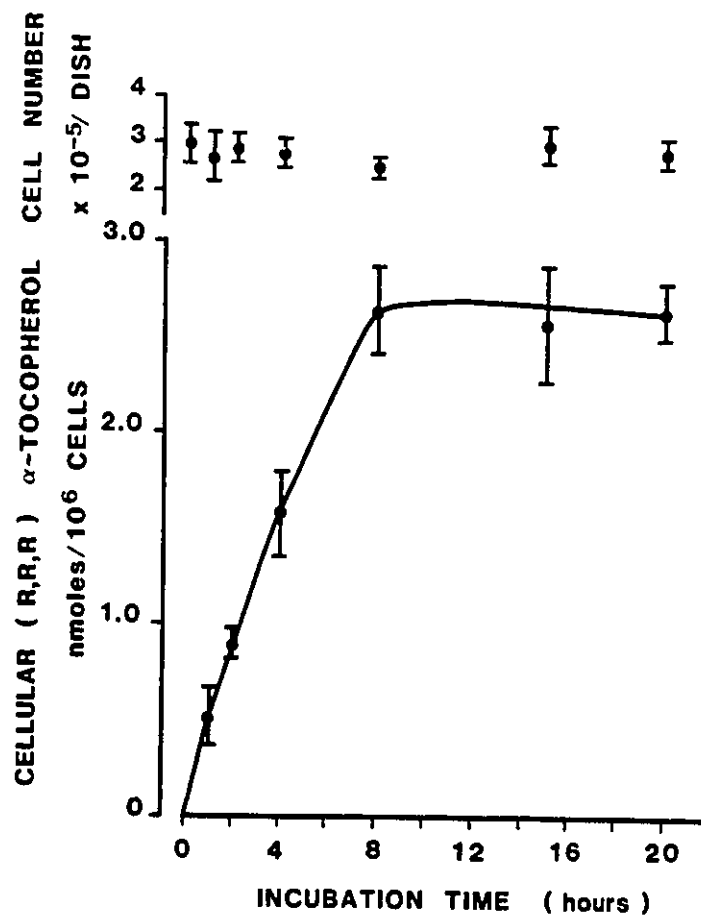


Figure 4

Figure 5 - Dose dependent uptake of R,R,R- α -tocopherol by human endothelial cells. *Confluent monolayers were incubated with indicated tocopherol concentrations for 20 h. Cells were harvested by trypsinization. Cellular lipid were extracted and tocopherol was determined by HPLC as described in Materials and Methods. Values are means $\pm$ S.D. of three dishes.*

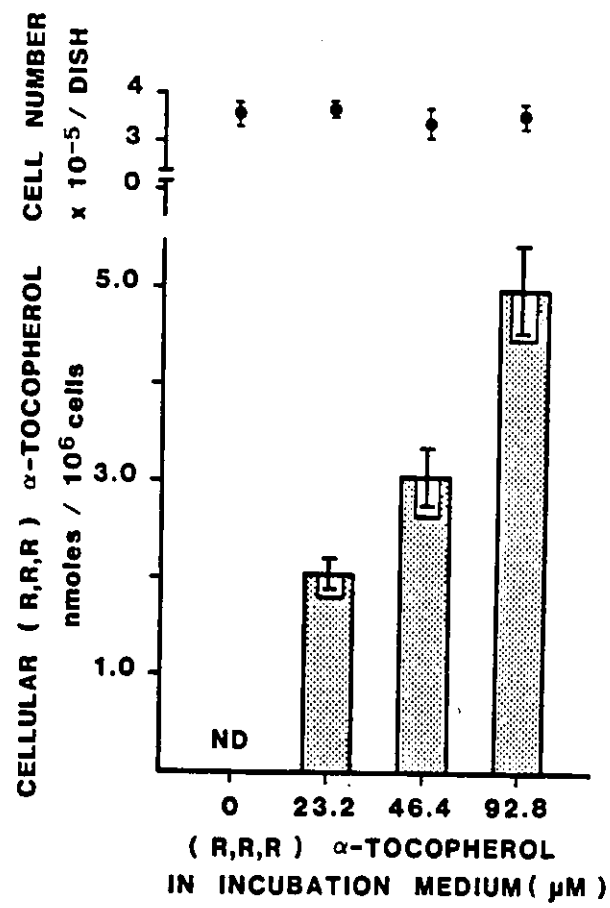


Figure 5

Table I

***Effect of added tocopherol on cellular tocopherol content
and cell number***

Values are means $\pm$ S.D. of 3 dishes, ND, not detectable. Confluent cells were incubated with different tocopherol concentrations for 5 or 20 hrs. Total lipids were extracted from cells and all-rac- α -tocopheryl acetate was added as internal standard. Tocopherol was quantitated by HPLC method as described in Methods.

Tocopherol in medium (μ M)	Tocopherol in cells, nmoles/dish (cell number $\times 10^{-5}$ /dish)	
	<u>5 h</u>	<u>20 h</u>
0	ND (3.62 $\pm$ 0.25)	ND (3.70 $\pm$ 0.31)
23.2	0.32 $\pm$ 0.04 (3.74 $\pm$ 0.11)	0.56 $\pm$ 0.08 (3.40 $\pm$ 0.40)
92.8	0.98 $\pm$ 0.09 (3.53 $\pm$ 0.35)	1.80 $\pm$ 0.10 (3.63 $\pm$ 0.24)

Subcellular distribution of cellular tocopherol

In order to determine the pool distribution of the incorporated tocopherol, monolayers were incubated with 23.2 μM tocopherol-enriched medium for 20h and subcellularly fractionated after carefully washing the cells with buffer containing 0.25% BSA to remove any non-specifically bound tocopherol. Table II shows that the distribution of tocopherol was found mainly associated with cellular membrane, and when tocopherol was expressed on the basis of membrane protein, the highest concentration was found in plasma membrane > mitochondria > endoplasmic reticulum > cytosol. Data presented in Table II represent the results obtained from one of three experiments which showed the same trend. Attempts to determine the enzyme markers associated with subcellular fractions were not successful. This was probably due to the limited cell number and the unexpressed activities of these enzyme markers after prolonged passages. However, the result in Table II clearly showed that tocopherol was found mainly in the cellular membranes which further supported its lipophylic property.

Rate of tocopherol disappearance

Disappearance of tocopherol from endothelial cells was next examined by incubating confluent monolayers with tocopherol (23.2 μM) for 4 h; at which time the tocopherol-enriched medium was removed and replaced with fresh culture medium which contained no added tocopherol. Cellular tocopherol content was monitored over the next 80 h. Under these conditions (figure 6), the tocopherol content of the cells showed a rapid disappearance during the initial 15 h (33.3 pmole/h/ 10^6 cells) but thereafter, the rate of tocopherol disappearance was much slower (6.7 pmoles/h/ 10^6 cells).

Advantages of the model

The tocopherol-deficient nature of the culture medium and the endothelial cells in culture enables us to control the level of tocopherol in these cells. Heat-inactivated bovine serum and the endothelial cells after a few days in primary culture contain no detectable level of α -tocopherol. The minimum level of tocopherol determined by reversed-phase HPLC was 40 pg . Therefore, cultured endothelial cells isolated from human umbilical veins are feasible for nutritional studies, particularly for the study of nutrient uptake or other fat-soluble vitamins.

Endothelial cells derived from human umbilical cords cannot be passaged more than 5 times at which time the cell population begins to senesce as indicated by the presence of large, dendritic, multinucleated cells and a reduction in the growth rate (unpublished observations). Routinely, the failure to keep the cells in many passages is not a major problem since fresh cords can easily be obtained.

It can be concluded that cultured human endothelial cells incorporated 2R,4'R,8'R- α -tocopherol in a dose and time-dependent manner. The incorporated tocopherol was found mainly associated with cell membranes. The half-time for the retention of tocopherol in endothelial cells was 63 ± 7 h.

Table II

***Subcellular distribution of (R,R,R)- α -tocopherol
in human endothelial cells***

Confluent monolayers were incubated with (R,R,R)- α -tocopherol, 23.2 μ M for 20 hrs. The cells were rinsed with buffer containing 0.25% BSA, harvested, homogenized and subjected to differential centrifugation for various subcellular fractions as described in Methods.

Fractions	Tocopherol (nmole)	Protein (mg)	Tocopherol/protein (nmole/mg)
Plasma membrane	1.10	0.06	18.3
Mitochondria	7.26	0.59	12.3
Endoplasmic Reticulum	2.46	0.36	6.8
Cytosol	1.15	5.56	0.2
Total cell homogenate	19.53	9.22	2.12

Figure 6 - Disappearance of R,R,R- α -tocopherol in human endothelial cells. *Confluent monolayers were incubated with 23.2 μ M of R,R,R- α -tocopherol for 4 h, after which the tocopherol-enriched medium was removed. The cells were gently rinsed with warm Hepes buffered saline containing 0.25 % of essential fatty acid free bovine serum albumin and they were reincubated with fresh culture medium without tocopherol (-E). At indicated time points, cells were harvested and cellular lipid were extracted to determine tocopherol as described in Materials and Methods. After 37 h of incubation, fresh medium was replaced in order to maintain cell viability (arrow). Values are means $\pm$ S.D. of three dishes.*

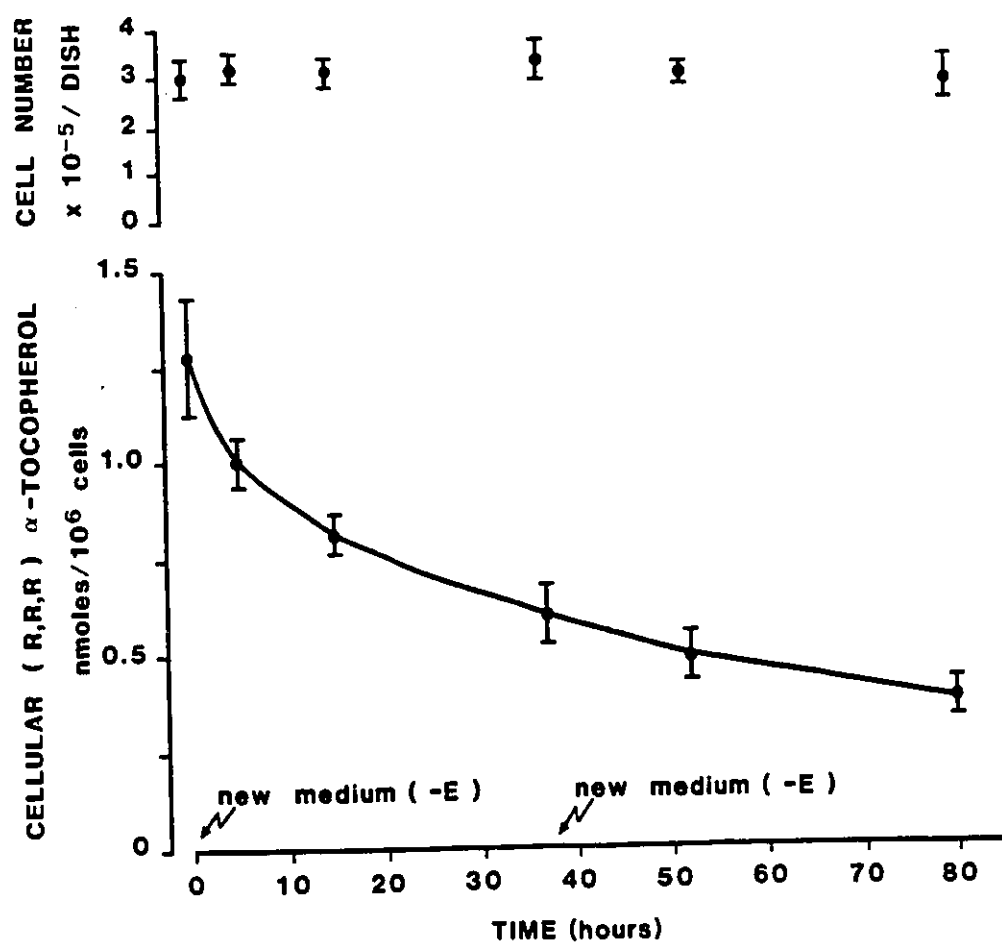


Figure 6

B. Effect of tocopherol enrichment on arachidonic acid release and cellular phospholipids in cultured human endothelial cells

This study was undertaken to better understand the involvement of α -tocopherol in the metabolic turnover of arachidonic acid in cultured human endothelial cells. In contrast to the previous reports, which showed that α -tocopherol inhibits rat platelets and myocardial phospholipase A₂ activity (25,26), the present study demonstrated that α -tocopherol stimulates arachidonic acid release in cultured human endothelial cells.

Distribution of incorporated labelled arachidonic acid in endothelial cell lipids

In order to study the effect of tocopherol on the turnover of arachidonic acid, the distribution of ^{14}C arachidonic acid in various cellular lipids must be first examined. The incorporation of labelled arachidonic acid into endothelial cell lipids after 5 or 20 h of incubation was studied. When labelled with 0.25 μCi or 3.8 μM of [$1\text{-}^{14}\text{C}$] arachidonic acid for 5 or 20 h, total radioactivity uptake by the cells was 34% and 64%, respectively. Incorporated radioactivity was found mainly in phosphatidylcholine (PC) and neutral lipid (NL) fractions; however, the distribution of incorporated radioactivity in various phospholipid classes was different after the two incubation periods (Table III). After 20 h of arachidonate labelling, there was a considerable shift of radioactivity from phosphatidylcholine (PC) and phosphatidylinositol fractions to those of phosphatidylethanolamine (PE) and neutral lipids (NL). This transfer of labelled arachidonic acid is consistent with the observation reported by Brown and coworkers (37).

- 32 -
Table III

Distribution of [1-¹⁴C] arachidonic acid in various lipid classes of human endothelial cells after 5 and 20 hours of incubation

Confluent endothelial cells were labelled with 0.25 μ Ci of [1-¹⁴C] arachidonic acid (3.8 μ M) for 5 or 20 hours. The dishes were rinsed with Ringer-Tyrode buffer, pH 7.4 containing 0.25% BSA. Cells were scraped in ice-cold methanol and cellular lipid was extracted and quantitated as described in Methods. Results are means $\pm$ S.D. of three dishes.

	Radioactivity, dpm x 10 ⁻⁴ /dish			
	5 hours	(%total)	20 hours	(%total)
Total uptake by cells	19.13 $\pm$ 0.07	(100)	35.48 $\pm$ 1.67	(100)
Phosphatidylcholine	8.94 $\pm$ 0.05	(46.7)	11.75 $\pm$ 0.42	(33.1)
Phosphatidylethanolamine	1.71 $\pm$ 0.15	(8.9)	6.15 $\pm$ 0.83	(17.3)
Phosphatidylinositol	2.56 $\pm$ 0.06	(13.4)	2.07 $\pm$ 0.27	(5.8)
Neutral lipid + free arachidonate	3.53 $\pm$ 0.08	(18.5)	11.31 $\pm$ 0.45	(31.9)

Experiment 1, effect of 2R,4'R,8'R- α -tocopherol on arachidonic acid release and cellular phospholipids

Arachidonic acid is the major C-20 polyunsaturated fatty acid (PUFA) of the blood and the vascular cells in mammals. It is also the main PUFA liberated during cell activation that results in the formation of many active products including the prostaglandins and leukotrienes. Therefore, this experiment was designed to determine whether or not cellular α -tocopherol has any effect on the release of arachidonic acid when the cells were stimulated with either thrombin or ionophore A23187.

Endothelial monolayers were enriched with indicated concentrations of α -tocopherol for 20 h followed by labelling with [1- 14 C] arachidonic acid for 5 h. Total uptake of radioactivity by the cells was $(19.11 \pm 1.4) \times 10^4$, $(19.46 \pm 1.1) \times 10^4$ and $(18.23 \pm 0.5) \times 10^4$ dpm which corresponded to 0, 23.2 and 92.9 μ M of tocopherol-enriched medium, respectively. Therefore, the incorporation of arachidonic acid in the endothelial cells was not significantly altered by tocopherol enrichment (Table I). Cells were rinsed with buffer containing 0.25% BSA to remove any nonspecific binding of arachidonic acid and then they were subjected to agonist stimulation. Figure 7A clearly showed that tocopherol enrichment significantly enhanced the release of arachidonic acid. This enhancement was directly proportional to the concentrations of tocopherol present in the medium. In addition, the potentiating effect of α -tocopherol on arachidonic acid release was similarly observed with the two agonists used. Ionophore A23187 (1 μ M) elicited a higher arachidonic acid release than thrombin (2.5 U/ml). In fact, in the absence of agonist, there was a small but significant increase in the release of arachidonic acid from cells that were enriched with the high concentrations of tocopherol (92.8 μ M).

Figure 7 - Experiment 1, dose-dependent stimulation by R,R,R- α -tocopherol on agonist-induced arachidonic acid release and radioactivity retained in cellular phospholipids. *Confluent monolayers were incubated with 0, 23.2 and 92.8 μ M of R,R,R- α -tocopherol-enriched medium for 20 h. Following the removal of medium, each dish was radiolabelled with 0.25 μ Ci of [1- 14 C] arachidonic acid for 5 h. Cells were rinsed with warm Ringer-Tyrode buffer containing 0.25 % BSA and they were stimulated with thrombin, 2.5 U/ml (■), ionophore A23187, 1 μ M (▲) or buffer alone (○). After 10 min stimulation, the medium was removed and an aliquot was taken for quantitation of total radioactivity release. Cellular lipids were extracted and phospholipids were resolved by thin-layer chromatography using CHCl₃/MeOH/HAc/H₂O (85:15:10:3.5, v/v/v/v) as solvent system. Identification of phospholipid class was achieved by I₂ vapor exposure and autoradiography with authentic phospholipid standards. The bands from TLC were scraped and radioactivity was determined by scintillation counting. Values are means $\pm$ S.D. of three dishes. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.*

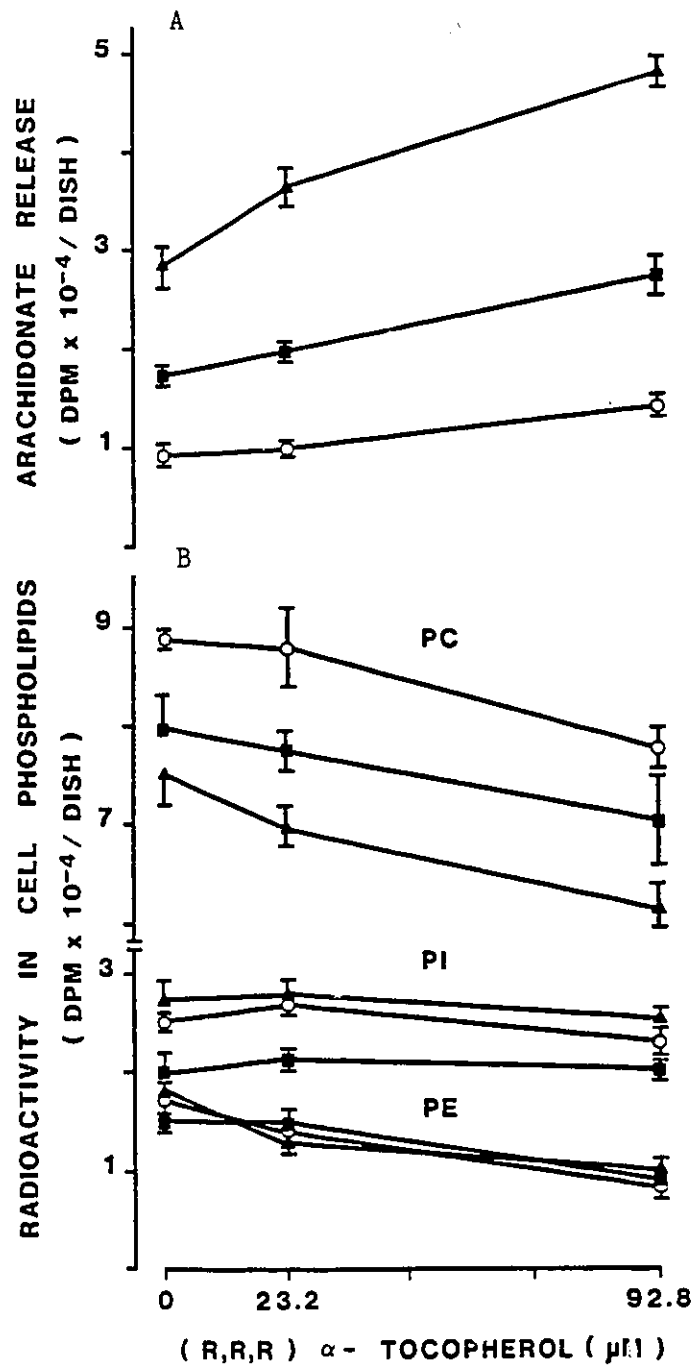


Figure 7 A and B

Analysis of the radioactivity remaining in the cellular lipids after agonist stimulation revealed that the principle source of arachidonic acid loss was PC (figure 7B). There was little loss of arachidonic acid from PE upon different agonist stimulation. Parallel comparison between the increase of arachidonic acid release in the medium in figure 7A and the decrease of radioactivity in PC in figure 7B clearly demonstrated that PC was the main source for the release. The radioactivity found in PI was unchanged by tocopherol status of the cells. Data from this experiment clearly indicated that α -tocopherol caused a significant increase of arachidonic acid release from PC in agonist-stimulated endothelial cells.

Experiment 2, effect of 2R,4'R,8'R- α -tocopherol on arachidonic acid release and cellular phospholipids

In order to determine whether the release of arachidonic acid was a direct effect of tocopherol on cellular lipids or whether the presence of tocopherol in the cells exerted a discrimination on the incorporation of arachidonic acid into any particular lipids, Experiment 2 was conducted in which the incubation order of tocopherol and arachidonic acid was reversed from that of Experiment 1. The cells were prelabelled with 0.25 μ Ci of [1- 14 C] arachidonic acid for 20 h and then incubated with varying tocopherol concentrations for 5 h. Among cellular phospholipids, PC again incorporated the highest radioactivity, a phenomenon that was similarly observed in Experiment 1. During the 5 h of tocopherol incubation, about 16% of the incorporated arachidonic acid radioactivity was released into the culture medium (ie. before tocopherol incubation: $(42.5 \pm 1.4) \times 10^4$ vs after 5 h of tocopherol incubation : $(35.3 \pm 3.8) \times 10^4$,

$(35.3 \pm 3.9) \times 10^4$ and $(35.0 \pm 3.6) \times 10^4$ dpm/dish for 0, 23.2 and 92.8 μM of tocopherol in medium, respectively). Analysis of cellular lipids showed that triacylglycerol was the principle source of this arachidonic acid release during 5 h of tocopherol incubation (data not shown).

When the cells were stimulated with ionophore and thrombin for 10 min, the tocopherol-induced enhancement of arachidonic acid release was again observed (Figure 8A). However, under these experimental conditions, the increased release of arachidonic acid leveled off at 23.2 μM of tocopherol enrichment and, in contrast to Experiment 1, no further increase of release could be detected at a higher (92.8 μM) level of tocopherol preincubation. This could be in part due to the shorter duration of tocopherol enrichment. The cellular tocopherol uptake in this experiment was 0.32 and 0.98 nmoles/dish for 23.2 and 92.8 μM of tocopherol in the medium. In contrast, uptake of cellular tocopherol in Experiment 1 (20 h of tocopherol incubation) was much higher, reaching 0.56 and 1.80 nmol/dish, as illustrated in Table I. Alternatively, the longer period of arachidonic acid labelling may result in chain elongation to adrenic acid, which has been demonstrated by Rosenthal and Hill (38) to be resistant to agonist-induced release.

The radioactivity retained in different phospholipid classes is presented in figure 8B. The loss of radioactivity by agonist stimulation was again found most prominent in PC, and the magnitude of radioactivity lost corresponded to the amount of arachidonic acid release. Results from this experiment confirmed the previous finding (Experiment 1), that α -tocopherol potentiates arachidonic acid release from PC in response to ionophore and thrombin stimulation.

Figure 8 (A) - Experiment 2, stimulation of arachidonic acid release by R,R,R- α -tocopherol. *Confluent monolayers were labelled with 0.25 μ Ci of [1- 14 C] arachidonic acid for 20 h. After removal of medium and rinsing with warm Ringer-Tyrode buffer containing 0.25 % BSA, the cells were incubated with 0, 23.2 and 92.8 μ M of R,R,R- α -tocopherol-enriched medium for 5 h. Cells were rinsed as indicated above and were stimulated with thrombin, 2.5 U/ml (●), ionophore A23187, 1 μ M (▲) or buffer alone (○). After 10 min stimulation, the medium was quickly removed and radioactivity was determined by scintillation counting. Values are means $\pm$ S.D. of three dishes.*

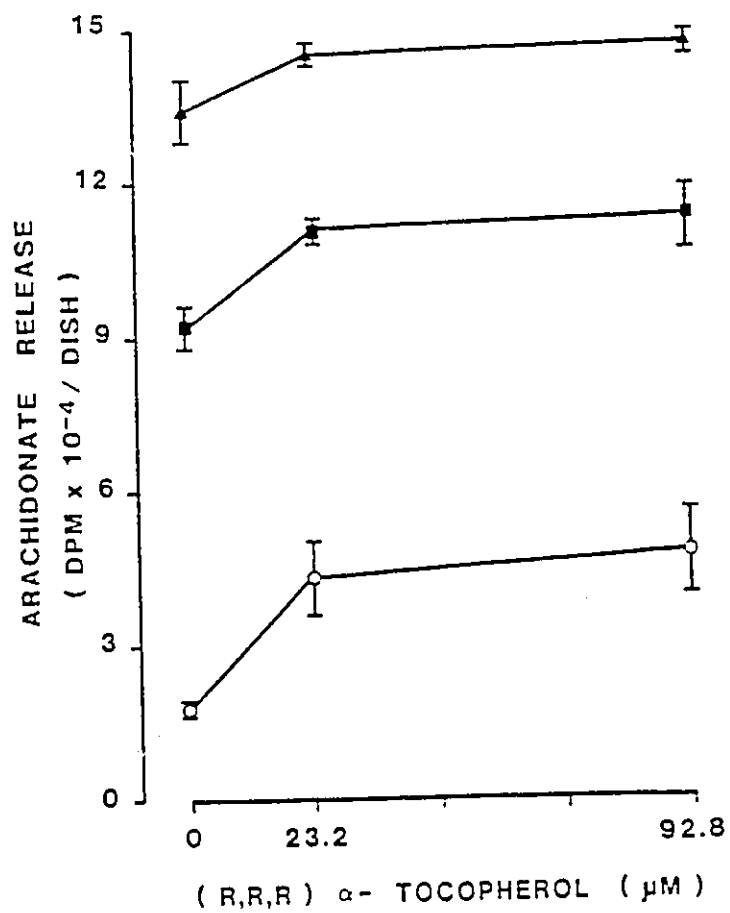


Figure 8 A

Figure 8 (B) - Experiment 2, radioactivity in cellular phospholipids after agonist stimulation. *Experimental conditions were identical to Fig. 8 (A).. After 10 min stimulation with thrombin, 2.5 U/ml (■), ionophore A23187, 1 μ M (▲) or buffer alone (○), cellular lipids were extracted and different phospholipid classes were quantitated as described in fig. 7. Values are means + S.D. of three dishes. PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol.*

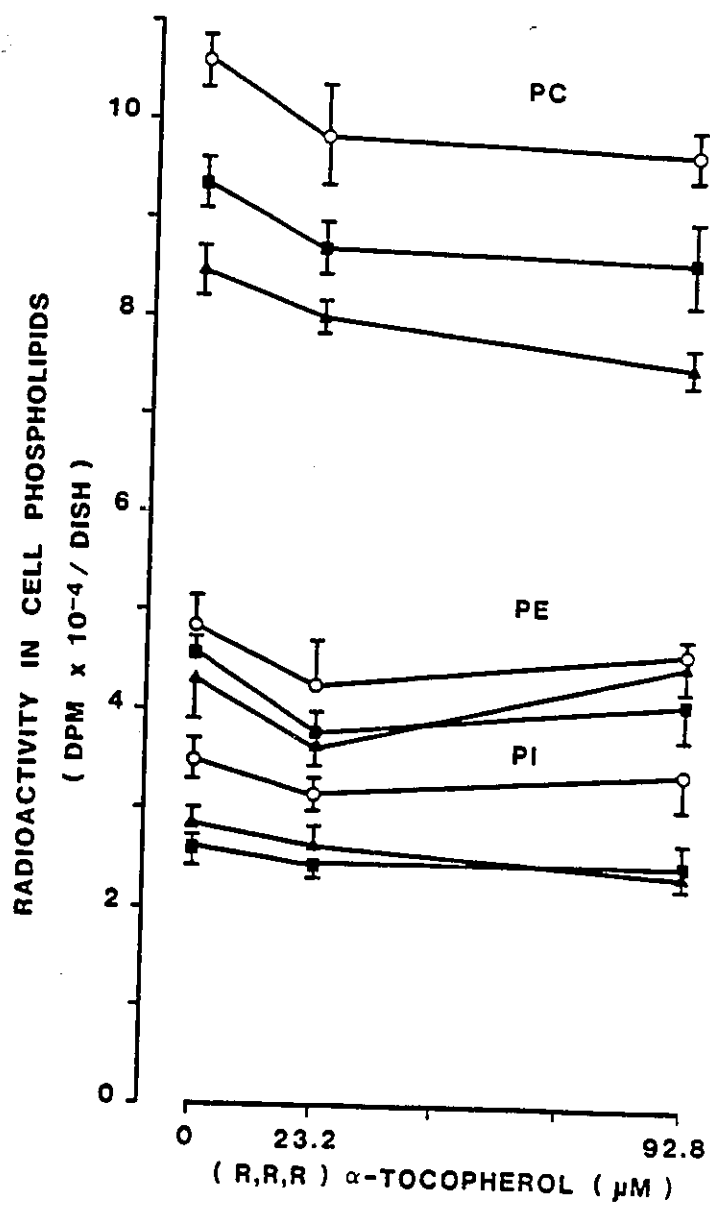


Figure 8 B

In order to determine whether or not α -tocopherol has any effect on cellular neutral lipids, aliquots of cellular lipid extract from Experiment 2 were subjected to quantitation of radiolabelled neutral lipid (Table IV). Radioactivity in cellular free arachidonic acid and diacylglycerol was unaffected by tocopherol enrichment while the radioactivity in triacylglycerol was slightly decreased with increasing concentration of tocopherol. This suggested that, in addition to phospholipase A₂, triacylglycerol lipase is also involved in arachidonic acid release potentiated by tocopherol. Under ionophore and thrombin stimulation, the radioactivity from both free arachidonic acid and diacylglycerol was higher compared to the control whereas the radioactivity of triacylglycerol was significantly decreased.

In the preceeding two experiments, I have shown that endothelial cells in culture incorporated arachidonic acid mainly into PC and neutral lipid fractions. There was a time-dependent redistribution of arachidonic acid between different phospholipid pools. Tocopherol in the medium as well as cellular tocopherol had no effect on the incorporation of arachidonic acid into cellular lipids. 2R,4'R,8'R- α -tocopherol potentiated the release of arachidonic acid mainly from PC, although triacylglycerol was also involved in contributing to the pool of released arachidonate.

Table IV

*Distribution of radioactivity in cellular neutral lipids
after agonist-stimulation.*

Aliquot of cellular lipid extract from Experiment 2 (see legend in Figure 8A and B) was used for quantitation of neutral lipids by thin layer chromatography as described under Materials and Methods. Values are means $\pm$ S.D. of three dishes.

	Tocopherol concentration in media, μ M		
	0	23.2	92.8
	dpm $\times 10^{-3}$ / dish		
Free arachidonate			
Control	0.81 $\pm$ 0.02	0.79 $\pm$ 0.01	0.77 $\pm$ 0.02
Thrombin	2.36 $\pm$ 0.14	2.35 $\pm$ 0.14	2.50 $\pm$ 0.23
A23187	1.75 $\pm$ 0.01	1.72 $\pm$ 0.10	1.79 $\pm$ 0.01
Diglyceride			
Control	2.93 $\pm$ 0.19	2.90 $\pm$ 0.19	2.81 $\pm$ 0.16
Thrombin	5.80 $\pm$ 0.23	6.02 $\pm$ 0.50	5.69 $\pm$ 0.17
A23187	4.60 $\pm$ 0.28	4.82 $\pm$ 0.20	4.65 $\pm$ 0.18
Triglyceride			
Control	49.21 $\pm$ 2.82	44.17 $\pm$ 4.49	41.93 $\pm$ 0.71
Thrombin	43.60 $\pm$ 2.62	41.90 $\pm$ 1.80	39.10 $\pm$ 0.80
A23187	38.63 $\pm$ 0.90	38.33 $\pm$ 3.53	34.60 $\pm$ 1.00

C. 2R,4'R,8'R- α -tocopherol potentiates prostacyclin release in human endothelial cells

Human umbilical vein endothelial cells in culture synthesize prostacyclin (PGI₂) as the predominant metabolite of arachidonic acid (AA) which is derived from the deacylation of phospholipids. Since α -tocopherol has been shown to potentiate the release of arachidonic acid in the previous section, it will be therefore interesting to examine whether or not the potentiating effect of α -tocopherol on arachidonic acid release is correlated with the synthesis of PGI₂ in endothelial cells. The following studies were designed to examine the effect of α -tocopherol on spontaneous and agonist-induced PGI₂ synthesis in human endothelial cells.

During the initial stage of these studies, I found that the prostacyclin (PGI₂) synthetic capacity of human umbilical vein endothelial cells decreased as the cells were passaged. Hence, in subsequent experiments, only cells from first passage were used in order to obtain reproducible results. I also found that when culture medium containing endothelial cell growth supplement was removed immediately prior to the study of PGI₂ synthesis, the amount of PGI₂ produced by the cells was extremely low. Exclusion of the growth supplement from the medium from 20 - 24 h "restored" the PGI₂ synthesis capacity. Therefore, in subsequent studies, confluent monolayers were replaced with fresh culture medium in the absence of growth supplement for 20 - 24 h before they were used. These two observations are consistent with those that were previously reported (39,40).

Effects of 2R,4'R,8'R- α -tocopherol enrichment on PGI₂ synthesis

When monolayers were preincubated with 23.2 μ M of 2R,4'R,8'R- α -tocopherol at different time points the release of PGI₂ and the uptake of tocopherol by endothelial cells were time-dependent (figure 9). This potentiating effect of tocopherol on PGI₂ release commences at 1 h of tocopherol incubation, continues to increase after 4 h and reaches maximum at 8 h which was also the time required for maximum cellular uptake of tocopherol (41,42). In order to determine the concentration of tocopherol that can elicit maximum PGI₂ release, monolayers were preincubated for 4 h with different tocopherol concentrations and the time course of basal PGI₂ release was monitored. Figures 10A and B clearly show that PGI₂ release was stimulated by tocopherol enrichment in a dose-dependent way. The maximum PGI₂ release was reached at 10 minutes and the maximum stimulatory tocopherol concentration was 46.4 μ M. This concentration of tocopherol is twice that of normal plasma level in North Americans who take no vitamin E supplement but is readily achievable by oral tocopherol supplementation. In all subsequent experiments, we routinely enriched the cells for 4 h with 23.2 μ M of tocopherol or its analogues.

Role of exogenous arachidonic acid and Ionophore A23187

To determine the mechanism by which tocopherol potentiates PGI₂ synthesis in human umbilical vein endothelial cells, experiments were designed to compare the effect of tocopherol on PGI₂ release in the presence of exogenous arachidonic acid and ionophore A23187.

Figure 9 - Time-dependent stimulation of tocopherol on the spontaneous PGI₂ release and uptake of tocopherol by human endothelial cells. *Confluent monolayers were incubated with R,R,R- α -tocopherol (23.2 μ M) at indicated times. After the cells were rinsed, they were incubated with Ringer-Tyrode buffer containing 1 mM Ca²⁺ for 10 min at 37 °C. PGI₂ release was quantitated from an aliquot of the supernatant by RIA and the tocopherol was determined by HPLC from total cellular lipid extract as described in Materials and Methods. Values are means of duplicate dishes and are derived after subtraction of basal PGI₂ release by corresponding cells incubated in the absence of tocopherol.*

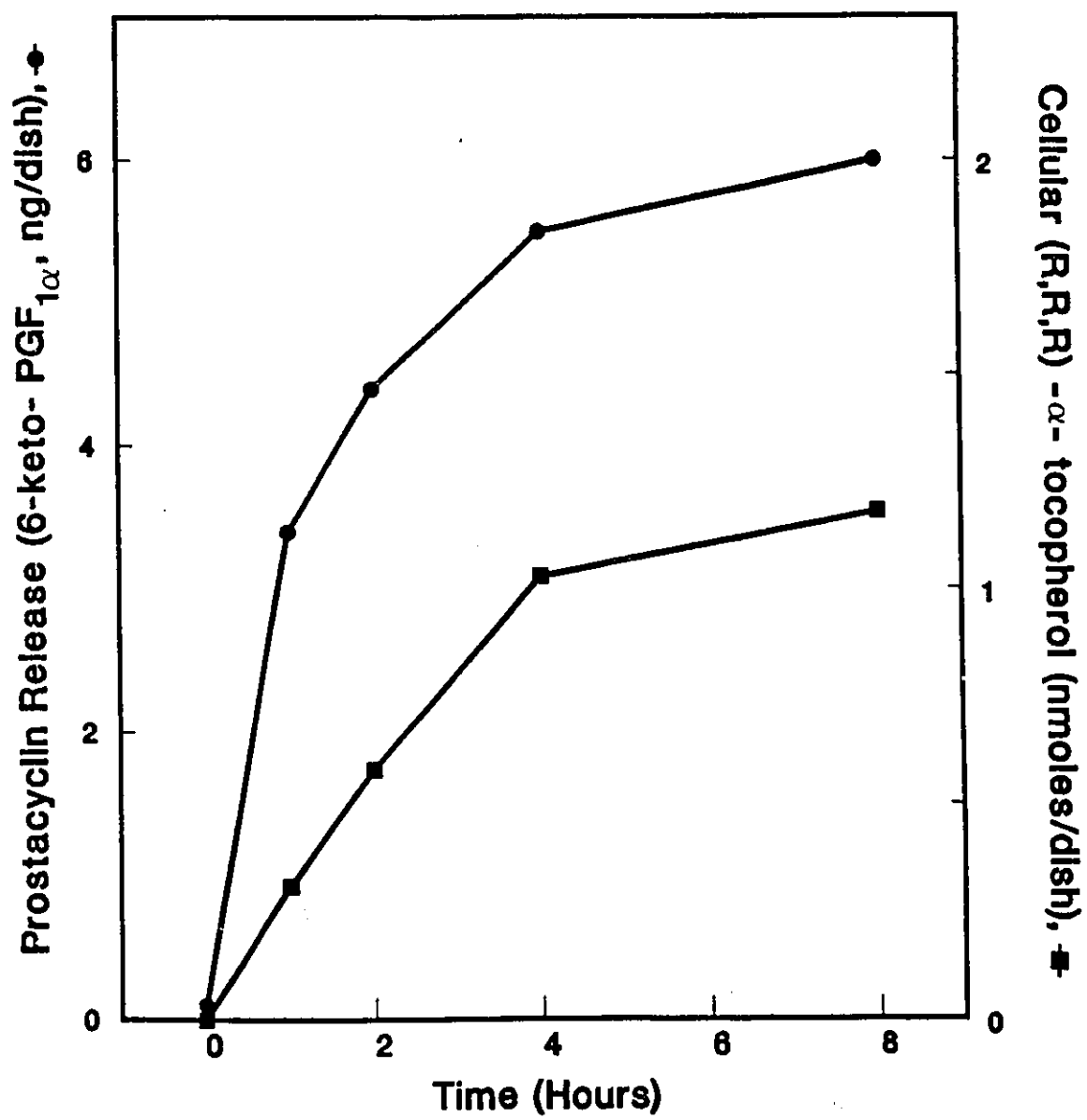


Figure 9

Figure 10 (A) - Time course of R,R,R- α -tocopherol-mediated prostacyclin production. Endothelial monolayers were incubated for 4 h at 37 °C with R,R,R- α -tocopherol at 0 (○), 11.6 (●), 23.2 (■), 46.4 (◇) and 92.8 (△) μ M. Cells were rinsed and then incubated with Ringer-Tyrode buffer containing 1 mM Ca^{2+} at the indicated times. PGI_2 was quantitated by RIA. Values are means $\pm$ S.D. of three dishes.

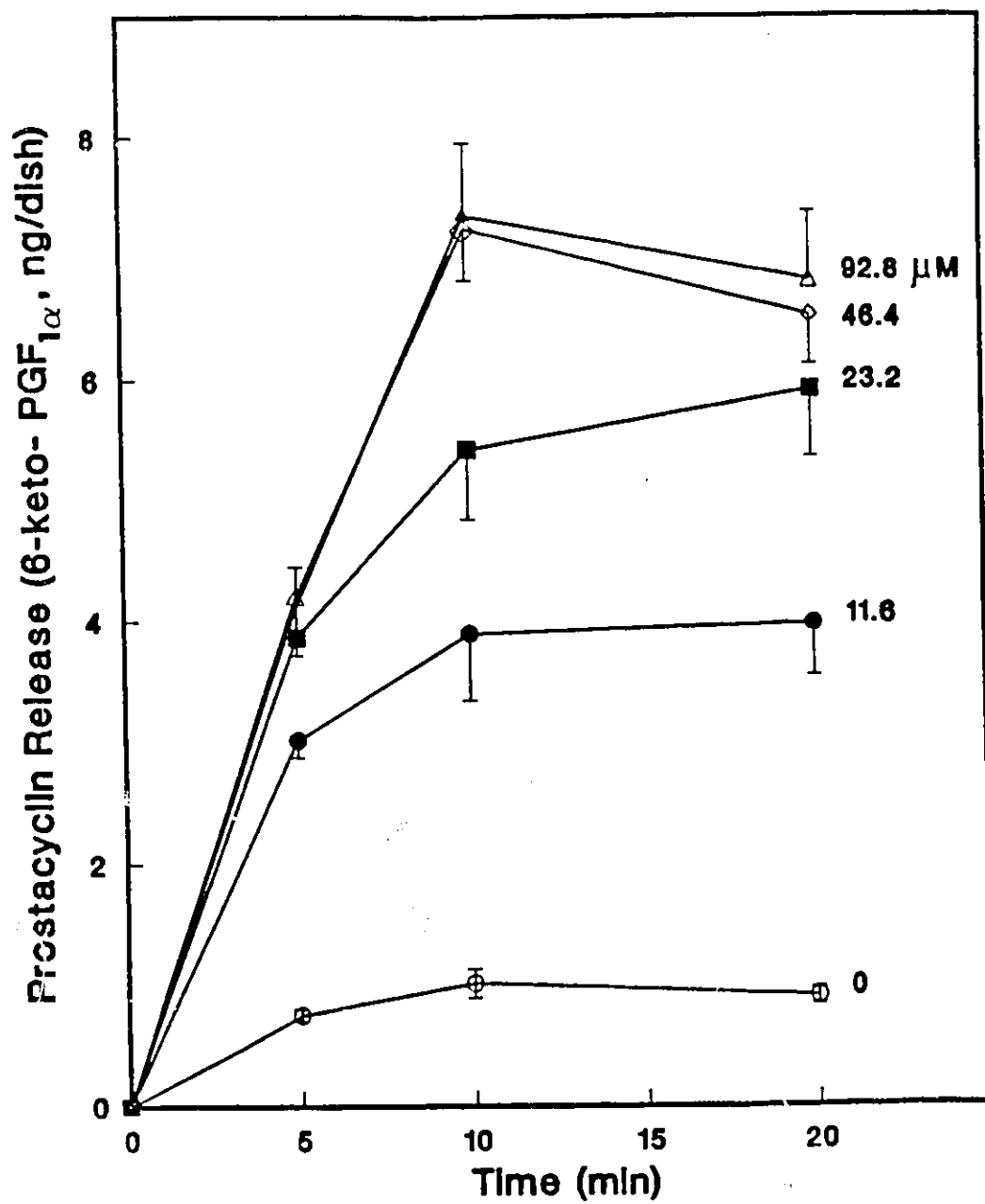


Figure 10 A

Figure 10 (B) - Dose-dependency of R,R,R- α -tocopherol-mediated prostacyclin release. *This figure was redrawn from the values in Figure 10 (A).*

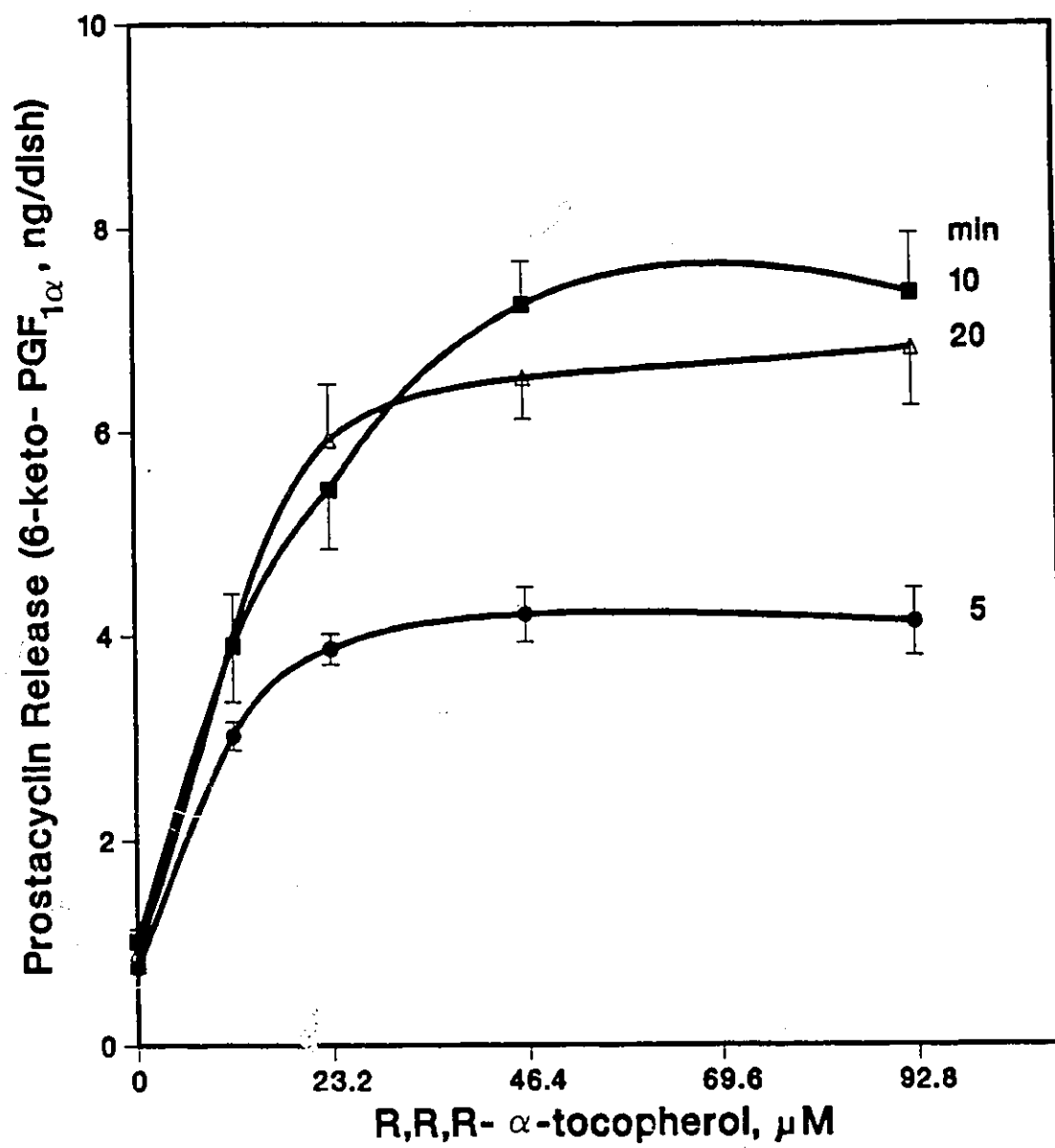


Figure 10 B

Ionophore A23187 was known to stimulate the cells to release endogenous arachidonic acid through the calcium-modulated phospholipase activation while exogenous arachidonic acid was used to bypass the step of arachidonic acid release.

Confluent monolayers were preincubated for 4 h with or without 23.2 μ M of 2R,4'R,8'R- α -tocopherol before they were stimulated for 10 min with varying concentrations of added arachidonic acid or ionophore A23187. Figure 11A shows that PGI₂ synthesis by human endothelial cells in response to exogenous AA was dose-dependent at low substrate concentrations (1-5 μ M). 2R,4'R,8'R- α -tocopherol-enriched cells synthesized consistently more PGI₂ than control cells that did not receive vitamin E-enrichment. However, the potentiating effect of vitamin E was greatly diminished when arachidonic acid was present at a higher concentration, ie. 50 μ M. This response to high level of exogenous arachidonate indicated that the cyclooxygenase - PGI₂ synthase system was fully operational irrespective of cellular tocopherol level. It further suggested that endogenous release of arachidonate may greatly be diminished by the presence of unsaturated fatty acid, which has been reported to be a strong inhibitor of phospholipase A₂ activity in platelets (101), neutrophils (102) and macrophages (103). In contrast, PGI₂ synthesis in response to A23187 stimulation showed a consistent dose-dependent increase at both low (20-100 nM) and high (1000 nM) concentrations (figure 11B). At all levels of A23187 tested, 2R,4'R,8'R- α -tocopherol- enriched cells synthesized significantly more PGI₂ than control cells. In fact, the greatest PGI₂ increase was found at the highest concentration of A23187 (1000 nM). Taken together, these data suggest that the potentiating effect of vitamin E on PGI₂ synthesis is predominantly mediated at the level of arachidonate release.

Figure 11 - Concentration dependence of exogenous arachidonic acid and ionophore A23187 on PGI₂ release by R,R,R- α -tocopherol. *Confluent monolayers were incubated in the absence (○) or presence (●) of 23.2 μ M R,R,R- α -tocopherol for 4 h at 37 °C. After the cells were rinsed, they were incubated with indicated concentrations of arachidonic acid (panel A) or A23187 (panel B) in Ringer-Tyrode buffer containing 1 mM Ca²⁺ for 10 min at 37 °C. PGI₂ was determined by RIA. Values are means $\pm$ S.D. of three dishes.*

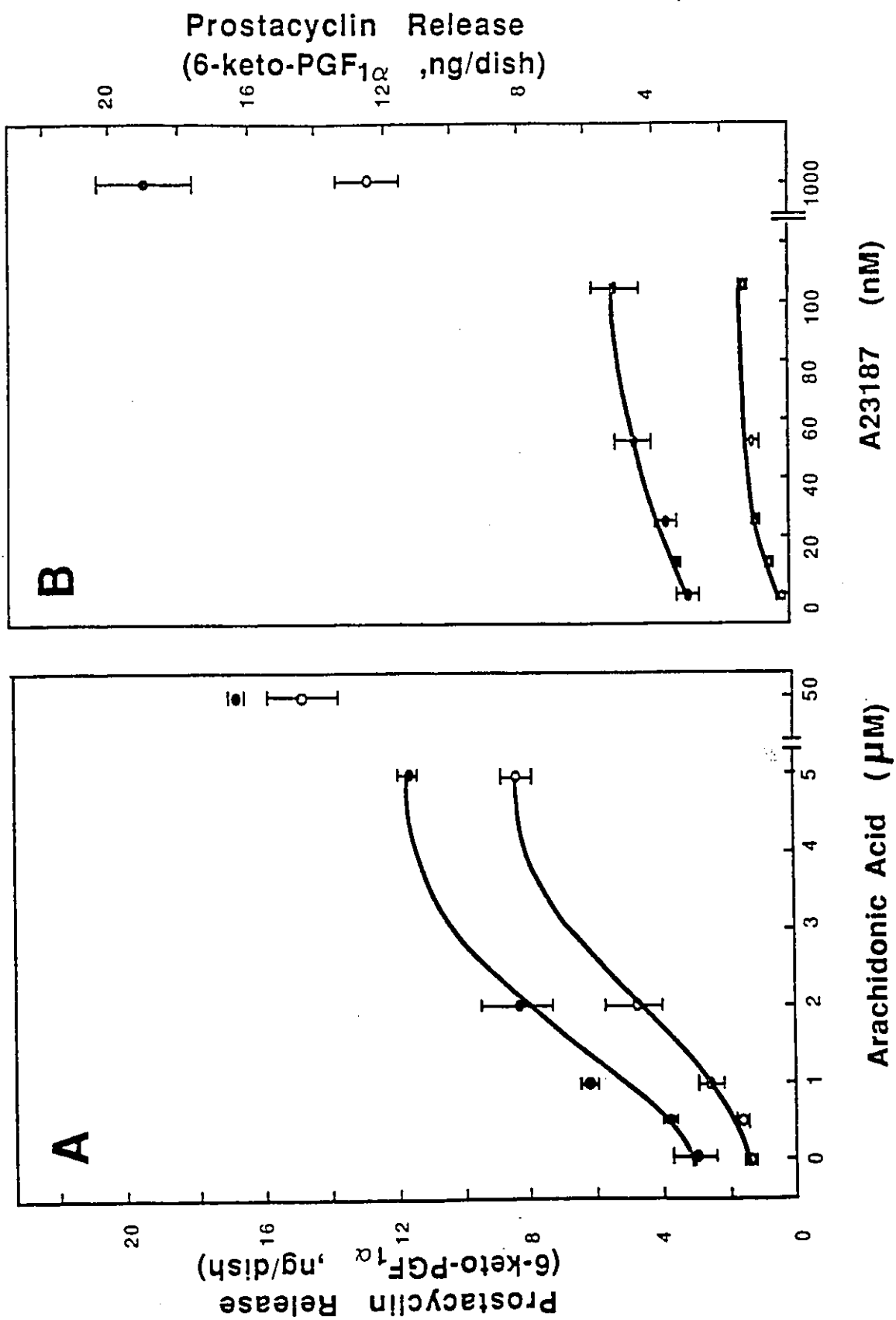


Figure 11

Effects of 2R,4'R,8'R- α -tocopherol on phospholipid metabolism

To discern the role of vitamin E on arachidonate release, my approach was to examine the deacylation-reacylation of phosphatidylcholine (PC) in human endothelial cells because PC is not only a major phospholipid in these cells but is also a significant pool for arachidonate release (see part B and reference 46). Possible mechanisms for the liberation of arachidonic acid are presented in figure 2.

Two experiments were conducted in which confluent monolayers were either labelled with [^3H]-methylcholine for 20 h prior to enrichment with 2R,4'R,8'R- α -tocopherol (23.2 μM) for 4 h or the cells were coincubated with [^3H]-methylcholine in the presence and absence of 2R,4'R,8'R- α -tocopherol for 20 h. After the removal of labelling compounds at indicated times, monolayers were rinsed to remove nonspecific label adhesion and were stimulated with ionophore A23187 (1 μM) for 10 min. Cellular lipids were extracted and the amount of labelled lysophosphatidylcholine was quantitated by liquid scintillation counting after different phospholipids were separated by thin layer chromatography. The incorporation of [^3H]-choline into PC during incubation was found to be unaffected by the presence of tocopherol in the cell or in the culture medium (data not shown). Figures 12A and B show that tocopherol-enrichment followed by ionophore A23187 stimulation significantly increased cellular labelled lysophosphatidylcholine content, irrespective of the sequence of choline labelling or tocopherol enrichment.

Figure 12 - Effect of R,R,R- α -tocopherol on ionophore A23187-stimulated lysophosphatidylcholine formation. *Confluent monolayers were labelled with 10 μ Ci/ml of [3 H]-choline for 20 h followed by enrichment of R,R,R- α -tocopherol (23.2 μ M) for 4 h (panel A), or the cells were coincubated with 10 μ Ci/ml of [3 H]-choline and R,R,R- α -tocopherol (23.2 μ M) for 20 h (panel B). Cells were rinsed and stimulated with 1 μ M A23187 in Ringer-Tyrode buffer containing 1 mM Ca^{2+} for 10 min.. Values are means $\pm$ S.D. of three dishes.*

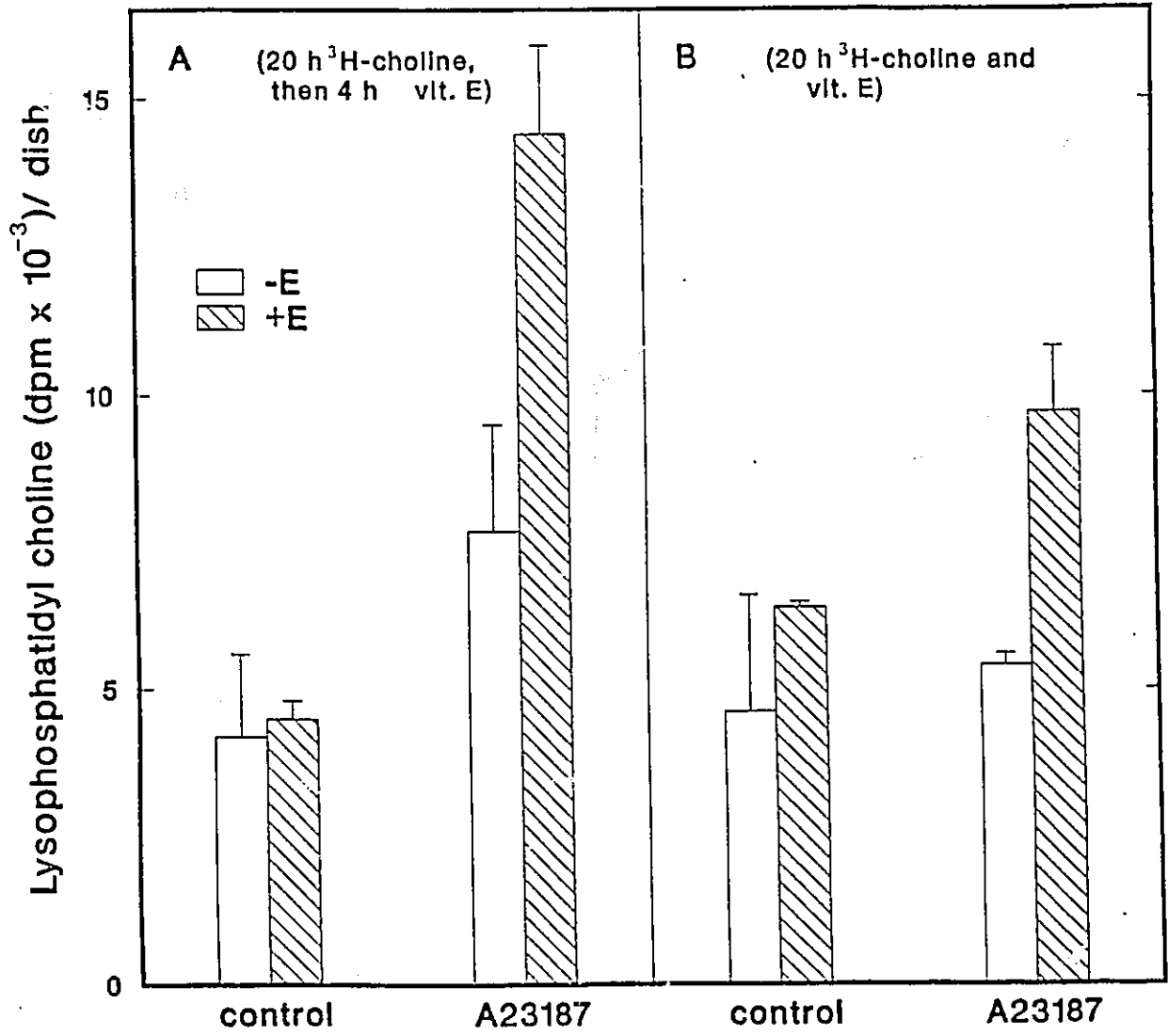


Figure 12

The increase in cellular lysophosphatidylcholine elicited by tocopherol can be attributed to an elevated phospholipase A activity or conversely, it may result from a change in enzymes that metabolize lysophosphatidylcholine. In order to differentiate the mechanism for the increase of cellular lysophosphatidylcholine, the activities of lysophospholipase and lysophosphatidylcholine-acylCoA-acyltransferase were determined in homogenates from monolayers pretreated with or without 2R,4'R,8'R- α -tocopherol (23.2 μ M) for 4 h. Table V clearly shows that the activities of these two lysophosphatidylcholine catabolising enzymes were essentially unchanged by vitamin E enrichment. Therefore, the elevated lysophosphatidylcholine observed in Figure 12 was primarily due to an increase in phospholipase A activity.

Direct analysis of phospholipase A₂ activity by using sn-1-palmitoyl-2-[1-¹⁴C] arachidonylphosphatidylcholine as substrate in the presence of 3 levels of Ca²⁺ shows that irrespective of the Ca²⁺ concentration, vitamin E-enrichment caused an enhanced activity of this enzyme (Table VI). These data suggest that the effect of vitamin E in potentiating the release of PGI₂ observed in Figure 9-11 is mainly mediated by an increased phospholipase A₂ activity.

Stimulatory effect of hydrogen peroxide (H₂O₂) and 2R,4'R,8'R- α -tocopherol on PGI₂ synthesis

The metabolism of endogenous or exogenous arachidonic acid by cyclooxygenase in cultured endothelial cells (99) generates fatty acid hydroperoxides which are important mediators that can activate or inactivate the enzymes required to convert arachidonic acid to PGI₂, depending on the concentration of peroxides. On a molar basis, ROOH are 1000-fold more potent than H₂O₂ in stimulating PGI₂ synthesis (67).

Table V

***Effect of R,R,R- α -tocopherol enrichment on
endothelial cell acylCoA acyltransferase and
lysophospholipase activities***

Confluent endothelial monolayers were incubated with or without tocopherol for 4 h. AcylCoA transferase and lysophospholipase activities were determined in sonicated cell homogenates (130 μ g protein) as described under Methods. Values shown are means $\pm$ SD of 3 dishes.

R,R,R- α -tocopherol enrichment μ M	AcylCoA acyltransferase		Lysophospholipase	
	nmole/mg protein	nmole/10 ⁶ cells	nmole/mg protein	nmole/10 ⁶ cells
0	8.68 $\pm$ 0.6	5.5 $\pm$ 0.3	31.8 $\pm$ 3.0	20.2 $\pm$ 1.8
23.2	8.50 $\pm$ 0.7	5.3 $\pm$ 0.4	29.7 $\pm$ 6.3	18.5 $\pm$ 4.0

Table VI

Effect of R,R,R- α -tocopherol on phospholipase A₂ activity

Endothelial monolayers were incubated with and without R,R,R- α -tocopherol for 4 h. Cells were harvested by scraping and sonicated in Tris-buffer, pH 9.3. Phospholipase A₂ activity was determined as described under Methods. Values shown are means of duplicates which varied within 5% of mean.

Ca ²⁺ (mM)	Fatty acid released		PLA ₂ activity	
	<u>dpm x 10⁵/mg protein/h</u>		<u>pmol/mg protein/h</u>	
	R,R,R- α -tocopherol, μ M		R,R,R- α -tocopherol, μ M	
	0	23.2	0	23.2
0	0.05	0.12	45	100
5.5	2.10	2.98	1740	2460
10.5	1.73	2.16	1410	1760

The potentiating effect of α -tocopherol on PGI₂ synthesis in cultured endothelial cells might be attributed at the level of cyclooxygenase and PGI₂ synthase in addition to its role at the level of arachidonic acid release, as previously presented. To address this hypothesis, an indirect study was conducted in which H₂O₂ has been used as the mediator for PGI₂ synthesis in both control and tocopherol-enriched cells. The purpose of this experiment is to determine whether or not α -tocopherol can perturb the effect of H₂O₂ with respect to PGI₂ synthesis.

Table VII clearly shows that α -tocopherol and H₂O₂ both stimulate the release of PGI₂ in cultured human endothelial cells. Both α -tocopherol (23.2 μ M) and H₂O₂ (35 μ M) were found to individually stimulate PGI₂ formation. α -Tocopherol enrichment caused a 10-fold increase in PGI₂ release whereas H₂O₂ stimulation caused a 4-5-fold increase when compared with control. The observation that H₂O₂ stimulates prostaglandin formation was consistent with the other reports (43-45). However, the intermediates of the cyclooxygenase reaction include carbon-centered radicals and hydroperoxides which have potential to inhibit cyclooxygenase activity by a feedback mechanism. Interestingly, in the presence of both H₂O₂ and α -tocopherol, the potentiating effect for PGI₂ synthesis was additive. Therefore it is possible that α -tocopherol may potentiate the stimulating effect of H₂O₂ on PGI₂ synthesis by protecting the catalytic suicide of the cyclooxygenase activity mediated by the intermediates of the reaction (radicals and hydroperoxides).

Table VII

***Stimulatory effect of hydrogen peroxide (H_2O_2) and
 R,R,R - α -tocopherol on PGI_2 synthesis.***

Confluent endothelial monolayers were incubated with or without tocopherol (23.2 μ M) for 4 h. The cells were rinsed with Ringer Tyrode buffer and stimulated in the presence or absence of 35 μ M H_2O_2 for 10 min. PGI_2 release was quantitated in the supernatant by RIA as described under Methods. Values are means $\pm$ SD of 3 dishes.

H_2O_2 / α -tocopherol	6-keto-PGF _{1α} ng/dish	%
- / -	0.56 $\pm$ 0.09	100
- / +	5.83 $\pm$ 0.26	1041
+ / -	2.70 $\pm$ 0.18	482
+ / +	8.35 $\pm$ 0.44	1491

Specificity of the tocopherol molecule in potentiating PGI₂ synthesis

In order to clarify whether the potentiating effect of tocopherol on PGI₂ synthesis is due to a non-specific lipid effect or if the response is specific to certain structural requirement of the tocopherol molecule, I have chosen to study the effect of several tocopherol analogues. These include : tocopherol acetate in which the free hydroxyl group on the chromanol ring responsible for its antioxidant property is blocked by acetylation; Trolox^R, which contains the complete chromanol ring but without the phytol side chain, and, phytol itself. Confluent monolayers were preincubated with or without physiological concentration of 2R,4'R,8'R- α -tocopherol (23.2 μ M) or with similar concentrations of the above-mentioned compounds for 4 h. After the enrichment medium was removed, basal PGI₂ release was monitored over time. Figure 13 shows that the PGI₂ potentiating effect of tocopherol exhibits an absolute requirement for both the free hydroxyl moiety as well as the phytol side chain since cells preincubated with tocopherol acetate, Trolox^R or phytol produced a similar amount of PGI₂ as control cells that were preincubated with carrier only.

To delineate the importance of different methyl substituents on the chromanol ring of the tocopherol molecule, cell monolayers were incubated for 4 h at 23.2 μ M with either 2R,4'R,8'R- α -tocopherol (2,5,7,8-tetramethyl), β -tocopherol (2,5,8-trimethyl), γ -tocopherol (2,7,8-trimethyl), δ -tocopherol (2,8-dimethyl), tocol (2-methyl), all-rac- α -tocopherol (2,5,7,8-tetramethyl), Trolox^R, all-rac- tocopherol acetate or phytol. Basal PGI₂ release was determined after removal of incubation medium. Table VIII clearly shows that the presence and location of methyl groups on the chromanol ring is not required for potentiating PGI₂ release since tocol, β , γ , and δ tocopherols all

induced a significantly higher PGI₂ release which is comparable to 2R,4'R,8'R- α -tocopherol. In addition, the synthetic all-rac-tocopherol, which contains 4 racemic pairs due to the presence of 3 chiral carbons on the tocopherol molecule, exhibited similar activity when compared to the natural 2R,4'R,8'R- α -tocopherol. The inability of tocopherol acetate, Trolox^R, and phytol to elicit basal PGI₂ release was similar to previous experiments (Table VIII). Collectively, these data demonstrate that in order to potentiate PGI₂ release, the hydroxyl moiety on the chromanol ring as well as the phytyl side chain on the tocopherol molecule are absolutely essential for this activity. However, the presence or absence of different methyl substituents on the chromanol moiety did not significantly alter the potentiating effect of the tocopherol molecule on the synthesis of PGI₂.

In summary, tocopherol potentiates the release of PGI₂ from human umbilical vein endothelial cells in culture in a dose- and time-dependent manner. The free hydroxyl group the phytyl side chain but not the methyl substituents, are absolutely required for the stimulatory activity of the tocopherol molecule. The effect of tocopherol is likely mediated through the activation/enhancement of phospholipase A₂ activity. Whether tocopherol directly activates phospholipase A₂ or indirectly removes some endogenous inhibitor(s) of phospholipase A₂ is not clear at present.

Figure 13 - Effect of tocopherol-related compounds on the time course of prostacyclin production. *Confluent monolayers were incubated for 4 h at 37 °C with 23.2 µM of various compounds : R,R,R-α-tocopherol (●), rac-α-tocopherol acetate (Δ), phytol (×) and Trolox^R (■). Cells were rinsed and then incubated with Ringer-Tyrode buffer containing 1 mM Ca²⁺ at the indicated times. Prostacyclin released by corresponding monolayers incubated in the absence of tested compounds was considered as controls (100 %). Values are means ± S.D. of three dishes.*

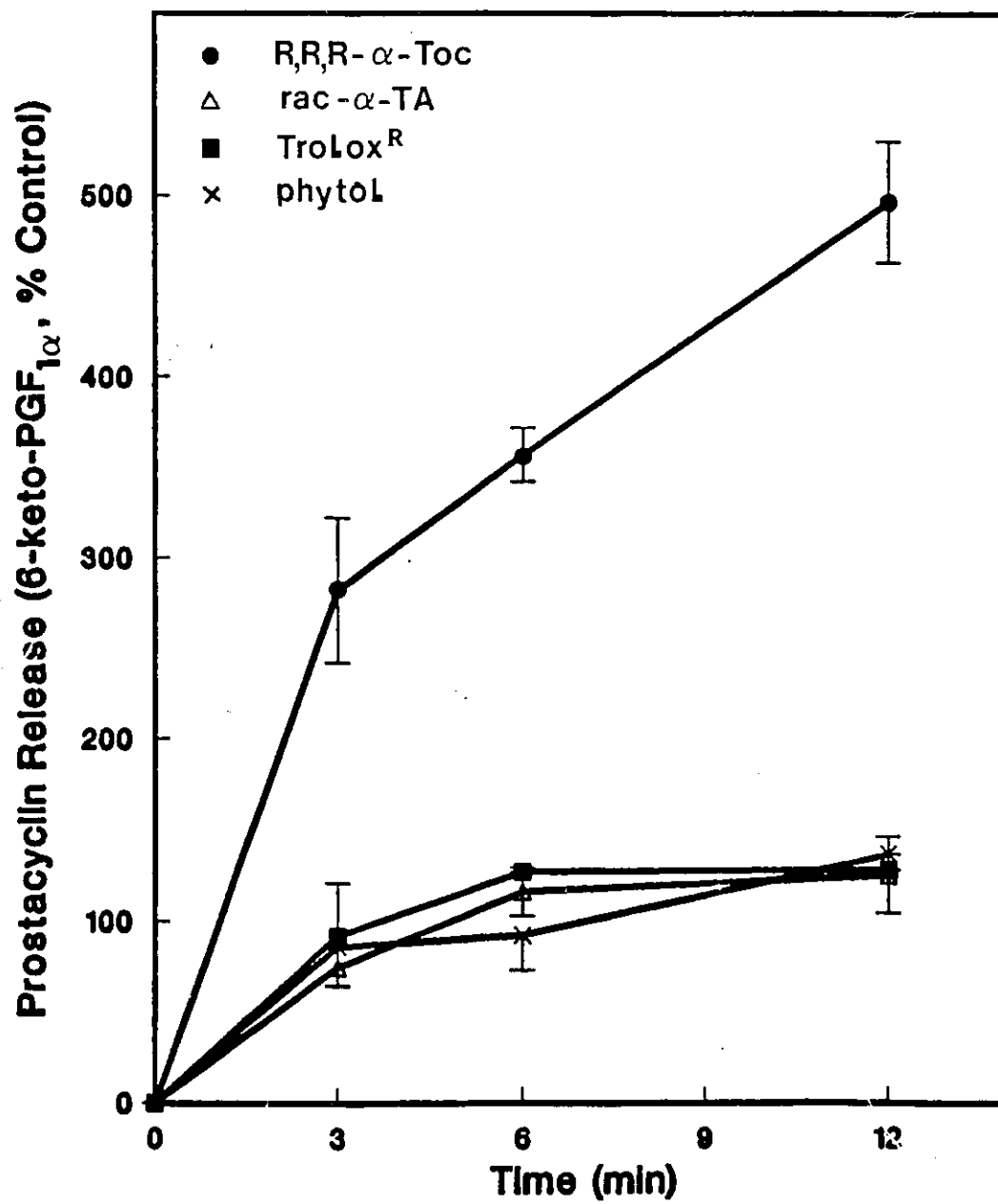


Figure 13

Table VIII

*Comparison of the potentiating effect of different
tocopherol analogues on PGI₂ release from
human endothelial cells*

Confluent monolayers were incubated with cultured medium containing 23.2 μ M of different tocopherol analogues. After 4 h, the incubation medium was removed and the dishes were rinsed with RT buffer. Cells were incubated for 10 min at 37 °C with 1 ml of RT buffer containing 1 mM Ca²⁺. The incubation buffer was collected and acidified to pH 3.6 with 1N HCl and 6-keto-PGF_{1 α} was quantitated by RIA as described in Methods. Values are means $\pm$ SD of 3 separate dishes.

Compound tested (23.2 μ M)	PGI ₂ release	
	ng/dish	%
R,R,R- α -tocopherol	7.5 $\pm$ 0.7	100
R,R,R- β -tocopherol	6.6 $\pm$ 1.1	88
R,R,R- γ -tocopherol	7.2 $\pm$ 0.5	96
R,R,R- δ -tocopherol	6.6 $\pm$ 1.0	88
rac- α -tocopherol	6.5 $\pm$ 0.5	87
Tocol	7.1 $\pm$ 1.3	95
rac- α -tocopherol acetate	2.0 $\pm$ 0.3	27
Trolox	2.1 $\pm$ 0.3	27
Phytol	2.3 $\pm$ 0.1	31
No addition	1.2 $\pm$ 0.1	16

DISCUSSION

Kinetics of cellular α -tocopherol uptake and depletion

The uptake of tocopherol was linear up to 8 h with an accumulation rate of about 300 pmoles/h/ 10^6 cells when the cells were incubated at physiological concentration (23.2 μ M or 1 mg/dl) of tocopherol (Figure 4). There was also a linear relationship between cellular content of tocopherol and the logarithm of tocopherol concentrations in the medium. The highest tocopherol level used in this study (92.8 μ M or 4 mg/dl) was 4-fold higher than the normal plasma level. This pharmacological concentration represents the maximum achievable plasma tocopherol when individuals are under high doses of vitamin E supplementation. The time- and concentration-dependences of tocopherol uptake by endothelial cells were similar to that observed in experimental animals in which tissues tocopherol responded logarithmically to tocopherol levels in the diet (47-50).

In the depletion study, cellular tocopherol content gradually diminished over time (figure 6), with a depletion rate much slower than the accumulation rate. The half life of tocopherol in the cells calculated from the lower linear portion of the depletion curve was approximately 2-3 days. In the studies of experimental animals given high level of dietary vitamin E, Machlin and Gabriel found that, with the exception of adipose tissue, the rate of depletion of tocopherol from most tissues (muscle, lung, heart, liver, platelets, red blood cells, brain) was more rapid than the rate of accumulation (51). The difference of the two observations was probably attributed to the difference in the experimental approaches (ie. in vitro vs in vivo), the different tissues studied or the dosage of tocopherol used. The observation that cellular concentration of toco-

pherol reached a plateau after 8 h of incubation (figure 4) suggests that the equilibrium is attained between the rate of uptake and release of tocopherol.

Vitamin E is believed to be localized in the cell membranes and subcellular organelles. This study showed that the majority of cellular tocopherol was indeed membrane-associated with very small amount present in the cytosol (Table II). The lipophilic nature of tocopherol is further elaborated by recent findings that the vitamin E content in brown adipose tissue was found at least five fold higher than that of white adipose tissue (52). This is attributed to a 100-fold difference in mitochondrial content between the two tissues. When present in the cytosol, the tocopherols are stored in the form of lipid droplets, whereas in the membrane, the main function of tocopherol is to protect polyunsaturated membrane lipids from oxidation (2,53,54).

In artificial lipid bilayers, Kagan and Quinn (55) found that α -tocopherol existed both in the monomeric and aggregated form. They postulated that the monomeric form is responsible for antioxidant and membrane stabilizing effects of tocopherol, whereas the aggregated form may act as a reservoir. Accumulation of large amounts of α -tocopherol in membranes was found to alter the phase behaviour, decreasing the stability and altering the permeability properties of the membrane (56,57); particularly, it has been suggested that α -tocopherol tends to form complexes with fatty acids and lysophospholipids (58-60). Whether or not α -tocopherol has any effect in altering the membrane dynamic and stability of biological membrane thereby affecting other membrane-associated biological functions remains to be elucidated.

Synthesis of Prostacyclin

Apart from serving as a selective barrier for nutrient clearance and transport, endothelial cells produce appreciable quantities of cyclooxygenase metabolites. Prostacyclin (PGI₂) is the major arachidonic acid metabolite of cultured human endothelium, although smaller amounts of PGF_{2 α} and PGE₂ have been reported to be released by stimulation of endothelial cells derived from microvessels of human foreskin (104,105). I found that 2R,4'R,8'R- α -tocopherol potentiates PGI₂ release in endothelial cells in a dose-dependent manner. The effective concentration of tocopherol required to elicit PGI₂ formation (figure 10) was below that of normal plasma value (23.2 μ M) in human. The maximum response was, however, saturable at 46.4 μ M. This concentration is twice that normally found in human plasma, but it is readily achievable by oral tocopherol supplementation.

The stimulatory activity of tocopherol in PGI₂ release has a specific structural requirement for both free hydroxyl moiety and the hydrophobic phytyl side chain (figure 13, Table VIII). Chemical modification of the tocopherol molecule by blocking the hydroxyl group or by removing the phytyl moiety greatly attenuated its PGI₂ potentiating activity. This observation confirms the importance of the phytyl side chain that is probably required for the anchorage and penetration of the molecule into the lipid bilayer, whereas the unmodified hydroxyl group is needed for the full effect of this vitamin (4,61). In contrast, the location and the presence of the methyl groups attached to the aromatic moiety of tocopherol are not required for its stimulating role in PGI₂ release. The synthetic rac- α -tocopherol which contains 4 racemic pairs of isomers present in equal molar amounts, possesses stimulatory activity that is comparable to the naturally occurring 2R,4'R,8'R- α -tocopherol. Therefore, the PGI₂ potentiating

property of tocopherol is independent of its isomeric forms. The stimulatory effect of the tocopherol molecules on PGI₂ release is likely mediated by an alternative pathway independent of its antioxidant property.

The observation that addition of H₂O₂ to endothelial monolayers increased PGI₂ formation was consistent with recent findings that a certain level of hydroperoxides, so-called tissue "peroxide tone" appears to be necessary for prostaglandin synthesis (63-67). Enzymatic oxidation of arachidonic acid leading to the formation of prostaglandin was catalyzed by prostaglandin H synthase (PHS). It catalyzes the primary cyclooxygenase reaction to form PGG₂ as well as the consecutive hydroperoxidase reaction to form PGH₂ (figure 2 and see Pace-Asciak and Smith (68) for review).

Generally, cyclooxygenase reaction is activated by hydroperoxides and self-inactivated by oxidants that are released during the reduction of PGG₂ to PGH₂. Hemler and Lands (65) found that low levels of hydroperoxides (i.e., < 1 μ M for lipid hydroperoxides or 100 μ M for H₂O₂) stimulated PHS, whereas high concentrations of peroxides resulted in inactivation of PHS. I also observed that H₂O₂ stimulated PGI₂ release in a dose-dependent manner but at concentrations above 100 μ M, H₂O₂ induced cytotoxicity (data not shown). An additional factor that affects the hydroperoxide activation is the length of prior hydroperoxide exposure before prostaglandin synthesis was measured. I noted that 35 μ M H₂O₂ resulted in a 5-fold increase in basal PGI₂ production by endothelial cells, but when the cells were rinsed and then stimulated with A23187 (1 μ M) + AA (20 μ M), the synthesis of PGI₂ was inhibited by 35%. This observation is consistent with the recent reports of Agar and Gordon (69) and Whorton et al (70). Taken together, these results suggested that a small amount of peroxide is necessary to initiate the cyclooxygenase activity, whereas excessive hydroperoxides resulted in the inactivation of cyclooxygenase (65).

H₂O₂ may also have other effects on intact cells that lead to elevated prostaglandin synthesis. It has been shown that H₂O₂ treatment results in a rapid K⁺ efflux with a time course which is similar to prostaglandin synthesis (69). The effect of H₂O₂ on the flux of Ca²⁺ has also been investigated in cultured endothelial cells (72). In this study, H₂O₂ was found to resemble the action of ionophore that caused Ca²⁺ influx. A net Ca²⁺ influx along with a net K⁺ efflux would certainly promote phospholipase activity, thereby increasing the level of free arachidonic acid and its subsequent transformation into prostaglandin (64, 73,74).

Both H₂O₂ and cellular α -tocopherol synergistically stimulated basal release of PGI₂ from endothelial cells (Table VII). I also found that α -tocopherol potentiates PGI₂ synthesis upon challenge with exogenous arachidonic acid (figure 11A). These results suggested that α -tocopherol apparently affects the cyclooxygenase-prostaglandin synthase in addition to its role in fatty acid release (discussed later). At present, I postulate that α -tocopherol behaves similarly to phenols or other oxidizable substances in preventing the PHS system from self-destruction. These antioxidants act as hydrogen donors and are cooxidized during the reaction catalyzed by PHS system (see Bruchhausen et al (71) for review). Hence, vitamin E apparently protects the cyclooxygenase from self-destruction and acts as cosubstrate during the reduction of PGG₂ to PGH₂.

The role of tocopherol on prostacyclin synthetase was not examined in our studies. In the perfused rabbit aorta, it has been found that both cyclooxygenase and PGI₂ synthase are extremely sensitive to metabolites of arachidonic acid, especially 15-hydroperoxyeicosatetraenoic acid (15-HPETE) (100). Some reports have suggested that vitamin E protects prostacyclin synthetase from inactivation by preventing the formation of hydroperoxide (29,30). Whether or not vitamin E has any effect on PGI₂

synthetase need to be reexamined by using PGG₂ or PGH₂ as substrates for PGI₂ synthetase in order to bypass phospholipase and cyclooxygenase enzymes.

The role of phospholipase A₂

In addition to its effect on the PHS system and PGI₂ synthetase, α -tocopherol had a direct effect on phospholipase A₂ from three lines of evidence according to the present study. First, tocopherol-enriched cells significantly enhanced labelled arachidonic acid release from PC upon challenge with thrombin or ionophore (figure 7 and 8). Second, ionophore stimulation resulted in an increase of cellular lysophosphatidyl-[³H]-choline in tocopherol-enriched cells (figure 12). The increase of cellular lysophosphatidylcholine elicited by tocopherol could not be attributed to an alteration of lysophospholipase or acylCoA-acyltransferase activities (Table V). Third, direct analysis of phospholipase A₂ demonstrated that tocopherol enrichment enhanced the activity of this enzyme (Table VI).

The release of arachidonic acid from phospholipids in intact cells is considered to be the rate limiting step in the biosynthesis of eicosanoids. Considerable evidence has accumulated to show that arachidonic acid can be released from many precursor phospholipid classes including phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine. It is now generally accepted that the release of arachidonic acid from phosphatidylinositol by the sequential actions of phospholipase C and diglyceride lipase or by a PI-specific phospholipase A₂ activity contribute to a significant mass of arachidonic acid released (75-81). In human endothelial cells, phospholipase A₂ activity has been reported to hydrolyze phosphatidylinositol, phosphatidylcholine and phosphatidylethanolamine (77,78).

Alpha-tocopherol did not alter the uptake and distribution of arachidonic acid in cellular phospholipids but tocopherol enrichment enhanced arachidonic acid release in response to thrombin and ionophore (figure 7 and 8). The potentiating effect of tocopherol on arachidonic acid release is compatible with its stimulation on PGI₂ release in endothelial cells. I have taken the approach to bypass the step of arachidonic acid release by direct incubation of endothelial cells with exogenous arachidonic acid. The dose-dependent increase in PGI₂ synthesis in response to added arachidonic acid presented in figure 11A is consistent with the concept that the release of arachidonic acid is a major rate-limiting step for eicosanoid biosynthesis (85) and it further implies that PGI₂ synthetic capacity in resting endothelial cells is highly depended on the level of endogenous arachidonate. In addition, the cells released PGI₂ in response to a wide range of ionophore (figure 11B). The observation that tocopherol increased arachidonate- and ionophore-induced PGI₂ release was similar to the potentiating effect of tocopherol in arachidonic acid release induced by phospholipase A₂-stimulated agonists. Direct analysis of phospholipase A₂ activity and indirect quantitation of lysophosphatidyl-[³H]-choline from choline-prelabelled cells further support the regulation role of vitamin E at the level of arachidonate release.

The mechanism by which tocopherol stimulates phospholipase A₂ activity in human endothelial cells is not clear at present. It is tempting to speculate that tocopherol may activate a mediator or remove a repressor which control acylhydrolase activity. In this respect, the lipoxygenase product 15-hydroperoxy-5,8,11,13-eicosatetraenoic acid, which was reported to stimulate phospholipase A₂ (82), could possibly be the mediator, because tocopherol-enrichment of human platelets have been shown to elicit a transitory increase in 12-hydroperoxy-5,8,10,14-eicosatetraenoic acid and

12-hydroxy-5,8,10,14-eicosatetraenoic acid when incubated with labelled arachidonic acid (83). Conversely, polyunsaturated fatty acids (PUFAs) are likely to be the repressor. High concentration of PUFAs, especially arachidonate and eicosapentaenoate, competitively inhibit phospholipase A₂ in macrophage (84). Tocopherol may abolish the PUFA-induced inhibition of phospholipase A₂ by either complexing with these PUFAs (58-60) or by channelling these PUFAs to be acylated in different intracellular lipid pools. The removal of the repressor will increase the activity of phospholipase A₂. Due to its lipophilic property, tocopherol would certainly change the phase behaviour of the membrane (56,57) and possibly alter the flux of certain ions such as Ca²⁺ and K⁺ that may result in a direct stimulation of certain membranous enzymes.

The stimulatory effect of vitamin E on phospholipase A₂ activity found in this study is in direct contrast with previous reports which showed that vitamin E inhibits rat platelets and myocardial phospholipase A₂ (25, 26). It appears, therefore, that the regulation of arachidonate release reaction by this vitamin is tissue- and probably species-specific.

CONCLUSION

In summary, human endothelial cells in culture is a feasible model for the direct study of vitamin E and it is possible that this model can potentially be used to study other fat-soluble vitamins and essential nutrients. The potentiating effect of vitamin E in the synthesis and release of PGI₂ from human endothelial cells is mediated by many possible mechanisms. This study demonstrated that vitamin E is able to regulate PGI₂ synthesis at the level of arachidonic release in which activation of PC-specific-phospholipase A₂ is likely to be one of the possible candidates. Arachidonic acid can also be released by the activation of phospholipase C followed by diglyceride lipase or through a combined actions of phospholipase D and phosphatidic acid-specific phospholipase A₂. Whether or not vitamin E has any effect on the last two sources of arachidonic acid release from mammalian cell system remains to be investigated. Additional in vitro studies are needed to determine whether or not stimulation of phospholipase A₂ is due to membrane perturbation by vitamin E. Apart from its role in the release of fatty acid and at the level of cyclooxygenase, it is also possible that vitamin E regulates PGI₂ synthesis by directly affecting the activity of PGI₂ synthetase or indirectly lowering the hydroperoxides concentration which is known to inhibit PGI₂ synthetase.

Other than its antithrombotic function, endothelial cells have been implicated to play a role in inflammatory reactions mainly through the synthesis of PGI₂ (15,86) and platelet-activating-factor (87, 88). The synthesis of these two lipid mediators requires activation of phospholipase A₂ activity (77, 79, 89). Recent evidence shows that vitamin E can influence the biosynthesis of platelet-activating-factor in rat polymorphonu-

clear leukocytes (90). Therefore, the function of vitamin E on the synthesis and remodelling of the ether-phospholipids in endothelial cells merits further investigation. Furthermore, endothelial-derived PGI₂ has recently been shown to modulate granulocyte responses (91-94), a process which is recognized to cause major destructive events in tissues during inflammatory diseases and myocardial reperfusion injury (95-98). Finally, the ratio of PGI₂/TXA₂ appears to be critical for the interactions between blood vessel and platelet during thrombosis formation. Whether the regulatory role of vitamin E on the synthesis of PGI₂, TXA₂, and PAF has any relevance in both normal and pathological conditions remain to be studied.

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