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EFFECTS OF NATURAL ANTIOXIDANT (VITAMIN E) ON PROTEIN
KINASE C ACTIVITY IN CULTURED SMOOTH MUSCLE CELLS.

CHUONG THANH HO

A thesis submitted to the School of Graduate Studies and Research of University
of Ottawa in partial fulfilment of the requirement for the degree of Master of
Sciences.

Department of Biochemistry
Faculty of Medicine
University of Ottawa
Ottawa, Canada



Chuong Thanh Ho, Ottawa, Canada, 1993



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Chuong Thanh Ho.

ABSTRACT

Since α -tocopherol, the active form of vitamin E, is an integral component of plasma membranes, the possibility that it may affect the activity of membrane-associated enzymes such as protein kinase C (PKC) is being investigated by a number of laboratories. In the present study, we examined the effects of vitamin E and another antioxidant, vitamin C, on PKC activity in A10 cells, an established smooth muscle cell line. Because several lines of evidence have suggested a possible involvement of oxygen free radicals in the activation of PKC, we attempted to elucidate the mechanism(s) by which vitamin E can influence PKC activity. To study the incorporation of vitamin E into A10 smooth muscle cells, cells have been treated with vitamin E - enriched medium for various times and at different concentrations; the results showed that cellular incorporation of vitamin E was increased in a time and dose-dependent manner. The incorporation of vitamin E did not show any effect on A10 cell number whereas it significantly diminished PKC activity in the membrane fraction. To investigate whether this decrease in membrane PKC activity was due to the effect of vitamin E on PKC translocation from cytosol to membrane, western immunoblotting was performed. The result showed that vitamin E treatment did not affect PKC translocation in our system.

In order to determine the role of oxygen radicals on PKC activity, A10 cells were treated with redoxcycling duroquinone which is known to generate oxyradicals. Cellular malondialdehyde was determined and used as an index for oxyradical-initiated lipid peroxidation. A time course study showed that duroquinone caused an increase in membrane-associated PKC activity as well as malondialdehyde formation. To ascertain the role of antioxidants on this duroquinone-induced PKC activation, cells were treated with duroquinone together with different combinations of vitamin E and C. Results showed that vitamin E alone significantly suppressed the duroquinone-induced PKC activation. The addition of vitamin C with vitamin E further suppressed PKC activation, whereas vitamin C alone did not have any effect.

Based on these observations, we suggested a model in which natural antioxidants, such as vitamins E and C, act in concert to inhibit PKC activity through their ability to quench cellular free radicals.

OBJECTIVES

General

To study the effects of antioxidants such as vitamin E (RRR- α -tocopherol) and vitamin C (ascorbate) on protein kinase C activity in A10 smooth muscle cells.

Specific

- to study the uptake of vitamin E in smooth muscle cells
- to study the involvement of oxygen radicals in protein kinase C activation
- to study the effects of vitamin E on malondialdehyde formation
- to study the effects of vitamin E and vitamin C on PKC activity

LIST OF ABBREVIATIONS

Ca ²⁺ :	Calcium
DAG:	Diacylglycerol
DNA:	Deoxyribonucleic acid
DMEM:	Dulbecco's modification of Eagle's medium
DMSO:	Dimethylsulfoxide
EDTA:	Ethylenediaminetetraacetic acid
EGTA:	Ethylenebis (oxyethylenitrilo) tetraacetic acid
HEPES:	[4-(2-hydroxyethyl)-1-piperazineethanosulfonic acid]
HPLC:	High performance liquid chromatography
HQ:	Hydroquinone
L [•] :	Fatty acid radical
LH:	Fatty acid
LO ₂ [•] :	Peroxy radical
LO [•] :	Alkoxy radical
LOH:	Alcohol
LOOH:	Hydroperoxide
MDA:	Malondialdehyde
NAD:	Nicotinamide adenine dinucleotide (oxidized)

NADH:	Nicotinamide adenine dinucleotide (reduced)
NADP:	Nicotinamide adenine dinucleotide phosphate (oxidized)
NADPH:	Nicotinamide adenine dinucleotide phosphate (reduced)
PBS:	Phosphate buffered saline
PKC:	Protein kinase C
PS:	Phosphatidylserine
PMSF:	Phenylmethylsulfonyl fluoride
PUFA:	Polyunsaturated fatty acid
Q:	Quinone
SDS:	Sodium dodecyl sulfate
SQ:	Semiquinone
TBA:	Thiobarbituric acid
VC:	Vitamin C (Ascorbate)
VC*:	Ascorbyl radical
VE:	vitamin E (in this thesis, it refers to α -tocopherol)
VE*:	Tocopheryl radical

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INTRODUCTION

Structure and Metabolism of Vitamin E

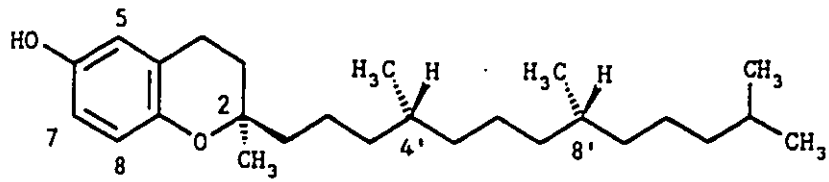
Since the discovery by Herbert Evans and Katherine Bishops in the early 1920's of a vitamin which influenced the reproduction of rats (hence the name tocopherol from the Greek tocos, meaning child birth, and the verb pherein, to bring forth), considerable progress was made to identify vitamin E and to determine its biological properties.

So far, at least 8 compounds that have vitamin E activity have been isolated from plant sources. All have a 6 chromanol ring structure and a side chain. The tocols have a phytyl side chain, and the trienols have a similar structure, with double bonds at 3', 7', and 11' positions of the side chain (Fig 1). Both tocols and tocotrienols occur as a variety of isomers that differ by the number and location of methyl groups on the chromanol ring of which the most active compound is 5,7,8 trimethyl tocol or α -tocopherol (Fig 1). The term vitamin E should be used as the generic description for all tocol or tocotrienol derivatives exhibiting the biological activity of α -tocopherol.

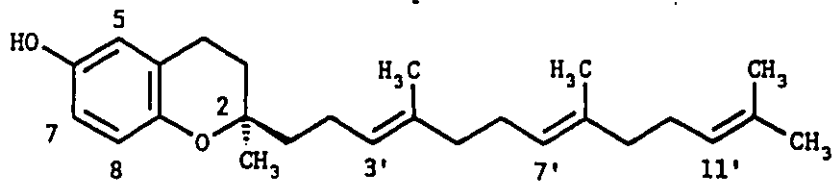
In man, absorption of vitamin E is incomplete, and it occurs maximally in the medium portion of the small intestine. Both bile acid and pancreatic juice are necessary for its optimal absorption. vitamin E is absorbed as a lipid bile micelle,

together with free fatty acids, monoglycerides and other fat soluble vitamins, by penetrating enterocytes at the brush border of the intestine, from where it enters the mesenteric lymphatics and is transported to the blood, and then to tissues . The vitamin is mostly concentrated in cell fractions rich in membrane such as mitochondria and microsome. Although all tocol and tocotrienol derivatives are readily absorbed and deposited in tissues, the structural difference of the chromanol ring as well as of the phytyl side chain are important for their variability in tissue retention and activity. In the membranes, α -tocopherol is oriented with the hydrophobic phytyl "tail" buried within the membrane, while the chromanol "head" is locate close to the surface of the membranes, thus explaining the interrelations between α -tocopherol and other water soluble agents such as ascorbate and glutathione. The absorbed α -tocopherol is stored mainly in adipose tissue, liver and muscle in its unmodified form and excreted mostly through fecal elimination.

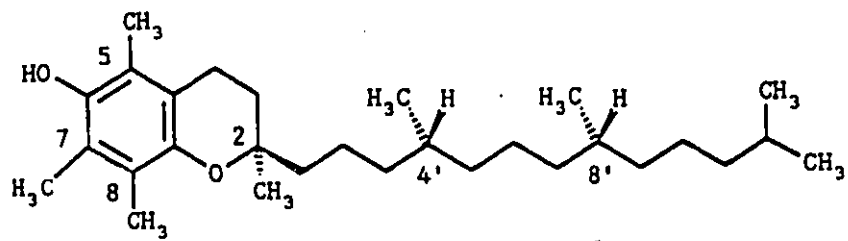
Figure 1 - Structure of Natural Tocopherol



Tocol



α -Tocotrienol



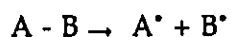
α -tocopherol
5, 7, 8-Trimethyltol

Figure 1

Free Radicals: Definition and Involvement

A free radical is any species capable of independent existence that contains one or more unpaired electrons (1).

For example, A and B are two atoms covalently bonded; if this covalent bond is broken and one electron from each of the pair remains with each atom, two radicals are formed:



The unpaired electrons have the tendency to look for a "partner", thus making the free radicals highly reactive.

These highly reactive substances may come from internal or external sources. Internally they are formed during oxygen metabolism and as a result of lipid peroxidation. External sources are radiations (solar and ionizing), ozone, food additives, air pollutants, cigarette smoke etc. Free radicals are thought to be linked to a number of pathological conditions such as radiation injury, aging, cancer and ischemia - reperfusion injury involving the lung, brain and cardiovascular system.

Functions of Vitamin E

Due to its solubility in lipid, vitamin E is located in the membranes of cells where it exerts its effect as an antioxidant. As summarized in Fig 2, the propagation of free radical generation from lipid peroxidation is terminated by

the presence of vitamin E. Therefore, vitamin E plays a unique role as the major lipid soluble antioxidant involved in protection against free radical-induced damage.

Apart from its antioxidant property, many studies have demonstrated that vitamin E interferes with arachidonic acid and prostaglandin metabolism, thus modulating the thrombotic and inflammatory processes (2-11). In addition, vitamin E is suggested to play a role in nucleic acid, protein (12) and lipid (13) metabolism as well as in the integrity of mitochondrial function (14) and in the stabilization of the membrane bilayer (15,16).

Although vitamin E deficiency has lead to undisputable clinical consequences in humans as listed in Table 1 (17), biochemical functions of vitamin E still remain a large field of research that is being pursued in several laboratories.

Figure 2 - Propagation of Free Radicals Generation
and Interrelationship of Vitamins E and C

(LH: fatty acid, L[•]: fatty acid radical, LO₂[•]: peroxy radical, LOOH: hydroperoxide,
VE: vitamin E, VE[•]: tocopheryl radical, VC: vitamin C, VC[•]: ascorbyl radical)

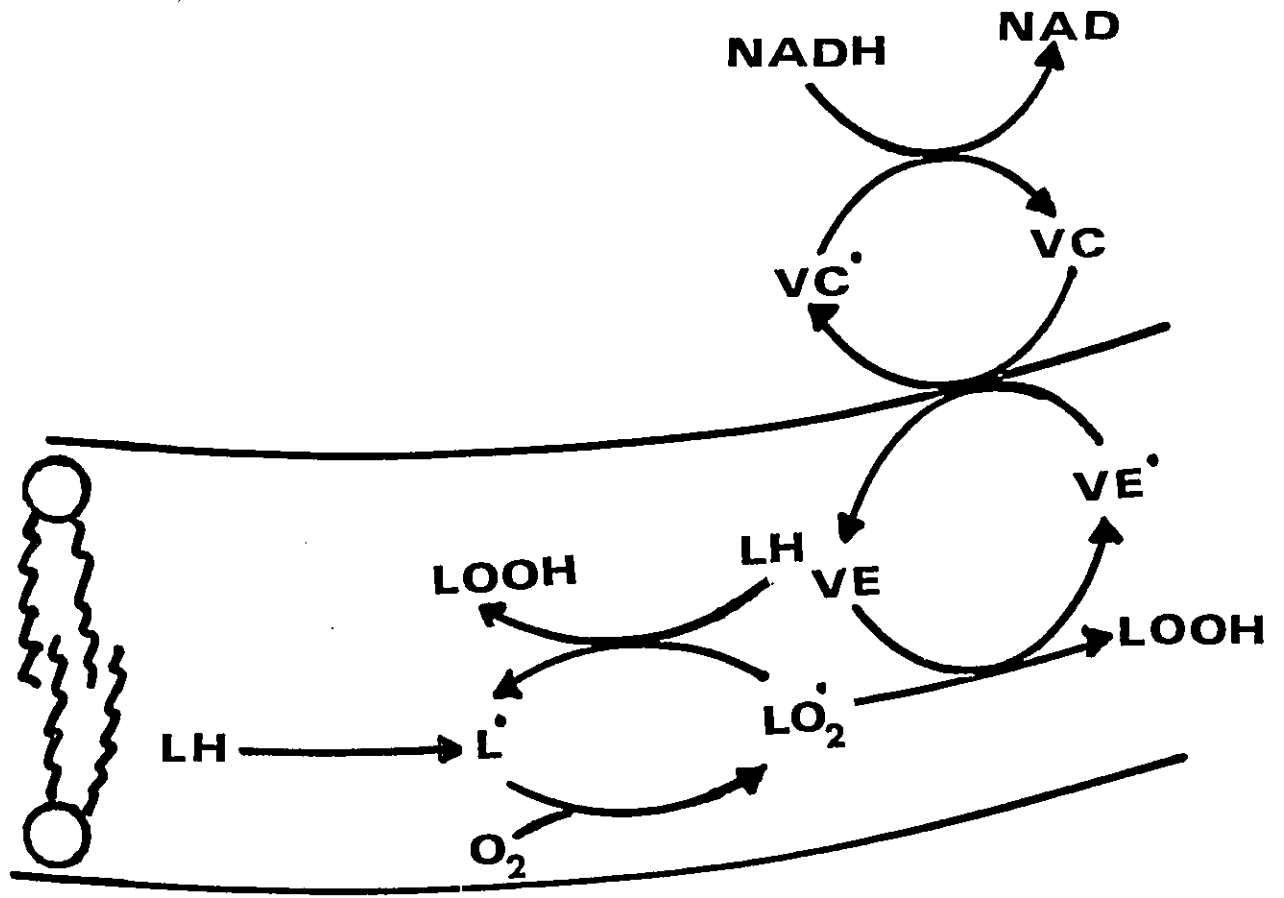


Figure 2

TABLE 1: CLINICAL CONSEQUENCES OF VITAMIN E DEFICIENCY IN HUMANS

- I Low Birth - Weight Infants (< 1500g)**
 - A) Hemolytic anemia
 - B) Thrombocytosis
 - C) Hyperaggregability of platelets
 - D) Greater risk of intraventricular haemorrhage
 - E) Increased severity of retinopathy of prematurity (ROP)
- II Children with Severe Malabsorption**
 - A) Reduced red blood cell half life
 - B) Axonal dystrophy
 - C) Neuromuscular deficits
- III Adults (Malabsorption, Malnutrition)**
 - A) Reduced red blood cell half life
 - B) Lipofuscinosis (ceroid in smooth muscle)
 - C) Neuromuscular deficit

Characteristics of Protein Kinase C (PKC)

PKC consists of a family of closely related enzymes widely distributed in animal tissues. The PKC molecule contains 2 domains: a regulatory domain which interacts with calcium (Ca^{2+}), phosphatidyl serine (PS) and diacylglycerol (DAG), and a catalytic domain with ATP and protein substrate-binding sites.

Activation of PKC requires the "unmasking" of the regulatory domain from the catalytic site. This conformational change is caused by the interaction of PKC with co-factors such as Ca^{2+} or PS. Addition of PS following preincubation of PKC with Ca^{2+} results in the formation of an active PKC - PS complex. A rise in intracellular Ca^{2+} triggers the interaction of PKC with the membrane where the kinase could be activated directly by DAG or converted to a membrane - inserted form that is still active after the Ca^{2+} and DAG signals dissipate. The membrane-bound form of PKC would be more sensitive to input signals than the cytosolic form, which becomes functional only after being translocated to the membrane. Interestingly, unsaturated fatty acids such as arachidonate and its metabolites have been recently found to activate PKC (18,21). Although PKC was originally characterized as a Ca^{2+} /DAG/phospholipid -dependent serine/threonine protein kinase, PKC is now recognized as a group of isoenzymes encoded by a family of genes, and some members of this PKC family have been found to be dependent on DAG/phospholipid but independent of Ca^{2+} (22). The molecular diversity of this enzyme family has been shown by the molecular cloning of several cDNAs of PKC (22,23,24) and by isolation of multiple PKC isozymes (25,26,27). So far,

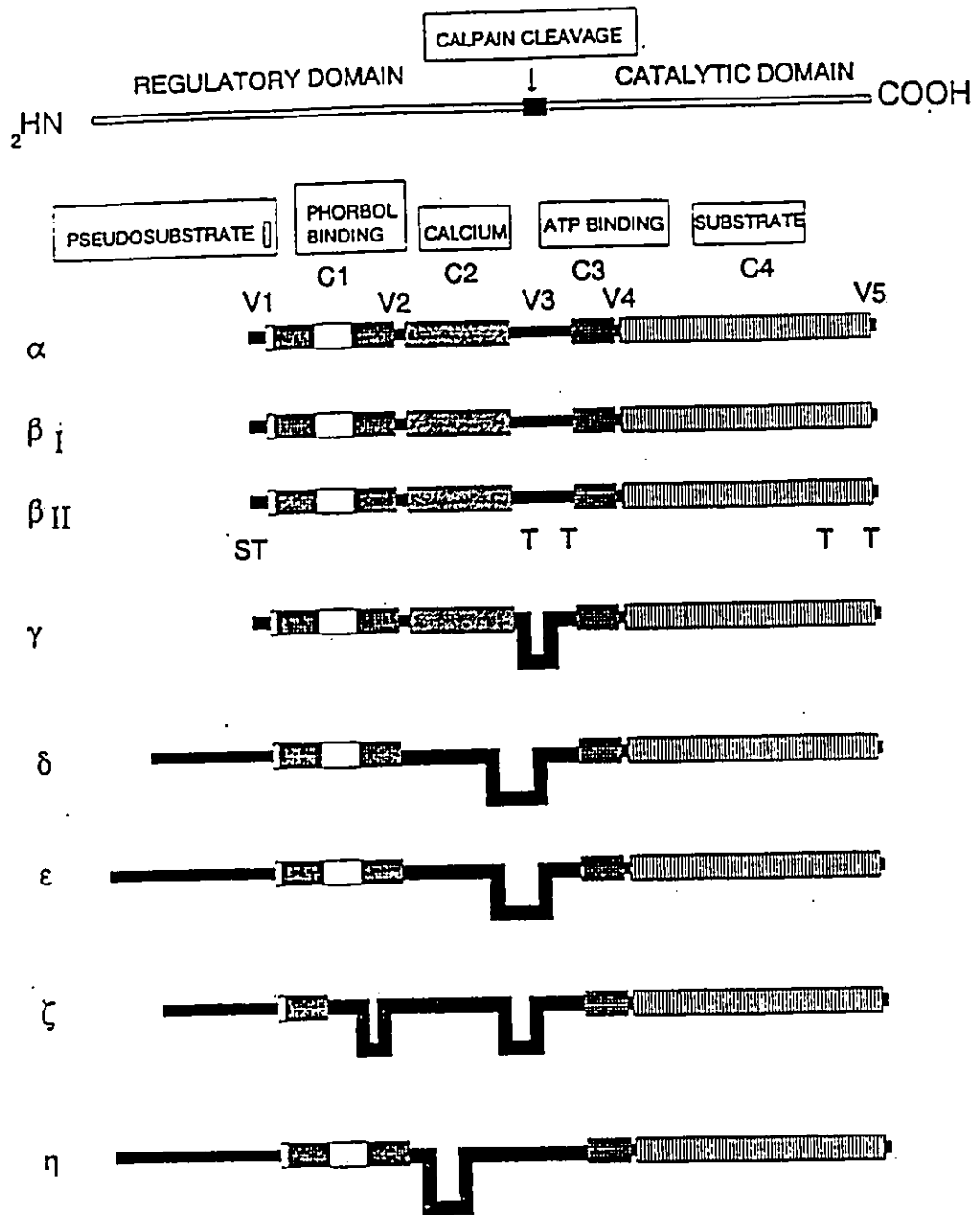
the cDNA coding for nine different PKC enzymes have been cloned from different tissues or cell lines. They can be divided into two main groups: the Ca^{2+} -dependent or conventional PKCs (cPKCs) and the Ca^{2+} -independent or novel PKCs (nPKCs). The PKC isoforms α , βI , βII and γ belong to the Ca^{2+} -dependent group, and the isoforms δ , ϵ , ζ , η and θ to the Ca^{2+} -independent group. The primary amino acid structure, deduced from the cDNA sequences available, can be divided into conserved and thus presumably functional domains (C_1 - C_4) which are separated by variable regions (V_1 - V_5), the functions of which are not yet evident. All PKC isoforms contain these constant and variable regions in a single subunit protein, although the nPKC isozymes differ in part from the structure of the cPKCs (Fig. 3). The C-terminal regions C_3 - V_5 have been defined in all PKC isozymes as the catalytic domain, which is separated by the V_3 region from the N-terminal regulatory domain. The nPKC genes differ from those of group A in the regulatory domain in which they do not have the conserved C_2 . Since the PKC isozymes encoded by cPKC genes are dependent on Ca^{2+} for activity whereas isozymes encoded by nPKCs are not (23,24), it is likely that the C_2 region is important for the Ca^{2+} dependence of PKC.

The tissue distribution of the PKC isozymes has been determined mostly by Northern blot analysis and, more recently, by Western blotting using isozyme-specific antibodies. PKC α , βI , βII , δ , ϵ and ζ seem to be ubiquitously distributed in brain, lung, spleen, thymus and skin, while PKC γ is exclusively found in the central nervous system. PKC η is strongly expressed in skin and lung and only slightly in brain and spleen. PKC θ is predominantly expressed in skeletal muscle and, to a clearly lower extent, in lung, spleen, skin and brain (reviewed in ref. 28).

The biochemical properties of these different PKC isozymes have been investigated, showing their differences with respect to the activation, autophosphorylation, proteolytic activation/degradation and substrate specificity (28).

**Figure 3 -Domain Structure of PKC Isoenzymes (excerpt
from a review of Hubert Hug et al.(28))**

All PKC isozymes consist of constant (C) and variable (V) regions. The cysteine rich repeats in the C1 region and the ATP binding site in the C3 region are indicated by arrowheads. The arrow points to the hinge region in the V3 domain which separates the regulatory from the catalytic domain.



T and S = serine and threonine phosphorylation sites

Figure 3

Functions of PKC

Since its discovery in 1977, PKC has become a focus of attention for researchers interested in the mechanisms by which external signals are transmitted to the interior of cells. A variety of hormones, neurotransmitters and growth factors express their biological activity by stimulating phospholipase C-mediated hydrolysis of phosphoinositides, generating two second messengers: inositol 1,4,5 - triphosphate, which mobilizes Ca^{2+} from intracellular stores, and DAG which, in the presence of phospholipid and Ca^{2+} , activates PKC. PKC in turn phosphorylates a range of cellular proteins, leading to a variety of cellular responses such as secretion, gene expression, proliferation, and muscle contraction (reviewed in ref. 29). Several lines of evidence suggest that PKC modulates membrane functions by regulating ion conductance via phosphorylation of membrane proteins such as channels, pumps, and ion exchange proteins. For example, it has been proposed that Ca^{2+} -transport ATPase is a possible target of PKC; similarly, a possible role of PKC in activating Na^{+} -transport ATPase in peripheral nerve have been suggested (30). In accordance with these suggestions, activation of PKC in the nervous system has been related to enhancement of neurotransmitter release (31,32), regulation of ion channels (33,34), control of growth and differentiation (35,36), and modification of neuronal plasticity (37). Interestingly, considerable evidence suggests that PKC activation participates in the regulation of vascular smooth muscle tone. Phorbol esters - which substitute for DAG as activators of PKC - produce slow, sustained contractions of vascular

smooth muscle (38,39). The vasorelaxant and hypotensive actions of flunarizine, an inhibitor of PKC, further illustrates the effect of PKC on blood pressure in spontaneously hypertensive rats (40).

Rationale of the Research

- I
- 1) Because PKC is known to play a crucial role in cellular signal transduction, proliferation and differentiation; and
 - 2) because PKC has also been suggested to play an important role in the multistep sequence that leads to smooth muscle contractility (41,42), and thus to arterial tone and blood pressure,

We sought to examine the properties of PKC in A10 cells, an established smooth muscle cell line. The proliferation of smooth muscle cells is known to contribute to the formation of plaques, hence the generation of atherosclerosis and hypertension (43-44).

- II
- 1) Because α -tocopherol is an integral component of cellular membranes; and because of the hydrophobic nature of the regulatory domain of PKC; and
 - 2) because α -tocopherol plays a unique role as the major lipid-soluble antioxidant involved in the protection of membranes against free radical-induced damage; and
 - 3) because of the increased involvement of PKC in the modulation of membrane functions,

We sought to examine the effect(s) of vitamin E on PKC activity.

III Because several lines of evidence have suggested a possible involvement of oxyradicals in the activation of PKC, such as:

- 1) in neutrophils, a highly significant correlation between the level of superoxide (O_2^-) production during the respiratory burst and PKC activation has been demonstrated (45); and
- 2) unsaturated fatty acids such as arachidonate and its metabolites have been found to activate PKC activity (18-21),

We sought to determine the effect of duroquinone, an oxyradical-generating substance, on PKC activity and to examine if the addition of antioxidants (vitamin E and C) could counteract the effect produced by duroquinone.

MATERIALS AND METHODS

Cell Culture and Treatment

A10 cells were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Gibco IBRL, Burlington, ON, Canada) supplemented with 10 mM HEPES, 100 mg/l gentamycin sulfate (Sigma Chemical Co. Ltd., St. Louis, MO, USA) and 10% newborn calf serum (Gibco IBRL) which contained negligible amounts of vitamin E. For experiments, cells were detached by brief exposure to 0.25% trypsin in phosphate buffered saline (PBS) and were replated in 60 mm plastic culture dishes (Nunc, USA; Sarstedt, Germany) and maintained at 37°C in a humidified atmosphere of 95% air / 5% CO₂. The culture media was changed every 3 days. Cells were treated with varying concentrations of vitamin E (donated by the Vitamin E Research Information Services, La Grange, IL, USA), duroquinone (250 µM) or vitamin C (100 µM) for different times using dimethyl sulfoxide (DMSO) or water as the vehicle; control cells received the vehicle alone. (All the reagents were purchased from Sigma Chemical Co. Ltd.)

Preparation of Vitamin E-Enriched Medium

An appropriate amount of vitamin E (RRR- α -tocopherol) was dissolved in DMSO. The amount of DMSO used was calculated so that its final concentration in the medium never exceeded 0.2%. Newborn calf serum, which represented 10% of the final medium volume, was prewarmed to 37°C and added to the vitamin E/DMSO mixture. This solution was vortexed and incubated at 37°C for another 15 minutes. Vitamin E incorporated by this method was determined by HPLC and was greater than 95%.

Vitamin E Enrichment of Cells and Determination of Vitamin E by HPLC

The vitamin E-enriched medium was added to confluent cells and after various incubation times, the medium was removed and the cells were rinsed twice with PBS containing 0.8 mM EDTA and 0.25% bovine serum albumin. Cells were detached by trypsinization and sedimented by centrifugation (500 x g) for 10 minutes. The cell pellet was washed, resedimented, and resuspended in Ringer's Tyrode buffer. An aliquot was taken for the determination of cell number using a hemocytometer. The total lipid in the remaining cell suspension was extracted by the method of Bligh and Dyer (46), and vitamin E was determined using an HPLC equipped with a UV detector (280 nm) and a reverse phase Bondapak column. The mobile phase contained: methanol/water/trifluoroacetic acid (99:1:0.1 v/v/v). Tocopherol acetate was added prior to lipid extraction as an internal standard.

Preparation of Membrane and Cytosolic Fractions

Cells were hypotonically lysed in PKC lysis buffer and a post-nuclear fraction was obtained by sedimenting the nuclear fraction at 500 x g for 5 minutes at 4°C. The post-nuclear fraction, which was the source of PKC activity, was separated into membrane and cytosolic fractions either by ultracentrifugation (150,000 x g for 60 minutes) or in a microfuge (14,000 x g for 15 minutes).

Assay of Protein Kinase C Activity

PKC activity was measured by phosphorylation of an 85 kDa protein isolated from murine S49 cyc-lymphoma cells as described by Chakravarthy (47). As reported by this group, this substrate is specific for PKC, and this assay has its advantage in that it permits the measurement of PKC activity in vitro while it is still in the native membrane-associated state and does not require detergent extraction, partial purification and/or reconstitution with phospholipid as do more conventional PKC activity assays.

For PKC activity determination, membrane and cytosolic fractions of A10 cells were added with an assay mixture consisting of 1 mM NaHCO₃, 50 mM Tris, HCl (pH 7.5), 5 mM MgCl₂, 100 μM CaCl₂, 100 μM NaVO₄ and 100 μM Na₄P₂O₇. The reaction was initiated by adding the 85 kDa protein substrate and γ[³²P]ATP (20 μM, 4000 cpm/pmol) (NEN Research Products, Dupont, Canada). Following incubation for 10 minutes at 37°C, the reaction was stopped by adding EGTA to

a final concentration of 10 mM. Membranes were removed by centrifugation (14,000 x g for 15 minutes) of the reaction mixture containing membrane fractions, and the proteins (primarily the 85 kDa protein) in the supernatant and in the cytosolic fractions were solubilized in SDS and separated on 10% polyacrylamide gels. The phosphorylated protein was visualized by autoradiography on Kodak X-OMAT AR film (Eastman Kodak Co., Rochester, NY, USA) for 48 hours. The 85 kDa bands were scanned with the densitometer (LKD 2220-020 ultrascan XL Enhanced Inser Densitometer, LKB Bromma), and PKC activity was expressed as area/ μ g protein.

Protein Determination

Protein from cell homogenate, membrane and cytosolic fractions were colorimetrically determined by the method of Lowry et al (48) using bovine serum albumin as standard.

Immunoblotting Assay

Cells were treated with or without α -tocopherol (50 μ M) for 1 hour and 24 hours. After preparation, samples of membrane fractions were resolved by SDS-polyacrylamide gel electrophoresis, then the proteins were electrophoretically transferred to nitrocellulose paper (Immobilon, Millipore) for 1 hour at 0.5 amps. To block nonspecific binding sites, the nitrocellulose was incubated with 0.5% skim milk in PBS for 2 hours. After being washed in 0.1% Tween-20 in PBS (6

times), the samples were analyzed for PKC by blotting with monoclonal anti-PKC antibody (Amersham Corp., Oakville, ON, Canada) at a 1:50 dilution in PBS for 2 hours at room temperature. This antibody has been shown to recognize α and β forms of PKC. Nitrocellulose papers were then washed in 0.1% Tween-20 in PBS (6 times) and incubated with biotinylated secondary antibody (goat antimouse Ig G) (Jackson Immunoresearch Laboratories, West Grove, PA, USA) for 1 hour at room temperature. Nitrocellulose papers were then washed with 0.1% Tween-20 in PBS (6 times), incubated in Streptavidin peroxidase (1/600 dilution in PBS - skim milk) (Jackson Immunoresearch Laboratories) for 20 min at room temperature, washed again with PBS Tween-20 (6 times), PBS (3 times). The colour was developed by incubating nitrocellulose papers in 4-chloro-1-naphthol in PBS (Aldrich Chemical Co Inc., Milwaukee, WI, USA) and then allowed to dry.

Thiobarbituric Acid Test (TBA)

Cells were incubated in the presence or absence of vitamin E (50 μ M) or duroquinone (250 μ M) for 24 hours. This was followed by the addition of 1.2 ml of 0.5% TBA solution to a final volume of 2.2 ml. Samples were capped and boiled for 10 minutes. MDA was detected as the pink chromophore by spectrophotometry at 532 nm. 1,1,3,3 tetraethoxypropane was used as standard.

RESULTS

The Incorporation Profile of Vitamin E by A10 Cells

In order to study the effects of vitamin E on PKC activity in A10 cells, it was necessary to first determine the profile of vitamin E incorporation by these cells. Two experiments were conducted in which cells were either incubated for 4 hours with different concentrations of vitamin E-enriched medium (range: 10-100 μM) or were incubated with 50 μM of vitamin E-enriched medium for different time periods (range: 1-24 hours). Fig. 4 shows that when A10 cells were incubated for 4 hours with increasing concentrations of vitamin E-enriched medium, there was a dose-dependent increase of cellular uptake of the vitamin. The level of vitamin E from controls cells that did not receive vitamin E-enriched medium was virtually undetectable. In our subsequent experiments, the dose of 50 μM of vitamin E will be used because, as shown in Fig. 4, half maximal uptake of vitamin E occurred at 50 μM and because the concentration of 50 μM is close to the plasma level (46 μM). When cells were treated with 50 μM of vitamin E-enriched medium for various time periods, there was a time-dependent linear increase of cellular incorporation of vitamin E up to 24 hours of incubation time (Fig. 5). These experiments showed that the incorporation of vitamin E by A10 cells is time and dose-dependent.

Figure 4 - Dose-Dependent Uptake of Vitamin E by
A10 Smooth Muscle Cells

Cells were treated with different concentrations of α -tocopherol for 4 hrs. The amounts of tocopherol were determined as described in "Methods". Values are means \pm SD from 3 dishes.

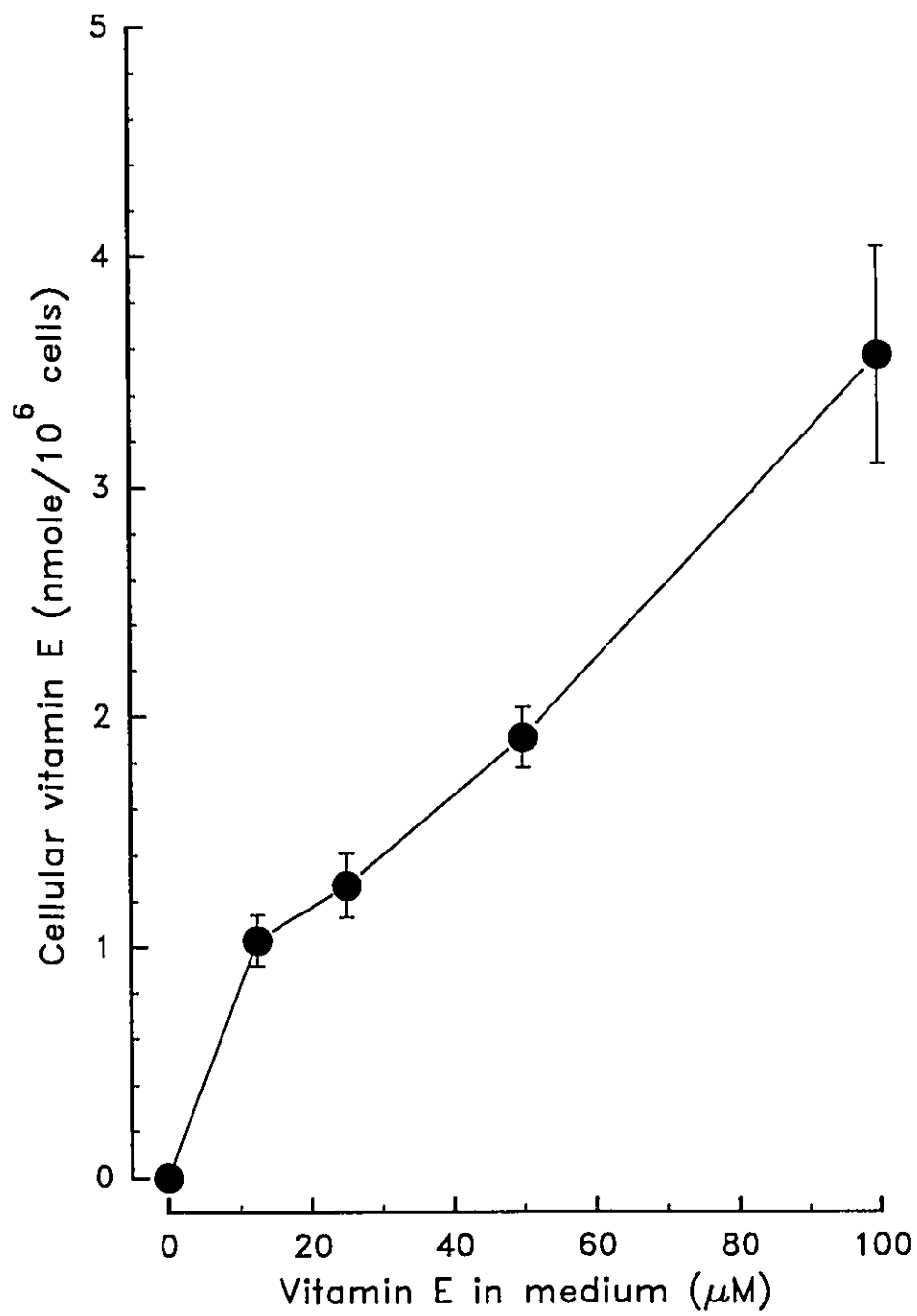


Figure 4

Figure 5 - Time-Dependent Uptake of Vitamin E
in A10 Smooth Muscle Cells

Cells were incubated with α -tocopherol (50 μ M) for indicated times, then amounts of α -tocopherol were determined as described in "Methods". Values are means \pm SD from 3 dishes.

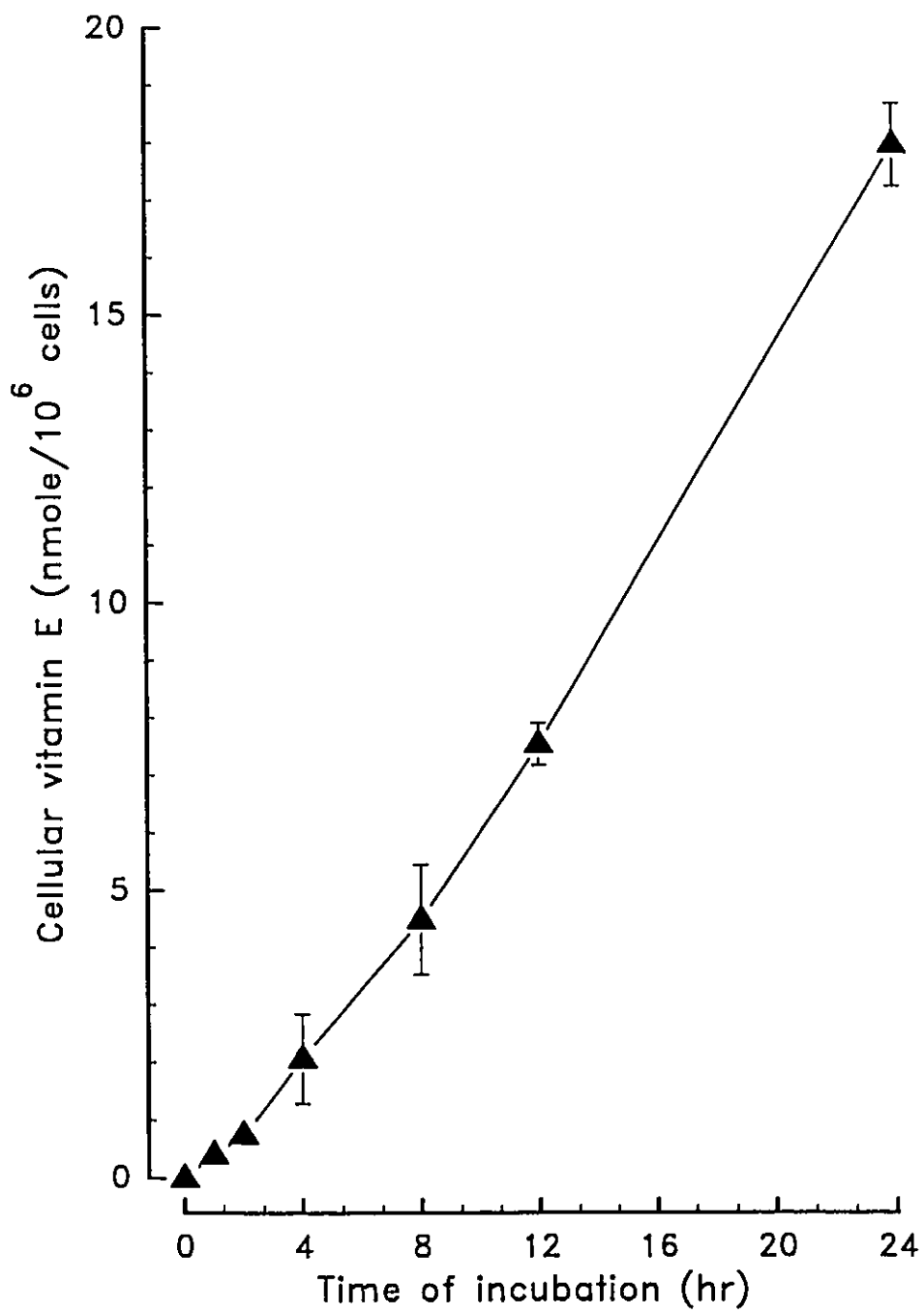


Figure 5

Effect of Vitamin E Enrichment on A10 Smooth Muscle Cells Number

The A10 smooth muscle cells used in our experiments have fibroblast-like morphology and they do not exhibit contact inhibition in their pattern of growth. Under our experimental conditions, the A10 cells were grown in the presence of 10% calf serum and therefore they would be randomly proliferating.

Vitamin E has recently been shown by Boscoboinik et al (49,50) to inhibit the proliferation of A7r5 cells, another smooth muscle cell line having similar properties to A10 cells. In order to determine if vitamin E would exert the same effect on our A10 cells, we conducted an experiment to study the effect of vitamin E-enrichment on cell number. Cells were incubated with normal medium or medium enriched with vitamin E (50 μ M) for various time periods (2hr, 4hr, 8hr, 12hr, 24hr). At each time point, cells were trypsinized and the cell number was determined by a haemocytometer as described under "Methods" section. Figure 6 shows that during this 24 hours period, cell number was increased similarly from 3.5×10^6 to 5.5×10^6 in cells treated with or without vitamin E. Vitamin E enrichment thus did not cause any change in A10 cell number during the 24 hour incubation period.

Figure 6 - Effect of α -Tocopherol Enrichment
on A10 Cell Number

Cells were incubated with α -tocopherol (50 μ M) for indicated times. At each time point, cells were trypsinized with 0.15% Trypsin + 0.3% EDTA and cells number was determined by hemacytometer. Means \pm SD from 3 dishes.

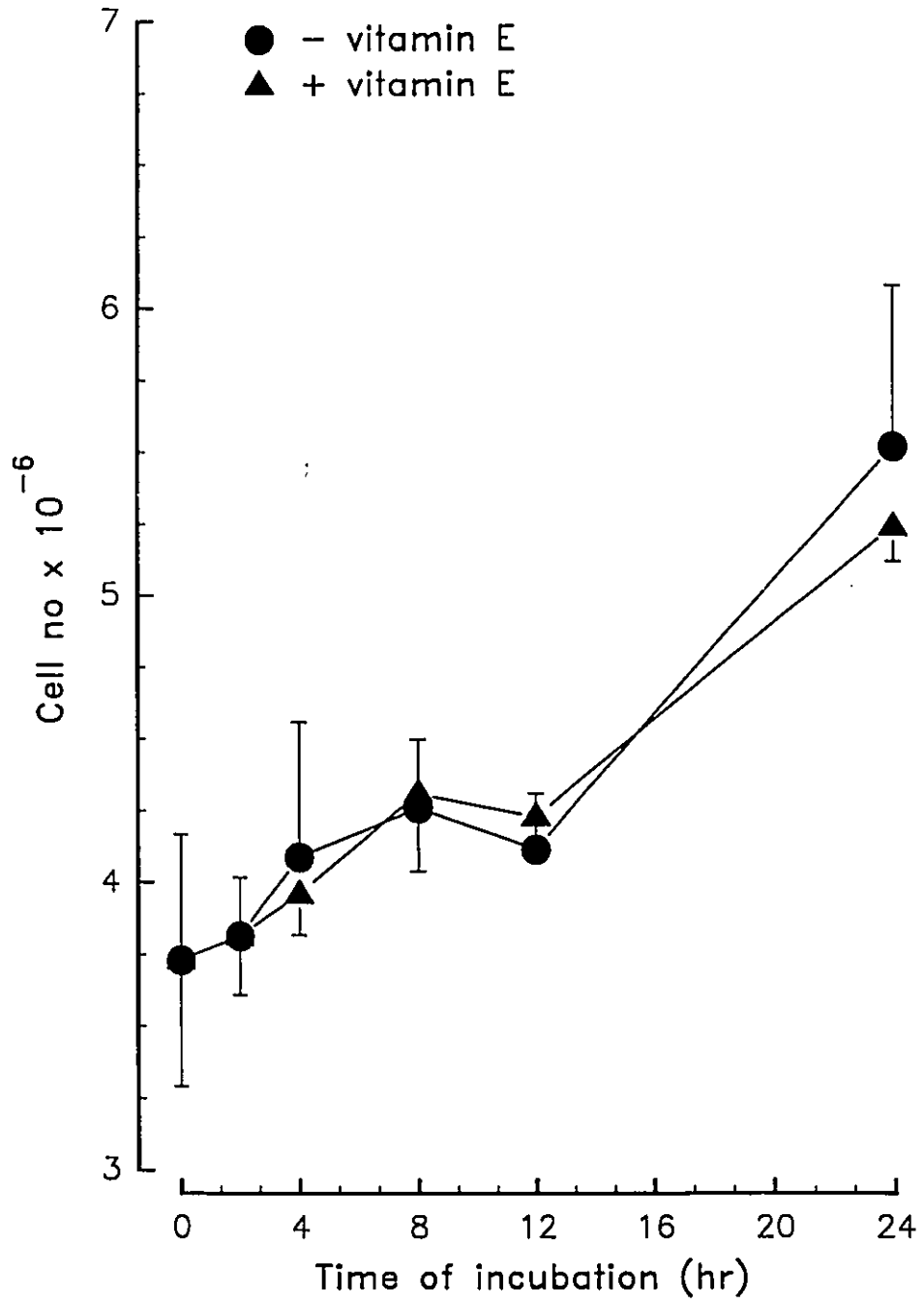


Figure 6

Effects of Vitamin E on PKC Activity (time-course)

In order to study the effects of vitamin E on PKC activity in A10 smooth muscle cells, we conducted a time-course experiment in which cells were enriched with vitamin E (50 μ M) for 1, 4 and 24 hours as described in "Methods". The phosphorylated proteins (primarily the 85 kDa proteins) in membrane and cytosol were solubilized in SDS, separated on 10% polyacrylamide gels and visualized by autoradiography (Fig. 7). The results showed that in the membrane, the 85 kDa protein was much less phosphorylated in cells incubated with vitamin E as compared to control cells that were not enriched with vitamin E. Maximum inhibition appeared to occur at the time of 1 hour of incubation, although this inhibitory effect of vitamin E on the phosphorylation of the 85 kDa substrate was also seen at 4 and 24 hours of incubation. In the cytosolic fractions, the inhibitory effect of vitamin E, although present, is not as pronounced as in the cytosol. To have a better overall view of the effects of vitamin E on PKC activity in both membrane and cytosolic portions, the 85 kDa phosphorylated bands on the autoradiogram were scanned for density (area) using a densitometer (LKD 2220-020 Ultrascan XL Enhanced Inset Densitometer, LKB Bromma), and PKC activities were expressed as area/ μ g of protein. As shown in Fig. 8, the activity of PKC was higher in the membrane than in the cytosolic fractions. In the membrane, this activity was inhibited by 50% within the first hour of incubation with vitamin E (50 μ M) and PKC activity remained significantly less than in vehicle treated cells for up to 24 hours. In contrast, in the cytosolic fraction, vitamin E

enrichment did not cause any significant changes in PKC activity when A10 cells were treated for 1, 4 and 24 hours. Because the results from this experiment showed the maximum effect of vitamin E on PKC activity at 1 hour of incubation, in subsequent experiments, the time of 1 hour of incubation with vitamin E was chosen for PKC activity determination.

**Figure 7 - Autoradiogram Showing Time-Dependent
Phosphorylation of the 85 kDa Substrate**

Cells were treated with or without Vitamin E (50 μ M) for the times indicated. The PKC activity in the membrane fraction was quantitated by the phosphorylation of an exogenous 85 kDa substrate specific for PKC as indicated in "Methods". The results are from 1 representative experiment, out of 2.

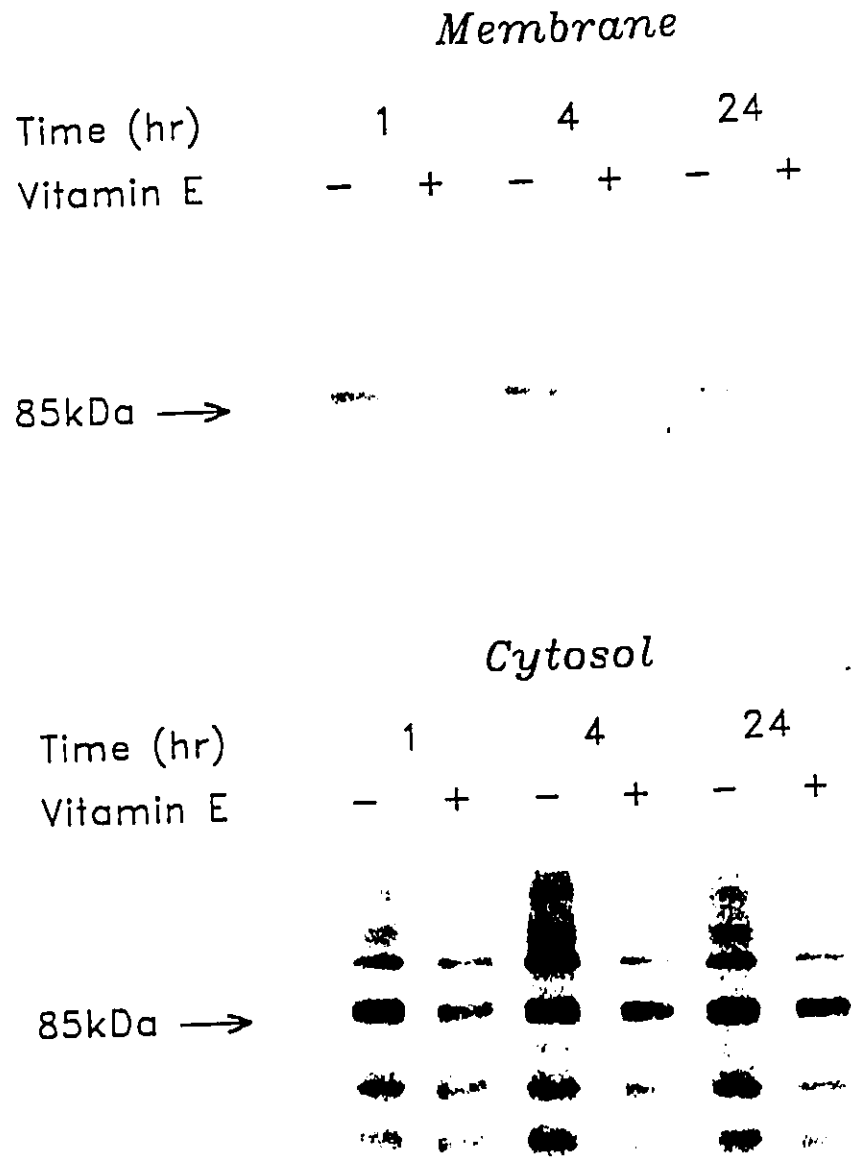


Figure 7

Figure 8 - Effect of Vitamin E on PKC

Activity (time-course)

Cells were treated with α -Tocopherol (50 μ M) for indicated times and PKC activity in membrane and cytosolic fractions was determined as described in "Methods". Values are averages from 2 experiments.

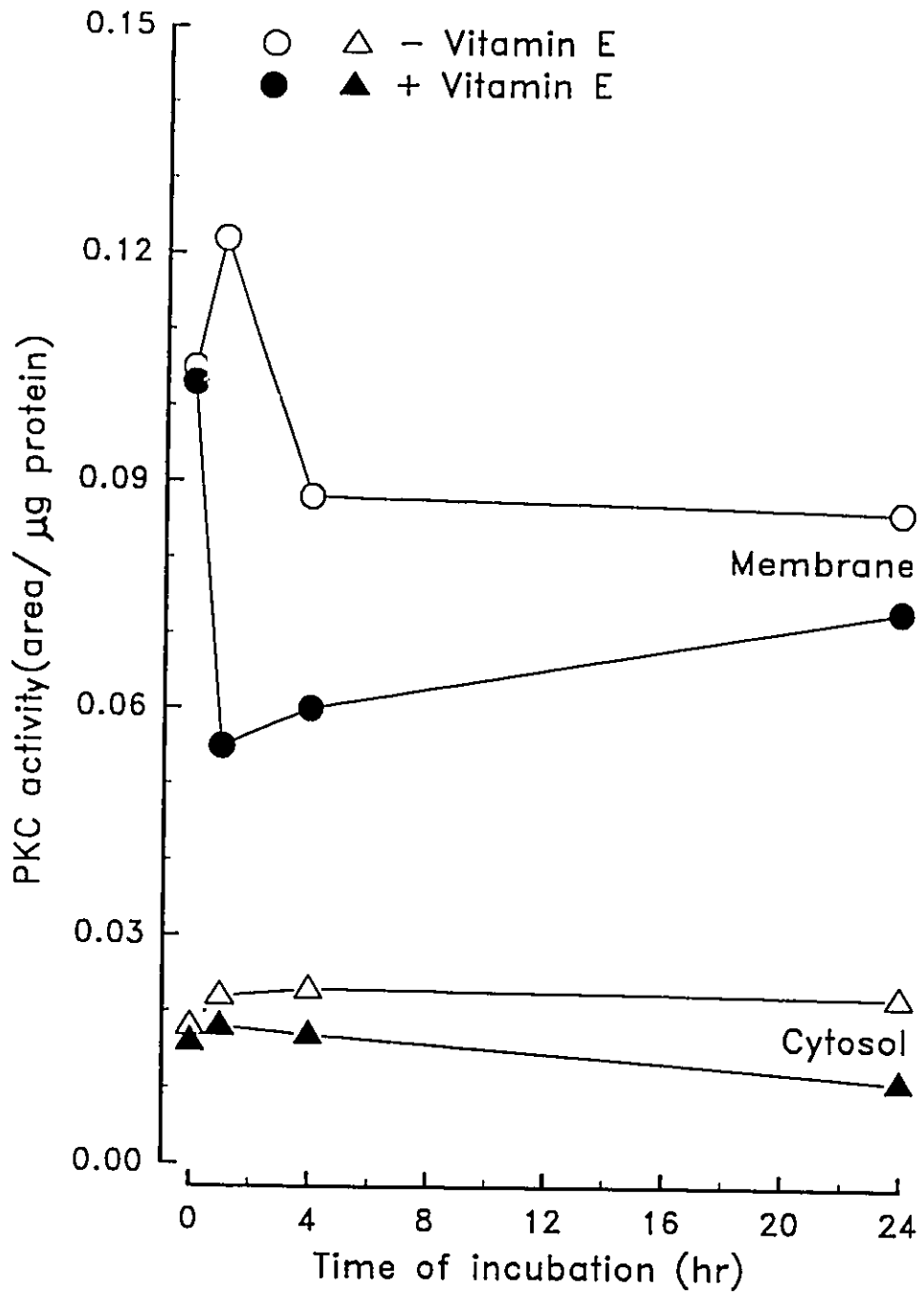


Figure 8

Effects of Vitamin E on PKC Activity (dose-dependent)

Based on the previous experiment which shows that the greatest inhibition exerted by vitamin E on PKC activity occurred in the membrane only after 1 hour of incubation, we sought to determine the effective doses of vitamin E by incubating A10 cells for 1 hour with different vitamin E concentrations ranging from 0 μM to 50 μM . The PKC activity in the membrane fraction was quantitated by the phosphorylation of an exogenous 85 kDa substrate specific for PKC. The autoradiogram (Fig. 9) showed clearly a decreased phosphorylation of the 85 kDa substrate in cells treated with increasing concentrations of vitamin E as compared with cells treated with vehicle only. When the phosphorylated 85 kDa bands were scanned densitometrically and PKC activities were expressed as area/ μg of protein as shown in Fig. 10, we saw that membrane-bound PKC activity was strongly inhibited by vitamin E in a dose-dependent manner. Maximum inhibition occurred at a range of 25 μM to 50 μM of vitamin E. Consistently with the previous experiment, 50 μM of vitamin E caused about 50% of inhibition on membrane-associated PKC activity after 1 hour of incubation.

**Figure 9 - Autoradiogram Showing Dose-Dependent
Phosphorylation of the 85 kDa Substrate**

Cells were treated with different concentrations of vitamin E for 1 hour. The PKC activity in the membrane fraction was quantitated by the phosphorylation of an exogenous 85 kDa substrate specific for PKC as indicated in "Methods". The results are duplicates from 1 experiment, out of 3.

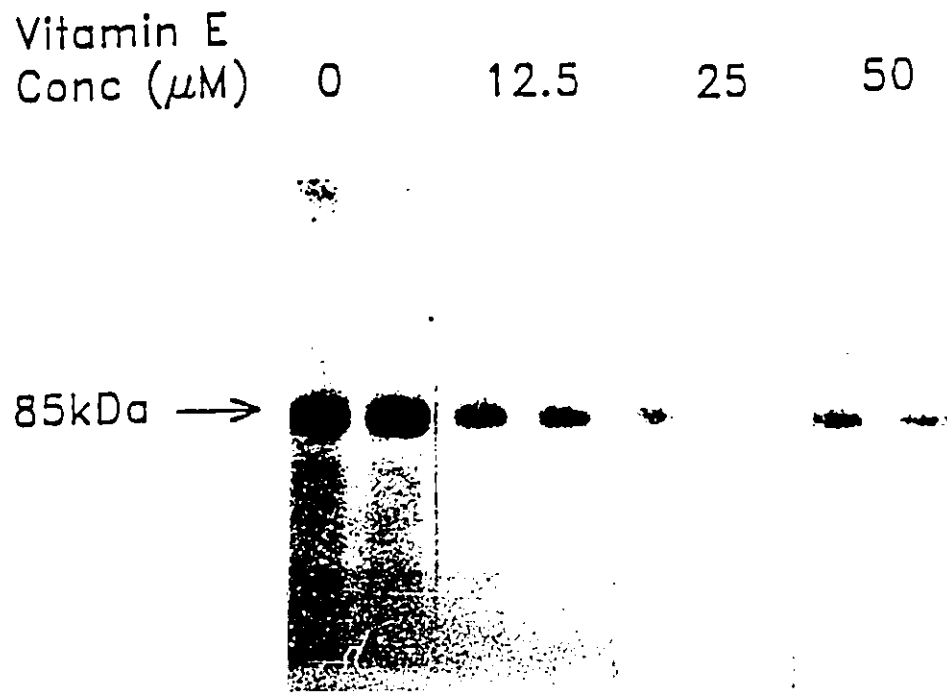


Figure 9

Figure 10 - Dose Response of Vitamin E
on Membrane PKC Activity

Cells were incubated with different concentrations of vitamin E for 1 hour and PKC activity in the membrane fraction was determined as described in "Methods". Values are means \pm SD from 3 experiments.

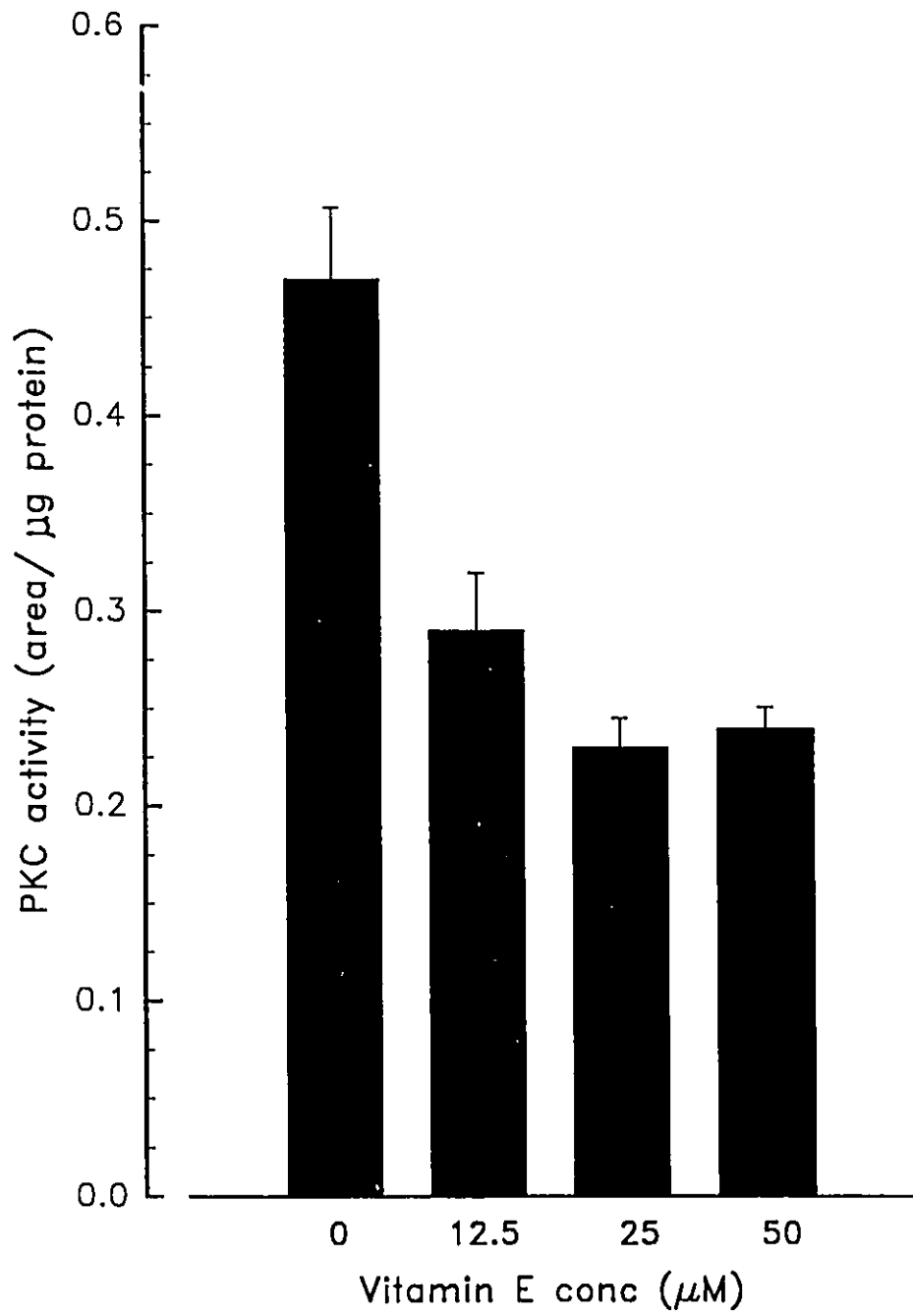


Figure 10

Effects of Vitamin E on PKC Amount in Membrane

In the previous experiments, we have demonstrated that the membrane-bound PKC activity in A10 cells was diminished significantly following incubation of the cells with different doses of α -tocopherol for various times. To further investigate whether this decrease in PKC activity in membrane fractions is due to a direct inhibitory effect of vitamin E on PKC activity in membranes or due to the effect of vitamin E on the redistribution of PKC within subcellular fractions, we performed a western immunoblotting assay in which A10 cells were treated with or without vitamin E (50 μ M) for 1 and 24 hours. At indicated times, membrane fractions were resolved by SDS - polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose, then analyzed for the amount of PKC by immunoblotting as described in "Methods".

Because PKC α and PKC β were identified as the principal Ca^{2+} / phospholipid-dependent PKC isozymes expressed in smooth muscle cells (51), we used a monoclonal anti-PKC antibody (Amersham Corp.) which has been shown to recognize α and β forms of PKC. From the results (Fig. 11), we observed that vitamin E did not cause any change in the amount of PKC isozymes α and β in membrane after 1 or 24 hours of incubation. In addition, we also observed that at 24 hours, the amount of PKC is less than that at 1 hour. We cannot explain this observation so far, except that DMSO as a carrier might have an effect on the level of PKC after long term of incubation. This study showed that vitamin E did not have any effect on the redistribution of PKC within subcellular fractions.

Figure 11 - Effects of Vitamin E on PKC

Amount in Membrane

Cells were incubated with α -tocopherol (50 μ M) for 1 hour and 24 hours and PKC amount in membrane fractions was visualized by immunoblotting assay as described in "Methods". The result is from a single experiment performed twice with similar results.

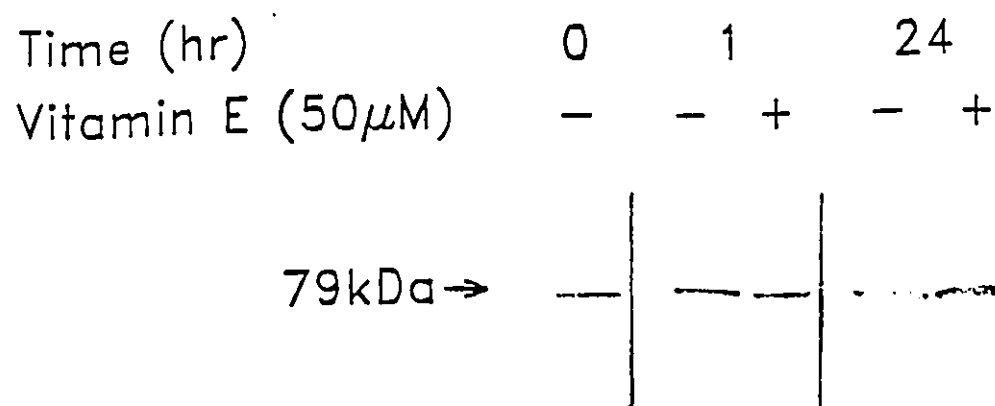


Figure 11

Effect of Vitamin E on Malonedialdehyde (MDA) Formation

It is a well established fact that oxygen radicals can induce lipid peroxidation in cell membranes leading to the formation of MDA as end product. To ascertain whether vitamin E enrichment of A10 smooth muscle cells will result in quenching of oxygen radicals, the membrane levels of MDA were determined in control cells and in cells that had been incubated with 50 μ M of vitamin E for 24 hours as indicated in "Methods". As can be seen from the results presented in Fig. 12, there was a significant formation of MDA in vehicle treated cells. However, treatment with vitamin E (50 μ M) for 24 hours suppressed MDA formation by 50%, reconfirming the role of vitamin E as a radical scavenger in A10 plasma membrane.

Figure 12 - Effect of Vitamin E on
Malonedialdehyde Formation

Cells were incubated with and without vitamin E (50 μ M) for 24 hrs and malonedialdehyde formation was determined as described in "Methods". Values are averages of 2 dishes.

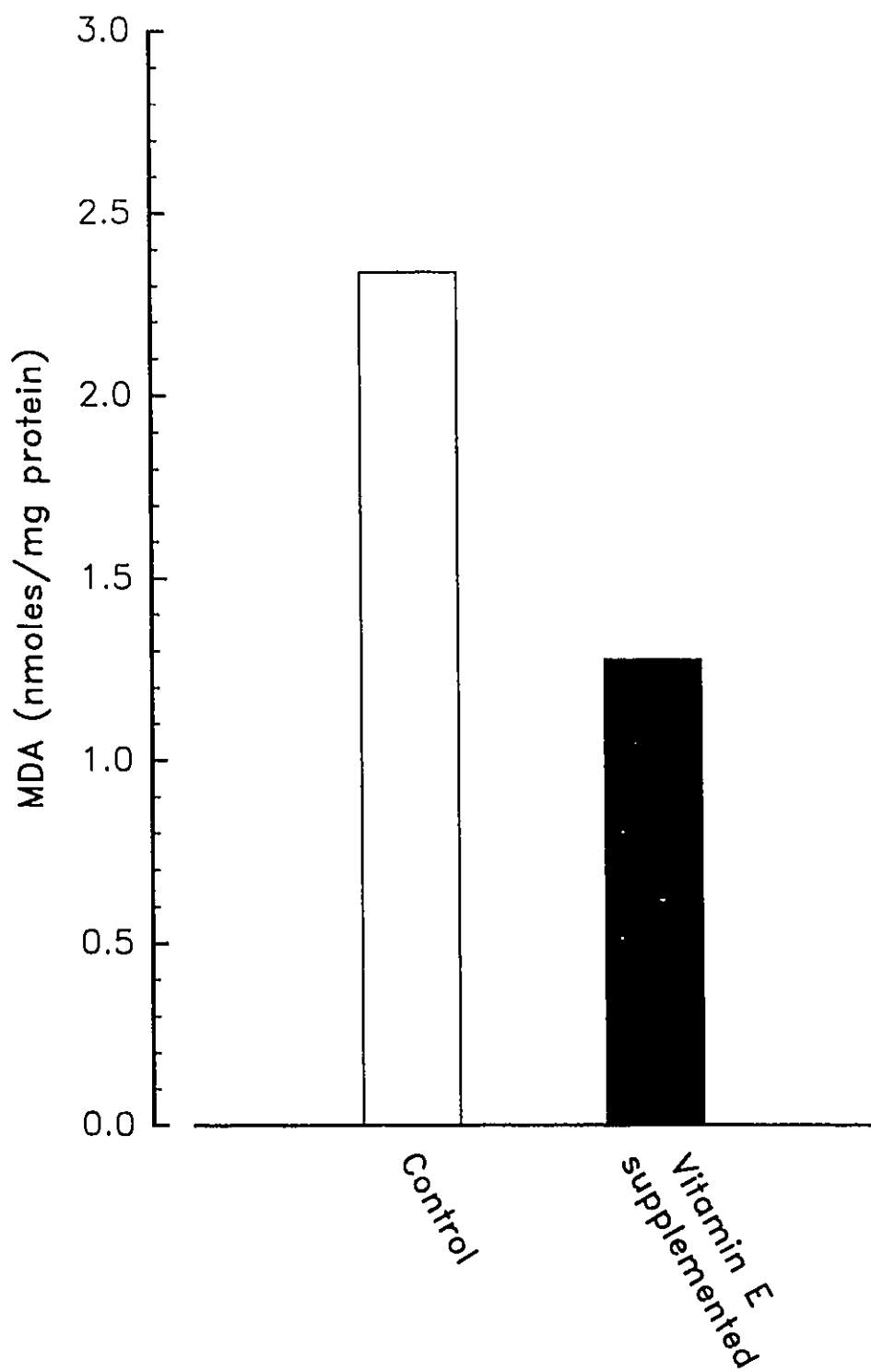


Figure 12

Effects of Duroquinone on MDA Formation and PKC Activity in Membrane

A10 Cells

Since several lines of evidence have suggested a possible involvement of oxygen free radicals in the activation of PKC, we put forward the hypothesis that, in our model, vitamin E inhibits membrane-associated PKC by its ability to act as a radical scavenger and thereby suppress the level of free radical in the cell membrane. If this is true, then generation of oxygen free radicals, as determined by an increase in MDA formation, should stimulate PKC activity. We therefore conducted experiments in which we preincubated the cells with duroquinone which is known to produce active oxygen species during its one electron reduction to semiquinone radicals that enter redox cycles with oxygen. A10 cells were incubated with duroquinone (250 μ M) for various times, ranging from 20 minutes to 24 hours. At each time point, MDA formation and PKC activity in membrane and cytosolic fractions were determined as described in "Methods". The results showed that treatment of cells with duroquinone for various times clearly caused an increase in the levels of MDA in the membrane in parallel with a significant increase in PKC activity (Fig. 13). Membrane-bound PKC activity was increased readily after 20 minutes of incubation, reached a peak at 1 hour and activity remained elevated for up to 24 hours of incubation with duroquinone. In contrast, treatment with duroquinone did not cause any change in MDA formation and PKC activity in the cytosol during different times of incubation (Fig. 14).

**Figure 13 - Effects of Duroquinone (250 μ M) on MDA
Formation and PKC Activity in Membrane**

Confluent cells were incubated with duroquinone (250 μ M) for indicated times. At each time point, MDA formation and PKC activity in membrane fractions were determined as described in "Methods". Values are average of 2 dishes. Upper and lower limits of the mean value are indicated.

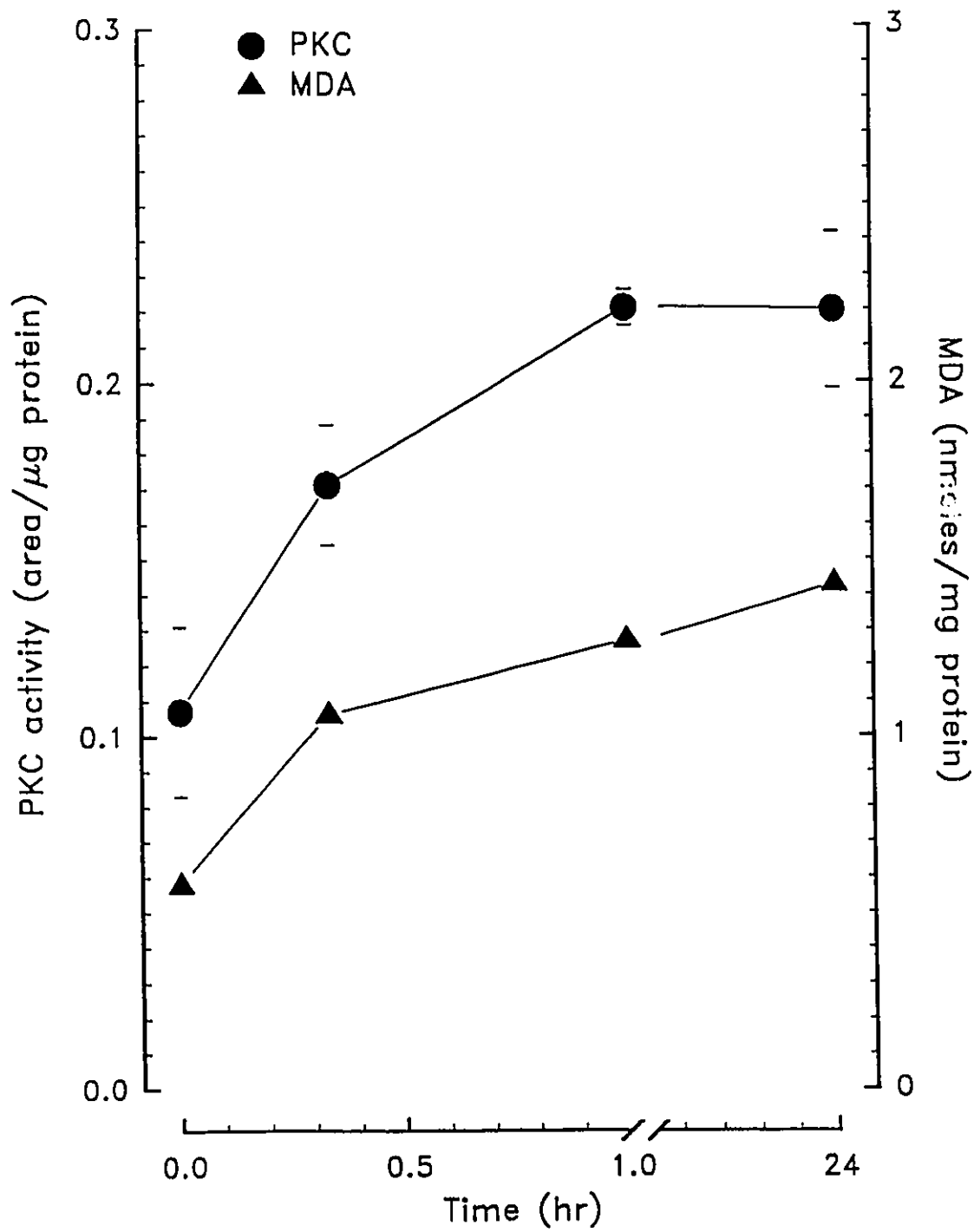


Figure 13

**Figure 14 - Effects of Duroquinone (250 μ M) on MDA
Formation and PKC Activity in Cytosol**

Confluent cells were incubated with duroquinone (250 μ M) for indicated times. At each time point, MDA formation and PKC activity in cytosolic fractions were determined as described in "Methods". Values are average of 2 dishes. Upper and lower limits of the mean value are indicated.

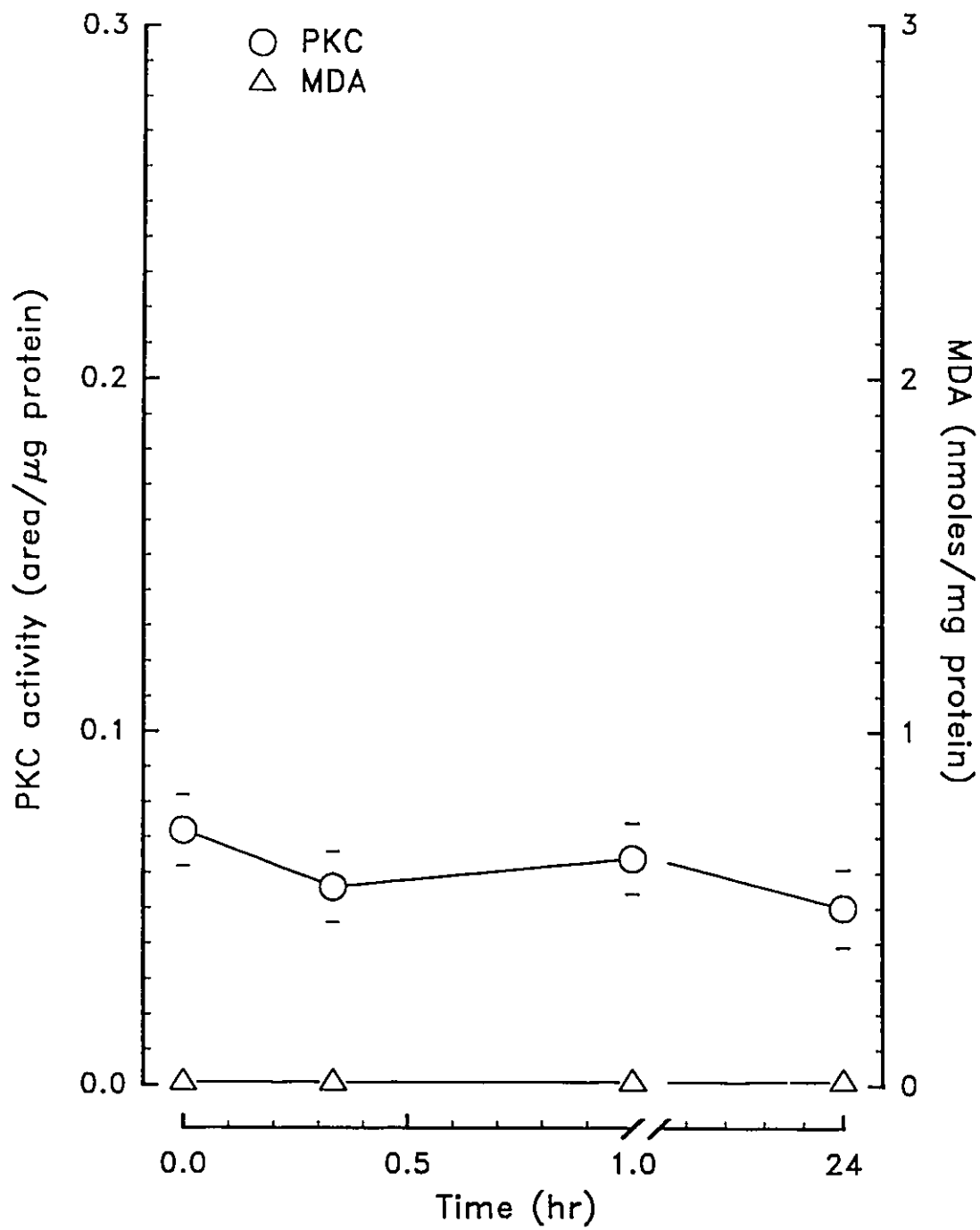


Figure 14

Effects of Duroquinone, Vitamin E and Vitamin C on PKC Activity

To further investigate the role of oxygen radicals in the process through which vitamin E inhibits PKC activity, the cells were treated with vitamin E (50 μM) alone or with duroquinone (250 μM) alone or with both vitamin E and duroquinone for 1 hour, and PKC activity in membrane fractions was determined. As shown in Fig. 15, vitamin E alone significantly diminished membrane-associated PKC activity as compared to the control, whereas duroquinone caused a clear activation of PKC activity. Interestingly, treatment of cells with both vitamin E and duroquinone decreased PKC activity by 50% when compared to the cells treated with duroquinone alone.

To ascertain the involvement of oxygen free radicals in the activation of PKC, we studied the effects of another natural antioxidant, vitamin C, on PKC activity. As shown in Fig. 15, treatment of cells with duroquinone (250 μM), vitamin E (50 μM) together with vitamin C (100 μM) for 1 hour further diminished PKC activity in membrane fractions as compared to cells treated with just duroquinone and vitamin E, whereas vitamin C alone did not show any effect on PKC activity. In other words, vitamin C further potentiates the effect of vitamin E on PKC activity.

Figure 15 - Effects of Duroquinone (250 μ M), Vitamin E (50 μ M) and Vitamin C (100 μ M) on PKC Activity in Membrane

Confluent cells were incubated with vitamin E (50 μ M) alone, or with duroquinone (250 μ M) alone, or with duroquinone (250 μ M) and vitamin E (50 μ M), or with duroquinone (250 μ M) and vitamin E (50 μ M) and vitamin C (100 μ M) or with vitamin C (100 μ M) alone for 1 hour. The PKC activity in the membrane fractions was determined as described in "Methods". Values are average of duplicates from a single experiment performed twice with similar results. Bars indicate upper limit of the mean value.

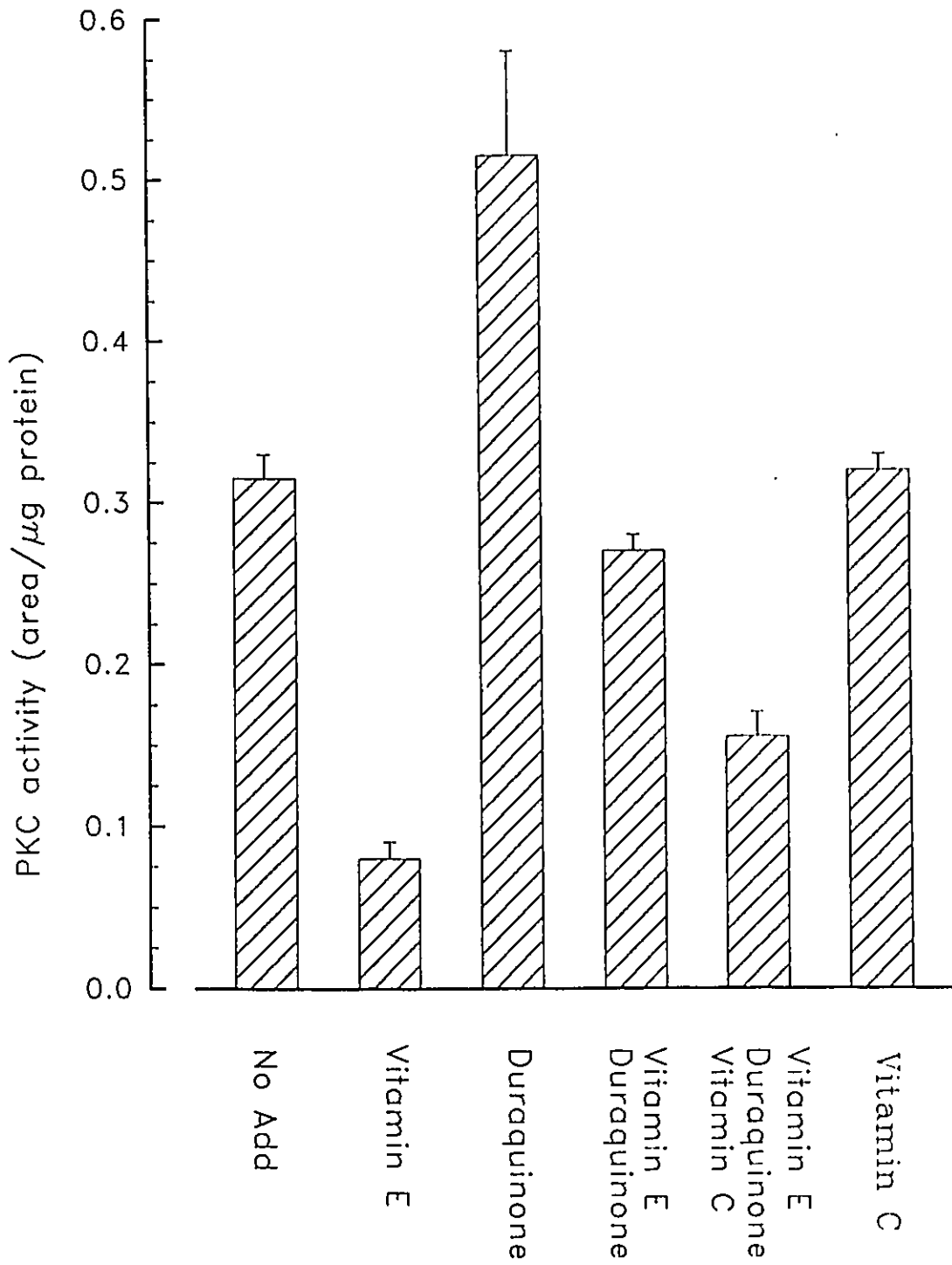


Figure 15

DISCUSSION

In the present study, we have examined the effect of vitamin E on PKC activity in rat aortic smooth muscle cells and we have investigated whether this effect of the vitamin was mediated through its ability to act as antioxidant in cell membranes.

A10 cells incorporated vitamin E in a dose- and time-dependent manner. When the cell culture medium, which contained no detectable vitamin E, was enriched with different concentrations of vitamin E, from 0 to 100 μM (equivalent to 0 to 45 $\mu\text{g}/\text{ml}$) and was treated to smooth muscle cells (A10), for 4 hours, we observed a linearly increased uptake of vitamin E by the cells. This is in agreement with the work of Lehmann et al who showed a linear uptake of vitamin E in human platelets and lymphocytes with plasma vitamin E ranging from 0 to 25 $\mu\text{g}/\text{ml}$ (52). Our group has also observed such a dose-dependent uptake of vitamin E in human endothelial cells (53).

The normal vitamin E level in human blood is 3.0 - 15.0 $\mu\text{g}/\text{ml}$ (or 7 - 35 μM) for children and 5.0 - 20.0 $\mu\text{g}/\text{ml}$ (or 11.6 - 46.4 μM) for adults. In our experiments, α -tocopherol at concentrations of 25 μM and 50 μM strongly inhibited the membrane-associated PKC activity over a 24 hours period, and the greatest inhibition occurred during the first hour of incubation. This observation suggests that the effect of α -tocopherol in the cell membrane was mediated

through a relatively rapid mechanism. Nevertheless, under the same conditions, α -tocopherol did not show any significant effect on cytosol-associated PKC activity. This selective effect of α -tocopherol on the membrane-associated PKC activity is to be expected due to the membrane location of vitamin E as well as to the hydrophobic nature of the PKC regulatory domain.

When the cells were treated with different concentrations of α -tocopherol for 1 hour, the membrane-associated PKC activity was again inhibited in a dose-dependent manner, and consistently, the concentration of 50 μ M of α -tocopherol gave about 50% of inhibition. In this experiment, the effect of vitamin E was already seen at low concentrations (12.5 μ M, 25 μ M), therefore when we compared with our previous experiment showing incorporation of different concentrations of vitamin E into the cells in which there was a linear increased uptake of vitamin E (Fig. 4), we can see that the effect of vitamin E on PKC activity was not correlated with its incorporation into the cells. One explanation is that the mechanism by which vitamin E exerts its effect (such as the quenching of intracellular radicals by vitamin E) might be saturated at low concentrations of the vitamin. Our results are in agreement with those of Mahoney & Azzi who found that in vitro, vitamin E inhibited EDTA/EGTA extractable (which would correspond to membrane-associated) PKC activity (54). However, in contrast with a number of studies carried out by the same group where they showed the inhibitory effect of α -tocopherol on the proliferation of a 7r5 cells (49,50), our experiments did not show any evidence of the effect of α -tocopherol on the A10 cell number. Nevertheless, in the studies carried out by Azzi et al. (54), the data also showed that different cell lines had different responses to α -tocopherol; the

inhibitory effect of α -tocopherol on cell proliferation was only observed with A7r5 cells. The proliferation of Balb/3 T₃ and Saas-2 cells was not inhibited by α -tocopherol. These observations suggested the cell specificity in regards to the sensitivity to α -tocopherol. In addition, the wide heterogeneity of PKC family as shown by different molecular cloning works (55,56) as well as from the purification of different isozymes of PKC (57,58) suggests that different isozymes of PKC may play distinct functions in a variety of cellular responses (59).

Since certain agents were known to translocate PKC to different cellular locations, we investigated whether the inhibitory effect of vitamin E on the membrane-associated PKC activity was mediated via a translocation mechanism. We therefore performed an immunoblotting assay in which aliquots of membrane portions of A10 cells were immunoblotted after the cells were treated with or without of vitamin E. We used a monoclonal anti-PKC antibody (Amersham Corp) which has been shown to recognize α and β forms of PKC.

The data showed that vitamin E treatment did not change the mass of PKC α and PKC β in A10 cell membranes. Based on a study reported by Singer et al. (51) that PKC α and PKC β were identified as the principal Ca²⁺ / phospholipid-dependent PKC isozymes expressed in smooth muscle cells with α isozyme the most abundant, we can say from our experiments that, in our models, the mass of PKC in the membranes was not affected by vitamin E treatment. Any effect of vitamin E on other PKC isozymes, if there is any, is not known at present. However, if it does occur, it will be regarded as a minor contribution to the change of the total mass. Therefore, together with the previous experiment, we can conclude that in our model, vitamin E inhibits PKC

activity in the membrane by mechanism(s) distinct from the redistribution of PKC within cellular fractions.

The fact that vitamin E exerts its effect on membrane-bound but not on cytosolic PKC activity, together with the observation that, in our model, the translocation of PKC is not affected by vitamin E made us investigate a possible involvement of free-radicals on the membrane-bound PKC activity. To test this possibility, we used duroquinone to generate free radicals. Quinones (Q), which are widely distributed in nature, are an important group of substrate for flavoenzymes and can undergo either one-electron reduction to the semiquinone (SQ) radicals or two-electron reduction to the hydroquinone (HQ) with subsequent generation of superoxide anion radicals (O_2^-) (60,61). When A10 cells were incubated with a non-cytotoxic level of duroquinone for various times, membrane-associated PKC activity was increased in a time-dependent manner, and the greatest stimulation was reached after 1 hour of incubation. In contrast cytosolic PKC activity was unchanged with duroquinone treatment. These results show that in A10 cells, membrane-PKC is activated by quinone-generated active oxygen species and, interestingly, the greatest activation occurred at 1 hour of treatment. This is consistent with the fact that the maximum inhibition caused by vitamin E enrichment also occurred at 1 hour of incubation. This activation of PKC activity by duroquinone in our model is in agreement with a number of studies dealing with the effects of different redox-cycling quinones on PKC activity (62). In fact, several lines of evidence have suggested effects of oxidants on PKC activity. McPhail et al. have shown that arachidonic acid and other unsaturated fatty acids activated PKC from human neutrophils (18), as well as in

from bovine cerebellum (19). These studies suggested that the release of arachidonic acid and its metabolites, which is triggered in many cell types by ligand-receptor interactions, could serve a second messenger function for activation of PKC. There is evidence showing that arachidonic acid can modulate neuronal Ca^{2+} channels in the brain via activation of PKC and generation of free radicals (63). This raises the possibility that arachidonate-induced free radicals affect ionic conductances, a phenomenon that can be ascribed to the activation of PKC. In a study on the effects of oxygen radicals on cellular Ca^{2+} homeostasis, Bellamo et al. presented evidence that oxidative stress produced in hepatocytes (64,65) and liver microsome (66) caused the mobilization of Ca^{2+} from intracellular stores and inhibited ATP-dependent microsomal sequestration. Interactions between free radicals and PKC have also been described in several systems (67,68,69). Larsson et al. (69) have shown that activation of PKC in mouse epidermal TB6 cells occurred at low concentrations of superoxide, conditions where no measurable Ca^{2+} fluxes take place, therefore suggesting a Ca^{2+} -independent mechanism for PKC activation process.

In our study, the observation that duroquinone activated membrane-but not cytosolic-PKC activity suggests that active oxygen species generated from this redox-cycling quinone exerted their effects via the lipid peroxidation process in the cellular membrane. If lipid peroxidation was indeed involved in the free radical-induced PKC activation, then its products should increase during cell treatment with quinone. In fact, we found that, following the incubation of A10 cells with duroquinone, the levels of malondialdehyde - the product of lipid peroxidation in cell membrane - were increased in parallel with the increased

membrane-associated PKC activity. Indeed, oxygen free radicals were shown to play a critical role in the pathogenesis of conditions involving cell membranes (70,71) such as ischaemia and starvation.

Although the critical involvement of lipids, notably phosphatidylserine and 1,2-diacylglycerol, in the regulation of PKC activity and cellular regulation is well-known (72), much remain unclear about the mechanism by which lipids regulate members of the PKC family. The weight of evidence furnished by our work implies that oxyradicals and its subsequent lipid peroxidation play a novel role in the activation of membrane-bound PKC activity.

The antagonizing effects of duroquinone and α -tocopherol on membrane PKC activity, together with the inhibition of malondialdehyde formation by α -tocopherol further support the involvement of active oxygen species in the mechanism by which α -tocopherol inhibits PKC activity.

Human cells, notably neutrophils, were shown to contain endogenous quinones (73) which stimulated oxygen consumption by intact cells and by disrupted cells, in the presence of NADPH or NADH. α -tocopherol, apart from its ability to scavenge active oxygen species, can form a semiquinone following abstraction of hydrogen by another free radical (74,75). This semiquinone might exert its inhibitory effect by disrupting the flow of electrons from NADPH to O_2 in the multi-component electron transport system.

The addition of vitamin C potentiated the antagonizing effect of vitamin E on duroquinone. This observation confirmed our previous findings concerning the regeneration of oxidized vitamin E by vitamin C (76,77). Vitamins E and C are synergistic in their antioxidant properties. Ascorbate can reduce the

tocopheroxy radical with the concurrent formation of an ascorbate radical which can be enzymatically reduced back to ascorbate by an NADH-dependent system. This will help to maintain vitamin E at the efficient concentration, thus potentiating its effect. Such an effect of vitamin C on PKC activity induced by duroquinone and vitamin E again strengthens the involvement of active oxygen species on PKC activation processes.

Apart from its role as a major effector of tumour promotion, many studies point to PKC as being an important factor involved in smooth muscle cell contractility (78). Whether PKC, which has been activated by active oxygen species, plays a role in maintaining smooth muscle tone via cellular Ca^{2+} levels remains to be determined. PKC has been shown to increase the sensitivity of ferret aorta to Ca^{2+} while maintaining its intrinsic myogenic tone by a mechanism the Ca^{2+} requirement of which is satisfied with the resting $[\text{Ca}^{2+}]_i$ (79). Accordingly, PKC has also been demonstrated to increase intracellular sensitivity to Ca^{2+} , thus enhancing stretch-induced cerebral artery tone (80). Therefore, bearing in mind that several anti-hypertensive compounds have been proven to inhibit PKC activity (81), and that PKC inhibitors, such as staurosporine, produce relaxation and lower blood pressure in rats (40), the use of antioxidants such as vitamin E and vitamin C could have clinical implications in hypertension therapy. A schematic presentation of the effects of oxidants and natural antioxidants on the regulation of PKC activity is presented in Fig. 16. In response to oxidation stress, cellular polyunsaturated fatty acids (PUFA) are oxidized to form alkoxy (LO^{\bullet}) and peroxy (LOO^{\bullet}) radicals. The process is termed lipid peroxidation. These active oxygen species are protein kinase C (PKC) activators which can transform protein

kinase C from an inactive (PKC_i) to an active (PKC_a) form. Vitamin E (VE), as a natural and potent chain breaking antioxidant, terminates the lipid peroxidation propagation by donating its phenolic hydrogen atom to LO[•] and LOO[•] to form alcohol (LOH) and hydroperoxide (LOOH), respectively. Vitamin E, after losing its hydrogen atom, becomes itself an unreactive tocopheryl radical (VE[•]) due to electron stabilization of the phenolic ring. Tocopheryl radical (VE[•]) can be converted back to its precursor molecule, tocopherol (vitamin E) by vitamin C (VC), a water soluble antioxidant, with the concurrent formation of vitamin C radical (VC[•]). The process is called regeneration of vitamin E. Vitamin C radical (VC[•]) can be enzymatically reduced back to vitamin C by an NADPH-dependent system.

**Figure 16 - Schematic Representation of the Effects of
Oxidants and Natural Antioxidants on the
Regulation of PKC Activity**

[PUFA: polyunsaturated fatty acid, LO[•]: alkoxyradical, LOO[•]: peroxyradical, LOH: alcohol, LOOH: hydroperoxide, PKC_i: protein kinase C inactive form, PKA_a: protein kinase C active form, VE: vitamin E, VE[•]: tocopherylradical, VC: vitamin C, VC[•]: ascorbyl radical, NADH: nicotinamide adenine dinucleotide (reduced), NAD: nicotinamide adenine dinucleotide (oxidized)]

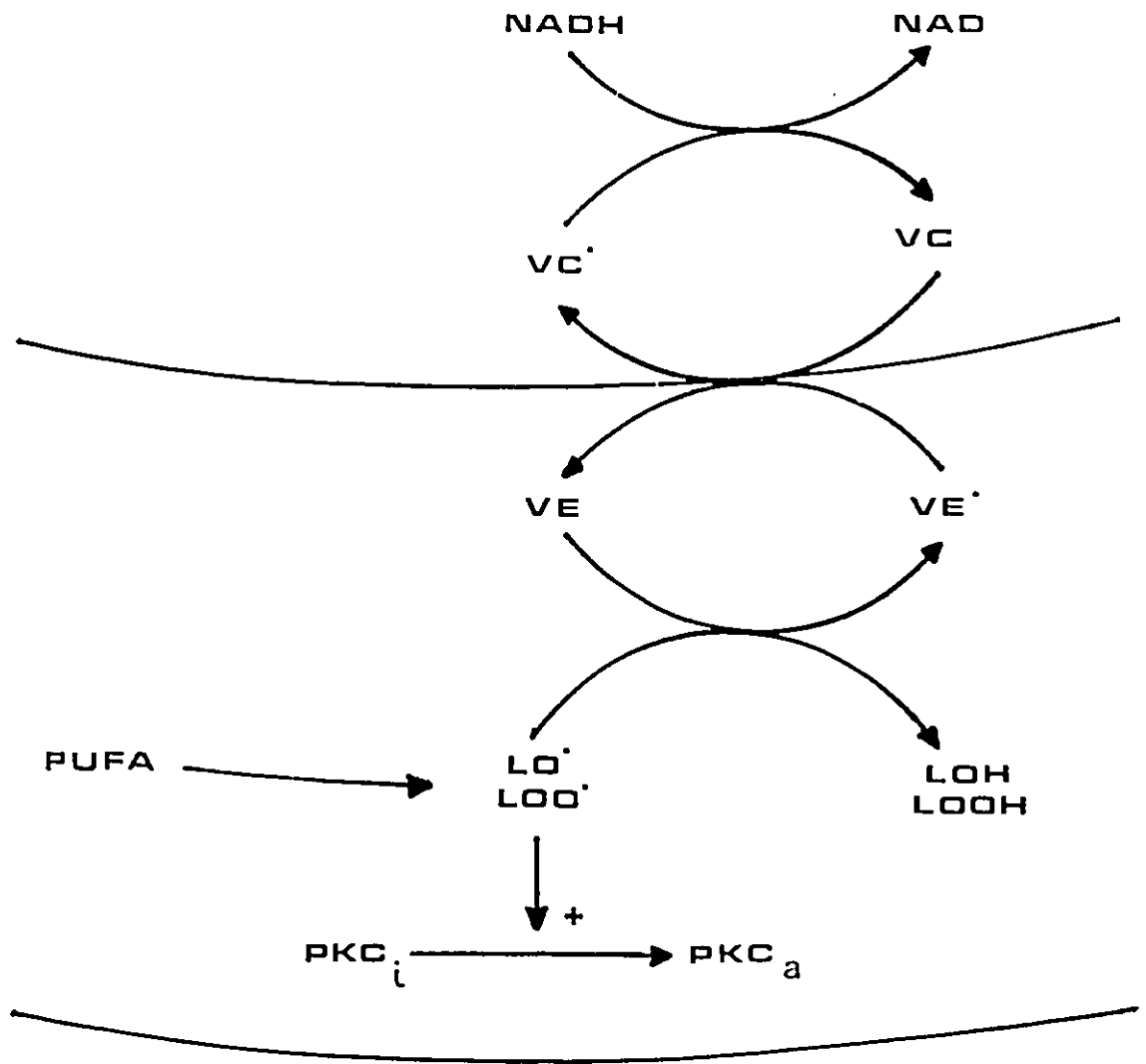


Figure 16

CONCLUSION

In this study, we have shown that free radicals and lipid peroxides caused the activation of membrane-associated PKC and that cells exposed to vitamin E-enriched media exhibited reduced membrane-associated PKC activity. This effect of vitamin E was also shown to be due to the ability of the vitamin to quench oxygen free radicals.

Because free radicals and lipid peroxides have been postulated to be involved in the etiology of cancer (82) and hypertension (83), while PKC is one of the earliest events in the cascade of events leading to a variety of cellular responses related to cancer and hypertension genesis such as gene expression, proliferation and muscle contraction, our finding suggests a novel mechanism by which free radicals might participate in the development of tumor initiation and hypertension. In addition, the antagonizing effect of natural antioxidants (vitamins E and C) and free radicals on PKC activity suggests the possibility that antioxidants may play a role in alleviating the above pathological conditions.

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