The Effects of Alpha Tocopherol Supplementation on Monocyte Function

Decreased Lipid Oxidation, Interleukin 1β Secretion, and Monocyte Adhesion to Endothelium

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Abstract

Low levels of alpha tocopherol are related to a higher incidence of cardiovascular disease and increased intake appears to afford protection against cardiovascular disease. In addition to decreasing LDL oxidation, alpha tocopherol may exert intracellular effects on cells crucial in atherogenesis, such as monocytes. Hence, the aim of this study was to test the effect of alpha tocopherol supplementation on monocyte function relevant to atherogenesis. Monocyte function was assessed in 21 healthy subjects at baseline, after 8 wk of supplementation with d-alpha tocopherol (1,200 IU/d) and after a 6-wk washout phase. The release of reactive oxygen species (superoxide anion, hydrogen peroxide), lipid oxidation, release of the potentially atherogenic cytokine, interleukin 1β, and monocyte-endothelial adhesion were studied in the resting state and after activation of the monocytes with lipopolysaccharide at 0, 8, and 14 wk. There was a 2.5-fold increase in plasma lipid–standardized and monocyte alpha tocopherol levels in the supplemented phase. After alpha tocopherol supplementation, there were significant decreases in release of reactive oxygen species, lipid oxidation, IL-1β secretion, and monocyte-endothelial cell adhesion, both in resting and activated cells compared with baseline and washout phases. Studies with the protein kinase C inhibitor, Calphostin C, suggest that the inhibition of reactive oxygen species release and lipid oxidation is due to an inhibition of protein kinase C activity by alpha tocopherol. Thus, this study provides novel evidence for an intracellular effect of alpha tocopherol in monocytes that is antiatherogenic. (*J. Clin. Invest.* 1996. 98:756–763.) Key words: atherosclerosis • antioxidants • lipid peroxidation • superoxide

Introduction

To date, much data have accrued to support the concept that oxidatively modified LDL can promote atherogenesis (1–3). In addition, several lines of evidence (1–3) support the in vivo existence of oxidized LDL. Hence, the role of dietary micronutrients such as alpha tocopherol in preventing LDL oxidation and atherosclerosis assumes great importance. In fact, several lines of evidence support a relationship between low levels of alpha tocopherol and increased cardiovascular morbidity and mortality (4–6) and increased intake with decreased cardiovascular morbidity (7–9). Numerous investigators have shown that alpha tocopherol supplementation decreases LDL oxidative susceptibility as evidenced by an increase in the lag phase of oxidation (10–12). In addition, studies have suggested that alpha tocopherol can have other beneficial effects in atherogenesis. Alpha tocopherol supplementation has been shown to decrease platelet adhesion and aggregation (13, 14). In vitro studies have shown that supplementation of endothelial cells with alpha tocopherol (15) decreases monocyte-endothelial cell adhesion and that alpha tocopherol in vitro decreases smooth muscle cell proliferation (16). Also, alpha tocopherol appears to protect endothelium-dependent vasodilation in cholesterol-fed rabbits (17, 18).

However, to date, there appears to be no data on the role of alpha tocopherol supplementation on monocyte function. The monocyte appears to be a crucial cell in early atherogenesis and fatty streak formation and it has been shown previously that monocytes can oxidatively modify LDL (19). Hence, the aim of this study was to test the effect of alpha tocopherol supplementation on the release of reactive oxygen (ROS)[1] species (superoxide anion and hydrogen peroxide), oxidation of an artificial lipoprotein emulsion, and the release of a potentially atherogenic cytokine, IL-1β. In addition, the effect of alpha tocopherol enrichment of monocytes on monocyte-endothelial cell adhesion was also investigated.

Methods

Subjects

The subjects for this study were 21 normal healthy controls who fulfilled the following inclusion criteria: (*a*) no recent infection in the last 6 wk; (*b*) nonsmokers; (*c*) no gastrointestinal disorders such as malabsorption; (*d*) not taking antioxidant supplements, oral contraceptives, hypolipidemic drugs, thyroxine, estrogen, or nonsteroidal antiinflammatory drugs for the past 6 mo; (*e*) alcohol consumption < 1 oz/d; and (*f*) normal blood count and renal and hepatic function. The mean age of the subjects was 28 ± 5.5 yr (range 23–44 yr) and the body mass index was 24.8 ± 5.6 kg/m^2 (range 23.2–25.7 kg/m^2). The study group comprised of 5 females and 16 males. This study was approved by the Institutional Review Board of the University of Texas Southwestern Medical Center.

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1. *Abbreviations used in this paper:* HPF, high power field; HRP, horseradish peroxidase; HUVEC, human umbilical vein endothelial cells; NFkb, nuclear factor kb; PKC, protein kinase C; ROS, reactive oxygen species; TBARS, thiobarbituric acid reactive substances.

Study design

Monocyte function was studied in 21 subjects at baseline (0 wk), after 8 wk of supplementation with alpha tocopherol (1,200 IU/d), and after a 6-wk washout phase when the alpha tocopherol was discontinued (14 wk). The alpha tocopherol was in the form of D-α tocopheryl acetate capsules and was provided by the Henkel Corporation (La Grange, IL). A washout phase was included to compensate for the omission of a placebo group. The placebo group was omitted because of the possibility of wide interindividual variability with monocyte studies (20). Throughout the study, the subjects were requested to adhere to their usual diet and physical activities. Monocyte function was assessed in terms of (*a*) release of ROS (superoxide anion and hydrogen peroxide); (*b*) modification of an artificial lipoprotein (fatty acid-BSA emulsion); (*c*) release of the cytokine, IL-1β; and (*d*) adhesion of monocytes to human umbilical vein endothelial cells (HUVEC).

Isolation of monocytes

Mononuclear cells were isolated from 120 ml of heparinized fasting venous blood by Ficoll-Hypaque centrifugation as described previously (21). 20 ml of blood (anticoagulated with 10 U/ml heparin) was layered carefully on 15 ml of Ficoll-Hypaque gradient (Sigma Immunochemicals, St. Louis, MO) and centrifuged at 500 *g*, without brakes at room temperature for 30 min. The mixed mononuclear band was aspirated and the cells were washed three times in phenol red RPMI 1640 medium containing 100 U/ml penicillin, 100 μg/ml streptomycin, and 2 mM glutamine and suspended in a known volume. Leukocyte count was performed on a Coulter counter and then cells were plated ($5–7 \times 10^6$ cells) in 6-well Primaria plates in RPMI 1640 medium. Incubation was carried out at 37°C for 2 h in 5% CO_2/95% air, after which nonadherent cells were removed after washing three times with phenol red–free RPMI 1640 medium. Nonspecific esterase staining revealed that 88.6% of the cells were monocytes (20). All the assays of monocyte activity were undertaken on the day of isolation. All reagents used to assay for monocyte function were tested for endotoxin contamination by the Limulus endotoxin assay and were found to have < 0.06 endotoxin units/ml. The viability of the monocytes was found to be 94% by Trypan blue exclusion (20). LPS was used to activate monocytes as reported previously by Cathcart et al. (20).

ROS

Superoxide. Superoxide anion generation in resting and LPS-activated cells was measured by the SOD-inhibitable reduction of ferricytochrome C (22, 23). Monocytes were incubated in Gey's balanced salt solution (GBSS), pH 7.4, for 60 min at 37°C, with and without SOD (100 μg/ml, final concentration), in the presence and absence of LPS (100 μg/ml) and 80 μM ferricytochrome C in a total volume of 1 ml. The reaction was stopped in melting ice and the absorbance of the supernatant was read at 550 nm. An extinction coefficient of 21.1 mM/cm was used for oxidized versus reduced cytochrome C. Results were expressed as nmol superoxide/min/mg cell protein. Cells were harvested using 0.1 N NaOH and the protein content was measured by the method of Lowry et al. (24).

Hydrogen peroxide. Hydrogen peroxide release in resting and LPS-activated cells was measured by the horseradish peroxidase (HRP)-scopoletin fluorescence method as described by Boveris et al. (25). Hydrogen peroxide is detected by its reaction with HRP, which can oxidize scopoletin with concomitant extinction of its fluorescence at 460 nm when activated at 350 nm. Briefly, the assay system consisted of GBSS, resting or activated cells, 0.01 ml scopoletin (4 μM, final concentration), 0.2 ml of HRP (0.24 μM, final concentration) in a final volume of 2.5 ml. Incubation was done at 37°C for 60 min after which the supernatant was aspirated and fluorescence was read at 460 nm. Hydrogen peroxide (0.25–5 μM) was used as standard and hydrogen peroxide release was expressed as μM H_2O_2/min/mg cell protein.

Lipoprotein oxidation by monocytes

To study lipoprotein modification by monocytes, resting and activated monocytes were incubated overnight in the presence of an artificial lipoprotein emulsion. An artificial lipoprotein emulsion was used in order to eliminate the variability that would arise when using three individual LDL samples over a 14-wk period. The artificial lipoprotein emulsion was prepared fresh on the day of the experiment according to the method of Ball et al. (26), with minor modification, using a nominal molar ratio of cholesteryl linoleate (CL), cholesteryl arachidonate (CA), cholesteryl oleate (CO)/bovine serum albumin (fatty acid free) of 60:1. The distribution of CL:CA:CO in mol% was 41:5:17, which corresponds to their distribution in human LDL (27). 50 μl of artificial lipoprotein emulsion was added to each culture dish in a total volume of 1.0 ml in Ham's F-10 medium, pH 7.4, and incubated for 18 h. Cell-free controls were also set up containing the emulsion in Ham's F-10 medium. At the end of the incubation period, in all experiments, the medium was aspirated out of the dish and centrifuged at 2,000 rpm for 10 min at 4°C and the supernatant was collected to assay for lipid peroxidation by the thiobarbituric acid reactive substances assay (TBARS). TBARS was measured by a modified fluorometric assay as described previously (28), using malondialdehyde as standard. Oxidative modification of the artificial lipoprotein by the monocytes was determined as the differences in absorbance between cells and cell-free controls. TBARS activity was expressed as malondialdehyde equivalents.

Release of ROS and lipoprotein oxidation was also tested in the lymphocyte-rich supernatant obtained after the 2-h incubation step during monocyte isolation since there was lymphocyte contamination (see above).

Release of IL-1β

The release of IL-1β was measured in resting and LPS-activated monocytes by ELISA using the human immunoassay kit (Biotrak Immunoassay; Amersham Corp., Arlington Heights, IL) (29).

Monocyte-endothelial cell adhesion

During the course of this study, an assay for monocyte-endothelial cell adhesion was validated and set up. Monocyte-endothelial cell adhesion was carried out in the last eight subjects entered in the study by the Rose-Bengal method (30, 31). Primary cultures of HUVEC were obtained from the laboratory of Dr. N. Oppenheimer-Marks at the University of Texas Southwestern Medical Center (32). Confluent monolayers of HUVEC were washed with 4 vol of incubation medium (DME/F-12 media supplemented with 15% FBS, heparin [90 mg/ml], and endothelial cell growth factor [150 μg/ml]). One set of HUVEC was incubated with LPS (3 ng/ml) for 3 h at 37°C (33). Monocytes were released from Petri dishes with 10 μM EDTA in PBS, pH 7.4 (21). After washing with RPMI 1640 medium, monocytes (1×10^5 cells/ml) were incubated in a 400 μl volume at 37°C in 5% CO_2/95% air with the HUVEC monolayers for 30 min in triplicate. Since adherence of monocytes to the Petri dishes results in some activation, to prevent further manipulation of the monocytes before incubation with endothelial cells, we chose to use the vital stain, Rose-Bengal, instead of chromium labeling of the cells. It has been shown previously that these two methods yield similar results (30, 34). After three washes with PBS to remove unbound cells, 200 μl of 0.02% Rose-Bengal in PBS was added and incubated at room temperature for 5 min. The excess dye was washed off using three washes of PBS with 10% FCS and then 400 μl of ethanol/PBS (1:1) was added and left at room temperature for 30 min. Monocyte-endothelial cell adhesion was calculated from the difference in absorbencies at 570 nm between wells that contained monocytes and HUVEC and wells that contained only HUVEC. Adhesion was also verified by counting the cells under a phase-contrast microscope. Cells were counted in five high power fields (HPF) by two individuals and the mean value was taken. Both methods revealed similar data.

The alpha tocopherol content of plasma and monocytes was measured after the medium was aspirated and the cells were harvested. For extraction of alpha tocopherol from cells, 1.0 ml of 0.1 M SDS was added to each dish and alpha tocopherol was extracted twice with 4 ml of hexane after ethanol precipitation (35). The hexane phase was

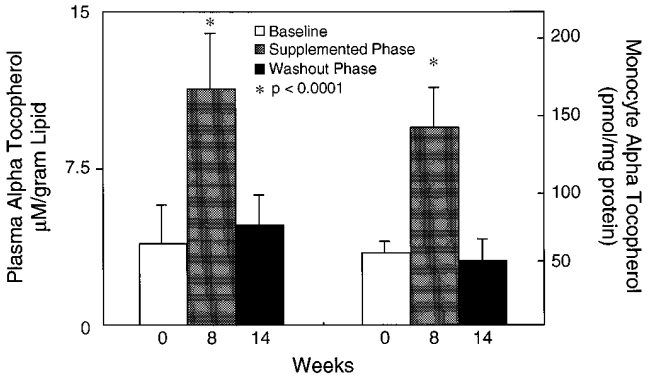

Figure 1. Alpha tocopherol levels in plasma and monocytes. Plasma lipid–standardized and monocyte alpha tocopherol content were measured at 0, 8, and 14 wk after ethanol precipitation and hexane extraction by reversed phase HPLC as described in Methods.

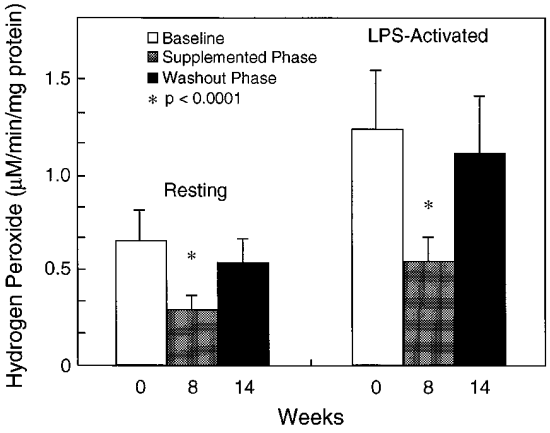

Figure 3. Effect of alpha tocopherol supplementation on hydrogen peroxide release. Hydrogen peroxide from resting and LPS-activated monocytes was measured at 0, 8, and 14 wk as described in Methods.

evaporated under nitrogen and reconstituted to 125 μl with ethanol. Alpha tocopherol was measured after ethanol precipitation and hexane extraction by reversed phase HPLC (36). Plasma alpha tocopherol levels were measured as described previously (36) and the data were lipid standardized as reported previously (12).

Statistical analysis was undertaken to assess the significance of the parameters tested with the help of the biostatistician from General Clinical Research Center. Repeated-measures ANOVA was used to assess differences between baseline, supplemented, and washout phases. Multiple comparisons were performed with (Bonferroni adjusted) paired *t* tests using the 0.01 level of significance to adjust for multiple testing. All data are expressed as mean±SD unless stated otherwise.

Results

After alpha tocopherol supplementation, plasma lipid–standardized alpha tocopherol levels were significantly increased when compared with baseline and washout phases (incremental increase, 187.1±14.2 and 136.4±8.3%, respectively, $P < 0.0001$) (Fig. 1). Also, alpha tocopherol supplementation resulted in a significant enrichment of alpha tocopherol within the monocytes when compared with baseline and washout phases (incremental increase, 169.3±52.3 and 198.9±51.4%, respectively, $P < 0.0001$).

Superoxide anion release from monocytes was assessed at baseline, after supplementation, and at the end of the washout phase. As shown in Fig. 2, there was a significant increase in superoxide anion release from LPS-activated monocytes at 0, 8, and 14 wk when compared with resting cells. Alpha tocopherol supplementation resulted in a significant decrease in superoxide anion release in resting monocytes compared with baseline and washout phases (51.7±3.1 and 50.7±5.0%, respectively, $P < 0.0001$). Also, alpha tocopherol supplementation resulted in a significant decrease in superoxide anion release from LPS-activated monocytes when compared with baseline and washout phases (60.8±6.3 and 59.7±6.7%, $P < 0.0001$). Similar to superoxide anion, there was also a significant increase in hydrogen peroxide release by LPS-activated monocytes ($P < 0.0001$) when compared with resting cells. Alpha tocopherol supplementation resulted in a significant decrease in hydrogen peroxide release from resting cells com-

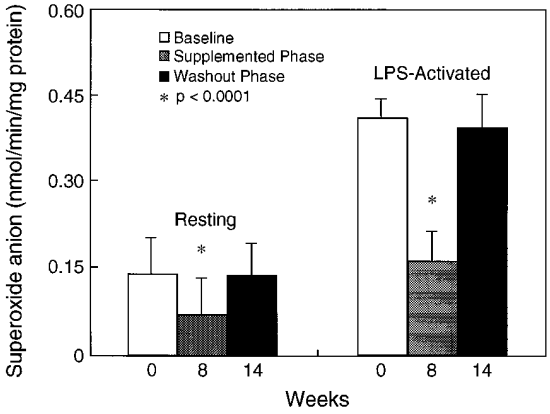

Figure 2. Effect of alpha tocopherol supplementation on superoxide anion release from monocytes. Superoxide anion release was measured in resting and LPS-activated (100 μg/ml) monocytes at 0, 8, and 14 wk as described in Methods.

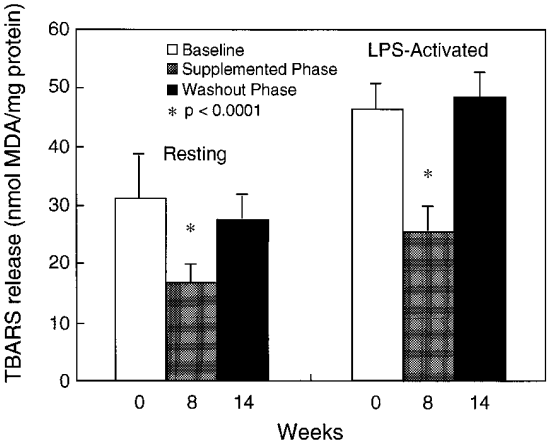

Figure 4. Effect of alpha tocopherol supplementation on lipid oxidation by monocytes. Monocytes, resting and LPS-activated, were incubated overnight with an artificial lipoprotein emulsion containing CL/CA/CO:BSA (60:1). Lipid oxidation by monocytes was measured by assaying for TBARS activity as described in Methods.

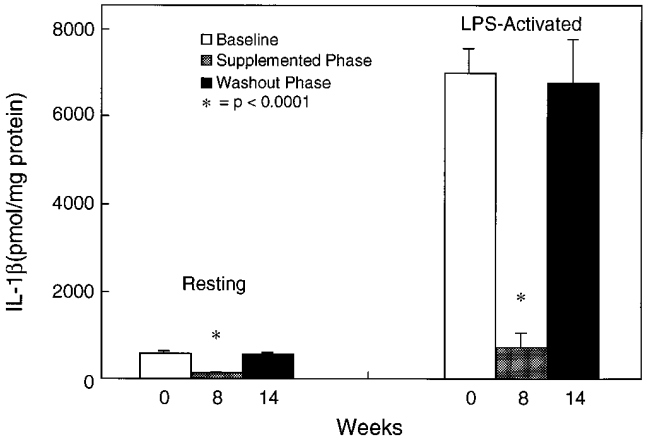

Figure 5. Effect of alpha tocopherol supplementation on IL-1β release from monocytes. IL-1β release from resting and LPS-activated monocytes was assayed in the supernatants at 0, 8, and 14 wk by a sandwich ELISA as described in Methods.

pared with baseline and washout phases (46.1±6.7 and 46.5±8.1%, respectively, $P < 0.0001$). As shown in Fig. 3, in LPS-activated monocytes also, alpha tocopherol supplementation produced a significant reduction in hydrogen peroxide release when compared with baseline and washout phases (56.3±12.3 and 51.0±12.1%, respectively, $P < 0.0001$).

Monocyte oxidation of the artificial lipoprotein emulsion was assessed at 0, 8, and 14 wk as depicted in Fig. 4. There was a significant increase in TBARS release after activation with LPS (60.8±7.4%, $P < 0.0001$). TBARS was significantly reduced after alpha tocopherol supplementation in resting monocytes when compared with baseline and washout phases (47.1±12.1 and 39.8±12.9% reduction, respectively, $P < 0.0001$). In LPS-activated monocytes, alpha tocopherol supplementation also produced a significant decrease in lipid oxidation by the activated monocytes when compared with baseline and washout phases (44.5±14.2 and 46.8±12.7% reduction, respectively, $P < 0.0001$).

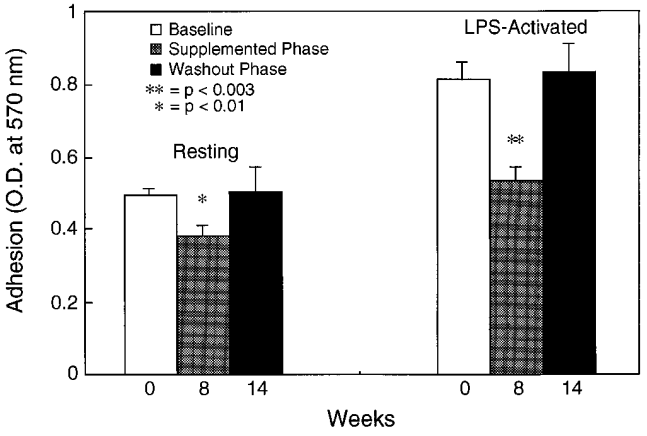

Figure 6. Effect of alpha tocopherol supplementation on monocyte-endothelial cell adhesion. HUVEC were incubated with LPS (3 ng/ml) 3 h before the assay. Monocytes were incubated with control and LPS-activated endothelial cells for 1 h followed by assay of adhesion using the Rose-Bengal method as described in Methods. Control wells consisted of HUVEC only. Cell counts were also done to assess monocyte-endothelial adhesion. For resting cells the number of cells attached per HPF to HUVEC at baseline, after supplementation, and at the end of the washout phase were 49.4±22.9, 37.1±15.9, and 52.9±22.7, respectively. After activation the cells attached per HPF at the three time points were 116.1±52.4, 76±38.4, and 120.1±55.6, respectively. For both resting and activated cells alpha tocopherol supplementation resulted in a significant reduction compared with baseline and washout phases ($P < 0.001$).

Since the monocyte preparation had some lymphocyte contamination, we determined the contribution of lymphocytes to the indices monitored. Superoxide and hydrogen peroxide release as well as lipid oxidation were tested in the lymphocyte-rich supernatant obtained after the 2-h incubation of the mixed mononuclear cell preparation at 37°C. There was less than a 10% increment in the release of either superoxide or hydrogen peroxide or lipid oxidation in the supernatant.

The effect of alpha tocopherol on IL-1β release is shown in Fig. 5. Release of IL-1β was tested in resting and LPS-activated cells. LPS-activated cells showed a 12.1-fold increase in IL-1β release when compared with resting cells ($P < 0.0001$). The IL-1β levels after alpha tocopherol supplementation were significantly decreased in resting monocytes when compared with baseline and washout phases (80.2±35.9 and 76.9±40.1%, respectively, $P < 0.0001$). Also, in LPS-activated monocytes, alpha tocopherol supplementation resulted in a significant reduction in IL-1β levels when compared with baseline and washout phases, respectively (90.3±43.1 and 88.7±47.5%, $P < 0.0001$).

The adhesion of monocytes to confluent HUVEC was also assessed at baseline, after supplementation, and after the washout phases, in the presence and absence of LPS. In spite of the monocytes being activated to some extent by adherence to the Petri dishes, there was a significant increase in adhesion to HUVEC after stimulation with LPS. LPS induced monocyte-endothelial cell adhesion 65.3±10.5% ($P < 0.0001$) when compared with resting cells. This prior activation of monocytes may also account for the high background adhesion. After alpha tocopherol supplementation, there was a significant decrease in monocyte-endothelial cell adhesion in resting cells (22.5±0.2 and 24.3±0.2% decrease, respectively, $P < 0.01$) and LPS-activated cells (34.6±0.2 and 36.3±0.2% decrease, respectively, $P < 0.0003$) when compared with baseline and washout phases (Fig. 6). As shown in the legend to Fig. 6, cell counts per HPF revealed similar findings.

It has been shown previously that alpha tocopherol inhibits protein kinase C (PKC) activity (37, 38) and that PKC activity is crucial for superoxide release and LDL oxidation by activated monocytes (39). To gain some mechanistic insights regarding the effect of alpha tocopherol on monocyte function, the effect of the specific PKC inhibitor, Calphostin C, on the parameters of monocyte function was tested. Since Cathcart and Li (39) have shown that 1–10 μM Calphostin C inhibited LDL oxidation by 15–25%, we tested the effect of 0.25 and 0.5 μM Calphostin C on copper-catalyzed LDL oxidation as described previously (12). At both concentrations, Calphostin C had no significant effect on the lag phase of LDL oxidation and maximum amount of oxidation as evidenced by the TBARS and lipid peroxide assay (data not shown). Since Calphostin C was not an antioxidant at 0.25 and 0.5 μM, the effect of these concentrations of Calphostin C on superoxide release, lipid oxidation, IL-1β release, and monocyte-endothelial adhesion was studied (Table I). Calphostin C (0.5 μM) pro-

Table I. Effect of Calphostin C on LPS-activated Monocyte Function

	Calphostin C (μM)		
	0 μM	0.25 μM	0.50 μM
Superoxide anion release (nmol/min/mg protein)	0.44±0.03	0.41±0.03	0.19±0.02*
Lipid oxidation TBARS (nmol/min/mg protein)	31.37±4.66	23.91±4.02*	18.27±2.99*
IL-1β release (pmol/mg protein)	3075±378	2762±178	3177±301
Monocyte-endothelial cell adhesion Abs 570 nm	0.44±0.03	0.45±0.01	0.45±0.01
Cells/HPF	80.3±17	80.3±13	78.0±15

Calphostin C was preincubated with the mixed mononuclear cell preparation for the 2 h and to the monocyte preparation for an additional 30 min. Thereafter, the cells were washed three times in RPMI 1640 before experiments. Values are given as mean±SD of three experiments in duplicate. *$P < 0.001$.

duced a significant decrease in superoxide release from activated monocytes ($P < 0.001$). Also, both concentrations of Calphostin C produced a significant decrease in lipid oxidation by the monocytes ($P < 0.001$). However, there was no significant decrease in IL-1β release or monocyte-endothelial cell adhesion in the presence of Calphostin C.

Discussion

Lower levels of alpha tocopherol have been associated with an increased prevalence of cardiovascular mortality and higher intakes of alpha tocopherol have been shown to be associated with decreased frequency of cardiovascular disease and decreased arterial lesion progression (4–9). Oxidative modification of the intimal lipoproteins is believed to be an essential component of the atherogenic process. In addition to decreasing LDL oxidative susceptibility in vitro, the potential exists that alpha tocopherol could partition in cells such as endothelial cells and monocytes and exert other intracellular effects that could be beneficial. The monocyte-macrophage is one of the crucial cell types in the arterial intima during the genesis of the atherosclerotic lesion and is present during all stages of atherogenesis. There are scanty data on the effect of antioxidant supplementation in vivo on the activity of pivotal cells in atherogenesis, such as endothelial cells and monocytes. The monocyte is the most accessible cell of the artery wall. To this end, we investigated the effect of alpha tocopherol supplementation on release of ROS and lipid oxidation by human monocytes, the release of an atherogenic cytokine, IL-1β, and monocyte-endothelial cell adhesion.

Monocytes have been shown to induce peroxidation of LDL lipids by generation of ROS such as superoxide and hydrogen peroxide (40). This study has shown that there is a twofold increase in the release of superoxide anion on activation with LPS and that alpha tocopherol produced a significant reduction in superoxide anion generation from resting and LPS-activated cells. Alpha tocopherol supplementation also significantly decreased hydrogen peroxide release from resting and LPS-activated monocytes. With regards to lipid oxidation, alpha tocopherol supplementation also resulted in a significant decrease in oxidation of the artificial lipoprotein emulsion in both resting and LPS-activated monocytes. The monocyte-macrophage is a crucial cell in modifying LDL in lesions. Hence, it is important to characterize LDL modification by monocytes and to see the effect of alpha tocopherol enrichment of monocytes on this process. To minimize assay variability, we chose an artificial lipoprotein which was prepared fresh on the day of the assay instead of LDL which would have to be isolated on three different occasions over 14 wk. We have chosen an artificial lipoprotein emulsion containing cholesteryl linoleate (CL), cholesteryl arachidonate (CA), and cholesteryl oleate (CO)/BSA in the proportion that would normally be present in human LDL (27). The CL/BSA emulsion has been shown to be avidly taken up by macrophages resulting in foam cell formation and ceroid accumulation (26). Mouse peritoneal macrophages and human monocyte-macrophages exposed to CL and CA/BSA rapidly accumulate lipid and oxidize the unsaturated esters (41). Antioxidants such as alpha tocopherol have been found to inhibit this macrophage-mediated lipid oxidation in vitro (42). In the presence of the artificial lipoprotein emulsion, there was a 1.5-fold increase in lipid oxidation by activated monocytes compared with resting cells. Thus, monocyte-mediated lipid oxidation might well contribute to a crucial step in the development of the atherosclerotic plaque. Enrichment of monocytes with alpha tocopherol significantly diminishes the ability of these cells to oxidize lipid and hence could preempt foam cell formation. From this study, it appears that supplementation with alpha tocopherol has dual effects in decreasing LDL oxidation. Numerous groups have shown that alpha tocopherol partitions into the LDL and reduces the oxidative susceptibility of LDL (10–12). The present study indicates that alpha tocopherol supplementation in addition results in enrichment in the monocyte with subsequent decrease in lipid oxidation.

To gain some insights on the effect of alpha tocopherol on monocyte function, we looked at the effect of a specific PKC inhibitor on the parameters of monocyte function studied since Cathcart and Li (39) have shown previously that PKC mediates superoxide release and LDL oxidation by monocytes. The PKC inhibitor, Calphostin C, was chosen since it has been reported to bind efficiently with the regulatory domain rather than the catalytic site and has been shown to be a potent inhibitor of PKC (IC_{50} = 50 nmol) (43). Calphostin C did not show any antioxidant properties or cytotoxicity at 0.25 and 0.5 μM and therefore these concentrations were used. Calphostin C produced a 51% decrease in superoxide anion release and a 32.3% decrease in lipid oxidation by activated monocytes, which could largely explain the inhibition seen after alpha tocopherol supplementation (59% in superoxide release and 40% decrease in lipid oxidation). Hence, it appears that the inhibition in superoxide anion release and lipid oxidation observed in the subjects after alpha tocopherol supplementation could be attributed to an inhibition of PKC activity rather than a general antioxidant effect.

Increasing evidence suggests that IL-1β participates either directly or indirectly in growth regulation and formation of atherosclerotic lesions in the arterial wall (44). Also, mRNA encoding for IL-1β has been found in atherosclerotic lesions (45). Individual cytokines such as IL-1β have been shown to modulate artery wall cell function, such as the induction of cell adhesion molecule expression (46), such as intercellular adhe-

sion molecule (ICAM) and vascular cell adhesion molecule (VCAM), and stimulation of smooth muscle cell proliferation via PDGF secretion (47). Recently, it has been reported that monokines such as IL-1 and TNF-α can prime PMNs to facilitate ROS production (48, 49). Together, these studies provide support for a potential role for IL-1β in atherosclerosis and suggest that modulation of cytokine production, e.g., by antioxidant supplementation, would affect the atherosclerotic process. Alpha tocopherol supplementation resulted in a 5- and 10-fold reduction in IL-1β levels in resting and activated cells. Akeson et al. (50) have shown in vitro that THP-1 cells respond to PMA activation by induction of IL-1β mRNA and induction of LDL-scavenger receptors. Concomitant with this induction are increased cholesterol esterification and loading by cells. In this in vitro system, probucol and alpha tocopherol were able to inhibit PMA-induced IL-1β secretion (50). The exact mechanism by which IL-1 release from cells is modulated is not well understood. PMA and other phorbol esters are thought to induce IL-1 activity through activation of cAMP and also via PKC (51, 52). The most potent stimulus for induction and release of IL-1 from monocytes is LPS (53). The lipid-A portion of LPS, which has all the endotoxin activity, is thought to interact with the cell membrane via a putative receptor of target cells and activate immune cell responses, including induction of IL-1 and ROS (54). Since alpha tocopherol is a known chainbreaking antioxidant (55) which provides cell membrane integrity, it could prevent induction of IL-1 release from monocytes by decreasing ROS. Kasama et al. (56) have shown that superoxide stimulates IL-1 release from monocytes and that both SOD and vitamin E inhibit IL-1 release, suggesting that IL-1 activity is enhanced by ROS. Another pathway by which LPS could stimulate IL-1β release is through the leukotriene pathway. Leukotriene B4 has been shown to increase IL-1 activity from monocytes (57) and it is possible that alpha tocopherol could reduce the release of IL-1β from monocytes by decreasing levels of leukotrienes (58). Furthermore, Shapira et al. (59) have shown that LPS-induced IL-1β production from human monocytes involves both PKC and protein tyrosine kinase. However, no appreciable decrease in IL-1β release from activated monocytes was observed in our studies using the specific PKC inhibitor, Calphostin C. A plausible mechanism via which ROS stimulate IL-1 release is through activation of transcription factors such as NFkb (60). Also, components of both lipid and the protein fraction of Ox-LDL have been shown to augment IL-1 release (61, 62). Thus, alpha tocopherol supplementation could conceivably decrease IL-1β release through an intracellular effect and also by partitioning in LDL and decreasing its oxidative susceptibility. Studies are in progress to elucidate the mechanism(s) by which alpha tocopherol modulates IL-1β activity from human monocytes.

Studies in nonhuman primates and other animal models have demonstrated that monocyte attachment to endothelial cells, migration, and subendothelial localization are early events in the pathogenesis of atherosclerosis (63). Monocyte enrichment with alpha tocopherol in the supplemented phase resulted in a significant reduction in monocyte-endothelial cell adhesion. Faruqi et al. (15) have observed that when endothelial cells were cultured in media containing alpha tocopherol as a nutritional supplement, there was less agonist-induced monocytic cell adhesion to EC when stimulated with IL-1. The inhibition correlated with a decrease in steady state levels of E-selectin mRNA and cell surface expression of E-selectin (15). The intracellular signaling events involved in mediating monocyte adhesion to activated EC are not fully defined. PKC activation may be necessary for this process and alpha tocopherol has been shown to inhibit PKC (37, 38). However, in this study, when monocytes were incubated with the PKC inhibitor, Calphostin C, it had no significant effect on monocyte-endothelial cell adhesion. Free radical–mediated injury, either direct or indirect (by generation of oxidized lipoproteins), in the microenvironment of the endothelium is another proposed mechanism by which EC are rendered atherogenic. Biologic response modifiers like IL-1, LPS, and PMA have been shown to induce monocyte-endothelial cell adhesion and these diverse substances may act by intracellular generation of ROS that serve as second messengers in gene activation (64). Immune response has been shown to be associated with expression of endothelial adhesion molecules such as E-selectin, VCAM, and ICAM-1, all of which are activated by the nuclear transcription factor, NFkb. NFkb is also activated by oxidative stress, such as hydrogen peroxide and lipid hydroperoxides. Furthermore, antioxidants have been shown to prevent NFkb activation (64). Suzuki and Packer (65) have shown a concentration-dependent inhibition of NFkb activation when human Jurkat T cells were incubated with alpha tocopherol acetate or succinate. Thus, alpha tocopherol may exert its attenuating effects at the transcriptional level by inhibiting NFkb-mediated gene activation in endothelial cells. Since ROS have been shown to increase transcription factors such as NFkb, it is possible that alpha tocopherol, by inhibiting the release of ROS, results in decreased monocyte-endothelial cell adhesion. However, it should be pointed out that Faruqi et al. (15) failed to demonstrate an effect of alpha tocopherol enrichment of endothelial cells on NFkb activation in spite of decreasing monocyte adhesion. Adhesion is mediated by integrins that bind to the endothelium, the most important ones being LFA-1 (CD11a/CD18), Mac-1 (CD11b/CD18), and VLA-4 (CD49d/CD29). While VLA-4 binds to VCAM, LFA-1 and Mac-1 bind to ICAM-1 and -2 on the endothelium (66). Future studies will be directed at the effect of alpha tocopherol supplementation on the expression of these counterreceptors on monocytes.

Thus, the novel observations in this study with respect to alpha tocopherol are that in addition to its effects in decreasing LDL oxidation, alpha tocopherol supplementation resulted in an intracellular effect that is antiatherogenic. It decreases the ability of the monocytes to release ROS (hydrogen peroxide and superoxide anion) and significantly reduces lipid oxidation by monocytes. This appears to be mediated by an inhibition of PKC activity. In addition, alpha tocopherol supplementation has other beneficial effects, such as suppression of a potentially atherogenic cytokine, IL-1β, and inhibition of a crucial event in atherogenesis, monocyte-endothelial cell adhesion. The release of IL-1β and monocyte-endothelial cell adhesion seem to be regulated via other mechanisms such as activation of transcription factors like NFkb. The inhibition of IL-1β release and monocyte-endothelial cell adhesion by alpha tocopherol is possibly due to its antioxidant effect and moderation of the intracellular oxidative stress. Further studies are being carried out to elucidate the mechanism(s) by which alpha tocopherol modulates some of these processes. However, this study provides significant information that strengthens the scientific basis for alpha tocopherol supplementation by clearly demonstrating an intracellular effect in addition to its

protective effect on lipoproteins. Obviously, clinical trials will prove to be the final arbiter in deciding whether alpha tocopherol emerges as an antiatherosclerotic therapeutic modality.

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