

# $\alpha$ -Tocopherol Inhibits Agonist-induced Monocytic Cell Adhesion to Cultured Human Endothelial Cells

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## Abstract

Antioxidants have been proposed to be anti-atherosclerotic agents; however, the mechanisms underlying their beneficial effects are poorly understood. We have examined the effect of  $\alpha$ -tocopherol ( $\alpha$ -tcp) on one cellular event in atherosclerotic plaque development, monocyte adhesion to stimulated endothelial cells (ECs). Human umbilical vein ECs were pretreated with  $\alpha$ -tcp before stimulation with known agonists of monocyte adhesion: IL-1 (10 ng/ml), LPS (10 ng/ml), thrombin (30 U/ml), or PMA (10 nM). Agonist-induced monocytic cell adhesion, but not basal adhesion, was inhibited in a time- and concentration-dependent manner by  $\alpha$ -tcp. The  $IC_{50}$  of  $\alpha$ -tcp on an IL-1-induced response was 45  $\mu$ M. The inhibition correlated with a decrease in steady state levels of E-selectin mRNA and cell surface expression of E-selectin which is consistent with the ability of a monoclonal antibody to E-selectin to inhibit monocytic cell adhesion in this system. Probucol (50  $\mu$ M) and N-acetylcysteine (20 mM) also inhibited agonist-induced monocytic cell adhesion; whereas, several other antioxidants had no significant effect. Protein kinase C (PKC) does not appear to play a role in the  $\alpha$ -tcp effect since no suppression of phosphorylation of PKC substrates was observed. Activation of the transcription factor NF- $\kappa$ B is reported to be necessary but not sufficient for E-selectin expression in EC. Electrophoretic mobility shift assays failed to show an  $\alpha$ -tcp-induced decrease in activation of this transcription factor after cytokine stimulation. It has been hypothesized that  $\alpha$ -tcp acts as an anti-atherosclerotic molecule by inhibiting generation of oxidized LDL—a putative triggering molecule in the atherosclerotic process. Our results point to a novel alternative mechanism of action of  $\alpha$ -tcp. (*J. Clin. Invest.* 1994. 94:592–600.) **Key words:** antioxidants • NF- $\kappa$ B • U937 cell • atherosclerosis

## Introduction

The role of monocytes in the pathogenesis of atherosclerosis is well recognized and the adhesion of blood-borne monocytes to the endothelium of lesion-prone regions of the vasculature is one of the earliest documented events in numerous animal mod-

els of atherosclerosis (1–7). Altered or dysfunctional endothelium expresses a number of cytokines, adhesion molecules, vasoactive compounds, and growth factors, which in turn attract, activate, and cause the transendothelial migration of monocytes (8–12). Monocyte adhesion to activated endothelial cells (ECs)<sup>1</sup> in vitro therefore serves as a useful model to simulate the early cellular events in the pathophysiology of atherosclerosis. The direct mechanism of EC activation in vivo is unknown. Free radical-mediated injury, either direct or indirect (by the generation of oxidized lipoproteins in the micro-environment of the endothelium), is one proposed mechanism by which EC are rendered pro-atherogenic (13–15). Biological response modifiers, such as IL-1, LPS, thrombin, and PMA, have also been shown to activate endothelium and cause enhanced monocyte adhesion in vitro. One way in which such diverse substances may act is by the intracellular generation of reactive oxygen intermediates that serve as second messengers in gene activation (16–20).

$\alpha$ -Tocopherol ( $\alpha$ -tcp) or vitamin E, a lipid-soluble vitamin and biological response modifier, is a chain-breaking tissue antioxidant (21). A ubiquitous molecule present at low concentrations in all biomembranes, it has been reported to be an anti-atherogenic agent (22). Though the mechanism of  $\alpha$ -tcp action has not been rigorously demonstrated, it has been hypothesized that it acts through inhibiting generation of oxidized LDL—a putative triggering molecule in the atherosclerotic process.  $\alpha$ -tcp is believed to act as a cytoprotective agent in a number of other pathophysiological processes (23).  $\alpha$ -tcp is also thought to be an immune modulator, enhancing both cell-mediated and humoral immunity (24, 25). The cytoprotective effect of  $\alpha$ -tcp is attributed to its ability to act as a scavenger of highly reactive oxygen radicals, thus stabilizing membranes against lipid peroxidation (26–29).

The intracellular signaling events involved in mediating monocyte adhesion to activated EC are not fully defined. Protein kinase C (PKC), a family of isoenzymes, is responsible for a number of intracellular events involved in receptor-mediated signal transduction, receptor down-regulation, cell activation, transformation, and proliferation (30, 31). We have previously reported that 1-(5-isoquinolinesulfonyl)-2-methylpiperazine dihydrochloride (H-7), an inhibitor of PKC, abolishes agonist-induced monocyte adhesion, suggesting that PKC activation may be necessary for this process (32). Recently,  $\alpha$ -tcp has also been shown to inhibit PKC in rat brain homogenates (33), permeabilized vascular smooth muscle cells and neuroblastoma cells (34). Another potential site of action of  $\alpha$ -tcp in abrogating agonist-induced monocyte adhesion is at the level of the tran-

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1. *Abbreviations used in this paper:*  $\alpha$ -tcp,  $\alpha$ -tocopherol; BHT, butylated hydroxytoluene; DPPD, N,N-diphenyl-1,4-phenylene diamine; EC, endothelial cells; MARCKS, myristoylated alanine-rich C kinase substrate; NAC, N-acetylcysteine; PKC, protein kinase C.

scription factor, NF- $\kappa$ B. E-selectin, an endothelial adhesion molecule first cloned and characterized by Bevilacqua et al. (35, 36), has been reported to be an important player in monocyte adhesion to EC (37, 38). The promoter region of the E-selectin gene contains a binding site for NF- $\kappa$ B (39). This transcription factor is thought to be necessary in regulating transcription of the E-selectin gene (40) and appears to be activated by reactive oxygen intermediates and PKC (41, 42), both of which can be potential target sites for the action of  $\alpha$ -tcp. In this study we have investigated the effect of  $\alpha$ -tcp on stimulated monocytic cell adhesion to EC and the sites along the signaling pathways of E-selectin expression that may be responsible for mediating the effect of  $\alpha$ -tcp.

## Methods

**Cell culture.** Human umbilical vein ECs were isolated by a technique similar to that described by Lewis et al. (43), as we have previously described (32). The collected cells were washed and seeded into fibronectin-coated flasks (5  $\mu$ g/ml rabbit fibronectin) under the conditions described by Thornton et al. (44) and grown in DME/F12 media (Irvine Scientific Co., Irvine, CA) supplemented with 15% FBS, heparin (90  $\mu$ g/ml), endothelial cell growth factor (150  $\mu$ g/ml), sodium bicarbonate (0.24%), MEM non-essential amino acids (0.1 mM), penicillin (100 U/ml), and streptomycin (100  $\mu$ g/ml). EC were used between the first and fourth subculture. U937 cells originally derived from a human histiocytic lymphoma were procured from the American Type Culture Collection (Rockville, MD) and grown in suspension culture in RPMI-1640 media (M.A. Bioproducts, Walkersville, MD) containing 5% FBS and routinely subcultured at a 1:5 ratio three times per week.

**Separation of human leukocytes.** Monocytes were separated from peripheral blood (100 U/ml heparin) by a modification of the method of Recalde (45) as we have recently described in detail (46). Briefly, total mononuclear cells were collected by Ficoll-Hypaque density gradient centrifugation (47), treated with NaCl, and re-isolated with Ficoll-Hypaque. The isolated monocytes were resuspended in MCDB 107 + 5% FBS in a Teflon beaker to prevent attachment during radioactive labeling. Isolated populations were routinely 78–90% monocytes by differential count with buffered Wright-Giemsa stain, and viability was always > 90% by Trypan blue dye exclusion.

**Assay for monocytic cell adhesion to endothelial cells.** U937 cell/monocyte adhesion to ECs was measured as previously described (48). Briefly, ECs were plated into 24-well plates 48 to 72 h before the assay. In nutritional experiments, the growth medium was supplemented with  $\alpha$ -tcp (45  $\mu$ M). When the ECs were confluent, the media was changed to MCDB 107 + 5% FBS with or without  $\alpha$ -tcp for the given pretreatment time. In some experiments the concentration of FBS in the pretreatment media was varied. On the day of the assay the media was once again changed and additional  $\alpha$ -tcp added prior to stimulation with the various agonists for 4 to 6 h at 37°C. U937 cells or human monocytes were labeled for 90 min at 37°C with 100  $\mu$ Ci  $^{51}$ Cr as sodium chromate (NEN, Boston, MA) in 1 ml culture media. The labeled cells were washed by centrifugation and  $10^6$  viable cells added per well of ECs after removal of the incubation media. This binding phase of the assay was performed at 4°C for 1 h, then the wells were washed and the cells lysed with 1% Triton X-100 and an aliquot removed for quantitation of radiolabel. The number of U937 cells or monocytes bound per well was calculated from the initial specific activity (cpm/cell). Spontaneous release of  $^{51}$ Cr from the monocytic cells during the assay incubation was < 5%. All presented data are representative of at least three experiments with determinations made in triplicate.

**Protein synthesis by ECs.** Protein synthesis by the ECs was determined by the incorporation of [ $^3$ H]leucine into TCA-precipitable material, as we have previously described (49). Briefly, EC were incubated for 4 h under the same conditions as for the monocytic cell adhesion assay in the presence of [ $^3$ H]leucine (2  $\mu$ Ci/ml). The cell-associated and

secreted protein synthesis was measured by TCA precipitation of the proteins, solubilization of the membranes with 0.25 N sodium hydroxide and determination of the radioactivity incorporated into the TCA-precipitable material.

**Measurement of E-selectin on EC surface.** E-selectin expressed on the surface of ECs was quantified using an anti-E-selectin monoclonal antibody (7A9) (50), as we have previously described (51). Briefly, ECs were cultured in 48-well plates for 48 h. At confluence, the media was changed to MCDB 107 + 5% FBS with or without  $\alpha$ -tcp for 72 h. On the day of the assay the media was changed once again and new  $\alpha$ -tcp added before stimulation with IL-1 for 4 h at 37°C. Following stimulation, the cells were washed twice with MCDB 107 media containing 1% bovine serum albumin (BSA). The primary monoclonal antibody 7A9 and secondary biotin-conjugated affinity-purified F(ab')<sub>2</sub> fragment goat anti-mouse IgG + IgM (Jackson Immuno Research Laboratories, Inc., West Grove, PA) were prepared in wash media (1% BSA) and added to the wells in a sequential manner for 30 min each, followed by  $^{125}$ I-streptavidin (5  $\mu$ Ci/ml) for 15 min. All incubations were performed at 4°C with the wells being washed 3–4 times between each incubation. ECs were lysed with 1% Triton X-100 and an aliquot removed for radiolabel quantitation. A nonspecific isotype control protein MOPC 21 (a mouse myeloma IgG1- $\kappa$ -protein), was included as a negative control to ascertain the specificity of the monoclonal antibody.

**Northern analysis.** Human umbilical vein EC were grown to confluence in 15-cm-diameter plates with MCDB 107 containing 15% FBS, 90 mg/ml heparin and 150 mg/ml ECGS at 37°C in 5% CO<sub>2</sub>. Once confluent, the cells were pretreated for 72 h with fresh media (MCDB + 5% FBS) with or without  $\alpha$ -tcp (45  $\mu$ M). On the day of the experiment, the medium was changed again and the monolayers treated with IL-1 (10 ng/ml) or media for 2 h at 37°C. Steady-state mRNA levels were determined by northern analysis as previously described (51). The conditioned medium was then aspirated; the cells were washed once with phosphate-buffered saline (PBS); and the total RNA extracted with guanidinium isothiocyanate. RNA was obtained by isopycnic centrifugation over CsCl (52) and separated by electrophoresis on a formaldehyde denaturing gel. RNA was then transferred from the gel to a Nytran (Schleicher & Schuell, Keene, NH) membrane by capillary transfer, UV crosslinked, prehybridized at 42°C for a minimum of 6 h and hybridized overnight at 42°C with  $^{32}$ P-dCTP random primer-labeled cDNA probe for E-selectin (1.85 kb cDNA in pCDN [amp<sup>r</sup>]) or a  $^{32}$ P-dCTP kinase-labeled oligonucleotide probe for the 18 S ribosomal RNA. The blots were then washed until the background counts were insignificant and subjected to autoradiography at –70°C.

**Electrophoretic mobility shift assay (EMSA).** The synthetic double-stranded oligonucleotide containing the consensus sequence for the NF $\kappa$ B DNA binding site in the E-selectin promoter, GGGGATTTC was synthesized using an Applied Biosystems 380B DNA Synthesizer and purified by gel filtration through a Sephadex G-25 column. The sequence of the 28-residue oligonucleotide was taken from the promoter region of the E-selectin gene (5'-AGGCCATT-GGGGATTTCCTCT-TTACTGG-3') (53). (The underlined sequence represents the consensus  $\kappa$ B region.) In competition studies an unrelated oligonucleotide representing the TF-IIIC consensus sequence (–5'-CCGGATCCGGGGTTC-GAACCCCGGCCCAA-3') was used (54). The oligonucleotide probes were annealed and labeled by a "filling-in" reaction, using  $\alpha$ -[ $^{32}$ P]-dCTP, unlabeled dTTP and the Klenow fragment of DNA polymerase I.

Nuclear extracts were prepared by a modified procedure of Dignam et al. (55). After 72 h of pretreatment with  $\alpha$ -tcp (90  $\mu$ M), EC were treated with IL-1 (10 ng/ml) for 1 h at 37°C in the presence of fresh  $\alpha$ -tcp. Cells were then washed twice with cold PBS, scraped and harvested by centrifugation. Cells were resuspended and incubated on ice for 15 min in hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.1 mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10  $\mu$ g/ml each of aprotinin, pepstatin A, leupeptin, and antipain) and then vortexed for 10 s with 0.6% NP-40. Nuclei were separated from the cytosol by centrifugation at 12,000 g for 60 s and were resuspended in buffer C (20 mM Hepes, pH 7.9, 25% glycerol, 0.4 M NaCl, 1 mM EDTA, 1

mM EGTA, 1 mM DTT, 0.5 mM PMSF, 10  $\mu$ g/ml each of aprotinin, pepstatin A, leupeptin, and antipain) and shaken for 30 min at 4°C. Nuclear extracts were obtained by centrifugation at 12,000 g and dialyzed against buffer D (20 mM Tris-Cl, pH 7.9, 0.1 M KCl, 0.2 mM EDTA, 0.5 mM DTT, 0.5 mM PMSF, 20% glycerol), overnight at 4°C. Protein concentration was measured using the Bradford assay (Bio Rad, Richmond, CA).

For binding reactions, nuclear extracts (5  $\mu$ g protein) were incubated in 15–25  $\mu$ l of total reaction volume, containing 20 mM Hepes, pH 7.9, 60 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 8% glycerol and 2.0  $\mu$ g of poly (dI-dC) (Pharmacia Fine Chemicals, Piscataway, NJ) and radiolabeled oligonucleotide (40,000–50,000 cpm), for 15 min at room temperature. In competition analysis studies, 100-fold molar excess of unlabeled oligonucleotide or an unrelated oligonucleotide (TF IIIC) was coincubated with the nuclear extract for 15 min before the binding reaction with the radiolabeled probe. Samples were loaded on non-denaturing 6% polyacrylamide gels in low-ionic-strength buffer (22.3 mM Tris, 22.2 mM borate, 0.5 mM EDTA) and electrophoresis performed at 15 V/cm with cooling. The gel was then dried and analyzed by autoradiography.

**Protein phosphorylation in ECs.** To study the effect of  $\alpha$ -tcp on PKC, protein phosphorylation assays were conducted using a modification of the method described by Rozengurt et al. (56). Briefly, EC were grown to confluence in 21-cm<sup>2</sup> cell culture dishes coated with fibronectin, as described above. Once confluent, the media was changed to MCDB 107 containing 5% FBS, with or without  $\alpha$ -tcp, for 72 h. All experiments were done in duplicate dishes. On the day of the assay the EC were metabolically labeled using <sup>32</sup>P-orthophosphate (200  $\mu$ Ci/ml) (ICN, Irvine, CA), in phosphate-free media with or without  $\alpha$ -tcp (45  $\mu$ M) for 2 h at 37°C. Half an hour prior to stimulation, some plates were treated with the PKC inhibitor, H-7 (100  $\mu$ M) (Seikagaku America, Inc., St. Petersburg, FL). The cells were then stimulated with PMA (10 nM), which served as a positive control, or IL-1 $\beta$  (10 ng/ml) for 5 min. The reaction was stopped by removing the incubation media and washing three times with ice cold PBS. This was followed by two 10-min 10% TCA washes and three subsequent PBS washes at 4°C. The TCA-insoluble material was solubilized with 0.25 ml of 1% NP-40 (Sigma Chemical Co., St. Louis, MO) in 10 mM Tris, 10 mM NaCl, and 2 mM MgCl<sub>2</sub>. The lysate was microcentrifuged (14,000 rpm for 15 min) at 4°C. The supernatant containing the cytoplasmic extract was transferred to another microcentrifuge tube, 50  $\mu$ l of 5 $\times$  Laemmli buffer added and the mixture boiled for 3 min before separation by SDS-PAGE, using a 10% gel as described by Laemmli (57). The gels were dried and subjected to autoradiography at –70°C.

**Reagents.** Purified human monocyte IL-1 $\beta$  was a gift of Otsuka America Pharmaceutical Co., Ltd. (Rockville, MD). Bovine  $\alpha$ -thrombin was obtained from U.S. Biochemical Corp (Cleveland, OH). The monoclonal antibody 7A9 against E-selectin and the cDNA probe for E-selectin were generous gifts of Dr. W. Newman (Otsuka America, Rockville, MD) (50). Probucol was supplied by Marion Merrell Dow Pharmaceuticals (Kansas City, MO). N,N-diphenyl-1,4-phenylene diamine (DPPD) was obtained from Aldrich Chemical Company, Inc. (Milwaukee, WI). Desferal was obtained from Ciba-Geigy (Summit, NJ). Ascorbic acid, butylated hydroxytoluene (BHT), tocopherol acetate and dl- $\alpha$ -tcp, heparin, LPS, PMA, and all media components not previously mentioned, were purchased from Sigma Chemical Co.

## Results

**$\alpha$ -tcp inhibits IL-1-induced monocyte adhesion to ECs.**  $\alpha$ -tcp inhibited IL-1-induced U937 cell adhesion to ECs in a time- (Fig. 1) and concentration- (Fig. 2) dependent manner. The IC<sub>50</sub> for  $\alpha$ -tcp was 45  $\mu$ M, a physiologically relevant concentration since plasma levels of this vitamin are normally in the range of 20–35  $\mu$ M (58). The optimal pretreatment time for the inhibitory effect was ~72 h. Unstimulated monocytic cell adhesion to ECs was negligible and unaffected by  $\alpha$ -tcp pretreatment of

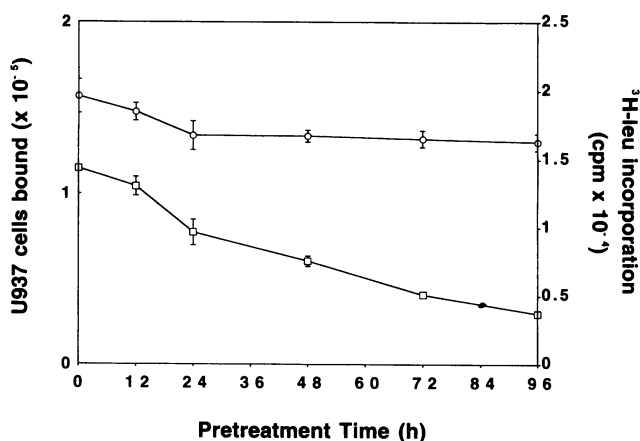


Figure 1. Effect of pretreatment time with  $\alpha$ -tcp on IL-1-induced U937 cell adhesion to (□) and protein synthesis (<sup>3</sup>H]leucine incorporation) by (○) ECs. ECs were pretreated with  $\alpha$ -tcp (45  $\mu$ M) for varying time periods prior to stimulation with IL-1 (10 ng/ml) for 4 h. Error bars represent standard error of the mean.

the ECs. The decrease in IL-1-induced monocytic cell binding to ECs varied in different EC isolates and ranged from 30–70% inhibition in the presence of 45  $\mu$ M  $\alpha$ -tcp. Protein synthesis by the ECs, as measured by [<sup>3</sup>H]leucine incorporation into TCA-precipitable material, was not significantly affected by the  $\alpha$ -tcp pretreatment, except for a slight reduction at the highest concentrations used (Fig. 2). Inhibition of IL-1-induced leukocyte adhesion to ECs was confirmed using freshly isolated blood monocytes (Fig. 3). In multiple experiments with normal monocytes  $\alpha$ -tcp-induced inhibition of adhesion was comparable to that seen with U937 cells.

To determine whether the inhibitory effect of  $\alpha$ -tcp was specific for IL-1-induced adhesion, we tested a series of agonists for the sensitivity of their stimulation to  $\alpha$ -tcp. ECs were treated with LPS (10 ng/ml), PMA (10 nM), and  $\alpha$ -thrombin (30 U/ml), for 4 to 6 h after 72 h pretreatment with  $\alpha$ -tcp (45  $\mu$ M). In every case  $\alpha$ -tcp significantly abrogated the agonist-induced response (Fig. 4).

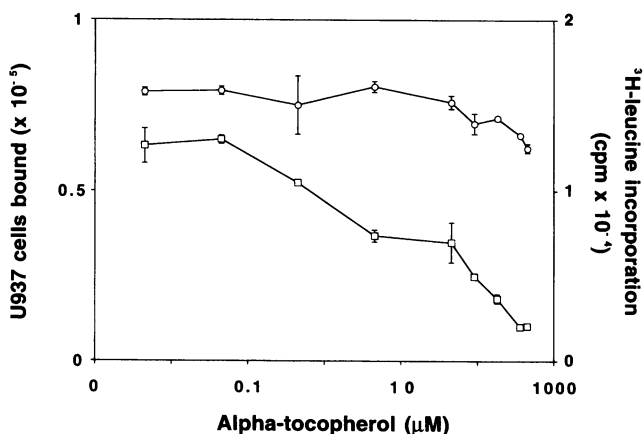


Figure 2. Effect of  $\alpha$ -tcp on IL-1-induced U937 cell adhesion to (□) and protein synthesis (<sup>3</sup>H]leucine incorporation) by (○) ECs. ECs were treated with  $\alpha$ -tcp for 72 h before stimulation with IL-1 (10 ng/ml) for 4 h.

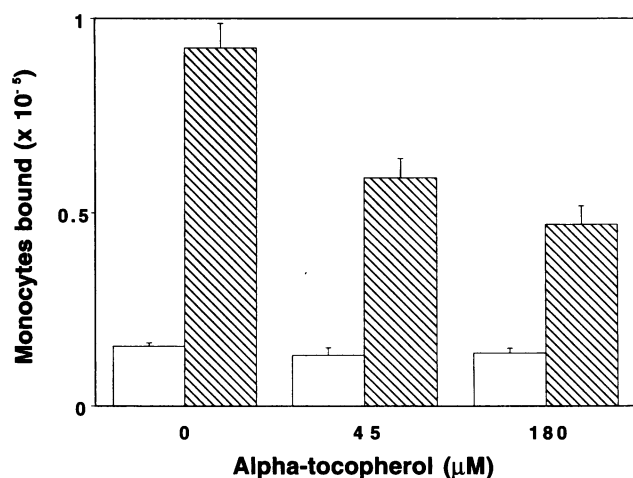


Figure 3. Effect of  $\alpha$ -tcp on IL-1-induced peripheral blood monocyte adhesion to ECs. Experimental conditions were identical to those described for Fig. 2. (□) Basal monocyte binding; (■) IL-1-stimulated monocyte binding. Error bars represent standard error of the mean.

To this point we had used  $\alpha$ -tcp as a pharmacological agent, pretreating the ECs only after they had reached confluence. To study the effect of  $\alpha$ -tcp when used as a growth supplement, ECs were grown to confluence in growth media (MCDB 107 + Heparin + ECGS + 15% FBS), in the presence or absence of  $\alpha$ -tcp (45  $\mu$ M). Upon reaching confluence (usually within 3 d), the media was changed to the pretreatment media (MCDB 107 + 5% FBS and no growth supplements), with or without  $\alpha$ -tcp, for 72 h before stimulation with IL-1. ECs grown with supplemental  $\alpha$ -tcp bound significantly less monocytic cells when stimulated with IL-1 as compared with ECs that had not

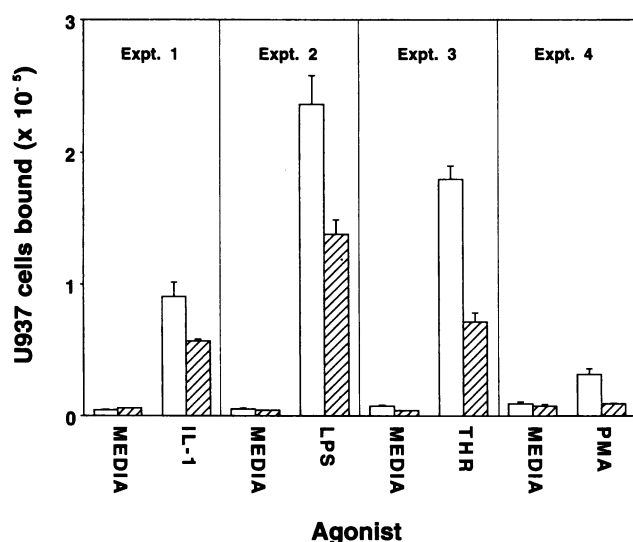


Figure 4. Effect of  $\alpha$ -tcp pretreatment on U937 cell adhesion to ECs in response to multiple agonists of monocyte adhesion. ECs were pretreated with  $\alpha$ -tcp (45  $\mu$ M) for 72 h before stimulation with the different agonists for 4–6 h. Each section represents different experiments, since the responsiveness of the ECs varied depending on isolate, passage number and agonist used. (□) ECs that were not pretreated with  $\alpha$ -tcp, and (■) those that were. Error bars represent standard error of the mean.

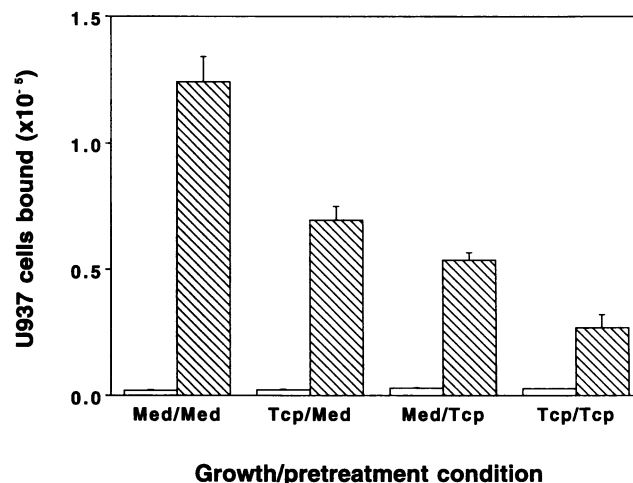


Figure 5. Effect of  $\alpha$ -tcp as a growth supplement on IL-1-induced U937 cell adhesion to ECs. ECs were grown in media with or without  $\alpha$ -tcp (45  $\mu$ M) as a growth supplement. Once confluent, ECs were pretreated for 72 h with or without  $\alpha$ -tcp before stimulation with IL-1 (10 ng/ml), for 4 h. (□) Basal; (■) IL-1-stimulated U937 cell binding. Error bars represent standard error of the mean.

been exposed to the vitamin (Fig. 5). This was true even if no  $\alpha$ -tcp was added during the pretreatment phase of the experiment. The most marked decrease in monocytic cell binding was observed with ECs that were treated with  $\alpha$ -tcp both in the “growth” and “pretreatment” phase of the experiment (Fig. 5). Neither the basal level of binding nor protein synthesis was affected by  $\alpha$ -tcp in either phase (data not shown).

**Effect of  $\alpha$ -tcp on EC expression of E-selectin.** It has been reported that E-selectin is involved in the adhesion of monocytes to cultured ECs (37, 38). We have verified this in our system by demonstrating the ability of an anti-E-selectin monoclonal antibody to dramatically inhibit U937 cell adhesion to IL-1-treated EC in the presence or absence of  $\alpha$ -tcp (Fig. 6). To determine the effect of  $\alpha$ -tcp on E-selectin expression, we quantified this glycoprotein on the EC surface using a mono-

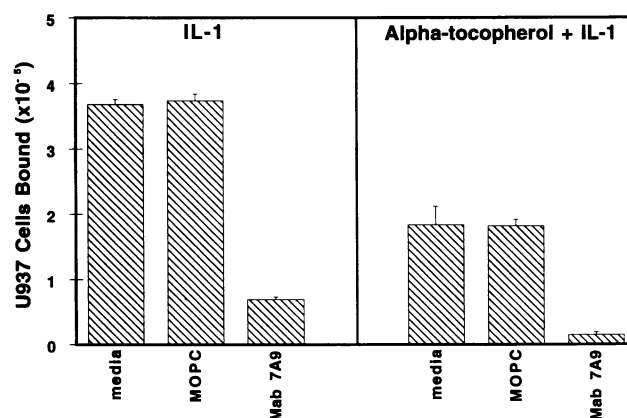
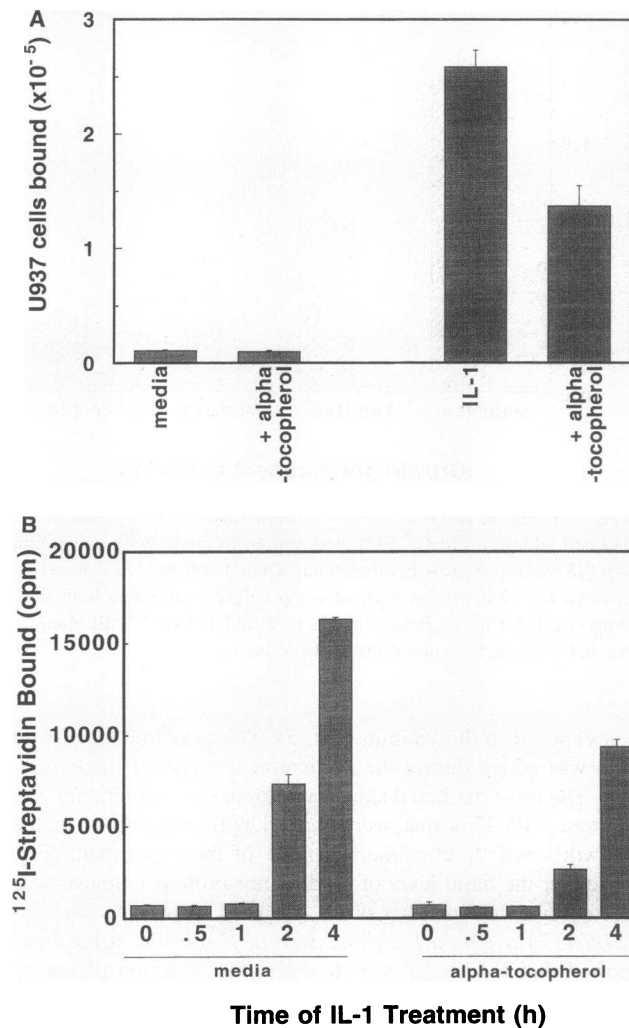
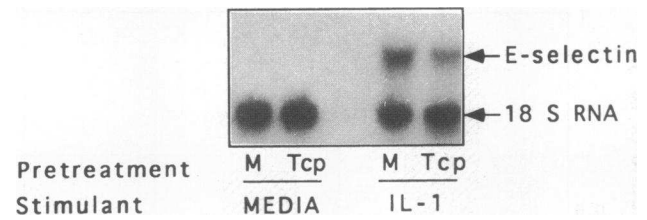


Figure 6. Effect of a monoclonal antibody (mAb 7A9) to E-selectin on U937 cell adhesion to IL-1-stimulated ECs. ECs were pretreated with or without  $\alpha$ -tcp (45  $\mu$ M) for 48 h before stimulation with IL-1 (10 ng/ml) for 4 h. The cells were rinsed and incubated with media, MOPC-21 (5  $\mu$ g/ml) or mAb 7A9 (5  $\mu$ g/ml) for 30 min at 4°C, before the U937 cell binding assay was performed.



**Figure 7.** Effect of  $\alpha$ -tcp on U937 cell adhesion and surface expression of E-selectin by ECs. ECs were pretreated with  $\alpha$ -tcp (45  $\mu$ M) for 72 h before stimulation with IL-1 (10 ng/ml) for 4 h for the adhesion assay and as shown for E-selectin expression. (A) The adhesion assay was performed as described in the legend to Fig. 1. (B) E-selectin surface expression was measured using a monoclonal antibody (7A9) against E-selectin, a biotin-conjugated secondary antibody and <sup>125</sup>I-streptavidin as described in Methods. Error bars represent standard error of the mean.

clonal antibody 7A9 in a sandwich-type radioimmunoassay. Under basal conditions or following  $\alpha$ -tcp pretreatment alone, EC did not express detectable levels of E-selectin. IL-1 stimulation of the ECs caused a marked induction of E-selectin expression which was significantly blunted with  $\alpha$ -tcp pretreatment ( $P < 0.01$ ) (Fig. 7). This decrease in E-selectin expression correlated well with the inhibition of agonist-induced monocytic cell adhesion by  $\alpha$ -tcp (Fig. 7). An unrelated immunoglobulin, MOPC 21, failed to show a corresponding increase in surface binding, indicating the specificity of 7A9 (data not shown). To address the issue of specificity with respect to E-selectin we have assessed the effect of  $\alpha$ -tcp on induced surface expression of ICAM-1. ICAM-1 was found to be constitutively high and only slightly induced at the time point under study which is consistent with the published results of others.  $\alpha$ -tcp had no significant effect on this induction (data not shown).



**Figure 8.** Autoradiograph showing the effect of  $\alpha$ -tcp on steady state message for E-selectin. Northern blot analysis was performed as described in Methods. The two left lanes represent basal E-selectin message, with and without  $\alpha$ -tcp (45  $\mu$ M) pretreatment, while the two right lanes represent IL-1-induced E-selectin message, with and without  $\alpha$ -tcp pretreatment (72 h). Arrows indicate the position of the E-selectin message and the control 18 S RNA band. M, media.

Northern analysis was performed to determine if the cytokine-induced steady state mRNA levels of E-selectin were affected by  $\alpha$ -tcp pretreatment. E-selectin mRNA was not detected under basal conditions in the presence or absence of  $\alpha$ -tcp. IL-1 stimulation of ECs for 2 h resulted in a marked induction of E-selectin mRNA and this induction was significantly abrogated by pretreating the ECs with  $\alpha$ -tcp for 72 h before stimulation (Fig. 8). mRNA levels were normalized to the amount of 18S ribosomal RNA to ensure equal loading of the lanes. The autoradiograph was subjected to densitometric evaluation which revealed a 35% reduction in E-selectin mRNA in response to  $\alpha$ -tcp. This decrease correlated well with the decreased expression of the glycoprotein and decreased agonist-induced monocytic adhesion to ECs.

**Effect of antioxidants on agonist-induced monocytic cell adhesion to ECs.** To determine whether  $\alpha$ -tcp was acting by its free radical scavenging ability, we tested other known antioxidants for their ability to inhibit agonist-induced monocytic cell adhesion. The results were variable; while some of the antioxidants did reduce induced-adhesion, others did not (Table I). Ascorbic acid, a water-soluble antioxidant, in fact appeared to further stimulate induced adhesion. None of the antioxidants tested had any significant effect on basal levels of adhesion. Among the other antioxidants tested only Probucol and N-acetylcysteine (NAC) significantly reduced IL-1-induced adhesion. Probucol (50  $\mu$ M), a lipid-soluble antioxidant, decreased agonist-induced adhesion by 45%; at higher concentrations Probucol was toxic to ECs. NAC (20 mM), a water-soluble antioxidant, reduced IL-1-induced monocytic cell adhesion by 36% without altering protein synthesis. Other antioxidants tested, such as DPPD, Tiron, and Desferal, failed to abrogate agonist-induced adhesion at nontoxic concentrations. When ascorbic acid and  $\alpha$ -tcp were used together, no net effect was observed.

In acting as a free radical scavenger,  $\alpha$ -tcp is itself oxidized. Since we pretreated ECs for 72 h, it was likely that oxidation products of  $\alpha$ -tcp accumulated in the microenvironment of the ECs during this time. We explored the possibility that the oxidation products of  $\alpha$ -tcp may be responsible for the inhibitory effect on stimulated adhesion. Copper sulfate, a known prooxidant, has been shown to accelerate the oxidation of low density lipoproteins (59). Incubation of copper sulfate with  $\alpha$ -tcp would presumably then accelerate oxidation of the vitamin over the 72 h pretreatment period. While copper sulfate alone had no effect on agonist-induced adhesion, when used in combination with  $\alpha$ -tcp, it diminished the inhibitory effect of the

Table 1. Effect of Antioxidants on U937 Cell Adhesion to ECs

Pretreatment	U937 Cells Bound ( $\times 10^{-4}$ )*		
	Control	IL-1	Percent bound compared with IL-1 treated
Experiment No. 1			
Media	0.2 (0.0)	8.1 (0.5)	100
$\alpha$ -tcp (45 $\mu$ M)	0.2 (0.0)	4.0 (0.3)	49
Experiment No. 2			
Media	0.5 (0.0)	9.1 (1.1)	100
Ascorbate (50 $\mu$ M)	0.7 (0.0)	13.6 (0.6)	149
Ascorbate + $\alpha$ -tcp	0.6 (0.0)	9.0 (0.3)	99
CuCO <sub>4</sub> (5 $\mu$ M)	0.7 (0.1)	9.2 (1.1)	101
CuSO <sub>4</sub> + $\alpha$ -tcp	0.8 (0.0)	7.4 (0.4)	79
BHT (45 $\mu$ M)	0.9 (0.1)	9.8 (0.3)	108
$\alpha$ -tcp (45 $\mu$ M)	0.6 (0.0)	5.7 (0.1)	63
Experiment No. 3			
Media	0.6 (0.1)	9.3 (0.3)	100
Probucol (50 $\mu$ M)	0.4 (0.0)	5.2 (0.1)	56
DPPD (1 $\mu$ M)	0.7 (0.1)	9.9 (1.2)	106
Experiment No. 4			
Media	0.5 (0.1)	42.8 (1.5)	100
Desferal (10 $\mu$ M)	—	36.5 (1.6)	85
Experiment No. 5			
Media	0.5 (0.1)	36.9 (0.9)	100
Tiron (100 $\mu$ M)	—	35.9 (1.6)	97
Experiment No. 6			
Media	0.4 (0.1)	12.8 (0.1)	100
NAC (20 mM)	0.2 (0.0)	8.2 (1.5)	64

\* Results represent the mean of triplicate determinations  $\pm$  SEM. Experiments represent one of three similar experiments.

latter, although the levels of adhesion remained lower than the IL-1-stimulated control levels (Table I).

**Effect of  $\alpha$ -tcp on NF- $\kappa$ B activation.** Certain antioxidants have been shown to prevent the activation of the transcription factor NF- $\kappa$ B in lymphoid cells (20, 41). The 5' flanking region of the E-selectin gene contains an NF- $\kappa$ B binding site and this transcription factor has been reported to be important in the regulation of E-selectin gene transcription (39, 53). Reactive oxygen intermediates and PKC have both been reported to activate NF- $\kappa$ B in other cell types (42). Hence,  $\alpha$ -tcp could potentially affect the activity of this transcription factor, either directly by its free radical scavenging property or indirectly by inhibiting PKC. We used EMSAs to determine whether  $\alpha$ -tcp was inhibiting agonist-induced monocytic cell adhesion in EC by blocking NF- $\kappa$ B activation. ECs stimulated with IL-1 (10 ng/ml) for 1 h contained nuclear proteins corresponding to NF- $\kappa$ B (Fig. 9). Pretreatment of the ECs with  $\alpha$ -tcp for 72 h before stimulation with IL-1 failed to demonstrate a decrease in nuclear protein binding to the labeled NF- $\kappa$ B oligonucleotide probe (Fig. 9). Gel shift bands corresponding to NF- $\kappa$ B were not observed in nuclear extracts from unstimulated ECs nor did  $\alpha$ -tcp pretreatment of the ECs induce the appearance of this band. Specificity of the NF- $\kappa$ B binding was verified by competition analysis studies. Coincubating the ECs nuclear extracts with a hundred fold molar excess of unlabeled NF- $\kappa$ B probe, before the binding reaction, caused the disappearance of the gel shift band observed in nuclear extracts from IL-1-stimulated ECs.

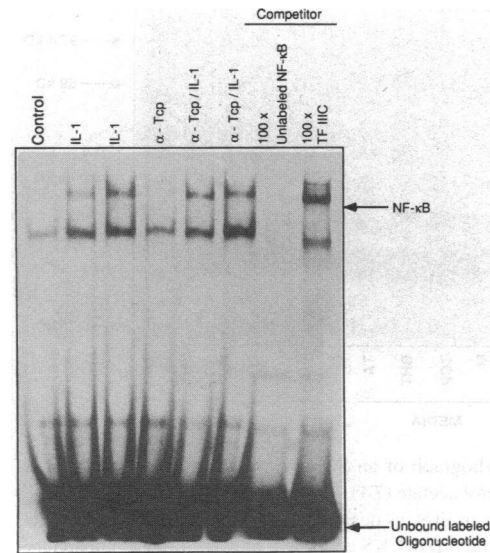
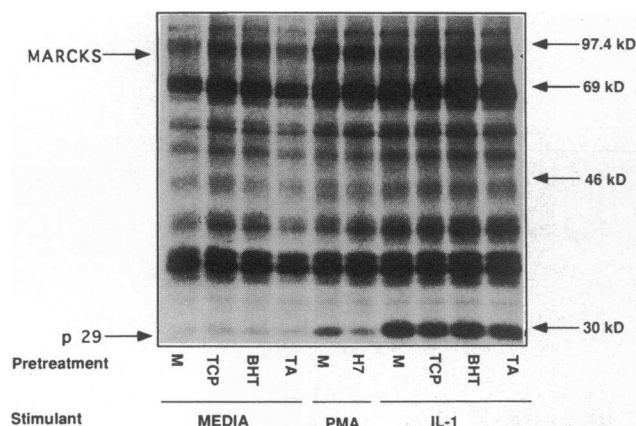


Figure 9. Autoradiograph showing the effect of  $\alpha$ -tcp on NF- $\kappa$ B activation in ECs as measured by electromobility shift assay. Lane 1, control ECs; lanes 2 and 3, IL-1-treated ECs; lane 4, ECs pretreated with  $\alpha$ -tcp (45  $\mu$ M for 72 h); lanes 5 and 6, IL-1-stimulated ECs that have been pretreated with  $\alpha$ -tcp (45  $\mu$ M for 72 h); lane 7, 100-fold excess of unlabeled NF- $\kappa$ B oligomer was preincubated with the nuclear extract before the binding reaction; lane 8, 100-fold excess of an unrelated oligonucleotide (TFIIIC) was preincubated with the nuclear extract prior to the binding reaction.

Similar coincubation with an unrelated oligonucleotide, TFIIIC, caused no change in the NF- $\kappa$ B band (Fig. 9). This result suggested that  $\alpha$ -tcp decreased E-selectin gene expression by an NF- $\kappa$ B-independent mechanism.

**Effect of  $\alpha$ -tcp on PKC.**  $\alpha$ -tcp has been reported to inhibit the activity of PKC in vascular smooth muscle cells and neuroblastoma cells (34). Since PKC is thought to play a role in mediating agonist-induced monocyte adhesion (32), we tested whether  $\alpha$ -tcp exerted its effect by suppressing PKC activity in ECs. We tested the effect of  $\alpha$ -tcp pretreatment of ECs on PKC activity, by quantifying the phosphorylation of the 80-kD, myristoylated, alanine-rich C kinase substrate (MARCKS) protein, that has been shown to be specifically phosphorylated by PKC (56, 60) and a good indicator of PKC activity (61–64). PMA, a well-established activator of PKC, served as a positive control for the phosphorylation of MARCKS. H-7 pretreatment of EC before PMA stimulation, served as a negative control, though the inhibition of MARCKS phosphorylation was not dramatic in this specific experiment. Under basal conditions  $\alpha$ -tcp did not affect the phosphorylation of MARCKS. PMA caused a significant increase in the phosphorylation of this protein. However, when the ECs were stimulated with IL-1, MARCKS phosphorylation was not significantly increased; nor was any decrease in protein phosphorylation observed after pretreatment of the ECs with  $\alpha$ -tcp (Fig. 10). This suggested that IL-1 induction of E-selectin was occurring independent of PKC.  $\alpha$ -Tcp was unable to inhibit the phosphorylation of the MARCKS in ECs induced by thrombin and PMA (data not shown), both of which are known to activate PKC. This suggested that in our intact cell system,  $\alpha$ -tcp appeared to mediate its inhibitory effect on agonist-induced adhesion independent



**Figure 10.** Autoradiograph of an SDS-PAGE showing the effect of  $\alpha$ -tcp (TCP), tocopherol acetate (TA), and butylated hydroxytoluene (BHT) on phosphorylation in ECs, as described in Methods. Arrows on the left of the figure represent MARCKS and p29. Lanes 1–4, basal phosphorylation of proteins in ECs, lanes 5 and 6, phosphorylation of proteins in response to PMA, with and without H7 pretreatment prior to stimulation; lanes 7–10, IL-1-stimulated phosphorylation of proteins in ECs. TA is an ester of  $\alpha$ -tcp that is known to have no effect on PKC.

of PKC. Additionally, a 29-kD protein (p29), initially reported by Levin and Santell to undergo PKC-independent phosphorylation in ECs in responses to IL-1 (65, 66), was also noted to be phosphorylated in our studies. This protein has been recently found to be identical to heat shock protein 27 (HSP27), the phosphorylation of which appears to be PKC dependent (67).  $\alpha$ -Tcp pretreatment of EC did not inhibit the phosphorylation of p29 in our experiment (Fig. 10).

## Discussion

$\alpha$ -tcp, a lipid-soluble vitamin, has been shown to protect the plasma membrane from oxidant stress by virtue of its ability to function as a naturally occurring, chain breaking antioxidant (21, 26).  $\alpha$ -tcp has also been reported to be an anti-atherogenic agent (22). Recently published epidemiological studies suggest a beneficial role for  $\alpha$ -tcp supplementation in the prevention of coronary events (68, 69), lending further support to experimental observations. While it is largely believed that  $\alpha$ -tcp serves a protective function by preventing the oxidation of LDL, a molecule believed to play a central role in the development of the atherosclerotic plaque, the exact mechanisms involved are incompletely understood. In this study we demonstrate that  $\alpha$ -tcp may also have effects at the level of vascular cell gene expression. Our results suggest an additional and novel mechanism by which  $\alpha$ -tcp may prevent the progress of atherosclerosis, that is by inhibiting induced monocyte adhesion to the endothelium.

We have demonstrated in this study that  $\alpha$ -tcp inhibits agonist-induced monocyte adhesion to EC with an  $IC_{50}$  of 45  $\mu$ M, consistent with normal plasma levels.  $\alpha$ -tcp was not cytotoxic, except at concentrations significantly higher than physiological levels. Furthermore, inhibition of the agonist-induced response occurred with multiple stimulators, suggesting that the site of action of  $\alpha$ -tcp was downstream from the agonist receptor. We also observed that when ECs were cultured in media containing  $\alpha$ -tcp as a nutritional supplement they were less adhesive to

monocytic cells after IL-1 treatment. This was true even if the ECs were not exposed to  $\alpha$ -tcp during the “pretreatment” phase of the experiment. Since  $\alpha$ -tcp has a half-life of 56–74 h in intact EC membranes (70), its presence in both phases of the experiment would conceivably have a cumulative effect, leading to an increase in effective concentration of  $\alpha$ -tcp in the membranes of ECs. This could account for the observation that ECs exposed to  $\alpha$ -tcp during both phases bound the least number of monocytic cells. A case could therefore be made for  $\alpha$ -tcp supplementation during procedures involving EC denudation, since in vitro experiments suggest that wounded ECs are especially adhesive to monocytes (48).

To determine whether the inhibitory effect of  $\alpha$ -tcp was a function of its antioxidant property, we tested other known lipid-soluble and water-soluble antioxidants to determine if they had similar inhibitory effects on agonist-induced monocyte adhesion. The results varied depending on the antioxidant tested. Ascorbate was found to be stimulatory, rather than inhibitory, which could be explained by the observation that under certain conditions ascorbate functions as a pro-oxidant, rather than an antioxidant, possibly due to the ability of ascorbic acid to release transitional metal ions from protein complexes leading to the generation of free radicals (71–77). Ascorbic acid has been shown to protect  $\alpha$ -tcp from oxidation and in doing so enhance the latter’s antioxidant activity (78–80). In our studies the addition of ascorbate plus  $\alpha$ -tcp led to no net effect on agonist-induced monocyte adhesion, which suggests the possibility that the  $\alpha$ -tcp reduced the enhancement in IL-1-induced adhesion caused by ascorbate alone.

The inhibitory effect of Probucol and NAC, two structurally unrelated antioxidants, could make a case for  $\alpha$ -tcp functioning as an antioxidant in exerting its inhibitory effect. This must, however, be interpreted with caution since several other antioxidants had no inhibitory effect on induced adhesion. Oxidation products of  $\alpha$ -tcp are probably not responsible for decreased monocytic cell adhesion since pretreatment of ECs with copper sulfate and  $\alpha$ -tcp together did not enhance the inhibitory effect of the latter. The degree of variability in the effects of the different antioxidants on agonist-induced monocyte adhesion to ECs may reflect the diverse free radical scavenging systems present within ECs.

To delineate the mechanisms through which  $\alpha$ -tcp may be acting, we have studied two possible targets along the signal transduction pathway of inducible adhesion molecules, i.e., PKC and the nuclear transcription factor NF- $\kappa$ B. The role of PKC as a mediator of cytokine-induced expression of adhesion molecules by, and leukocyte adhesion to ECs remains controversial, with some studies indicating a role for the enzyme, and other reports contesting this (32, 53, 81–85). PMA and thrombin stimulation of ECs did cause an increase in phosphorylation of the MARCKS protein and  $\alpha$ -tcp did not inhibit this event (data not shown), suggesting a PKC-independent mode of action of the vitamin. Further evidence supporting this conclusion is the inability of  $\alpha$ -tcp to prevent the IL-1-induced phosphorylation of a 29-kD protein in EC (Fig. 10), the phosphorylation of which has been reported to be PKC dependent (67).

A number of different cytokines and biological response modifiers have been shown to exert their cellular effects through the activation of nuclear transcription factors, a notable example of which is the trimolecular complex, NF- $\kappa$ B (42). Sequence analysis of the 5’ flanking region of the E-selectin gene in ECs, has revealed an NF- $\kappa$ B binding site (39). The oxidation of NF-



$\kappa$ B by free radicals has been hypothesized as one mechanism by which this transcription factor is activated (20, 41, 86). Furthermore, IL-1, TNF- $\alpha$ , LPS, and PMA have all been reported to increase intracellular levels of free radicals (86). Although a "cause-effect" relationship has not been directly demonstrated, certain thiol-containing antioxidants have been reported to prevent the activation of NF- $\kappa$ B in some lymphoid cell lines (20, 41), suggesting a role for free radicals in gene activation. It is conceivable then, that  $\alpha$ -tcp was acting similarly in our EC system, inhibiting E-selectin gene transcription by preventing the activation of NF- $\kappa$ B by free radicals generated within the cells. While we have confirmed the activation of NF- $\kappa$ B by IL-1 in EC,  $\alpha$ -tcp did not affect the activation of this transcription factor at vitamin concentrations that caused marked decreases in E-selectin mRNA and protein expression. Our oligomer probe for these studies was derived from the sequence of the E-selectin promoter and therefore the nuclear factor we quantitated was of direct relevance to our other observations. It must be kept in mind, however, that a family of NF- $\kappa$ B proteins are known to exist and that some of these might be much more sensitive to the presence of antioxidants. A report appeared during revision of this manuscript that supports the contention that antioxidants, in their case PDTC, do not suppress activation of NF- $\kappa$ B using the E-selectin consensus sequence in a gel shift assay (87).

The mechanism by which  $\alpha$ -tcp exerts its effects remain to be elucidated. However, our study points to a novel target site for the protective action of  $\alpha$ -tcp in which this vitamin acts at the cellular level to decrease E-selectin gene expression in ECs with a consequent decrease in leukocyte adhesion. In so doing,  $\alpha$ -tcp may prove beneficial in multiple pathological situations involving the adhesion of leukocytes, including inflammation, and atherosclerosis.

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