ELSEVIER

# Vitamin E protects human aortic endothelial cells from cytotoxic injury induced by oxidized LDL in vitro

**Antonio Martin, Dayong Wu, Simin N. Meydani, Jeffrey B. Blumberg, and Mohsen Meydani**

*From the Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, Boston, MA, USA*

*Human aortic endothelial cells (HAEC) were used to investigate the protective role of vitamin E (α-tocopherol) against oxLDL in vitro. HAEC exposed to oxLDL (200 mg LDL protein/mL) significantly decreased cell viability by 36%. HAEC presupplemented with 14, 28, and 53 μM α-tocopherol in the media dose-dependently increased cell resistance to cytotoxic injury from oxLDL (200 μg protein/mL) as reflected by the number of cells remaining attached to the flask: $3.3 \times 10^5$, $3.8 \times 10^5$, $4.9 \times 10^5$, and $5.2 \times 10^5$, respectively, versus $5.1 \times 10^5$ in control cultures. Prostacyclin ($PGI_2$) produced by HAEC increased after 12-hr incubation with oxLDL, correlating positively with cell damage and negatively with α-tocopherol concentration in the cells. Under the same conditions after a 22-hr incubation, a significant decrease in $PGI_2$ production was observed only in HAEC pretreated with a high α-tocopherol concentration [unsupplemented HAEC: 839 ± 296 pg/mL versus α-tocopherol supplemented (53 μM): 241 ± 79 pg/mg]. Interleukin-1β production was not detected in HAEC under control conditions or after exposure to oxLDL. HAEC supplemented with α-tocopherol but not exposed to oxLDL maintained significantly higher α-tocopherol concentrations compared with those exposed to oxLDL. Under phase contrast microscopy, HAEC showed a pronounced elongation of their normal cobblestone morphology after oxLDL exposure; this change was reduced by α-tocopherol pretreatment. Thus, supplementation with α-tocopherol protected HAEC by reducing the cytotoxic effect of oxLDL, modulating $PGI_2$ production without having any effect on IL-1β.* (J. Nutr. Biochem. 9:201–208, 1998) *© Elsevier Science Inc. 1998*

**Keywords:** α-tocopherol; oxidized low-density lipoprotein; prostacyclin; interleukin-1; human aortic endothelial cells

## Introduction

The risk of atherosclerosis is increased when plasma levels of LDL-cholesterol exceed 160 mg/dL.[1] However, the cellular and molecular mechanisms linking hypercholesterolemia and elevated LDL to atherosclerosis remain unclear.

This project has been funded at least in part with Federal funds from the U.S. Department of Agriculture, Agricultural Research Service under contract number 53-K06-01. The contents of this publication do not necessarily reflect the views or policies of the U.S. Department of Agriculture, nor does mention of trade names, commercial products, or organizations imply endorsement by the U.S. government.
Address correspondence and reprint requests to Dr. Mohsen Meydani, Vascular Biology Laboratory, or Dr. Antonio Martin, Neuroscience Laboratory, Jean Mayer USDA Human Nutrition Research Center on Aging at Tufts University, 711 Washington Street, Boston, MA 02111 USA.
Received September 12, 1997; accepted December 10, 1997.

The oxidative modification of LDL (oxLDL) may play a critical role in the pathogenesis of atherosclerosis[2] as suggested by in vivo observations including the presence of epitopes of oxLDL in atherosclerotic lesions,[3,4] and elevated titers of circulating auto-antibodies against oxLDL in patients with carotid atherosclerosis.[5]

The modification of LDL seems to be a prerequisite for its uptake by macrophages;[2] when LDL is incubated with cultured endothelial or smooth muscle cells, it is taken up much more rapidly.[6] oxLDL is taken up by macrophages via scavenger receptors,[7] which can lead to a massive accumulation of cholesterol ester and the formation of a foam cell.[2,8] LDL is susceptible to free radical-mediated oxidation both in vitro and in vivo.[9,10] The modification introduced in LDL during incubation with all major cell types present in the arterial wall,[6,11,12] and in cell free systems[13] is attributable to the oxidation of lipids that subsequently modify the protein.[13,14]

Nutritional Biochemistry 9:201–208, 1998
© Elsevier Science Inc. 1998
655 Avenue of the Americas, New York, NY 10010

0955-2863/98/$19.00
PII S0955-2863(97)00185-X

Injury to endothelial cells (EC) represent one of the early events in the pathogenesis of atherosclerosis.[15,16] Injury to vascular endothelium increases vessel wall permeability and accumulation of macromolecules in the subendothelial space.[17] The close association of lipid peroxidation events with cytotoxicity observed in vitro suggest that this phenomenon may be an important mechanism in the development of atherosclerosis. oxLDL has been shown to be cytotoxic for EC[18] and smooth muscle cells[14] and to impair endothelium-dependent arterial relaxation.[19] Furthermore, oxLDL enhances the adherence of monocytes to EC[20] and stimulates transcription and secretion of monocyte chemotactic protein-1 (MCP-1) by cultured human aortic EC (HAEC) and smooth muscle cells.[21] In response to oxidative stress induced by oxLDL EC produce prostacyclin ($PGI_2$), which is a vasodilator and potent anti-aggregator of platelets.[22–24] Therefore, formation of oxLDL in the arterial wall may have a profound effect on HAEC production of chemotactic factors, cytokines, growth factors, and $PGI_2$, known to play important roles in the development and progression of atherosclerosis.

Epidemiologic data, clinical trials, and studies of animal models suggest that dietary and supplemental vitamin E reduce the risk of cardiovascular disease.[25] Vitamin E is the principal fat-soluble antioxidant in membranes, where it serves to protect lipids and membrane-bound enzymes from oxidative modification[26–28] and also has modulatory effects on the oxidative signal transduction pathway in the EC.[29,30] Vitamin E also protects EC from oxidative stress damage induced by lipid peroxides in vitro.[31] Investigations exploring the effect of oxLDL on aortic endothelial cells from healthy human, i.e., HAEC, one of the principal vascular cells directly involved in the atherosclerotic process, are lacking. In addition to its role as an antioxidant in LDL,[13] vitamin E may contribute to the reduction of risk of atherosclerosis by increasing HAEC resistance to oxLDL cytotoxicity. Therefore, the goal of this study was to investigate the role of vitamin E on protecting HAEC when exposed to oxLDL.

## Methods and materials

### *Human aortic endothelial cell culture*

HAEC were purchased from Clonetics Laboratories (San Diego, CA USA) and cultured in M-199 medium (Gibco, Grand Island, NY USA) containing 5% fetal bovine serum (FBS) (Sigma Chemical Co., St. Louis, MO USA). Cultured media also contained 5 ng/mL endothelial cell-derived growth factor (EDGF) prepared from bovine retina, 100 μg/mL heparin (Sigma), 100 U/mL penicillin, 100U/mL streptomycin, and 1.25 μg/mL amphotericin B (Sigma). Cells were seeded into T-25 flasks (Corning, Corning, NY USA) coated with 1% gelatin; grown to confluence in 5% CO2, 20% O2; and balance N2 at 37°C; and passaged using calcium- and magnesium-free Hanks balanced saline solution (HBSS) (Gibco), 0.05% trypsin, and 0.02% EDTA (Sigma). The culture medium was replaced every 2 days until the cells attained confluence. HAEC were cultured in gelatin-coated six-well plates (Corning), examined by phase contrast microscopy, and photographed to monitor cell growth and confluence. The 24-hr post-confluent HAEC from Passage 6 were used for experiments. HAEC were characterized by the presence of von Willebrand factor antigen using immunofluorescent microscopy.[32] The HAEC had been tested by the vendor (Clonetics Laboratories) for sterility, mycoplasma, HIV and HBV infection, normal morphology, and rate of proliferation.

After treatment of HAEC with trypsin 0.05% for 3 min or until 80% of the cells detached, cell viability was determined using a hemocytometer after an aliquot of suspended cells was mixed with trypan blue. Cell viability was expressed as the percent of cells showing exclusion of trypan blue.

### *Vitamin E supplementation of HAEC*

A stock solution of d-α-tocopherol (Kodak, Springfield, NJ USA) containing 3–10 mg/mL in ethanol was prepared and stored at −70°C. HAEC were supplemented with d-α-tocopherol by drying the required amount of stock solution and redissolving with 100% ethanol to achieve a final concentration of 0.05% ethanol in the culture media. The d-α-tocopherol-ethanol solution was mixed with FBS (10% final concentration in the media) and incubated at 37°C for 15 min, mixing gently every 5 min. The FBS solution containing d-α-tocopherol and ethanol was mixed with M-199 culture media. HAEC were incubated with the medium containing d-α-tocopherol for 24 hr.

### *Experimental design*

Confluent HAEC grown in six-well plates were incubated in media M-199 containing 0–54 mM d-α-tocopherol for 24 hr. The media were subsequently removed, HAEC washed twice with M-199, and new media containing oxLDL (200 μg LDL protein/mL) added. HAEC were incubated for 12 or 22 hr then washed with HBSS and removed with 0.05% trypsin. Cells were counted, and after the addition of 1 mM EDTA and 0.01% BHT, stored at −70°C until vitamin E analysis. Supernatant from the culture was collected in aliquots and stored at −70°C for later determination of vitamin E, prostacyclin ($PGI_2$), and interleukin (IL)-1β.

### *Vitamin E determination*

The vitamin E (α-tocopherol) content of HAEC was measured by reverse-phase HPLC. Briefly, after sonication, HAEC were saponified with 30% KOH in the presence of pyrogallol at 50°C for 30 min. Tocol (a gift from Hoffmann-La Roche) was added to the mixture as an internal standard. Tocopherols were extracted into 2.5-mL hexane containing 0.002% butylated hydroxy toluene (BHT), dried under nitrogen gas, and reconstituted in 40-μL methanol. Tocopherols were separated using a 3-μm C18 reverse-phase column with 100% methanol as the mobile phase.[33] Eluted peaks were detected with a Perkin-Elmer 650-15 fluorescence spectrophotometer (Norwalk, CT USA) set at 292 nm excitation and 330 nm emission. Peaks were integrated with a Waters 860 System, and vitamin E concentration expressed as nmol/mg protein. α-Tocopherol concentration in the media was measured by HPLC after extracting of 100 μL media (mixed with 100 μL ethanol containing tocol) with 0.5 mL hexane containing 0.002% BHT.

### *$PGI_2$ and IL-1β determination*

After the HAEC were incubated with oxLDL for 22 hr, 200 μL aliquots of media were collected and stored at −70°C for $PGI_2$ and IL-1β determination. $PGI_2$ was analyzed by radioimmunoassay (RIA) for 6-keto-PGF1a, the principal hydration product of $PGI_2$, as described by Hwang et al.[34] and McCosh et al.[35] IL-1β in the media was measured by RIA according to Endres et al.[36] and Lonnemann et al.[37] IL-1β concentration was expressed per mL media and $10^6$ cells.

### *Isolation of LDL*

LDL (1.019 to 1.063 g/mL) was isolated from plasma by single vertical spin discontinuous density gradient ultracentrifugation as described by Chung et al.[38] Blood was obtained from normolipidemic individuals (<160 mg LDL-cholesterol/dL), in a vacutainer containing sodium heparin, and centrifuged for 15 min at 2,000 rpm. The density of the plasma was adjusted to 1.21 g/mL by the addition of solid KBr (0.365 g/mL). Tubes loaded with sample and gradient were immediately placed in a near vertical tube (NVT) 90 rotor, and centrifuged in a Beckman L7–80M ultracentrifuge at 7°C and 70,000 rpm for 90 min with slow acceleration and deceleration modes. This procedure yields three lipoprotein fractions banded distinctly, with VLDL at the top, LDL in the upper middle, and HDL in the lower middle portion of the tube.[38] The isolated LDL was dialyzed using membrane with a cut molecular weight of 8000 against 150 mM NaCl, 1 mM EDTA at pH 7.4, filtered and stored at 4°C under nitrogen until used within 4 hr after isolation. Protein in LDL was measured by the method of Lowry et al.,[39] using bovine serum albumin as standard. The concentration of cholesterol in isolated LDL was measured using a commercial diagnostic kit (Boehringer Mannheim, Indianapolis, IN USA).

### *Oxidative modification of LDL (oxLDL)*

LDL (600 μg LDL protein/mL) was incubated with 10 mM 2,2Õ-azobis-2-amidinopropane (AAPH) at 37°C for 2 hr then dialyzed against normal saline containing 1 mM EDTA at 4°C for 8 hr by using a membrane with the cut molecular weight of 8,000. After dialysis, the LDL was sterilized by passing it through a 0.2-μm filter. The extent of oxidative modification of LDL was determined by measuring its phosphatidylcholine hydroperoxide (PC-OOH) content. The concentration of AAPH (10 mM) used to oxidize LDL and the length of the incubation are conditions that generate a mild oxidized LDL, as other researchers have shown previously.[40] LDL modification was assessed by measuring the formation of PC-OOH. PC-OOH is oxidation product of phosphatidylcholine, which is formed along with cholesterolester hydroperoxides during LDL oxidation.[41] This is the one of the most sensitive and specific assay to detect very low level of peroxidation product of polyunsaturated fatty acids of phospholipids in LDL particle. The extent of LDL protein modification was characterized by gel electrophoresis of native LDL and oxLDL (see below).

### *Determination of phosphatidylcholine hydroperoxide (PC-OOH)*

LDL or oxLDL aliquots were extracted with redistilled chloroform:methanol (2:1, vol/vol) containing 0.02% BHT. The ratio of extraction solvent to plasma was 4:1. After centrifugation, the bottom layer was collected and dried in a rotary evaporator. The residue was reconstituted in 60 μL chloroform:methanol (2:1, vol/vol) and 20 μL injected into the HPLC. PC-OOH was measured by HPLC post-column derivatization and detection of chemiluminescence.[42] Briefly, the sample was injected into a 25-cm silica column packed with 5 μm particles. PC-OOH was eluted by applying a mobile phase containing 90% methanol and 10% 2-propanol at a flow rate of 1.4 mL/min. The eluted PC-OOH was reacted with a chemiluminescence reagent mixture containing 1.21 mM cytochrome C and 8.47 mM luminol (3-aminophthalhydrazide) (Sigma) at pH 10.7. The chemiluminescence was measured with a Tohoku CL-110 detector (Tohoku Electronic Ind., Sendai, Japan). The detected peaks were integrated with a Waters 860 system. The PC-OOH standard was synthesized from L-α-phosphatidylcholine, β-linoleoyl-g-palmitoyl (Sigma) using rose bengal as a photosensitizer and air to generate singlet oxygen.[43] The mixture was irradiated with a high-intensity visible light beam condensed and guided by fiber optics (American Optical, Southbridge, MA USA) for 12 hr at ambient temperature. The concentration of standard was calibrated with cumene hydroperoxide using potassium iodide and the starch reaction.[43] A calibration curve was generated by injecting different concentrations of PC-OOH standard into the HPLC-chemiluminescence system for each set of LDL analysis for PC-OOH analysis.

### *Electrophoresis*

Native LDL and oxLDL were examined for Apo B100 modification by a denaturing gel system using 5–15% polyacrylamide gradient gels (Gibco). Five μg LDL protein samples were loaded onto gels. The running buffer contained 40 mM Tris-HCl, pH 7.4, 20 mM sodium acetate, 3 mM EDTA, and 0.2% SDS. Electrophoresis was performed at 125 V for 70 min. Gels were stained for 4 hr in 25% methanol, 10% acetic acid, and 0.1% coomasie brilliant blue R-250, and destained overnight in the same solution without dye.[44]

### *Statistical analysis*

Analysis of variances followed by Students' *t*-test comparison was used to assess the significance of differences of the measured parameters between treated and untreated HAEC. Data are presented as mean ± SD with $P < 0.05$ was considered significant and denoted by asterisk.

## Results

### *α-Tocopherol supplementation of HAEC*

We have shown previously that α-tocopherol is incorporated into HAEC in a concentration-dependent manner.[30] Similarly, HAEC were incubated in the media, which were supplemented with 0, 14, 28, and 53 μM α-tocopherol for 24 hr showed concentration-dependent increases in α-tocopherol (*Figure 1*, solid bars). However, when these cells were exposed to oxLDL, α-tocopherol was consumed by 64, 44, 35, and 46% respectively ($P < 0.05$) (*Figure 1*, hatched bars). oxLDL used in these experiments contained significant concentration of PC-OOH (6–8 nmol/mg LDL protein), compared with the control LDL no exposed to AAPH. The agarose gel electrophoresis of AAPH-oxidized LDL showed a significant shift in the mobility with appearance of second small band, indicating changes in charges of protein and some protein fragments attributable to oxidation (data not shown).

### *Effect of oxidized-LDL on cell viability*

After incubation of HAEC with oxLDL (200 μg protein/mL) for 22 hr, cell viability was significantly reduced from $5.1 \pm 0.3 \times 105$ cells to $3.3 \pm 0.4 \times 105$ cells ($P < 0.05$) (*Figure 2*). Concentrations of oxLDL from 100 to 300 mg LDL protein/mL were tested, and the 200 μg/mL dose was found the most suitable based on cell viability test for these experiments, without causing extreme cell cytotoxicity (data not shown). When HAEC were enriched with α-tocopherol in the media and then exposed to oxLDL, cell damage was significantly less and cell viability was higher than in the unsupplemented cells (*Figure 2*). HAEC en-

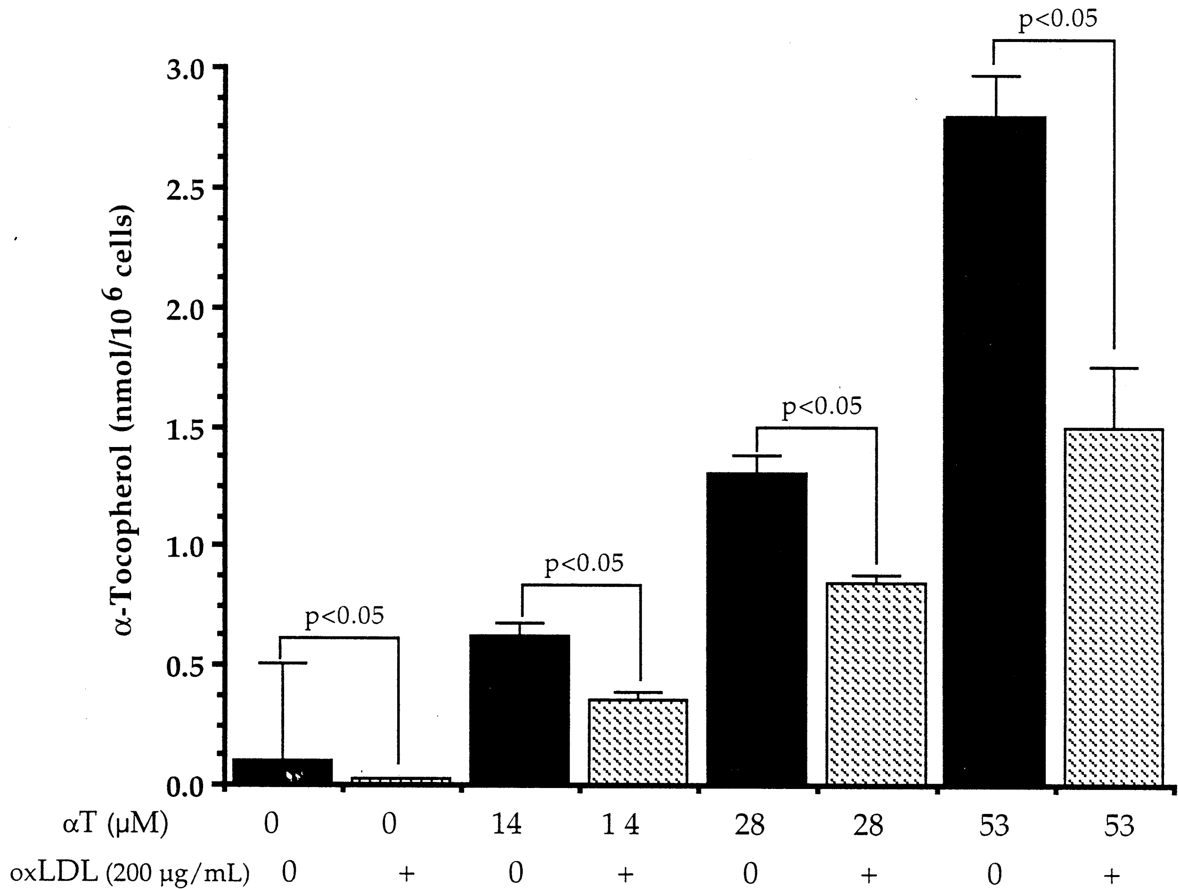


**Figure 1** α-Tocopherol concentration (mean ± SD) in HAEC with or without oxLDL and α-tocopherol. Each point represents the mean of two independent experiments, with triplicates in each experiment.

riched with 53 μM α-tocopherol were fully protected against the cytotoxic effect of oxLDL.

### *α-Tocopherol consumption during oxLDL exposure*

The concentration of α-tocopherol in unsupplemented HAEC after incubation with oxLDL was decreased by 64%. In HAEC presupplemented with 14, 28, and 53 μM α-tocopherol, exposure to oxLDL reduced the α-tocopherol content of the cells by 44, 35, and 46%, respectively, compared with HAEC supplemented with α-tocopherol but not exposed to oxLDL ($P < 0.05$) (*Figure 1*). Even though the percentages of α-tocopherol consumed were similar, they represent different concentrations of total α-tocopherol consumed, being greater in those cells that had higher concentrations before exposure to oxLDL. The higher α-tocopherol doses may be associated with a larger number of α-tocopherol molecules located in the HAEC membrane interacting with oxLDL to reduce its cytotoxic effect. Thus, the increased protection provided by α-tocopherol against oxLDL in a concentration-dependent manner was accompanied by an increased utilization of α-tocopherol.

### *Effect of oxLDL and α-tocopherol on $PGI_2$ and IL-1β production by HAEC*

Exposure of HAEC to oxLDL for 12 hr increased $PGI_2$ production by 6-fold compared with unexposed controls

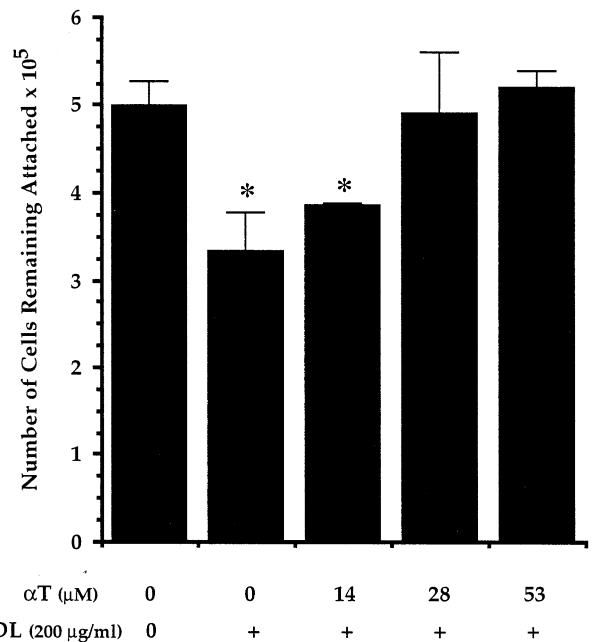


**Figure 2** Effect of oxLDL on HAEC cytotoxicity as expressed by number of cells remaining attached to the flask (mean ± SD from triplicates wells) after 22 h incubation at 37°C. *$P < 0.05$ compared to no oxLDL addition. Each point represents the mean of three independent experiments, with triplicates in each experiment.

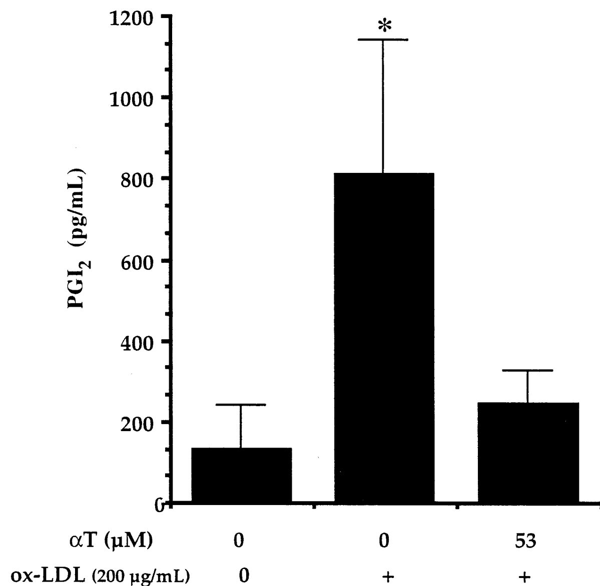


**Figure 3** $PGI_2$ production (mean ± SD) by HAEC after 12 hr incubation with oxLDL and/or α-tocopherol. $*P < 0.05$ compared to other treatments. Each point represents the mean of three independent experiments, with triplicates in each experiment.

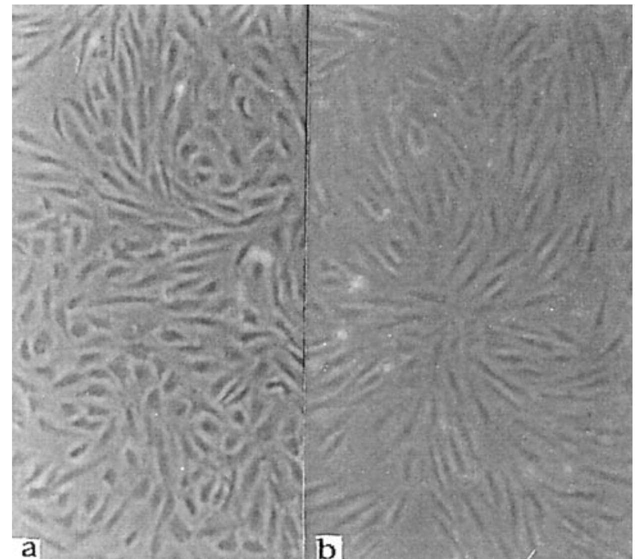


**Figure 4** Phase contrast photomicrographs of HAEC incubated for 12 hr without oxLDL (a), or with oxLDL (b). Each phase contrast photomicrograph is representative of four independent experiments, with triplicates in each experiment.

(839 ± 296 vs. 129 ± 114 ng/mL, $P < 0.05$) (*Figure 3*). In contrast, the oxLDL-induced production of $PGI_2$ by HAEC pretreated with 53 μM α-tocopherol was significantly lower than unsupplemented HAEC. However, increasing incubation time with oxLDL to 22 hr, $PGI_2$ production by HAEC increased by 9-fold (*Table 1*). Presupplementation of HAEC with 14 and 28 μM α-tocopherol was not effective in reducing oxLDL-induced $PGI_2$ production relative to controls. Whereas, HAEC presupplemented with 53 mM α-tocopherol completely prevented oxLDL-induced $PGI_2$ production (*Table 1*). Despite substantial variation in $PGI_2$ data obtained here, the values are consistent with those reported for human saphenous EC exposed to oxLDL.[45] IL-1β production by HAEC (<4 pg/mL) was not detected in control cultures nor after α-tocopherol and/or oxLDL treatment.

### *Effect of oxidized-LDL on HAEC morphology*

After 12 hr of incubation with oxLDL (200 μg protein/mL), HAEC showed changes in their morphology as detected by phase-contrast microscopy, specifically a pronounced elongation of their normal cobblestone appearance (*Figure 4*). However, after 22 hr of incubation with oxLDL, HAEC showed dramatic changes in morphology. This was associated with a significant increase in cell death and reduction in the number of viable cells in the culture (*Figure 5b*). Although morphological changes were evident in all oxLDL-exposed cells, the extents of the changes and cytotoxicity attributable to a longer exposure to oxLDL were relatively lower with increased pre-enrichment of HAEC with α-tocopherol (*Figure 5*c–e).

**Table 1** Prostacyclin ($PGI_2$) production by HAEC after 22-hr incubation with oxLDL

| Treatment group | oxLDL (μg/mL) | Presupplemented vitamin E (μM) | $PGI_2$ (pg/mL)[1] |
|---|---|---|---|
| a (control) | 0 | 0 | 129 ± 114 |
| b | 200 | 0 | 1128 ± 978* |
| c | 200 | 14 | 2280 ± 1482* |
| d | 200 | 28 | 941 ± 1307 |
| e | 200 | 53 | 195 ± 189 |

[1]Values are mean ± SD.
$*P < 0.05$ *compared to control and treatment e.*

## Discussion

HAEC presupplemented with α-tocopherol, protected the cells from cytotoxic injury induced by oxLDL and decreased their production of $PGI_2$, in a dose-dependent manner. The range of α-tocopherol concentrations selected for this study (14 to 53 μmol/L) are comparable to plasma concentrations common among healthy adults (12 to 60 μM with >22 μmol/L usually achieved by supplemental intake).[46,47] α-Tocopherol has been shown to have a protective effect against free radical-induced oxidative damage in several in vitro cell systems including umbilical vein,[48] porcine pulmonary artery,[49] rat heart muscle,[50] and rat aortic smooth muscle.[51] However, the in vitro model used here, HAEC and oxLDL as a stimulus for oxidative stress, is particularly relevant to atherosclerosis in human.

As part of the "oxidative modification hypothesis" of atherosclerosis, oxLDL in addition to its cytotoxic effect on the EC, it can stimulate HAEC to produce monocyte chemotactic protein 1 (MCP-1), and increases recruitment of monocytes into the arterial wall.[52] As a corollary of this hypothesis, antioxidants that protect HAEC from oxLDL-induced effects, should limit the development of atherosclerosis.[25,53]

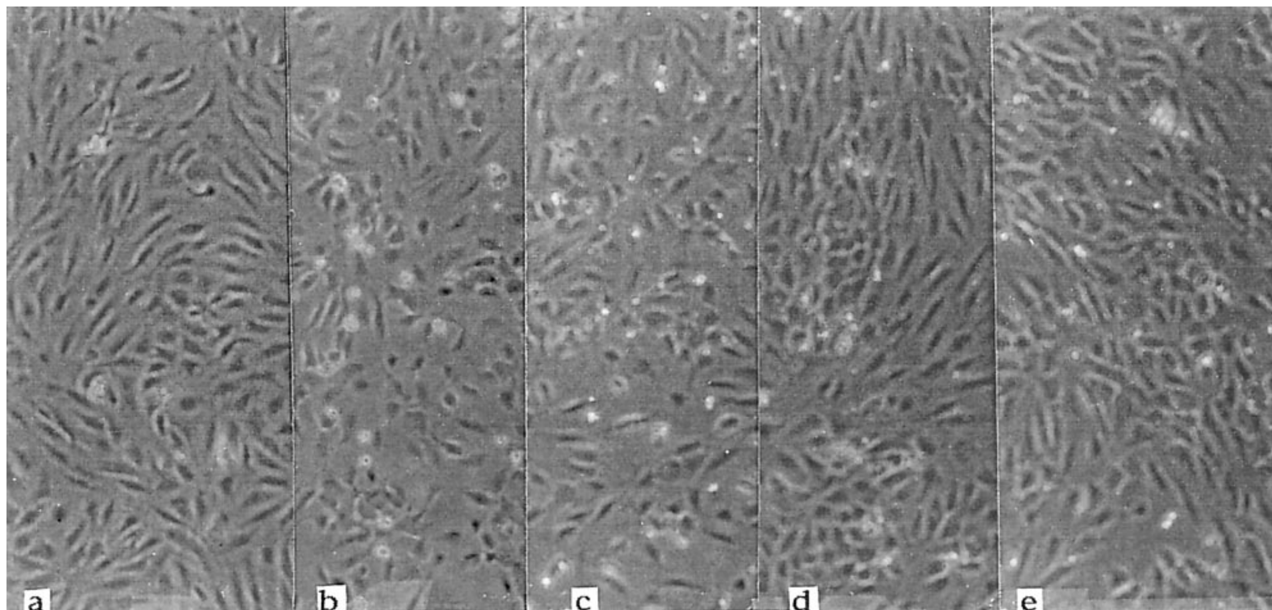


**Figure 5** Phase contrast photomicrographs of HAEC incubated for 22 hr without oxLDL (a), or with oxLDL (b), and/or pre-supplemented with α-tocopherol in media at concentrations of 14 mM (c), 28 mM (d), and 53 mM (e). Each phase contrast photomicrograph is representative of four independent experiments, with triplicates in each experiment.

Cell-mediated oxidation of LDL has been demonstrated by all the major cell types present in the vascular wall including EC, smooth muscle cells, and monocyte/macrophages.[54,55] Cell-mediated oxidation of LDL in cell culture systems is dependent on the presence of trace amounts of transition metal ions in the medium.[56] Consistent with in vitro studies, atherosclerotic lesions contain $Fe^{3+}$ and $Cu^{2+}$ sufficient to promote lipid peroxidation.[57] AAPH-mediated LDL oxidation results in the formation of significant amounts of phospholipid and cholesterolester hydroperoxides in the absence of metal ions, which could interfere with the effect of oxLDL on HAEC. Oxidation of phospholipids to PC-OOH and related compounds like lysophosphophatidylcholine has been shown to stimulate EC to produce adhesion molecules.[58] PC-OOH has been found to be augmented in plasma from individuals with hypercholesterolemia.[59] Therefore, oxLDL used in these experiments contained significant amounts of PC-OOH and other oxidatively modified products known involved in the pathogenesis of atherosclerosis.

$PGI_2$ produced by EC has anti-aggregatory and vasodilatory effects on the vascular system and decreases adhesion of polymorphonuclear cells (PMN) to EC in vitro.[60] $PGI_2$ may serve to protect against noxious stimuli that induce inflammatory and necrotic responses.[61] Under normal physiological conditions the secretion of $PGI_2$ is extremely low in humans[62] but its production is increased by stresses associated with atherosclerosis and platelet activation.[24,62] The increased production of $PGI_2$ in response to stimuli reflects an active protective process against stress and/or cell injury. This is supported by studies using drugs[24,63] and sheer stress.[64] Consistent with these observations, the production of $PGI_2$ by HAEC in response to oxLDL exposure in this study also supports the concept of a protective response of EC to stress. The levels of $PGI_2$ secreted by HAEC under these conditions are consistent with those reported for human saphenous EC exposed to oxLDL.[45]

IL-1β is a mediator of cell defense and has been implicated in the pathogenesis of vascular disease.[65,66] IL-1β, through autocrine or paracrine signaling, may induce EC secretion of chemotactic factors and adhesion of monocytes and lymphocytes to the arterial EC and accumulation in the intima.[66–68] We have reported that HAEC production of IL-1β increases with oxidative stress and pretreatment of the cells with α-tocopherol reduces the subsequent injury along with decreases in IL-1β production.[69] Thomas et al.[70] reported that oxLDL may preferentially stimulate human blood mononuclear cells to release IL-1β. In this study even though 12 to 22 hr exposure to oxLDL induced cytotoxicity in HAEC, we observed no changes in IL-1β production. It is possible that oxLDL might have inactivated IL-1β gene through inactivation of oxidant sensitive nuclear transcription factor-kB signaling pathway,[71] which remains to be determined.

Dietary vitamin E supplementation has been shown to increase α-tocopherol concentrations and antioxidant capacity of LDL, blood cells, and other tissue.[72–74] α-Tocopherol may possess antiatherogenic properties unrelated to its action on LDL oxidation.[75] Our observation indicate that α-tocopherol protection of HAEC against cytotoxic effect of oxLDL is another potential mechanism by which generous intake of vitamin E may contribute to the reduction of risk of cardiovascular disease as reported by epidemiological and clinical trials.[76]

In conclusion, our data demonstrate that enrichment of HAEC with α-tocopherol protects these cells from the cytotoxic effect induced by oxLDL and modulates $PGI_2$ production without any significant effect on IL-1β production. Although the significance of oxLDL as a causal factor in the pathogenesis of atherosclerosis has yet to be estab-

lished in vivo, these findings may be important in defining the mechanism of protective effect of α-tocopherol for atherosclerosis as indicated by epidemiological studies.

## Acknowledgment

The authors would like to thank Timothy S. McElreavy, M.A., for preparation of this article.

## References


1 Levine, G., Keaney, J.J., and Vita, J. (1995). Cholesterol reduction in cardiovascular disease. *N. Engl. J. Med.* **332,** 512–521
2 Steinberg, D., Parthasarathy, S., Carew, T., Khoo, J., and Witztum, J. (1989). Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N. Engl. J. Med.* **320,** 281–284
3 Yla-Herttuala, S., Palinski, W., Rosenfeld, M.E., Parthasarathy, S., Carew, T.E., Butler, S., Witztum, J.L., and Steinberg, D. (1989). Evidence for the presence of oxidatively modified low density lipoprotein in atherosclerotic lesions of rabbit and man. *J. Clin. Invest.* **84,** 1086–1095
4 Jurgens, G., Chen, Q., Esterbauer, H., Mair, S., Ledinski, G., and Dinges, H.P. (1993). Immunostaining of human autopsy aortas with antibodies to modified apolipoprotein B and apoprotein(a). *Arterioscler. Thromb.* **13,** 1689–1694
5 Salonen, J., Yla-Herttuala, S., Yamamoto, R., Butler, S., Korpela, H., Salonen, R., Nyyssonen, K., Palinski, W., and Witztum, J.L. (1992). Autoantibody against oxidized LDL and progression of carotid atherosclerosis. *Lancet* **339,** 883–886
6 Henriksen, T., Mahoney, E., and Steinberg, D. (1981). Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells: Recognition by receptors for acetylated low density lipoproteins. *Proc. Natl. Acad. Sci. USA.* **78,** 6499–6503.
7 Rohrer, L., Freeman, M., Kodama, T., Penman, M., and Krieger, M. (1990). Coiled fibrous domains mediate ligan binding by macrophage scavenger receptor type II. *Nature* **343,** 570–573
8 Steinberg, D. and Witztum, J. (1990). Lipoproteins and atherogenesis: Current concepts. *JAMA* **264,** 3047–3052
9 Morel, D.W., Hessler, G.M., and Chisolm, G.M. (1983). Low density lipoprotein cytotoxicity induced by free radical peroxidation of lipid. *J. Lipid Res.* **24,** 1070–1076
10 Palinsky, W., Rosenfeld, M., Yla-Herttuala, S., et al. (1989). Low density lipoprotein undergoes modification in vivo. *Proc. Natl. Acad. Sci. USA* **86,** 1372–1376
11 Heinecke. J., Baker, L., Rosen, H., Chait, A. (1986). Superoxide-mediated modification of low density lipoprotein by arterial smooth muscle cells. *J. Clin. Invest.* **77,** 757–761
12 Steinbrecher, U., Parthasarathy, S., Leake, D., Witztum, J., and Steinberg, D. (1984). Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc. Natl. Acad. Sci. USA* **81,** 3883–3887
13 Esterbauer, H., Gebicki, J., Puhl, H., and Jurgens, G. (1992). The role of lipid peroxidation and antioxidants in oxidative modification of LDL. *Free Radic. Biol. Med.* **13,** 341–390
14 Morel, D., DiCorleto, P., and Chisolm, G. (1984). Endothelial and smooth muscle cells alter low density lipoprotein in vitro by free radical oxidation. *Arteriosclerosis* **4,** 357–364
15 DiCorleto, P. and Chisolm, G. (1986). Particpation of the endothelium in the development of the atherosclerotic plaque. *Prog. Lipid Res.* **25,** 365–374
16 Ross, R. (1986). The pathogenesis of atherosclerosis, an update. *N. England J. Med.* **314,** 488–500
17 Conn, H.J., Kostis, J., and Saviano, G. (1985). *Diseases of Blood Vessels*. Lea and Febiger, Philadelphia, PA, USA
18 Hessler, J., Robertson, A., and Chisholm, G. (1979). LDL-induced cytotoxicity and its inhibition by HDL in human vascular smooth muscle cells and endothelial cells in culture. *Atheriosclerosis* **32,** 213–216
19 Kugiyama, K., Kerns, S., Morrisett, J., Roberts, R., and Henry, P. (1990). Impairment of endothelium-dependent arterial relaxation by lysolecithin in modified low-density lipoproteins. *Nature* **344,** 160–162
20 Berliner, J., Territo, M., Sevanian, A., Ramin, S., Kim, J.A., Bamshad, B., Esterson, M., and Fogelman, A.M. (1990). Minimally modified low density lipoprotein stimulates monocyte endothelial interactions. *J. Clin. Invest.* **85,** 1260–1266
21 Cushing, S., Berliner, J., Valente, A., et al. (1990). Minimally modified low density lipoprotein induces monocyte chemotactic protein 1 in human endothelial cells and smooth muscle cells. *Proc. Natl. Sci. USA* **87,** 5134–5138
22 Massotti, G., Poggesi, G., and Galanti, G. (1981). *Clinical Pharmacology of Prostacyclin*. Raven Press, New York, NY USA
23 Pace-Asciak, C. and Gryglewski, R. (1983). *The Prostacyclins*. Elsevier Science Publishers, Amsterdam
24 Voss, R., Ten Hoor, F., and Matthias, F. (1983). Prostacyclin production and atherosclerosis of the rabbit aorta. *Adv. Prostaglandin Thromboxane Leukotriene Res.* **11,** 469–474
25 Rimm, E., Stampfer, M., Ascherio, A., Giovannucci, E., Colditz, G., and Willett, W. (1993). Vitamin E consumption and the risk of coronary heart disease in men. *N. Engl. J. Med.* **328,** 1450–1456
26 Machlin, L. and Bendich, A. (1987). Free radical tissue damage: Protective role of antioxidant nutrients. *Faseb J.* **1,** 444–445
27 Niki, E., Yamamoto, Y., and Takahashi, M. (1988). Free radical-mediated damage of blood and its inhibition by antioxidants. *J. Nutr. Sci. Vitaminol.* **34,** 507–512
28 Burton, K. (1989). Evidence of direct toxic effect of free radicals on the myocardium. *Free Radic. Biol. Med.* **4,** 15–24
29 Faruqui, R., de la Motte, C., and DiCorleto, P. (1994). α-tocopherol inhibits agonist-induced monocytic cell adhesion to cultured human endothelial cells. *J. Clin. Invest.* **94,** 592–600
30 Martin, A., Zulueta, J., Hassaun, P., Blumberg, J., and Meydani, M. (1995). The effect of vitamin E on human vascular endothelial cells response to oxidative injury induced by hypoxia/reperfussion. *Free Radic. Biol. Med.* **20,** 99–105
31 Henning, B., Enoch, C., and Chow, C. (1987). Protection of vitamin E against endothelial cell injury by linoleic acid hydroperoxides. *Nutr. Res.* **7,** 1253–1259
32 Jaffee, E., Hoyer, L., and Nachman, R. (1974). Synthesis of von Willebrand factor by cultured human endothelial cells. *Proc. Natl. Acad. Sci. USA* **71,** 1906–1913
33 Meydani, M., Chon, J., Macauley, J., McNamara, J., Blumberg, J., and Schaefer, E. (1989). Postprandial changes in the plasma concentration of α- and γ-tocopherol in human subjects fed fat-rich meal supplemented with fat-soluble vitamins. *J. Nutr.* **119,** 1252–1258
34 Hwang, D., Mathia, M., Dupont, J., and Meyer, D. (1986). Linoleate enrichment of diet and prostaglandin metabolism in rats. *J. Nutr.* **105,** 995–1002
35 McCosh, E., Meyer, D., and Dupont, J. (1976). Radioimmunoassay of prostaglandins E1, E2 and F2 alpha in unextracted plasma, serum, and myocardium. *Prostaglandins* **12,** 471–486
36 Endres, S., Ghorbani, R., Lonnemann, G., Van der Meer, J., and Dinarello, C. (1988). Measurement of immunoreactive interleukin-1β for human mononuclear cells: Optimization of recovery, intrasubject consistency and comparison with interleukin-1α and tumor necrosis factor. *Clin. Immunol. Immunopath.* **49,** 424–438
37 Van Der Meer, J., Endres, S., and Lonnemann, G. (1978). Concentrations of immunoreactive human tumor necrosis factor alpha produced by human momonuclear cells in vitro. *J. Leukocyte Biol.* **43,** 216–223
38 Chung, B. (1986). Single vertical spin isolation of LDL. *Methods Enzymol.* **128,** 181–209
39 Lowry, O., Rosebrough, N., Farr, A., and Randall, R. (1951). Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193,** 265–269
40 Lynch, S.M. and Frei, B. (1994). Natural antioxidants in human health and disease. In *Natural Antioxidants in Human Health and Disease*. Frei Be, ed., p. 353–385, Academic Press, San Diego, CA USA
41 Frei, B., Stocker, R., and Ames, B. (1988). Antioxidant defenses and lipid peroxidation in human blood plasma. *Proc. Natl. Acad. Sci. USA* **85,** 9748–9752
42 Miyazawa, T. (1989). Determination of phospholipid hydroperoxides in human blood plasma by a chemiluminiscence-HPLC assay. *Free Radic. Biol. Med.* **7,** 209–217

43 Van Kuijk, F. (1987). Detection of phospholipid peroxides in biological samples. *Free Radic. Biol. Med.* **3,** 349–354
44 Harlow, E. and Lane, D. (1988). SDS-polyacrylamide gel electrophoresis. In *Antibodies: A Laboratory Manual*. Laboratory CSH, New York, NY USA
45 Triau, J., Meydani, S., and Schaefer, E. (1988). Oxidized low density lipoprotein stimulates prodtacyclin production by adult human vascular endothelial cells. *Arteriosclerosis* **8,** 810–818
46 Meydani, M. (1995). Vitamin E. *Lancet* **345,** 170–175
47 Meydani, S.N., Meydani, M., Blumberg, J.B., Leka, L.S., Siber, G., Loszewski, R., Thompson, C., Pedrosa, M.C., Diamond, R.D., and Stollar, B.D. (1997). Vitamin E supplementation enhances cell-mediated immunity in healthy elderly: A dose-response study. *JAMA,* **277,** 1380–1386
48 Kaneko, T., Nakano, S., and Matsuo, M. (1991). Protective effect of vitamin E on linoleic acid hydroperoxide-induced injury to human endothelial cells. *Lipids* **26,** 345–348
49 Henning, B., Gilbert, A., and Wang, Y. (1989). Protective effects of vitamin E in aged-related endothelial cell injury. *Internat. J. Vit. Nutr. Res.* **59,** 273–279
50 Janero, D. and Burghardt, B. (1989). Oxidative injury to myocardial membrane: direct modulation by endogenous alpha-tocopherol. *J. Molecular Cellular Cardiov.* **21,** 1111–1124
51 Boscoboinik, D., Szewczyk, A., Hensey, C., and Azzi, A. (1991). Inhibition of cell proliferation by alpha-tocopherol. *J. Biol. Chem.* **266,** 6188–6194
52 Navab, M., Imes, S.S., Hama, S.Y., Ross, L.A., Bork, R.W., Valente, A.J., Berliner, J.A., Drinkwater, D.C., Laks, H., and Fogelman, A.M. (1991). Monocyte transmigration induced by low density lipoprotein in cocultures of human aortic wall cells is due to induction of monocyte chemotactic protein1synthesis and is abolished by high density lipoprotein. *J. Clin. Invest.* **88,** 2039–2046
53 Restkey, K.L., Freeman, M.W., and Frei, B. (1993). Ascorbic acid oxidation product(s) protect human low density lipoprotein against atherogenic modification. Antirather than prooxida nt activity of vitamin C in the presence of transition metal ions. *J. Biol. Chem.* **268,** 1304–1309
54 Heinecke, J.W., Rosen, H., and Chait, A. (1984). Iron and copper promote modification of low density lipoprotein by human arterial smooth muscle cells in culture. *J. Clin. Invest.* **74,** 1890–1894
55 Sparrow, C. and Olszewski, J. (1993). Cellular oxidation of low density lipoprotein is caused by thiol production in media containing transition metal ions. *J. Lipid Res.* **34,** 1219–1228
56 Martin, A. and Frei, B. (1997). Both intracellular and Extracellular vitaminC inhibit atherogenic modification of LDL by human vascular endothelial cells. *Arterioscler. Thromb. Vasc. Biol.* **17,** 1583–1590
57 Smith, C., Mitchinson, M., Aruoma, O., and Halliwell, B. (1992). Stimulation of lipid peroxidation and hydroxil radical generation by the contents of human atherosclerotic lesions. *Biochem. J.* **286,** 901–905
58 Kume, N., Cybulsky, M., and Gimbrone, M.J. (1992). Lysophosphatidylcholine, a component of atherogenic lipoproteins, induces mononuclear leukocyte adhesion molecules in cultured human and rabbit arterial endothelial cells. *J. Clin. Invest.* **90,** 1138–1144
59 Miyazawa, T., Yasuda, K., Fujimoto, K., and Kaneda, T. (1988). Presence of phosphatidylcholine hydroperoxide in human plasma. *J. Biochem.* **103,** 744–746
60 Boxer, L., Allen, J., Schmidt, M., Yoder, M., and Baehner, R. (1980). Inhibition of polymorphonuclear leukocyte adherence by prostacyclin. *J. Lab. Clin. Med.* **95,** 672–678
61 Boxer, L., Allen, J., and Baehner, R. (1980). Diminished polymorphonuclear leukocyte adherence: Function dependent on release of cyclic AMP by endothelial cells after stimulation of B-receptors by epinehrine. *J. Clin. Invest.* **66,** 268–274
62 FitzGerald, G., Friedman, L., Miyamori, I., O'Grady, J., Lewis, P. (1979). A double blind placebo controlled crossover study of prostacyclin in man. *Life Sci.* **25,** 665–672
63 Bermylen, J., Chamone, D., and Verstraete, M. (1979). Stimulation of prostacyclin release from vessel wall by Bay g 6575, an antithrombotic compound. *Lancet* **i,** 518–520
64 Grabowski, E., Jaffe, E., and Beksler, B. (1985). Prostacyclin production by cultured endothelial cell monolayers exposed to step increases in shear stress. *J. Lab. Clin. Med.* **105,** 36–43
65 Dinarello, C.A. (1989). Biology of interleukine-1. *Faseb J.* **2,** 108–115
66 Bevilacqua, M., Pober, J., Wheeler, M., Cotran, R., and Gimbrone, M.J. (1985). Interleukin-1 acts on cultured human vascular endothelium to increase the adhesion of polymorphonuclear leukocytes, monocytes, and related leukocyte cell lines. *J. Clin. Invest.* **76,** 2003–2011
67 Libby, P., Warner, S., and Friedman, G. (1988). Iinterleukin-1: A mitogen for human vascular smooth muscle cells that induces the release of growth-inhibitory prostanoids. *J. Clin. Invest.* **88,** 487–498
68 Akeson, A., Mosher, L., Woods, C., Schroeder, K., and Bowlin, T. (1992). Human aorta endothelial cells express the type I but not the type II receptor for interleukin-1 (IL-1). *J. Cell. Physiol.* **153,** 583–588
69 Martin, A., Wu, D., Baur, W., Meydani, S., Blumberg, J., and Meydani, M. (1996). The effect of vitamin E on human aortic endothelial cell responses to oxidative injury. *Free Radic. Biol. Med.* **21,** 505–511
70 Thomas, C. (1994). Multiple lipid oxidation products in low density lipoproteins induce interleukin-1 beta release from human blood mononuclear cells. *J. Lipid Res.* **35,** 417–427
71 Shackelford, R., Misra, U., Florine-Casteel, K., Thai, S., Pizzo, S., and Adams, D. (1995). Oxidized low density lipoprotein suppresses activation of NFkB in macrophages via a pertussis toxin-sensitive mechanism. *J. Biol. Chem.* **270,** 3475–3478
72 Meydani, S., Barklund, P., and Liu, S. (1990). Effect of vitamin supplementation on immune responsiveness of healthy elderly subjects. *Am. J. Clin. Nutr.* **52,** 557–563
73 Mickle, D., Weisel, R., Burton, G., and Ingold, K. (1991). Effect of orally administered alpha-tocopheryl acetate on human myocardial alpha-tocopherol levels. *Cardiovasc. Drugs Ther.* **5,** 309–312
74 Meydani, M., Fielding, R.A., Cannon, J.G., Blumberg, J.B., and Evans, W.J. (1997). Muscle uptake of vitamin E and its association with muscle fiber type. *J. Nutr. Biochem.* **8,** 74–78
75 Keaney, J.F. Jr., Gaziano, J.M., Xu, A., Frei, B., Curran-Celentano, J., Shwaery, G.T., Losculzo, J., and Vita, J.A. (1993). Dietary antioxidants preserve endothelium-dependent vessel relaxation in cholesterol-fed rabbits. *Proc. Natl. Acad. Sci. USA* **90,** 11880–11884
76 Meydani, M. and Meisler, J.G. (1997). A closer look at vitamin E. *Postgrad. Med.* **102,** 199–207