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α-Tocopherol prevents apoptosis of vascular endothelial cells via a mechanism exceeding that of mere antioxidation

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Abstract

α-Tocopherol has been reported to exert an anti-atherogenesis effect. We attempted to clarify the effect of α-tocopherol—both as an antioxidant and as a nonantioxidant—on apoptosis induced by oxidized low-density lipoprotein (LDL) or oxysterols. Oxidized LDL and oxysterols induced necrosis and/or apoptosis of vascular endothelial cells. The induction of apoptosis was associated with increased caspase-3 activity and the generation of intracellular reactive oxygen species, both the effects of which were attenuated by α-tocopherol. Apoptosis was also decreased by β-tocopherol or intracellular radical scavengers, but these suppressive effects were less than those of α-tocopherol. Neither β-tocopherol nor the scavengers had pronounced effect on caspase-3 activity, but each of them decreased the generation of reactive oxygen species to the same extent as α-tocopherol. Our study suggests that α-Toc protects against apoptosis not only by scavenging reactive oxygen species, but also by inhibiting caspase activity, which means that its activity may exceed that of a mere antioxidant.

Keywords: LDL (low-density lipoprotein), oxidized; α-Tocopherol; Apoptosis

1. Introduction

Oxidized low-density lipoproteins (LDLs) are thought to play a key role of triggering molecules in the earliest steps of atherosclerosis (Steinberg et al., 1989), i.e., the expression of adhesion molecules on endothelial cells (Kume et al., 1992), the decrease in the production of endothelial cell-derived relaxing factor (Mangin et al., 1993), the transformation of macrophages and smooth muscle cells to foamy cells (Henriksen et al., 1981) and the proliferation and migration of vascular cell surface (Auge et al., 1996). The end result of these steps is the growth of atherosclerotic plaque and the propensity of the diseased vessel toward vasospasm. Recent studies have shown that oxidized LDL is cytotoxic not only to smooth muscle cells or macrophages, but also to vascular endothelial cells (Naito et al., 1993, 1994; Reid et al.,1993). Injury of smooth muscle cells and endothelial cells leads to two distinct types of cell death: apoptosis and necrosis. Oxidized LDLs or their constituent oxysterols have been also reported to induce apoptosis in both these cell types, and to activate the suicide pathway leading to apoptosis of endothelial cells (Chien-Cheng et al., 2001; Claudio et al., 1996; Dayuan et al., 2000; Jovinge et al., 1997).

Recently, it has been shown that the signal transduction leading to apoptosis is characterized by a complex array of biochemical pathways in which caspase, a member of the interleukin-1β-converting enzyme-like protease family, appears to play a central role (Enari et al., 1996; Nicholson et al., 1995). Further, the generation of reactive oxygen species has been demonstrated to be involved in mediating apoptosis via activation of the cell death program in numerous cell lines (Jacobson, 1996; Li et al., 1997; Sugiyama et al., 1996).

Vitamin E, a lipid-soluble vitamin, is a chain-breaking tissue antioxidant (Burton and Ingold, 1989) that is present in all cell membranes in low concentrations, and is reported to be an anti-atherogenic agent (Janero, 1989). α-Tocopherol is believed to have a cytoprotective effect, which is attributed to its ability to prevent leukocyte-endothelial interactions (Terasawa et al., 2000; Yoshida et al., 1999, 2000), and to act as a scavenger of highly reactive oxygen radicals in various

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pathophysiological processes (Chow, 1991; Kagan, 1989; Kaneko et al., 1991; Packer and Lamdvik, 1991; Urano and Matsumoto, 1989). Nonetheless, the mechanism of its action has not been rigorously demonstrated.

The aim of the present study was to investigate which components induce endothelial cell injury leading to which types of cell death, i.e., apoptosis or necrosis, and to identify the mechanism of the protective effect of α-tocopherol, which acts as an antioxidant or a "nonantioxidant", against cell injury induced by oxidized LDLs or their components.

2. Material and method

2.1. Preparation of LDL

Isolation of human LDL ($d = 1.019-1.063$) was performed as previously described in detail (Noguchi et al., 1993). The LDL was sterilized through a 0.45- and a 0.22-mm filter, and had a protein concentration of approximately 2 mg/ml. It consisted of more than 95% apolipoprotein B and less than 5 pg/ml endotoxin. Oxidatively modified LDL was obtained by incubation of native LDL with 10 μM $CuSO_4$ at 37 °C for 3 h. The extent of oxidation of the LDL preparations was measured using a Determinor LPO (lipid peroxide) assay kit (Kyowa Medex, Tokyo, Japan). The extent of oxidation of preparations used in these experiments was as follows: native LDL, 0.277 ± 0.104; oxidized LDL, 100–200 nM hydroperoxide/mg protein.

2.2. Endothelial cell culture

Human umbilical vein endothelial cells were isolated and harvested by collagenase treatment of umbilical cords according to established procedures (Jaffe et al., 1973). Endothelial cells were plated in gelatin-coated tissue-culture flasks (Nunc, Roskilde, Denmark) with Medium 199 (GIBCO, Great Island, NY) containing 10% heat-inactivated fetal bovine serum (GIBCO), 2 mM L-glutamine (GIBCO), 10 U/ml heparin (Mochida Pharmaceutical, Tokyo, Japan), 2.4 mg/ml thymidine (Sigma, St. Louis, MO), antibiotics (penicillin G/streptomycin/fungizon) (GIBCO) and 100 μg/ml endothelial cell growth supplement, a mitogen (Biological Technologies, Stoughton, MA). Endothelial cells were incubated at 37 °C in a humidified atmosphere with 5% CO_2 and expanded by brief trypsinization (0.025% trypsin in phosphate-buffered saline). Cells passaged two to three times were plated on 35-mm culture dishes or 96-well plates coated with gelatin (0.1%), and used when subconfluent.

2.3. Reagents

D-α-tocopherol and D-β-tocopherol (Eisai, Tokyo, Japan) were diluted with ethanol to a concentration of 100 mg/ml and stored at 4 °C. 7-Ketocholesterol (5-cholesten-3β-ol-one), 7β-hydroxycholesterol (5-cholesten-3β, 7β-diol), lysophosphatidylcholine, Hochest33342 dye, propidium iodide and 2-phenyl-1,2-benzisoselenazol-3[2*H*]-one (ebselen) were purchased from Sigma. Mn(III) tetrakis (1-methyl-4-pyridyl) porphyrin pentachloride (Mn-TMPyP) was purchased from Cayman Chemical (Ann Arbor, MI). 5 (and 6)-carboxy-2′,7′-dichlorofluorescin diacetate (cDCFH-DA) was purchased from Molecular Probes (Eugene, OR).

2.4. Measurement of cell injury

2.4.1. Trypan blue dye exclusion assay and modified MTT assay

After incubation of endothelial cells with various concentrations of the reagents—i.e., native LDL, oxidized LDL, 7-ketocholesterol, 7β-hydroxycholesterol or lysophosphatidylcholine—with or without tocopherols for 24–72 h at 37 °C, endothelial cell injury was assessed by Trypan blue dye exclusion assay and by modified 3-(4,5-dimethyl-thiazole-2-yl)-2,5-diphenyl tetrozolium bromide (MTT) assay (Cell Counting Kit, Dojindo, Kumamoto, Japan), as recently described (Ishiyama et al., 1993; Paull et al., 1994), to determine whether cells were dead or alive. In the case of the Trypan blue dye exclusion assay, cells in 35-mm dishes were detached by trypsinization, then collected and suspended in 500 μl of medium containing trypan blue. Dead cells were counted with a chamber. In the modified MTT assay, 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium, monosodium salt, was added in 96-well plates and then incubated in a CO_2 incubator for 2 h. The plates were read at 450 nm in a Microplate Reader (MPR-A4I, Tosoh, Tokyo) to quantify the number of living cells.

2.4.2. Hochest33342–propidium iodide double staining

The morphology of cell death, i.e., apoptosis or necrosis, was determined by fluorescent-microscopical findings after

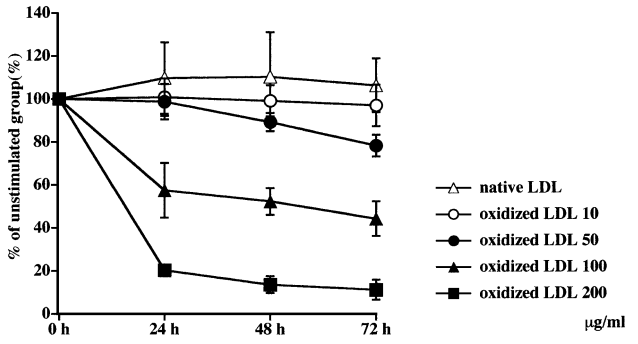

Fig. 1. Time course and dose dependency of cytotoxicity in human umbilical vein endothelial cells induced by oxidized LDL, examined with Trypan blue exclusion dye. After incubation of endothelial cells with various concentrations of native LDL or oxidized LDL for 24–72 h at 37 °C, vascular endothelial cell injury was assessed by Trypan blue dye exclusion assay. Data are shown as a percentage of the result for unstimulated cells.

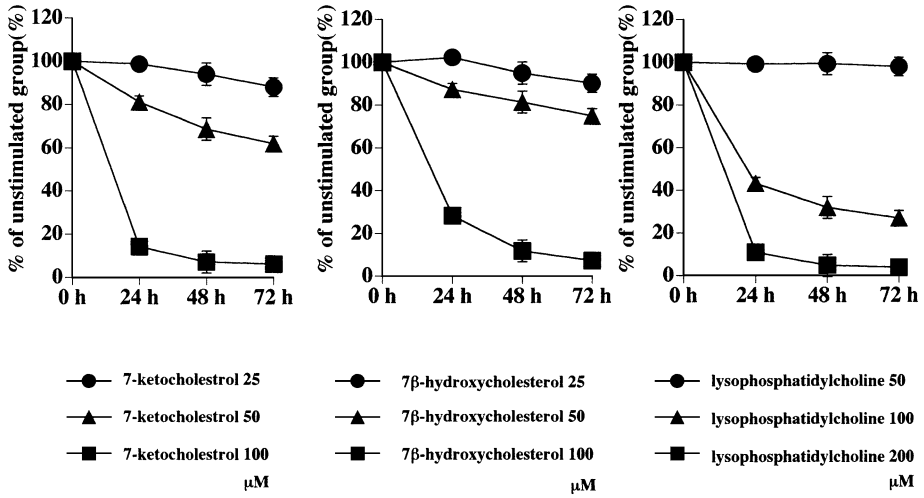

Fig. 2. Time course and dose dependency of cytotoxicity in human umbilical vein endothelial cells induced by 7-ketocholesterol, 7β-hydrooxycholesterol or lysophosphatidylcholine, as examined with Trypan blue exclusion dye. After incubation of endothelial cells with various concentrations of 7-ketocholesterol, 7β-hydrooxycholesterol or lysophosphatidylcholine for 24–72 h at 37 °C, vascular endothelial cell injury was assessed by Trypan blue dye exclusion assay. Data are shown as a percentage of the result for unstimulated cells.

labeling with Hochest33342 and propidium iodide as previously described (Nakajima et al., 1995). Endothelial cells harvested from 35-mm dishes were treated with a different concentration of native LDL, oxidized LDL, 7-ketocholesterol, 7β-hydroxycholesterol or lysophosphatidylcholine. After 24-h incubation (37 °C, 5% CO_2), cells were treated with 10 μg/ml of Hochest33342 dye for 15 min and then with 10 μg/ml of propidium iodide for 10 min. After washing and centrifugation at 300 × *g*, the collected pellet was resuspended in Hanks' Balanced Salt Solution and examined. The morphological findings were recorded by a color camera mounted on a fluorescence microscope with the illumination filter in 100 microscopic fields, and then the extent of apoptosis was analyzed. Experiments were done at least three times in triplicate.

2.4.3. Measurement of DNA fragmentation

DNA fragmentation was analyzed as described previously (Haendeler et al., 1996a,b) using a cell death detection assay kit (Boehringer-Mannheim Biochemicals, Mannheim, Germany). Subconfluent monolayers of endothelial cells on 96-well plates were treated with various concentrations of native LDL, oxidized LDL, 7-ketocholesterol, 7β-hydroxycholesterol or lysophosphatidylcholine, with or without tocopherols. After 24 h of treatment (37 °C, 5% CO_2), plates were washed and the medium was aspirated. The centrifuged (200 × *g*, 10 min) cells were resuspended in 200 μl lysis buffer for 30 min. After centrifugation (200 × *g*, 10 min), the supernatants containing the cytoplasmic fractions were pipetted into streptavidin-coated microtiter wells. A mixture of anti-histone–biotin and anti-DNA-peroxidase

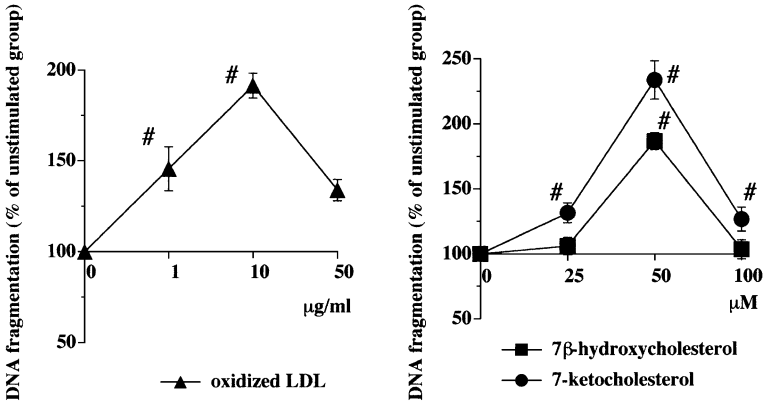

Fig. 3. Changes in DNA fragmentation in human umbilical vein endothelial cells induced by oxidized LDL, 7-ketocholesterol and 7β-hydrooxycholesterol at various concentrations. Endothelial cells were exposed to oxidized LDL, 7-ketocholesterol and 7β-hydrooxycholesterol at various concentrations for 24 h. Subsequently, the amount of DNA fragmentation was measured with a Cell Death Detection Kit. Data are shown as a percentage relative to the result for unstimulated cells and expressed as the means ± SE of three experiments performed in triplicate. #$P < 0.01$ vs. unstimulated endothelial cells.

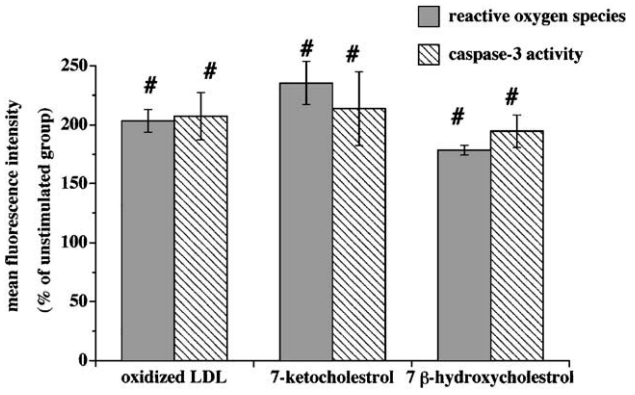

Fig. 4. Elevation of intracellular reactive oxygen species and activation of caspase-3 elicited by oxidized LDL (10 μM), 7-ketocholesterol (50 μM) or 7β-hydrooxycholesterol (50 μM). Reactive oxygen species were measured after a 3-h stimulation, using cDCFH-DA. Activation of caspase-3 after stimulation for 6 h was investigated by caspase-3 fluorometric assay. Data are shown as a percentage of the result for unstimulated cells, expressed as means ± SE of three experiments performed in triplicate. #$P < 0.01$ vs. each unstimulated endothelial cells.

was subsequently added and incubated for 2 h. Then 2,2-azino-di-[3-ethylbenzthiazoline sulfonate] was added as a substrate. The plates were read at 450 nm in a Microplate reader (MPR-A4I; Tosoh) to quantify the amount of DNA fragmentation. Experiments were done at least three times in triplicate.

2.5. Measurement of caspase-3 activity

Subconfluent endothelial cells monolayers seeded in 35-mm culture dishes were treated with various concentrations of oxidized LDL, 7-ketocholesterol or 7β-hydroxycholesterol with or without tocopherols, ebselen or Mn-TMPyP. After 6 h of incubation (37 °C, 5% CO_2), the cells collected by detachment and centrifugation (200 × g, 10 min) were suspended in a lysis buffer and incubated on ice for 10 min. caspase-3 activity was detected by measurement of the proteolytic cleavage of the fluorogenic substrate, 7-amino-4trifluoromethyl-coumarin (AFC)-DEVD, using a fluorometric assay kit (Bio Source International, Camarillo, CA) and a Microplate reader (Bio-Rad Laboratories, Hercules, CA) with excitation at 405 nm and emission at 505 nm. Experiments were done at least three times in triplicate.

2.6. Measurement of intracellular reactive oxygen species production

It has been reported that the intracellular level of reactive oxygen species is parallel to the fluorescence intensity measured with cDCFH-DA (LeBel et al., 1992). This probe is a stable nonpolar compound that readily diffuses across the cell membrane and is enzymatically hydrolyzed by intracellular esterases to nonfluorescent DCFH, then trapped and oxidized to a highly fluorescent compound (DCF) when hydrogen peroxide or low-molecular-weight peroxides are present in the cells. Endothelial cells were seeded and cultured in 96-well plates and then treated with oxidized LDL, 7-ketocholesterol or 7β-hydroxycholesterol, with or without tocopherols, ebselen or Mn-TMPyP. After 3-h incubation, endothelial cells were loaded with 50 μM cDCFH-DA dissolved in dimethyl sulfoxide for 30 min (37 °C, 5% CO_2) in the dark. After removal of residual cDCFH-DA, the fluorescence intensity of DCF was measured with a microplate reader (Bio-Rad Laboratories) with excitation at 485 nm and emission at 530 nm. Experiments were done at least three times in triplicate.

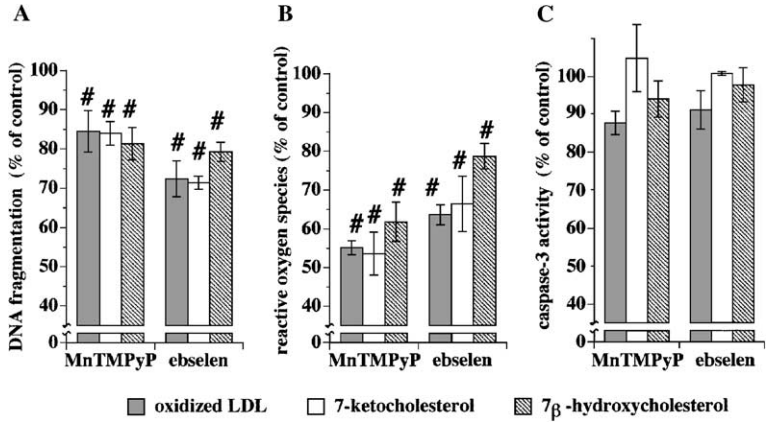

Fig. 5. Effect of Mn-TMPyP (5 μM) and ebselen (20 μM) on DNA fragmentation, reactive oxygen species production or the caspase-3 activity in human umbilical vein endothelial cells stimulated by oxidized LDL (10 μM), 7-ketocholesterol (50 μM) or 7β-hydrooxycholesterol (50 μM). (A) DNA fragmentation stimulated by various reagents for 24 h was measured with a Cell Death Detection Kit. (B) Intracellular reactive oxygen species production stimulated by various reagents for 6 h was measured according to fluorescence intensity using cDCFH-DA. (C) Caspase-3 activity stimulated by various reagents for 3 h was investigated by caspase-3 fluorometric assay. Data are shown as a percentage of the result for each control (stimulated) group, expressed as means ± SE of three experiments performed in triplicate. #$P < 0.01$ vs. stimulated control.

2.7. Statistical analysis

Results are presented as the means ± S.E.M. Data were analyzed using analysis of variance (ANOVA) followed by Scheffe's test. Level of $P < 0.05$ was considered to indicate statistical significance.

3. Results

3.1. Oxidized LDLs or their components induced vascular endothelial cell injury, resulting in necrosis or apoptosis

Oxidized LDL, 7-ketocholesterol, 7β-hydroxycholesterol and lysophosphatidylcholine, but not native LDL, induced vascular endothelial cell injury in a time- and dose-dependent manner, as measured by Trypan blue exclusion dye test (Figs. 1 and 2). The type of cell injury leading to death, i.e., apoptosis or necrosis induced by oxidized LDL, 7-ketocholesterol, 7β-hydroxycholesterol or lysophosphatidylcholine, was detected by double-staining with Hochest33342 and propidium iodide, or by DNA fragmentation detection assay. Low concentrations of oxidized LDL (1–10 μg/ml), 7-ketocholesterol and 7β-hydroxycholesterol (25–50 μM) induced cell shrinkage, chromatin condensation and fragmentation, as detected by staining with Hochest33342 and propidium iodide (photograph not shown) with apoptotic features, whereas higher oxidized LDL (100 μg/ml), 7-ketocholesterol and 7β-hydroxycholesterol (100 μM) concentrations led to necrosis. The DNA fragmentation detection assay after 24 h of incubation revealed the following: (a) cells exposed to lower doses (< 10 μg/ml) of oxidized LDL underwent dose-dependent apoptosis, which reached 180 ± 6.85% at the dose of 10 μg/ml; (b) exposure to lower doses (< 50 μM) of 7-ketocholesterol and 7β-hydroxycholesterol also induced apoptosis in a dose-dependent manner, with a maximum of 229.3 ± 14.8% in 50 μM of 7-ketocholesterol and 186.5 ± 6.5% in 50 μM of 7β-hydroxycholesterol; whereas (c) exposure to higher doses of oxidized LDL (50 μg/ml), 7-ketocholesterol (100 μM) or 7β-hydroxycholesterol (100 μM) decreased the rate of apoptosis (Fig. 3). Lysophosphatidylcholine induced necrosis rather than apoptosis at all concentrations used in each assay (data not shown).

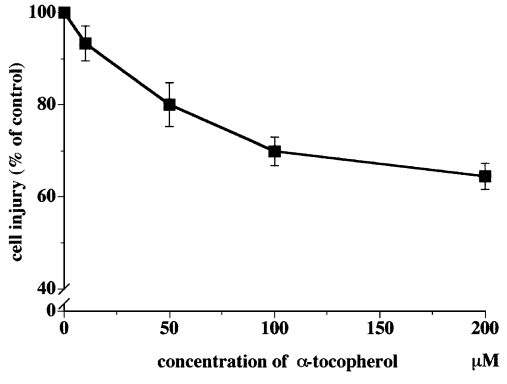

Fig. 6. Concentration-dependent effect of α-tocopherol on the protection of human umbilical vein endothelial cells against injury induced by oxidized LDL. Endothelial cells were incubated with α-tocopherol at various concentrations for 24 h. After washing endothelial cells to remove α-tocopherol, endothelial cells were stimulated with oxidized LDL at a final concentration of 100 μM for 24 h. Subsequently, after removal of oxidized LDL, cell injury was measured with a Cell Counting Kit. Data are shown as a percentage relative to the result for control group without α-Toc preincubation and expressed as the means ± SE of three experiments performed in triplicate.

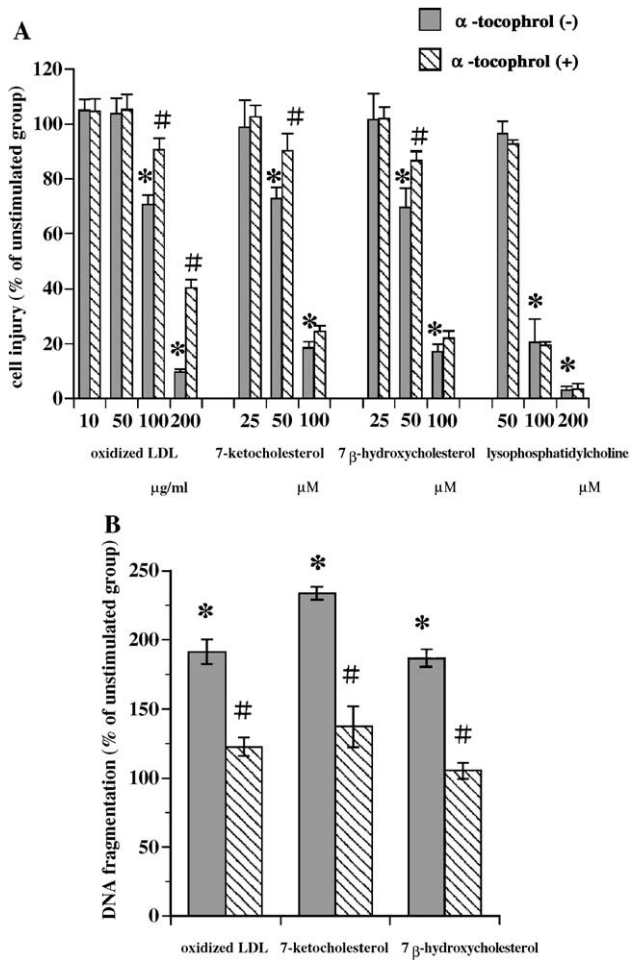

Fig. 7. Effect of α-tocopherol on human umbilical vein endothelial cell injury induced by oxidized LDL, 7-ketocholesterol, 7β-hydrooxycholesterol or lysophosphatidylcholine. Endothelial cells were incubated with α-tocopherol at a final concentration of 100 μM for 24 h. After washing endothelial cells to remove α-tocopherol, endothelial cells were stimulated with various concentrations of reagents for 24 h. Subsequently, after removal of these stimulants, (A) living cells were counted with a Cell Counting Kit. (B) DNA fragmentation was measured with a Cell Death Detection Kit. Data are shown as a percentage of the result for unstimulated cells and expressed as the means ± SE of three experiments performed in triplicate. *$P < 0.01$ vs. unstimulated endothelial cell, #$P < 0.05$ vs. Endothelial cell preincubated without α-tocopherol before exposure to the same concentration of each stimulant.

3.2. Participation of caspase-3 or intracellular reactive oxygen species in oxidized LDL- or oxysterol-induced apoptosis

To investigate the pathway of apoptosis under stimulation of oxidized LDL, 7-ketocholesterol, 7β-hydroxycholesterol, caspase-3 activity and intracellular reactive oxygen species were measured. Caspase-3 activity induced by incubation with a low dose of oxidized LDL (10 μg/ml) for 6 h, determined by a fluorometric assay kit, was elevated to 207.4 ± 20.2% compared with that in untreated endothelial cells. Such activity was also increased to 213.9 ± 31.3% by 7-ketocholesterol (50 μM) treatment, and to 194.7 ± 13.7% by 7β-hydroxycholesterol (50 μM) treatment, as shown in Fig. 4. Next, the production of intracellular reactive oxygen species was evaluated using fluorescent molecules of cDCFH-DA. The mean fluorescent intensity after 3 h of exposure to oxidized LDL (10 μg/ml) was elevated to 203.3 ± 9.5% compared with that in untreated endothelial cells. 7-ketocholesterol (50 μM) treatment also increased the intensity to 235.4 ± 18.3%, and 7β-hydroxycholesterol (50 μM) treatment increased it to 178.7 ± 4.1% (Fig. 4).

3.3. Relationship between caspase-3 and intracellular reactive oxygen species on oxidized LDL- or oxysterol-induced apoptosis

Oxidized LDL, 7-ketocholesterol and 7β-hydroxycholesterol significantly increased the activity of caspase-3 and intracellular reactive oxygen species, resulting in apoptosis, as previously described. The relationship between these signal transduction pathways and apoptosis induced by oxidized LDL or the oxysterols was also investigated. The intracellular reactive oxygen species scavengers Mn-TMPyP (5 μM), which is reported to scavenge intracellular superoxide anion, and ebselen (20 μM), which is thought to scavenge intracellular hydrogen peroxide, decreased apoptosis elicited from exposure to oxidized LDL, 7-ketocholesterol or 7β-hydroxycholesterol (Fig. 5A). Next, the production of intracellular reactive oxygen species stimulated by oxidized LDL or the oxysterols was reduced by Mn-TMPyP (5 μM) and ebselen (20 μM) (Fig. 5B), whereas these scavengers did not attenuate the activation of caspase-3 by oxidized LDL or the oxysterols (Fig. 5C).

3.4. Protective effect of α-tocopherol on oxidized LDL- or oxysterol-induced vascular endothelial cell injury

Pretreatment of endothelial cells with α-tocopherol for 24 h decreased oxidized LDL (100 μg/ml))-induced injury in a concentration-dependent manner, as measured with the Cell Counting Kit (Fig. 6). Therefore, in the subsequent experiments, we used α-tocopherol in a concentration of 100 μM with 0.05% ethanol, which caused no injury to endothelial cells. 7-ketocholesterol- or 7β-hydroxycholesterol- (but not lysophosphatidylcholine) induced endothelial cell injury was significantly attenuated by 24 h of pretreatment with 100 μM of α-tocopherol (Fig. 7A). The increased apoptosis induced with a low dose of the stimulants, oxidized LDL (10 μg/ml), 7-ketocholesterol (50 μM) or 7β-hydroxycholesterol (50 μM) was also significantly reduced by pretreatment with α-tocopherol (100 μM) (Fig. 7B).

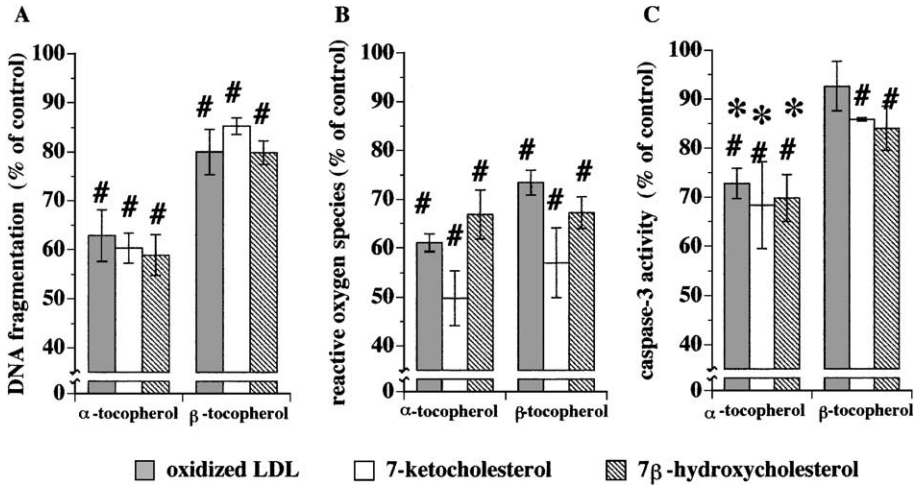

Fig. 8. Effect of pretreatment with α-tocopherol (100 μM) or β-tocopherol (100 μM) on DNA fragmentation, reactive oxygen species production and the caspase-3 activity in human umbilical vein endothelial cell stimulated by oxidized LDL (10 μM), 7-ketocholesterol (50 μM) or 7β-hydrooxycholesterol (50 μM). (A) DNA fragmentation stimulated by various reagents for 24 h was measured with a Cell Death Detection Kit. (B) Intracellular reactive oxygen species stimulated by various reagents for 3 h was measured according to fluorescence intensity using cDCFH-DA. (C) Caspase-3 activity stimulated by various reagents for 3 h was investigated by caspase-3 fluorometric assay. Data are shown as a percentage of the result for each control (stimulated) group, expressed as the means ± SE of three experiments performed in triplicate. #$P < 0.01$ vs. stimulated control. *$P < 0.01$ vs. β-tocopherol pretreatment.

3.5. Protective mechanism of α-tocopherol against oxidized LDL- or oxysterol-induced apoptosis

The effect of α-tocopherol on the activation of caspase-3 or the production of intracellular reactive oxygen species in response to exposure to oxidized LDL, 7-ketocholesterol or 7β-hydroxycholesterol, which participate in the apoptotic pathway, was investigated. Pretreatment for 24 h with α-tocopherol (100 μM) significantly reduced the increase in both caspase-3 activity and intracellular reactive oxygen species induced by oxidized LDL (10 μg/ml), 7-ketocholesterol (50 μM) or 7β-hydroxycholesterol (50 μM) (Fig. 8B and C). Next, to investigate the mechanisms of α-tocopherol for protection against apoptosis, we used β-tocopherol, which is one form of tocopherol homologues but has an antioxidative action as strong as that of α-tocopherol. Apoptosis induced by oxidized LDL (10 μg/ml), 7-ketocholesterol (50 μM) or 7β-hydroxycholesterol (50 μM) was not as greatly suppressed by pretreatment with β-tocopherol (100 μM) as by pretreatment with α-tocopherol (Fig. 8A). The increase in caspase-3 activity stimulated by oxysterols was slightly suppressed, but that stimulated by oxidized LDL, was not suppressed by β-tocopherol (100 μM) (Fig. 8C). The fluorescent intensity of cDCFH-DA, which was a response to the increase of intracellular reactive oxygen species elicited by oxidized LDL or oxysterols, was significantly inhibited by β-tocopherol (100 μM) (Fig. 8B).

4. Discussion

It has been reported that both oxidized LDL and its constituent oxysterols induce apoptosis (Dimmeler et al., 1997; Kenji et al., 1997; Miyashita et al., 1997). The present results also indicate that oxidized LDL or its components, 7-ketocholesterol and 7β-hydroxycholesterol, induce cell death in a dose- and time-dependent manner, with the mechanism of cell death being apoptosis at lower concentrations of these compounds and necrosis at higher concentrations. Lysophosphatidylcholine, which is also one of the major components of oxidized LDL, is regarded as a triggering molecule for atherogenesis, and has been reported to induce the expression of adhesion molecules on ECs (Kume et al., 1992) and growth factors (Kume and Gimbrone, 1994), resulting in necrosis, but not apoptosis, at all concentrations used in the present study. Thus, the main components of oxidized LDL that induce apoptosis may be regarded as the oxysterols, 7-ketocholesterol and 7β-hydroxycholesterol.

It has been reported that apoptosis occurs through activation of the ICE family, the caspase cascade (Enari et al., 1996; Nicholson et al., 1995), and production of reactive oxygen species (Chien-Cheng et al., 2001; Jacobson, 1996; Li et al., 1997; Sugiyama et al., 1996) in various cell lines. It has also been reported that oxidative stress induces activation of caspase-3, resulting in apoptosis (Hampton, 1996), which may indicate that there are some interactions between reactive oxygen species and caspase in the apoptotic pathway. In this study as well, we showed that oxidized LDL- or oxysterol-induced apoptosis in human umbilical vein endothelial cells occurred through the activation of caspase-3 or the production of intracellular reactive oxygen species (Fig. 4). In addition, the intracellular radical scavengers Mn-TMPyP, which directly scavenges intracellular superoxide anion (Faulkner et al., 1994), and ebselen, which indirectly scavenges intracellular hydrogen peroxide, reduced apoptosis and intracellular reactive oxygen species elicited in response to exposure to oxidized LDL or oxysterols, whereas these scavengers did not attenuate the activation of caspase-3 (Fig. 5). These findings mean that oxidative stress by oxidized LDL or oxysterols stimulated the generation of intracellular reactive oxygen species (superoxide anion and hydrogen peroxide) and the activation of the caspase cascade, which independently led to apoptosis through each pathway. Recent reports have indicated that mitochondrial function, including the release of cytochrome *c*, is important for the activation of the caspase cascade for apoptosis (Liu, 1996; Reed, 1997). We will also need to investigate the participation of cytochrome *c* in oxidized LDL- or oxysterol-induced apoptotic pathways.

α-Tocopherol is known to be a strong antioxidant and to exert various cytoprotective effects (Azzi et al., 2001; Sylvester et al., 2001; Terasawa et al., 2000; Yoshida et al., 1999, 2000), although γ-tocopherol has recently been reported to show protective effects against oxidized LDL (Li et al., 1999). In this study, we showed that α-tocopherol attenuates necrosis or apoptosis in vascular endothelial cells induced by oxidized LDL or oxysterols (Fig. 7), and that it also decreases the generation of intracellular reactive oxygen species and the activation of caspase-3 (Fig. 8). We next investigated whether these effects of α-tocopherol occurred via an antioxidative or nonantioxidative action. For this purpose, we used β-tocopherol, which is one form of tocopherol homologues and which is regarded to have an antioxidative action as strong as that of α-tocopherol. Although β-tocopherol reduced the production of reactive oxygen species to the same extent as α-tocopherol, it did not attenuate apoptosis or the activation of caspase-3 more than α-tocopherol (Fig. 8). These observations suggest that α-tocopherol acted to reduce the activation of both apoptotic pathways not only as an antioxidant but as a nonantioxidant.

In summary, we can conclude that the cytotoxic effect of oxidized LDL, 7-ketocholesterol, and 7β-hydroxycholesterol on vascular endothelial cells leads to necrosis at a high dose or to apoptosis at a low dose, and one of the mechanisms leading to apoptosis is the generation of intracellular reactive oxygen species and the activation of the caspase cascade, which are independent mechanisms. In these processes, α-tocopherol plays a role in attenuating the

cytotoxicity of apoptosis via a mechanism exceeding that of mere antioxidative effects, which may also contribute to the prevention of atherogenesis.

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References

Auge, N., Andrieu, N., Negre-Salvayre, A., Tiers, J.C., Salvayre, R., 1996. The sphingomyelin–ceramide signaling pathway is involved in oxidized low density lipoprotein-induced cell proliferation. J. Biol. Chem. 271, 19251–19255.

Azzi, A., Breyer, I., Feher, M., Ricciarelli, R., Stocker, A., Zimmer, S., Zingg, J., 2001. Nonantioxidant functions of alpha-tocopherol in smooth muscle cells. J. Nutr. 131, 378–381.

Burton, G.W., Ingold, K.U., 1989. Vitamin E as an in vitro and in vivo antioxidant. Ann. N.Y. Acad. Sci. 570, 7–22.

Chien-Cheng, H., Mao-Hsiung, Y., Chia-Hung, Y., Ying-Tung, L., 2001. Oxidized low density lipoprotein induces apoptosis via generation of reactive oxygen species in vascular smooth muscle cells. Cardiovasc. Res. 49, 135–145.

Chow, C.K., 1991. Vitamin E and oxidative stress. Free Radic. Biol. Med. 11, 215–232.

Claudio, N., Oswald, Q., Filomena, D.N., Pasquale, A., Christopher, K.G., Wulf, P., 2000. Mildly oxidized low density lipoprotein activates multiple apoptosis signaling pathways in human coronary cells. FASEB 14, 1996–2007.

Dayuan, L., Tom, S., Jawahar, L.M., 2000. Effect of α-tocopherol on Ox-LDL-mediated degradation of IκB and apoptosis in cultured human coronary artery endothelial cells. J. Cardiovasc. Pharmacol. 36, 297–301.

Dimmeler, S., Haendeler, J., Galle, J., Zeiher, A.M., 1997. Oxidized low-density lipoprotein induces apoptosis of human endothelial cells by activation of CPP32-like proteases. Circulation 95, 1760–1763.

Enari, M., Talanian, R.V., Wong, W.W., Nagata, S., 1996. Sequential activation of ICE-like and CPP32-like proteases during Fas-mediated apoptosis. Nature 380, 723–726.

Faulkner, K.M., Liochev, S.I., Fridovich, I., 1994. Stable Mn(III)porphyrins minic superoxide dismutase in vitro and substitute for it in vivo. J. Biol. Chem. 269, 23471–23476.

Haendeler, J., Mebmer, U.K., Brune, B., Neugebauer, E., Dimmeler, S., 1996a. Endotoxic sock leads to apoptosis in vivo and reduces Bcl-2. Shock 6, 405–409.

Haendeler, J., Zeiher, A.M., Dimmeler, S., 1996b. Vitamin C and E prevent lipopolysaccharide-induced apoptosis in human endothelial cells by modulation of Bcl-2 and Bax. Eur. J. Pharmacol. 317, 407–411.

Hampton, M.B., 1996. Dual regulation of caspase activity by hydrogen peroxide: implications for apoptosis. FEBS Lett. 414, 552–556.4.

Henriksen, T., Mahoney, E.M., 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. U. S. A. 78, 6499–6503.

Ishiyama, M., Mizoguchi, M., Shiga, M., Sasamoto, K., 1993. A new tetrazolium salt that produces a highly water-soluble dye. Chem. Pharm. Bull. 41, 1118.

Jacobson, M.D., 1996. Reactive oxygen species and programmed cell death. TIBS 21, 83–86.

Jaffe, E.A., Nachman, R.L., Becker, C.G., Minick, C.R., 1973. Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria. J. Clin. Invest. 52, 2745–2756.

Janero, D.R., 1989. Therpeutic potential of vitamin E in the pathogenesis of spontaneous atherosclerosis. Free Radic. Biol. Med. 11, 129–144.

Jovinge, S., Crisby, M., Thyberg, J., Nilsson, J., 1997. DNA fragmentation and ultrastructural changes of degenerating cells in atherosclerotic lesions on smooth muscle cells exposed to oxidized LDL in vitro. Arterioscler. Thromb. Vasc. Biol. 17, 2225–2231.

Kagan, V.E., 1989. Tocopherol stabilize membrane against phospholipase A, free fatty acid, and lysophospholipids. Ann. N.Y. Acad. Sci. 570, 121–135.

Kaneko, T., Nakano, S.I., Matsuo, M., 1991. Protective effect on vitamin E on linoleic acid hydroperoxide-induced injury to human endothelial cells. Lipids 26, 345–348.

Kenji, H., Shun, I., Toshiyuki, M., Jun-ichi, O., Hiroki, Y., Ken, O., Yoshiro, Y., Nobuhiro, Y., 1997. Bcl-2 protein inhibits oxysterol-induced apoptosis through suppressing CPP32-mediated pathway. FEBS 411, 63–66.

Kume, N., Gimbrone Jr., M.A., 1994. Lysophosphatidylcholine transcriptionally induces growth factor gene expression in cultured human endothelial cells. J. Clin. Invest. 93, 907–911.

Kume, N., Cyblesky, M.I., Gimbrone Jr., M.A., 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.

LeBel, C.P., Ischiropoulos, H., Bondy, S.C., 1992. Evaluation of the probe 2, 7-dichlorofluorescin as an indicator of reactive oxygen species formation and oxidative stress. Chem. Res. Toxicol. 5, 227–231.

Li, P.F., Dietz, R., Von Harsdorf, R., 1997. Reactive oxygen species induce apoptosis of vascular smooth muscle cell. FEBS Lett. 1404, 249–252.

Li, D., Saldeen, T., Mehta, J.L., 1999. Gamma-tocopherol decreases ox-LDL-mediated activation of nuclear factor-kappaB and apoptosis in human coronary artery endothelial cells. Biochem. Biophys. Res. Commun. 259, 157–161.

Liu, X., 1996. Induction of apoptotic program in cell-free extracts: requirement for dATP and cytochrome *c*. Cell 86, 147–157.

Mangin, E.L., Kugiyama, K., Nguy, J.H., Kerns, S.A., Henry, P.D., 1993. Effect of lysolipids and oxidatively modified low density lipoprotein on endothelium-dependent relaxation of rabbit aorta. Circ. Res. 72, 161–166.

Miyashita, Y., Shirai, K., Ito, Y., Watanabe, J., Urano, Y., Murano, T., Tomioka, H., 1997. Cytotoxicity of some oxysterols human vascular smooth muscle cells was mediated by apoptosis. J. Atheroscler. Thromb. 4, 73–78.

Naito, M., Hayashi, T., Yamada, K., Asai, K., Yoshimine, N., Iguchi, A., 1993. Protective effect of idebenone on vascular endothelial cells against toxicity induced by oxidatively modified density lipoprotein. Artery 20, 314–323.

Naito, M., Yamada, K., Hayashi, T., Asai, K., Yoshimine, N., Iguchi, A., 1994. Comparative toxicity of oxidatively modified low-density lipoprotein and lysophosphatidylcholine in cultured vascular endothelial cells. Heart Vessels 9, 183–187.

Nakajima, H., Park, H.L., Henkart, P.A., 1995. Synergistic roles of granzymes A and B in mediating target cell death by rat basophilic leukemia/mast cell tumors also expressing cytolysin/perforin. J. Exp. Med. 181, 1037–1046.

Nicholson, D.W., Ali, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., 1995. Identification and inhibition of the ICE/CED-3 protease necessary for mammalian apoptosis. Nature 1376, 37–43.

Noguchi, N., Gotoh, N., Niki, E., 1993. Dynamics of the oxidation of low density lipoprotein induced by free radicals. Biochem. Biophys. Acta 1168, 348–357.

Packer, L., Lamdvik, S., 1991. Vitamin E: introduction to biochemistry and health benefits. Ann. N.Y. Acad. Sci. 570, 335–344.

Paull, K.D., Shoemaker, R.H., Boyd, M.R., Parsons, J.L., Risbood, P.A., Barbara, W.A., Sharma, M.N., Baker, D.C., Hand, E., Scubiero, D.A., Monks, A., Alley, M.C., 1994. The synthesis of XTT: a new tetrazolium

reagent that is bioreducible to a water-soluble formazan. J. Heterocycl. Chem. 25, 911.

Reed, J.C., 1997. Cytochrome *c* cannot live with it—cannot live without it. Cell 91, 559–562.

Reid, V.C., Mitcinson, M.J., Skeppe, J.N., 1993. Cytotoxicity of oxidized low density lipoprotein to mouse peritoneal macrophages. J. Pathol. 171, 321–328.

Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C., Witztum, J.L., 1989. Beyond cholesterol: modification of low density lipoprotein that increases its atherogenicity. N. Engl. J. Med. 320, 915–924.

Sugiyama, H., Kashihara, N., Makino, H., Yamasaki, Y., Ota, Z., 1996. Reactive oxygen species induce apoptosis in cultured human mesangial cells. J. Am. Soc. Nephrol. 7, 2357–2363.

Sylvester, P.W., McIntyre, B.S., Gapor, A., Briski, K.P., 2001. Vitamine E inhibition of normal mammary epithelial cell growth is associated with a reduction in protein kinase C(alpha) activation. Cell Prolif. 34, 347–357.

Terasawa, Y., Manabe, H., Yoshida, N., Uemura, M., Sugimoto, N., Naito, Y., Yoshikawa, T., Kondo, M., 2000. Alpha-tocopherol protects against monocyte Mac1 (CD11b/CD18) expression and Mac1dependent adhesion to endothelial cells induced by oxidized low-density lipoprotein. BioFactors 11, 221–233.

Urano, S., Matsuo, M., 1989. Membrane stabilizing effect of vitamin E. Ann. N.Y. Acad. Sci. 570, 524–526.

Yoshida, N., Yoshikawa, T., Manabe, H., Terasawa, Y., Kondo, M., Noguchi, N., Niki, E., 1999. Vitmin E protects against polymorphonuclear leukocyte-dependent adhesion to endothelial cells. J. Leukoc. Biol. 65, 757–763.

Yoshida, N., Manabe, H., Terasawa, Y., Nishimura, H., Enjo, F., Nishino, H., Yoshikawa, T., 2000. Inhibitory effects of vitamin E on endothelial-dependent adhesive interactions with leukocytes induced by oxidized low density lipoprotein. BioFactors 13, 279–288.