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γ-Tocopherol Decreases Ox-LDL-Mediated Activation of Nuclear Factor-κB and Apoptosis in Human Coronary Artery Endothelial Cells

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γ-Tocopherol, produced by many plants, is the major form of tocopherol in the United States diet. It is an effecient protector of lipids against peroxidative damage. Epidemiologic studies show that supplementation of diet with γ-tocopherol is inversely related to the risk of death from cardiovascular disease. This study was conducted to examine the role of γ-tocopherol in oxidized LDL (ox-LDL)-induced nuclear factor (NF)-κB activation and apoptosis in human coronary artery endothelial cells (HCAECs). Cultured HCAECs were treated with ox-LDL (10-40 μg/ml). Incubation of HCAECs with ox-LDL resulted in apoptosis of HCAECs, as determined by TUNEL and DNA laddering. Ox-LDL degraded IκB protein and activated NF-κB in HCAECs (both $P < 0.01$ vs control), as determined by Western blot. Treatment of cells with γ-tocopherol attenuated ox-LDL-mediated degradation of IκB and activation of NF-κB (both $P < 0.01$ vs ox-LDL alone). The presence of γ-tocopherol also reduced ox-LDL-induced apoptosis ($P < 0.01$ vs ox-LDL alone). A high concentration of γ-tocopherol (50 μmol/L) was more effective than the low concentration of γ-tocopherol (10 μmol/L) in this process. These observations show that ox-LDL induces apoptosis of HCAECs at least partially by activation of NF-κB signal transduction pathway. γ-Tocopherol significantly decreases ox-LDL-induced apoptosis of HCAECs by inhibiting the activation of NF-κB.

Key Words: **γ-tocopherol; endothelial cells; oxidized LDL; apoptosis; NF-κB.**

The membrane antioxidant vitamin E consists of two major forms, alpha-tocopherol and gamma-tocopherol. Gamma-tocopherol is the principal form found in the lipid fraction of many plants and nuts (1,2), and accounts for as much as 70% of the total intake of tocopherols in the United states diet (3). In traditional bioassays, gamma-tocopherol constitutes only 10-20% of the vitamin E activity of commercial preparations (4), but it is very efficient as an antioxidant (5). The serum levels of gamma-tocopherol average one fifth those of alpha-tocopherol in humans (6) despite the higher dietary intake of gamma-tocopherol (3) and similar rates of absorption from the gastrointestinal tract (7). Gamma-tocopherol is preferentially taken up by human endothelial cell, yet it disappears from the cells at a faster rate, resulting in low serum concentration (8).

Epidemiological studies show alpha-tocopherol to be protective against cardiovascular disease in some studies (9), but not in others (10). A study by Kushi et al (11) showed that vitamin E supplementation (all as alpha-tocopherol) had no effect on the risk of death from coronary artery disease (CAD), whereas when supplied in the diet (mainly as gamma-tocopherol), it was strongly related to decrease in CAD risk. Notably, patients with CAD have lower serum levels of gamma-tocopherol, and not alpha-tocopherol (12). A recent experimental study (13) shows that gamma-tocopherol inhibits phospholipid oxidation and oxidation of LDL components, more potently than alpha-tocopherol. Taken together, these observations suggest that gamma-tocopherol may play an important role in the inhibition of progression of atherosclerosis and CAD. However, there is little information on the pathophysiological significance and mechanisms of gamma-tocopherol on vascular function.

Endothelial dysfunction elicited by ox-LDL has been implicated in the pathogenesis of atherosclerosis (14). Recent studies show that apoptosis, which is commonly seen in atherosclerosis, is induced by ox-LDL in a variety of cell types (15-18). A recent study (15) showed that ox-LDL-mediated apoptosis of human coronary

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artery endothelial cells (HCAECs) is associated with a decrease in Bcl-2 expression and an increase in Fas expression as well as activation of protein kinase C (PKC). Dimmeler et al (16) found that ox-LDL initiates apoptosis of human endothelial cells by enhancing CPP-32-like protease activity.

NF-κB, an oxidative stress-responsive transcription factor (19), is present in cytosol as a heterodimer composed of NF-κB1 (P50) and Rel (P65) subunits bound to an inhibitor protein, IκB. After activation, NF-κB translocates from the cytosol to the nucleus of the cells, binds to specific DNA sequences and initiates transcription. Proteolytic degradation of IκB plays a pivotal role in NF-κB activation in endothelial cells. Maziere et al (20) showed that ox-LDL activates NF-κB in fibroblasts and endothelial and smooth muscle cells and causes cell injury. Brand et al (21) demonstrated NF-κB activity in endothelial cells in atherosclerotic plaques in vivo. Hernan dez-Presa et al (22) also confirmed the activation of NF-κB in accelerated atherosclerosis in rabbits.

The present study shows that gamma-tocopherol decreases ox-LDL-mediated NF-κB activation and subsequently reduces ox-LDL-induced apoptosis of HCAECs.

MATERIALS AND METHODS

Cell Culture

The methodology for culture of HCAECs has been described previously (15). In brief, initial batch of HCAECs was purchased from Clonetics Corporation. The endothelial cells were pure based on morphology and staining for factor VIII and acetylated LDL. These cell were 100% negative for alpha actin smooth muscle expression. Microvascular endothelium growth medium consisted of 500 ml of endothelial cell basal medium, 5 ng of human recombinant epidermal growth factor, 5 mg of hydrocortisone, 25 mg of gentamycin and 25 μg of amphotericin B, 6 mg of bovine brain extract, and 25 ml fetal bovine serum. HCAECs in 5 ml medium were seeded in a 25 cm^2 flask (4,000 cells/cm^2), incubated at 37°C in 95% air-5% CO_2. Fifth generation HCAECs were used in this study.

Groups of HCAECs were incubated with ox-LDL (10,20, and 40 μg/ml) to determine apoptosis. Additional groups of HCAECs were incubated with ox-LDL (40 μg/ml) alone or with gamma-tocopherol (10 or 50 μM) plus ox-LDL (40 μg/ml) to observe degradation of IκB protein, activity of NF-κB, and apoptosis.

Preparation of Lipoproteins

Native LDL and oxidized LDL were prepared as described earlier (15). In brief, human native LDL was isolated from human blood plasma by discontinuous centrifugation. It was further purified via ultracentrifugation (1.063-1.210 g/ml) to homogeneity determined on agarose gel electrophoresis. LDL was oxidized by exposure to $CuSO_4$ (5 μmol/L free Cu^{2+} concentration) in phosphate-buffered saline at 37°C for 24 hours. The TBARS content of ox-LDL was 18.2 ± 0.28 versus 0.56 ± 0.16 nmoles/100 μg protein in the native-LDL preparation ($P < 0.01$). LDL, ox-LDL were kept in 50 mM Tris-HCl, 0.15 M NaCl and 2 mM EDTA at pH 7.4, and were used within 10 days of preparation.

Determination of Apoptosis

In situ nick end-labeling (TUNEL) and propidium iodide (PI) staining. To detect DNA fragmentation in situ, nick end-labeling was performed by the method described by Gavrieli et al (23) and by us (15). Briefly, the cells plated on slides were fixed with 4% methanol-free formaldehyde, pH 7.4 for 25 minutes at 4°C and washed with PBS. The slides were incubated with 3% H_2O_2 for 5 minutes for inactivation of endogenous peroxidase and equilibrated with terminal deoxynucleotidyl transferase (TDT) buffer (Promega) for 10 minutes at room temperature (RT). The slides were covered with 0.3 U/μl TDT and 0.04 nmol/μl fluorescein-12-dUTP (Promega) in TDT buffer for 60 minutes at 37°C. Unincorporated fluorescein-dUTP was removed, and the slides were immersed in 1 μg/ml of propidium iodide in PBS for 15 minutes at RT and washed, and analyzed under a fluorescence microscope (green fluorescence at 520 nm and red fluorescence of propidium iodide at >620 nm). The negative controls were performed without TDT enzyme. The positive controls were performed in samples pretreated with DNase I.

DNA fragmentation gel electrophoresis (DNA ladder). HCAECs pellets were lysed with buffer (1% NP-40 in 20 mM EDTA, 50 mM Tris-HCl, pH 7.5), the supernatants were treated for 2 hours with RNase A (final concentration 5 μg/μl) at 56°C followed by digestion with proteinase K (final concentration 2.5 μg/μl) for 2 hours at 37°C. After addition of 1/2 volume of 10 M ammonium acetate, DNA fragmentation was precipitated with 2.5 volume of absolute ethanol. DNA fragmentation was recovered by centrifugation at 12,000 g for 10 min and dissolved in gel loading buffer. DNA fragmentation was separated by electrophoresis in 1.6% agarose gel with ethidium bromide (15,24).

Preparation of Nuclear Extracts and Western Blot for NF-κB

Nuclear extracts were prepared as described by Alksnis et al (25). Briefly, the HCAECs were lysed in 300 μl lysis buffer (10 mM HEPES, pH 7.3, 10 mM KCl, 1.5 mM $MgCl_2$, 0.4% Nonidet P-40, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, and 15 μg/ml aprotinin). After a 10 min incubation at 4°C, nuclei were collected by centrifugation for 1 min at 8000g, and the pellets washed in 1 ml of 20 mM KCl buffer (20 mM HEPES, pH 7.3, 22% glycerol, 20 mM KCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, 1 mM PMSF, 1 μg/ml leupeptin, and 15 μg/ml aprotinin). The isolated nuclei were resuspended in 100 μl of 484 mM KCl buffer. Nuclear proteins were extracted by incubation on ice for 30 min. After centrifugation for 15 min at 8000g, tsupernatant containing nuclear proteins was transferred and quantitated.

Monoclonal antibody (MAb) to the P65 subunit of NF-κB from mouse to mouse hybrid cells was purchased from Boehringer Mannheim. The antibody recognizes an epitope overlapping the nuclear location signal of the P65 subunit and, therefore, selectively binds the activated form of NF-κB. Equal amounts of protein (15 μg) from nuclear extract from each group were separated by 12% SDS-PAGE, transferred to nitrocellulose filters (Sigma). After incubating in blocking solution (3% nonfat milk, Sigma), membranes were incubated in buffer containing 7.5 μg/ml of the MAb to P65 subunit of NF-κB. Anti-mouse alkaline phosphatase-conjugated antibody was used as a secondary antibody at 1:3000 dilution. Protein band of interest (65 kD) was detected by ECL system, and relative intensities of protein bands were analyzed by MSF-300G Scanner (Microtek Lab) (15).

Western Blot for IκB

HCAEC lysates from each experiment (20 μg per lane) were separated by 12% SDS-PAGE, and transferred to nitrocellulose membranes. After incubation in blocking solution (4% non-fat milk, Sigma), membranes were incubated with 1:1000 dilution of rabbit

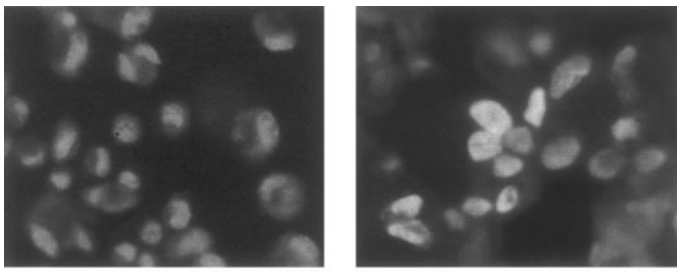

FIG. 1. Evidence of apoptosis in cultured HCAECs treated with ox-LDL (40 μg/ml), as assessed by fluorescence microscopy. The apoptotic cells are those with green fluorescence as fluorescent-12-dUTP is incorporated at the 3′-OH ends of fragmented DNA. The cytoplasm is stained pink (original magnification $\times$100).

polyclonal anti-IκB (Santa Cruz Biotechnology) in TBS (25 mM Tris, pH 7.4, 137 mM NaCl, 2.7 mM KCl) with 0.1% Tween 20. Excess primary antibody was removed by washing the membranes in TBS with 0.1% Tween 20. The membranes were incubated with 1:3000 dilution second antibody (Santa Cruz Biotechnology) for 1 hr. IκB protein was detected and quantitated as described above.

Data Analysis

Data presented in this paper represent mean of duplicate samples from six independently performed experiments. Data are presented as mean ± SD. Statistical significance was determined in multiple comparisons among independent groups of data in which ANOVA and the F test indicated the presence of significant differences. A P value $\leq$0.05 was considered significant.

RESULTS

Ox-LDL, Gamma-Tocopherol, and Apoptosis in HCAECs

Since a small number of cells normally die during culture or are damaged during processing, 1 to 5% (3.8 ± 1.9%) of control cells stained positive. Treatment with ox-LDL caused a marked increase in apoptosis compared with control ($P < 0.01$). Results of a representative experiment upon treatment of HCAECs with 40 μg/ml of ox-LDL are shown in Fig. 1. The pro-apoptotic effect of ox-LDL was concentration-dependent. The presence of gamma-tocopherol in the culture medium before the cells were exposed to ox-LDL reduced the number of apoptotic cells in HCAECs compared with ox-LDL alone ($P < 0.01$). High dose of gamma-tocopherol (50 μM) was more effective than alpha-tocopherol (10 μM) in these effects ($P < 0.05$). Data from multiple experiments are summarized in Fig. 2.

Apoptosis, measured as DNA laddering on gel electrophoresis, was also enhanced by treatment of HCAECs with ox-LDL. Treatment with gamma-tocopherol before the cells were exposed to ox-LDL reduced DNA laddering. Pattern of DNA laddering in a representative experiment is shown in Fig. 3.

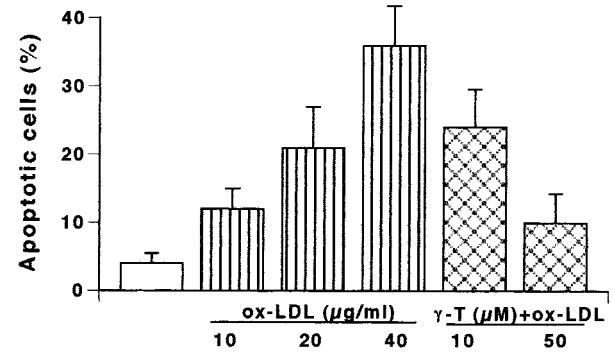

FIG. 2. Effect of ox-LDL on apoptosis in HCAECs. The increase in the number of apoptotic cells is dependent on the concentration of ox-LDL (10-40 μg/ml). γ-Tocopherol decreases the number of ox-LDL-induced apoptotic cells ($P < 0.01$ vs ox-LDL alone). High concentration of γ-tocopherol (50 μM) was more effective than the low concentration (10 μM) on ox-LDL (40 μg/ml)-induced apoptosis. Data are from six separate experiments and are shown as mean ± SD.

Ox-LDL, Gamma-Tocopherol and Degradation of IκB Protein and Activation of NF-κB

Western analysis ($n = 6$) showed that ox-LDL (40 μg/ml) completely degraded IκB ($P < 0.01$ vs control) and caused activation of NF-κB ($P < 0.01$ vs control) (Fig. 4). Gamma-tocopherol inhibited ox-LDL-mediated degradation of IκB and activation of NF-κB (both $P < 0.01$ vs ox-LDL alone). High concentration of gamma-tocopherol (50 μM) was more effective than the low concentration of gamma-tocopherol (10 μM) in these effects ($P < 0.05$).

DISCUSSION

This study conducted in human coronary artery endothelial cells shows that ox-LDL induces degradation

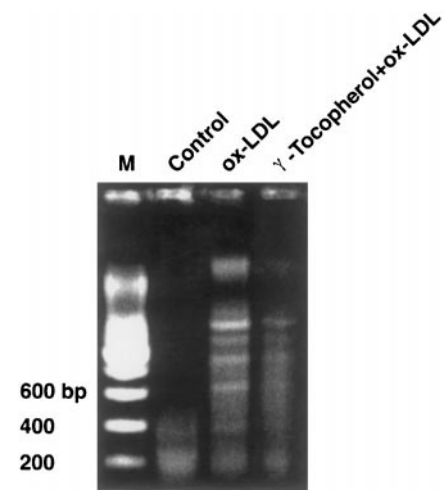

FIG. 3. DNA laddering on gel electrophoresis. The typical laddering pattern is induced in ox-LDL (40 μg/ml)-treated HCAECs, suggestive of apoptosis. DNA laddering is markedly decreased by γ-tocopherol (50 μM). This figure is representative of six separate experiments.

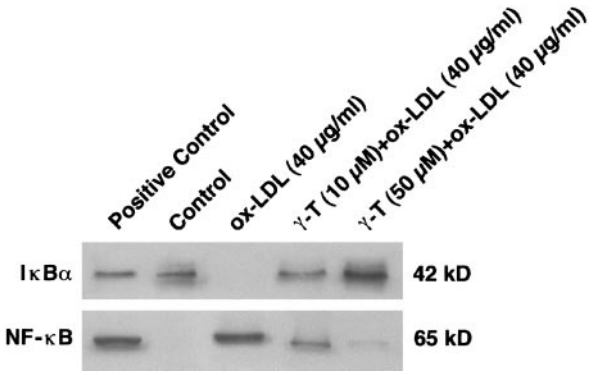

FIG. 4. Identification of IκB protein in cytoplasm by Western analysis (upper panel). IκB protein in cytoplasm is degraded in HCAECs treated with 40 μg/ml ox-LDL. However, γ-tocopherol inhibits ox-LDL-mediated degradation of IκB ($P < 0.01$ vs ox-LDL alone). Identification of NF-κB protein in the nuclear extracts by Western analysis (bottom panel). NF-κB is activated in HCAECs treated with ox-LDL (40 μg/ml) ($P < 0.01$ vs control). Treatment of HCAECs with γ-tocopherol inhibits ox-LDL-mediated activation of NF-κB ($P < 0.01$ vs ox-LDL alone). This figure is representative of six separate experiment. Abbrev.: γ-T, γ-tocopherol. The *P* values are based on densitometric analysis.

of IκB and activation of NF-κB and simultaneously causes apoptosis in these cells. Importantly, gamma-tocopherol reverses the effect of ox-LDL on degradation of IκB and activation of NF-κB and prevents apoptosis in HCAECs. These observations in human endothelial cells may have a bearing the beneficial effects of gamma-tocopherol observed in clinical studies.

Ox-LDL, and not the native-LDL, is thought to be a key factor that causes injury to the endothelium early in atherogenesis (14,15). Ox-LDL causes coronary endothelial dysfunction, loss of myocardial contractile function, and damage to the cardiac ultrastructure (26). Apoptosis is a distinct mode of cell death often seen in atherosclerotic tissues (27,28), and has been identified in human endothelial cells (15,16), rabbit vascular smooth muscle cells (17), and human monocytes/macrophages (18) exposed to ox-LDL. Ox-LDL decreases bcl-2 and increases Fas protein expression, and enhances CPP 32-like protease activity in human endothelial cells (15,29). Our previous studies in cultured endothelial cells showed that ox-LDL treatment causes release of oxidant species in conjunction with apoptosis and altered gene expression (15). Ox-LDL also increases intracellular cellular calcium to trigger apoptosis (30). The precise signal transduction pathway/s of ox-LDL-mediated apoptosis, however, need to be further defined.

Increasing evidences suggests that NF-κB plays a key role in signal transduction in endothelial cells (31,32). Two major mechanisms that are likely to play a role in its activation are: phosphorylation of the inhibitory molecule IκB (31) and free radical-dependent oxidation (19). It is well established that NF-κB elements are tightly controlled by several inhibitors belonging to the IκB family (31). Ox-LDL activates NF-κB in fibroblasts and endothelial and smooth muscle cells and causes cell injury (20). Oxidative activation of endothelial cell transcription factors, especially NF-κB, has been suggested as a mechanism for initiation of atherosclerotic lesions (32). Importantly, NF-κB activation has been demonstrated in endothelial cells in early atherosclerotic plaques (21,22).

In the present study, we confirmed our previous observations (15) that ox-LDL induces apoptosis of HCAECs. The results of the TUNEL and DNA laddering methods used in this study complement the features of apoptosis on transmission electron microscopy and the alterations in apoptosis-specific gene proteins (15). We extended these observations of induction of apoptosis of HCAECs by ox-LDL, and now show that ox-LDL (40 μg/ml) markedly degrades IκB protein and activates NF-κB. We also found that gamma-tocopherol markedly inhibits the effect of ox-LDL on degradation of IκB protein and activation of NF-κB, and concurrently, it attenuates the degree of apoptosis. These novel findings indicate that ox-LDL-mediated apoptosis in human endothelial cells is, at least partially, mediated by activation of transcription factor NF-κB.

Although gamma-tocopherol is the principal form of vitamin E in the United States diet (1,2), it has not received much attention, perhaps related to the fact that alpha-tocopherol levels in blood plasma and most other tissues are about 5-fold higher than gamma-tocopherol (6). However, alpha-tocopherol is much less efficient as an antioxidant as shown in previous studies (5). Although some studies suggest that alpha-tocopherol supplement decreases the risk of CAD (33), other studies have failed to demonstrate a similar protective effect (10,11). Furthermore, high doses of alpha-tocopherol can increase tumor formation in animals (34,35) and displace gamma-tocopherol in plasma and other tissues (36). Keaney et al (37) found that low dose of alpha-tocopherol improves and high dose of alpha-tocopherol worsens endothelial vasodilator function in cholesterol-fed rabbits. Together these data indicate a need to reevaluate the potential of the two tocopherols alter vascular biology. Christen et al (13) investigated the efficacy by which alpha-tocopherol and gamma-tocopherol inhibit peroxynitritic-induced lipid peroxidation and found that gamma-tocopherol was more effective than alpha-tocopherol in this context. Cooney et al (38) found that nitrogen dioxide ($^{\cdot}NO_2$)-mediated nitrosation of morpholine is inhibited effectively only by gamma-tocopherol and not by alpha-tocopherol. Evidence is now emerging that gamma-tocopherol may be at least as important as alpha-tocopherol in the prevention of degenerative and cardiovascular diseases (12,38).

It is noteworthy that many studies which employed dietary alpha-tocopherol supplementation have failed to demonstrate cardioprotective effect (10,11,33). Interestingly, in one of the studies (11), vitamin E appeared to offer protective effect against CAD only when taken in the from of diet and not when taken as commercial supplements, which consist primarily of alpha-tocopherol. The strongest correlation of cardioprotective effect of vitamin E was shown when vitamin E was in dietary form [consumption of margarine, nuts, and seeds; all these are excellent sources of gamma-tocopherol (11)]. Interestingly, clinical evaluation of individuals suffering from CAD shows decreased serum levels of gamma-tocopherol, but not alpha-tocopherol (12).

In the present study, we found that gamma-tocopherol attenuates ox-LDL-mediated apoptosis of HCAECs, perhaps via inhibition of ox-LDL-mediated NF-κB activation. The attenuation of ox-LDL-induced degradation of IκB and activation of NF-κB as well as apoptosis shown in the present study may provide a unique mechanism for the beneficial effect of gamma-tocopherol in coronary heart disease.

In summary, ox-LDL induces degradation of IκB and activation of NF-κB pathway as well as apoptosis in human coronary artery endothelial cells. Gamma-tocopherol significantly attenuates ox-LDL-mediated activation of transcription factor NF-κB pathway and apoptosis. These data provide novel insight into the potential benefit of gamma-tocopherol in cardiovascular disease.

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