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Modulation by α- and γ-Tocopherol and Oxidized Low-Density Lipoprotein of Apoptotic Signaling in Human Coronary Smooth Muscle Cells*

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ABSTRACT. Apoptosis may play an important role in atherogenesis. Oxidized low-density lipoprotein (oxLDL) promotes apoptosis in the arterial wall in addition to several other proatherogenic effects. Tocopherol supplements have been suggested to protect against coronary heart disease (CHD) in epidemiological studies. The effects of oxLDL and α- and γ-tocopherol on apoptotic signaling pathways are poorly understood. Thus, the goal of the study was to investigate these pathways in the presence of copper-oxidized LDL and tocopherols in human coronary smooth muscle cells (SMC). We showed that oxLDL-mediated apoptosis, assessed by DNA fragmentation, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay, and caspase activation stimulated several transcription factors and proapoptotic dynamic movements of the Bcl-2 family proteins through the mitogen-activated protein kinase (MAPK) and Jun kinase pathways. α-Tocopherol and γ-tocopherol significantly reduced these molecular events and cell death effectors caspase-3 and -8. Under our experimental conditions, α-tocopherol was significantly more effective than γ-tocopherol, and oxLDL-mediated apoptosis increased c-Jun, cyclic AMP-responsive element-binding, Ets-like element kinase-dependent 7, and activating transcription factor-2 proteins as well as nuclear activity of the activated protein-1 complex in human coronary SMC. Moreover, our results demonstrate that tocopherols may exert their antiatherogenic effects at least in part via reduction of the MAPK and JunK cascade together with a protective profile of apoptotic genes of the Bcl-2 family. These data are consistent with the beneficial effects of tocopherols on atherogenesis seen in experimental studies and on CHD in epidemiological surveys. BIOCHEM PHARMACOL **59**;11:1477–1487, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. LDL; oxidation; α-tocopherol; γ-tocopherol; apoptosis, SMC; CHD

Apoptosis may play an important role in the pathophysiology of atherogenesis by inducing cell death primarily in endothelial cells and SMC** (reviewed in [1]). oxLDL promotes apoptosis in the normal arterial wall and in atherosclerotic lesions [1–5]. It is well known that oxLDL plays a pivotal role in atherogenesis (reviewed in [6]), and it is an important pathogenic factor in early atherogenesis in fetal [7] and childrens' [8] arteries. The corollary of the oxidation theory of atherogenesis is that antioxidants may have beneficial effects on atherosclerosis [6]. Vitamin E consists of two major forms, α-tocopherol, the most abundant form in plasma and on LDL, and γ-tocopherol, which is found in many plants and accounts for more than 50% of the total intake of tocopherols in Western-type diets [9, 10]. γ-Tocopherol is avidly taken up by endothelium, disappearing at a faster rate [11] and reaching serum concentrations on average one-fifth those of α-tocopherol [10].

Epidemiological surveys have shown that α-tocopherol may exert advantageous actions against CHD (reviewed in [12]) as well as in its secondary prevention [13]. Plasma α-tocopherol is inversely correlated with the manifestation of angina pectoris [14], and supplemental α-tocopherol is

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** *Abbreviations:* SMC, smooth muscle cells; LDL, low-density lipoprotein; oxLDL, oxidized low-density lipoprotein; CHD, coronary heart disease; MAPK, mitogen-activated protein kinase; JunK, Jun amino-terminal kinase; MEK1, MAP kinase kinase; ERK-1, extracellular regulated kinase-1; TUNEL, terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labelling; PMSF, phenylmethylsulfonyl fluoride; FACS, fluorescence-activated cell sorting; PARP, poly(ADP-ribose) polymerase; NF-kappaB, nuclear factor kappa B; CREB, cyclic AMP-responsive element-binding protein; ATF-2, activating transcription factor-2; ELK-1, Ets-like element kinase-dependent 1; AP-1 complex, activated protein-1 complex; and IgG, immunoglobulin G.

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inversely associated with the development of CHD in both men [15] and women [16]. However, α-tocopherol appears to be less effective in lowering the risk of death due to CHD with respect to γ-tocopherol in postmenopausal women [17], and patients with CHD often have low levels of γ-tocopherol [18]. α-Tocopherol decreases the oxidation of LDL and the accumulation of oxLDL in the arterial wall as well as preventing endothelial alterations via inhibition of protein kinase C (reviewed in [19]). Preliminary data indicate that α-tocopherol reduces oxLDL-induced apoptosis in human endothelial cells [20].

Currently, the effects of oxLDL and α- and γ-tocopherols on the signaling pathways of coronary cells and the events involved in the transduction of programmed death signal into the nucleus (reviewed in [21]) are poorly understood. Here, we analyzed not only oxLDL-induced apoptosis in coronary cells by DNA fragmentation and TUNEL assay, caspase activation, PARP degradation, and Bcl-2 family proteins (see below), but also the apoptotic involvement of protein kinases and many transcription factors [21]. In fact, the progressive understanding of the apoptosis genetic machinery has identified the MAPK cascade and in turn the enhanced Jun amino-terminal kinase (JunK) as downstream targets controlling cellular growth and apoptosis [22, 23]. Once activated, MAPK and JunK can then phosphorylate several transcription factors (e.g. ATF-2, ELK-1, c-Jun, CREB, AP-1 complex, and others, see below) [23]. Since multiple cell-cycle control signals, which also involve apoptosis [21, 24], converge on NF-kappaB [24], we also investigated its nuclear activation in the presence of oxLDL and tocopherols. Thus, the goal of the present study was to explore these apoptotic signaling pathways in the presence of oxLDL and tocopherols in human coronary SMC.

MATERIALS AND METHODS

Low-Density Lipoprotein Isolation and Oxidation

Plasma was obtained from healthy non-smoking males (N = 4, age 21 ± 2 years), and LDL was quickly isolated by two consecutive steps of discontinuous density ultracentrifugation in a KBr gradient, as previously described in detail [25]. A Sephacryl S-300 column (5 × 0.9 cm, equilibrated with 150 mM NaCl–PBS containing 1 mM EDTA) was used to desalt and remove low molecular weight components. Fresh LDL preparations were used within a few hours to decrease spontaneous peroxidation [25]. LDL purity and relative agarose electrophoretic gel mobility with respect to the baseline (REM) were checked both by agarose gel electrophoresis under non-denaturing conditions and by SDS–PAGE performed on a 5–16% linear gradient slab gel [25]. Protein content was measured by the Lowry method [26]. LDL (300 μg/mL) was incubated for 12 hr at 37° with 1 μM copper sulphate, as previously described [27], in the presence of γ- or α-tocopherol. Malondialdehyde (MDA) content was assayed by thiobarbituric acid, as previously described in detail [25, 27]. LDL was first oxidized in the absence or presence of tocopherols, dialyzed against phosphate buffer at 4° for 2 hr, and then incubated for 24 hr with coronary SMC at 37° under 95% air, 5% CO_2 (see below). To preserve tocopherols entirely from oxidation induced by copper, LDL was first oxidized by copper, and both dialyzed oxLDL and fresh tocopherols were then added to the culture medium.

Cell Culture

Primary human coronary SMC were cultured from coronary arteries obtained during by-pass surgery, essentially as previously described [28]. Cell suspensions were flushed through a series of sieves ranging from 60 to 400 mesh. The cells retained by the 400-mesh sieve were washed with 50 mL of HEPES buffer (pH 5.5) and centrifuged for 3 min at 250 *g*. Cells were transferred to culture flasks preflushed with 0.2% gelatin and incubated at 37° for 4 days in an incubator in a humidified atmosphere of 95% air and 5% CO_2. The incubation medium (delipidated Dulbecco's modified Eagle's medium) was supplemented with 10% human delipidated serum, 10 ng/mL of human endothelial growth factor, penicillin/streptomycin, amphotericin B, and glutamine [28]. At the end of the incubation period, the cells were washed with HEPES buffer, centrifuged, and supplied with new incubation medium without growth factors. Cell viability was detected by trypan blue exclusion [28]. Cell suspensions with approximately 10^5 cells/mL were used for all experiments.

For FACS analysis experiments, cells were scraped, transferred into a 15-mL Falcon tube, and centrifuged at 250 *g* for 5 min. After removal of the supernatant, the cell pellet was resuspended in 3 mL PBS and centrifuged again under the same conditions, twice. After incubation with propidium iodide (PI), cells were analyzed by FACS, as described below.

Preparation of Nuclear Extracts

Nuclear extracts were prepared by a modification of the method of Wu [29]. Briefly, cells were washed twice in PBS and resuspended in 10 volumes of a homogenization solution consisting of 10 mM HEPES (pH 7.9), 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, and 0.5 mM dithiothreitol (DTT). Cells were disrupted by a forced passage through a 26-gauge needle. Nuclei were then collected by centrifugation at 250 *g* and resuspended in 1.2 volumes of extraction solution consisting of 10 mM HEPES (pH 7.9), 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.1 mM EGTA, 0.5 mM DTT, and 5% glycerol. Nuclear proteins were gently shaken at 4° to facilitate elution. Nuclei were then pelleted again by centrifugation at 9000 *g*, and the supernatant was stored at −70° until use. Both homogenization and extraction solutions contained the following protease inhibitors: 5 mM leupeptin, 1.5 mM aprotinin, 2 mM PMSF, 3 mM pepstatin A, and 1 mM benzamidine. Protein concentrations were determined by the Lowry assay [27].

Electrophoretic Mobility Shift Assay (EMSA)

Nuclear extracts (2.5–5 μg of proteins) were preincubated for 10 min at room temperature in 20 μL of a solution consisting of 20 mM HEPES (pH 7.5), 40 mM KCl, and 5% glycerol containing 1 μg poly(dI-dC) and 5 mM spermidine. After addition of the probe (see below), binding reactions were incubated for an additional 15 min. The sequences of the oligonucleotide probes were: consensus binding site for NF-kB: 5′-AGTTGAGGGGACTTTC-CCAGGC-3′ (Cod.sc-2511, Santa Cruz Biotechnology); binding site for the AP-1 complex: 5′-CGCTTGAT-GACTCAGCCGGAA-3′ (Cod. sc-2514, Santa Cruz Biotechnology). Samples were then separated on 8% native polyacrylamide gels.

Western Blot Analysis

Whole-cell extracts were prepared according to Zimarino and Wu [30], as follows. Cells were scraped in cold PBS (4°) and lysed in cold buffer consisting of 0.5% Nonidet P-40 (Sigma) in 50 mM HEPES (pH 7.5), 250 mM NaCl, 5 mM EDTA, 50 mM NaFl, 0.5 mM sodium orthovanadate, 0.5 mM PMSF, 5 μg/mL of aprotinin, and 5 μg/mL of leupeptin. Fifty micrograms of proteins separated by 12.5% SDS–PAGE were transferred to Immobilon-P transfer membranes (Millipore). Western blot analysis was performed according to the standard procedure [31]. Membranes were blocked with 5% nonfat milk proteins and incubated for 1 hr at room temperature with the primary antibodies listed below. The following were purchased from Santa Cruz Biotechnology: antibodies to Bcl-2 (goat polyclonal IgG, #N-19), Bax (goat polyclonal IgG, #N-20), PARP (rabbit polyclonal IgG, #H250), caspase-3 (CPP32) (rabbit polyclonal IgG, #H-277 or mouse monoclonal IgG, #E-8), caspase-8 (Mch5) (goat polyclonal IgG, #C-20), c-Jun (rabbit polyclonal IgG, #H-79), CREB-1 (rabbit polyclonal IgG, #C-21), the phosphorylated form only of CREB (mouse monoclonal IgG, #Ser133), an antibody to the carboxy terminus of human ATF-2 that recognizes both forms of ATF-2 (phosphorylated and non-phosphorylated) (rabbit polyclonal IgG, #C-19), an antibody against the carboxy terminus of ELK-1 that recognizes both forms of ELK-1 (rabbit polyclonal IgG, #I-20), and an antibody to the carboxy terminus of Bad that binds both forms of Bad (rabbit polyclonal IgG, #R-20). An antibody against the phosphorylated form of c-Jun (mouse monoclonal, #420110-S) was purchased from Calbiochem Signal Transduction. Optimal antibody concentrations were determined in pilot assays (usually 1:1000 dilution). Antibodies bound to their respective antigen in the membrane were visualized using species-specific monoclonal secondary antibodies against the Fab region of the primary antibody labeled with horseradish peroxidase. After adding substrate, luminescence was detected by enhanced chemiluminescence (Amersham) and exposed to autoradiograph film for 2 min. To ascertain that blots were loaded with equal amounts of protein lysates, the same membranes were also incubated with a polyclonal antibody against γ-tubulin protein (Sigma). Semiquantitative densitometric scanning analysis of the Western blots was done using a Scan LKB (Pharmacia Produckter AB, Sweden), as previously described in detail [25].

Assessment of DNA Fragmentation

The degradation of DNA into internucleosomal fragments (i.e. DNA fragmentation) was assessed on agarose electrophoresis gels as proposed by Yi *et al.* [32]. Adherent and non-adherent cells were collected, washed twice with cold PBS, resuspended in lysis buffer containing 10 mM Tris–HCl (pH 7.4), 10 mM NaCl, 10 mM EDTA, 1% SDS, and 0.1 mg/mL of proteinase K. Cell suspensions were incubated at 4° for 30 min and then centrifuged at 13,000 r.p.m. for 10 min. Supernatants were extracted with 1:1 (v/v) phenol/chloroform (24:1 isoamylic) and precipitated with 2.5 volumes of pure ethanol. DNA pellets were resuspended in Tris–EDTA buffer and applied to a 1.2% agarose gel in TBE in order to evaluate DNA fragmentation (i.e., "DNA laddering").

TUNEL Assay

Apoptosis in cultured cells was also assessed by the TUNEL technique [33], using the *in situ* cell death detection kit (Boehringer Mannheim). The TUNEL assay was chosen because it is more sensitive with greater signal intensity than *in situ* nick translation using DNA polymerase in discriminating apoptosis from necrosis and primary DNA strand breaks. This technique exploits the fact that single strand breaks ("nicks") in high molecular weight DNA resulting from cleavage of genomic DNA during apoptosis can be identified by labeling free 3′-OH termini with fluorescein-containing nucleotides in an enzymatic reaction. TdT, which catalyzes polymerization of nucleotides to free 3′-OH DNA ends in a template-independent manner, is used to label DNA strand breaks. Incorporated fluorescein is then detected by an alkaline phosphatase-labeled sheep antifluorescein antibody (Fab). After substrate reaction, stained cells are analyzed under light microscope. Briefly, cells mounted on slides were rinsed twice with PBS and 50 mL of TUNEL reaction mixture (containing DNA polymerase, TdT, and labeled nucleotides) and incubated for 60 min at 37° in a humidified chamber. After 3 rinses with PBS, 50 mL of Converter AP (alkaline phosphatase-labeled Fab antibody) was added and incubated for 30 min at 37° in a humidified chamber. Slides were again rinsed 3 times with PBS, and 50 mL of substrate solution (Fast Red) was added. After 10-min incubation at room temperature, the slides were mounted under glass coverslip and analyzed under the visible light microscope (Zeiss).

TABLE 1. Characteristics of copper-oxidized LDL in the presence of tocopherols added before copper-induced LDL oxidation

	MDA (nmol/mg prot)	REM
LDL	0.8 ± 0.5	0.5 ± 0.2
oxLDL	15.6 ± 3.2*	2.2 ± 0.3*
oxLDL + 10 μM α-tocopherol	9.3 ± 2.1*†	1.4 ± 0.2*†
oxLDL + 50 μM α-tocopherol	5.4 ± 1.8*†	1.2 ± 0.2*†
oxLDL + 10 μM γ-tocopherol	11.6 ± 2.4*	1.8 ± 0.3*
oxLDL + 50 μM γ-tocopherol	9.0 ± 1.8*†	1.5 ± 0.2*†

MDA: malondialdehyde; REM: relative electrophoretic mobility on agarose gel with respect to the baseline. See Methods for more details. Results are the means ± SD of 6 different lipoprotein preparations in duplicate.

*$P < 0.001$ vs LDL.

†$P < 0.05$ vs oxLDL.

Fluorescence-Activated Cell Sorting Analysis (FACS)

Cells were prepared as described above. DNA was stained with 50 μg/mL of propidium iodide (PI) and analyzed using a FACScan flow cytometer with fluorimetric detection of both frontal and lateral diffusion (FACS Vantage™, Becton Dickinson) interfaced with a Hewlett-Packard computer, as described by Tortora *et al.* [34]. Cell-cycle data analysis was performed by the CELL-FIT program (Becton Dickinson). Apoptotic cells represent the Sub-G1 phase.

MAPK Assay

In the MAPK assay, cells were preincubated for 1 hr with 50 μg/mL of the MAPK synthetic inhibitor PD 98059 [35] (513000-S, Calbiochem Signal Transduction, purity ≥98% by HPLC). PD 98059 ($C_{16}H_{13}NO_3$; (2′-amino-3′-methoxyphenyl)-oxanaphthalen-4-one; MW 267.32) selectively inhibited the MAPK-activating enzyme, MAPK/ERK kinase (MEK1), without significant inhibitory activity of MAPK itself; inhibition of MEK1 by PD 98059 prevented activation of MAPK and subsequent phosphorylation of MAPK substrates both *in vitro* and in intact cells [35]. The culture medium was then changed, and LDL prepared in the absence or presence of tocopherols was then added to control cells or PD 98059-treated cells. After 3 and 24 hr of incubation at 37° under 95% air and 5% CO_2, cells were lysed with PBS containing 1% Nonidet P-40 (Sigma), 0.5% sodium deoxycholate, 0.1% SDS, 100 μg/mL of protease inhibitor PMSF, 30 μg/mL of aprotinin A, and 100 mM sodium orthovanadate [36]. Protein concentration was determined by the Lowry assay [26]. MAPK activity was determined on immunoprecipitates essentially as described by Wan *et al.* [36], using the MAPK Scintillation Proximity Assay (SPA) kit (Amersham). Briefly, 400 μg of protein was immunoprecipitated by incubation for 60 min at 4° with 1–2 μg of the ERK-1 and -2 antibodies (from hamster cell pellet; Biosignal for Amersham). Proteins were then collected and incubated overnight at 4° with 20 mg protein A-Sepharose (Pharmacia). The pellets were washed four times with lysis buffer containing 50 mM Tris–HCl (pH 7.4) and 10 mM $MgCl_2$ and resuspended in 25 μL of a buffer containing 50 mM HEPES (pH 7.5), 0.1 mM EDTA, 0.1 mg/mL of BSA, 0.15 M NaCl, and 0.1% mercaptoethanol [36]. This was followed by addition of 5 μg of myelin basic protein as substrate and 10 μL of ATP mix solution (930 μL kinase buffer, 6 μL of 50 mM ATP (pH 7.0), 20 μL of 2 mM $MgCl_2$, 44 μL of [γ-^{32}P]ATP-10 mCi/mL) [36]. After 20-min incubation at 30°, reactions were stopped by adding one volume of 2 × Laemli buffer. After boiling for 5 min and SDS–PAGE electrophoresis, the incorporated radioactive phosphate was determined using a PhosphorImager (GS-525 BioRad) interfaced with a Hewlett-Packard computer. Results were expressed as % of MAPK activity calculated from the relative increase in radioactivity compared to the control.

RESULTS

The effects of copper ions on LDL and/or tocopherols under our experimental conditions are summarized in Table 1. As expected, copper induced the oxidative modification of LDL, and 50 μM of both tocopherols significantly reduced it. Since 10 μM γ-tocopherol similarly exerted little or no effect in other experimental settings, several experiments throughout the study were conducted using both 10 and 50 μM α-tocopherol and 50 μM γ-tocopherol alone.

The apoptotic effects of oxLDL and tocopherols added before LDL oxidation were tested on SMC. Figure 1 shows the data with the TUNEL assay and DNA fragmentation. OxLDL induced incorporation of nucleotides in apoptotic cells detected by TUNEL positivity (upper panel) with a parallel increase in DNA laddering (lower panel). These phenomena were progressively reduced by the addition of tocopherols, especially α-tocopherol (Fig. 1). Addition of fresh tocopherols to cells and oxLDL produced similar qualitative results in both TUNEL and DNA laddering experiments (P = NS vs experiments with tocopherols added during LDL oxidation, N = 5). Apoptosis was also investigated by FACS, a more sensitive method directly investigating DNA content of single cells among a population of 10^5 cells. FACS showed the accumulation of apoptotic cells in the Sub-G1 fraction without any block in cell-cycle progression. Figure 2 shows a typical experiment after exposure to oxLDL in the presence of tocopherols

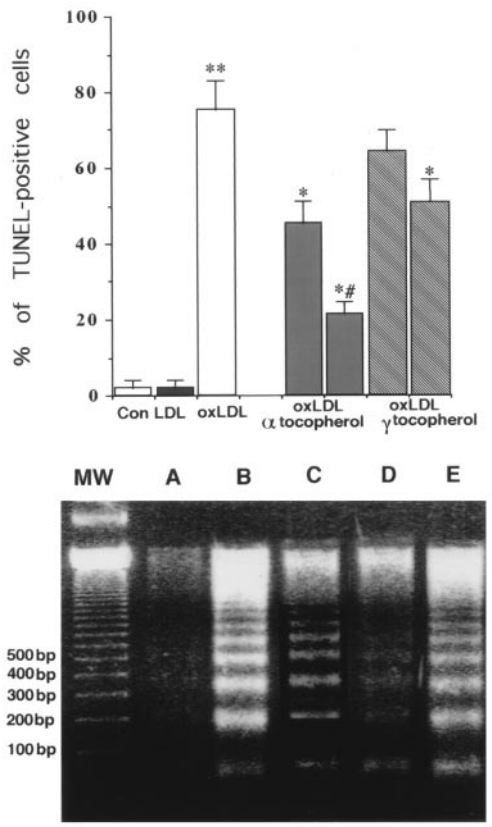

FIG. 1. Effects of oxLDL and both tocopherols added before LDL oxidation (bars represent 10- and 50-μM concentrations, respectively) in human coronary SMC detected by TUNEL assay (upper panel; mean ± SD of six different experiments, *P < 0.05 vs oxLDL, **P < 0.0001 vs LDL, and #P < 0.05 vs 10 μM α-tocopherol and vs 50 μM γ-tocopherol). Effects of oxLDL and both tocopherols added before LDL oxidation in human coronary SMC on DNA laddering (lower panel). In DNA laddering experiments, MW represented the respective molecular weights; A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol.

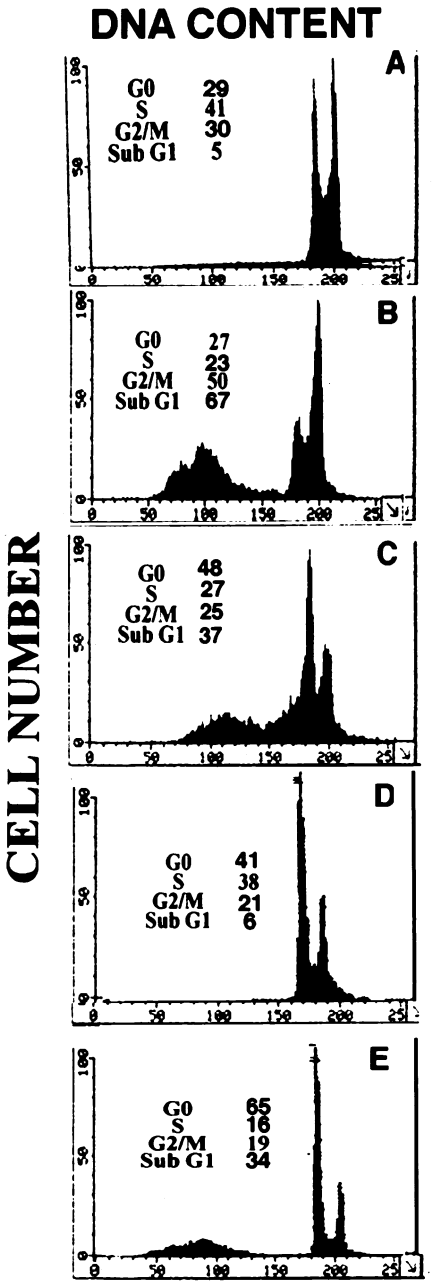

FIG. 2. FACS analysis to determine the percentage of apoptotic human coronary SMC exposed to oxLDL and both tocopherols added before LDL oxidation (Sub-G1 fraction). A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol.

added before LDL oxidation. OxLDL increased the number of apoptotic cells, which were progressively reduced by tocopherols. Addition of fresh tocopherols to cells and oxLDL produced a greater reduction in apoptotic cells (mean −9%, range 7–12%, N = 4) than that achieved with tocopherols added before LDL oxidation (P = NS), with a higher rate of reduction achieved with α-tocopherol than γ-tocopherol.

To study the molecular events involved in the transduction of death signal from extracellular membrane through the cytoplasm and inner nucleus, we first investigated the role of the MAPK cascade. Table 2 shows the MAPK activity of coronary SMC in the presence of oxLDL and tocopherols with the MAPK-MEK1 inhibitor PD 98059. This inhibitor was added to cells which were treated 1 hr thereafter with oxLDL and tocopherols in turn added before LDL oxidation. We found that PD 98059 was able to significantly block the MAPK activity induced by oxLDL at both 3 and 24 hr. Results were qualitatively similar for both incubation times, although MAPK activity was significantly higher at 3 hr (Table 2). Similarly, using the TUNEL assay, we observed that the percent of apoptotic cells induced by oxLDL was significantly reduced by PD 98059 at 24 hr (45 ± 8% reduction in TUNEL-positive cells exposed only to oxLDL). Taken together, these data show that MAPK activity has an important role in apoptosis promoted by oxLDL. Tocopherols, especially α-tocopherol, also contribute to the reduction in kinase activity via

TABLE 2. Assay for MAPK activity of human coronary smooth muscle cells pretreated with the MAPK-MEK1 inhibitor PD 98059

Samples	% MAPK activity at 3 hr (Arbitrary units)	% MAPK activity at 24 hr (Arbitrary units)
Control untreated cells	1.6 ± 1.2	2.0 ± 1.5
LDL	2.0 ± 1.3	2.1 ± 1.5
oxLDL	24.8 ± 4.5*¶	18.5 ± 3.8*
50 μM α-tocopherol + oxLDL	11.9 ± 3.1*†	9.2 ± 2.2*†
50 μM γ-tocopherol + oxLDL	15.8 ± 2.3*†	13.5 ± 1.8*†
PD 98059	0.8 ± 0.3	0.2 ± 0.2
PD 98059 + oxLDL	11.2 ± 2.8*§	9.4 ± 3.2*§
PD 98059 + oxLDL + 10 μM α-tocopherol	8.3 ± 1.6*§‡	7.5 ± 2.1§‡
PD 98059 + oxLDL + 50 μM α-tocopherol	5.2 ± 2.8§‡	4.5 ± 3.4§‡
PD 98059 + oxLDL + 10 μM γ-tocopherol	9.6 ± 2.5§*	9.2 ± 3.0*§
PD 98059 + oxLDL + 50 μM γ-tocopherol	7.9 ± 1.8§‡	7.6 ± 2.1§‡

Human coronary SMC were pretreated with PD 98059 and then incubated for 3 and 24 hr with oxLDL and tocopherols added before copper-induced LDL oxidation (see Materials and Methods for further details).

*$P < 0.001$ vs controls and LDL.

†$P < 0.05$ vs oxLDL.

§$P < 0.01$ vs oxLDL.

‡$P < 0.05$ vs PD 98059 + oxLDL.

¶$P < 0.05$ vs oxLDL at 24 hr.

reduction of the degree of LDL oxidation. Again using PD 98059, the addition of fresh tocopherols to cells produced similar results both at 3 and 24 hr (P = NS vs experiments with tocopherols added before LDL oxidation, N = 4). OxLDL also determines through MAPK the activation of JunK, which in turn mediates the phosphorylation of transcription factor c-Jun. We found high levels of the phosphorylated form of c-Jun protein (p-Jun) in extracts of cells exposed to oxLDL when compared to controls (Fig. 3, A and B). c-Jun transcription was reduced either by tocopherols added before LDL oxidation (Fig. 3A) or after addition of fresh tocopherols to oxLDL and cells (Fig. 3B), identical results being obtained by semiquantitative densitometric scanning blot analysis (P = NS for all lanes, N = 6). Similarly, c-Jun protein transcription showed an increase after exposure to oxLDL, one that was reduced by tocopherols (Fig. 3, A and B).

Transduction of the apoptotic signal is dependent on the death effector domain that interacts with additional downstream molecules, triggering a cascade of caspases together with a degradation of the intracellular substrate PARP. We analyzed the activation of the Class I procaspase-8 and the Class II procaspase-3 by Western blot (Fig. 4). Caspase-3 (CPP32) and Caspase-8 were strongly activated by oxLDL as shown by the presence (liberation) of their respective peptides (subunits) p17 and p18 which contain the catalytic site. Caspase activation was also supported by PARP degradation (presence of the band of 85 kD, Fig. 5). More importantly, both tocopherols added before LDL oxidation reduced caspase activation (Fig. 4). Although there was a slight trend toward a minor degree of activation of both caspases, addition of fresh tocopherols to cells and oxLDL produced similar qualitative results by semiquantitative densitometric scanning blot analysis (P = NS for all lanes, N = 4).

Cells exposed to oxLDL and tocopherols added before or after LDL oxidation were analyzed for the dynamic movements of the members of the Bcl-2 family proteins. We found the disequilibrium between the antiapoptotic genes

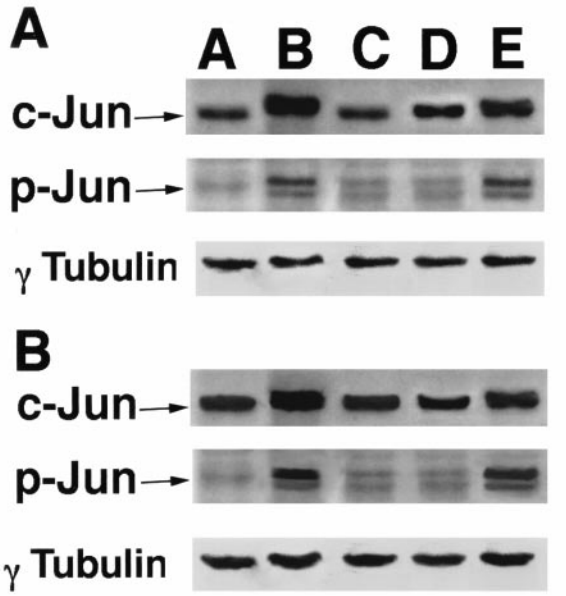

FIG. 3. Western blot analysis of transcription factor c-Jun and its activated phosphorylated form (p-Jun) in human coronary SMC. Panel A represents experiments in which tocopherols were added before LDL oxidation: A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol; normalization by γ-tubulin. Panel B depicts experiments in which fresh tocopherols were added to cells and oxLDL after copper-induced LDL oxidation: A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol; normalization by γ-tubulin.

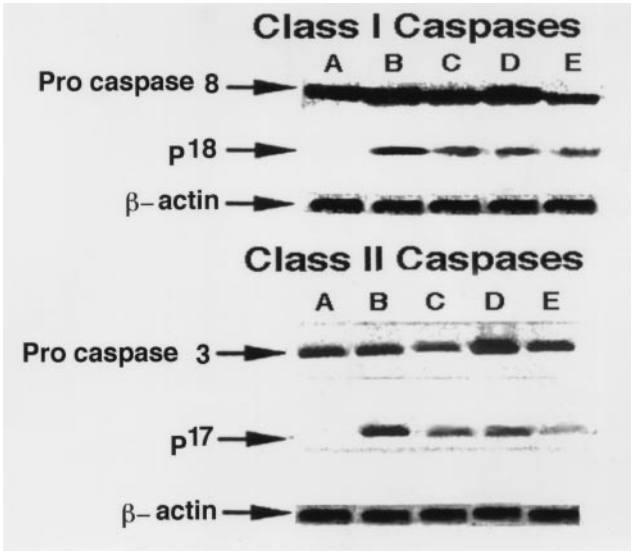

FIG. 4. Western blot analysis of the relative contribution of Class I caspase (caspase-8) and Class II caspase (caspase-3) as cell death effectors induced by oxLDL and both tocopherols added before LDL oxidation in human coronary SMC. A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol; normalization by β-actin.

(Bcl-2) and the proapoptotic genes (Bax) more evident in cells treated with oxLDL; tocopherols progressively attenuated the phenomenon (Fig. 6, A and B). In contrast, the proapoptotic factor Bad was not affected by different treatments. These results were qualitatively comparable when tocopherols were added before (Fig. 6A) or after (Fig. 6B) LDL oxidation (P = NS for all lanes by semiquantitative densitometric scanning blot analysis, N = 5).

The binding shift of the nuclear extracts of SMC showed an oxLDL-dependent increase in NF-kappaB activity (left panel, Fig. 7) and the AP-1 complex (right panel, Fig. 7) when compared to control. Tocopherols added before oxidation partially reduced NF-kappaB and AP-1 nuclear activation. We acquired comparable results by semiquantitative densitometric scanning analysis when fresh tocopherols were added to cells after LDL oxidation. However, we found that NF-kappaB activity was further reduced when 50 μM of fresh tocopherols was added to cells after LDL oxidation, revealing an "oxidation-sensitive" mechanism of NF-kappaB activation (mean further reduction of 18 + 3% and 21 + 4% with γ- and α-tocopherol, respectively; P = 0.072 [NS] and P = 0.059 [NS] vs tocopherols added before LDL oxidation, N = 6).

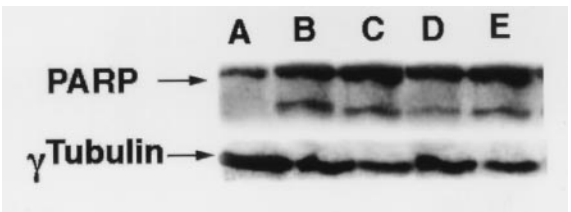

FIG. 5. Western blot analysis of the substrate PARP of caspases in human coronary SMC exposed to oxLDL and both tocopherols added before LDL oxidation. A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol; normalization by γ-tubulin.

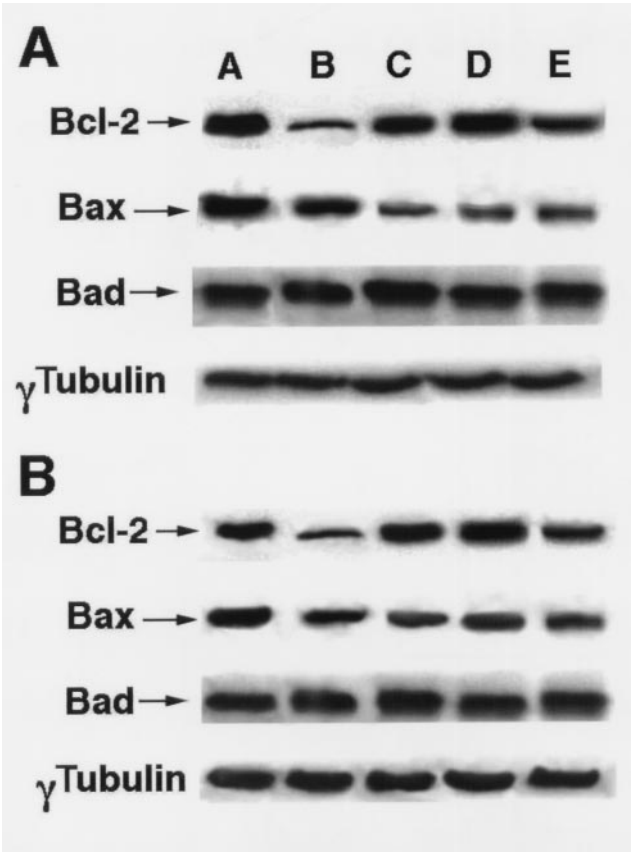

FIG. 6. Western blot analysis of apoptotic factors (Bcl-2, Bax, and Bad) in human coronary SMC. Panel A represents experiments in which tocopherols were added before LDL oxidation: A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol; normalization by γ-tubulin. Panel B depicts experiments in which fresh tocopherols were added to cells after copper-induced LDL oxidation: A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol; normalization by γ-tubulin.

When the binding shift was technically inappropriate due to repetitive DNA sequences for the components of the same family (e.g. the ETS family of transcription factors to which ELK-1 belongs has similar DNA motifs that share a centrally located 5′-GGAA-3′ element) [23], we performed Western blot analysis using specific antibodies. The transcription factor ELK-1 depends on the MAPK-MKK3-p38 or MAPK-MEK1-ERK1-ERK2 pathways and on the JunK-JunK1-JunK2-JunK3 pathway [24]. Accordingly, ELK-1 protein was strongly accumulated by oxLDL compared to control, and both tocopherols added before LDL oxidation induced an attenuation of its transcription (Fig. 8). Similarly, the transcription of ATF-2 and pCREB was stimulated by oxLDL and lowered by tocopherols added before LDL oxidation (Fig. 8). Analogous data were obtained by semiquantitative densitometric scanning blot analysis when

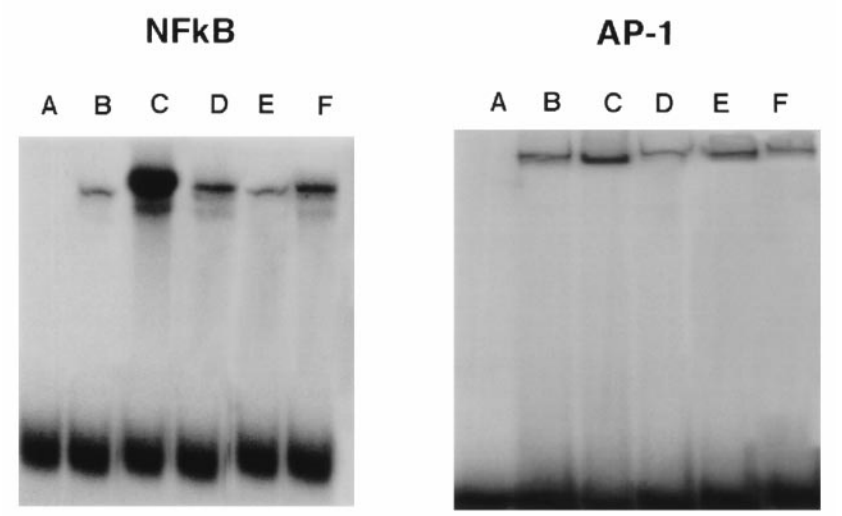

FIG. 7. Binding shift analysis for assessing activity of NF-κB (left panel) and of the AP-1 complex (right panel) in nuclear extracts from human coronary SMC exposed to oxLDL and both tocopherols added before LDL oxidation. A: sham; B: LDL; C: oxLDL; D: oxLDL + 10 μM α-tocopherol; E: oxLDL + 50 μM α-tocopherol; F: oxLDL + 50 μM γ-tocopherol.

fresh tocopherols were added to cells and oxLDL (P = NS for all lanes, N = 4).

DISCUSSION

Whether deterministically activated or triggered by physiological or pathophysiological conditions, apoptosis requires a complex combination of signals that can either originate within the cell or be delivered by exogenous stimuli. In the present study, we have shown that oxLDL stimulates the apoptotic program in coronary SMC through the MAPK and Jun kinase pathways coupled to activation of several transcription factors and apoptotic genes and that tocopherols significantly reduce these molecular events. Specifically, we demonstrate for the first time that copper-oxidized LDL-mediated apoptosis enhances the transcription of c-Jun, CREB, ELK-1, and ATF-2 and the nuclear activity of the JunK-dependent AP-1 complex.

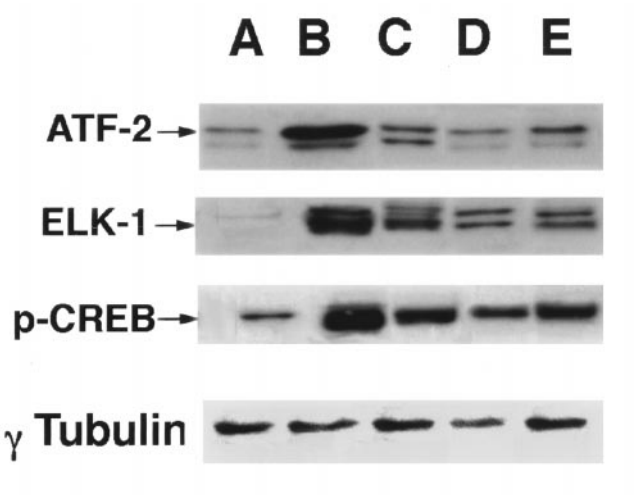

FIG. 8. Western blot analysis of transcription factor ELK-1, the phosphorylated form of CREB, i.e., p-CREB, and ATF-2 in human coronary SMC exposed to oxLDL and both tocopherols added before copper-induced LDL oxidation. A: LDL; B: oxLDL; C: oxLDL + 10 μM α-tocopherol; D: oxLDL + 50 μM α-tocopherol; E: oxLDL + 50 μM γ-tocopherol; normalization by γ-tubulin.

The ATBCCP study [37] was a primary prevention trial designed to verify whether supplements of vitamin E and beta-carotene had any effect on cancer rates. After a follow-up period of 6.1 years, this study demonstrated no effect on cardiovascular death, but these results should be not considered relevant, as the dose of vitamin E was well below (50 mg/day) the usual dosages employed in epidemiological studies. The Chinese Cancer Prevention Study [38] investigated four different combinations of several different substances (vitamins A, C, E, beta-carotene, zinc, niacin, riboflavin, and selenium). After a follow-up period of 5.2 years, a reduction of 13% in total mortality was observed. However, because of the various combinations used, it is not possible to establish the relative contribution of vitamin E. The CHAOS study [13] conducted on patients with CHD showed that vitamin E (400–800 IU/day) reduced non-fatal and fatal myocardial infarction up to 510 days of follow-up (41 episodes in the vitamin group vs 65 in the placebo group). Finally, the GISSI-Prevenzione trial [39] showed that vitamin E had no benefit in the secondary prevention of cardiovascular events and death up to 3.5 years of follow-up in patients surviving myocardial infarction. The discrepancies seen in these trials may depend on the variable characteristics of the population tested, on the great number and dosages of vitamin tested, and whether they were administered alone or in multiple regimens [40]. At least six major clinical trials on the effect of vitamins on CHD are currently underway in an attempt to clarify these issues (reviewed in [41]).

Biochemically, human LDL has a molecular mass of 2.5 million, containing on average 1300 molecules of polyunsaturated fatty acids; the predominant antioxidant in LDL is α-tocopherol, with 6 molecules in each LDL particle, other antioxidants being γ-tocopherol, β-, and α-carotene [42]. Each is present in amounts of only 1/20th to 1/300th

that of α-tocopherol [42]. During LDL oxidation, antioxidants are rapidly reduced, but daily supplements of 150 to 1200 IU α-tocopherol increased the LDL α-tocopherol content and the oxidation resistance from 118 to 175%, respectively [42]. We showed that the addition of 10 μM γ-tocopherol before or after LDL oxidation did not significantly prevent the activation of several apoptotic-related genes with respect to 10 μM α-tocopherol. Micromolar concentrations of tocopherols are severalfold higher than those reached in human plasma and on LDL [42]. Thus, incubation with these high concentrations before LDL oxidation might result in residual non-oxidized tocopherols. This might explain why we observed similar effects on signaling pathways when fresh tocopherols were added to cells and oxLDL. At the same time, we showed that high concentrations of tocopherols before LDL oxidation are able to preserve LDL from oxidative modifications which per se promote apoptosis in coronary SMC. Although γ-tocopherol plays a minor role in these phenomena, it may exert additional actions. For example, when tocopherols react with nitrogen dioxide, this leads to the formation of a nitrosating agent from α-tocopherol, but not from γ-tocopherol [43]. Furthermore, γ-tocopherol is a more potent inhibitor of ~neoplastic transformation in murine fibroblasts than is α-tocopherol [43], whereas the latter decreased both radiation-induced apoptosis and DNA fragmentation in mice splenocytes [44]. This is according to the antiapoptotic effects of α-tocopherol seen in the present study. Finally, α-tocopherol supplements decrease plasma γ-tocopherol in humans [45], while cholesterol-fed rabbits receiving 110 IU/day α-tocopherol demonstrated preserved endothelial function, whereas animals receiving 10 times this dose demonstrated an impairment of endothelium-dependent arterial relaxation [19]. Taken together, these considerations lead to avoiding a clinical indiscriminate use of high doses of supplements of α-tocopherol. Indeed, antioxidant defenses play a crucial role in modulating the ambient steady-state levels of reactive oxygen species, which also have important physiological signaling functions when produced in a controlled fashion. Moreover, our data show that administration of tocopherol may interfere with signaling pathways regardless of the effects on plasma LDL oxidation. These effects may at least in part be responsible for its antiatherogenic properties. Indeed, it is well known that α-tocopherol is easily incorporated into the vascular wall [12, 19] and into atherosclerotic plaques [46]. Furthermore, α-tocopherol influences leukocyte adhesion and targeting to endothelial cells, monocyte transmigration, and oxidant-mediated cytotoxicity [19]. α-Tocopherol is also known to inhibit cell proliferation by inhibiting protein kinase C in SMC [47]. The effect on kinase C may be related to the interference on apoptosis intracellular signaling (in particular on MAPK) seen in the present study and in earlier studies [48–50]. We found that PD 98059 reduced apoptosis induced by ox-LDL. Since the MEK1 inhibitor also blocks the ERK pathway, we suggest ERK involvement in apoptosis. However, several recent reports are rather conflicting on the exact nature of the apoptotic role of the ERK pathway. Indeed, the ERK cascade is considered both as rather antiapoptotic [51, 52] and as having proapoptotic effects [22, 53, 54].

We also provide evidence here that ox-LDL activates the MAPK-dependent transcription factors CREB and ATF-2 [23, 55]. The ATF/CREB family consists of a series of transcription factors that function through binding to the CRE palindromic octanucleotide, TGACCTCA. As described, scaffolding of MAPK-MEKK4-MKK4-MKK7 activates JunK-mediated signaling [23], which is also selectively involved in apoptosis [56]. We describe here for the first time that oxLDL stimulates JunK, activating the transcription of the phosphorylated form of c-Jun (p-Jun) [23] and the nuclear binding-shift activation of the AP-1 complex [57]. As described, ELK-1 belongs to the ETS family of transcription factors [23]. The activation of all subunits of transcription factor ELK-1 depends on both MAPK (ERK-1 and -2 and p38 pathways) and JunK pathways [23]. Indeed, we have also shown here the oxLDL-mediated transcription of ELK-1 protein. However, the biological and/or pathophysiological significance of the oxLDL-induced activation of these factors remains to be determined.

In the present study, binding-shift analysis showed that NF-kappaB was also activated in the nucleus. NF-kappaB exists in the cytoplasm in an inactive form associated with inhibitory proteins called IkappaBs, and NF-kappaB activation is achieved through the signal-induced proteolytic degradation of IkappaBs in the cytoplasm [24]. It is well known that NF-kappaB is activated by oxygen radicals and oxidative compounds [58] and that simultaneous multiple signals of cell proliferation and apoptosis converging on this factor [24]. Our findings are in line with previous studies which have described that oxLDL stimulated the activation of NF-kappaB in mononuclear phagocytes [59] and the apoptosis of endothelial cells [60]. This latter study also described beneficial effects of γ-tocopherol in preventing apoptosis via attenuated oxLDL-mediated degradation of the IkappaBs and activation of NF-kappaB. Whether enhanced nuclear activity of NF-kappaB represents a proapoptotic or a proliferative protective signal from oxLDL-induced apoptosis [24] remains to be established.

Apoptosis is a genetically regulated, cellular suicide mechanism that plays a crucial role in the development and the defense of homeostasis [61]. Members of the Bcl-2 family of proteins interact dynamically to regulate programmed cell death. Bcl-2 blocks cell death following a variety of stimuli, whereas Bax p21 (i.e. Bcl-associated X protein), has extensive aminoacid homology and forms homodimers with Bcl-2, countering the death repressor activity of Bcl-2 [61]. In the present study, we found a disequilibrium between Bcl-2 and Bax which was more evident in cells treated with oxLDL, whereas the proapoptotic Bad was not affected by different treatments with tocopherols. Since the propensity of a cell to undergo apoptosis may be dependent on the ratio of these positive

and negative regulators within the cell, our data show that oxLDL-induced apoptosis in human coronary SMC depends on a Bcl-2/Bax dependent-mechanism. Both tocopherols, especially when added after LDL oxidation, reduced the expression of apoptotic Bcl-family proteins and, more importantly, the activation of cell death effector caspases-3 and -8.

In conclusion, our data indicate that tocopherols (mainly α-tocopherol) may exert their protective effects at the molecular level by reducing kinase cascade activation (MAPK and JunK) in the cytoplasm and also by decreasing nuclear activities of several transcription factors. These phenomena may preserve the intima of the arterial wall, thereby reducing the rate of apoptotic cells in human coronary SMC. Our evidence is consistent with the protective effects of tocopherols on CHD seen in epidemiological studies.

This paper is dedicated to the memory of Dr. Russel Ross who passed away in March 1999. The authors acknowledge their indebtedness to Drs. W. Palinski, O. Quehenberger, C. K. Glass, L. J. Ignarro, and M. Mancini for valuable discussions. We also apologize for omitting all the appropriate primary references in order to reduce the size of the reference list. This research was supported by Grant ISNIH.99.56980 (C.N.).

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