α-Tocopherol Inhibits the Respiratory Burst in Human Monocytes

ATTENUATION OF p47phox MEMBRANE TRANSLOCATION AND PHOSPHORYLATION*

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Odile Cachia‡§, Jamel El Benna¶, Eric Pedruzzii, Bernard Descomps‡,
Marie-Anne Gougerot-Pocidalo¶, and Claude-Louis Leger‡

From the ‡Institut de Biologie, Faculté de Médecine, Laboratoire de Biologie Biochimie des Lipides,
Boulevard Henri IV, 34000 Montpellier and ¶INSERM U 479, Faculté de Médecine Xavier Bichat,
16 rue Henri Huchard, 75018 Paris, France

Vitamin E (α-tocopherol), one of the most important natural antioxidants, is assumed to be beneficial in the prevention of cardiovascular diseases. α-Tocopherol exhibits acyl-peroxyl-radical scavenger properties and exerts cell-mediated actions in the hemovascular compartment, such as inhibition of superoxide anion (O2•−) production by leukocytes. The aim of this study was to examine the mechanism underlying the inhibitory effect of α-tocopherol on O2•− production by human monocytes. In activated monocytes O2•− is produced by the NADPH-oxidase enzyme complex. The oxidase activation elicited by phorbol myristate acetate (PMA) requires membrane translocation of several cytosolic factors. We found that in human PMA-stimulated adherent monocytes, α-tocopherol (but not β-tocopherol) inhibited O2•− production in intact cells but had no effect on a membrane preparation containing activated NADPH-oxidase, suggesting that α-tocopherol impairs the assembly process of the enzyme complex. We showed that translocation and phosphorylation of the cytosolic factor p47phox were reduced in monocytes preincubated with α-tocopherol. We verified that the tryptic phosphopeptide map of monocyte p47phox was similar to that of neutrophil p47phox, indicating that several serine residues were phosphorylated. Peptides whose phosphorylation is dependent on protein kinase C (PKC) were phosphorylated to a lesser degree when p47phox was immunoprecipitated from α-tocopherol-treated monocytes. In vitro, the activity of PKC from monocytes was inhibited by α-tocopherol in a specific manner compared with that of β-tocopherol or Trolox®. Membrane translocation of PKC was not affected. These results show that α-tocopherol inhibits O2•− production by human adherent monocytes by impairing the assembly of the NADPH-oxidase and suggest that the inhibition of phosphorylation and translocation of the cytosolic factor p47phox results from a decrease in PKC activity.

Oxidative modification of low density lipoprotein (LDL)3 appears to be a key event in the early stages of atherogenesis (1). Monocyte release of superoxide anion (O2•−), a reactive oxygen species (ROS), induces LDL oxidation (2). Vitamin E (α-tocopherol), a lipophilic molecule present in plasma lipoproteins, is one of the most important natural antioxidants (3). It is able to scavenge acyl-peroxyl radicals in membrane structures and may prevent or delay cardiovascular diseases (4, 5), possibly through its capacity to increase LDL resistance to oxidative modification (6). The possibility that α-tocopherol has additional antioxidant effects by inhibiting ROS generation has received little attention. We (7) and others (8–10) have shown that α-tocopherol inhibits O2•− production by human monocytes (7), rat macrophages, and neutrophils (8–10), but the mechanism(s) underlying this inhibitory effect are unknown.

The system responsible for O2•− production in phagocytic cells is the multicomponent enzyme NADPH-oxidase. This complex includes membrane-bound cytochrome b558 and cytosolic proteins (p47phox, p67phox, Rac1/2, and p40phox) (11) that translocate to the membrane during stimulation to form a catalytically active oxidase (12). During NADPH-oxidase activation, p47phox is phosphorylated on several serine residues (13). Protein kinase C (PKC) is involved in NADPH-oxidase activation and can phosphorylate p47phox (14, 15). PKC is a family of Ca2+- and phospholipid-dependent serine/threonine kinases that play a pivotal role in agonist-stimulated cell functions (16). Several PKC isoforms have been described (17). The conventional PKCs αPKC and βPKC are present in monocytes and are activated by phorbol esters such as phorbol 12-myristate 13-acetate (PMA) (18). In human monocytes and neutrophils, PMA induces the production of O2•− and the phosphorylation of several proteins, one of which is p47phox (14).

The purpose of this study was to explore the underlying mechanism of α-tocopherol-induced inhibition of O2•− production by monocytes. We found that α-tocopherol treatment of human adherent monocytes inhibited PMA-induced translocation and phosphorylation of the cytosolic oxidase component p47phox.

EXPERIMENTAL PROCEDURES

Materials—dl-α-Tocopherol, dl-β-tocopherol, and PMA were from Sigma; SDS-PAGE reagents were from Bio-Rad; and [32P]orthophosphate and [γ-32P]ATP were from NEN Life Science Products. Anti-p47phox antibody was a kind gift from Dr. Babior (Scripps Research Institute). Anti-PKC antibody was from Santa Cruz Biotechnology.

Monocyte Preparation—Mononuclear cells were obtained from healthy subjects by dextran sedimentation and Ficoll-Paque fractionation of freshly drawn blood. The cells were resuspended in RPMI medium + 10% fetal calf serum and then plated in Petri dishes for 2 h at 37 °C (20 × 106 cells/10 ml) in humidified air with 5% CO2. The adherent monocytes remaining after washing with RPMI (about 5 × 105 cells/dish) were incubated with α-tocopherol or ethanol in basal conditions for 30 min and then stimulated with PMA (30 ng/ml) for 10 min. For the phosphorylation experiments, cells were resuspended in phosphate-free Dulbecco’s modified Eagle’s medium, treated with 2.7 mM...
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disopropylfluorophosphate on ice for 10 min, and then washed. After 2 h in Petri dishes, non-adherent cells were removed, and the remaining monocytes were incubated in phosphate-free Dulbecco’s modified Eagle’s medium containing 250 μCi of [32P]orthophosphate/10^6 cells/ml for 1.5 h at 37 °C. a-Tocopherol and PMA were then added as described above. The purity and viability of the monocyte preparations after the adherence step were higher than 95 and 90%, as assessed by trypan blue dye exclusion and neutral red staining, respectively.

Superoxide Production Measurement—The effect of a-tocopherol on O_2^- production was measured in a lucigenin-enhanced chemiluminescence assay. Adherent monocytes purified in a 48-well plate were pre-incubated with various amounts of a-tocopherol or its ethanol vehicle for 30 min before stimulation with 60 ng/ml PMA in the presence of 100 μM lucigenin. Luminescence was measured by an ultrasensitive photomultiplier counting imaging camera monitored by a computer-assisted image processor (Argus 100, Hamamatsu Photonics, Japan). Photon emission was recorded for 60 min after PMA stimulation and was proportional to O_2^- production (7). We also tested the effect of a-tocopherol on an acellular system producing O_2^- by using the superoxide dismutase-inhibitable cytochrome c reduction method (19). The membrane fraction containing active NADPH-oxidase was obtained by sonication of stimulated human neutrophils or monocytes (1 μg/ml PMA) in 0.34 M sucrose + 1 mM PMSF and ultracentrifugated (100,000 × g for 30 min at 4 °C in a Beckman TL 100). The pellet (membrane fraction) was solubilized in 0.34 M sucrose. The production of O_2^- was measured (20) in the presence of a-tocopherol or ethanol.

p47^{phox} and PKC Translocation—Resting and stimulated cells were scraped from the Petri dishes (5 × 10^6 cells/ml), sonicated on ice (2 × 10 s) in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl_2, 10 mM PIPES, 1 mM EGTA, 10 μg/ml pepstatin, 10 μg/ml leupeptin, 0.5 mM PMSF), and centrifuged (600 × g for 10 min at 4 °C) to remove nuclei and unbroken cells; the supernatant was then ultracentrifuged (100,000 × g for 30 min at 4 °C). Pellets were washed in relaxation buffer, dissolved in Laemmli sample buffer, and submitted to 10% SDS-PAGE (21) and then blotted onto nitrocellulose using the Towbin protocol (22). The upper part of the gel was revealed with anti-p47^{phox} antibody (1/1000 dilution), and the lower part with anti-PKC antibody (1/1000 dilution) and labeling by secondary antibodies (1/2000), followed by ECL detection.

RESULTS

a-Tocopherol Inhibits O_2^- Production in Intact Human Adherent Monocytes—PMA-stimulated production of O_2^- by monocytes was depressed in the presence of a-tocopherol in a concentration-dependent manner (Fig. 1). The inhibition was not complete, reaching a maximum of 50% at 45 μg/ml a-tocopherol. It is noteworthy that the basal activity of unstimulated monocytes (representing 15 ± 5% (n = 4) of the activity of stimulated cells) was not inhibited by a-tocopherol. O_2^- production by xanthine/xanthine oxidase was not decreased by a-tocopherol (data not shown), suggesting that inhibition did not result from scavenging of O_2^- This lack of O_2^- scavenging was previously found even in the presence of LDL (7), showing that it is not due to the absence of a lipid phase in the medium. a-Tocopherol also failed to decrease O_2^- production in a membrane preparation containing NADPH-oxidase (Fig. 1). This showed that a-tocopherol did not directly interfere with activated NADPH-oxidase but may instead have inhibited an event preceding oxidation activation. In contrast to this depressive effect of a-tocopherol, β-tocopherol enhanced O_2^- production by monocytes. These results provide additional evidence for an action of a-tocopherol that is independent of its scavenging properties and suggest that a-tocopherol has a specific inhibitory effect.

a-Tocopherol Inhibits p47^{phox} Translocation—As p47^{phox} translocation is a key event in NADPH-oxidase activation (23), we investigated the effect of a-tocopherol on this process by using Western blot analysis of the membrane fractions. We chose a concentration of a-tocopherol that clearly inhibited O_2^- production (Fig. 1). When monocytes were stimulated with PMA, pretreatment with 20 μg/ml a-tocopherol inhibited membrane translocation of p47^{phox} (Fig. 2). In resting monocytes, p47^{phox} was barely detectable in the membrane fraction, and a-tocopherol alone did not induce detectable movement of p47^{phox} between the cytosolic and membrane compartments (Fig. 2). Western blots were scanned, and the combined densitometry data indicated that in the presence of a-tocopherol the level of p47^{phox} translocation was 70.9 ± 4.3% of control (n = 4) (p < 0.05).

2 N. Kadri-Hassani and C. L. Leger, unpublished observations.
PKC Translocation Is Insensitive to α-Tocopherol—To assess whether α-tocopherol inhibits p47phox translocation and phosphorylation by modulating PKC activation, we used Western blot analysis to visualize the translocation of PKC from cytosol to membrane. The antibody used to reveal PKC cross-reacted with the heavy chain of the IgG antibody and not with PKC. This provides further evidence that the inhibitory effect of α-tocopherol is mediated by PKC.

α-Tocopherol Inhibits PKC Activity in Vitro—We then tested the effect of α-tocopherol on PKC activity. A PKC preparation from the cytosolic fraction was incubated in the presence of PS/DG liposomes, with or without incorporated α-tocopherol. Fig. 6 shows that α-tocopherol strongly inhibited PKC activity, whereas β-tocopherol was less effective and Trolox had a weak effect; this is consistent with a specific action of α-tocopherol.

DISCUSSION

In this study α-tocopherol depressed the PMA-induced respiratory burst of human adherent monocytes without affecting the assembled oxidase activity of membrane preparations originating from PMA-activated monocytes or neutrophils. This strongly suggests that the α-tocopherol inhibition of O₂⁻ production is at least in part related to a functional impairment of the NADPH-oxidase assembly. Indeed, we found that α-tocopherol inhibited p47phox translocation to the membrane and impaired p47phox phosphorylation. In human neutrophils, oxidase activation by PMA is believed to result from the translocation of p47phox to the membrane and impaired phosphorylation. Indeed, we found that α-tocopherol inhibited p47phox translocation to the membrane and impaired phosphorylation. In human neutrophils, oxidase activation by PMA is believed to result from the translocation of p47phox to the membrane and impaired phosphorylation.
PKC activity (%)

control alpha-T beta-T trollox

PKC

p47phox

Cytosol Membrane

**Fig. 5.** α-Tocopherol does not modify PKC translocation in PMA-treated human monocytes. Cells were treated as described in Fig. 1. After SDS-PAGE and blotting of cytosol and membrane fractions, PKC and p47phox were detected with their respective antibodies and revealed with the ECL technique. Concentrations used are as follows: PMA 30 ng/ml; α-tocopherol (α-toc) 20 µg/ml. Data are representative of five experiments.

**Fig. 6.** Effect of α-tocopherol, β-tocopherol, and Trolox on PKC activity of monocyte cytosol. Cytosol fraction was separated from resting monocytes by sonication and ultracentrifugation. PKC activity was measured by PS/DG- and Ca2+-dependent transfer of [γ-32P]ATP to histone III-S. α- and β-tocopherol were integrated in PS/DG liposomes, with a final concentration in the assay mixture of 20 µg/ml. Trolox was added at 11.6 µg/ml (46 µM). Activity was measured on 6.5 µg of cytosolic protein. Values are expressed as the percentage of control activity (mean ± S.E., n = 3). Control (100%) corresponds to 6080.35 ± 290.4 pmol of [32P]Pi per min.

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